

An analytical method to investigate the estrogen metabolism in women taking combined oral contraceptives

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The same boiling water that softens the potato hardens the egg. It's about what you're made of, not the circumstances.

Unknown

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Abstract

The human body is exposed to numerous (endogenous and exogenous) toxins daily. The body has a protective mechanism to help detoxify certain toxins, through biotransformation processes. However, when exposure exceeds the capacities of the biotransformation systems, it may result in pathophysiological conditions. The additional intake of estrogens are a good example thereof, where exposure (through intake) might contribute to a disturbance in the balance between the different phases of liver detoxification and allow toxic metabolites to be circulated in the blood (rather than quickly excreted in the urine). Evidence suggests that the estrogen metabolism and an imbalance thereof could be associated with an increased risk of breast cancer development. The intake of estrogens through combined oral contraceptives is one of these exogenous sources of estrogens. A large part of the South African population might be unknowingly affected by the additional intake of estrogens, as more than half of married or in-union females use this type of contraceptive method. Determining the effect of exogenous estrogens, on the normal physiological detoxification capacities, requires certain markers to be monitored. A link between the urinary estrogens and relative cancer risk has been made in previous studies, and evidence has shown that the evaluation of the estrogen-related metabolites in the urine, might aid in breast cancer risk evaluation. This study focused on the need for a non-invasive method for the quantification of the estrogen metabolites excreted in the urine. These metabolite concentrations and their ratios of excretion, resulting from either normal estrogen metabolism or an additional estrogen intake, could be evaluated as possible markers for breast cancer development risks. Previous methods for the quantification of some of these metabolites have been developed but limitations still exist and in-house implementation always requires adaptation of previously developed methods due to variation in factors such as the availability of infrastructure, analytical standards and the sensitivity of the available instrumentation. This study, therefore, aimed to develop a highly sensitive and selective urine-based liquid chromatography–electrospray tandem mass spectrometry method to investigate the estrogen metabolism in women.

Outcomes of this study included the development of analytical methods to measure the metabolic products of estrogen metabolism in the urine. The first part of the study comprised of the development of a sensitive, specific, accurate and precise liquid chromatography tandem mass spectrometry (LC-MS/MS) method to quantify the levels of estrogens and related metabolites in the urine. A non-derivatised method was developed, however, during validation, the limitations of the capacity of the method became known. This was then followed by the development of a derivatised method that enabled the quantification of 27 estrogen and related metabolites. This was achieved after a single sample clean-up with solid phase extraction, derivatisation through dansylation, and two different (polar and non-polar) analyses on the LC-MS/MS instrument. The metabolites that could be accurately quantified with the developed methods included parent

estrogens (E1, E2, E3), their hydroxylated forms (2OH, 4OH, 16OH), metabolites of the 16 α -hydroxyestrogen pathway, sulphate and glucuronide conjugated forms, methylated forms (2 & 4-methoxy), a precursor (testosterone and androstenedione) and related steroid hormones (progesterone). The developed method was then validated and allows quantification in the high picogram and low nanogram per millilitres range. The developed method is now available for laboratory use and for use in a research setting to investigate estrogen metabolism and the effect that exogenous sources of estrogens, for examples combined oral contraceptives (COCs), might have on metabolic profiles. Furthermore, in a more clinical setting, the method could be beneficial for monitoring the relative estrogen metabolite-related cancer risk in females over time. The study was concluded by testing the utility of the developed method on actual clinical samples, merely to confirm detection and quantification of each of the metabolites in urine samples and to compare the concentrations obtained through urine analysis to those of published reference ranges.

Key terms: Biotransformation, estrogen metabolism, combined oral contraceptives, breast cancer risk, LC-MS/MS, method development, dansylation

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List of symbols and abbreviations

A4	Androstenedione
ACN	Acetonitrile
AIDS	Acquired immune deficiency syndrome
AMP	Adenosine mono phosphate
APCSI	Atmospheric pressure chemical ionisation
APZ	4-(4-Methyl-1-piperazyl)-3-nitrobenzoyl azide
ATP	Adenosine triphosphate
b	Slope of regression line
BSEP	Bile salt export pump
BSTFA	N, O-Bistrifluoroacetamide
C	Carbon
C18	Octadecylsilane
C8	Octasilane
CA	California
CAV	Cell accelerator voltage
CE	Catechol estrogen
CH ₃	Methyl group
CO ₂	Carbon dioxide
CoA	Coenzyme A
COCs	Combined oral contraceptives
COMT	Catechol-ortho-methyltransferase
COOH	Carboxyl group
CV	Coefficient of variance
CYP	Cytochrome P450
D	Deuterium

Da	Dalton
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DNA	Deoxyribonucleic acid
DNSCL	Dansyl chloride
E1	Estrone
E1-2,3-Q	Estrone-2,3-Quinone
E1-3,4-Q	Estrone-3,4-Quinone
E2	Estradiol
E2-2,3-Q	Estradiol-2,3-Quinone
E2-3,4-Q	Estradiol-3,4-Quinone
E3	Estriol
EC	End capped
EE	Ethinyl estradiol
EMV	Electron multiplier voltage
ER	Estrogen receptor
ER α	Estrogen receptor alpha
ER β	Estrogen receptor beta
ESI	Electrospray ionisation
eV	Electron volts
F ⁻	Fluoride ion
FA	Fusaric acid
FDA	Food and drug administration agency
Fe ²⁺	Iron ion
FMPTS	2-Fluoro-1-methylpyridinium-p-toluenesulfonate
FSH	Follicle-stimulating hormone

G	Continuous extension to the naming of hormones to indicate its conjugation to glucuronide
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GSH	Glutathione
GSSG	Glutathione disulphate
GST	Glutathione-S-transferases
H ₂ O	Water
HCL	Hydrogen chloride
HeLa	Cervikal cell line (derived from Henrietta Lacks 1951)
HMG-CoA	3-Hydroxy-3-methylglutaryl Coenzyme A
IQR	Interquartile range
IS	Internal standard
K _M	Michaelis-Menten constant
LC	Liquid chromatography
LC-MS	Liquid chromatography- mass spectrometry
LH	Luteinizing hormone
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass to charge ration
ME	Methoxy estrogen
ME1	Methoxyestrone
ME2	Methoxyestradiol
MeOH	Methanol

MF	Matrix factor
Min	Minutes
mL	Millilitres
Mmol	Millimolar
MPPZ	1-(2,4-dinitro-5-fluorophenyl)-4,4-dimethylpiperazinium iodide
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MTBE	Methyl tert-butyl ether
N ₂	Nitrogen
NAcCys	N-acetyl cysteine
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NaHCO ₃	Sodium bicarbonate
ng	Nanograms
NH ₂	Amino group
NIST	National Institute of Standards and Technology
NQO	NAD(P)H dehydrogenase quinone
OH	Hydroxyl
OHE1	Hydroxyestrone
OHE2	Hydroxyestradiol
P4	Progesterone
PA	Picolinic acid
PED	N-(5-Fluoro-2,4-dinitrophenyl)-N,N-dimethyl-1,2-ethanediamine
PFBBBr	Pentafluorobenzyl bromide
PFP	Pentafluorophenyl
pH	Potential of hydrogen

PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
Pmol	Picomole
ppb	Parts per billion
ppm	Parts per million
PPZ	1-(2,4-Dinitro-5-fluorophenyl)-4-methylpiperazine
PSCI	Pyridine-3-sulfonyl chloride
psi	Pounds per square inch (6.89kPa)
Q	Continuous extension to the naming of hormones to indicate its conjugation to quinones
Q1	First quartile (the 25th percentile of the data)
Q3	Third quartile (the 75th percentile of the data)
QC	Quality control
QQQ	Triple quadrupole
r^2	Represents the percentage of variation in the data explained by the linear model
rcf	Relative centrifugal force
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRF	Relative response factor
RRHD	Rapid resolution high definition
RSD	Relative standard deviation
RT	Retention time
S	Continuous extension to the naming of hormones to indicate its conjugation to sulphate
S/N	Signal-to-noise ratio
S_a	Standard deviation of the response from the regression line of calibration curve

SB	Stable bond
SNP	Single nucleotide polymorphisms
SOD	Superoxide dismutase
SPE	Solid phase extraction
SQ	Semiquinone
SULT	Sulfotransferase
T	Testosterone
TB	Tuberculosis
TIC	Total ion chromatogram
TMA	Trimethylamine
TMCS	Trimethylsilyl chloride
U.S.	United states
UGT	Diphosphate-glucuronosyltransferase enzyme
ULOQ	Upper limit of quantification
USA	United states of America
Uv	Ultraviolet
V	Volts
v/v	Volume/volume %
%	Percentage
°C	Degrees celsius
µg	Microgram
µl	Microliter
α	Alpha
β	Beta
π	Pi

CHAPTER 1

INTRODUCTION

1.1 Background and motivation

Increasing evidence indicates that estrogen metabolites are involved in the initiation of cancers, especially breast cancer (Cavalieri *et al.*, 2017; Gaikwad *et al.*, 2008). Estrogen has even been added to the list of suspected carcinogens by the national institute of environmental health sciences (Miller, 2003; Twombly, 2003). An increase in estrogen exposure, due to various factors (early menstruation, continuous high estrogen levels, late menopause, exogenous estrogen exposures), has been associated with an increased risk to develop breast cancer (Yager & Davidson 2006). There are two mechanisms by which estrogens may be responsible for the initiation of breast cancer, a hormonal mechanism where increased cell proliferation causes cancer (Nandi *et al.*, 1995; Preston-Martin *et al.*, 1990) and the non-hormonal pathway. In the non-hormonal pathway, estrogens metabolised to catechol estrogens (estrogens hydroxylated at the 2-or-4-carbon position) may become a highly reactive species if not detoxified, i.e. inactivated and consequently excreted (Cavalieri, 2000). These reactive molecules form when catechol estrogens are oxidised and form semiquinones (SQ) or quinones (Q), which can then readily react with deoxyribonucleic acid (DNA) to form depurinating DNA adducts. These adducts can lead to the formation of mutations in the DNA that may be involved in the initiation of certain cancers (Cavalieri & Rogan, 2014; Cavalieri, 2000). The estrogens initiating this cancerous effect can come from various sources, including endogenous estrogen biosynthesis (reviewed in (International Agency for Research on Cancer, 1987) or exogenous estrogen exposure (Cavalieri, 2000), for example, from using combined oral contraceptives (COCs). Many women are already using COCs from a young age, and in South Africa alone, 64% of married or in-union women aged 15 to 49 are using some type contraceptive (hormonal and non-hormonal) as reported in 2015 (United Nations, Department of Economic and Social Affairs, Population Division, 2015). The additional intake of estrogens in the form of COCs might contribute to an imbalance in estrogen metabolism and biotransformation (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). Previous studies have shown that the urinary estrogen profile of women might be indicative of their relative cancer risk (Ziegler *et al.*, 2015). Analysis of urinary estrogens and estrogen metabolites in women will, therefore, be of great value to evaluate how these COCs affect estrogen metabolism and biotransformation pathways, and thereby also the activation of toxic metabolic pathways and the production of metabolites that may directly influence cancer risk. The estrogen biotransformation homeostasis may also be altered by other conditions (apart from COC-use) (Yager & Davidson 2006), and therefore, screening methods that are inexpensive and non-invasive for regular screening in South Africa will be beneficial. Nationally, most cancer patients go undiagnosed and untreated, which could even lead to premature and unnecessary deaths due to economical and locational factors. Such a method might also allow early detection of an increased risk to develop estrogen-related cancer. Detecting cancer in the early stages could allow positive prognosis and early treatment.

Most previously developed methods for the evaluation of estrogen metabolites and biotransformation products, either use conventional, less specific methods, have incomplete profiling methods (only including parts of the estrogen metabolism), or were developed for other biological matrixes. Comprehensive evaluation of the estrogen metabolic products together with the parent estrogens would be beneficial, as developed by (Gaikwad *et al.*, 2008). No in-house method for the evaluation of estrogens and related metabolites exists that would enable the evaluation of the estrogen biotransformation and consequently estrogen-related cancer risk. Due to variations in the available infrastructure and analytical standards between the methods in published literature and the current area of study, method development is necessary. Rather than implementation of previously developed methods, a new in-house method with the currently available resources will therefore be developed, to also include related metabolites previously omitted such as progesterone, estradiol precursors, sulfate and glucuronide conjugates, and metabolites from the 16-hydroxylation metabolic pathway of estrogens. Previous literature will still be the basis of all experiments and decisions, and be consulted throughout the method development proceedings.

1.2 Dissertation layout

Chapter 2 contains a concise literature review on the steroid hormone estrogen, its origin, metabolism and biotransformation. This chapter furthermore discusses the method of choice for this urine analysis and literature regarding the partial validation of an analytical method. Chapter 2 concludes with the problem statement, aim and objectives to be achieved in this study. Chapter 3 contains a description of all materials and methods used throughout the course of the study to develop and partially validate the analytical method. Chapter 4 and Chapter 5 contain all the results and discussions of method development and partial validation of the underivatised and derivatised methods, respectively, and Chapter 6 the results and discussion of the validated method implemented on the urine samples of female participants. Chapter 7 is the concluding chapter that summarises all the finding and gives further prospects. Annexures (Annexure A & B) are attached at the end of the dissertation. Annexure A contains literature references for the estrogen metabolite physiological reference ranges, and Annexure B, the supplementary results. Most results, illustrated as figures or graphs, are illustrated (as Figure S... or Table S...) in the supplementary results and are referred to throughout Chapters 4 and 5.

CHAPTER 2

LITERATURE REVIEW

2.1 Cholesterol

Cholesterol, the precursor of steroids inside the human body, contains 27 carbon atoms, 46 hydrogen atoms, and 1 oxygen atom, after the combination of six isoprene units and removal of three methyl groups. The oxygen group can be in the hydrocarbon tail to form a hydroxyl group or it can be in a ring structure region with 4 hydrocarbon rings (Wrona *et al.*, 2015). Most cells in the human body are capable of cholesterol biosynthesis, depending on the organ function and the cell type (Hu *et.al.*, 2010). The cholesterol biosynthesis as described by Wrona *et al.* (2015), is summarised in Figure 2.1, and will shortly be explained. The mevalonate pathway is a collective term to describe the multi-step *de novo* synthesis process of cholesterol. In the mevalonate pathway, two acetyl-CoA molecules form acetoacetyl-CoA and combine with another acetyl-CoA to be hydrated to 3-hydroxy-3-methylglutaryl CoA (HMG-CoA), catalysed by HMG-CoA synthase (Hu *et.al.*, 2010). The HMG-CoA is then reduced to mevalonate by HMG-CoA reductase and this process requires nicotinamide adenine dinucleotide phosphate (NADPH). Mevalonate is the key intermediate in the cholesterol biosynthesis pathway, and this conversion to mevalonate is the reaction regulated by increased uptake of cholesterol from the blood into the cells. Mevalonate is then converted into isopentenyl-5 pyrophosphate in the endoplasmic reticulum, through various reactions that require sufficient adenosine triphosphate (ATP). The formed farnesine is then the precursor of squalene, which can be converted into lanosterol, and from lanosterol cholesterol is then synthesized, in several reactions. Cholesterol can then be transported from the endoplasmic reticulum to the plasma membrane and intracellular organelles by lipid transport proteins, or through the circulation. Cholesterol can also additionally enter the gastrointestinal tract from the diet, bile, or intestinal mucosa. Cholesterol molecules are insoluble in water and are rather transported in circulation on glycoproteins as lipoproteins, which are particles that contain lipids, proteins and cholesterol to be circulated in water-based mediums (Wrona *et al.*, 2015). The five major classes of lipoproteins are very low-density lipoproteins, intermediate density lipoproteins, low-density lipoproteins, high-density lipoproteins, and chylomicrons (Hu *et.al.*, 2010).

Although homeostasis between cholesterol synthesis and cholesterol breakdown occurs in most cases (Mcdonnell *et al.*, 2014), it can be disturbed by an increased dietary intake of high cholesterol foods, disruption of *de novo* cholesterol synthesis, usage or disruption of excretion and recycling (Wrona *et al.*, 2015). Previous studies have shown that high levels of cholesterol might be a cause of breast cancer (Mcdonnell *et al.*, 2014). The mechanism of action proposed was through the formation of oxysterol 27-hydroxycholesterol, a cholesterol derivate that not only acts as a ligand for the liver X receptor to maintain cellular cholesterol homeostasis but also as an agonist of the estrogen receptor, which has been shown to play a role in carcinogenesis through the stimulation of cell proliferation (Mcdonnell *et al.*, 2014; Nandi *et al.*, 1995; Preston-Martin *et al.*, 1990). A second mechanism that has been proposed for cholesterol-induced cancer,

where estrogen receptors may be linked to abnormalities such as cancer, will be described in detail at a later stage (section 2.5.3). This mechanism is based on the increased production of estrogens by aromatase in adipose tissue (McDonnell *et al.*, 2014). Cholesterol is also a precursor of steroid hormones. However, it has been suggested that steroid hormones can be used as a treatment possibility for atherosclerosis (Adams *et al.*, 1990) and hypercholesterolemia (Dzuga & Smith, 2002), by normalizing or improving the serum levels of total cholesterol. It is hypothesized that hypercholesterolemia downregulates steroid hormone synthesis and that decreased steroid hormone production increases cholesterol production to help restore the homeostasis by a mechanism of precursor loading of synthetic pathways with cholesterol (Dzuga & Smith, 2002). Furthermore, in patients treated with anti-cholesterol medication (statins), there was no significant decrease in steroid hormones when compared to controls (Braamskamp *et al.*, 2015; Moggs & Orphanides, 2001). The statins not only decrease cholesterol synthesis but initiate the upregulation of low density lipoprotein-receptors that might be able to incorporate sufficient low density lipoprotein cholesterol to synthesize steroid hormones (Braamskamp *et al.*, 2015).

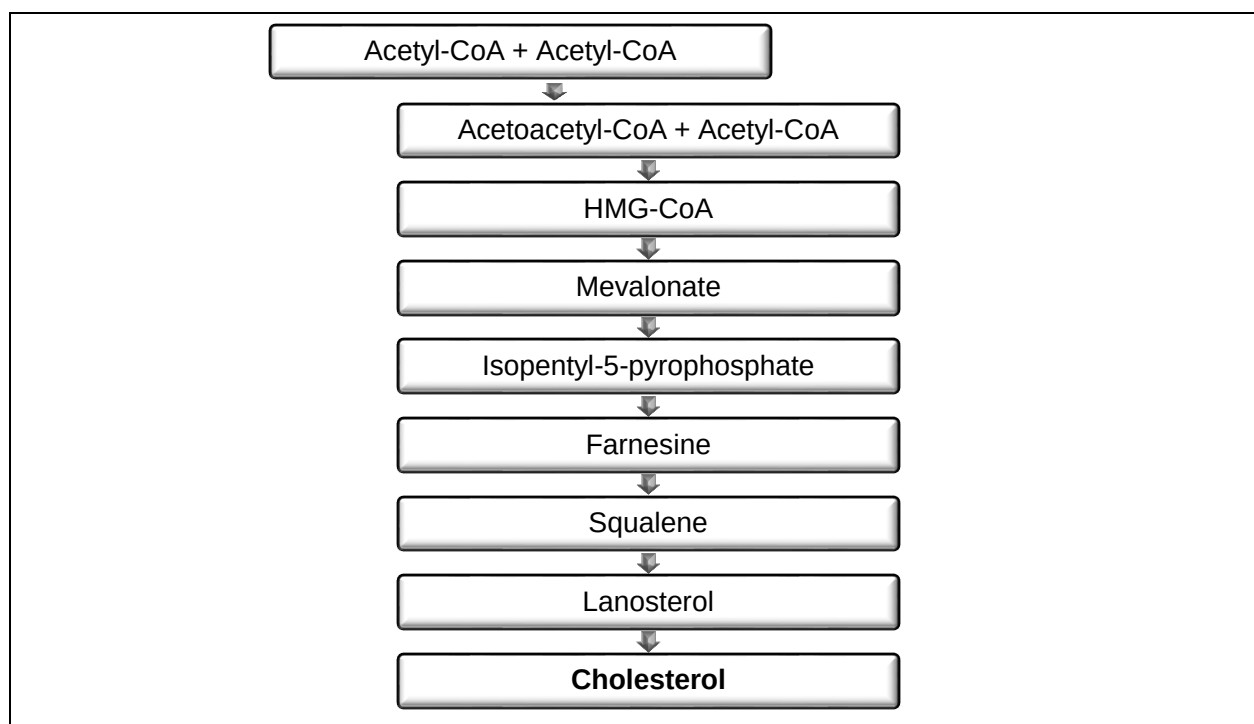


Figure 2.1: A summarised diagram of the pathway of reactions leading to cholesterol biosynthesis within the mevalonate pathway.

2.2 Steroid hormones

Steroid hormones are low molecular weight lipophilic compounds with numerous important physiological roles (Fabregat *et al.*, 2013) such as differentiation, metabolic homeostasis, and growth (Barkhem *et al.*, 1998). Steroid synthesis from cholesterol starts inside the mitochondria,

in the inner mitochondrial membrane, where the ability of the cholesterol to move into mitochondria will determine the efficiency of steroid hormone production (Rone *et al.*, 2009). They are mostly synthesised in endocrine glands such as the adrenals and gonads (Fabregat *et al.*, 2013). The production of steroids regulated by adrenocorticotrophic hormone and luteinizing hormone (LH) (Rone *et al.*, 2009) results in five groups of steroid hormones classified according to their activity and structure (Fabregat *et al.*, 2013). The five groups include androgens, estrogens, progestogens, glucocorticoids and mineralocorticoids. The progestogens play an important role in human reproduction. The glucocorticoids have primarily carbohydrate mobilizing properties, mineralocorticoids are the group of steroid hormones responsible for sodium retention inside the renal tubules and the estrogen and androgen groups are essential for female and male secondary sexual characteristics, respectively (Miller, 1988). After transportation of cholesterol into mainly the mitochondrion, a process regulated by steroidogenic acute regulatory protein (Samavat & Kurzer, 2015), the conversion of cholesterol to pregnenolone, takes place. This reaction is catalysed by the cholesterol side-chain cleavage enzyme, also termed as desmolase. The detailed flow of events from cholesterol to the five groups of steroid hormones can be viewed in Figure 2.2. Desmolase or cholesterol side-chain cleavage enzyme is one of the members of the cytochrome P450 (CYP) superfamily of enzymes encoded by the CYP11A1 gene (family 11, subfamily A, polypeptide 1) (Tsuchiya *et al.*, 2005).

Pregnenolone can be converted to the progestogen group, by conversion to progesterone, catalysed by hydroxy- dehydrogenase enzyme. This progesterone (P4) can then be either converted into deoxycorticosterone of the mineralocorticoids group, 17-OH progesterone (precursor of glucocorticoids and androgens), or it can be converted into various different progestogens. These progestogens include pregnanediol, allopregnanolone (which can be interconverted to allo-pregnanediol), 3 α -dihydroprogesterone and 20 α -dihydroprogesterone, which are all catalysed by hydroxysteroid dehydrogenase enzymes. The reaction to form deoxycorticosterone, the first mineralocorticoids, is catalysed by 21-hydroxylase. Deoxycorticosterone is then converted to corticosterone by 11 β -hydroxylase and then to aldosterone through aldosterone synthase. Conversion of 17-OH progesterone to 11-deoxycortisol, the first steroid in the group of glucocorticoids are also catalysed by 21-hydroxylase. 11-Deoxycortisol is then converted to cortisol by 11 β -hydroxylase and cortisol is converted to tetrahydrocortisol by 5 β -reductase or 3 α -hydroxysteroid dehydrogenase. Cortisol can also be reversibly metabolised to cortisone by 11 β -Hydroxysteroid dehydrogenase, to be converted into tetrahydrocortisone by 5 β -reductase or 3 α -hydroxysteroid dehydrogenase (Kushnir *et al.*, 2011; Lønning *et al.*, 2011; ZRT Laboratories, 2015).

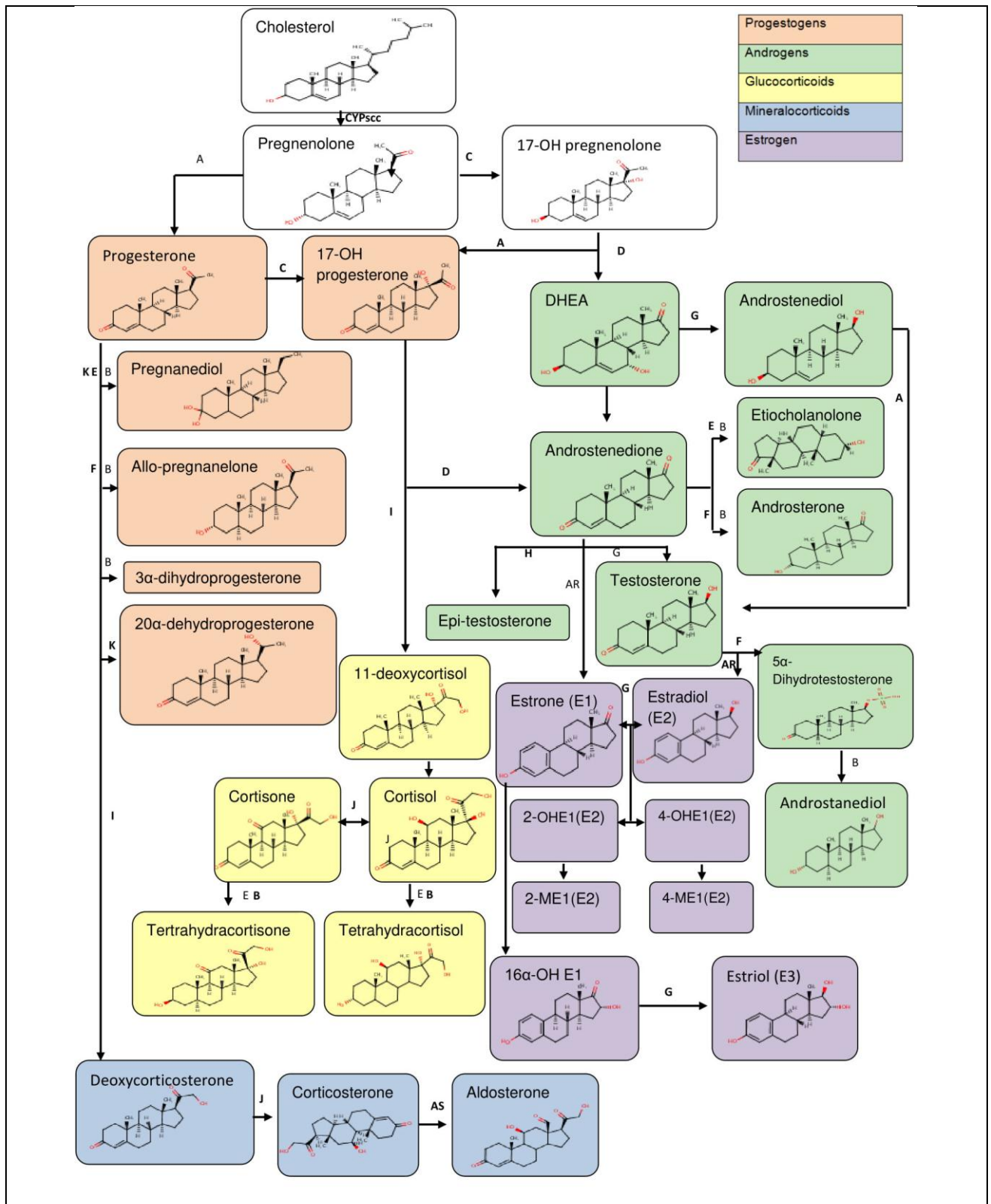


Figure 2.2: A schematic representation of the conversion of cholesterol into five groups of steroid hormones. Orange represents progestogens, green represents androgens, yellow represents glucocorticoids, blue represents mineralocorticoids and purple represents estrogens. The enzymes are as follows: A-3 β -hydroxysteroid dehydrogenase, B-3 α -hydroxysteroid dehydrogenase, C-17 α -hydroxylase, D-17, 20-Lyase, E- 5 β -reductase, F-5 α -reductase, G-17 β -hydroxysteroid dehydrogenase, H-17 α -hydroxysteroid dehydrogenase, I- 21-hydroxylase, J-11 β -hydroxysteroid dehydrogenase, K-20 α -hydroxysteroid dehydrogenase, AR- aromatase, AS-aldosterone synthase. Adapted from Eliassen et al. (2012); Gaikwad et al. (2008); ZRT Laboratories (2015).

Dehydroepiandrosterone (DHEA), the first in the group of androgens, can be synthesized from 17-OH pregnenolone, a precursor also for 17-OH progesterone, catalysed by a 3 β -hydroxysteroid dehydrogenase. Conversion of 17-OH pregnenolone to DHEA is catalysed by 17, 20-lyase. DHEA can then be either converted into androstenediol by 17 β -hydroxysteroid dehydrogenase or to androstenedione by 3 β -hydroxysteroid dehydrogenase. Androstenedione can also be formed from 17-hydroxy (OH) progesterone by 17, 20-lyase to be further converted into either epi-testosterone or testosterone by 17 α - and- 17 β -hydroxysteroid dehydrogenase, respectively, or to estrone in the estrogen group of steroids, by the aromatase enzyme. Androstenedione can additionally be converted into etiocholanolone by 5 β -reductase or 3 α -hydroxysteroid dehydrogenase; or to androsterone by 5 α -reductase or 3 α -hydroxysteroid dehydrogenase (Kushnir *et al.*, 2011; ZRT Laboratories, 2015). Androstenediol, in turn, can also be converted to testosterone by 3 β -hydroxysteroid dehydrogenase. Testosterone is then a precursor for either 5 α - Dihydrotestosterone by 5 α -reductase, to be converted into 5 α -androstenediol by 3 α -hydroxysteroid dehydrogenase; or the precursor for 17 β -estradiol, another estrogen catalysed by aromatase. This heme protein is then responsible for the binding of the carbon 19 androgenic steroid substrates to catalyse certain reactions leading to the phenolic a-ring formation (Simpson & Davis, 2001), which is characteristic of all estrogens. The metabolism of estrone (E1) and estradiol (E2) will be described later in more detail (section 2.5.2). In short, the estrone and estradiol (which can be interconverted) can also be converted to 2-OH and 4-OH estrogens, to be converted into 2-methoxy and 4-methoxy estrogens (2ME1[E2] & 4ME1[E2]). Alternatively, estrone can also be converted into 16 α -hydroxyestrone (16 α OHE1) by the CYP3A4 or CYP1A2 enzyme and then to estriol by 17 β -hydroxysteroid dehydrogenase (Lønning *et al.*, 2011; ZRT Laboratories, 2015).

Once these steroid hormones are synthesized in their independent tissues from cholesterol as a precursor, they are released into the bloodstream. Steroids can be delivered to target sites or organs through paracrine or endocrine secretion into the bloodstream (You, 2004). Inside the target tissues or cells, the steroid hormones bind to highly ligand-specific hormone receptors to act as transcriptional activators of genes responsive to steroids (Ascenzi *et al.*, 2006; Kushnir *et al.*, 2011), or they can exert actions that do not require any alteration in gene transcription (You, 2004). Receptors of steroids contain an amino-terminal domain for transactivation of gene expression, a C-domain with two zinc-fingers for receptor specific DNA binding and then also a carboxyl-terminal, which is the ligand binding domain (Barkhem *et al.*, 1998). Most steroids also bind reversibly with proteins before or after reaching the target tissue to regulate the availability of biologically active steroids at any given moment, at target sites. These binding proteins inactivate steroids towards the steroid hormone receptors and allow steroids to be transported through circulation (You, 2004). After the steroid hormones lead to the initiation of transcription of

specific cellular products, the steroid receptors degrade. This allows the steroid hormones to either repeat the process by binding to another steroid hormone receptor or to re-enter into the circulation system to be eliminated from the body through the liver, intestinal mucosa wall or other tissues. This process of elimination is termed biotransformation (Liska *et al.*, 2006). Assessing the efficiency of steroid metabolism/biotransformation can be of significant value, for example, to detect imbalances or identify biomarkers that are associated with pathological conditions or hormone abuse (Fabregat *et al.*, 2013).

2.3 Biotransformation

Xenobiotics is a term to describe molecules that are not essential for maintenance of the human body, but can modulate or damage physiological processes. The damage caused by xenobiotics usually occurs when they are present in high concentrations, or when the body is exposed to them for a prolonged time (Petzinger & Geyer, 2006). The lipophilic character of xenobiotics contributes to the difficulty of excreting these substances through glomerular filtration by the kidneys. For this reason, an enzyme system termed biotransformation is in place, to convert them into more hydrophilic, biologically inactive compounds that can be eliminated from the human body (Ioannides, 2001). This conversion of toxic substances into non-toxic metabolites, and their subsequent excretion, is known as biotransformation (Liska *et al.* 2006). Detoxification, another term to describe biotransformation processes, is also explained as processes that decrease the negative impact of xenobiotics on the normal body function. These two terms (biotransformation and detoxification) will be used interchangeably throughout this manuscript. Biotransformation processes take place mainly in the liver and intestinal mucosa wall, but may also occur inside other tissues to a lesser extent (Liska *et al.*, 2006). It is not only xenobiotics, as briefly explained, that can undergo biotransformation, but endobiotics also undergo biotransformation processes to ensure that sufficient homeostatic control occurs inside the body. Endobiotics are the metabolites, chemicals, metabolic products and harmful reacting molecules that form within the body. These endobiotic molecules form during normal physiological or during abnormal pathological processes. A few examples of endobiotics include steroids, neurotransmitters, eicosanoids, glucocorticoids and metabolic waste products from healthy or diseased tissues (Burcham, 2014). The biotransformation process is not just one reaction, but rather multiple reactions involving multiple players (Liska *et al.*, 2006) and can be divided into 4 phases (Figure 2.3.1) (Liska, 1998). Some hormones, as well as other signalling molecules, are not detoxified solely for elimination but are rather metabolised into other active signalling molecules and this allows variation in the activities of such important substances (Liska *et al.*, 2006).

Biotransformation reactions are also highly influenced by varying factors that can either induce biotransformation to contribute to optimal biotransformation processes, or inhibit the processes and lead to inadequate biotransformation of toxic substances. These factors include diseases such as obesity and diabetes, medication that might act as competitive inhibitors for detoxifying enzymes, age, gender, genetic polymorphisms, exposure to xenobiotics in the environment, as well as diet and lifestyle (Liska *et al.*, 2006). Lifestyle factors that might cause disturbances in the biotransformation pathways include smoking and alcohol use as only two of the many well-investigated examples (Castro & Castro, 2014).

2.3.1 Phase 0 biotransformation

Phase 0 of biotransformation terms the process of the uptake of xenobiotics across the basolateral membrane into the liver or other biotransformation tissue cells. Some also consider the intracellular transport of the xenobiotic following its uptake, to deliver the xenobiotic from the site of metabolism to the membrane transporters, or the other way around, as part of the Phase 0 biotransformation process (Döring & Petzinger, 2014). For the transport of xenobiotics across the basolateral membrane, a family of solute carriers is primarily involved (Petzinger & Geyer, 2006). These transporters, located on the luminal and basolateral membranes of cells, include organic anion or cation transporters and organic transporting polypeptides and are multi-specific with overlapping preferences for substrates (Grundemann *et al.*, 1994; Hagenbuch & Meier, 2003; Hediger *et al.*, 2004). These Phase 0 transport processes can ultimately influence the compound allocation and may influence the overall effectiveness of biotransformation (Döring & Petzinger, 2014; Petzinger & Geyer, 2006).

2.3.2 Phase I biotransformation

Phase I of the biotransformation process is usually the body's first defence against toxic compounds and involves hydrolysis and/or oxidation and reduction reactions. These reactions either expose or add functional groups including hydroxyl groups (-OH), amino groups (-NH₂) or a carboxyl group (-COOH), depending on the molecular structure. The Phase I reactions are primarily responsible for initiation of the biotransformation process to detoxify hazardous compounds. Most Phase I activity is localised at the membrane, and any influence on the integrity of the cell membrane may influence the activity of Phase I (Liska *et al.*, 2006). Some Phase I compounds are excreted after this phase of biotransformation, but most of these compounds also undergo Phase II conjugation before excretion (Liska *et al.*, 2006). The cytochrome P450 family of enzymes, primarily responsible for catalysing Phase I detoxifying reactions, have an extremely broad range of substrate specificities. There are at least 57 human cytochrome P450 enzyme isoforms, encoded by a variety of genes, highly subjected to polymorphisms (Liska *et al.*, 2006). CYP is expressed in the liver and small intestine and is primarily bound to the smooth endoplasmic reticulum membrane, however, numerous steroid metabolising CYPs are found

inside the mitochondria (Testa & Krämer, 2007). The cytochrome P450 enzymes consist of a heme moiety as a prosthetic group (iron-protoporphyrin IX) and an apoprotein (Testa & Krämer, 2007). These enzymes use oxygen and nicotinamide adenine dinucleotide hydrogen (NADH) as co-factors to add a hydroxyl radical, which is a highly reactive group, to the toxin. Other enzymes that might also catalyse oxidation reactions include peroxidases, xanthine and amine oxidases, dehydrogenases and flavin monooxygenases (Zamek-Gliszczyński *et al.*, 2006). The products yielded by this phase can either be biotransformed, stable (but toxic) metabolites, electrophiles, reactive oxygen metabolites or free radicals (Roškar & Lušin, 2012). These oxidation reactions lead to the formation of molecules that are more reactive than the parent molecules and are able to react with DNA, ribonucleic acid (RNA), and proteins inside the cell if they are not further detoxified through Phase II biotransformation (Liska *et al.*, 2006). An imbalance between Phase I and Phase II biotransformation may lead to a build-up of toxic metabolites, which may have severe physiological consequences.

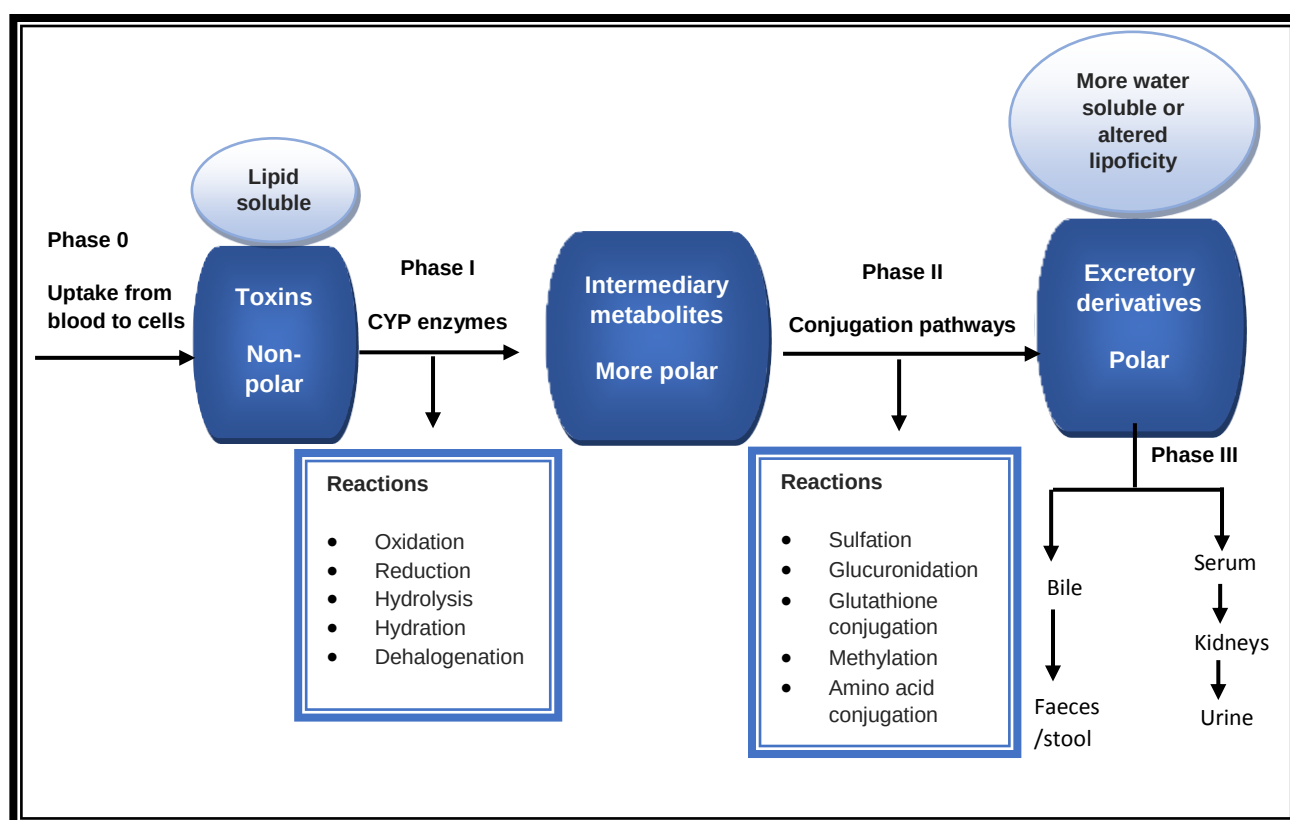


Figure 2.3.1: A schematic diagram representing the flow of events during biotransformation of toxic compounds to more water soluble molecules. The toxins that are non-polar first go through Phase I biotransformation, catalysed by CYP enzymes, to give more polar intermediary metabolites. These metabolites can then undergo conjugation through Phase II of biotransformation to give polar derivatives, to be excreted either in the urine or in the stool. Adapted from (Liska *et al.*, 2006)

2.3.3 Phase II biotransformation

Phase II biotransformation inside the human body is the group of conjugation reactions that yield non-toxic products to be excreted from the body. These conjugation reactions include sulphate

conjugation, glucuronide conjugation, where glucuronic acid is conjugated, glutathione conjugation where electrophilic groups are conjugated with glutathione (GSH), amino acid conjugation, acetylation and methylation, where acetyl and methyl groups are conjugated to toxins, respectively (Liska *et al.*, 2006). Conjugation to a fatty acid carrier molecule known as carnitine has also been described and serves to remove acyl groups that have accumulated inside the mitochondria (Liska, 1998). Phase II of biotransformation combines toxic molecules or Phase I products with endogenous hydrophilic compounds to produce molecules that are more hydrophilic or have changed lipoficity in order to increase their excretion from the body (Liska *et al.*, 2006). Phase II occurs in the cytoplasm of the cell and while Phase I biotransformation reactions require sufficient antioxidants for effective excretion of xenobiotics, Phase II requires sufficient energy in the form of ATP and nutrients that are replenished from the diet, to support the processes (Liska *et al.*, 2006). For most conjugation reactions, cofactors derived from the organism's metabolism are involved. These include sulphate, glucuronic acid, glutathione, and amino acids such as glycine and methylation cycle substrates. For conjugation reactions to occur successfully, there must be at least one suitable functional group to anchor the moiety or endogenous molecule. These reactions are classified as anabolic or synthetic reactions to produce high molecular weight products (Testa & Krämer, 2008). The four major conjugation reactions in steroid hormone biotransformation (sulfation, glucuronidation, glutathione conjugation, and methylation) will be discussed briefly.

Sulfate conjugation (sulfation) is one of the most common conjugation reactions and results in more hydrophilic molecules for excretion in bile or urine. Sulfation reactions, although classified as a Phase II conjugation reaction, can occur directly on the xenobiotic parent compound without Phase I biotransformation, or follow oxidation in Phase I (Zamek-Gliszczynski *et al.*, 2006). These reactions occur when the OH-group of the xenobiotic compound nucleophilically attacks the sulfur (S) -atom of the cofactor 3'-phospho-adenylyl sulphate and produces the by-product adenosine 3,5-biphosphate, while donating the sulfate group to the xenobiotic compound (Testa & Krämer, 2008). Hepatotoxins causing covalent microsomal protein binding (e.g. phenacetin), DNA-binding carcinogens (e.g. safrole) and prodrugs (e.g. minoxidil), can also be activated when hepatic xenobiotic sulfation occurs (Liska, 1998; Zamek-Gliszczynski *et al.*, 2006). For the catalysis of sulfation reactions, sulfotransferase (SULT) enzymes are essential. The SULT enzymes have a lower Michaelis-Menten constant value (K_M -value) for estrogens than the glucuronidation diphosphate-glucuronosyltransferase (UGT) enzymes (Technische University, 2016) and consequently result in more rapid sulfation reactions and faster depletion of sulfation capacities (Zamek-Gliszczynski *et al.*, 2006). Interestingly, high concentrations of substrates, such as 17 β -estradiol, can act as inhibitors of the SULT and UGT enzymes, where high concentrations of these metabolites cause a decrease in the activity of the enzyme (Krämer &

Testa, 2009). It is important to note, though, that these conjugation reactions are reversible. Sulfatase enzymes, for example, can desulphate sulphate-conjugates to be re-conjugated in a cycle known as the futile cycle (Zamek-Gliszczynski *et al.*, 2006). Although sulphate conjugation is one of the most common mechanisms used during Phase II to increase the water solubility of toxins, it is quickly saturated. The substrates for sulfation can also undergo glucuronic acid conjugation (glucuronidation) when they are present in high concentrations (Zamek-Gliszczynski *et al.*, 2006). Glucuronidation, however, does not occur as readily in the liver as the sulfation cycle (Zamek-Gliszczynski *et al.*, 2006). The glucuronidation reactions have high capacity and low affinity (Testa & Krämer, 2008), and occur only inside the microsomal membranes. A superfamily of UGT enzymes are required for catalysing these reactions (Raftogianis *et al.*, 2000). Substrates for these enzymes usually contain an electrophilic group and the reaction requires the co-factor uridine diphosphate-glucuronic acid (Testa & Krämer, 2008). If this cofactor is limited, glucuronidation might be impaired (Zamek-Gliszczynski *et al.*, 2006). Glucuronidation products can be grouped into either sulphur (S)- glucuronides, nitrogen (N)- glucuronides, carbon (C)- glucuronides or oxygen (O)- glucuronides, where estrogen steroid hormones are predominantly O-glucuronidated (Testa & Krämer, 2008). These conjugation reactions are also reversible by deconjugation, either in an acidic environment or through a β -glucuronidase enzyme.

The tripeptide glutathione is involved in a wide range of metabolic reactions and cellular functions, including reduction of hydroperoxides and maintenance of the thiol-disulfide status of proteins. In addition, it is also responsible for the biotransformation of electrophiles (Bellomo *et al.*, 1992). Glutathione conjugation, usually with potent electrophiles as substrates, is one very important mechanism of protection because if not conjugated, the electrophiles will bind to, and damage, macromolecules or DNA. Xenobiotics, Phase I and even Phase II products can act as substrates for glutathione conjugation (Zamek-Gliszczynski *et al.*, 2006). These reactions can either occur spontaneously inside the liver (Testa & Krämer, 2008) or are catalysed by the glutathione conjugation enzyme, glutathione-S-transferases Mu 1 and Theta 1 (Cavalieri & Rogan, 2014; Ritchie *et al.*, 2001). GSTs in the membrane or in cytosolic fractions may have varying functions. Approximately 20 types of cytosolic GST are responsible for the conjugation of xenobiotics to glutathione by making the sulfhydryl group of the GSH active and more susceptible to electrophilic xenobiotic binding. The membrane-bound GSTs can also be involved in biotransformation processes and include mitochondrial and also microsomal GSTs (Zamek-Gliszczynski *et al.*, 2006). The Glutathione tripeptide consists of glutamate, cysteine and glycine, and exists inside the body in redox equilibrium. Glutathione can be either oxidised to glutathione disulphate (GSSG) or reduced to the known GSH form. In rat liver, the glutathione compartmentalisation is 10-20% inside the mitochondrial matrix, while cytosolic GSH depends on synthesis and transport to the mitochondria. There is also a pool of GSH present inside the nucleus, maintained by active

gradient mechanisms to protect DNA and other nuclear parts from chemical and oxidative injury (Bellomo *et al.*, 1992). Although the cytosol is the primary site for glutathione conjugation, some of the reactions also occur in the endoplasmic reticulum (Zamek-Gliszczynski *et al.*, 2006). As a radical scavenger in the body, glutathione reacts with radicals and oxides to form a glutathionyl radical (Testa & Krämer, 2008). After glutathione conjugation, the conjugates can then either act as substrates for transport reactions (substrates for carriers and export pumps) or be further catabolised for excretion (Testa & Krämer, 2008). For the latter, γ -glutamyl transpeptidase liberates the glutamyl moiety of the conjugated glutathione to yield a cysteine-glycine conjugate, which can be hydroxylated by cysteinyl glycanase and then acetylated to form a N-acetylcysteine conjugate (Todorovic *et al.*, 2001), also referred to as mercapturic acid. The N-acetylation of cysteine conjugates is catalysed by amidases in a reversible reaction (Testa & Krämer, 2008).

Methylation, catalysed by methyltransferases, conjugates a methyl group (CH_3) from S-adenosyl-L-methionine to the xenobiotic in the presence of magnesium to give a methylated product, together with demethylated S-adenosyl-L-homocysteine (Tunbridge *et al.*, 2006). Although O-methylation, N-methylation and S-methylation are known methylation reactions, the methylation of catechols, like catechol estrogens by O-methylation, will be focused on (Testa & Krämer, 2008). The primary enzyme responsible for O-methylation, catechol O-methyltransferases (COMT), is present in cells in two isoforms. The first is the membrane-bound isoform, with a 150 base pair extension at the 5' of the second, soluble isoform (Jiang *et al.*, 2003; Xie *et al.*, 1999). The COMT enzyme methylates epinephrine, nor-epinephrine, dopamine and catechol estrogens; thus it is of great importance (Dauvilliers *et al.*, 2015; Testa & Krämer, 2008). Interestingly, women have a lower COMT activity than men (20-30%) and it is hypothesised that it might be due to estrogens being endogenous agents downregulating COMT gene expression (Jiang *et al.*, 2003). The COMT gene contains estrogen responsive elements, and in cell cultures, estradiol down-regulates the overall expression of the COMT enzymes, reducing the levels of soluble COMT proteins. This downregulating mechanism seems to only be applicable in receptor positive and not receptor negative cells (Xie *et al.*, 1999). The COMT 5' region has a high amount of methylation sites (regulated in neoplastic tissue) and silencing of the COMT gene can occur through methylation at these sites (Tunbridge *et al.*, 2006). Data from other studies indicates that the binding of the COMT promoter regions to estrogen receptor alpha may be an integral factor, which will cause the estrogens to suppress the expression of the COMT gene (Jiang *et al.*, 2003).

2.3.4 Phase III biotransformation

Before the last phase of biotransformation (Phase III), some intracellular shuttles (cytoskeletal structures and cytosolic binding proteins) transport metabolites from the endoplasmic reticulum to the basolateral or luminal membrane (Petzinger & Geyer, 2006). The final phase of biotransformation is an active type of transport reaction over the mammalian cell membranes

without any chemical modification (Döring & Petzinger, 2014). Phase II products are usually too hydrophilic to diffuse across the basolateral membrane into the sinusoidal blood (blood vessels in the liver) or across the luminal membrane into the bile (Figure 2.3.2). Thus the transportation reaction requires carrier-mediated transport to cross the diffusional barrier (Zamek-Gliszczynski *et al.*, 2006). For basolateral (sinusoidal) excretion, most carriers are familial to the adenosine binding cassette located on the luminal membranes, apical membranes or in some cases, the basolateral membranes (Petzinger & Geyer, 2006; Zamek-Gliszczynski *et al.*, 2006). In the liver, multidrug resistant proteins 3-6 are present and primarily responsible for the transport of xenobiotics and endobiotics from hepatocytes into the sinusoidal blood (Döring & Petzinger, 2014; Zamek-Gliszczynski *et al.*, 2006). For biliary excretion, several other transporters are involved, namely multidrug resistant proteins 1 and 2, bile salt export pump, Adenosine binding cassette sub-family G5 and G8, and recently the breast cancer resistance protein was also identified as a hepatic canalicular drug transporting protein (Zamek-Gliszczynski *et al.*, 2006). All these uphill transport systems, with direct ATP consumption, comprise the final step in the biotransformation process transporting the biotransformed conjugates from the cells into the bile or urine, and under certain conditions, back into the blood. This transport process is also called the antiporter system, since it is able to pump conjugated as well as unconjugated xenobiotics out of the cells. (Liska, 1998). By being able to also pump unconjugated compounds from the cells back into the intestinal lumen, this mechanism allows xenobiotics that have not been metabolised during Phase I to be reabsorbed, thereby enhancing the biotransformation process. The role of the antiporter system in promoting and supporting biotransformation is further motivated by indications that it is co-regulated with the CYP3A4 enzyme in the intestine (Liska, 1998).

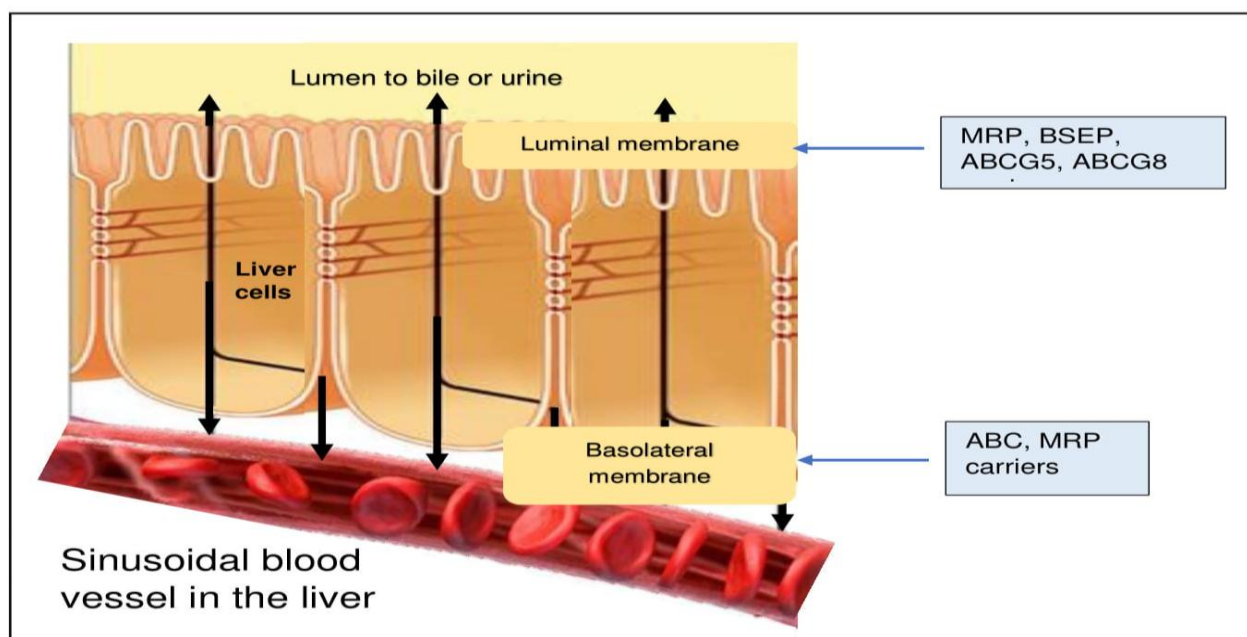


Figure 2.3.2: Visual illustration of the transportation of Phase II products during Phase III of biotransformation. The figure illustrates the different membranes (basolateral or luminal) to be absorbed either into the sinusoidal blood, or to be excreted through the bile or urine. Adapted from Hall (2015).

Both Phase I and Phase II of the biotransformation of steroid hormone metabolites play important roles in cellular processes through nongenomic and genomic mechanisms (Fabregat *et al.*, 2013). Dysfunction may occur when biotransformation systems are overloaded or imbalanced and thus, as previously mentioned, it is essential for Phase I and Phase II biotransformation to be balanced. Products of Phase I biotransformation may be more reactive and toxic than the parent molecules (a phenomenon that is commonly referred to as bioactivation) (Liska *et al.*, 2006) and can cause a wide variety of discrepancies. However, these discrepancies can be avoided if the balance is maintained. Imbalances in biotransformation processes can occur when one of the phases are either induced or inhibited. There are various scenarios that may lead to the induction of Phase I and Phase II enzyme activities, one of which is high xenobiotic load, where inducers selectively upregulate the enzymes for biotransformation reactions. These inducers only upregulate one enzyme or one phase of biotransformation and disturb the biotransformation balance. Some examples of this induction are the intake of flavonoids in fruits and vegetables, amines in charbroiled meats and polycyclic hydrocarbons from cigarette smoke (Liska *et al.*, 2006). Inhibition activities of Phase I and Phase II can also occur by competitive inhibition, in which there is competition between different compounds for the exact same detoxifying enzyme. Some inhibitors might be found in the human diet and pharmaceuticals, although any xenobiotic may compete for a detoxifying enzyme (Liska *et al.*, 2006). Phase II enzymes may furthermore also be inhibited by the depletion of cofactors essential for successful conjugation, especially amino acid conjugation (Liska *et al.*, 2006). Sulphate conjugation is also highly susceptible to inhibition. Most of the sulphate necessary for Phase II reactions comes from inorganic sulphate that is absorbed by the body, or from sulphate that is produced through synthesis from cysteine. Serum sulphate levels are regulated by the balance between sulphate production and absorption, and sulphate conjugation and excretion. Sulphate levels can thus be upregulated by increased dietary intake of inorganic sulphate or sulfur-containing amino acids (Liska *et al.*, 2006). Other nutrients that can cause inhibition of biotransformation processes when depleted include vitamin A, proteins for sulfation reactions, magnesium for high integrity lipid bilayer, (necessary for glucuronidation), vitamin B6, vitamin B12, magnesium and folate for glutathione cofactor synthesis (Liska *et al.*, 2006).

Disruptions in the balance between different biotransformation phases can lead to the covalent binding of the reactive molecules to proteins, which may then result in conformation change of receptors, enzymes, and carrier proteins. Covalent binding of reactive molecules to lipids can form lipid-soluble xenobiotics and may initiate lipid peroxidation, whereas the covalent binding of nucleic acid results in irreversible binding to DNA and initiation of carcinogenesis (Cavalieri, 2000). The link between biotransformation disruption and the development and proliferation of different types of cancers has been investigated (Fabregat *et al.*, 2013). The biotransformation

processes can also lead to the (increased) production of reactive oxygen species (ROS) by oxidizing cytochrome P450 isoenzymes and result in oxidative stress (Liska *et al.*, 2006). These ROS may include superoxide anions, hydroxyl radicals and hydrogen peroxide. Another way in which biotransformation processes may lead to an increase in oxidative stress is by quenching cellular antioxidants when reactive electrophiles react with these antioxidants (Valko *et al.*, 2007). Depletion of antioxidant capacities, such as the capacity of superoxide dismutase, catalase and glutathione may, therefore, contribute to an increase in oxidative stress (Liska *et al.*, 2006) and ultimately contribute to disease development, particularly cancer (Aruoma, 1998; Brown & Bicknell, 2001; Knowles & Harris, 2001; Tas *et al.*, 2005).

2.4 Cancer

Cancer, a highly common disease, kills more people globally than tuberculosis (TB), acquired immune deficiency syndrome (AIDS) and malaria combined (National Cancer Registry, 2011). Most cancers originate from mutations of critical regulatory genes, also known as oncogenes or tumour suppressor genes, but certain cancers might even originate due to endogenous carcinogens (Cavalieri *et al.*, 1997). Generally, an agreement about cancer has been reached within the scientific community that cancer is a genetic disease due to the fact that cancer is mostly triggered by a genetic mutation in critical regulatory genes. The effect of these mutations is abnormal DNA replication and DNA repair processes, resulting in altered differentiation, cell proliferation and cell death (Cavalieri & Rogan, 2006). Results from previous studies concluded that depurinating DNA adduct formation is responsible for the generation of mutations in oncogenes. These adducts are removed from the DNA by the cleavage of bonds between the DNA purine base and the deoxyribose. Mutations may then occur due to misreplication of the unrepaired (or ill-repaired) apurinic sites (Cavalieri *et al.*, 1997). A common characteristic of the molecules that form these DNA adducts, are that they are electrophilic, and this led the group of Geikwad *et al.* (2008) to postulate that there is an association between the electrophilic (estrogen) metabolites and many human cancers, including endometrial, ovarian, prostate and breast cancer (Cavalieri *et al.*, 1997). In 2011 Rappaport reported that genetic factors contributing to cancer are about 10%, while non-genetic and environmental factors are responsible for about 90% of chronic diseases including cancer. Epidemiological and clinical studies have shown that endocrine factors, such as steroids, are implicated in the initiation and development of mammary tumorigenesis. Breast cancer, the most prevalent cancer in women, is currently an overwhelming health burden with an estimated 522 000 breast cancer-related deaths reported in 2012 among women worldwide (Jemal *et al.*, 2016). In the U.S., an estimated 249,260 new breast cancer cases was expected to be reported in 2016 alone (Samavat & Kurzer, 2015; Schneider *et al.*, 1982; Siegel *et al.*, 2015). Although the statistics are not up to date, the cancer association of

South Africa reported that 30.52 of every 100 000 females in South Africa developed breast cancer in 2011 (National Cancer Registry, 2011). There are a variety of risk factors for breast cancer, the highest being age. Other risk factors include family history, breast cancer gene mutations, reproductive history, high-dose radiation to the chest, obesity, alcohol consumption and also high endogenous estrogens and hormone therapy (Samavat & Kurzer, 2015). The latter has been receiving growing attention in recent years, since several lines of epidemiologic evidence and literature show that the duration of exposure to premenopausal ovarian hormones as well as levels of circulating estrogens is associated with an increased risk of developing breast cancer (Arslan *et al.*, 2009; Cauley *et al.*, 2003; Cavalieri & Rogan, 2011; Clemons & Goss, 2001; Colditz, 1998; Kabat *et al.*, 2006; Muti *et al.*, 2000; Rogan *et al.*, 2003). Previous studies are even indicative that prenatal hormone levels may increase the breast cancer development or the relative risk to develop cancer. From 1950-1960, a synthetic estrogen hormone, Diethylstilbestrol has been used in pregnant women and their offspring showed an greater breast cancer occurrence than those not exposed at all Palmer (Palmer *et al.*, 2006). These findings together with other studies shows that hormonal exposure from exogenous sources can influence the relative breast cancer risk. This, in turn, has led to an increasing research interest into estrogen hormone metabolism.

2.5 Estrogens

2.5.1 Overview

Estrogens are aromatic, carbon 18 steroids and play essential roles in developing as well as maintaining secondary sexual characteristics, long-bone maturation, growth modulation in different tissues, cardiovascular apparatus, the immune and nervous system, and pregnancy (Ascenzi *et al.*, 2006; Qin *et al.*, 2008). As previously discussed, the biosynthesis of estrogens originates from cholesterol in the ovary (Tsuchiya *et al.*, 2005), although estrogen exposure from exogenous sources can also occur (Andersson & Skakkebaek, 1999). The absorption of exogenous estrogens occurs in three phases. The first phase of absorption allows estrogens with a 2-hour half-life to be circulated, followed by Phase 2 absorption resulting in 24 or more hours of sustaining estrogen levels. Lastly, enterohepatic reabsorption (Phase 3) after 50 hours also occurs, where after they are excreted into the bile (Testa & Krämer, 2008). In biosynthesis, cholesterol is converted to androstenediol, the precursor of E1, which is further converted to testosterone to form E2, the most potent and dominant estrogen in humans (Ascenzi *et al.*, 2006). Both these conversions to estrogens are catalysed by the aromatase enzyme (CYP19) (Tsuchiya *et al.*, 2005). Each estrogen molecule consists of a benzene ring and a phenolic hydroxyl group at the carbon 3-position. 17 α / β -E2 has a hydroxyl group at position C17, while E1 has a ketone group at the same position (Samavat & Kurzer, 2015). E1 and E2 are the most abundant

circulating estrogens, followed by estriol (E3), mostly found in pregnant women. Estrone and estradiol can be interconverted by the 17 β -estradiol dehydrogenase enzyme. In pre- and postmenopausal women, the importance of different estrogens differs. In pre-menopausal women, estradiol is the most important estrogen, while in post-menopausal women, estrone is more prominent (Samavat & Kurzer, 2015). This corresponds with evidence that 17 β -E2 has an antioxidant effect, inhibiting the oxidation of low density lipoprotein cholesterol inside the plasma (Epstein *et al.*, 1999). The levels of circulating E2 in premenopausal woman range from 100 pmol/L to 1000 pmol/L, while the circulating E2 levels in postmenopausal woman range from 10 pmol/L to 20 pmol/L (Folkerd *et al.*, 2014). Estrogen levels also vary significantly over a pre-menopausal woman's monthly cycle (Fortner *et al.*, 2013). Estrogens diffuse in and out of cells until retained in target cells with high specificity and affinity, by an intranuclear binding protein known as the estrogen receptor (ER) (Kuiper *et al.*, 1997). The physiological working of the alpha (α) and beta (β) 17-estradiol subtypes differs. The main divergence is in the fact that 17 α -estradiol is a short-acting estrogen, together with estrone and estriol, whereas 17 β -estradiol is not. These short-acting estrogens cause nuclear binding of the estrogen receptor complex for only a short period of time, allowing a 12 to 80 times lower effectivity of the estrogens than that of 17 β -estradiol (Hall, 2015). Most of the estrogens' biological effects result from their interaction with the estrogen receptors. The estrogen receptors include estrogen receptor α (ER α) and estrogen receptor β (ER β) (Kuiper *et al.*, 1997) products from the estrogen receptor 1 and 2 genes (Ascenzi *et al.*, 2006). Once the ER is bound to estrogens, it undergoes conformational changes that allow the estrogen receptor to bind to chromatin for the modulation of transcription of target genes (Kuiper *et al.*, 1997). Although ER α and ER β have 96% identical amino acid identities in the DNA-binding domains of these receptors, they have only a 53% identical sequence in their ligand-binding domains (Yager & Davidson, 2006).

The binding affinity of estrogens and estrogen metabolites or precursors to these receptors, therefore, varies (Table 2.1), with a more than 5 times higher affinity of 17 α -E2 for the ER α protein (Kuiper *et al.*, 1997). *In vitro*, the two ERs form heterodimers with each other and this causes ER β to decrease the sensitivity of the ER α for estrogens (Clemons & Goss, 2001), explaining why some studies suggest that ER β can act as an antagonist of ER α in mice liver (Granata *et al.*, 2009). The balance between these two receptors is critical as more ER α have been found in tumours than in the normal breast tissues, and carcinogenesis can be dependent on these balances (Clemons & Goss, 2001). Estrogen-related receptors are receptors binding to estrogen responsive elements but not to E2, and they might also be involved in estrogen signalling pathways and play regulatory roles in cell growth (Ascenzi *et al.*, 2006). Additionally to the estrogen precursors and metabolic products that may interact with estrogen receptors as seen in Table 2.1, estrogens from exogenous sources (xenoestrogens) (Andersson & Skakkebaek, 1999)

or chemicals with hormone disruptive properties may also interact with estrogen receptors (Kuiper *et al.*, 1997) to primarily act as agonists and initiate estrogen-like reactions or act as antagonists to inhibit estrogen-like reactions (Zava *et al.*, 1997). This collection of compounds also forms part of the so-called exposome. The exposome, as defined by Christopher Wild in 2005 and cited by Rappaport (2011), is the “totality of exposures from conception onwards, of a quantity of critical interest for understanding environmental causes of disease”. Environmental estrogens mimic the endogenous estradiol through receptor-mediated mechanisms, yet they can sometimes also have effects on the hypothalamus and pituitary in non-hormonal ways (Arcand-Hoy *et al.*, 1998). Some of the compounds interacting with estrogen receptors include genistein, coumestrol, diethylstilbestrol, hexestrol, dienestrol, moxestrol, 4-OH-tamoxifen, nafoxidine, clomifene, tamoxifen and various others that are not mentioned here (Kuiper *et al.*, 1997). Dietary exposure to estrogens includes mycoestrogen found in fungi, phytoestrogens found in certain plants, and estradiol, progesterone and testosterone used in the agricultural practice of most countries (Kuiper *et al.*, 1997). Another factor that may contribute to the levels of active estrogens in the human body, independent from estrogen biosynthesis, is through the bacteria found in the human digestive tract (Hawksworth *et al.*, 1971; Liska, 1998). In a process called enterohepatic recirculation, the gut microflora can remove conjugated moieties to convert active molecules back to its original form (Liska, 1998). These *Escherichia coli* (Enterococci, Lactobacilli, Nagler-positive Clostridia, Bacteroides species and Bifidobacteria) groups produce β -glucuronidase, the enzyme responsible for deconjugation of glucuronide conjugates. It is thus probable that estrogen glucuronide conjugates are then deconjugated by the bacterial β -glucuronidase, which allows an

Table 2.1: The relative binding affinity of various estrogens and estrogen metabolites as well as precursors of estrogens. The relative binding affinity for estrogen receptor (ER) α and β are shown individually where the binding affinity of 17 β -estradiol is represented as 100, and all other binding affinities are expressed relative to the value of 100. Adapted from Gaikwad *et al.* (2008); Kuiper *et al.* (1997)

Estrogen compound	Relative binding affinity for ER α	Relative binding affinity for ER β
17 α Estradiol	58	11
17 β Estradiol	100	100
Estriol	14	21
4-OH-Estradiol	13	7
2-OH-Estradiol	7	11
5-Androstenedione	6	17
3 β -Androstenedione	3	7
4-Androstenediol	0.5	0.6

increase in circulating unconjugated and active estrogens (Hawksworth *et al.*, 1971) leading to a consequential increased toxic load (Liska, 1998).

2.5.2 Estrogen metabolism

Estrogens, regardless of their source, play an important role, especially in female biological systems. Apart from the natural functions mentioned in 2.5.1, they are also widely used in pharmaceutical applications as contraceptive drugs, for the prevention of osteoporosis, and for treatment of hormonal imbalances (Arcand-Hoy *et al.*, 1998). As described for all steroids, estrogen is released back into circulation after activating transcription. These estrogens are then converted via biotransformation (Figure 2.3.1) to estrogen inactive metabolites, mainly inside the liver and kidneys or a specific target tissue, and eliminated from the body (Tsuchiya *et al.*, 2005). The first line of defence the body engages to protect against estrogenically active molecules, occurs mainly inside the liver where the conjugation of estrone or estradiol, either by sulfation or glucuronidation, can occur. This reaction then leads to the formation of either estrogen sulphates, the most abundant circulating estrogen with a much longer half-life than the parent estrogens, (Raftogianis *et al.*, 2000) or estrogen glucuronidates, which are less abundant in circulation due to its higher urine secretion rates (Raftogianis *et al.*, 2000). For sulfation of estrogens, the SULT2A1 isoform is essential for catalysing the sulphate conjugation reactions in endogenous steroids (Testa & Krämer, 2008). Although these conjugated estrogen metabolites are estrogenically inactive, they can be hydrolysed back to parent estrogens by the enzymes arylsulfatase (E1) and 17-hydroxysteroid dehydrogenase (E2) (Raftogianis *et al.*, 2000). These parent estrogens can then actively bind to estrogen receptors to initiate an effect.

Parent estrogens (E1 & E2) can also be oxidised as part of the biotransformation process. This occurs at the 2-, 4- or 16-position of the estrogen's carbon skeleton and yields 2-hydroxylated estrogens (2-OHE1[E2]), 4-hydroxylated estrogens (4-OHE1[E2]) or 16-hydroxylated estrogens (16-OHE1[E2]) (Key *et al.*, 2002). 2 & 4 Hydroxy estrogens are collectively known as catechol estrogens (CE) (Cavalieri *et al.*, 2006; Siddique *et al.*, 2005; Tsuchiya *et al.*, 2005) and the 2-OHE1(E2) are the predominant hydroxylation reaction, resulting in 80% of the total hydroxyestrogens. The different cytochrome P450 enzymes that are responsible for catalisation of these reactions are CYP1A1 for conversion to 2-hydroxy estrogens, CYP1B1 for hydroxylation at the 4 position of the estrogen carbon skeleton, and CYP3A4 and CYP1A2 for conversion to 16-hydroxylated estrogens (Figure 2.5.1) (Reding *et al.*, 2014). The CYP3A family of enzymes, including CYP3A4, are particularly sensitive to hormones. Regulation of this enzyme by the female hormone progesterone or the ratio of progesterone to estrogens has previously been suggested, since pre-menopausal women show a higher activity of the CYP3A4 enzyme than post-menopausal women and men (Liska *et al.*, 2006). CYP1A1 activity for E2 occurs at the C-2,

C15a and C6-a positions, whereas CYP1B1 primarily hydroxylates estradiol at the C-4 position, although hydroxylation can also occur at the C-2 position. 16-Hydroxyestrone can be further metabolised by oxidation or reduction at the 16- and 17- positions to give 16, 17-epiestriol and estriol as products (Key *et al.*, 2002). The 2-OHE1(E2) and 4-OHE1(E2) estrogens can be methylated by the COMT enzyme to prevent their conversion to more toxic estrogen quinones by cytochrome P450 and peroxidase (Todorovic *et al.*, 2001). The O-methylation of the 2-hydroxylated catechol estrogens takes place more readily than that of the 4-hydroxylated estrogens, increasing the toxic potential of the latter (Tsuchiya *et al.*, 2005).

Catechol estrogens and the methoxy estrogens can also be conjugated with glucuronic acid and sulphate for their inactivation (Samavat & Kurzer, 2015). If not conjugated at this point, the catechol estrogens are oxidised to 2- or 4- estrogen semiquinones (Raftogianis *et al.*, 2000). These semiquinones can be further oxidised to quinones, E1(E2)-2,3-Q and E1(E2)-3,4-Q, by the enzyme peroxidase or a cytochrome P450 family member (Cavalieri *et al.*, 2017; Gaikwad *et al.*, 2008). The quinones can be reduced back into semiquinones by the enzymes NAD(P)H dehydrogenase quinone reductase 1 and 2 (NQO1 and NQO2), where molecular oxygen is reduced to a superoxide anion radical. This anion radical is then converted to hydrogen peroxide by superoxide dismutase (SOD) and further transformed into a hydroxyl radical in the presence of ferrous ions (Fe^{2+}) (Cavalieri & Rogan, 2014). The estrogen quinones are highly reactive molecules and when in contact with DNA, they cause adenine and guanine DNA adducts. Previous studies have shown that 4-hydroxyestrone and its quinone are mutagenic in both *in vitro* and *in vivo* assays (Cavalieri & Rogan, 2006). Additionally, some of these DNA adducts lead to toxic mutations and increase cancer risk, a concept that will be described later. At the quinone level, protection can still occur by conjugation of the quinones with GSH via S-transferases. Glutathione conjugates are then catabolised through enzymatic hydrolysis of GSH tripeptide via γ -glutamyl transpeptidase, to yield CE-cysteine, which is then hydrolysed by cysteinyl glycanase for acetylation to form the CE-N-acetylcysteine conjugate (Todorovic *et al.*, 2001). Alternatively, the quinones can be inactivated by reduction thereof back to catechol estrogens by the quinone reductase enzyme (Li *et al.*, 2004).

Estrogen homeostasis is maintained by three different enzymes and a feedback mechanism. These enzymes include the i) COMT enzyme, which methylates the catechol estrogens, preventing their oxidation to quinones, ii) glutathione-S-transferase, which catalyses the reaction between catechol estrogen quinones and GSH, and the iii) NQO1 and NQO2 enzymes that limit the interaction between DNA and the quinones by reduction back to catechol estrogens (Cavalieri & Rogan, 2011). In the feedback inhibition, the methoxy estrogens inhibit the expression of CYP1A1 and CYP1B1 and might then assist to regulate the levels of catechol estrogens produced

(Cavalieri & Rogan, 2011). Removal of these estrogens from the body through the bile or the urine follows these biotransformation processes. Biliary excretion of the conjugated estrogens occurs only when the molecules exceed a threshold of 450-500 Da and accounts for 20% of the estrogen conjugate excretion. All other conjugates or metabolite products (the remaining 80%) are excreted in the urine (Hall, 2015; Testa & Krämer, 2008). Among the natural estrogens excreted in the urine, the parent estrogens (E1, E2 & E3 [free and conjugated]) make up about 66-82% of all the estrogen metabolites excreted and of the remaining 18-44%, 4.42-9.67% are the hydroxylated estrogens with 2-OHE1 having the highest excretion level, compared to the other hydroxyl estrogens (Liu *et al.*, 2009).

2.5.3 Estrogens and cancer

It has been hypothesised that estrogens can be associated with cancer through two different mechanisms, by causing estrogen receptor-positive or estrogen receptor-negative cancer (Figure 2.5.2) (Wellejus *et al.*, 2005). The first is a hormonal mechanism, in hormone-dependent organs, where estrogen binds to estrogen receptors and initiates cell proliferation (Cavalieri & Rogan, 2014). Rapidly proliferating cells are more susceptible to developing genetic errors and mutations during replication processes that can lead to malignancies when not corrected during DNA repair processes (Rogan *et al.*, 2003). Excess estrogens may be associated with increased cell proliferation, a characteristic of cancer, due to prolonged initiation of cell proliferation in estrogen receptive tissues (Embrechts *et al.*, 2003; Schneider *et al.*, 1982). 16 α -Hydroxyestrone is a predominant estrogen in the first mechanism of cancer initiation, by binding covalently to the DNA and consequently causing genotoxic DNA damage. It can also alkylate amino acid residues on proteins and activate parent estrogen receptors to cause additional proliferation after triggering growth promoting genes (Huang *et al.*, 1998; Zhu & Conney, 1998). 2-Hydroxylated estrogens in the breast tissue can, on the other hand, reduce the risk of breast cancer due to the reduced concentration of bioactive parent estrogens in the breast circulation (Ziegler *et al.*, 2015). Therefore, a 2-hydroxyestrone:16 α -hydroxyestrone ratio equal to or above 2:1 will reduce the risk of receptor positive estrogen-related cancer (Zhu & Conney, 1998).

In the non-hormonal mechanism, it is hypothesised that estrogens act as chemical carcinogens that have carcinogenic, transforming, genotoxic and mutagenic potential (Yager & Davidson, 2006) that can initiate or cause the progression of cancer through the DNA adducts formed, if effective degradation of estrogens (Figure 2.5.1) does not occur. This mechanism is based on disruption in the homeostatic balance that can occur between activating as well as deactivating pathways. These imbalances may contribute to the even higher oxidation of estrogens to quinones and consequently their reaction with DNA (Cavalieri & Rogan, 2006). The 4-OHE1 (E2) DNA adduct forms when 4-hydroxylated estrogen quinones react with DNA, and is more potent

than the 2-OHE1 (E2) DNA adduct. The reason for this is that 2-OHE1(E2) has a poor competitive ability compared to that of the 4-OHE1(E2) adducts (Rogan, 2007), and also that the 4-OHE1(E2) forms higher levels of the depurinating adduct. In short, the estradiol-3,4-quinone reacts with the DNA via proton assisted 1,4-Michael addition, while the estradiol-2,3-quinone rearranges to a para-quinone methide (1,6-Michael addition). Estradiol-2,3-quinone does not react that readily with DNA and mutations caused by these adducts are more likely to be repaired (Cavalieri & Rogan, 2014; Cavalieri & Rogan, 2011). The term *depurinating adducts* refers to DNA adducts that form apurinic sites when an adenine or guanine is lost from the DNA strand, due to destabilisation of the glycosyl bond (Cavalieri *et al.*, 2006). 4-HydroxyE1(E2)-1-N7Guanine and 4-HydroxyE1(E2)-1-N3Adenine are the two DNA adducts formed by E1(E2)-3,4-Q, whereas E1(E2)-2,3-Q form much lower levels (<1%) of adducts known as 2-hydroxyE1(E2)-6-N3adenine (Gaikwad *et al.*, 2008; Markushin *et al.*, 2006). Erroneous repair of the apurinic sites caused by DNA adduct formation may lead to mutations that could initiate cancer, including breast, ovarian, prostate, endometrial, and possibly brain cancers (Cavalieri & Rogan, 2014; Cavalieri *et al.*, 1997).

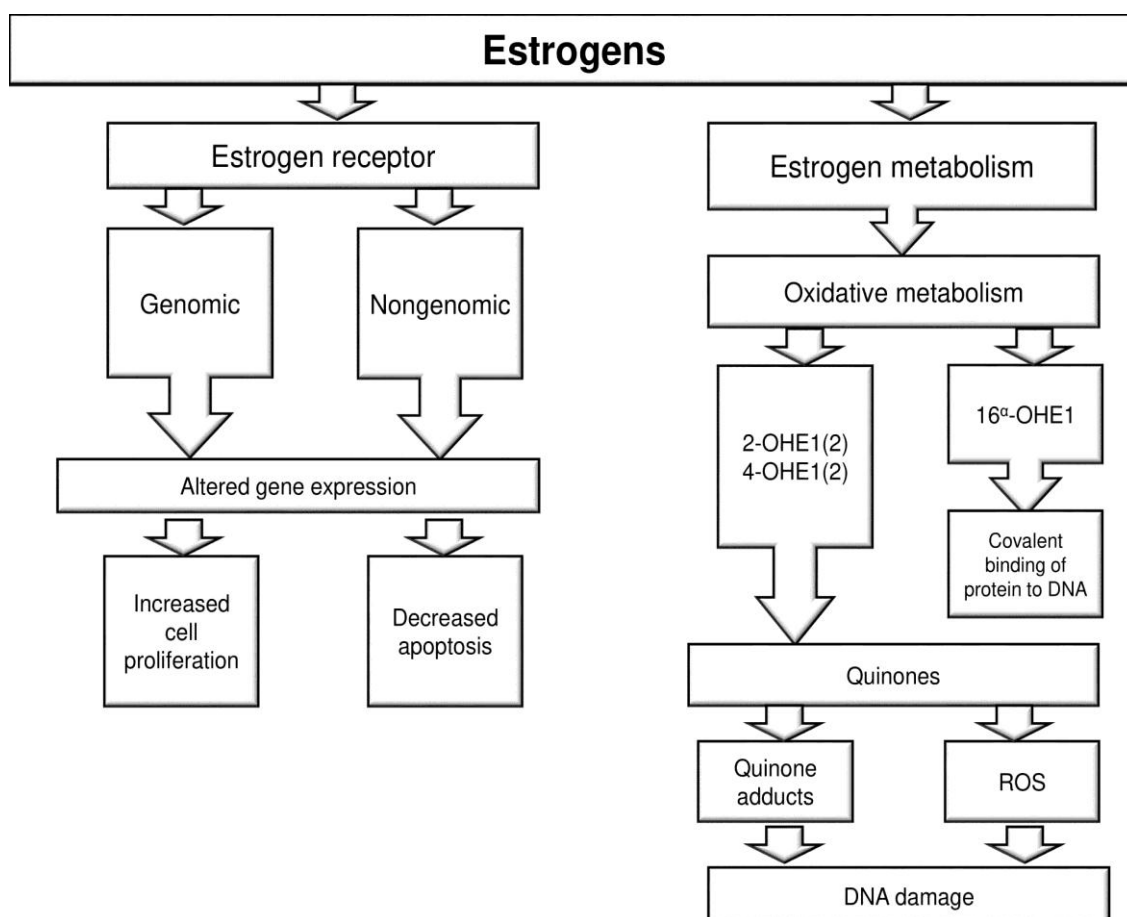


Figure 2.5.2: A schematic representation of the different pathways by which estrogens can act as carcinogens. The hormonal pathway is represented on the right and the non-hormonal pathway on the left. The hormonal pathway evidently leads to decreased apoptosis and increased cell proliferation. The non-hormonal carcinogenic pathway ends in oxidative DNA damage. Adapted from Yager and Davidson (2006).

These depurinating DNA adducts can also travel out of the cells to be excreted in the urine, making them an ideal marker of carcinogenicity of estrogens (Cavalieri & Rogan, 2011). Previous studies also concluded that higher concentrations of carcinogenic catechol estrogen metabolites might be present in the liver if high levels of estrogens are present. This is not only due to the higher levels of CE precursors (estrogens), but might be due to the inhibitory effect of estrogens on the COMT enzyme activity. This increase in CE can then lead to more DNA adducts and more carcinogenicity (Xie *et al.*, 1999). On the other hand, studies that have concluded that 2-methoxyestradiol can inhibit certain cell line proliferation *in vitro* showed that the human breast tissue cancer cells are highly sensitive to the cytotoxic effect of 2-methoxyestradiol. This methylated product of estradiol has also been shown to disrupt the microtubule function and acts as an angiogenesis inhibitor (Zhu & Conney, 1998). This was proven to be true for both estrogen receptor positive and estrogen receptor negative cell lines. These findings may lead to a hypothesis that it is not only the levels of the carcinogenic estrogen metabolites that may have an effect on the total cancer risk but that the levels of metabolites such as the 2-methoxyestradiol that may decrease the total cancer risk, are also of importance.

Estrogen metabolism is also implicated in the formation of ROS, in the form of hydroxyl radicals. Since the methylation of the 4-hydroxylated estrogens is slower, a higher rate of ROS is formed in the form of free radicals (Tsuchiya *et al.*, 2005). These ROS can also cause oxidative stress when the oxidants produced exceed the antioxidant capacity (Tas *et al.*, 2005). This may then lead to DNA damage through oxidation of DNA bases (Brown & Bicknell, 2001), and may be responsible for cellular death, both of which may be implicated in cancer as well as the initiation of other physiological abnormalities (Castro & Castro, 2014; Khan & Khan, 2013). ROS can cause breaks in the DNA strands, induce abasic sites in DNA by hydrogen abstraction from the sugar backbone of nucleic acid (Chen *et al.*, 2011), cause alterations in thymine and guanine bases and also alterations in the sister chromatid exchange; it may also inactivate tumour suppressing genes and upregulate the expression of proto-oncogenes (Brown & Bicknell, 2001). The toxicity of ROS depends on the balance between ROS production rates and ROS removal rates. Furthermore, the damage caused by ROS repair and oxidative stress can increase the malignant potential of a tumour (Brown & Bicknell, 2001). Superoxide dismutase, glutathione peroxidase and catalase are enzymes that provide cellular protection against oxidative stress, together with antioxidants such as vitamin A, vitamin C, vitamin E and glutathione (Castro & Castro, 2014). The hydroxyl radicals produced by the redox cycling of quinines to semiquinones is also widely implicated in the production of lipid hydroperoxides (cofactors of cytochrome P450 enzymes). These cofactors are unregulated and may cause an abnormal increase in oxidation of hydroxylated estrogens (Cavalieri & Rogan, 2006). Progressive oxidative stress is generated through the increase in lipid hydroperoxides as well as the oxidation of hydroxyl estrogens that form more semiquinones;

consequently, more quinones are reduced back and even more hydroxyl radicals released (Cavalieri & Rogan, 2006). *In vitro* cell proliferation is also promoted by sub lethal oxidative stress, since mitosis and progression through the cell cycle have been shown to be stimulated by ROS (Brown & Bicknell, 2001). Figure 2.5.3 illustrates only a few of the consequences of increased free radicals leading to oxidative stress.

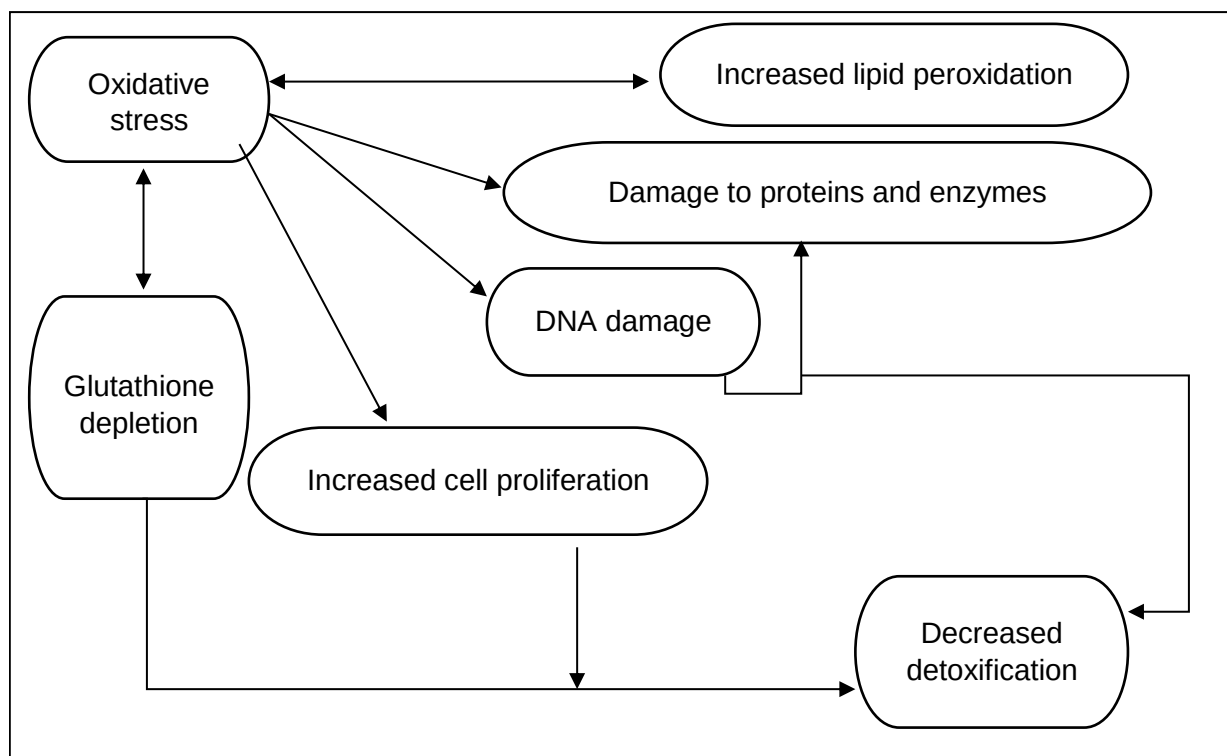


Figure 2.5.3: Additional consequences to increased oxidative stress conditions. All the consequential effects of oxidative stress lead to decreased detoxification in some way by antioxidant depletion, protein and enzyme damage or by further increasing oxidative stress. Adapted from Aruoma (1998)

2.5.4 Factors that influence estrogen metabolism and relative cancer risk

Inter-individual factors, which are the factors that differ between different organisms, but stay constant in an organism over its lifetime, in other words, factors based on the organism's genetic code (Krämer & Testa, 2008) might be one of the factors responsible for variations in the metabolism and detoxification of estrogen-related metabolites. Genetic polymorphism is a good example of the genetic variability in the drug metabolism of individuals (Krämer & Testa, 2008). Intra-individual factors, on the other hand, include inhibitions or induction of enzymes by different pathophysiological conditions, physiological conditions (age, sex, pregnancy, ethnicity) and environmental influences (exposure, food, drugs) (Krämer & Testa, 2008), and are also extremely important in variations in metabolisms. Some examples of intra-individual factors that might affect the detoxification pathways, include diet as seen in Table 2.2, and also smoking, which may alter the levels of circulating estrogens with the hypothesis that the COMT genotype may be modified with increased cigarette smoking (Millikan *et al.*, 1998). Furthermore, some detoxification

enzymes such as the CYP3A4 and some antiporter activities are present in high concentrations in the intestine at the tip of the villi; thus, the integrity of the gut mucosa may also influence the biotransformation profile (Liska *et al.*, 2006). Asthma can also act as a disruptor of the detoxification processes as it may cause imbalances in redox status and depletion of the antioxidant capacity (Bowler & Crapo, 2002). The abundant use of medication, increased use or exposure to toxic chemicals and history of sensitivity to exogenous toxic exposures, might additionally also contribute to variations in the detoxification capacity of an individual (Liska *et al.*, 2006; Roškar & Lušin, 2012). Previous studies have shown an 11% of lower urinary estrogens in women with high physical activity (Ziegler *et al.*, 2015). Table 2.3 also shows the effect of different pathological conditions on drug metabolism, including conditions such as diabetes, cardiovascular and liver disease, inflammation and infection (Krämer & Testa, 2009). When evaluating biological samples of an individual it would be ideal to evaluate both inter- and intra-individual factors.

Table 2.2: The influences of different food and drinks on the detoxification pathway as tested predominantly in rats and mice. Adapted from Krämer and Testa (2009)

Diet and food constituents	Effect on the detoxification metabolism
Vitamin A & coffee	Increases the activity of CYP1A enzymes
Vitamin E	Increases the activity of CYP3A4 enzymes
Garlic, sulphide	Increases the activity of CYP2B and NQO enzymes
High fructose and glucose	Decreases the hepatic cytochrome P450 levels
High fat	Increases the hepatic cytochrome P450 levels
Fat free	Decreases the hepatic cytochrome P450 levels
High protein	Increases the activity of CYP1A2 enzyme
Low protein	Decreases the activity of CYP1A2 enzyme
High carbohydrate	Decreases the activity of CYP1A2 enzyme
Low carbohydrate	Increases the activity of CYP1A2 enzyme

There is a wide range of evidence available, indicating that certain SNPs and DNA mutations may also be associated with varying enzyme activity in the estrogen biotransformation pathway (Krämer & Testa, 2008; Samavat & Kurzer, 2015; Tsuchiya *et al.*, 2005; Zahid *et al.*, 2014; ZRT Laboratories, 2015). These SNPs and mutations are mostly associated with the CYP enzymes, COMT enzymes and SULT enzymes. The COMT gene has been known to be polymorphic in much of the human population, especially in Caucasians with 25% homozygous genes for the low

activity polymorphic enzyme (Lavigne *et al.*, 1997). The cytosolic SULT enzyme has long been recognised to have variants between individuals, although the effect thereof on drug detoxification still needs additional investigation (Krämer & Testa, 2008). Given the important role of enzymes in estrogen metabolism for detoxifying reactions, genetic variations in enzymes may increase the risk of breast cancer initiation by disturbing the balance in estrogen biotransformation (Samavat & Kurzer, 2015). If estrogen homeostasis is additionally disturbed by endogenous or exogenous factors, such as diet, environment and ageing, the degradation of toxic estrogens may be even further disrupted and have physiological consequences (Cavalieri *et al.*, 2006). Deficient expression of certain protective enzymes involved in biotransformation has also been suggested to contribute to breast cancer, and it is hypothesised that there may be interactions between different polymorphisms, having a non-additive or synergistic effect on breast cancer pathogenesis (Cavalieri *et al.*, 2006; Ritchie *et al.*, 2001). Another estrogen-related possible cause of breast cancer is the use of oral contraceptives. There is evidence that the use of oral contraceptives later in the female life is associated with increased breast cancer cases (Key *et al.*, 2001). The effect of using oral contraceptives earlier in life by pre-menopausal women has not been thoroughly investigated enough to draw concise conclusions.

Table 2.3: The effect of different pathological conditions on the general drug metabolism and detoxification mechanisms, adapted from Krämer and Testa (2009)

Condition	Effect on the drug metabolism
Inflammation or infection	Decreased expression and activity of most enzymes
Liver disease and liver tumors	Decreased activity of many of the detoxification enzymes
Cardiovascular disease	- Reduces blood flow to the liver, and thus decreased liver metabolism - Decreases the catalytic activity of some of the Cytochrome P450 enzymes
Diabetes	Increases the expression of most detoxification enzymes
Celiac disease	Decreases the intestinal cytochrome P450 levels

2.5.5 Possible estrogen-related cancer interventions

There are various interventions, some hypothesised and other tested to treat or prevent breast cancer caused by estrogens. Two of these interventions are tamoxifen and aromatase inhibitors, that specifically target the receptor pathway and reduce the receptor-ligand interactions (Yager &

Davidson, 2006). Tamoxifen, a selective estrogen receptor modulator, has been shown to reduce both reassurance of breast cancer as well as the risk of developing breast cancer for the first time. The mechanism by which tamoxifen works, is that its primary effect is mediated through the estrogen receptor pathway, and is shown to be effective for ER α types of cancers. Aromatase inhibitors, on the other hand, suppress the production and synthesis of estrogens to restore imbalances in homeostasis, however, it was also just proven to work in receptor positive types of cancers (Yager & Davidson, 2006). Development of therapeutic strategies targeting the non-hormonal mechanisms of cancer initiation caused by estrogens, for example, by inactivating quinones, or inhibition of certain carcinogenic steps in the estrogen metabolism, might be advantageous. Using antioxidants to help with the balancing of the estrogen homeostasis includes the use of N-Acetylcysteine (NACys) and resveratrol. NACys is a cysteine derivative also found in the antioxidant glutathione, while resveratrol is a more natural antioxidant found in the skin of red grapes. These two antioxidants have shown to be effective for inhibiting the formation of DNA-adducts in the estrogen metabolism pathway and have anticarcinogenic effects (Cavalieri & Rogan, 2011).

2.6 Female reproductive cycle

The female reproductive or menstrual cycle (Figure 2.6) describes a series of events that prepare the endometrium for implantation of a fertilised egg cell (embryo). If fertilisation does not occur, the endometrium is broken down, resulting in menstrual bleeding (Nelson, 2007). Most premenopausal women have reproductive cycles that last approximately 24 to 35 days and consist of a series of events that end in ovulation. This cycle can be divided into four different phases including the follicular phase, ovulatory phase, luteal phase and lastly, the menstrual phase (Nelson, 2007). Each phase is associated with specific changes in the serum levels of the follicle-stimulating hormone (FSH), luteinising hormone, estradiol and progesterone.

2.6.1 Follicular phase

This phase of the menstrual cycle ranges from 10 to 17 days and is associated with follicular growth (Hall, 2015; Nelson, 2007). During the follicular phase, gonadotropin-releasing hormone is released by the hypothalamus, which results in pulses of other hormones known as FSH and LH. In day one to five of this phase, FSH stimulates the granulosa to proliferate and produce estradiol. This initiates follicle growth, and more FSH receptors are induced, allowing even more estradiol to be produced, in the end causing a negative feedback on FSH secretion (Nelson, 2007). One of the follicles becomes dominant in days five to seven. The dominant follicle produces the most estradiol and has the most FSH receptors. After day seven, the dominant follicle, which is at this point the only surviving follicle matures and still produces high levels of estradiol. The

LH stimulates the stromal tissue to raise androgen levels late in the follicular phase to increase libido in an aim to also increase sexual activity (Nelson, 2007)

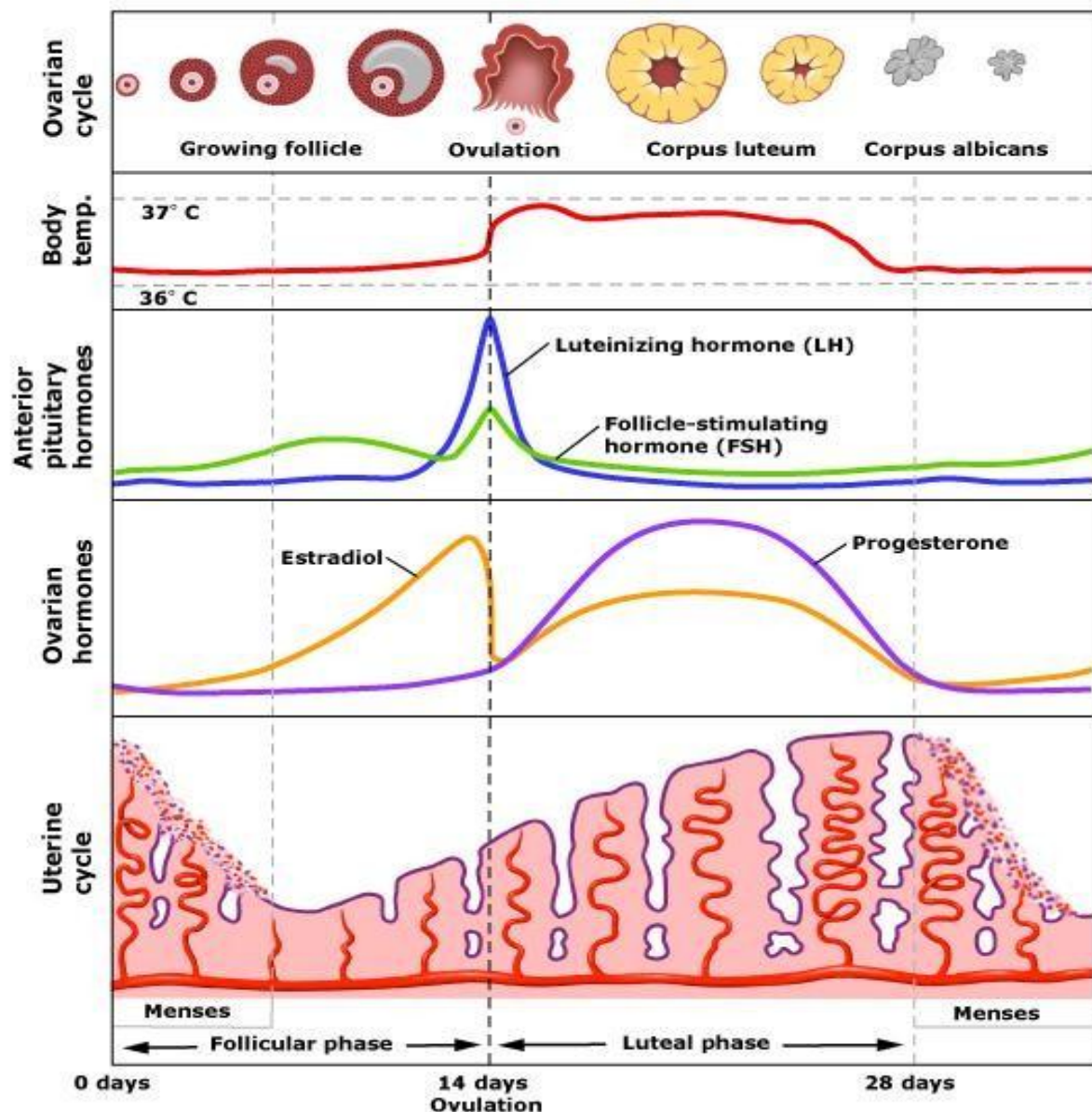


Figure 2.6: Schematic representation of the female reproductive cycle. Showing the occurrences in the ovaries, body temperature fluctuations, hormone secretions and also occurrences inside the endometrium The Roding Reproductive Centre (2016).

2.6.2 Ovulation

In the ovulatory phase or commonly known as the ovulation phase, the oocyte is released from the follicle (Hall, 2015). This only occurs when the estradiol levels are extremely high for two to three days and cause a positive feedback on the pituitary glands, causing LH and FSH hormone levels to increase drastically. About 24 hours before ovulation occurs, the estradiol levels peak and the increased levels of LH lead to the luteinisation of the granulosa cells, causing the

production of progesterone and further meiosis of the oocyte. The follicle then breaks out of the oocyte, which is discharged from the body within a few days (Nelson, 2007).

2.6.3 Luteal phase

The luteal phase starts off with the formation of the corpus luteum and instead of estradiol being the predominant hormone, progesterone takes the dominating role in the luteal phase (Nelson, 2007). These increased levels of progesterone cause growth suppression of new follicles and also allow secretory changes to occur inside the endometrium. Seven to eight days after the LH surge in the ovulation phase, the progesterone levels peak, and this is approximately also the time that implantation will occur. If pregnancy does not occur, the luteal phase is approximately 14 days in length (Nelson, 2007).

2.6.4 Menses

The phase ending the menstrual cycle, if pregnancy did not occur, is the menstrual phase, with a duration of 4 to 6 days. The decline in the corpus luteum and consequently the decrease in progesterone and estradiol levels are the first sign that the menstrual phase is starting. The decline in the estrogen and progesterone levels causes a decreased blood flow to the endometrium and shrinks the endometrial height (Nelson, 2007). Vasoconstriction following vasodilatation in the endometrium leads to the interstitial haemorrhage known as the menstrual flow or bleeding. During this phase, approximately 20 to 80 ml of blood is lost (Nelson, 2007). After the last day of the menstrual cycle has occurred, the estradiol levels start to rise again to initiate the next follicular phase.

2.7 Combined oral contraceptives

Since the 1950's, female sex hormones have been used as contraceptive agents (Rivera *et al.*, 1999) and it is estimated that since the start of use, 200 million women worldwide have used them (Daniels *et al.*, 2013). The most frequently used type of hormonal contraceptive contains a combination of both estrogens and progestogens (Collaborative Group on Hormonal Factors in Breast Cancer, 1996) to primarily prevent ovulation (Rivera *et al.*, 1999). COCs are available as monophasic formulations, where each hormone-containing pill contains the exact same doses of progestins and estrogens than all other active hormone containing pills; or as multiphasic formulations, where the amounts of hormones in the active hormone pills vary (Nelson, 2007). Conventional COC treatment consists of a 21-day contraceptive phase, followed by a 7-day pill-free phase, in order to mimic the natural $\pm$ 28-day menstrual cycle. Non-contraceptive benefits of oral contraceptives may include its assistance in premenstrual dysphoric disorder, acne, menorrhagia, certain cancers and dysmenorrhea (Petitti, 2003; Shoupe, 2011). The mechanism involved in the treatment of these conditions will be discussed at a later stage. For contraceptive

function, the progestins with a higher androgenic activity block the midcycle rise of LH secretion (Rivera *et al.*, 1999) to inhibit ovulation and are combined with the estrogens to reduce some of the effects that the estrogens have on the female reproductive cycle under normal conditions (Nelson, 2007). COCs inhibit ovulation, follicular development and consequently, the formation of the corpus luteum (Rivera *et al.*, 1999). The estrogen compounds that are most frequently used in combined oral contraceptives are ethinyl estradiol (EE) and mestranol (Nelson, 2007). The latter is a precursor drug, which is demethylated by the liver into biologically active EE. Ethinyl estradiol is much more long-acting and more potent than the endogenously produced estradiol and thus the metabolic impact of EE is higher than that of E2 (Nelson, 2007). Structurally, ethinyl estradiol is very similar to 17 β -estradiol (the dominant estrogen produced in premenopausal women), except for the substitution of hydrogen with an ethyl group at the 17 carbon position, making it less susceptible to biotransformation in the liver (Arcand-Hoy *et al.*, 1998). Ethinyl estradiol is completely absorbed after ingestion, with the initial metabolism of EE occurring inside the gut. Here, it is converted to ethyl estradiol sulphate, which reaches a peak within 2 hours of administration and has a half-life varying from 6 to 20 hours (Arcand-Hoy *et al.*, 1998). Ethinyl estradiol can further be metabolised by hydroxylation, D-homo annulation, or by removing the ethyl group (Zhang *et al.*, 2007). Hydroxylation occurs most commonly at the 2-carbon position, although hydroxylation at the 4, 6 or 16 positions can also occur to a lesser extent (Nelson, 2007). During D-homo annulation, the second carbon is removed as a carbon dioxide (CO₂) group after rearrangement of the ethyl group into the EE D-ring (Zhang *et al.*, 2007). As with endogenous estrogens, the catechol estrogens from EE can be further oxidised to quinones (Rogan, 2007). All the EE that is taken in as part of the contraceptive is metabolised completely, allowing no excretion of the unchanged form (Zhang *et al.*, 2007).

Progestin is a term commonly used to describe synthetic progestogens. The progestins used in these oral contraceptive pills include drospirenone, norethindrone, norethindrone acetate, ethynodiol diacetate, norgestrel, levonorgestrel, desogestrel and norgestimate. The latter two are known as third generation progestins, which maintain increased progestational activity and have less androgenic activity (Nelson, 2007). The progestins are responsible for most of the contraceptive effects, yet the estrogens are also important to suppress ovulation. The COCs suppress ovulation by providing a negative feedback on the hypothalamic-pituitary system, which then causes a decrease in the gonadotropin-releasing hormone pulsatility (pituitary responsiveness to gonadotropin-releasing hormone stimulation), suppresses LH and FSH production and consequently, leads to the inhibition of mid-cycle LH efflux (Nelson, 2007). Other ways in which the COCs act as birth controlling agents is by thickening the mucus inside the cervix. This prevents sperm from entering into the upper part of the genital tract, by reducing the motility inside the fallopian tubes, thereby intervening with fertilisation. COCs may also lead to

atrophy of the endometrium, which changes the function of the endometrium vessels and may inhibit implantation by localising oedema of the endometrium or by altering the metalloproteinase content in the endometrium (Nelson, 2007).

As mentioned earlier, COCs are effectively used in a wide range of applications besides birth control. Acne, for example, is reduced among patients using oral contraceptives, thereby causing major increases in the sales of many low-dose contraceptive formulations. The mechanism behind the reduction in acne presumably involves the reduction of free testosterone (Petitti, 2003). Other conditions for which contraceptives also work include abnormal uterine bleeding, heavy bleeding, painful menses and oligomenorrhea (infrequent periods). It reduces heavy bleeding as well as the prevalence of anaemia by increasing the haemoglobin levels in anaemic women and reducing the severity of uterine bleeding. The role COCs play in cancer development is quite controversial and seems to depend on the specific tissue involved. COC-use has been shown to reduce ovarian and endometrial cancer, possibly through the suppression of ovulation and progestin suppression of estrogen-induced endometrial cell proliferation, respectively (Petitti, 2003). Although COCs are very effective, with a good safety profile, the individual hormonal components can have various broader metabolic effects, including effects on the carbohydrate and lipid metabolism as well as haemostatic variables (Klipping & Marr, 2005). Synthetic sex steroids mostly have a low affinity for sex hormone-binding globulin and are thus unbound in the circulation system. Endogenous steroids, on the other hand, bind 98-99% of carriers, making the steroids inactive. Thus, even if serum concentrations of synthetic and endogenous estrogens are equal, the synthetic estrogens will have a much stronger action (Andersson & Skakkebaek, 1999). Risks associated with the use of oral contraceptives include myocardial infarction, ischemic stroke, venous thromboembolism, cerebral venous thrombosis, as well as breast and cervical cancer (Petitti, 2003). The World health organization, together with the International agency for research on cancer, declared in 2005 that COCs have carcinogenic potential and increase liver, cervical, and especially breast cancer (World Health Organization, 2016). A number of clinical studies also demonstrated that EE in COCs, may lead to the inhibition of detoxification enzymes, especially cytochrome P450 isoenzymes. This inhibition is due to the acetylenic group on the EE that changes not only the metabolism and detoxification of these contraception pills, but may also alter the detoxification capacity of other drugs and exogenous factors as well (Zhang *et al.*, 2007). Previous studies concluded that there is an increase in breast cancer risk in users of combined oral contraceptives (Yager & Davidson, 2006) or woman who have used them in the past 10 years (Calle *et al.*, 1996). Meta-analysis in 1996 showed a 24% increased risk of breast cancer in women using COCs (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). Further analysis of the data from this investigation showed that users who began the use of COCs before the age of 20, have a relatively higher risk of having breast cancer diagnosed while using (until 5 years after use), as women that began using at an older age (Calle *et al.*, 1996; Collaborative

Group on Hormonal Factors in Breast Cancer, 1996). These results were obtained through combining data collected from 53 297 women with breast cancer and 100 239 women without breast cancer, by observing and evaluating the effect of COCs on the relative risk of breast cancer, retrospectively (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). A standardised method to evaluate cancer risk through biological markers of estrogen metabolism may provide a means of determining such a risk before actual tumorigenesis and provide an opportunity to take preventative action, if necessary.

2.8 Measuring estrogens in urine

The consequence of drug intake and metabolism can either be favourable and lead to detoxification, or it can lead to toxification (unwanted products). Screening for intermediates, together with assessing toxicity are, therefore, useful tools for prognosis (Testa & Krämer, 2009). Identifying specific biomarkers associated with estrogen-related abnormalities, including the disruption of successful biotransformation, would be of enormous value in a clinical setting. It would not only improve the assessment of cancer and other related diseases, but also give an indication, if optimal biotransformation of estrogens occur (Pruthi *et al.*, 2012). Additionally, it would enable investigation of side effects of COCs or excessive exposure to exogenous estrogens. There are various definitions of what a biomarker is. A biomarker, as defined by the international program on chemical safety (2001), is “any substance, structure, or process that can be measured in the body or in its products, and influence or predict the incidence or outcome of disease” (Who International Programme on Chemical Safety, 2001). It may also refer to a “broad subcategory of medical signs that are objective indications of medical state observed from the outside of a patient, which can be measured accurately and reproducibly” (Strimbu & Tavel, 2010). Biomarkers can be classified into either markers of exposure, markers of effect or markers of susceptibility (Who International Programme on Chemical Safety, 2001), yet can only serve as replacements for clinical endpoints when the normal physiology of the biological process is completely understood (Strimbu & Tavel, 2010).

An ideal biomarker for estrogen metabolism-related abnormalities should be metabolites; or markers that will clearly indicate up- or downregulations in estrogen hormone metabolism, or a disruption in estrogen homeostasis (Gaikwad *et al.*, 2008). The estrogen metabolite levels in the urine (including parent, hydroxy, methoxy and conjugated estrogens, as well as depurinating DNA adducts) may be related to the biotransformation capacity and expression level of detoxifying enzymes (Cavalieri & Rogan, 2014). Estrogen metabolism varies highly between persons, and thus, individual profiles of estrogens together with their metabolites may provide information on variations in estrogen metabolism, and hence, also cancer risk (Qin *et al.*, 2008). The highly

accurate and precise measurement of estrogens and their metabolites may also make it possible to further study the roles that the metabolites play in estrogen-related physiological processes (Qin *et al.*, 2008). Endogenous estrogens are excreted under normal biological conditions in the urine. The levels of these estrogens are dependent upon the female's reproductive cycle, age, and reproductive status. Normal estradiol excretion in the luteal phase is 10-80 µg a day and 25-100ug/day at ovulation (Arcand-Hoy *et al.*, 1998), however, the concentrations can vary from <5 pg/ml to 2000 pg/ml, depending on the therapy patients are using (Keevil, 2016).

Previously used methods for quantification of steroids in biological samples of patients, included radioimmunoassay, enzyme immunoassays and enzyme-linked immunosorbent assays; bioassays that rely on recombinant yeast models and HeLa cells; gas chromatography/mass spectrometry (GC-MS) and liquid chromatography/mass spectrometry (LC-MS) (Faupel-Badger *et al.*, 2010b; Key *et al.*, 2002; Ziegler *et al.*, 2010). From as early as 1982, Fishman and Bradlow did studies on the association between breast cancer and estrogen metabolites, by measuring the metabolites in blood through radiometric procedures (Schneider *et al.*, 1982). The limitations of radioimmunoassay, enzyme immunoassays and enzyme-linked immunosorbent assays include the fact that each hormone should be analysed independently, questionable reproducibility (Nguyen, 2011), the need for large sample volumes per hormone analysis, as well as high costs (Key *et al.*, 2002). No structural validation is achieved by these methods and sometimes antisera do not exist for all the hormones and hormonal metabolites (Penning *et al.*, 2010). These methods can also require radioactive materials to be used and it has been reported that artefacts occur due to the nonspecific binding of some of the radioactive labels (Nguyen, 2011). Both the radioimmunoassay methods, one including extraction and chromatography and the other being direct radioimmunoassay, lack accuracy and sensitivity to monitor estradiol levels (Faupel-Badger *et al.*, 2010a). radioimmunoassays also show a lack of specificity, which may be due to exogenous and endogenous interferences and low antibody specificity for specific steroid hormone structures (Caron *et al.*, 2015). Cross-reaction during these methods can then occur due to similar structures of which the estrogens consist (Qin *et al.*, 2008), and the low antibody specificity mentioned (Caron *et al.*, 2015). Bioassays, on the other hand, are more sensitive than radioimmunoassay, however, it also lacks specificity, as well as convenience, due to it being technically demanding (Folkerd *et al.*, 2014; Ziegler *et al.*, 2010). The lack of specificity in these conventional methods was confirmed after absolute concentration studies were carried out (for the ratio of 2-hydroxyestrone to 16α-hydroxyestrone). The authors concluded that enzyme immunoassays was not as specific as expected and that interfering compounds may be involved (Faupel-Badger *et al.*, 2010b). The GC-MS method provides specificity, sensitivity and accuracy, nevertheless, in previous studies, the coefficient of variations (CVs) was not as high as for the LC-MS methods (Ziegler *et al.*, 2010). Additionally, because estrogen metabolites range from

polar to non-polar and have low volatility; hydrolysis, derivatisation and additional steps of sample preparation are required for GC-MS analysis. Some of these additional procedures might be labour intensive, time consuming, and introduce artefacts, which is not ideal for clinical analysis (Qin *et al.*, 2008).

High-performance liquid chromatography electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS) has been successfully used for the determination of estrogens and estrogen-related metabolites (Qin *et al.*, 2008). Zang *et al.* (1999) followed this alternative route to the conventional measurement of parent estrogen, where they developed a method to evaluate the presence and concentrations of estrogen-sulfate conjugates using an LC/MS technique (Zhang & Henion, 1999). In 2005, Xu *et al.* also published a novel method for measuring 15 endogenous estrogens in urine and serum, using LC-MS/MS; from there, the use of LC-MS/MS in steroid and estrogen analysis dramatically increased. Advantages of using the LC-MS/MS method, is its accuracy, sensitivity, selectivity and high precision. Furthermore, the procedure is simple, rapid and requires lower volumes of urine sample per run (Key *et al.*, 2002). Tandem mass spectrometry offers high specificity, even in complex matrices such as the urine, and also allows minimal sample preparation (Rauh, 2010). This method allows high throughput and together with its simplicity, it can easily be routinely employed in clinical environments (Rauh, 2010). Previous results thus suggest that LC-MS/MS methods are ideal and preferred for comparison of relative or absolute amounts, or changes in the levels of estrogens and estrogen metabolites (Faupel-Badger *et al.*, 2010b). Estradiol might, however, be difficult to measure using LC-MS/MS methods due to the fact that it is not easily ionised and will preferentially ionise in the negative ionisation mode (Keevil, 2016). Furthermore, the method to be developed should be able to overcome some other obstacles. High specificity is required because steroid hormones (especially estradiol) are multi plastic, meaning that they can be converted into different unconjugated or conjugated metabolites (Caron *et al.*, 2015). Others include limited sample volumes and the fact that the estrogens and its conjugates have different polarities. Conjugation of metabolites may cause different ionisation characteristics between the two types (parent and conjugated) of metabolites (Zhao *et al.*, 2014). It is also critical that the mass spectrometry methods are subjected to at least partial validation, to assess against any known physiological effects of estradiol (Qin *et al.*, 2008).

2.9 LC-MS/MS method

Liquid chromatography together with triple quadrupole mass analysers has been proven to be a highly sensitive and selective method for quantification of estrogen steroids in complex biological samples (Nguyen, 2011). Due to the fact that the urine contains various electrochemically active

compounds, it may require some extensive pre-purification prior to an LC method (Todorovic *et al.*, 2001). The urine is a highly polar matrix and contains salts, phosphates, urates, proteins, sugars, metal ions, creatinine and metabolic products, including the estrogen metabolites interested in. The estrogen metabolites range from relatively polar (sulphate and glucuronic conjugates), less polar (catechol estrogens and precursor steroid hormones), to non-polar (parent estrogens and methoxyestrogens) (Todorovic *et al.*, 2001). A clean-up step such as SPE, liquid-liquid extraction (LLE) or protein precipitation, can then be used to decrease ion-suppression that is usually caused by co-eluting biomatrix components (Athanasiadou *et al.*, 2013; Kushnir *et al.*, 2011); it would, however, have to be sufficient for all metabolite polarities. Disregarding such a clean-up step would decrease performance in more complex matrixes such as urine (Van Eeckhaut *et al.*, 2009).

Protein precipitation is ideal for high concentration steroid hormones, like cortisol and dehydroepiandrosterone; SPE and LLE are more ideal methods of sample preparation for steroid hormones that are less abundant inside biological matrixes (Keevil, 2016). Liquid-liquid extraction is based on the mechanism that two immiscible liquids are used to separate analytes by the partitioning of the analytes, including the metabolites of interest and all other matrix contents, in separate phases, based on their polarity. These two liquids are usually an aqueous phase, preferred by the hydrophilic compounds, and an organic phase, preferred by the hydrophobic compounds. The analytes in the aqueous phase can sometimes be directly injected, while the analytes in the organic phase can then be recovered by the evaporation of the organic solvent (Wal *et al.*, 2010). Some problems that might be encountered during LLE processes, include the formation of emulsion, strong adsorption of analytes to particulates, binding of analytes to compounds that have a high molecular weight and also the mutual solubility of the two phases. Solid phase extraction, described in previous estrogen investigation studies as the chosen method of sample clean-up, allows greater sensitivity while reducing sample matrix effects and complexity of samples (Kushnir *et al.*, 2011). During the SPE process, the sample contents, again containing the metabolites of interest and all other matrix contents, are partitioned between the solid phase sorbent or the liquid phase (aqueous or organic). To extract steroids from complex biological matrixes, polymeric or silica-based packing materials have been used for SPE. Before sample preparation, using SPE, the sorbent in the SPE cartridge is firstly conditioned, to activate it. After dilution and centrifugation of the samples, to discard all solids into a pellet, the supernatant is applied to the SPE column. This will allow the metabolites of interest to be retained by the sorbent, while weakly- and non-retained molecules are removed by washing them off the sorbent with a more polar aqueous washing solution (Sargent, 2013). The sorbent is then washed with organic solvents, to elute the targeted metabolites (Kushnir *et al.*, 2011). As a sample clean-up method, SPE has numerous advantages over LLE, including higher recoveries, less solvent

consumption, no emulsion problems and the requirement of smaller sample sizes (Roškar & Lušin, 2012). Both LLE and SPE allow concentration of the extraction product by reconstitution in a lower volume than the initial biological sample volume (Sargent, 2013). Whether a sample would be concentrated or diluted, depends on the sensitivity of the LC-MS method, which is based purely on the signal to noise ratio (S/N) at a certain concentration. The S/N mainly depends on the assay requirements, although a S/N of about 10 is usually acceptable. The linearity of the assay in a certain concentration range will also play an important role in determining whether the sample should be diluted or concentrated (Sargent, 2013). Internal standards (IS) can be added to samples to monitor estrogen derivatisation (if necessary), sample extraction or purification. This can then contribute to the correction of any analytes that may be lost during sample preparation or derivatisation (Cerdeira, 2010).

For liquid chromatography (LC), a stationary phase, offering sufficient separation and retention of the steroid hormone metabolites, is necessary. With regard to estrogen analysis, LC is usually executed on reverse phase stationary phase columns such as octasilane (C8), octadecylsilane (C18) and phenyl bonded phase columns (Nguyen, 2011). The choice of the mobile phase depends highly on the polarity of the ionisation (positive/negative) after the LC run. For both positive and negative ionisation, mobile phase A consists of an aqueous solution and mobile phase B an organic solution (acetonitrile and/or methanol). For positive ionisation, a mobile phase modifier, like formic acid or ammonium formate in water (and/or organic solvent) (Xu *et al.*, 2005) is used to acidify the mobile phase and protonate the targeted estrogen metabolites and derivatives (Nguyen, 2011). Negative ionisation, on the other hand, works ideally with ammonium acetate, ammonium fluoride or ammonium hydroxide as modifiers (Keevil, 2016; Zhao *et al.*, 2014). The two mobile phases are usually used in gradient, where the separation between metabolites based on their polarities is necessary, starting with a higher percentage of water (with modifier) and low percentage of acetonitrile/methanol. The percentage of water is then decreased while the organic mobile phase is increased to ensure sufficient separation (Gaikwad *et al.*, 2008).

The ionisation source commonly used to give highly specific quantitative data on steroid hormones is ESI. This method of ionisation gently converts the complex biological molecules into ions in the gas phase (Key *et al.*, 2002). In ESI, the urine sample is firstly dissolved in a solvent that is able to exist in an ionised form (e.g. mobile phase A/B with mobile phase modifier transporting the sample). The solution is then pumped through a capillary, which is raised to a high potential and allows charged droplets to be sprayed from the capillary into the gas phase, and travel down a gradient of potential and pressure into the MS high vacuum system (Griffiths *et al.*, 2001). These droplets reduce in size until surface forces overcome the surface tension. This allows the small droplets to break into even smaller droplets. The process is repeated until

the solvent is completely removed or an ion desorbs from the small droplet. The ions that are produced in this way can now be sampled by the MS (Griffiths *et al.*, 2001). Most additional chemical noise is thus removed to reduce false negative or false positive results (Nguyen, 2011). The application of ESI-MS for the analysis of steroids may be limited because the moieties in the steroid molecules are not easily ionisable. This is specifically true for ionisation of anabolic steroids, which contain carbonyl and hydroxyl groups with low proton affinity (Athanasiadou *et al.*, 2013). Chemical derivatisation might be a solution to this problem by adding an ESI responsive moiety to the steroid's keto, carbonyl and hydroxyl functional groups. Derivatisation aims to modify the physical and chemical properties as well as the structure of the analyte, to increase the detection characteristics of the analyte (Athanasiadou *et al.*, 2013). This will then yield derivatives with higher sensitivity, to be formed for analysis due to the addition of more easily ionisable, or even permanently charged species (Athanasiadou *et al.*, 2013; Nguyen, 2011). Derivatisation reagents for steroids, include, but are not limited to, fusaric acid (FA) combined with 2-methyl-6-nitrobenzoic anhydride, 4-dimethylamino pyridine and trimethylamine (Yamashita *et al.*, 2010), 2-fluoro-1-methyl pyridinium-toluenesulfonate (FMPTS) in the presence of trimethylamine (TMA) (Faqehi *et al.*, 2016), N-(5-Fluoro-2,4-dinitrophenyl)-N,N-dimethyl-1,2-ethanediamine (PED), 1-(2,4-Dinitro-5-fluorophenyl)-4-methylpiperazine (PPZ), 1-(2,4-Dinitro-5-fluorophenyl)-4,4-dimethylpiperazinium iodide (MPPZ), 4-(4-Methyl-1-piperazyl)-3-nitrobenzoyl azide (APZ), dansyl chloride (DNSCL), pyridine-3-sulfonyl chloride (PSCI) combined with picolinic acid (PA), and pentafluorobenzyl bromide (PFBBBr) (Lin *et al.*, 2007). Others have also derivatised with 4-(1H-pyrazol-1-yl)benzene sulfonyl chloride (Wang *et al.*, 2015; Xu & Spink, 2008), N-methyl-nicotinic acid N-hydroxysuccinimide ester (Yang *et al.*, 2008) and isomers of 1,2-dimethylimidazole-sulfonyl chloride (Keski-Rahkonen *et al.*, 2015; Xu & Spink, 2008). Previous studies recommended dansyl chloride to be used due to its impressive limit of detection (Nguyen, 2011). Another advantage of derivatisation is that it allows the use of smaller sample volumes. Furthermore, previous results with the use of derivatisation methods showed approximately 10%-20% improvement in results for estradiol than non-derivatising results (Soldin & Soldin, 2009). Derivatisation, however, adds additional sample preparation to the analysis, making it a more labour intensive option. Although most methods for estrogen quantification without derivatisation have been unsuccessful, it was discovered that the addition of ammonium fluoride to the mobile phase, makes it possible to increase the sensitivity of these methods and allow detection of low concentration estrogens (Keevil, 2016). It is thought that a fluoride adduct that is easily cleaved, allows a greater yield of products, allowing quantification of estrogens without the need for derivatisation (Keevil, 2016).

Due to the fact that some estrogens and estrogen metabolites are chemically indistinguishable, tandem mass spectrometry is particularly useful for the identification of these metabolites. This is because the ions that are generated by the first fragmentation undergo a second fragmentation

and separation step (Key *et al.*, 2002). This allows not only greater sensitivity and specificity, but also increases the dynamic range (Kushnir *et al.*, 2011). In tandem mass spectrometry, the precursor ion is filtered by the mass spectrometer 1 (MS1), based on its mass and is then fragmented in the collision cell to enable filtering based on structurally significant product ions by mass spectrometer 2 (MS2) (Griffiths *et al.*, 2001). Inside the collision cell, a collision occurs between an inert gas and the precursor ions, allowing precursor ions to be converted to product ions. This process is termed collision induced dissociation (Griffiths *et al.*, 2001). The triple quadrupole MS system can be used in different modes of acquisition. These different modes include product ion scan, precursor ion scan, neutral loss and multiple reaction monitoring (MRM). The latter is the most commonly chosen mode for quantitative analysis because the MS is filtering for various, but selective, mass to charge ratios (m/z) over the entire time period, making it more sensitive. Monitoring of the metabolite precursor m/z and the product ion m/z increases its specificity (Kushnir *et al.*, 2011).

Several methods for the quantification of the parent and/or related estrogen metabolites on the LC-MS/MS have been developed, validated and published. Most of these methods were developed for the use on serum, included only the parent metabolites or only a few metabolic products (Cerdeira, 2010; Keevil, 2016; Keski-Rahkonen *et al.*, 2015; Keski-Rahkonen *et al.*, 2013; Lin *et al.*, 2007; Pruthi *et al.*, 2012; Wang *et al.*, 2015). In 2003, Rogan *et al.* already evaluated 32 different estrogens and estrogen metabolites in the breast tissue of women with carcinoma (Rogan *et al.*, 2003). Although 32 metabolites exceeded the quantities of estrogen-related metabolites from previous studies to investigate disruptive estrogen homeostasis, body fluid (especially urine) is the product of metabolism making it more applicable and less invasive for biotransformation product evaluation (Rogan *et al.*, 2003). Another, is the method developed by Xu *et al.* (2005), which allowed the quantification of 15 estrogen metabolites (E1, E2, E3, 2-OHE1(E2), 4-OHE1, 16 α -OHE1, 2-ME1(E2), 4-ME1(E2), 16-epiestriol, 17-epiestriol, 2-OHE1-3-methyl ether and 16-ketoE2) after dansyl chloride derivatisation. Although this method did not contain all the related metabolites, it allowed the quantification of metabolites with concentrations below 1 ng/ml (Xu *et al.*, 2005). In 2008, Gaikwad *et al.* published a paper on 40 urinary markers that could be used to determine the risk of breast cancer (Gaikwad *et al.*, 2008). Collectively, they investigated estrogen precursors (androstenedione, testosterone), parent estrogens (E1, E2, E3), catechol estrogens (2-OHE1(E2), 4-OHE1(E2), 16-OHE1) conjugated estrogens (E1-S, 2-ME1(E2), 4-ME1(E2), 2-OH-3-ME1, 2-OHE1(E2)-1-glutathione, 4-OHE1(E2)-2-glutathione, 2-OHE1(E2)-4-glutathione) and some of their metabolites (2-OHE2-(1+4)-Cysteine, 2-OHE1(E2)-2-cysteine, 2-OHE1(E2)-1-N-acetylcysteine, 2-OHE1(E2)-4-N-acetylcysteine, 4-OHE1(E2)-2-cysteine, 4-OHE1(E2)-2-N-acetylcysteine). Lastly, they also included estrogen-related DNA-adducts (4-OHE1(E2)-1-N7-guanine, 4-OHE1(E2)-1-N3-adenine, 2-OHE1(E2)-6-N3-adenine)

(Gaikwad *et al.*, 2008). This broader field of investigation of not only estrogens or DNA-adducts, but rather a collaboration of free estrogens and biotransformation products as well as products formed because of some of the biotransformation products, allows a more holistic approach to the estrogen metabolism investigation.

2.9.1 Parameters for consideration during method development

When biological samples are analysed for the quantification of estrogen and its metabolites (which is present in very low concentrations), ion suppression, ion enhancement and matrix effect might be observed. This is primarily caused by other endogenous species that are present in the background matrix (Nguyen, 2011). The biological matrix is a material of biological origin, for example, blood, plasma, serum, urine, saliva or faeces that can be reproducibly sampled and processed (Imre *et al.*, 2008). The matrix effect occurs when matrix components co-elute with the analyte/component of interest (Kushnir *et al.*, 2011), and is sometimes overlooked during the method development process (Rauh, 2010). Despite the fact that LC-MS/MS analysis has high specificity, the matrix effect has been recognised to cause interference (Kushnir *et al.*, 2011) and result in a specific compound responding differently when analysed in a biological matrix, than when analysed in a standard solution (Van Eeckhaut *et al.*, 2009). Due to the matrix effect, this response may be either suppressed or enhanced. The potential of interference in steroid analysis is higher than for other molecules, due to the fact that a large number of isobars (two or more isotopes of different elements, with the same atomic weight) and endogenous isomers are present (Kushnir *et al.*, 2011). The matrix effect can be evaluated (described as part of the method validation process further on) to ensure that ion enhancement or suppression does not affect the results. Furthermore, the ionisation efficiency of analytes in the liquid phase can also differ and may affect the response factor of analytes, resulting in low sensitivity of the method. Ionisation efficiency depends on factors, including the mobile phase stability (which is influenced by pH, solvent composition and additives), characteristics of the analytes and spray conditions (spray rate and spray voltage) (Nguyen, 2011). Another challenge in quantifying estrogens and estrogen-related metabolites is related to the structural diversity of estrogen metabolites. These metabolites are sometimes isomeric, exhibiting a wide range of glucuronide, hydroxyl and sulphate groups (Nguyen, 2011), and have differing polarity and hydrophilicity or hydrophobicity, which makes chromatographic method development even more challenging. Thus, measuring a group of estrogens together in an analytical run, may be troublesome due to varying optimal analytical conditions for varying functional groups (Nguyen, 2011).

Quantification, by the use of internal standard, improves precision due to the avoidance of any sample preparation, derivatisation or injection uncertainties (Wal *et al.*, 2010). Internal standards refer to test compounds that are either structurally similar to the compounds of investigation, or may also be one or more of the compounds of investigation stable labelled (stable isotopes).

These internal standards or stable isotopes are then added to samples at a known concentration to allow the quantification of the target analytes (Imre *et al.*, 2008). Adding internal standards will also allow correction of instrument drift and variations in sample injection (Sargent, 2013). Introducing stable isotopes as internal standards to observe and consequently reduce variation through correcting for recovery problems and signal irreproducibility, may be a valid method to approach effects that could alter quantification results. The benefits of using an isotopically labelled internal standard are that it will have a similar chromatographic retention time, with similar extraction recovery, as well as ionisation in the mass spectrometer (Wal *et al.*, 2010). Additionally, stable isotopes may overcome the drawbacks of bad ionising or difficult detectable metabolites at low concentrations. Stable isotopes can also increase the amount of metabolite labelled, at times, above the detection limit, even when the endogenous compound levels are lower than detectable. However, distinction of the endogenous and labelled metabolites is still possible, allowing quantification of the naturally occurring metabolites overcoming matrix effect and increasing the accuracy of the method. However, internal standards and stable isotopes do not completely eliminate variation and all the above-mentioned problems, thus, the analysis should be completed carefully, with partial method validation (Nguyen, 2011) to ensure the accuracy and diagnostic usability of the method (Rauh, 2010).

2.9.2 Method validation

Although any LC-MS/MS method has high-resolution separations, with a wide dynamic range and enhanced sensitivity, and specificity, it still needs comprehensive standardisation and at least partial validation (Koal *et al.*, 2012) prior to implementation. This standardisation will minimise any analytical variabilities (inter- and intra-laboratory), imprecision and inaccuracy, and improve the overall quality of the quantitative results (Koal *et al.*, 2012). The experimental procedure of method validation demonstrates the reliability, precision and accuracy of the specific analytical method, ensuring that it is sufficient for the appropriate task. Furthermore, it gives an indication of the quality of the work done by the analyst, confirming that results and conclusions from the data to be obtained through the method, will be reliable and could be reported (Imre *et al.*, 2008). Validation of mass spectrometry based tests should include an evaluation of the range, linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy, selectivity, specificity and matrix effects (Zhang & Henion, 1999).

Linearity

The linearity of any analytical procedure is the ability to obtain variable data and test results within the given range, directly proportional to the sample concentration or amount of analyte in the sample (Chan *et al.*, 2004). The working sample concentration should be in a linear range to obey Beer's law of quantitation of analytes. The primary method of demonstrating linearity is by the direct dilution of standard stock solutions, where at least five concentrations of the sample should

be used. Linearity is achieved when r^2 (representation of the percentage of variation in the data explained by the linear model) is ≥ 0.997 (Chan *et al.*, 2004). An example of such a linear result is illustrated in Figure 2.9.

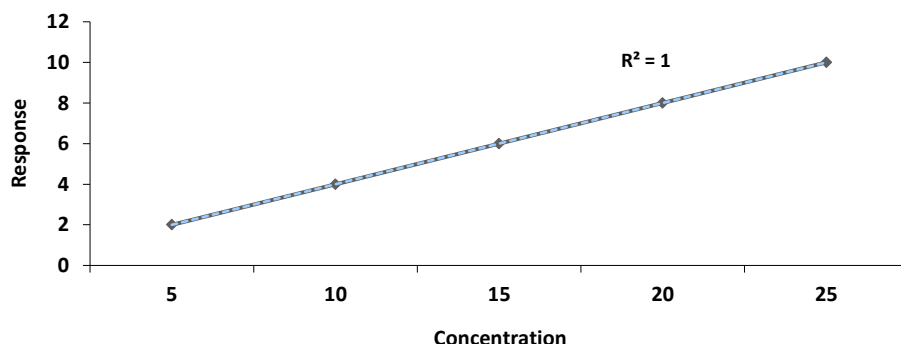


Figure 2.9: Example of linearity with a correlation coefficient greater than 0.997

Limit of detection

The limit of detection is defined as the lowest concentration of analyte present in a sample that can be differentiated from background noise to be detected (Imre *et al.*, 2008). The ratio of noise response to analyte response should be at least 1:3 to allow analytes to be successfully detected (Sanagi *et al.*, 2009). According to Westgard *et al.* (1999), this method of determining the detection limit is not completely reliable, and a regression method calculation might also assist in determining the limit of detection. Using the calibration data, the limit of detection can also be calculated by the linear regression method. This method predicts the lowest concentration at which an analyte can be detected and by using deviations from the data-points that best fits the linear line (Shrivastava & Gupta, 2011). For a linear calibration curve, as would be expected in the case of hormonal metabolites, the response of the instrument (y) is linearly related to x the concentration as in the equation of a linear line ($y=a+bx$). The equation derived from the linear line is used as seen below in Equation 1. S_a represents the standard deviation of the responses (calculated by standard deviations of y-intercepts and y-residuals of the regression line) and b represents the slope of the calibration curve (Shrivastava & Gupta, 2011).

Equation 1: The formula for the calculation of the lower limit of detection

$$LOD = 3 \times S_a / b$$

Limit of quantification (LOQ)

The lower limit of quantification refers to the lowest amount of analytes that can be analysed quantitatively with acceptable accuracy and precision (Chan *et al.*, 2004). To allow quantification, the noise-to-analytes response should be at least 1:10 (Sanagi *et al.*, 2009). As with the limit of detection, the LOQ can also be calculated through a linear regression method (Shrivastava & Gupta, 2011). The equation for these calculations can be seen in Equation 2. The upper limit of quantification is the highest concentration of analytes in biological samples that can be determined quantitatively with acceptable accuracy and precision (Imre *et al.*, 2008). The upper limit of quantification is usually accepted as the highest concentration in the calibration/linearity curves that are within the linear range (Kushnir *et al.*, 2011).

Equation 2: The formula for the calculation of the lower limit of quantification

$$LOQ = 10 \times S_a/b$$

Precision

Precision is one of the main performance characteristics and represents the reproducibility of repeated analysis (Zhao *et al.*, 2014). Precision is usually investigated for repeatability, reproducibility and intermediate (variation within the same laboratory) precision levels. Samples for precision evaluation are prepared as a standard and inside a matrix that is similar to that of targeted clinical samples to be analysed in triplicate on approximately five separate occasions. Precision measurements can be calculated by evaluation of the standard deviation, CVs or relative standard deviation (RSD) in, and between runs, and should be evaluated from samples with analyte concentrations below, above and around clinical levels (Zhao *et al.*, 2014). The percentage of RSD should not exceed 15% for all values except for the LLOQ where an RSD percentage below 20% is acceptable (González *et al.*, 2014). For intermediate precision; equipment variation, day-to-day variation and analyst variation are investigated and ideal results would be in the same range or less than repeatability variation (Chan *et al.*, 2004). A quality control (QC) sample can be used for precision experiments. A quality control sample is a sample spiked with quantified standards of metabolites, to be used for monitoring the performance of an analytical method to enable the assessment and the validity of the results for samples analysed in individual batches. It additionally also contributes to the calculation of the accuracy and precision of the method (Imre *et al.*, 2008). The QC sample is usually prepared in a large amount and is analysed at different and regular intervals. The use of QC samples also allows the monitoring of assay biasness (Sargent, 2013).

Accuracy

Accuracy is the term used to describe the closeness or nearness of agreement between the accepted reference value and the value from the results. It can also refer to the comparison of how close the measured concentration is to the concentration known to be present, more commonly referred to as the recovery of the method. Accuracy can be affected by the matrix effect, sample preparation error and calculation error (Chan *et al.*, 2004). Conventionally, accuracy is reported as a recovery percent, by performing the analytical procedure on a standard solution with known concentration, as seen in Equation 3 below. Ideally, nine determinations should be performed at three different concentration levels to cover the specified range. For acceptable accuracy, deviation of the mean concentration should be below 15% for all values, except the LLOQ, where below 20% was excepted (González *et al.*, 2014). The expectancy of accuracy would thus be between 85% to 115% for quantitative analytical methods (Chan *et al.*, 2004).

Equation 3 : Equation for the calculation of accuracy

$$\text{Overall accuracy} = \frac{\text{Response relative o metabolite calculated}}{\text{Response relative substance in theory or in standard}}$$

Dynamic range

The interval between the lower and upper concentrations of analytes in the biological sample is termed the range of an analytical procedure of which the accuracy and precision has been successfully validated. For best validation of range, low and high levels of analytes should be included in precision experiments (Chan *et al.*, 2004).

Selectivity

Selectivity of a bioanalytical method is its ability to measure as well as differentiate between analytes in the presence of components expected to be present in biological samples (Imre *et al.*, 2008). The selectivity of a method is usually assessed by evaluation of a blank matrix sample for the interference of the matrix at the retention times of the metabolites of interest, to be compared with the response of the analytes in six different matrix samples, spiked with a concentration that represents the LLOQ (all in triplicate) (FDA CDER, 2001; Imre *et al.*, 2008).

Stability

Stability of the metabolites in both stock and matrix solutions are of great importance, as this might be a limiting factor of the developed method. Good stability ensures the reliability of the results that the analytical method provides (Pawula *et al.*, 2013). Stability may include all the

situations that samples may encounter, both during preparation, analysis and storage (González *et al.*, 2014). These might include short-term and long-term storage, workbench waiting after sample preparation and freeze thaw cycles. The food and drug administration agency (FDA) guideline currently does not have acceptance criteria for the results of stability evaluations, but for all other validation analysis, deviation above 15% was unacceptable (FDA CDER, 2001; González *et al.*, 2014).

Matrix effect

The matrix effect, as previously briefly mentioned, is defined as the influence of other matrix components on the successful evaluation and quantification of the metabolites of interest (González *et al.*, 2014). Matrix effect can be overcome by injecting smaller volumes of the samples or dilution of the samples, if the sensitivity of the instrument remains adequate (Van Eeckhaut *et al.*, 2009). Optimisation of sample preparation and chromatographic parameters is another method to reduce matrix effect, or by the use of internal standards to compensate for signal alteration (Van Eeckhaut *et al.*, 2009). The matrix effect can be measured by calculation of the matrix factor (MF), which is the ratio of the response of an analyte in a biological matrix to the response of the analyte without any matrix (González *et al.*, 2014) (Trontelj, 2012) (Equation 4). At least 5 matrixes from individuals are necessary for the determination of the relative matrix effect, as there is inter-individual variability between samples and their matrixes (Trontelj, 2012).

Equation 4: Equation for Matrix factor calculation

$$MF = \frac{\text{Response of analyte in matrix}}{\text{Response of analyte in standard or solution}}$$

2.10 Method development and/or implementation

Most previously developed methods, as explained in section 2.8-2.9, used the conventional less specific methods for estrogen quantification, were developed for tissue or serum analysis, or they included only parts of the estrogen metabolism. For the comprehensive evaluation of the estrogen biotransformation, metabolic products should also be included. A more extended approach might allow the evaluation of not only parent estrogens in relation to their metabolites, but also products of estrogen metabolism, including those related to the biotransformation pathway. The most comprehensive method published, is the method as described by Gaikwad *et al.* (2008). Most of the metabolites evaluated in this method are not commercially available and the authors performed synthesis thereof. They investigated 40 estrogen-related metabolites without any derivatisation. However, in addition to these metabolites, one steroid hormone proposed to be added to this holistic approach, is progesterone. It has been reported that the steroid hormone,

progesterone, might be implicated in the regulation of a 16-hydroxyestrogenic pathway enzyme (CYP3A4), explaining why premenopausal women show a much higher activity of the CYP3A4 enzyme than postmenopausal women and men (Liska, 1998). Furthermore, inclusion of the metabolites from the 16-hydroxylation pathway, including 17-epiestriol, 16-epiestriol, estriol, 16-ketoestradiol and 16 α -hydroxyestradiol, will assist in assembly of a comprehensive profile. Sulfate and glucuronide conjugation usually omitted, evaluated separately or evaluated after deconjugation, could also be included into the profile. Additional precursors of parent estrogens might also be beneficial for expansion of the profiling.

The development of a new method, or implementation of an already developed method are dependent on a few factors (Ceglarek *et al.*, 2010; Keevil, 2016). The aim of the method, available infrastructure and availability of analytical standards, are some of these factors. The available infrastructure can include anything from the analytical instrumentation (LC-MS/MS) and the supplier thereof, column and mobile phase availability and sample preparation and desolvation instrumentation, and derivatization reagents (Chan *et al.*, 2004). The aim of the developed method may also vary; where running time or low cost might be a priority for some, low quantification limits and high sensitivity might be more important to others. The availability of certain reagents and analytical standards may also influence the method development or implementation process. When analytical standards of a compound are not readily available, some might choose to preliminarily exclude it from their method (until it becomes commercially available), while others might choose to rather synthesise the standard themselves (or initiate synthesis thereof by suppliers). The same would be the case for derivatisation reagents, mobile phase modifiers and sample preparation reagents and consumables. All of these factors would then play a part in how a method is developed, what is included and excluded, and whether implementation of a previously developed method would be a possibility (Wal *et al.*, 2010).

2.11 Problem statement

Although numerous studies have focused on the necessity and advantages of estrogens in the human reproductive system (Epstein *et al.*, 1999; Fortner *et al.*, 2012; Sunyer *et al.*, 1999), the association between estrogen metabolites in the urine and the risk of cancer development is still an growing field of investigation (reviewed (Ziegler *et al.*, 2015). Various literature points towards estrogens being initiators of cancers, especially breast cancer, and estrogens have even been added to the list of possible carcinogens (Miller, 2003; Twombly, 2003). A link between the urinary estrogens and relative cancer risk has been made, and evidence has shown that the evaluation of estrogen-related metabolites in the urine might aid in cancer risk evaluation (Ziegler *et al.*, 2015). In previous studies on the estrogen-cancer relation, little focus has been directed towards the role of exogenous estrogens in the form of combined oral contraceptives on the estrogen

metabolism and especially biotransformation profiles. A few studies that have evaluated the relationship between oral contraceptives and the risk of breast cancer (Calle *et al.*, 1996; Collaborative Group on Hormonal Factors in Breast Cancer, 1996), have to our knowledge only obtained physical and clinical data that lack biomedical confirmation or evaluation. Moreover, the use of metabolic markers in biofluids (e.g. urine) is less invasive, less expensive, has high throughput and could be useful in monitoring patients over time and in clinical settings. The measurement of urinary estrogen metabolite profiles in women that use COCs would, therefore, be of great value to compare their relative breast cancer risk to those of non-users, and to evaluate what part of their estrogen metabolism would be effected (as indicated by metabolic profiles). No in-house method or even similar methods to previously developed estrogen evaluating methods exist at the Centre for Human Metabolomics Potchefstroom. The available infrastructure and standards are not identical to those in previously developed methods and, therefore, an in-house method development with the currently available resources was necessary, rather than implementation of previously developed methods.

2.12 Aim and objectives

2.12.1 Aim

The aim of the study was therefore to develop a highly sensitive and selective urine-based liquid chromatography–electrospray tandem mass spectrometry method to investigate the estrogen metabolism in women.

2.12.2 Objectives

Firstly, to fully utilise the capabilities of the LC-MS/MS instrument to develop a highly sensitive and selective high-performance method for quantifying estrogens and estrogen-related metabolites in urine. This will be achieved by optimizing the sample preparation method, the liquid chromatography parameters as well as the mass spectrometry parameters.

Secondly, to validate the developed method to ensure optimal quantification of metabolites when biological samples are analysed. The validation parameters will include accuracy, precision, stability and selectivity as well as matrix effect and dynamic range.

Thirdly, to implement the method for the analysis of urine samples from control women and women using COCs in order to confirm that the validated method can be used to detect all the various estrogen-related metabolites in both groups of women. These results will confirm if the method would allow both detection and quantification of metabolites in urine samples.

2.12.3 Experimental strategy

Figure 2.12 illustrates the experimental strategy employed during the study to satisfy the aim of the study. The aim of the study will be achieved by developing an analytical method, consequently optimising the fundamental parameters of the LC-MS/MS system (objective 1). The method development will include the choice of the mobile phases, chromatographic column and gradient choice as well as precursor and product ion selection. Choice of sample preparation method will also be performed. Optimisation may include the mass spectrometry voltages and temperatures, mobile phase flow and pH, the run times and chromatographic separation as well as the sample preparation steps. To ensure the reliability and to investigate the limitation, the developed and optimised method will then be subjected to method validation (objective 2). These validation parameters will include the measurement and calculation of the lower limits of detection and quantification, the linearity of the analysis as well as the accuracy, precision, matrix effect and stability. Additionally carry-over and selectivity will also be analysed. Lastly, the utility of the method will be evaluated (objective 3) by the analysis of female urine samples on the developed method. These analysis results will not be used to draw any conclusions on the effect that the use of COCs have on the estrogen metabolism, but rather to confirm the successful detection of the metabolites in the presence of biological variation.

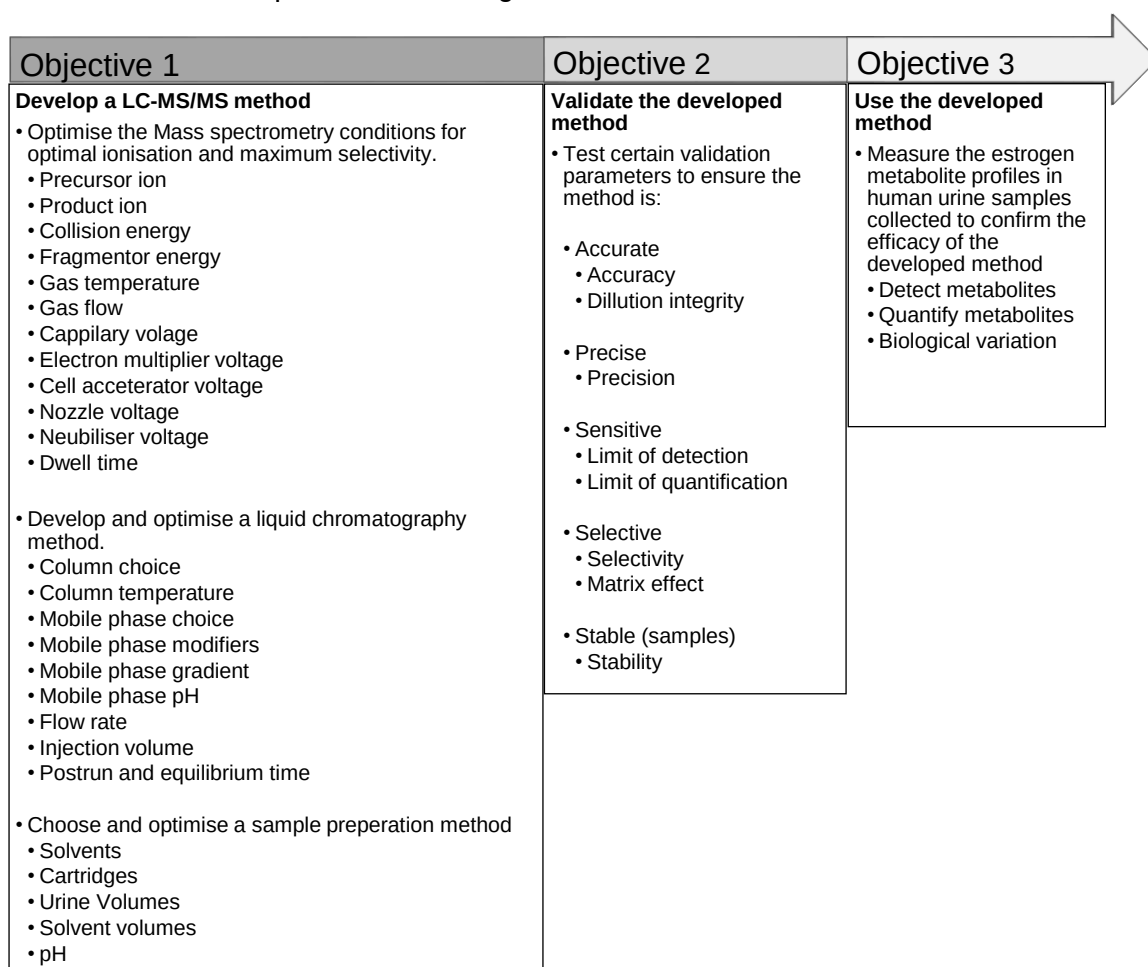


Figure 2.12: The Experimental strategy depicting the objectives of the study. This study was divided into three different objectives. The first is to develop a method for estrogen metabolite analysis in urine, the second to validate the developed method and lastly to apply the method on a group of samples.

CHAPTER 3

MATERIALS AND METHODS

3.1 Analytical standards, solvents and chemicals

The following hormone analytical standards (with catalogue codes as indicated) were purchased from Sigma Aldrich Co., now ©Merck KGaA, Darmstadt, Germany: E1 (46573), 17 α -E2 (46542), 17 β -E2 (E1132), progesterone (1568007), androstenedione (46033), testosterone (46923), dehydroepiandrosterone (D4000), dehydroepiandrosterone sulphate (D5297), E3-3-sulphate (E6375), E2-3-sulphate (E9505), E1-3-sulphate (E0251), E1-3-glucuronide (E1752), E2-3-glucuronide (E2127), E3-16-glucuronide (E1877). Analytical standards for 2-OHE1 (E1130-000), 4-OHE1 (E1170-000), 16 α -OH-E1 (E1250-000), 2-ME1 (E1148-000), 4-ME1 (E1175-000), 2-OHE2 (E2470-000), 4-OHE2 (E2500-000), 2OHE1-3-methyl ether (E1151-000), 2-ME2 (E2490-000), 4-ME2 (E2510-000), estriol (E2600-000), 16-epiestriol (E2850-000), 17-epiestriol (E2570-000), 16-ketoestradiol (E1400-000), and estradiol-17-sulphate (E1103-000) were purchased from Steraloids Inc., Newport United States. Isotopes of E1 (E2300-010), E2 (E0950-014), E3 (E2600-016), progesterone (Q2600-014) and DHEA (A8500-009) were purchased, through a South African supplier, Stargate scientific, while isotopes of 2&4-OHE1 were purchased from Toronto research chemicals (TRC), Ontario, through Industrial analytics. Dansyl chloride (03641), formic acid (94318), ammonium fluoride (338869), ammonium formate (70221), ammonium hydroxide (44273), sodium hydroxide (S8045), hydrochloric acid (320331), acetic acid (27225), 2-acetamidophenol (A7000), chlorotrimethylsilane (TMCS, 89595) pyridine (270970) and N,O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA, 15222) were purchased from Sigma Aldrich Co, while ammonium acetate (32301) was purchased from Riedel-de Haën. Ammonium (A2536) was purchased from BDH limited, Poole, England. Solvents used on the LC-MS/MS instrument from Honeywell, Burdick & Jackson, supplied by Anatech, South Africa, included high purity water (365-4), methanol (230-4), acetonitrile (017-4), acetone (010-4) and isopropyl alcohol (323-4).

3.2 Instrumentation

For the sample preparation, a Phenomenex SPE column vacuum manifold (AHO-6024) with SPE cartridges including Phenomenex, Strata X (8B-S100-UBJ) and Strata C18 (8B-S002-FBJ) was purchased through South African supplier Separations. From Agilent Technologies, Santa Clara, CA, SPE columns, Bond elute PH (12113031) and Bond elute C18 (14102001), were purchased through Chemetrix as national supplier. For the LC-MS/MS analysis, the LC system used was an Agilent 1290 Infinity, with a binary pump (G4220A), vacuum degasser (G1330B), thermostat autosampler (G4226A) and a temperature-controlled column compartment (G1216C). For UV evaluation, an Agilent 1200 series variable wavelength UV detector (G1315C) was coupled to the LC. For all other analyses, the LC system was coupled to an Agilent 6460 triple quadrupole mass spectrometer (G6460A), with a jet stream ESI source. For the brief GC analysis, an Agilent Gas chromatogram (7890A) with mass spectrometer inert XL MSD and capillary GC-MS column (30

x 0.25 x 0.25; JW Scientific, DB1-MS) was used. The Agilent instruments were supplied by Agilent Technologies, Santa Clara CA, USA, though local supplier Chemetrix. Data acquisition and quantification of the analytes was done using Agilent MassHunter workstation software, these included 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). Additional software programs used for ionisation optimisation conditions included Agilent technologies MassHunter workstation software Optimizer for 6400 series triple quadrupole (B.07.01) and Source and iFunnel Optimizer for 6400 triple quadrupole (B.07.01) for source optimisation.

For the chromatographic separation of the analytes, various columns were compared, including an Infinity lab Agilent Poroshell 120 end capped (EC) -C18 column (2.1 mm x 50 mm, 2.7 micron particle size; 95974-902), an Agilent Poroshell 120 EC-C18 column (2.1 mm x 100 mm, 2.7 micron particle size; 695775-902), Agilent Zorbax Stable Bond– Phenyl column (2.1 mm x 100 mm, 1.8 micron particle size; 828700-912), Agilent Zorbax Eclipse column plus C8 columns (2.1 mm x 100 mm, 1.8 micron particle size; 95976-906), Agilent Zorbax Eclipse plus C18 column (2.1 mm x 100 mm, 1.8 micron particle size; 95976-902), rapid resolution high definition Agilent Zorbax Eclipse plus C8 column (2.1 mm x 100 mm, 1.8 micron particle size; 959758-906) and Agilent Pursuit 3 pentafluorophenyl (PFP) column (3 mm x 100 mm, 3 micron particle size; A3051100X030). These columns were also supplied by Agilent Technologies, Santa Clara CA, USA. Additional supportive instrumentation included an Eppendorf centrifuge (5414R), purchased from Merck ; a Heraeus multifuge X3R centrifuge, supplied by Thermo scientific; a Mermle centrifuge (2206A) supplied by Lasec/SA, Agela Technologies; BFC nitrogen evaporator purchased from Stargate scientific; a WX vortex and RC series heating plate from Velp Scientific, as supplied by Amanzi Tech; a Modop Digital sonicator; a WTW series inolab pH meter 720 and a Mettler Toledo classic plus balance (AB265-s/fact). A Rotor Torque rotator for evaluation of LLE for sample preparation and Virtis freeze dryer were also additionally used.

3.3 Methods

3.3.1 Preparation of standard stock solutions

Quantifying unknown concentrations can be quite complex and thus samples with known concentrations are prepared first, in order to quantify the unknown (Sargent, 2013). The stock solutions of all the estrogens were prepared at concentrations of 0.1 mg/mL (100 ppm) in methanol, and further diluted to 10 ppm and 1 ppm stock solutions in MeOH. The standards were then stored at -80°C until used for method development.

3.3.2 Preparation of working stocks and calibration standards

Before preparation of working and calibration standards, the physiological concentrations of each of the metabolites in human urine were investigated from different sources. Where possible, concentrations in the luteal phase of the reproductive cycle were used. Table A1 (Annexure A) summarises the reference ranges and the literature source from which the data was obtained to enable calculation of average reference ranges to be used throughout the rest of the study. Working and calibration standards, and standard solutions, are usually solutions of an analyte, of which the amount of analyte in a suitable matrix or solvent is known (Sargent, 2013). For method development and validation, both urine and solvent-based standard mixtures were prepared. For solvent-based working solution and calibration standard preparation, the stock solutions were diluted to the concentrations required. For the matrix-based working solution and calibrations, in the relevant matrix, urine was prepared, in which a known amount of analyte was spiked in order to develop the method and eventually construct calibration curves. These samples, as well as the blank samples, should have a similar composition and similar properties as the biological samples that will be analysed, in other words, they must contain the same matrix in order to yield the same response (Sargent, 2013). Due to the metabolites of interest being naturally present in female urine (also in low concentrations in most male urine), the urine samples were all evaluated as unspiked samples as well. Methanol-prepared standard solutions with the spiked concentration, without matrix influence, were also evaluated. This allowed compensation of naturally occurring metabolites. All working stocks and sample clean-up assays were investigated by preparing a standard mixture with a final concentration of 50 ng/ml in methanol. The concentration was chosen in such a way that it would be relatively in the middle of the expected range for most of the metabolites after sufficient concentration during sample preparation, without overloading the sorbents.

3.3.3 Freeze drying

Freeze drying, a method to dry aqueous solvents that are less likely to evaporate under nitrogen with lower freezing points such as water, was performed throughout the study. Before freeze drying, the sample or eluent was frozen into a solid for at least 12 hours at -80°C, followed by the drying process. For freeze drying, each sample tube or cap was punctured to allow the vacuum into the sample. Samples were then placed in the freeze dryer flasks that were individually attached and the quick seal valve was closed. The freeze-drying process was allowed for 18-36 hours, depending on the sample volume.

3.3.4 Sample preparation

Sample preparation methods, including solid phase extraction and liquid-liquid extraction methods, will only be described briefly, and adjustments and expansions will be discussed later on. For SPE, the SPE cartridge was placed inside the vacuum manifold and the vacuum set in

such a way as to allow an approximate flow rate of 3 ml/min. The adsorbent that was inside the SPE cartridges was then washed with MeOH, followed by two column volumes of water for sorbent conditioning. The adsorbent bed was not allowed to run dry before the next step, which included sample loading. Before loading, the samples (standard or matrix) were centrifuged and only the supernatant was diluted with water to a total volume twice the SPE column capacity, and unless indicated otherwise, the pH was adjusted to 7. The sample was then loaded in two volumes onto the adsorbent, again without allowing it to run dry. The adsorbent was washed with water or higher organic composition solvents and the sorbent was allowed to dry for 10 min under full vacuum. The last step of the SPE included the elution of the metabolites of interest into a polypropylene tube at approximately 1 ml/min with methanol or another organic solvent, of at least two column volumes. Again, the sorbent was allowed to dry. Any of the washing or eluent fractions collected, containing solvent, were dried under a gentle stream of nitrogen gas and those that contained water were freeze dried. If both were present, the organic solvent was evaporated first, followed by freeze drying the remaining aqueous solution. During LLE sample preparation, the specific volume of sample (1-5 ml) was added to a cimax tube. To this sample, two larger volumes of highly organic, water immiscible solvents were added. The sample was then either mixed on a rotor-torq mixer, or vortexed before being centrifuged for 10 min at 2800 g. The organic solvent was then transferred to a clean cimax tube with a glass Pasteur pipet and the process was repeated with the aqueous phase and another organic solvent. Afterwards, the combined organic solvent products were evaporated to dryness under a gentle stream of nitrogen gas. Once resuspended, all samples were filtered through 0.2 µm Nylon Spin-X filters for LC, after which the supernatant was transferred to inserts in LC-MS analysis vials.

3.3.5 Ionisation following direct infusion

The direct infusion into the ionisation source method was performed with the Agilent auto sampler, where 0.1 µl of each prepared standard was injected directly into the ionisation source. The mobile phase consisted of water (H₂O) with the chosen modifier as solvent A and acetonitrile (ACN), methanol (MeOH), or a combination of the two as solvent B, operating at a flow rate of 0.2 ml/min. A 50:50 combination of solvent A and B was employed. The general mass spectrometer conditions included ESI as ion source with positive ionisation (0 V nozzle voltage and 3000 V capillary voltage) and negative ionisation (nozzle voltage 1000 V and 4000 V capillary voltage) using nitrogen gas with sheath gas flow of 8 L/min and a drying gas flow of 11 L/min. The sheath gas temperature was set at 350°C and the drying gas temperature at 250°C. The mass spectrometer was set to do scans from 100 to 1000 m/z in MS². All the parameters are in the default range for the instrument as in the informational user manual. After identification of the metabolites ionised, the same analytical standard solutions were also evaluated in the Optimizer program to indicate which of the metabolites product ions were detected. The Source and iFunnel Optimizer program were also used to optimise the source conditions as described in Chapter 4.

3.3.6 LC-MS/MS analysis of samples

Liquid chromatography was carried out on a reversed-phase packing column to evaluate and compare the columns and to optimise conditions for the chosen sample. The column temperature was maintained at 40°C unless stated otherwise. Five microliters of each sample was injected into the column for separation. Mobile phase A consisted of water with the chosen mobile phase modifier as solvent A and acetonitrile, methanol or a combination of the two as solvent B, operating at a flow rate of 0.3 ml/min. A gradient of solvent A:B from 10:90 to 0:100 was employed. The general mass spectrometer conditions included the conditions as described for default direct infusion, and the mass spectrometer was set to MRM mode with nitrogen as the collision gas. Unless indicated otherwise, the EMV was +300 V and -300 V, CAV was 7 V, and the dwell time for each compound was 100.

CHAPTER 4

UNDERIVATISED METHOD DEVELOPMENT

4 Introduction

Bioanalytical methods for the quantification of drug metabolites in physiological matrixes such as blood and urine, can be helpful in both research and in clinical pharmacology (Wal *et al.*, 2010). These methods, however, need to be developed and validated before any application can occur. For the LC-MS/MS method to fully exploit the capabilities of the instrumentation and software, and give sensitive and selective quantification results, conditions of the method need to be optimal. Method development, therefore, include as illustrated in Figure 4 the optimisation of the LC conditions to ensure sufficient separations of the compounds, optimisation of the MS conditions, as well as sample preparation procedures, which is then followed by method validation procedures.

Method development outline:

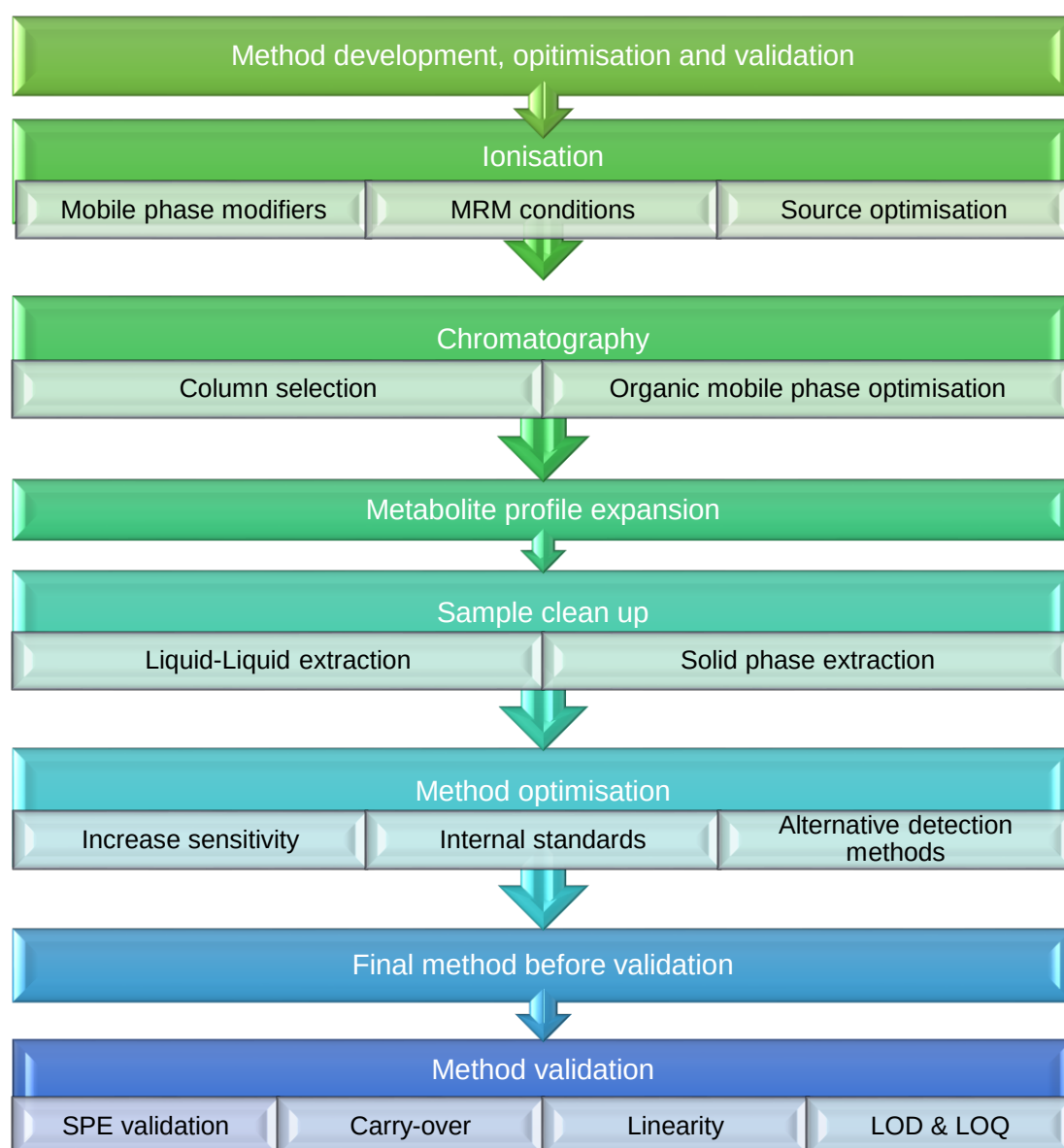


Figure 4: Experimental outline for the non-derivatised method development part of the study. This illustration shows the method development outline for this study.

The parameters that were optimised, optimisation results and a short discussion of each result will be given throughout this chapter. Although previous articles were published on 'methods to investigate a profile of estrogen metabolites in urine' (Gaikwad *et al.*, 2009; Xu *et al.*, 2005), these methods cannot simply be adopted and implemented. Variation in the available infrastructure, and in the instruments and columns available, requires method development to be done on the instruments that are available and under the conditions that are present in a specific laboratory. During method development, the literature was consulted in choices of certain factors, however, most were still evaluated and compared to optimise the final method and to satisfy the aim of this study. For this study, an underivatised method was developed, optimised and partially validated to a point. From the results, sensitivity issues were observed and a derivatisation method, as described in Chapter 5, was consequently developed, optimised and validated.

4.1 Mobile phase modifiers

The initial steps in LC-MS/MS analysis, where mass spectrometers are used as a sensitive detection source, are to determine whether an analyte or analytes will ionise under certain conditions or not (Sargent, 2013). The mobile phase acting as a carrying medium to pump the analytes through the instrument should also aid in the ionisation process. In order for the mass spectrometer, as a detector, to detect any analyte, the analyte should first be ionised through ESI, either by receiving or donating a proton. This accepting or donating of a proton by the metabolites of interest, is a mechanism which the so-called modifiers inside the mobile phase might assist with. The best ion polarity (positive/negative), for maximum detection of each metabolite, was also determined during the comparison of different mobile phase modifiers for ionisation capacities.

Formic acid (Gaikwad *et al.*, 2008; Gaikwad *et al.*, 2009), ammonium acetate (Sargent, 2013; Zhao *et al.*, 2014), ammonium fluoride (Hindle, 2013; Yanes *et al.*, 2011) and ammonium hydroxide (Fayad *et al.*, 2013; Naldi *et al.*, 2016) were evaluated and compared as possible mobile phase modifiers. This was performed using 10 ppm stocks of the 20 most relevant estrogen and related standards, following the direct infusion method and scan conditions as described in Chapter 3 with mobile phases water and acetonitrile, and the concentration of modifier, as described in the literature. The scans allowed precursor ion identification for each metabolite, which was then used in the optimiser program to identify the most abundant product ions for each precursor ion. Although previous articles indicated that 0.1% formic acid, as mobile phase modifier, gave detection in positive ionisation, the same success was not obtained for the metabolites of interest. Only two of the metabolites produced product ions with this modifier in positive ionisation mode; for negative ionisation, again, only two metabolites produced sufficient product ions. Ammonium acetate, which is a more aggressive proton-donating modifier, gave

better ionisation of the metabolites of interest than the formic acid in ionisation. With 5 mM ammonium acetate in both mobile phases, product ions for three metabolites were detected in positive ionisation mode and for seven metabolites in negative ionisation mode. For more reproducible results, ammonium fluoride (1 mM) is stable in a range of different solvents as a mobile phase modifier. Due to the very basic properties of the fluoride ion (F^-), previous reports show it to increase deprotonation of neutral steroids during ESI (Yanes *et al.*, 2011). After evaluation of ammonium fluoride as mobile phase modifier, 16 of the metabolite standards gave detectable product ions (2 in positive and 14 in negative ionisation mode). Ammonium hydroxide as modifier, did not seem to increase detection any more than the ammonium fluoride; 3 metabolites were detected in positive ionisation mode and only nine metabolites gave sufficient product ions for negative ionisation. A comparison of the metabolites that were successfully ionised for each mobile phase modifier, is presented in Table S1 (supplementary results).

Literature suggests that the presence of lower ammonium salt concentrations in the mobile phases may restrict the effect of interactions of the basic compounds with the column silica surface, by acting as a counter ion (Sargent, 2013). Despite this, ammonium fluoride as mobile phase modifier, still gave the most sufficient ionisation results. To confirm that it will result in detection of the four metabolites not yet detected, the injection volume was adjusted to 0.5 μ l instead of 0.1 μ l; the remaining four metabolites gave product ions with high abundances. These comparative results led to the choice of 1 mM ammonium fluoride to be used as the modifier in the mobile phases for the analysis of the chosen 20 estrogenic metabolites and their chromatographic separations. The concentration of the modifier was also optimised to be as low as possible without disturbing the ionisation potential. This was, however, done only after column separation was evaluated and is described in section 4.7.

4.2 Optimisation of ionisation conditions

After the choice of 1 mM ammonium fluoride as the modifier in the mobile phase A and the provisional choice of 100% acetonitrile as mobile phase B, the ionisation and MRM conditions could be optimised by using the Agilent MassHunter Optimizer software. This optimisation was performed on 0.5 μ l of 10 ppm standards, as described in Chapter 3.3.5. The optimiser settings and parameters evaluated, included fragmentor voltage at coarse range from 40 V to 200 V in fine steps of 5, and collision energy range from 0 eV to 50 eV. The processed results of the Optimizer software can be seen in Table 4.1, indicating the choice of ionisation mode, the fragmentor voltage, the most abundant fragments, and the collision energy for each fragment. For each of the metabolites, the most abundant product ion was chosen, except for 2-

hydroxyestradiol, 2-methoxyestradiol, and 2-hydroxyestrone-3methyl ether, where only one product ion was obtained.

Table 4.1: The processed results of the optimiser program. The results shown include the formula, chosen polarity for ionisation, precursor ion, fragmentor voltage, two most abundant product ions, and collision energies for optimal ionisation of the estrogen metabolites.

Compound Name	Formula	Polarity	Precursor m/z	Fragmentor energy (V)	Product m/z	Collision energy (eV)
16-Epiestriol	C ₁₈ H ₂₄ O ₃	Negative	287.2	148	171.1	36
16-Ketoestradiol	C ₁₈ H ₂₂ O ₃	Negative	285.1	163	145.1	40
16 α -Hydroxyestrone	C ₁₈ H ₂₂ O ₃	Negative	285.1	148	145.1	40
17-Epiestriol	C ₁₈ H ₂₄ O ₃	Negative	287.2	148	171.1	40
17 α -Estradiol	C ₁₈ H ₂₄ O ₂	Negative	271.2	168	145.1	40
17 β -Estradiol	C ₁₈ H ₂₄ O ₂	Negative	271.2	158	145.1	44
2-Hydroxyestradiol	C ₁₈ H ₂₄ O ₃	Negative	287.2	183	147.0	28
2-Hydroxyestrone	C ₁₈ H ₂₂ O ₃	Negative	285.1	163	161	40
2-Hydroxyestrone-3-methyl ether	C ₁₉ H ₂₄ O ₃	Negative	299.2	119	284.1	20
2-Methoxyestradiol	C ₁₉ H ₂₆ O ₃	Negative	301.2	121	286.1	20
2-Methoxyestrone	C ₁₉ H ₂₄ O ₃	Negative	299.2	114	284.1	20
4-Hydroxyestradiol	C ₁₈ H ₂₄ O ₃	Negative	287.2	124	255.1	12
4-Hydroxyestrone	C ₁₈ H ₂₂ O ₃	Negative	285.1	153	161.1	44
4-Methoxyestradiol	C ₁₉ H ₂₆ O ₃	Negative	301.2	109	286.1	16
4-Methoxyestrone	C ₁₈ H ₂₄ O ₃	Negative	299.2	109	284.1	16
Androstenedione	C ₁₉ H ₂₆ O ₂	Positive	287.2	109	97.1	20
Estradiol 17-sulphate	C ₁₈ H ₂₃ O ₅ S	Negative	351.2	158	97	44
Estriol	C ₁₈ H ₂₄ O ₃	Negative	287.2	163	171.1	36
Estrone	C ₁₈ H ₂₂ O ₂	Negative	269.1	156	145.1	40
Testosterone	C ₁₉ H ₂₈ O ₂	Positive	289.2	109	97.1	20

4.3 Optimisation of source conditions

After obtaining the optimal fragmentor and collision energy voltages for each steroid hormone metabolite, the optimal mass spectrometry source conditions were evaluated, using the Agilent MassHunter Source and iFunnel Optimizer software. The source parameters needed to be optimised in such a way that the temperatures chosen produced enough heat to evaporate the solvent under the ESI conditions but not that high that decomposition occurs. The flow rate should also go along with sufficient temperatures to ensure they have enough time for evaporation

(Sargent, 2013). The software permitted the optimisation of the drying gas flow and temperature, the sheath gas flow and temperature, the capillary and nozzle voltages as well as the nebuliser voltage. Direct infusion, as described in Chapter 3.3.5, was done with the exception that the mass analysers were set to multiple reaction monitoring (MRM) configuration instead of scan mode. All parameters were kept on default except for the parameter being investigated, and the specific fragmentor and collision energy voltages. The MRMs for each metabolite were prepared with the collision energy and fragmentor energies as seen in Table 4.1. The details on the parameters that were varied during the source optimisation process, can be seen in Table 4.2, which points out the range of the parameter as well as the steps taken within the ranges. The raw data of the source optimiser was processed to get one most suitable temperature, voltage or flow for all the metabolites. Processing was primarily based on ionisation capacities and physiological concentrations of each metabolite, to ensure detection of all the metabolites under the chosen conditions. The final conditions chosen for the 20 hormone metabolites include a capillary voltage of 4000 V, drying gas and sheath gas flows of 8 ml/min and 12 ml/min, respectively. A Nebuliser voltage of 45 V and a nozzle voltage of 500 V were proven to be most optimal, accompanied by a drying gas temperature of 200°C and a sheath gas temperature of 400 °C. These source parameters were from this point on, used throughout all analyses, unless indicated otherwise.

Table 4.2: The table demonstrating the parameters varied during the source optimisation. This includes voltages such as capillary voltage, nozzle and nebuliser voltage, drying gas and sheath gas flow, drying and sheath gas temperatures and the increments of change within each range.

	Starting value	End value	Increments
Capillary voltage (V)	2000	4500	500
Nozzle voltage (V)	0	2000	500
Nebuliser voltage (V)	25	50	5
Drying gas flow (L/Min)	8	12	2
Drying gas temperature (°C)	150	350	25
Sheath gas temperature (°C)	200	400	50
Sheath gas flow (L/min)	6	12	2

4.4 Liquid chromatographic separations

The first step in the development and optimisation of the liquid chromatographic separation part of the analytical method, was to choose a chromatographic column that will successfully separate

the estrogen metabolites based on their polarity. Together with the column choice, the organic mobile phase was chosen, as its composition affects chromatographic separations. The columns used in previously described studies, especially regarding estrogenic metabolites, were investigated and compared to see if they might result in sufficient separation. As described, the MRM method was followed according to the ionisation and MRM conditions, mobile phase A modifiers and source conditions, as optimised in section 4 up until this point. For the column choice, the separation of the 18 estrogens in negative ionisation mode was investigated first and the separation of the two metabolites with positive ionisation was confirmed in the end, using the same method employed for negative ionisation. For each column, 3 different organic mobile phases were evaluated: 100% acetonitrile, 100% methanol and a 1:1 mixture of acetonitrile:methanol. Methanol has a weaker elution strength of the analytes than acetonitrile, thus, more effective desolvation can occur with methanol as an organic mobile phase to enhance the ESI response. Acetonitrile, on the other hand, is less viscous than methanol and will not cause the higher back pressure associated with methanol, and the earlier elution of analytes may result in a shorter run time (Sargent, 2013). Subsequently, three different mobile phase compositions were evaluated. For each column, a few different mobile phase gradients were evaluated, based on a principle published by Wal *et al.* (2010), to determine whether an isocratic or gradient mode would be optimal. It is recommended that a gradient is initially run, where after the ratio between the difference in gradient, between the last and the first analyte to the total gradient time, is calculated. Any ratio >0.25 indicates that a gradient will be more beneficial for separation than isocratic conditions (Wal *et al.*, 2010). This was also applied here, in order to try and find optimal separation on the column for that specific mobile organic phase. A 0.1 µl of the standard solution (100 ppb) was injected into the chromatographic column (35°C), with varying binary pump flow rates for each column. The same flow rates to that of 100% acetonitrile mobile phase were used, unless indicated otherwise. Lower flow rates were applied for methanol (mobile phase B), to reduce the extremely high binary pump back-pressure caused by methanol. In comparison to methanol, acetonitrile can give an up to 2 times lower pressure than methanol (Wal *et al.*, 2010). The analytes were evaluated, based not only on their retention times but also by statically scanning only for the specific precursor and product ions (transition from the precursor m/z to the product m/z). Provided that the transitions differ and the MS is able to distinguish between a metabolite and co-eluting metabolites, co-elution should not in large contribute to interference in the ionisation process (Sargent, 2013).

The versatility of Octadecyl silane (C18) reverse phase stationary materials made this an obvious option for the separation of hormone-related metabolites. In various articles and application notes, C18 columns were used for separation of underivatised steroid hormones, including Acquity ultra performance liquid chromatography BEH C18 (Gaikwad *et al.*, 2008; Gaikwad *et al.*, 2009),

Agilent Poroshell 120 C18 (Roškar & Lušin, 2012) reverse phase Phenomenex Luna C18 column (Qin *et al.*, 2008; Todorovic *et al.*, 2001), Keystone Betasil C18 column (Zhang & Henion, 1999) and an Agilent Zorbax Extend-C18 (Agilent-Technologies, 2011; Naldi *et al.*, 2016). These and many more references led to the investigation of columns with C18 packing materials. For the study, Agilent Poroshell 120 EC-C18 column (2.1 x 50 mm, 2.7 μ M), Agilent Poroshell 120 EC-C18 column (2.1 x 100 mm, 2.7 μ M) and an Agilent Zorbax eclipse plus C18 (2.1 x100 mm, 1.8 μ M) columns were available. C18 columns are in general, the more widely used type of column because they can resolve a wide variety of compounds due to high plate counts and their high selectivity (Wal *et al.*, 2010). The Agilent Poroshell 120 EC-C18 column (2.1 x 50 mm, 2.7 μ M) and Agilent Zorbax Eclipse plus C18 (2.1 x100 mm, 1.8 μ M) were evaluated using the MRM method as previously described. With acetonitrile as solvent B, a few different mobile phase gradients were applied with 0.4 ml/min (Poroshell) or 0.25 ml/min (Zorbax) flow. The combination of methanol and acetonitrile in mobile phase B, gave the best separation for the Poroshell 120 EC-C18 column. Although some peak broadening was detected, the column gave good initial separation of metabolites with identical transitions. Good separation and sharp peaks were obtained with the Zorbax eclipse column, with the best separation on 100% acetonitrile as the organic mobile phase. When chromatograms were compared to those obtained from the Poroshell column, quite identical separations were obtained, although the 1:1 methanol:acetonitrile combination on the Poroshell 120 EC-C18 column (2.1 x 50 mm, 2.7 μ M) gave slightly better separation between metabolites with the same MRM transitions.

Although separation on the C18 columns seemed adequate, the results were compared to those from other columns, the first being the Agilent Zorbax SB – Phenyl column (2.1 x 100 mm, 1.8 μ M). According to the literature and an Agilent application note, the presence of π - π interactions inside phenyl bonded phase columns gives these columns the ability to provide excellent alternatives to the C18 columns (Hunter & Sanders, 1990). This is especially true for difficult separation of closely related compounds (Mack, 2009), such as the estrogen metabolites. An Agilent Pursuit 3 PFP column (3 x 100 mm, 3 μ M) was also investigated for sufficient separation. In an application note published by Agilent on the improved analysis of trace hormones in drinking water by LC/MS/MS, using the Agilent 6460 Triple quadrupole LC/MS, an Agilent Poroshell 120 phenyl-hexyl column (3.0 x 100 mm, 2.7 μ M) was used and proved to be ideal for the separation of aromatic compounds (Hindle, 2013). Just like the phenyl-hexyl material, pentafluorophenyl (PFP) bonded phase columns (with pentafluorophenyl groups bound to the silica surface) also have selectivity for aromatic characteristics. The increased sensitivity is due to highly electronegative fluorine atoms on the periphery of each phenyl ring (Phenomenex, 2016a). Furthermore, positional isomers show increased separation on PFP phases. This, as well as the

increased sensitivity when separating aromatic compounds, made the PFP column worth investigating.

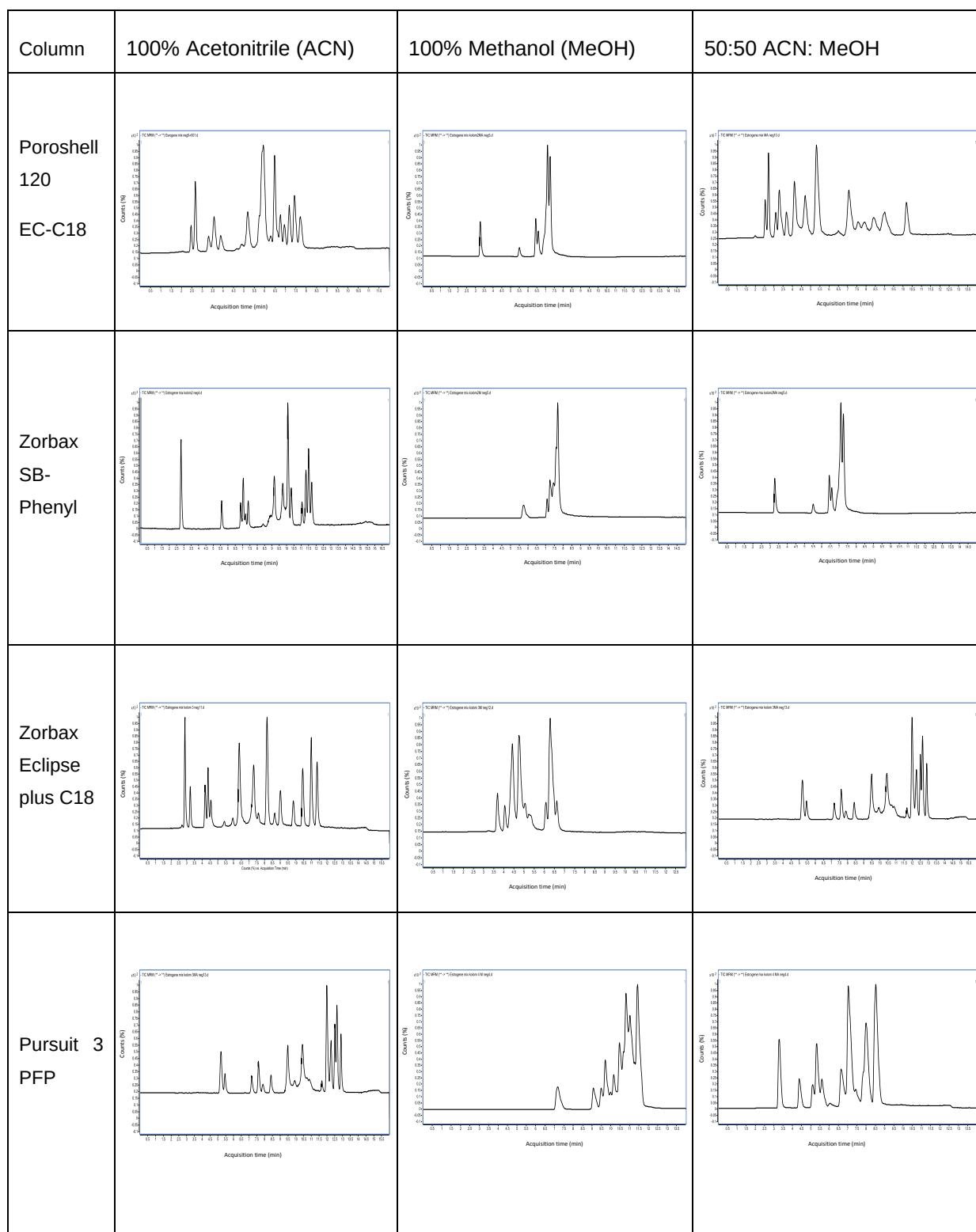


Figure 4.4.1: Summary of the results of the total ion chromatograms of the four columns investigated with the three variations in the organic mobile phases. A concentration of 100 ppb standard solution was injected onto a Poroshell 120 EC-C18, a Zorbax eclipse C18, a Zorbax SB-phenyl and Pursuit 3 PFP columns. The mobile phase B compositions included 100% acetonitrile, 100% Methanol and 50:50 acetonitrile:methanol.

For testing of the columns mentioned above, the MRM method described for the previously investigated columns was used, with mobile phase binary pump flow rates of 0.3 ml/min and 0.4 ml/min, respectively. For the Zorbax SB column, 100% acetonitrile gave the best metabolite separation results, although the separation was not as effective as with the C18 columns. Overall, the PFP column gave very broad peaks with poor separation, especially with methanol and a 50:50 mixture of methanol and acetonitrile. In an attempt to make the peaks sharper and increase separation, a lower volume of 0.1 μ l was injected (in case of column overloading), and alternatively, the temperature was reduced to 25°C, however, none of these variations gave satisfactory separation when compared to the other columns. Comparisons of the total ion chromatogram results for these columns can be viewed in Figure 4.4.1.

Comparison of the four columns indicated that there were two columns that gave better separation for the 20 estrogen metabolites. These two columns included the Poroshell 120 EC-C18 (50 mm) with the organic phase of 1:1 acetonitrile:methanol and the Zorbax Eclipse plus C18 column with 100% acetonitrile as the organic phase. For both of these columns, the peaks were extracted using the MassHunter Workstation Quantitative Analysis software (©Agilent Technologies, Inc. 2008), which gives the peak separation of the metabolites with the same MRM transitions. These results were then used to aid in the choice of which of these columns should be used. Some of the results, used as motivation in the final column choice, can be seen in Figure 4.4.2. For the transition of 285.15- $\rightarrow$ 161.10, it is visible that the Zorbax Eclipse plus gave two peaks, while the Poroshell 120 gave even better separation, yielding three peaks for that specific transition. The same trend was observed for the 287.16 > 147.00 transition. However, as seen for the transition 271.17- > 145.10 (which is the precursor and product ion m/z for 17 α -estradiol and 17 β -estradiol), the Zorbax Eclipse gave sharper peaks than those of the Poroshell 120. The separation between all other metabolites (with identical transitions) were very similar between the two different columns as Figure 4.4.2 illustrates for transition 287.16- $\rightarrow$ 145.10 and 285.15- $\rightarrow$ 145.10. The results led to the choice of using the Poroshell 120 (50 mm) instead of the Zorbax Eclipse plus. The Poroshell gave quite broader peaks, a problem solved by adjusting basic parameters in the methods or by using a longer (100 mm) available column. Although the Zorbax Eclipse yielded very neat sharp peaks, it did not result in sufficient separation for certain transitions with the preliminary gradient tests (a more difficult problem to solve) and consequently, was not chosen for separation of the 20 estrogen metabolites.

Using a longer column or a column with smaller particle size increases the number of theoretical plates and, therefore, peak resolution. However, it may also increase the total time of analysis (Wal *et al.*, 2010). A longer Poroshell 120 C18 column was available for comparison with the Poroshell 120 EC-C18 (2.1 x 100 mm, 2.7 μ M); the gradient of mobile phase B as described for

the 50 mm Poroshell 120 was adjusted as proposed by the Sigma-Aldrich LC method transfer calculator (Sigma-Aldrich Co., 2017). The longer poroshell C18-EC column gave good and sufficient separation, with sharp individual peaks and a total run time of 20 min. To ensure that the same mobile phase for the shorter column is also suitable for the longer column, the exact same gradient, as well as an adapted gradient was applied for all three variations of the organic mobile phases. However, separation was not as ideal as with the 1:1 methanol:acetonitrile mobile phase B. Therefore, a 1:1 composition was chosen as the organic mobile phase. Separation on the Poroshell 120 EC-C18 (2.1 x 100 mm, 2.7 μ M) gave sharper peaks in comparison to the shorter column, even when compared to the results of adjusted parameters (steeper gradient, increased flow rate of 0.45 ml/min, temperature decrease to 25°C) on the 50 mm Poroshell.

Conclusion

After the evaluation and comparison of four different columns (the Agilent Poroshell 120 EC-C18 column [2.1 x 50 mm, 2.7 μ M], Agilent Zorbax Eclipse plus C18 [2.1 x100 mm, 1.8 μ M], Agilent Zorbax stable bond [SB] – Phenyl [2.1 x 100 mm, 1.8 μ M] and Agilent Pursuit 3 PFP column [3 x 100 mm, 3 μ M]) and three different options for mobile phase B (100% acetonitrile, 100% methanol, or 1:1 acetonitrile:methanol); two columns and mobile phases yielding the best separation of the 20 estrogen metabolites were identified. The two columns included the Poroshell 120 with 1:1 acetonitrile:methanol as mobile phase B and the Zorbax Eclipse plus with 100% acetonitrile as mobile phase B. Subsequently, separation on these two columns was compared for each of the metabolites and finally, the Poroshell 120 was identified as the best column to be used out of the four columns with 1:1 acetonitrile:methanol as mobile phase B. Next, the separation ability of the 100 mm Poroshell 120 column was compared to that of the 50 mm Poroshell. The results indicated that the longer column gave the sharpest peaks and is consequently, more optimal. The Agilent Poroshell 120 EC-C18 column (2.1 x 100 mm, 2.7 μ M) was, therefore, chosen for all further analyses. Finally, testing of this column with 100% acetonitrile, 100% Methanol and 1:1 methanol:acetonitrile, indicated that the combination of acetonitrile and methanol seemed to be the best composition for the organic mobile phase.

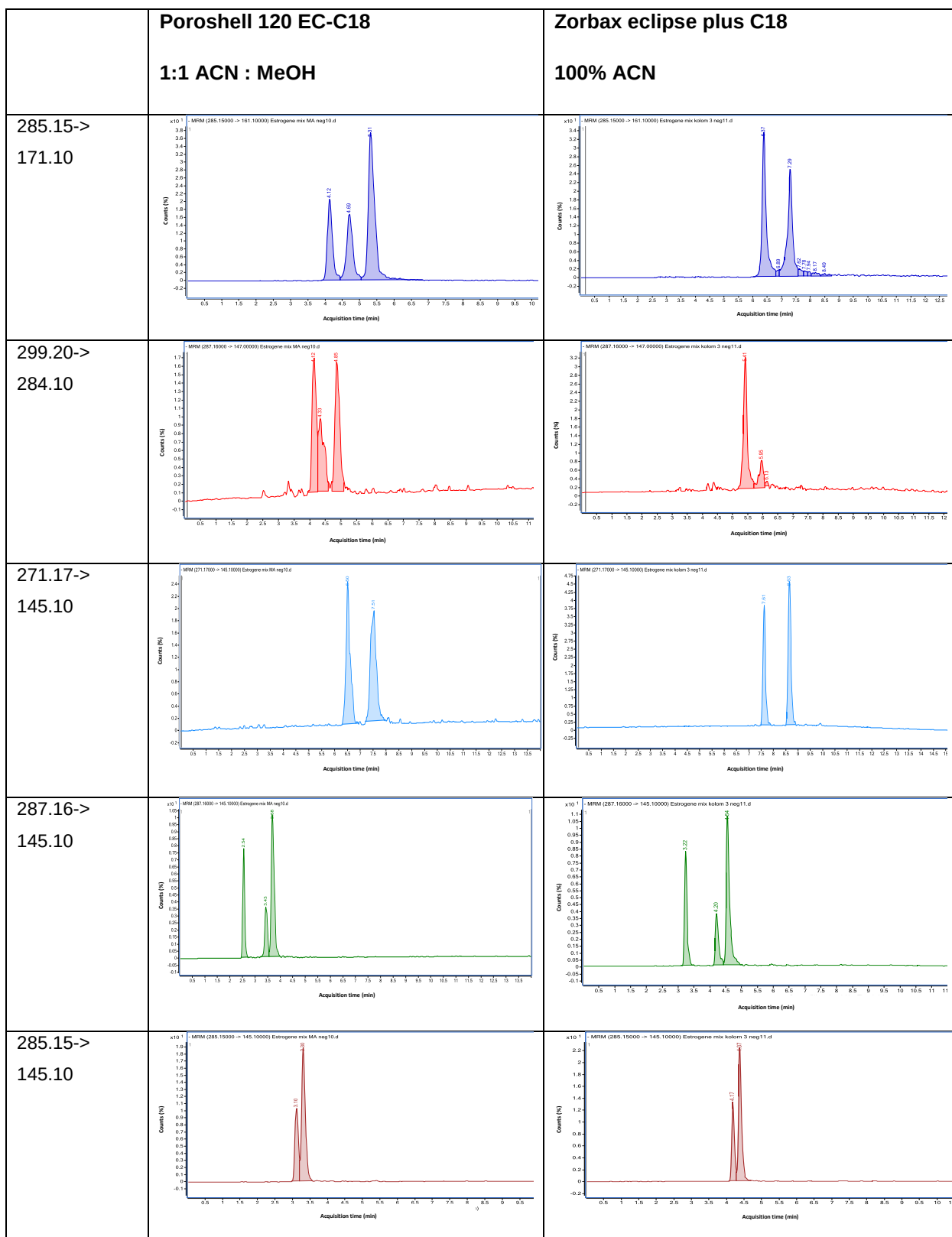


Figure 4.4.2: Comparison of the chromatographic separation of a few of the transitions between the Poroshell 120 EC-C18 and the Zorbax Eclipse plus C18 column.

4.5 Expansion of the estrogen metabolite profile

The 20 estrogen metabolites investigated to this point were the major estrogens, including the parent estrogens, hydroxyl, methoxy estrogens and estrogens from the 16 α -hydroxy pathway. This study aimed to look at a total of approximately 30 estrogens in total and therefore, a few estrogen-related metabolites were added to the current profile. In addition to the 20 above-mentioned metabolites, a few other estrogen-related metabolites were then added to extend the profile in order to make decisions regarding the modifiers, organic mobile phase, source conditions and columns to be used. The sulphate (S) and glucuronide (G) estrogen conjugate groups, can either be analysed directly or indirectly. In the indirect estrogen conjugate analysis, the conjugates are transformed back into their free unconjugated form, usually through enzymatic hydrolysis, although it can be done through chemical-deconjugation processes as well (Liu *et al.*, 2015). Another study, however, showed that enzymatic-deconjugation with sulfatase or glucuronidase decreases the levels of parent estrogens, other conjugates and DNA adducts (Gaikwad *et al.*, 2009). They observed that this phenomenon was ascribed to the hydrolysis incubation of 8 hours at 37°C. A second drawback of enzymatic-deconjugation is that hydrolysis is never complete. To ensure that complete hydrolysis occurs, additional experiments will be needed for calculation of the deconjugation efficiency (%) (Liu *et al.*, 2015). Furthermore, not all glucuronides are cleaved by the commercially available enzymes and these enzymes can react with other matrix components, reducing the binding efficiency to estrogen conjugates (Trontelj, 2012). Finally, the rate, ratio of decretion and specific adjustments to be made in the measured parent estrogen concentration are not known and compensation, therefore, might then be inaccurate. In the direct method, as for all of the 20 previously mentioned metabolites, the investigation would then be for each individual metabolite. Although this has previously not been the preferred method, the progress that has been made in the development of methods and standards, and the ability to analyse broader ranges of metabolites, now makes it possible to investigate conjugated estrogens collectively in urine and in environmental samples (Liu *et al.*, 2015). The direct analysis of the estrogen conjugates is the more desirable method of the two, as one gains information about which specific conjugates are present and in what concentrations these conjugates are present in a biological sample. This might be useful information if the conjugates have different pharmacological activities. Other significant benefits include better accuracy and precision, and possibly quicker sample preparation (Trontelj, 2012). Although previous studies deconjugated and desulphated biological samples to collectively measure the parent estrogens (conjugated and unconjugated) through an indirect quantification approach (Gaikwad *et al.*, 2008; Gaikwad *et al.*, 2009; Xu *et al.*, 2005), it was still decided to use the direct method of measurement of the estrogen sulphate and glucuronide conjugates without deconjugation. The direct method was then applied for conjugates that have authentic analytical standard solutions available for purchase, including E3-3-sulphate, E1 & E2 – 3-glucuronide and

E3-16 α -glucuronide metabolites. Additionally, E3-3-sulphate, E2-3-sulphate and E1-3-sulphate was also included among these conjugates. Other metabolites added to the hormone profile, included dehydroepiandrosteronedione (DHEA, a precursor of androstenedione), dehydroepiandrosteronedione sulphate (DHEAS, its water soluble sulphate conjugate) and, progesterone, the other steroid hormone found in COCs.

Table 4.3 The precursor and product ions as well as fragmentor and collision energy of the nine estrogen metabolites added to the estrogenic profile.

Compound Name	Formula	Ion polarity	Precursor ion (m/z)	Fragmentor energy (V)	Product ion (m/z)	Collision energy (eV)
DHEA	C ₁₉ H ₂₈ O ₂	Positive	287.2	89	213.1	16
DHEAS	C ₁₉ H ₂₈ O ₅ S	Positive	418.1	94	102.2	4
Estradiol-3-glucuronide	C ₂₄ H ₃₁ O ₈	Negative	446.6	146	271.1	44
Estradiol-3-sulphate	C ₁₈ H ₂₃ O ₅ S	Negative	350.5	146	271.1	32
Estriol-16 glucuronide	C ₂₄ H ₃₂ O ₉	Negative	463.2	168	287.1	28
Estriol-3-sulphate	C ₁₈ H ₂₃ O ₆ S	Negative	366.5	148	287.1	36
Estrone-3-glucuronide	C ₂₄ H ₂₉ O ₈	Negative	444.6	131	113.1	16
Estrone-3-sulphate	C ₁₈ H ₂₁ O ₅ S	Negative	348.2	143	269.1	28
Progesterone	C ₂₁ H ₃₀ O ₂	Positive	315.2	109	97.1	20

For each of the metabolites, the method was repeated as for the first 20 estrogens described in section 4.1- 4.4. A mobile phase modifier of ammonium acetate is most commonly used on estrogen sulphate and glucuronide conjugates (Emmanuelle Bichon, 2008; Qin *et al.*, 2008; Zhao *et al.*, 2014) and therefore, ammonium fluoride as well as ammonium acetate were evaluated as mobile phase modifiers. Selective ion monitoring of these estrogens was performed, followed by the evaluation of precursor and product ions, using the Optimizer software. The results of a comparative assay between the two different modifiers showed that the most abundant product ions were produced with the ammonium fluoride as mobile phase modifier; consequently, it was the chosen mobile phase additive for the collective method of the now 29 estrogen metabolites from this point forward. Previously described (section 4.3) source conditions were chosen, and these metabolites were then evaluated using the source optimiser software to ensure that the conditions chosen were in an optimal range for them as well. Hereafter, standards of these metabolites were also combined with those of previously investigated estrogens, to a final concentration of 100 ppb to ensure sufficient separation in the previously chosen column, gradient and organic mobile phase. All parameters evaluated for these metabolites are summarised in Table 4.3. The source conditions for the method that was preliminarily optimised in section 4.3, remained unchanged, as the newly added compounds were mostly optimal under those

conditions and the column separations were also sufficient for the added metabolites, so no changes were made in that respect either.

Peak identification and preliminary retention sequence

To confirm the retention times of metabolites, especially those with overlapping MRM transitions, the 29 estrogen metabolite standards were injected individually on the MRM method as described in section 4 up to this point. This allowed the identification of peaks in correspondence to its analytical standard to confirm which specific metabolite each peak represents, and which retention time is associated with which metabolite. The standard samples were each diluted to give 100 ppb standards and 1 µl of this was injected. The retention order of the metabolites can be viewed in Table 4.7 in section 4.8.2, where all the metabolites and accurate retention times in the urine matrix are indicated and a visual illustration of the retention sequence and chromatographic results can be seen in Figure S1 (Supplementary results).

4.6 Sample clean-up

A step to clean up biological samples is necessary to ensure that the sample matrix does not cause any decrease in performance of the method and that ion suppression of matrix components does not occur (Athanasiadou *et al.*, 2013; Kushnir *et al.*, 2011; Van Eeckhaut *et al.*, 2009). Since protein precipitation is more likely to be used on high concentration steroid hormones and samples, which contain higher amounts of problematic protein containing matrix components, only liquid-liquid extraction and solid-phase extraction was evaluated as possible sample clean-up methods. Although liquid-liquid extraction is less often used on the metabolites of interest, and likely not ideal for more polar and hydrophilic metabolites such as sulphates and glucuronic acid conjugates, it was still investigated for the sake of including a simpler and less laborious method. Two different LLE methods were investigated including one published by Xu *et al.* (2005), and another in an application discussion by Agilent technologies (Szczesniewski, 2012). In the first mentioned method, 7 ml dichloromethane was added to 1 ml of the standard solution, mixed well, centrifuged, the top layer discharged and the bottom layer transferred to a clean cimax tube, where after it was dried under a gentle stream of nitrogen and resuspended in 100 µl of methanol. This method was published as part of a method development article to quantify 15 endogenous estrogens, including a parent, hydroxyl, methoxy and keto estrogens. The second LLE method, as proposed by Agilent, was discussed in an application note on the analysis of endogenous steroids in plasma (Trontelj, 2012). This method was also investigated on the urine samples, to which 1 ml of standard solution and 4 ml methyl tert-butyl ether (MTBE) was added before the mixture was vortexed, centrifuged and the top layer transferred to a clean tube. The MTBE extracted negligible amounts of one of the most interfering matrix components (lyso-phosphatidyl

choline), making it a good choice for LLE solvent. To remove additional urine matrix components not present in blood and serum samples, a second extraction step was added where hexane:dichloromethane was added to the remaining bottom layer and the vortex and centrifuge steps were repeated, after which both top layers were combined, dried under a gentle stream of nitrogen and reconstituted in 100 µl of MeOH. Both LLE procedures are summarised in Figure 4.6.1

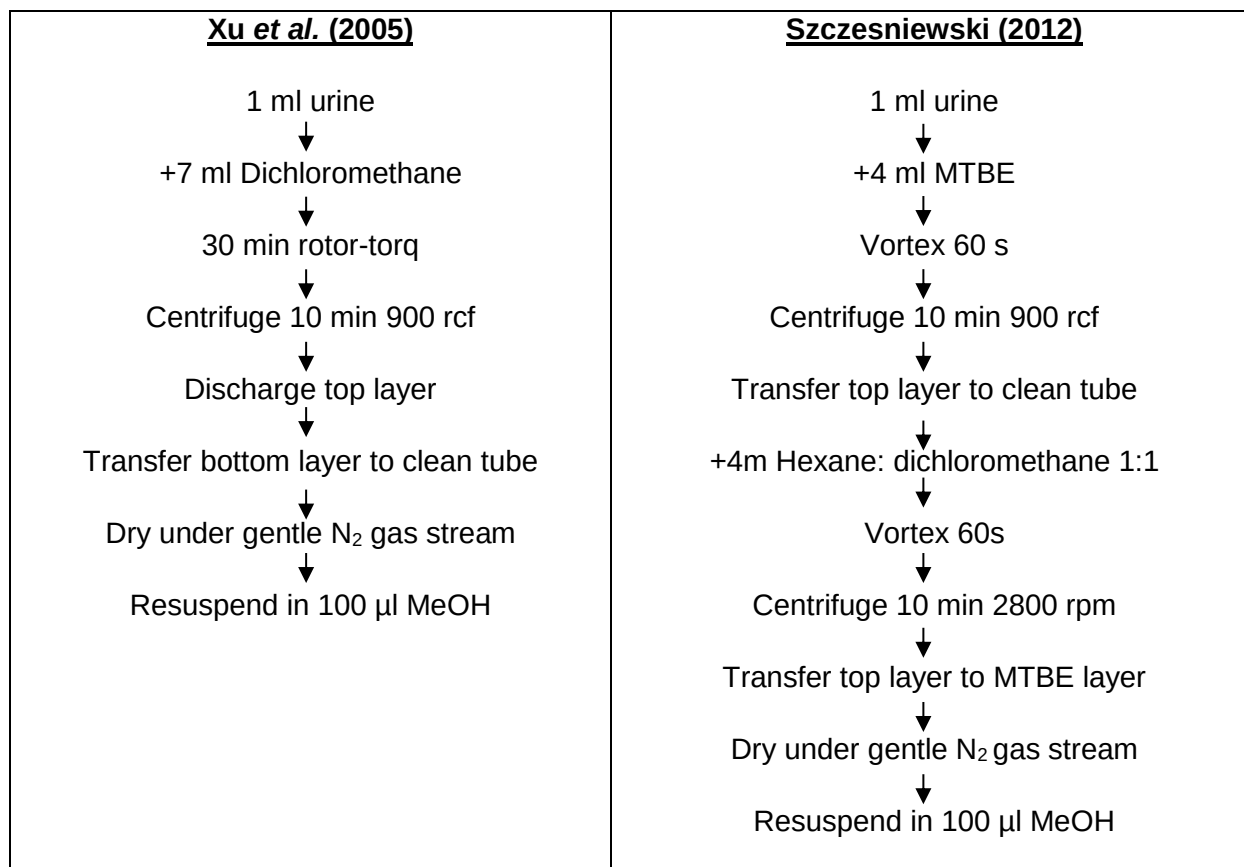


Figure 4.6.1: Comparison of the two different LLE methods. A flow diagram of events during the LLE sample preparation procedures. Both extractions were started with 1 ml urine and reconstituted in a final volume of 100 µl of methanol.

Both samples were injected onto the LC-MS/MS system, using the method as developed and optimised up until this point, and the abundances of the chromatographic peaks for each analyte were compared to a standard stock solution of the same prepared concentration. The more polar metabolites, such as the sulphate and glucuronic acids, were unfortunately not detected in these samples and only the most non-polar metabolites were recovered in acceptable percentages, which was the case for both LLE protocol results. The recoveries of the metabolites in the two methods were quite similar with acceptable recoveries for non-polar metabolites and low recoveries for more polar metabolites. As can be seen in Figure S2 (Supplementary results), the metabolite responses were not sufficient for either of the LLE methods to be used solus,

necessitating two different sample extraction methods (one for polar and one for non-polar metabolites) – a possibility that was initially being avoided as far possible for reasons of laboriousness and time constraint.

Gaikwad *et al.* (2008), the publishers of a method to investigate 40 different estrogen metabolites and steroid hormones, as well as most other articles investigating hormones in the urine, used solid phase extraction for sample clean-up (Gaikwad *et al.*, 2008; Kuehnbaum & Britz-Mckibbin, 2011; Naidong *et al.*, 2002; Qin *et al.*, 2008). The solid phase extraction method performed and evaluated in this study was developed based on literature, the SPE selection tools, and advice from Phenomenex, (a SPE cartridge supplier) (Gaikwad *et al.*, 2009; Majors, 2013; Phenomenex, 2016b). The sample preparation fundamentals for chromatography published by Agilent, another supplier of SPE products were also considered (Gaikwad *et al.*, 2009; Majors, 2013; Phenomenex, 2016b). The main objective during development of the SPE method, was to achieve the highest possible reproducibility, high recovery and sufficient matrix clean-up. The SPE procedure, as described in Chapter 3, is summarised in Figure 4.6.2 below. In the first step, methanol is commonly used for reverse phase SPE sorbents (Majors, 2013), followed by a buffering aqueous solvent. For the latter, water with 10 mM ammonium formate (pH7) was chosen since it has been used on estrogen metabolites before (Gaikwad *et al.*, 2009). Although most previous studies investigating different estrogen metabolites used the Oasis HLB cartridges, provided by Waters (Kuehnbaum & Britz-Mckibbin, 2011; Naidong *et al.*, 2002; Qin *et al.*, 2008; Revilla-Ruiz *et al.*, 2007; Silcock *et al.*), its comparable phases (polymeric and silica-based) (Agilent-Technologies, 2012; Peng; Smith, 2010), was investigated. The Strata C18 SPE column (100 mg) as silica-based reversed phase sorbent, and Strata-x (also reversed phase), as polymeric sorbent (60 mg), were then evaluated as possible sample clean-up cartridges on the standard mixture. In their SPE method, Gaikwad *et al.* (2008). used a phenyl sorbent SPE column and therefore, a phenyl SPE cartridge sorbent was also included in this study to be evaluated in the form of an Agilent bond elute-PH (100 mg). For washing, the wide range of polarities among the metabolites restricted the use of organic solvent in the washing medium. Therefore, a solution of 100% water with 10 mM ammonium formate as buffer was initially chosen for washing, to prevent wash out of any of the metabolites of interest. The washing solution was later optimised by increasing the percentage of the organic phase in the washing steps, to enable extra matrix clean-up without washing out the metabolites of interest. An evaluation of the eluting solvents for the specific SPE cartridges was done, and the most frequently used elution solvent, methanol, was then chosen as the elution solvent for optimisation. As recommended, the flow through the SPE was kept at a flow below 5-6 ml/min during the whole SPE process (Majors, 2013) and 2 ml/min during elution (Thomas *et al.*, s.a.). The abundance of the mass spectrum peak for each of the hormone metabolites was compared to those of other SPE sorbent methods performed.

The results of the three different sorbent phases were processed (Figure S3 & S4 supplementary results) and showed that the C18 column gave the best recovery of all the sorbents.

Conditioning:	6 ml MeOH 6 ml 10 mM ammonium formate (pH7)
Load:	1:1 urine: water
Rinse:	6 ml 10 mM ammonium formate (pH7)
Elute:	6 ml MeOH
Dry:	Under gentle nitrogen gas stream at 45°C
Resuspend:	In 200 µl MeOH

Figure 4.6.2: Flow diagram indicating the flow of events during solid phase extraction procedures.

A urine sample (including the matrix effect) was spiked with a standard solution, to investigate whether any of the metabolites of interest were lost during the SPE method due to the matrix effect. The matrix effect was analysed on 2 ml of the urine sample, spiked with an initial concentration of 10 ppb and 50 ppb of standard mixture and then prepared through SPE on the C18 column, together with an unspiked urine sample. The samples were analysed on the LC-MS/MS apparatus and the raw data was investigated on the MassHunter Workstation Qualitative analysis software (©Agilent technologies, Inc. 2008). Although the spiked urine samples gave high abundances for all the metabolites, the unspiked urine sample did not give sufficient results. Consequently, the method was not sensitive enough to be used on clean urine samples, as the concentration of the metabolites in such a sample might be too low. The SPE method was then further optimised to increase the sensitivity and reduce the matrix effect.

SPE adjustment and optimisation

All the optimisation steps discussed below were followed by analysing the urine sample of a control patient to ensure that the method would be sufficient to quantify these metabolites. Spiked (50 ppb estrogen metabolite mixture) and unspiked urine samples were investigated. The first optimisation step was to evaluate different methods and steps of elution. This optimisation was performed, not because the elution step was insufficient, but rather to speed up the drying process, a very time-consuming process. Elution with methanol alone, acetone alone and an

acetone and methanol mixture was investigated. The results of the elution variations showed the best recovery with 2 x 2 ml acetone followed by 2 ml methanol. This elution combination was then used for all further SPE analyses. The suppliers, however, recommended that when acetone is being used as an elution solvent, the conditioning phase should also use a stronger solvent, therefore, 6 ml acetone additional to 6 ml methanol was used during conditioning. Furthermore, they advised to use methanol for elution first, followed by 4 ml acetone. Both these recommendations were then implemented and compared to the normal method, and since no variations were observed, the latter was then applied.

The sample preparation as well as the LC-MS/MS method, as described up to this point, resulted in the detection of 10 of the 29 metabolites to be investigated. Subsequently, it was decided that the urine samples be more concentrated to improve detection. This led to higher urine volumes to be tested on larger sorbent mass SPE cartridges. SPE cartridges included a 500 mg phenyl column (500 mg Stata-c18-E) and a 200 mg Strata-x (as it is a polymeric based cartridge, lower sorbent mass is required). A higher volume of 5 ml urine was, therefore, employed to increase the concentration of the metabolites of interest, to ensure that the best choice was made with regards to which higher volume SPE column sorbent should be used. A urine volume of 5 ml spiked with physiological concentrations (5 x that in the 1 ml urine) of all the metabolites were used for each of the different SPE columns. Acetone (6 ml), followed by 6 ml 10 mM ammonium formate, was used for column equilibration. This was then followed by loading a 1:1 urine:water mixture, washing with 6 ml 10 mM ammonium formate and drying under vacuum for 10 minutes. Lastly, the metabolites were eluted with 2 ml methanol and 2 x 2 ml acetone. The eluents were then evaporated to dryness under nitrogen (N₂) gas at 45°C and resuspended in 200 µl methanol. This permitted a concentration factor of 25 times, as 5 ml urine is the starting volume and 200 µl the final volume. These urine samples were then analysed by the method developed up to this point, and the areas under the peaks were again, compared. As with the smaller sorbent mass SPE columns, the C18 again gave the best abundances for the metabolites of interest. As seen in the results in Figure S4 (Supplementary results), C18 gave the highest abundances for most metabolites and also gave the highest average recovery of the three sorbents evaluated. Consequently, the 500 mg strata C18-E SPE columns were chosen as the sample clean-up cartridge and SPE as the sample preparation method, instead of LLE. A relative comparison between the C18 SPE and the LLE method results (Figure S5 supplementary results) permitted the final choice.

Due to lower abundances of metabolites that were obtained in matrix spiked samples, compared to abundances from the standard mixtures, the question arose of whether the matrix compounds in the urine weren't causing competition for SPE sorbent binding as well as ionisation in the MS

source, leading to damping of the signal of the metabolites of interest. This was firstly evaluated by using the 500 mg SPE columns as chosen up to this point, and loading 1 ml, 2 ml, 3 ml, 4 ml and finally, the normal 5 ml of urine on them. The more concentrated samples (5 ml), however, gave an even lower response for some of the metabolites than the 1 ml or 3 ml (Figure S6, supplementary material). From these results, it is therefore, clear that the urine sample still has quite a lot of matrix components, causing a decreased response from the metabolites of interest. Therefore, the decision was made to rather use 3 ml of urine instead of 5 ml and to apply this in further sample clean-up steps. In this way, it is possible to both sufficiently increase the concentrations of the metabolites of interest and limit the matrix effect. Furthermore, the reconstitution volume was adjusted to 100 μ l, in accordance with the lower urine volume used, to allow a concentration factor of 30 times.

A variety of wash approaches was also evaluated by repeating the SPE procedure as described and optimised to this point, on spiked matrix samples, but instead of keeping the wash solution a 10 mM ammonium formate buffer, the MeOH concentrations in the ammonium formate solution were adjusted. These included 5%, 10%, 20%, 40%, 70%, and 90% MeOH in the washing solution. For this evaluation step, both the wash and the elution from the SPE cartridges was collected separately, dried and reconstituted in 100 μ l MeOH. As can be seen from the results in Figures S7 a-ac for most of the metabolites, either a 20% or a 40% MeOH wash solution would be necessary to achieve the most optimal elution results. This could be due to the high amount of matrix components present in the urine, which can cause an increased matrix effect or a decreased ionisation of the metabolites of interest. The results of the washing step, however, showed that during a 40% MeOH wash, a number of the more non-polar metabolites eluted, causing a significant loss of these metabolites (Figure S7 a-ac). Therefore, a 20% MeOH wash was used from this point onwards. Variations in loading, wash and elution volumes were also investigated. These included 6 ml, 9 ml and 12 ml loading volumes (Figure S8a); a 6 ml, 9 ml and 12 ml wash with 10 mM ammonium formate and 20% MeOH (Figure S8b); a 6 ml load 6 ml wash volume and finally, a 9 ml load 9 ml wash volume (Figure S8c). The results as shown in Figures S8 a-c, indicates that there were very little differences in the washing for most of the metabolites, but the most consistent results over the whole profile were obtained from the normal 6 ml load, 6 ml wash and normal elution, which was then chosen for all following analyses.

The use of a buffering agent such as the 10 mM ammonium formate was also investigated, where a sample with and without this buffer, and with adjusted pH levels was evaluated. Varying the pH during the whole SPE process by eluting with a more acetic solvent, or conditioning with a more acetic solvent and consequently eluting with an alkaline solvent (Gaikwad *et al.*, 2009; Kuehnbaum & Britz-Mckibbin, 2011), was also investigated and compared. The overall results

showed that both buffering and clean water without any pH adjustments gave comparable results (blue and purple bars, Figure S9, supplementary results). However, when the pH was varied for conditioning and elution steps (green and red bars, Figure S9), the results were lower for most metabolites than the pH7 or no pH set procedures. Since there were no significant differences between a buffering solution and a pure water solution observed for future analyses, only the pH of the urine samples was adjusted to pH 7 with 1 M NaOH. All other conditions, equilibration and wash steps were performed using distilled water and methanol or acetone. Finally, to ensure that the SPE can be used as described up until this point, the SPE method was also evaluated in terms of breakthrough, repeatability and recovery, this was performed after the LC-MS/MS method was optimised for more reliable results.

4.7 LC method Optimisation

Before any optimisation on the preliminary developed method was done, the methods as developed up to this point for both positive and negative ionisation polarities were combined. The Agilent 6460 Mass spectrometer has the ability of switching polarity between positive and negative ions, therefore, the two methods were combined, and the response of both the positive and negative ionising metabolites was evaluated in all further optimisation procedures. To complete the total metabolite profile and consequent quantification of the metabolites of interest, internal standards were included in the method. An internal standard mixture consisting of a range of isotopes, including estradiol-d4, estrone-d4, DHEA-d2, progesterone-d9 and estriol-d3 (Table 4.4), were combined to a final concentration of 2 ppm. The precursor and product ions for each of these isotopes were calculated and confirmed with the Optimizer software and tested in MRM mode with only the isotope mixture being injected. The polarity, collision energy and fragmentor energy of the isotope parent molecules were used, and all parameters for each of the isotopes can be seen in Table 4.5. Before any further analyses, 10 µl of the internal standard mixture was added to samples or working standards to be analysed with the samples at a 100 ppb/200 µl concentration.

The LC-MS/MS method was then further optimised by increasing the sample volume that can be detected by injecting 0.5 µl, 1 µl, 2.5 µl and 5 µl of the same working standard into the LC system. From the comparative results in Figure S10a (supplementary results), it is evident that the highest volume of 5 µl gave the best results. The increase in response obtained from a larger injection volume, was again acquired by injecting 1 µl, 5 µl, and 10 µl volumes of the exact same standard. Although 10 µl seems quite a lot for the column, Agilent technologies have injected 10 µl and even higher volumes using the exact same column (as used in this study), in application notes and column comparisons (Agilent-Technologies, 2015). The 10 µl injection volume gave the

highest and best response (Figure S10b), more than the 1 μ l, and was then chosen as the injection volume to be used from there on out. Furthermore, the time period of 100% organic phase solvents at the end of the run, to ensure that all matrix components of the run are washed off the column, were also evaluated.

Table 4.4: A table containing schematic representations of the parent molecules as well its deuterium (D) labelled isotopes. For each metabolite (parent and isotope) the precursor and product ion are also included.

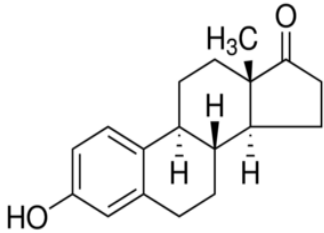
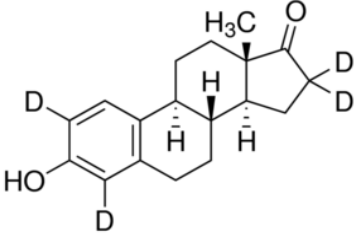
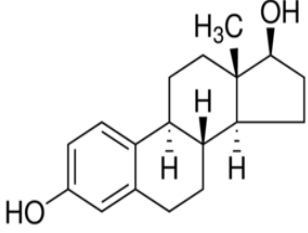
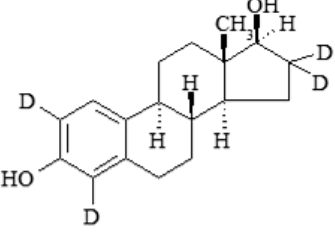
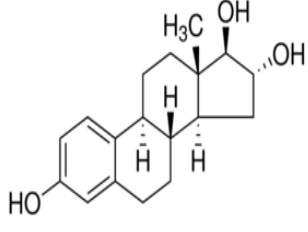
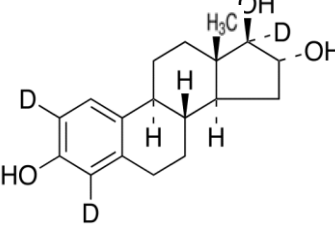
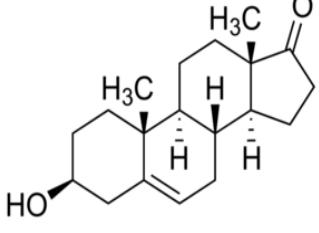
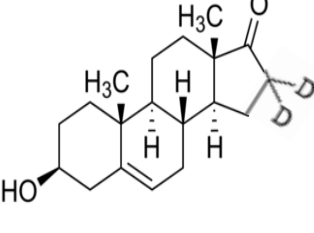
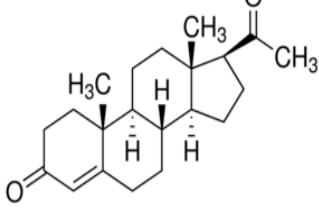
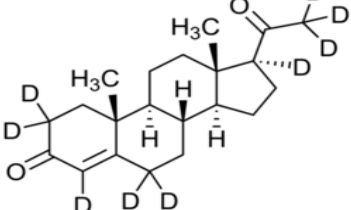
Parent molecule		Isotope	
Estrone 269.1>145.1		Estrone-d4 273.1>147.1	
B- Estradiol 271.1>145.1		B-estradiol-d4 275.17>147.1	
Estriol 287.2 >171.1		Estiol-d3 290.16>173.1	
DHEA 289.2>213.1		DHEA-d2 291.2>213.1	
Progesterone 315.2>97.1		Progesterone-d9 324.23>97.1	

Table 4.5 Synoptic table with the retention times (RT), precursor ions, product ions, collision energy, fragmentor energy and polarity of each of the isotopes used as internal standards.

Isotope	RT (min) <i>*before optimisation</i>	Precursor ion (m/z)	Fragmentor energy (V)	Product ion (m/z)	Collision energy (eV)	Polarity
DHEA-d2	12.30	291.22	89	213.10	16	Positive
Estradiol-d4	11.13	275.17	40	147.1	168	Negative
Estriol-d3	4.15	290.16	163	173.10	36	Negative
Estrone-d4	11.88	273.15	163	147.10	40	Negative
Progesterone-d9	13.88	273.15	109	147.10	20	Positive

In addition, the post run time was also evaluated, during which the mobile phase composition is kept for a certain period of time at the starting composition to give the chromatographic column time to equilibrate again before the next injection. Both these parameters were investigated on a standard estrogen metabolite mixture and a matrix spiked sample. The length of time that mobile phase B should be kept on 100%, was investigated by executing a positive and negative scan, and evaluating the amount of time taken for the baseline of the scan results to return to the original baseline value. Returning baseline values is an indication that all metabolites were eluted from the column and that very little or no metabolites enter the mass detector after stabilisation to baseline value has occurred. The results showed that after 4 min of 100% Mobile phase B, the baseline returned back to normal and as a result, the time for 100% mobile phase B was adjusted to 4 min. The sufficiency of the post run time was also evaluated. The post run, as previously described, is not only for equilibration and conditioning of the column before the next injection is done, but also to allow the pressure of the system and especially the column to stabilise. The optimal post run time was evaluated by doing an MRM as optimised up until this point, but with a post run of 20 min to see the length of time taken for the pressure to completely stabilise. The results indicated that a 5-6 min post run would be more than sufficient to stabilise the binary pump pressure completely and in other words completely equilibrate the LC column. The needle wash was also increased from 5 to 10 times, to ensure that no carry over occurs due to insufficient wash time or the fact that the needle is not contaminant free.

As part of the optimisation of the method, a few other parameters that were kept constant up until this point were also investigated to ensure that the most optimal conditions were chosen before attempting any type of quantification. One of these parameters is the ratio of acetonitrile to methanol in mobile phase B. Although 100% methanol and 100% acetonitrile were already investigated, it was thought that it might be beneficial to see what the effect of a higher concentration of each of these in the final organic phase might be. Therefore, a ratio of

methanol:acetonitrile of 50:50, 55:45, 45:55 and 40:60 was evaluated and compared by applying the method as described up until this point on a standard mixture and evaluating each peak separation individually on MassHunter Qualitative analysis software to ensure that the most optimal composition is chosen. Most peak separations remained unchanged (Figure S11 supplementary results) and some minor changes in the separations that varied between different ratios were not significant, therefore, a MeOH:ACN ratio of 50:50 was chosen due to minimal differences in the separation of transitions. After evaluation of the composition of mobile phase B, mobile phase A (water with ammonium fluoride) was also evaluated by investigating the effect of different ammonium fluoride concentrations. For this purpose, the method, as described up until this point, was used with concentrations of 1 mM, 0.5 mM, 0.25 mM and 0.125 mM ammonium fluoride (AF) in mobile phase A. Again, the response (area under the peaks) for each metabolite was compared in prepared working methanol standards and in urine matrix. As seen in the processed results in Figure S12 (supplementary results), the lowest ammonium fluoride concentration showed the best results for most metabolites. This was also the case for the sulphate and the glucuronide conjugates in both the urine (Figure S12) and the standards (not shown in results). To achieve maximum recovery, the ammonium fluoride concentration was then adjusted to 0.125 mM in water as mobile phase A. Since previous literature indicated that 1 mM ammonium fluoride gives the highest sensitivity in global metabolite profiling, and that increasing the concentration to 5 mM and above introduces background to the mass spectra (Yanes *et al.*, 2011), concentrations higher than 1 mM ammonium were not evaluated as part of this study.

The pH of mobile phase A was also investigated, as this affects the chromatographic separation and the quality of the chromatographic peaks. According to Wal *et al.* (2010), a low pH value gives the best peak shapes for the more basic metabolites on a silica-based column. The reason for this is that the silica silanols are protonated fully, causing positively charged compounds to not have a strong interaction with the column (Wal *et al.*, 2010). In the mobile phase A, used up until this point, a pH of approximately 7 (6.9) was measured and consequently, lowered to pH 5 and pH 6 with 1M HCl. Lowering the pH too much might affect the chromatographic separations, and although aiming to make the method more optimal, the aim was not to change the method much from the developed method up until this point. The responses of a standard sample were then compared between a normal mobile phase pH range of approximately 7 and the two lower pH values. From these three pH values, a pH of 7 gave the best results (Figure S13a), therefore, the pH of mobile phase A was not decreased. At a mid to higher pH, the basic compounds may have a positive charge and the silica silanol surface a negative charge. By covering as many silanols as possible, the peak shape might be improved at these higher pH values (Wal *et al.*, 2010). The response of estrogen metabolites standard mixture at higher pH values of pH 7.5 and pH 8 was then compared to those at a pH 7 value, and again the best ionisation and chromatographic

responses was achieved at pH 7. The results for the higher pH ranges can be viewed in Figures S13 b in the supplementary results, where the responses were compared between the different pH ranges of mobile phase A. Figure S14 also illustrates the effect of the pH changes on the chromatograms for certain transitions.

To further optimise the developed method and ensure that no unnecessary metabolites enter the mass spectrometer and affect the ionisation of the metabolites of interest, the first minute of elution from the column was set to go directly to waste and not enter the ionisation chamber. This was possible because none of the metabolites of interest elutes during this time period. Preventing additional metabolites from entering the MS might reduce the number of metabolites being ionised in the source, resulting in the metabolites of interest being more easily ionised. In addition, it protects the source from the effect of some additional matrix effects. To increase the detection of the metabolites of interest and to improve the detection limit of the analytical apparatus, the electron multiplier voltage (EMV) was increased. The electron multiplier improves detection of selected mass ions in the form of signals from the mass analysers. The delta-EMV refers to a voltage of energy by which the current of the tuning multiplier voltage, as selected in the tune file, is multiplied to give an exponential increase in the signal or response (Agilent-Technologies, 2014). It is important to note that when increasing the signal of the metabolites, the signal of all background molecules and products present in the biological matrix is also being increased. The implication is that if a sample is not properly cleaned up, it may cause an increase in noise peaks, but might still be extremely beneficial if metabolites that are present in very low concentrations are investigated. As internal standards will also be added to each sample and analysed on the exact same EMV as the metabolites of interest, no concentration calculations will be affected by this voltage increase. The method as described and optimised up until this point was followed, with EMV of 0 V, 100 V, 200 V and 300 V. Although the analytical apparatus can be set to an EMV of 0 V - 500 V, increasing the EMV might affect the lifespan of the multiplier tube. The lowest possible multiplier setting without compromising on sensitivity would, therefore, be the most economical. As expected, the results (Figure S15 supplementary results) showed an exponential increase with an EMV of 300 giving a 5-10 times higher response than a 0 V EMV. Therefore, 300EMV was chosen to be used for all further analyses.

Before validation of the analytical method, the chromatogram of each metabolite was again confirmed, evaluating retention times, peak shapes and signal to noise ratios. One observation of these final evaluations was that certain metabolites give chromatograms with more than one, but close eluting peaks. These metabolites, included E2-3-G, E1-3-G, DHEAS, E2-3-S, E1-3-S, 2&4-OHE2, 2 & 4 OHE1, 16-ketoestradiol, 16&17-epiestriol and 16 α -hydroxyestradiol. Some examples of the double peaks can be seen in Figure S16 in the left-hand column (supplementary

results). Quite a few troubleshooting options were evaluated, the first of which was to make up new mobile phases to ensure that a measuring error did not occur. Unfortunately, the results remained the same and were followed by spiking a physiologically spiked sample even more, with only the one metabolite of interest, to see if only one or both peaks increased. The results for all metabolites revealed that both peaks increased, indicating that both peaks represent one metabolite and that it might be a chromatographic phenomenon that was causing the double peaks to form. Therefore, the gradient was changed in such a way that the metabolites were caused to elute faster and preferably only form a single peak. The gradient was adjusted to 8 different variations, but it only caused poor separation of all the peaks, and double peaks were still formed. Next, the temperature was adjusted. This is also a parameter that will result in metabolites eluting faster and giving sharper peaks. The temperature was then adjusted from 35°C to 40°C, 45°C and 50°C. Again, this did not solve the problem and instead caused previously separated isomers to co-elute. Next, a clean standard solution of some of these metabolites dissolved in methanol, was injected onto the column (0.1 µl of 1 ppm). This resulted in very neat single sharp peaks per compound and led to the conclusion that it might be either due to the high volume injection, the high concentration on the column, or a decrease in the stability of the metabolites over time. The volume effect was the first to be evaluated. Of the same 100 ppb standard, 0.1 µl, 1 µl and 10 µl was injected onto the column and the results were visually compared. The results (as seen in Figure S16) show that for the transition 185>145, 1 µl gave only one peak per metabolite and 10 µl gave two peaks per compound. The concentration effect was further investigated by injecting 10 µl of a 10 ppb standard onto the column, but the results remained exactly the same and each metabolite gave two different peaks. The conclusion was that the volume of 10 µl seemed to be the problem causing the double peaks to occur. This could either be because the columns can't handle 10 µl all at once, or because resuspension of the dried metabolites occurred in 100% methanol, which might interfere with the chromatographic separation, especially at the beginning of the spectrum. The latter was investigated by drying a volume of the 100 ppb standard, resuspending it in the starting mobile phase (90:10, A: B), and injecting 10 µl thereof onto the column. The results show that it then caused chromatographic separations in such a way that each compound only gives one peak (Figure S16). Therefore, the composition of resuspending fluid was then identified as the problem for the double peaks for certain compounds. The dried sample clean-up products were then rather reconstituted in 100 µl of the initial mobile phases for the chromatographic run during all following analyses.

Increasing the peak shape to sharp and well-defined peaks where possible, will give better sensitivity. A lower flow rate can lead to a prolonged retention time, which leads to broader peaks. This may be solved either by increasing the flow rate or gradient, or by changing the organic mobile phase to a stronger solvent (Stephanie A. Schuster, 2013). Changing the gradient so that

a higher percentage of the organic solvent is reached earlier in the chromatographic run, allows the compounds to elute faster from the C18 column, resulting in narrower peaks, that are more easily detectable for the most sensitive method. An increased temperature might also result in narrower, higher chromatographic peaks, provided that there is sufficient separation between the peaks. The effect of an increasing mobile phase B percentage was evaluated by increasing each binary pump gradient step by 5%, 10% and 20% organic phase B, so that all chromatographic processes occur faster. This was performed on a working standard mixture. The results showed that for some of the metabolites, sufficient separation was compromised by increasing the %B too early. This phenomenon was especially seen with the 10% and 20% increases. The chosen final gradient, very similar to the originally developed gradient, can be seen in section 4.8.2 where the total final method is described. When the flow is increased, all separations on the column from a certain mobile phase composition also occurs faster, giving thin sharp peaks and also reducing the run time. When the flow rate is increased and all other parameters are kept unchanged, there is a significant increase in the binary pump pressure and consequently, this high pressure may affect the chromatographic pressure. Although the column may withstand a certain amount of backpressure, increasing the temperature might reduce the pressure of a higher flow rate, back to that of a normal flow rate. The increased temperature can also sharpen the peaks as the mobile phase viscosity plays a role in the peak shape and decreases by an increase in the temperatures (Stephanie A. Schuster, 2013). The normal MRM method, as described up until this point, was followed on a working standard mixture of all the metabolites. Column compartment temperatures of 35°C, 50°C and 60°C were investigated as well as flow rates, 0.3 ml/min, 0.35 ml/min, 0.4 ml/min, 0.45 ml/min and 0.5 ml/min. The results showed that the conditions of 0.4 ml/min with a column temperature of 50°C, gave the best results in terms of peak shape and peak width, and as a result, both the temperature and the flow rate was adjusted. From this point forward, a 0.4 ml/min binary pump flow rate, and 50°C as the controlled column compartment temperature, were used.

From the chromatographic response for each metabolite standard analysed, the compounds with the lowest response was found to be the hydroxyestrogens and DHEAS. Due to the fact that the exact same amount of these standard samples gives a lower response than other metabolites, indicates that these metabolites do not ionise as well as other metabolites investigated. Because a broad range of metabolites (more polar to less polar) were investigated, with a number of metabolites in one method, it was aimed to win sensitivity from as many variables as possible for these metabolites. Firstly, because all chromatographic changes were already done, segments could now be built into the method to result in looking at certain metabolites at specific times. These selected segment divisions can be seen in Table 4.6. Increasing the EMV for the one segment in which these metabolites elute, were one of the methods by which sensitivity was

gained. Although this caused the background noise to increase, a corresponding increase in signal for metabolites could be achieved. Furthermore, in the segment of metabolites that are not ionising well, the dwell times of the hydroxyestrogens as well as DHEAS was increased to 300, whereas other metabolites had a dwell of 100 or less. By increasing the dwell times, the mass analyser is set at a longer static setting for a specific m/z , which may increase sensitivity. The cell accelerator voltage (CAV) is another parameter evaluated for these metabolites. The CAV of the collision cell refers to the gradient of the voltage between the two ends of the hexapole assembly (in the collision cell). This value determines the velocity of ions travelling through the collision cell and consequently, the average time the ions are in the cell (Agilent-Technologies, 2014). Increasing this voltage, gives fast ion drift times. In some extreme cases, increased CAV can lead to ion loss in the collision cell. On the other hand, low CAV values could lead to the stalling of ions in the cell and eventual loss. Although the default is a CAV (7 V) works for the majority of compounds, giving excellent performance with no crosstalk, some compounds prefer a CAV of 1-3. These compounds are most likely to produce fragile product ions, which do not survive at such a high CAV value. In an optimisation of a method to detect steroids in water, a lower CAV gave a better response for estrogens (Cullum, 2012). Therefore, MRMs for these metabolites were created in such a way as to test a CAV of 1 V, 3 V and 7 V for each of them. The results (Table S2, supplementary results) showed that the CAV of 7 was, by default, the best choice for DHEAS, while 4OHE2 gave higher response values with 3 V; 2OHE2 and 2&4OHE1 gave higher responses on a CAV of 1 V. These parameters were then also changed in the method as currently used for all analyses. For these hydroxyestrogens and DHEAS, the source optimisation program was again used to ensure that there aren't source conditions that are more optimal for these metabolites than the source conditions currently being used and optimised for the combination of metabolites. The voltages that were investigated, include nozzle voltage, nebuliser voltage and the capillary voltages. The direct infusion method as described in Chapter 3, with voltage in the exact same ranges as described previously in section 4, were performed on analytical standards for each of these metabolites. The results thereof were that most of these voltages that were chosen before were already optimal and the only change made was the nebuliser voltage, which was adapted to 50 V, because DHEAS gave almost 5 times higher response values under these conditions.

Although the method was not validated to confirm that the metabolites would be detectable in the human urine of control females and of COC users and that the method has a good repeatability, other detection methods were briefly evaluated to compare the results with the already developed method. This was done to ensure that the responses couldn't be improved further and lower concentrations weren't detectable. Firstly, Agilent 1200 series Variable Wavelength UV Detector was added to the system so all metabolites would pass through the UV cell after the chromatographic separation before MS evaluation. A literature study was performed on the

analysis of estrogen metabolites in a UV spectrum to enable the choice of wavelengths. The most common wavelengths used in general were 254 nm for catechol estrogen, 210 nm-220 nm for parent estrogen metabolites, and 280 nm and 240nm for typical steroid metabolites (Fisher & Lopez; Hanai, 1991; Kasal *et al.*, 2010; Kautsky, 1981; Lima *et al.*, 2001).

Table 4.6: The segments created for more optimal detection of metabolites of interest.

Time segment	Time (min)	Polarity	Precursor m/z	Compound Name	RT (min)
1	0			Waste	
2	2	Negative	366.5>287.1	Estriol-3-sulphate	2.28
		Negative	446.6>271.1	Estradiol-3-glucuronide	2.89
		Negative	463.2>287.1	Estriol-16 glucuronide	2.9
		Negative	444.6>113.1	Estrone-3-glucuronide	3.02
		Negative	351.2>97.0	Estradiol 17-sulphate	3.82
		Negative	287.2>171.1	Estriol	4.15
		Negative	290.16>173.10	Estriol-d3	4.2
		Negative	350.5>271.1	Estradiol-3-sulphate	4.28
3	4.8	Negative	285.1>145.1	16-Ketoestradiol	5.32
		Negative	348.2>269.1	Estrone-3-sulphate	5.53
		Negative	287.2>171.1	16-Epiestrinol	5.98
		Negative	285.1>145.1	16 α -Hydroxyestrone	6.08
		Negative	287.2>145.1	17-Epiestrinol	6.48
4	7	Negative	285.1>161.1	4-Hydroxyestrone	7.85
		Negative	287.2>255.1	4-Hydroxyestradiol	8.32
		Negative	287.2>147.0	2-Hydroxyestradiol	8.85
		Positive	418.1>102.2	DHEAS	9.03
5	9.2	Negative	285.1>161	2-Hydroxyestrone	9.66
6	10	Negative	271.2>145.1	17 β -Estradiol	11.16
		Negative	275.17>147.1	17 β -Estradiol-d4	11.16
		Negative	301.2>286.1	4-Methoxyestradiol	11.61
		Positive	289.2>97.1	Testosterone	11.73
		Negative	271.2>145.1	17 α -Estradiol	11.808
		Negative	269.1>145.1	Estrone	11.89
		Negative	273.15>147.1	Estrone-d4	11.92
		Positive	287.2>97.1	Androstenedione	11.99
		Negative	301.2>286.1	2-Methoxyestradiol	12.01
		Negative	299.2>284.1	4-Methoxyestrone	12.22
		Positive	287.2>213.1	DHEA	12.3
		Positive	291.22>213.1	DHEA-d2	12.35
		Negative	299.2>284.1	2-Methoxyestrone	12.36
		Negative	299.2>284.1	2-Hydroxyestrone-3methyl ether	12.65
7	13	Positive	315.2>97.1	Progesterone	13.57
		Positive	273.15>147.1	Progesterone-d9	13.60

The detection process was performed on a standard metabolite mixture and gave acceptable results. These results were not as good as with the MS, but each of the hydroxyl metabolites gave a peak of abundance, which would allow confirmation of these reduced ionising metabolites. Unfortunately, when repeated in a spiked urine sample of a control participant, the UV absorbance values were extremely low, the separation between the UV detected peaks were not sufficient and this type of detection would not improve the currently developed method (Figure S17- Supplementary results). Another method of detection that was also evaluated as part of this method development and optimisation was atmospheric pressure chemical ionisation (APCI). The APCI ionisation, as with the ESI, is one of the most commonly used methods for ionisation of steroids. The APCI ionisation has been known to give a more selective ionisation that might reduce the matrix effect by using a higher temperature source for ionisation and a corona discharge region for gas phase chemical reactions to take place (Keevil, 2016). The ESI source was replaced by the APCI source, and the method as developed up until now was executed on a standard mixture. The source conditions for this evaluation were set as suggested by literature (Keski-Rahkonen *et al.*, 2013; Naldi *et al.*, 2016; Zavitsanos, 2002). The results showed that the response of the metabolites decreases quite dramatically in comparison to the results of the same sample after ESI ionisation and detection. Some chromatograms of transitions are shown in the supplementary results, Figure S18, to show visual differences. The sulphates, methoxy estrogens and parent estrogen metabolites gave lower/no response in the APCI, in comparison to the ESI and consequently ESI ionisation was again chosen, as none of the other ionisation or detection methods gave better results than the ESI ionisation.

4.8 The final method as described and optimised up until this point before validation:

4.8.1 Sample preparation

The Strata C18-E SPE columns were conditioned with 6 ml acetone, 6 ml methanol and 6 ml distilled water. The urine sample (3 ml, pH7) was then diluted 1:1 with water, centrifuged for 10 min at 2400 g, and the supernatant loaded onto the SPE columns. Each column was then washed with 3 ml water followed by 6 ml 20% MeOH in water and dried under vacuum for 10 min, where after the sample was eluted with 2 ml MeOH and 2 x 2 ml acetone. The SPE eluate was then evaporated to dryness under a gentle stream of nitrogen and resuspended in 200 µl of the initial mobile phase combination. All resuspended samples were then filtered through 0.2 µm Nylon Spin-X filters for LC, where after the supernatant was transferred to inserts in LC analysis vials.

4.8.2 LC-MS/MS analysis

The resuspended samples (10 µl) were then injected onto the Poroshell 120 EC-C18 chromatographic column at a controlled temperature of 50°C. Mobile phase A consisted of water with 0.125 mM ammonium fluoride and mobile phase B 50:50 methanol:acetonitrile. The mobile phase gradient started at 10% B and increased to 30% in 0.5 min. From 30% it was further increased to 40%, 45% and 50% at 3 min intervals each. There after, it was further increased to 100% B in 1 min where it was kept isocratically for 4 min before it was reduced to 10% once again. The post time for the run was set at 5 min for a final run time of 21 min. The gas temperature of 250°C, the gas flow of 8 ml/min, nebuliser at 50 psi, sheath gas of 400°C and 12 ml/min flow, and a capillary voltage of 4000 V for positive and negative ionisation, a nozzle voltage of 0 V for positive and 1000 V for negative ionisation were used. MRMs, as seen in Table 4.7, were conducted with specific transitions, collision and fragmentor settings, and cell accelerator voltages for each of the metabolites.

4.8.3 Data analysis

The LC-MS/MS data were then extracted to the MassHunter Quantitative data analysis software for each of the metabolites to be quantified relative to the stable isotopes added. The transitions and retention times for each of the metabolites in the final MRM method can be seen in Table 4.7 and all data analyses were also conducted from these parameters for each compound.

4.9 Partial method validation

A range of parameters can influence the performance of the method and these parameters should be evaluated to establish and document the performance of the final method, ready for clinical use. Method validation, a process of demonstrating that a certain method has the capacity of producing results fit for the purpose for which it was intended (Sargent, 2013), was then performed on both standard samples, and on biological matrixes and spiked matrixes as well, to take interpatient matrix variability into account. For this, charcoal stripped urine was not used as in most previously published articles, as the charcoal stripping also removes matrix effects (vitamins and cholesterol products) that might affect the results in the biological samples.

4.9.1 SPE Validation

The evaluation of the purity of the samples after SPE clean-up, was done by doing an SPE on the control urine sample as described throughout. The resuspended sample was then divided into two different parts, one to be analysed on the LC-MS/MS through doing a positive ionisation, and then negative ionisation scans. These scans gave the whole background in the urine samples as well as the metabolites of interest and the intensity of the background, which are not of interest during an MRM. This information might be of value because these are the molecules in the

ionisation source that are in competition for ionisation and might decrease the sensitivity of the method. The results of these scans can be seen in Figures S19.1 a-c (supplementary results). Although it seems as if there were quite a few different molecules detected during scans, the time during which most of the molecules (including background) elute from the chromatographic columns were in the time frame of 2 - 5 min and after 13 min.

Table 4.7: The final compound polarity, retention times, precursor and product ion, as well as collision and fragmentor energies used for MRM setup and also for data analysis.

Compound Name	RT	Polarity	Precursor m/z	Fragmentor energy (V)	Product m/z	Collision energy (eV)
Estriol-3-sulphate	2.28	Negative	366.5	148	287.1	36
Estradiol-3-glucuronide	2.89	Negative	446.6	146	271.1	44
Estriol-16 glucuronide	2.9	Negative	463.2	168	287.1	28
Estrone-3-glucuronide	3.02	Negative	444.6	131	113.1	16
Estradiol 17-sulphate	3.82	Negative	351.2	158	97	44
Estriol	4.15	Negative	287.2	163	171.1	36
Estriol-d3	4.2	Negative	290.16	163	173.10	36
Estradiol-3-sulphate	4.28	Negative	350.5	146	271.1	32
16-Ketoestradiol	5.32	Negative	285.1	163	145.1	40
Estrone-3-sulphate	5.53	Negative	348.2	143	269.1	28
16-Epiestriol	5.98	Negative	287.2	148	171.1	36
16 α -Hydroxyestrone	6.08	Negative	285.1	148	145.1	40
17-Epiestrol	6.48	Negative	287.2	153	145.1	44
4-Hydroxyestrone	7.85	Negative	285.1	153	161.1	44
4-Hydroxyestradiol	8.32	Negative	287.2	124	255.1	12
2-Hydroxyestradiol	8.85	Negative	287.2	183	147.0	28
DHEAS	9.03	Positive	418.1	94	102.2	4
2-Hydroxyestrone	9.66	Negative	285.1	163	161	40
17 β -Estradiol	11.16	Negative	271.2	158	145.1	44
Estradiol-d4	11.16	Negative	275.17	40	147.1	168
4-Methoxyestradiol	11.61	Negative	301.2	109	286.1	16
Testosterone	11.73	Positive	289.2	109	97.1	20
17 α -Estradiol	11.808	Negative	271.2	168	145.1	40
Estrone	11.89	Negative	269.1	156	145.1	40
Estrone-d4	11.92	Negative	273.15	163	147.10	40
Androstenedione	11.99	Positive	287.2	109	97.1	20
2-Methoxyestradiol	12.01	Negative	301.2	121	286.1	20
4-Methoxyestrone	12.22	Negative	299.2	109	284.1	16
DHEA	12.3	Positive	287.2	89	213.1	16
DHEA-d2	12.35	Positive	291.22	89	213.10	16
2-Methoxyestrone	12.36	Negative	299.2	114	284.1	20
2-Hydroxyestrone-3methyl ether	12.65	Negative	299.2	119	284.1	20
Progesterone	13.57	Positive	315.2	109	97.1	20
Progesterone-d9	13.60	Positive	273.15	109	147.10	20

Only progesterone elutes after 13 min and as this is a very good ionising metabolite, with no problems of detection, background molecules eluting at that time period were not a matter of concern. From 2 - 5 min, there were a number of metabolites of interest that eluted from the column, which probably includes the very polar sulphate and glucuronide metabolites. These metabolites might interfere with ionisation and although the levels of the sulphates and glucuronic acid conjugates are expected to be quite high in the urine, the effect of the matrix will again be investigated during linearity evaluations. The other half of the sample was evaporated to dryness and derivatised with pyridine, TMCS and BSTFA before being injected into a GC-MS instrument (Agilent Gas chromatogram, 7890A, with Mass spectrometer inert XL MSD), to be analysed by means of a full scan. The scan parameters included oven temperature increase from 70°C to 325°C in 10 min, with 1 µl, 1:10 split injection into a capillary GC-MS column and helium gas as a carrier, scanning in the MS from 40-450 m/z. The results, in this case (Figure S19.2 a&b), showed only a few molecules at high abundances to be detected as interference molecules. These include Ethanolamine, 3-methylphenol, glycerol, indole-3-acetic acid, glucuronic acid, inositol, palmitic acid and stearic acid. These compounds were identified by using the Chemstation MSD GC software, comparing the spectrums with those on the national institute of standard technology (NIST) database and only metabolites with >90% certainty was mentioned above. The GC-MS analysis also confirmed that the SPE method cleans the sample from urea. Urea is not ideal for LC-MS/MS analysis as it also increases the cleaning necessary on the apparatus and ionisation source, and elute with very little to zero retention, together with some of the salts.

4.9.2 Carry over

The carry-over amount was calculated to an estimate by evaluating the response of a blank methanol injection before and after the analyses of both a standard solution and a matrix spiked solution to ensure that autosampler and column carryover, as well as residue adhering, does not affect the quantification of metabolites (Sargent, 2013). The results showed that no carry-over occurred throughout and during the analysis and all further analyses were done with the assumption that no carry-over will occur.

4.9.3 Repeatability

Repeatability of the autosampler's injections was then evaluated by injecting pure standard samples quintuple (5) times followed by methanol blanks, and investigating the deviations in the standard for the retention times and relative responses (response/IS response). This was also repeated for the 50 ppb spiked urine sample to incorporate the effect of the matrix into the evaluation. The results showed that the retention times were highly repeatable with a coefficient of variation (CV) below 15%. The %CV on the abundances, however, was not acceptable for all the metabolites, especially Estrone-3-glucuronide, 17Epiestriol, DHEAS and the hydroxyl metabolites (Table 4.8). Most other deviations on the abundances were within acceptable range.

Although initially attempting to evaluate the repeatability of the autosampler injections, these results might indicate that the ionisation of some of the metabolites is not repeatable under the set conditions (metabolites with CV>15%) and for these metabolites, unideal linear ranges were expected.

4.9.4 Calibration and linearity

Before linearity was investigated, a literature investigation was performed to evaluate the average concentrations of the estrogen metabolites (and other steroid hormones to be investigated) in the human urine. Extreme variations in these levels can occur due to environmental and other factors, as described in Chapter 2, as well as intramonthly variations in the female reproductive cycle. Preliminary concentration ranges were obtained from literature as displayed in Table A1 (Annexure A). Each sample would be concentrated during drying and resuspension of the SPE product and the concentration range was chosen in such a way that the concentration factor was considered. This finally led to the choice of a broad concentration range to be chosen to determine the most linear concentration region to be used. Linearity and calibration were preliminarily done on standards redissolved in the starting mobile phase of 90:10 mobile phase A:B, before attempting to reproduce the results in matrix spikes. Calibration samples were prepared as described in Chapter 3, and 50 ppb of the internal standard mixture was added to each calibration sample and redissolved in the starting mobile phase. Linearity was then evaluated by using a total of 10 calibration standards, all analysed in replicate, to construct the calibration curves by plotting response/IS response against the concentration of metabolite/concentration of the internal standard. The concentration ranges evaluated for each metabolite can be seen in Table 4.9. The least square linear regression model was used where the equation of the trend line best fitting the data was determined. The equation of the line was then used to calculate the predicted concentration of the sample. The correction coefficient (r) was then used to determine and evaluate the line fitting where the value closest to 1 indicated the best fit (Sargent, 2013). The response of the calibration curves were linear for most metabolites, with correlation coefficients ranging from low values (0.760 & 0.912) to 1. The concentration range evaluated, concentration range that gave the most linear results and r^2 values are shown for each metabolite in Table 4.9.

4.9.5 Limit of detection and quantification

Using the calibration data, the limit of detection and limit of quantification, were calculated by the linear regression method. The linear regression method predicts the lowest concentration at which an analyte can be detected and quantified accurately, by using deviations from the data-points that best fit the linear line (Shrivastava & Gupta, 2011). The equations used are as seen below in Equation 1 & 2, where S_a represents the response's standard deviation (calculated by standard deviations of y-intercepts and y-residuals of the regression line) and b represents the slope of the calibration curve. The LOD and LOQ values for each of the metabolites were

calculated and can be seen in Table 4.9. Although the correlation coefficient for most of the metabolites, excluding progesterone, 2 & 4-hydroxyestrone, and 4-hydroxyestradiol, looked quite promising, the LOD and LOQ values were less so. These results led to a conclusion that the method might not be able to detect low concentrations of metabolites and that quantification of these metabolites may have a poor repeatability.

Equation 1: The formula for the calculation of the lower limit of detection

$$LOD = 3 \times S_a/b$$

Equation 2: The formula for the calculation of the lower limit of quantification

$$LOD = 10 \times S_a/b$$

Table 4.8: The table summarising the coefficient of variance for each metabolite's relative response between different injections done on the exact same sample.

Metabolite	%CV of relative metabolite response	Metabolite	%CV of relative metabolite response
16-Epiestriol	6.17	Androstenedione	0.75
16-Ketoestradiol	5.19	DHEA	1.39
16 α -Hydroxyestrone	4.09	DHEAS	20.07
17-Epiestriol	172.23	Estradiol 3 glucuronide	5.75
17 α -Estradiol	2.96	Estradiol 3 sulphate	1.55
17 β -Estradiol	5.71	Estradiol-17-sulphate	3.70
2-Hydroxyestradiol	160.50	Estriol	1.52
2-Hydroxyestrone	157.02	Estriol 16 glucuronide	5.89
2-Hydroxyestrone-3-Methyl ether	7.39	Estriol 3 sulphate	6.90
2-Methoxyestradiol	1.65	Estrone	1.91
2-Methoxyestrone	1.41	Estrone 3 glucuronide	20.47
4-Hydroxyestradiol	146.16	Estrone 3 sulphate	7.03
4-Hydroxyestrone	5.39	Progesterone	1.79
4-Methoxyestradiol	2.94	Testosterone	0.14
4-Methoxyestrone	4.50		

The results as seen in the Table 9.4, indicate high LLOQ results and in some cases, LLOQ values were not in the linear range of the analytes. Repeatability also gave less than ideal results and may be the reason for the unexplainable bad results. Due to the results, a decision was made that the analytical method would either have to go through additional optimisation steps to increase the sensitivity even more before additional validation is done on the method, or alternative methods should be investigated as the limitations of the equipment available did not give reliable results on the method and means of ionisation as developed up to this point. No further validation steps were then followed.

4.9.6 Discussion of the validation results

Because all developed methods need at least partial method validation before implementation to ensure certain parameters, validation on the developed methods was initiated. In an attempt to validate the developed method, carry over, repeatability, linearity, LLOD and LLOQ were evaluated. The carryover results showed that the post-run and needle wash was sufficient and no carry over occurred. However, the repeatability of the injection (auto-sampler) and ionisation (MS) after multiple injections of the exact same sample, gave worrying results. For metabolites 17-Epiestriol, 2-hydroxyestradiol, 2-hydroxyestrone and 4-hydroxyestradiol, the coefficient of variation was well above 100%. Estrone-3-glucuronide and DHEAS also gave CV values above the expected 15%. All other metabolites gave a CV<15%. These results indicate that the ionisation of these metabolites with high variation results, do not occur repeatedly and quantification of these concentrations of metabolites would, therefore, not be possible on the developed method. The r^2 values for most of the metabolites were fairly linear, with 4-hydroxyestradiol (0.912) and progesterone (0.760) having the lowest values. The LLOQ and LLOD concentration values were also fairly high, ranging from 2.99ng/ml for DHEAS to 4379.64 ng/ml for progesterone, confirming that the method would not be usable for quantification of low concentration metabolites as in the human urine.

Table 4.9: A table with the concentrations evaluated, that gave a linear response, the r^2 values as well as the LOD and LOQ values of each metabolite as calculated by equation 1 & 2 previously described.

Metabolite	Concentration range evaluated (ng/ml)	Concentration range linear (ng/ml)	r^2 value	LLOD	LLOQ
16-Epiestriol	0.30-150.00	1.2-37.5	0.999	1.53	5.12
16-Ketoestradiol	0.5-300.00	2.3-75	0.997	9.34	31.15
16 α -Hydroxyestrone	2.00-1200.00	2.3-300	0.997	18.62	62.08
17-Epiestrol	0.30-150.00	0.6-37.5	0.998	8.42	28.07
17 α -Estradiol	1.20-600.00	4.60-600	0.998	16.95	56.52
17 β -Estradiol	1.20-600.00	4.7-600	0.996	7.04	23.46
2-Hydroxyestradiol	1.20-600.00	18.8-600	0.998	32.16	107.22
2-Hydroxyestrone	0.5-300.00	2.3-300	0.955	29.61	98.73
2-Hydroxyestrone-3methyl ether	0.5-300.00	2.3-300.00	0.998	29.10	97.01
2-Methoxyestradiol	0.5-300.00	2.3-300	0.997	28.38	94.60
2-Methoxyestrone	0.5-300.00	2.3-300	0.991	14.21	47.38
4-Hydroxyestradiol	0.30-150.00	4.7-150	0.912	12.77	42.58
4-Hydroxyestrone	0.30-150.00	0.3-37.5	0.979	6.19	20.65
4-Methoxyestradiol	0.30-150.00	1.20-150.00	0.993	40.52	135.08
4-Methoxyestrone	0.30-150.00	1.2-150.00	0.991	15.02	50.09
Androstenedione	15.00-7500.00	58-7500.00	0.992	167.05	556.83
DHEA	5.00-3000.00	23-3000.00	0.996	90.19	300.65
DHEAS	0.30-150.00	2.3-37.5	0.998	0.89	2.99
Estradiol 17-sulphate	0.5-300.00	2.3-300	0.993	9.84	32.82
Estradiol-3-glucuronide	0.30-150.00	1.2 -37.5	0.999	20.86	69.56
Estradiol-3-sulphate	0.30-150.00	1.2-37.5	1	24.31	81.04
Estriol	1.20-600.00	4.7-150	0.993	6.78	22.62
Estriol-16 glucuronide	0.30-150.00	0.3-37.5	0.999	2.35	7.84
Estriol-3-sulphate	1.80-900.00	7-225	0.986	4.65	15.52
Estrone	1.20-600.00	4.60-600	0.992	13.43	44.79
Estrone-3-glucuronide	0.30-150.00	0.3-37.5	0.998	4.43	14.78
Estrone-3-sulphate	3.00-1500.00	11.7-37.5	0.992	30.32	101.09
Progesterone	100-600.00	470-7500	0.760	1313.89	4379.64
Testosterone	1.20-600.00	4.60-600.00	0.994	10.70	35.67

4.10 Conclusion Chapter 4

The method to analyse 29 estrogen and related steroid metabolites was developed. This development included mobile phase and mobile phase modifiers, the chromatographic column and conditions, and also the MS source conditions. The sample cleanup in the form of an SPE method was also developed and optimised to evaluate matrix spiked samples. The LC-MS/MS method was then optimised to the best of the analyst's ability to increase the sensitivity, especially for the metabolites that don't ionise well, including the hydroxyl estrogens and DHEAS. As part of validation of the developed method; carry over, repeatability, linearity, LLOD and LLOQ were evaluated. Unfortunately, the results from standards (excluding matrix interference) showed that the method could not be validated in the concentration ranges that are relevant for quantification of estrogen metabolites in urine. In comparison to the literature published by Gaikwad *et al.* (2008) with LOD values as low as 14 fmol and quantifying from 0.05 pmol/mg creatinine, the results obtained during this study, were not as successful and alternative methods will be evaluated to hopefully give better ionisation, detection and sensitivity (Gaikwad *et al.*, 2008; Gaikwad *et al.*, 2009). Additionally, the sample preparation methods might be optimised even more to permit more concentration of the samples without increasing the matrix effect, something that has not been evaluated.

CHAPTER 5

METHOD DEVELOPMENT FOR DERIVATISED STEROID HORMONES

5 Introduction

After development and partial validation of the non-derivatised method, as described in Chapter 4, and obtaining less than ideal validation results, the literature was once again investigated to compare the results and findings of the developed method (Chapter 4) with those of other scientists. To still reach the aim of the study, the two options were to either try and adjust the developed method (SPE and LC-MS) for more sensitivity or to develop an alternative method that would be more sensitive and would result in the quantification of even lower metabolite concentrations in the urine. Due to the vast amount of optimisation already done on the developed method and relatively poor validation results, the decision was made to rather develop a more sensitive method by using other techniques, principals or chemical reactions to increase the sensitivity. The most recommended approach to increase sensitivity and selectivity is through derivatisation (Athanasiadou *et al.*, 2013; Faqehi *et al.*, 2016). The derivatisation of metabolites increases the sensitivity by increasing the mass of most of the relevant analytes, thereby resulting in better discrimination based on mass. The derivatisation reaction adds a certain chemical group to the parent molecules causing their molecular mass to increase. This results in a decrease in the number of molecules that may cause interference, since more low molecular weight molecules are naturally present in matrix compared to higher molecular weight molecules, and only certain molecules and compounds will derivatise, thereby eliminating some of the effects of non-derivatised molecules (Faqehi *et al.*, 2016). Secondly, the derivatisation procedure also changes the polarity of metabolites, usually making it more non-polar. This decrease in the polarity can increase the chromatographic column retention and help to obtain more efficient separation of more polar metabolites. In addition, more efficient separation usually contributes to improved ionisation (Athanasiadou *et al.*, 2013; Nishio *et al.*, 2007). In this section, a derivatisation method was firstly chosen, followed by the exact same procedures of method development as for the non-derivatised method, and rationale for each step was not explained in detail again, except for deviations, which will be motivated.

5.1 Derivatisation

Choice of derivatisation

As the validation results of Chapter 4 illustrated, alternative methods to the non-derivatised method may be necessary. The first decision to be made regarding the development of a more sensitive method, is which derivatisation method to use to increase the sensitivity and selectivity. A derivatisation reagent ideal for ESI ionisation and MS/MS detection should firstly not have too high a molecular weight and should consist of either a moiety that is permanently charged or a proton-affinitive moiety. Additionally, the reagent should be moderately hydrophobic and have a fragmentable moiety for a specific product ion that is characteristic of the derivatised steroids.

Lastly and probably most importantly, the derivatisation reaction should occur under mild conditions for steroid metabolites, especially those containing hydroxyl and keto groups (Higashi & Ogawa, 2016). Additional factors that were taken into account in the deciding of derivatisation methods were cost, labour intensity and efficiency. Fusaric acid, together with 2-methyl-6-nitrobenzoic anhydride, 4-dimethylaminopyridine and trimethylamine is one of the derivatisation reagent combinations for the detection of hydroxysteroids in sub-femtogram concentrations on a chromatographic column (Yamashita *et al.*, 2010). The downfall for this method of derivatisation is that they have only used the method on DHEAS, testosterone, pregnenolone and 17 α -hydroxypregnenolone. Although it is expected to work for all hydroxysteroids, the use of this derivatisation method for the other metabolites of interest and the hydroxyestradiol compounds has not been tested. Other drawbacks include that it is a bit more laborious and time-consuming, and an increased amount of reagents is used, making it a more costly method. An advantage of this method is the increased sensitivity to allow the detection of sub-femtogram concentrations. Furthermore, the recommended sample preparation was done after derivatisation to ensure that the the derivatisation reagents were not injected into the chromatographic column and ionisation source. By derivatisation of estrone and estradiol alone, Faghihi *et al.* (2016) have detected 2 picogram (pg) in human serum and plasma after derivatisation with FMPTS in the presence of TEA. The authors suggested that this method of derivatisation might also be used for other naturally occurring phenolic steroids, however, they emphasised that the samples are not stable once derivatised. PED, PPZ, MPPZ and APZ are some of the other derivatisation reagents used on estrone, estradiol and DHEA. Their drawback, however, is that the above-mentioned needs to be synthesised beforehand, making it an extremely laborious and costly procedure (Nishio *et al.*, 2007).

In a review on the chemical derivatisation of samples for the enhanced sensitivity of steroids during LC-ESI-MS/MS, Higashi and Ogawa (2016) compared different types of derivatisations for the steroid hormone analysis. Although the derivatisation of all types of steroids is explained and differs, they state that the usage of DNSCL as derivatisation reagent for the dansylation reaction to occur, is the most practical procedure. It gives pg/ml LLOQ values for more than 16 estrogen metabolites, is not laborious at all and includes a derivatisation procedure of approximately 10 min in total. Derivatisation with PSCI and PA with femtogram (fg) detection limits for parent estrogen metabolites were also discussed as part of the review. In a paper published in 2007, derivatisation methods on estrogen hormones were compared, where DNSCL, FMPTS and PFBBBr were used as derivatisation reagents. The results showed that DNSCL derivatisation is the best option for the detection of estrogens in sample matrixes, if sufficient sample preservation is performed beforehand (Lin *et al.*, 2007). Other derivatisation reagents for steroids and estrogens include 4-(1H-pyr-azol-1-yl) benzene sulfonyl chloride (Wang *et al.*, 2015; Xu & Spink,

2008), N-methyl-nicotinicacid N-hydroxysuccinimide ester (Yang *et al.*, 2008) and isomers of 1,2-dimethylimidazole-sulfonylchloride (Keski-Rahkonen *et al.*, 2015; Xu & Spink, 2008), each with its own advantages and disadvantages. Dansylation is the derivatisation method for the estrogen-related steroid hormones that is repeatedly recommended universally. This approach, where the secondary amine moiety of the dansyl chloride reagent reacts with the phenolic hydroxyl group of the steroid metabolite through nucleophilic aromatic substitution, can be viewed in Figure 5.1. Disadvantages of this derivatisation method are that the product ions are not specific for the analyte, but rather a product that was generated by a derivative moiety (Faqehi *et al.*, 2016). With the LC-MS/MS apparatus, however, specificity is achieved not only by the product ion mass but also by chromatographic retention times, and precursor ion and product ion selective monitoring. Secondly, potential pitfalls are limited by switching the chromatographic eluent to waste to reduce the amount of dansyl chloride that enters the ionisation source.

Dansylation method

A derivatisation method in the form of dansylation was chosen due to the low cost, little sample preparation and limited time required for derivatisation reaction to complete. Although some of the results obtained through the underivatised method development will be used as guidelines in the optimisation and validation of the derivatised method, the ionisation and even chromatographic separations will differ and, therefore, the method development process was repeated (as done in Chapter 4). The derivatisation method was described in a number of articles and application notes of estrogens measured in the human urine or serum by LC-MS/MS (Li & Franke, 2015; Xu *et al.*, 2005), thus, there is sufficient literature on which to base choices. Initially, 50 μ l Dansyl chloride (1 mg/ml) and 50 μ l 1 M sodium bicarbonate (NaHCO_3) buffer was added to dried samples/standards and incubated at 60°C for 10 min as described in the literature (Szczesniowski, 2012; Xu *et al.*, 2005). The concentration of the derivatising reagent, the buffer pH, derivatisation temperature and time, as well as the addition of ascorbic acid during derivatisation was later optimised, once the method for evaluation of the responses was developed.

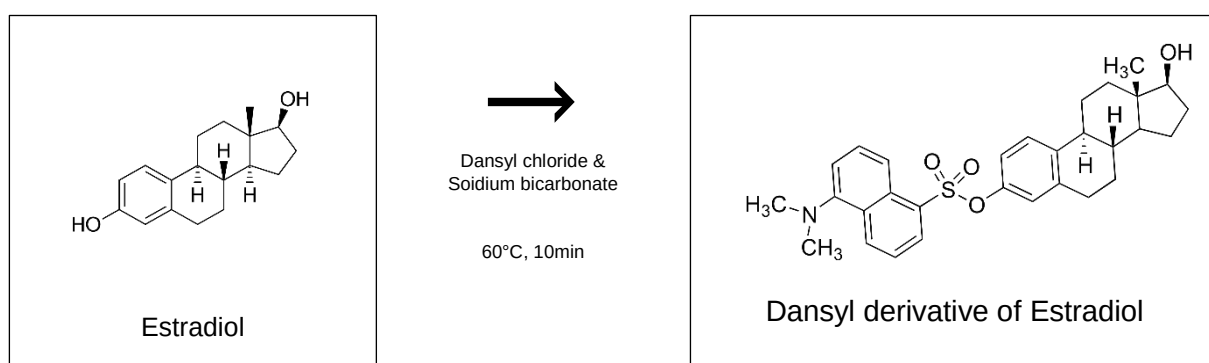


Figure 5.1: A Structural illustration of the conversion of estradiol to dansyl-estradiol, by addition of dansyl chloride during a 10 min, 60°C incubation period.

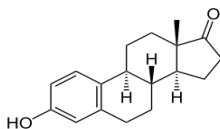
5.2 Mobile phase modifiers

The mobile phase modifier responsible for aiding in ionisation was one of the variables that was investigated. Although the same estrogen-related metabolites were evaluated, the derivatisation will cause the once negatively ionised metabolites to predominantly ionise positively, due to the dansyl group replacing a proton on one of the hydroxyl moieties of the steroid hormones. For positive ionisation, a mobile phase modifier with proton donating properties, such as ammonium formate (Szczesniowski, 2012) or formic acid (Xu *et al.*, 2005; Ziegler *et al.*, 2010), would be ideal. Although formic acid is the most preferred positive ionisation mobile phase modifier, note was taken that not all the metabolites will derivatise, causing possible mixed mode (positive and negative ionisation) possibilities. For the dansylation reaction to occur, the parent molecule should have either an amine or a phenol moiety with which the dansyl group can interact. Figure 5.2 shows the structures of all 29 metabolites to be investigated, grouped either as amine/phenol-containing, or non-containing metabolites.

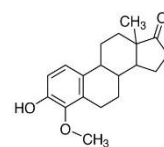
The effect of the two mobile phase modifiers, ammonium formate and formic acid on the abundance of precursor and product ions detected, were compared by using 10 ppm stocks of the 29 estrogen working standards and derivatising them, as briefly explained in section 5.1. After derivatisation of only the metabolite standards that will be able to form dansyl derivatives (Figure 5.2), the samples were injected through direct infusion as described in Chapter 3.3.5, using the full scan conditions. The concentration of modifier, as described in the literature, included 5 mM ammonium formate and 0.1% formic acid (Szczesniowski, 2012; Xu *et al.*, 2005). Although previous articles indicated that formic acid as mobile phase modifier will give the best detection in positive ionisation, note was taken that the conjugate metabolites will most probably still be ionised best in the negative ionisation mode. The results for the formic acid were as predicted and gave good ionisation for most of the positive ionising metabolites, including the dansyl derivatives. However, ionisation (positive and negative) of the conjugates was poor (if any at all), as reflected by the very low to no precursor ions detected in the full scans and in the Optimizer program. With ammonium formate, on the other hand, all metabolites were detected: the derivatised and previously positive ionised metabolites in positive ionisation, and the sulphate and glucuronide conjugates (some in positive ionisation but all in negative ionisation).

Phenol containing metabolites

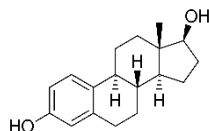
Estrone



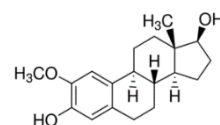
4-Methoxyestrone



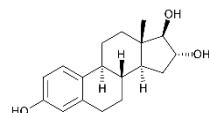
17-(α/β) Estradiol



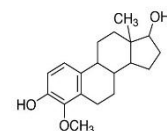
2-Methoxyestradiol



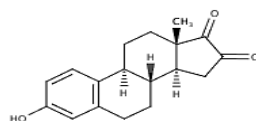
Estriol



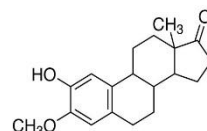
4-Methoxyestradiol



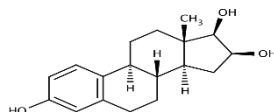
16 ketoestradiol



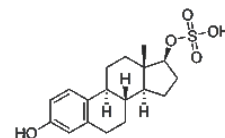
2-Hydroxy-3Methylether



16-Epiestriol

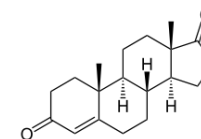


Estradiol-17-sulphate

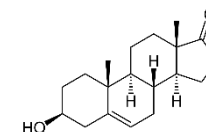


Metabolites not containing any phenol groups

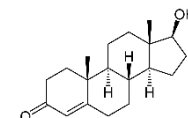
Androstenedione



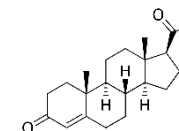
DHEA



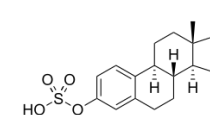
Testosterone



Progesterone



Estrone-3-sulphate



17-Epiestriol		Estriol-16-glucuronide		Estradiol-3-sulphate	
16-Hydroxyestrone		2-Hydroxyestradiol		Estriol-3-sulphate	
2-Hydroxyestrone		4-Hydroxyestradiol		Estrone-3-glucuronide	
4-Hydroxyestrone		2-Methoxyestrone		Estradiol-3-glucuronide	
				DHEAS	

Figure 5.2: Sorting of the 29 estrogen metabolites into either phenol-containing or non-containing metabolites as visually illustrated in their structures.

The only two metabolites that gave less than ideal ionisation results with the ammonium formate as mobile phase additive, were DHEA and DHEAS. Up until this point, it was aimed to analyse the metabolites in a single run and if not possible to do so, to then rather analyse the metabolites on one single mobile phase combination to decrease the labour involved in making and changing the mobile phases and also to make it as economically as possible on both the solvents, and the column equilibration times and column life. Even though the DHEA and DHEAS do not ionise as well in the ammonium formate modifier additive, the choice was still made to use ammonium formate, which resulted in the detection of all other metabolites. The source conditions could still increase the ionisation potential for these metabolites, and if in the end they would still be a problem, all parent and important metabolites would be detected and secondary methods for the additional non-estrogenic metabolites such as DHEA and DHEAS, could, be developed later on and performed on samples with abnormal estrogen concentration results. A small percentage of mobile phase B (5% acetonitrile) were also included in mobile phase A (water with ammonium formate) to prevent any ammonium initiated growth in the mobile phase A bottle.

5.3 Optimisation of ionisation conditions

Mass spectrometric evaluation of the estrogen and related metabolites, with and without the formation of the dansyl chloride derivatives, vary in a few more ways. Other than the previously mentioned change in ionisation polarity for most of the metabolites, the precursor, product and ionisation conditions may change. As previously mentioned, the dansylation reactions occur on the hydroxyl moiety of phenolic groups. For some of the metabolites, that includes only one possible dansylation position, while for others there may be two. The hydroxyl metabolites are good examples, since they can form either one or two dansyl derivatised products. Additionally, dansylated steroid hormones can also form sodium adducts. These sodiated molecules $[MNa^+]$ formed less abundantly than the major protonated molecule $[MH^+]$, but were still evaluated on full scans to ensure that the most optimal positively ionised molecules were selected for quantification. After deciding to use 5 mM ammonium formate as the modifier in the mobile phase A and mobile phase B preliminary as 100% acetonitrile, direct infusions for full scans were repeated, although, this time it was for precursor ion evaluations. Dansylated standards (0.5 μ l of 10 ppm) were injected and the MS was set to full scan to evaluate firstly, the precursor ions that formed and secondly, evaluate which formed predominantly. Once the most abundant ionised molecules were identified, the ionisation and MRM conditions could be optimised by using the optimiser software. This optimisation was also performed on 0.5 μ l of 10 ppm dansylated standards. These results can be viewed in Table 5.1 and were used to make decisions about the ionisation mode, the most informative fragments, as well as the fragmentor and collision energy to be used. The catechol estrogens all produced a 170 m/z product ion, while the other

metabolites gave the product ion of 171 m/z, both due to the dansyl group being added during the dansylation process.

5.4 Optimisation of source conditions

Identification of the optimal ionised conditions (precursor ion, most abundant fragment ion, and fragmentor and collision energy for each steroid hormone metabolite), then allowed for the optimal source conditions to be evaluated. The optimal source conditions were obtained by using the MassHunter source optimiser software, which runs the optimisation of the drying gas flow and temperature, the sheath gas flow and temperature, the capillary and nozzle voltages, as well as the nebuliser voltage. The direct infusion was performed with all parameters except for the parameter being investigated, and the fragmentor and collision energies were set by default. The fragmentor and collision energies, together with the precursor and product ions for the MRMs, were set as displayed in Table 5.1. The ranges for the parameters that were varied (identical to those in Chapter 4) can be seen in Table 5.2, which includes the steps taken within the ranges. The results from the source optimiser were then processed and the final conditions chosen for the 29 hormone metabolites, which can be seen in Table 5.3.

Table 5.1: The table summarising the most optimal and chosen polarity, precursor ion and fragmentor energy as well as product ion and collision energy values. The table additionally also indicates the dansylated formula for most of the metabolites. For metabolites not dansylated, their analytical standard formula is indicated.

Compound Name	Formula dansylated	Polarity	Precursor ion (m/z)	Fragmentor energy (V)	Product ion (m/z)	Collision energy (eV)
16-Epiestriol	C ₃₀ H ₃₅ NO ₅ S	+	522	170	171	36
16-Ketoestradiol	C ₃₀ H ₃₃ NO ₅ S	+	520.38	166	171.1	44
16 α -Hydroxyestrone	C ₃₀ H ₃₃ NO ₅ S	+	520.38	166	171.1	44
17-Epiestrol	C ₃₀ H ₃₅ NO ₅ S	+	522	170	171	36
17 α - Estradiol	C ₃₀ H ₃₅ NO ₄ S	+	506.19	148	171.1	40
17 β - Estradiol	C ₃₀ H ₃₅ NO ₄ S	+	506.19	148	171.1	40
17 β - Estradiol-d4	C ₃₀ H ₃₁ D ₄ NO ₄ S	+	510	210	171.1	40
2-Hydroxyestradiol	C ₃₀ H ₃₅ NO ₅ S	+	755.01	205	170..1	44
2-Hydroxyestrone	C ₃₀ H ₃₃ NO ₅ S	+	753.37	173	170.1	36
2-Hydroxyestrone-3methyl ether	C ₃₁ H ₃₅ NO ₅ S	+	534.4	146	171.1	36
2-Methoxyestradiol	C ₃₁ H ₃₇ NO ₅ S	+	536.42	114	171.1	44
2-Methoxyestrone	C ₃₁ H ₃₅ NO ₅ S	+	534.4	146	171.1	36
4-Hydroxyestradiol	C ₃₀ H ₃₅ NO ₅ S	+	755.01	205	170.1	44
4-Hydroxyestrone	C ₃₀ H ₃₃ NO ₅ S	+	753.37	173	170.1	36
4-Methoxyestradiol	C ₃₁ H ₃₇ NO ₅ S	+	536.42	114	171.1	44
4-Methoxyestrone	C ₃₁ H ₃₅ NO ₅ S	+	534.4	146	171.1	36

Androstenedione	C ₁₉ H ₂₆ O ₂	+	287.2	135	97.1	24
DHEA	C ₁₉ H ₂₈ O ₂	+	289.22	120	213.2	20
DHEAS	C ₁₉ H ₂₈ O ₅ S	+	418.1	94	102.2	4
Estradiol 17-sulphate	C ₁₈ H ₂₃ O ₅ S	-	351.2	168	97	44
Estradiol-3-glucuronide	C ₂₄ H ₃₁ O ₈	-	446.6	146	271.1	44
Estradiol-3-sulphate	C ₁₈ H ₂₃ O ₅ S	-	350.5	146	271.1	32
Estriol	C ₃₀ H ₃₅ NO ₅ S	+	522	170	171	36
Estriol-16 glucuronide	C ₂₄ H ₃₂ O ₉	-	463.2	168	287.1	28
Estriol-3-sulphate	C ₁₈ H ₂₃ O ₆ S	-	366.5	148	287.1	36
Estriol-d3	C ₃₀ H ₃₂ D ₃ NO ₅ S	+	525	170	171	36
Estrone	C ₃₀ H ₃₃ NO ₄ S	+	504.0	114	171.1	16
Estrone-3-glucuronide	C ₂₄ H ₂₉ O ₈	-	444.6	131	113.1	16
Estrone-3-sulphate	C ₁₈ H ₂₁ O ₅ S	-	348.2	143	269.1	28
Progesterone	C ₂₁ H ₃₀ O ₂	+	315.23	109	97.1	20
Progesterone-d9	C ₂₁ H ₂₁ D ₉ O ₂	+	324.3	141	100.1	24
Testosterone	C ₁₉ H ₂₈ O ₂	+	289.22	121	97.1	20

Table 5.2: The parameters varied during the source optimisation with the ranges of the parameters as well as increments of change within the range. These included the capillary voltage, nozzle voltage, nebuliser voltage as well as the drying gas and sheath gas flows, and temperatures.

	Starting value	End value	Increments
Capillary voltage (V)	1000	4500	500
Nozzle voltage (V)	0	2000	500
Nebuliser voltage (V)	15	50	5
Drying gas flow (L/min)	2	8	2
Drying gas temperature(°C)	100	350	25
Sheath gas temperature (°C)	100	400	50
Sheath gas flow (L/min)	4	12	2

Table 5.3: The processed results of the source optimisation, showing the most practical conditions for all metabolites. Due to the fact that positive and negative ionisation polarities may have different capillary and nozzle voltages, positive and negative conditions are shown individually.

	Capillary voltage(V)	Drying gas flow (L/min)	Drying gas temperature (°C)	Nebuliser (V)	Nozzle (V)	Sheath gas flow (L/min)	Sheath gas temperature (°C)
Positive ionisation	2000	8	300	25	500	8	300
Negative ionisation	3000	8	300	25	500	8	300

5.5 Liquid chromatographic separations

5.5.1 Chromatographic column and mobile phase choice

Rather than comparing various different chromatographic column packing materials, the literature suggested that C18 columns are the preferred choice for most of the derivatised metabolites (Caron *et al.*, 2015; Szczesniewski, 2012; Xu *et al.*, 2005; Zhao *et al.*, 2014). The C18 columns available for the study were evaluated for sufficient separation and compared for optimal peak shape. These columns included the Agilent Poroshell 120 C18-EC (2.1 x 100 mm x 2.7 μ M) as the chosen column to use for the underivatised metabolites and the Agilent Zorbax Eclipse plus C18 (2.1 x 100 mm x 1.8 μ M). The chromatographic separation of both of these columns was evaluated during the non-derivatised method development, resulting in the best separations and peak shapes of all the columns evaluated. The dansyl derivatives are, however, larger molecules and chromatographic separations might be influenced due to altered metabolite shapes and polarities. A standard 100 ppb mixture of the estrogenic metabolites was derivatised and 1 μ l thereof was injected onto the chromatographic columns with source and ionisation conditions, as previously discussed. At a flow of 0.3 ml/min and a column temperature of 40°C, the exact same mobile phase gradients as for the underivatised method were evaluated and again compared to 100% MeOH, 100% acetonitrile and a 50:50 mixture of MeOH: ACN. Once the results were reviewed, a few different gradient adaptations were made for each column to optimise the chromatographic separation. The most important considerations were peak shape and sufficient separation. Both columns gave comparable results in terms of peak shape and separation. Unfortunately, sufficient separation of the hydroxyl estrogens were obtained for neither of the columns, and with none of the mobile phase B choices or the gradient changes. The results of the separation of the 2 and the 4-hydroxy estrone and estradiol metabolites can be seen in Figure 5.5 for both columns. Although the hydroxy metabolites, as previously mentioned, can be derivatised at two positions, results indicate that complete dansylation did occur and all the hydroxyl metabolites were derivatised at both positions, resulting in high m/z precursor ions.

As Figure 5.5 depicts, sufficient separation of these catechol estrogens was not obtained with either C18 chromatographic columns, and additional columns for sufficient separation were evaluated. Although few literature references discussed insufficient separation of these metabolites and very little used any columns other than the C18 packing materials, all options were still evaluated to find a solution for the problem at hand. A Zorbax eclipse C8 column was evaluated. Although the retention to the column sorbent is decreased on C8 columns and this usually leads to less retention, resulting in a decrease in the separation capabilities, the problem at hand might be resolved by the lower retention factor. On both the C18 columns, the retention of the hydroxyl metabolites occurs only once a very high percentage of the organic composition is used. Once 100% of the organic mobile phase is reached, chromatographic retention cannot

be controlled anymore, with no further changes in mobile phase gradient possible; this often leads to insufficient separation. A less steep gradient was a possibility, although the shorter runtime and the peak shapes would have been compromised for sufficient separation. The Zorbax Eclipse C8 (2.1 x100 mm, 1.8 μ M) column, with identical dimensions to the Zorbax C18 column used, was then evaluated, injecting 1 μ l of 100 ppb of the exact same standard mixture as before. This was first done under the same conditions and gradient as for the C18 column, despite the retention capacity variations between the two columns. The separation of all the metabolites was satisfactory, including the hydroxyl estrogens, after a few gradient alterations were made due to decreased retention on the column. The three combinations of organic mobile phases were again compared, including methanol, acetonitrile and a combination of 50:50 methanol acetonitrile. The best results were obtained for 100% acetonitrile. Although all the combinations gave satisfactory separations, the acetonitrile allowed a higher flow rate of 0.4 ml/min (evaluated as part of the mobile phase B choice to ensure that higher flow rates would be compatible with the mobile phase), without increasing the backpressure to a non-ideal high value. The higher flow rate contributed to overall better peak shapes. The other two combinations of organic mobile phases did not permit high flow rates as the backpressure (due to the presence of MeOH) increased dramatically. The results of the hydroxyestrogen separation on the C8 column can be seen in Figure 5.5 and all the other chromatographic separations on the Zorbax Eclipse plus C8 column are included in the supplementary results, Figure S20.

Conclusion

The Agilent Zorbax Eclipse plus C8 column was chosen for chromatographic separations from this point on to ensure sufficient separation of all the metabolites and it gave the most optimal peak shapes. The organic mobile phase was also chosen to be 100% acetonitrile, as 100% methanol and a 50:50 methanol:acetonitrile combination did not improve the chromatographic separations. Furthermore, a flow rate of 0.4 ml/min was preliminarily chosen instead of the initial 0.3 ml/min as it gives optimal separations with the best defined peaks.

5.5.2 Peak identification and preliminary retention sequence

Once again (as in the previously developed method), to confirm the retention times of metabolites, especially those with overlapping MRM transitions, the metabolite standards were injected individually, and combined in the MRM method developed and optimised in this section: with mobile phase modifiers as in section 5.2; MRM transitions, ionisation conditions and MS source conditions as in section 5.3 - 5.4; and column, mobile phase B and flow rate choice as in section 5.5. Identification of the peaks, in comparison to its analytical standard, was then used to confirm which specific metabolite each peak represents, and which retention time is associated with which metabolite. The working standards (100 ppb) were derivatised and 1 μ l of this was injected. The visual illustration of chromatographic separation, preliminary retention time and retention order of

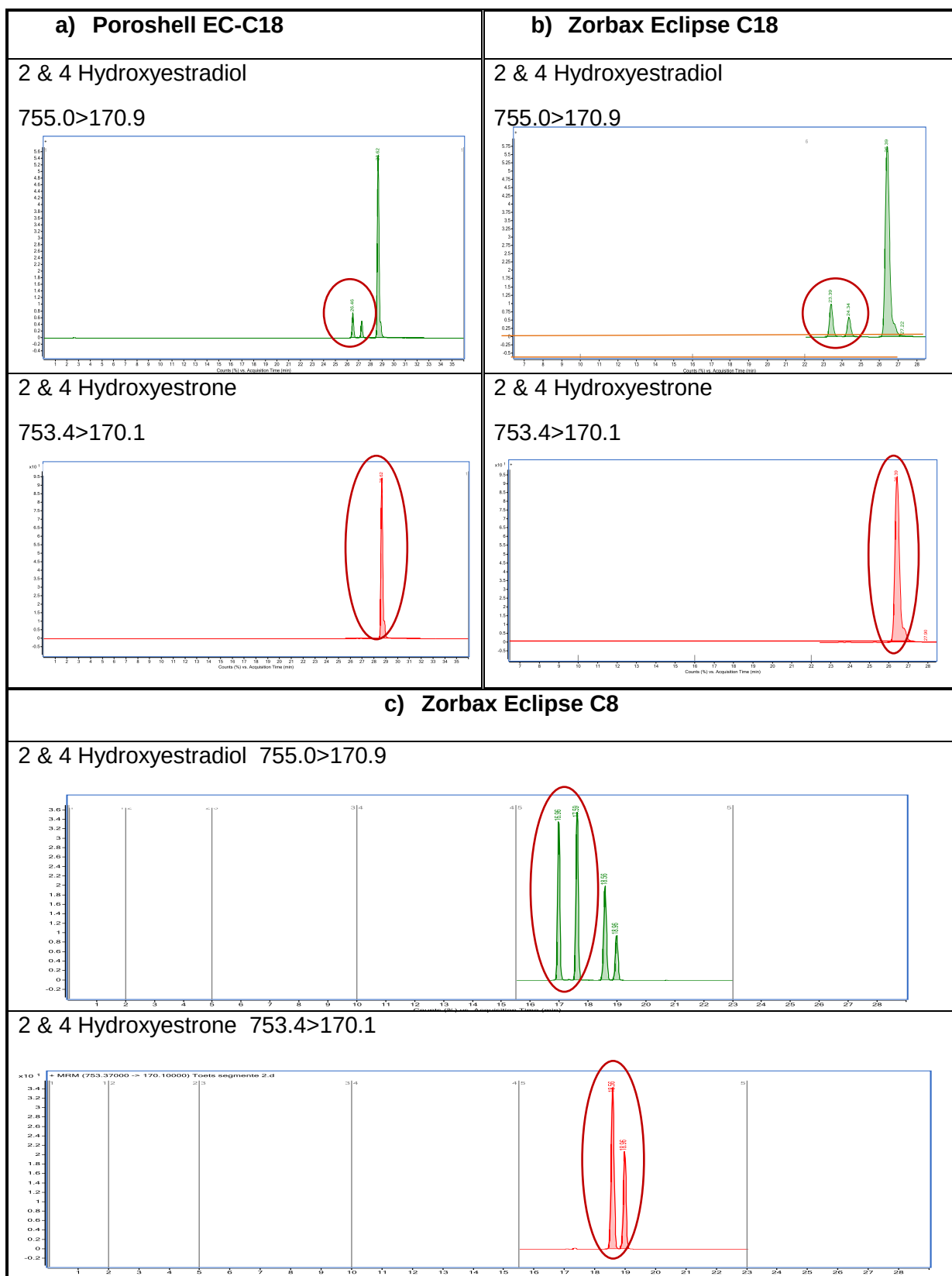


Figure 5.5: Chromatographic illustrations of the separation of 2&4-hydroxy estrogen metabolites on a) Poroshell-C18-EC, b) Zorbax Eclipse plus C18 and c) Zorbax Eclipse plus C8. The transition of 755.0>170.1 is for the hydroxyestradiols and transition 753.4>170.1 for hydroxyestrone metabolites.

the metabolites summarised as per MRM transition, can be viewed in Figure S20. The final retention times after optimisation are indicated in Table 5.6 (section 5.7), where all the metabolites and accurate retention times in the urine matrix are indicated.

5.6 Final method optimisation

5.6.1 Sample clean-up adjustment and optimisation

Although metabolites were now derivatised after sample preparation and analysed chromatographically using another method, the sample preparation was still sufficient, so there was no need to change it. This was evaluated by performing the SPE method as described in Chapter 4.8, followed by the LC-MS/MS as developed in section 5 on a spiked matrix sample. This spiked matrix sample was created by drying 20 µl of a 1 ppm standard mixture and resuspending it in a control urine sample, before SPE, to represent the matrix. The SPE eluent was dried, derivatised and analysed. The response from a spiked sample matrix was compared to that of a derivatised standard mixture of the same concentration (concentration spiked with). The results showed that the sample preparation, as developed up until this point, was not sufficient. Firstly the urine volume was reduced to 1 ml to decrease the matrix effect of larger urine volumes. This volume reduction improved metabolite response. The sample preparation method was then further optimised, for more optimal results. Due to the wide range of the metabolites' polarities, (very polar to less polar) and the fact that only the less-polar and non-polar metabolites derivatise, as explained in Figure 5.2 with the results in Table 5.1, evaluation of the more polar metabolites separately from the non-polar metabolites were investigated as a possible method. Standard samples (100 ppb) were loaded onto eight different SPE cartridges, which were then washed with 5%, 10%, 15%, 20%, 25%, 30%, 35% and 40% organic solvent in the aqueous washing step to give an indication of which metabolites will elute at which stage. Both the wash and eluting steps were collected and investigated using the LC-MS method developed to this point (after drying and derivatisation). As Figure S21 in the supplementary results depicts, the response of the non-polar eluents from a 25-40% organic solvent (in the washing mixture), were very much the same with very little changes or variation and admissible differences, if any. Furthermore, the response from the higher percentage of organic washes (25%-40% MeOH in the washing step) were higher than those washed with only a lower organic percentage (5-20%), indicating that a higher percentage of organic solvent in the washing solution causes more matrix interference to be washed out, consequently, giving higher responses. When evaluating the responses of the metabolites that were washed out during the washing step of the SPE (supplementary Figure S22), the following trend was observed for most of the metabolites. With 5-15% methanol in the wash step, few metabolites were washed out; with 20-25% methanol, more metabolites were washed out in comparison to other percentages and from 30% - 40% methanol in the washing step, the response is again lower. This could be an indication that either a lower

amount of metabolites were washed out during those washing steps or that in the washing step a large number of interfering matrix components were washed out together with the metabolites, decreasing their ionisation capacity and ultimately their response.

For the more polar estrogen conjugates, the same tendency was visible, where the different washing conditions give minor if any differences in the response of metabolites (Figure S23, supplementary). For most metabolites, 5-10% MeOH as well as washing with 35-40% MeOH in the washing solvent, gave the lowest response of metabolites washed out. The latter can once again be ascribed to either fewer metabolites being washed out during those washing steps or a large quantity of interfering matrix components being washed out decreasing detection capabilities of the MS. From the elution results (Figure S24, supplementary) a tendency is visible that when washing the SPE column with a low percentage of organic solvent, the highest response from the eluent was obtained. When increasing the percentage, however, the response seems to decrease to a minimum at 20% organic solvent, where after the response of the eluent seems to be relatively unchanged, except for 40% giving either no or lower responses. The comparison of the two figures, S23 & S24 led to the choice of washing the SPE columns with only 5% MeOH in water, to wash out matrix affecting compounds and polar salts, without washing away large amounts of estrogen conjugates. Thereafter, it was decided that the estrogen conjugates be washed out with a 20% MeOH solvent mixture. Although this might not result in the elution of all sulphate and glucuronide conjugates, based on the previous data (high response in the washing step and low response in the elution after washing step), it might be the best percentage to use in order to get as many of these metabolites out from the urine sample on the SPE cartridge, without a large amount of additional matrix components. Due to the fact that the response for the non-polar metabolites was at their lowest after a 20% MeOH wash, a higher percentage of MeOH in the washing step was then considered. Based on the identical results obtained from a 25% - 40% MeOH in the washing step, additional experiments were performed to decide which percentage would be more optimal.

The SPE method was then repeated on standards. Firstly, all loaded standards, were washed with a 5%, followed by a 20% organic solvent, and then consequently followed by 30%, 40% and 50% MeOH in water composition for another washing step. Again, all steps were collected and the response of only non-polar metabolites was then investigated. Figure S25 contains the results for the response of all metabolites that washed out in the washing step and Figure S26 shows the response for each metabolite eluted after the varying washing steps. The results obtained were quite puzzling. Figure S25 shows that the two lower organic solvent compositions (30-40%) gave comparable responses for most metabolites, but a 50% organic mobile phase gave a more than double response for most metabolites washed out of the SPE column. Despite the fact that more

metabolites were detected as they washed out during the 50% wash, the elution results for all the washing compositions (30%, 40% & 50%) gave very similar responses. Only four metabolites (T, E3, 16 α -OHE1 and 16KetoE2) gave lower elution responses with a 50% wash. All other metabolites still gave high response values from the fraction eluted, even though intermediary response values were obtained from the fractions washed out during washing steps. Therefore, it was decided that the comparison of the 30% and 40% MeOH in the washing steps on a matrix spike sample be repeated. The results (Figure S27) showed 40% gave the highest responses for all metabolites, except the 2- hydroxyestrogens, which are a bit more polar. Because a 40% wash was thought to be the best choice for all the metabolites, except the hydroxyestrogens, the option of rather eluting the hydroxyestrogens with the very polar conjugates. The non-polar metabolites could then be washed with a higher percentage of organic solvent for cleaner samples. This was evaluated by washing a matrix, spiked with standards, loaded on the SPE columns with 40%, 45%, 50% and 55% MeOH and collecting both washing eluents and elution with acetone-methanol, then drying and derivatising all the samples and comparing the results. The collective response of the elution step for all the metabolites showed that for most metabolites, there is little variation in the elution response between the different washing solvents (Figure S28, Supplementary). For metabolites such as progesterone, estriol, 16-hydroxyestosterone, 17-epiestriol and the other hydroxyestrogens, a higher percentage of the solvent in the washing step seemed to slightly reduce the response of the metabolites in the eluent, indicating that a high percentage may have washed some of these metabolites out. For most other metabolites, comparable results were obtained for the other percentages. The hydroxyestrogens, expected to wash out as more polar metabolites would, were still retained to the SPE columns and a much higher response was obtained from their eluent results, than from fractions that washed out (with 55% MeOH as an exception). Figure S29 (supplementary results) shows that with even higher organic solvent compositions, higher responses were still obtained for the hydroxyestrogens from the elution, than the washing steps. This may be indicating that the higher organic solvents cause more matrix components to wash out of the SPE column, resulting in the better detection of the metabolites of interest. Therefore, the SPE method was adjusted accordingly, by washing with 5% MeOH, to wash out most polar salts after conditioning and loading of the sample onto the SPE cartridge. After this, a 20% MeOH washing solution (20% MeOH and 80% H₂O) through the columns, to wash out the estrogen conjugates. This step was then followed by another washing step of 45% MeOH in water to clear some additional matrix components before final elution of all the remaining non-polar metabolites with methanol and acetone.

SPE results showed that not all the metabolites were washed out or eluted during each of the washing or elution steps. Some metabolites were lost during the washing step for the elimination of matrix components. The washing may have resulted in less of the metabolites of interest, but

still increased their responses, because of better ionisation. This resulted in better detectable responses at low concentrations. Initially, it was not thought that the matrix effect might have such an enormous impact on the ionisation capacities of the metabolites of interest. The matrix effect was reduced by performing sample clean-up (centrifugation, SPE and filtering) and the LC separation, based on size and polarities, which will cause only fractions of the sample to enter the ionisation source and act as competition for ionisation. The best sample clean-up method and the best LC separations are, therefore, of great importance. The lost metabolites will, however, be largely compensated for by the addition of internal standards in the form of stable isotopes. This form of compensation will be further discussed as part of the method validation section where metabolite to internal standard relative response factors will be calculated as a result of SPE losses. Following the SPE washing composition experiments, the washing volumes were evaluated. The SPE method as described and optimised to this point was performed on 100 ppb standard solution and urine spiked with the same concentrations of standard mixtures, with either 3 ml water and methanol for all the washing volumes, or 6 ml washing volumes. The SPE eluents were then dried, derivatised and analysed. The results of the non-polar metabolites are represented as relative response values (Figure S30-S31, supplementary). For the non-polar metabolites, a clear tendency that the 3 ml washing volumes give higher responses for all metabolites, was seen for the standard mixture (Figure S30 a&b). The matrix spiked samples, however, showed a tendency to the exact opposite side, where higher responses for all metabolites (with the exception of DHEA) were obtained with a 6 ml washing volume (Figure S31 a&b). This again illustrates how significant the effect of the sample matrix is. Unfortunately, a specific tendency is not that clear for the polar metabolites; the last washing step volume would not be of any concern as they already elute after the second washing step with 20% MeOH in the solution. The results as in Figure S32 show that for the standard mixture, almost no metabolites were eluted with the 3 ml 20%, although all eluted with 6 ml. On the matrix spike sample the metabolites were eluted in both steps; for metabolites E3-3-S, E3-16-G, E2-3-G and E2-17-S, the 6 ml washing volumes gave higher responses, while for the remaining metabolites, E1-3-G, E2-3-S and E1-3-S, a 3 ml wash volume gave the best response values. Despite the inconsistent results of the polar metabolite responses from the matrix spike sample, a washing volume of 6 ml was chosen for all three washing steps, including the 5%, 20% and 45% MeOH in water washes. All other SPE parameters were kept as initially decided, unless MS sensitivity required better SPE optimisation.

5.6.2 Derivatisation optimisation

As previously mentioned, the derivatisation method chosen (dansylation), still needed further optimisation before final application. Some parameters that may affect the derivatisation capacities, metabolite of interest stability and dansylation efficiency was investigated and are discussed here. These parameters included the time of derivatisation incubation, the temperature

at which the derivatisation incubation is done (high enough for optimal reaction of the dansyl chloride with the metabolites, but low enough not to disrupt the metabolites of interest), the reaction pH, the volume and concentration of the dansyl chloride metabolite, and the addition or exclusion of ascorbic acid during the dansylation reaction (Xu *et al.*, 2005). A standard mixture (20 μ l, 1 ppm) was dried for each analysis, resulting in a final concentration of 100 ppb in the final 200 μ l volume. All derivatised samples were injected into the LC-MS/MS system using the method as developed up to this point. Incubation temperature variations included 30°C, 40°C, 50°C, 60°C, 70°C and 80°C, with all other parameters kept identical. In the end, 60°C, which was used up until this point, was chosen. Although the different temperatures did not really seem to affect the derivatisation capacities (except lower responses obtained from a 40°C incubation temperature), the good repeatability at 60°C was known as it was used up until this point and did not affect the non-derivatising metabolites during the 10 min incubation period (Figure S33, supplementary). Furthermore, time intervals of 5 min, 10 min, 15 min, 20 min and 30 min for incubation at 60°C were evaluated and compared. Again, very little variation between different time intervals was observed, except higher responses obtained from most metabolites after only the short incubation time of 5min. (Figure S34, supplementary). Because there were small visual differences between 5-30 min, with 5min resulting in the highest response, 5 min was chosen to ensure that sufficient time is given for the reaction to take place without unnecessarily increasing the duration of the procedure. The normal reaction pH was measured at pH 10.38 $\pm$ 0.02. Due to previous studies that concluded that no pH adjustments made any differences in dansylation reactions and consequent responses, the pH was kept at approximately 10.38 for all further analyses. The volume of the dansyl chloride solution was also not evaluated, as it would only cause additional concentration or dilution of the metabolites. Instead, the concentration of the dansyl chloride in the final mixture was evaluated. Ideally, the lowest concentration to give the best results would be chosen. Concentrations of, 0.1 mg/ml, 0.25 mg/ml, 1 mg/ml and 2 mg/ml were evaluated and compared. The results as in Figure S35, (supplementary) showed that there were very little differences in the response between different concentrations used. This may be indicating that the lower concentrations already caused sufficient dansylation to occur and increasing the concentrations might not be necessary. The concentration of 0.25 mg/ml in the final 200 μ l (50 μ l of 1 mg/ml) was then chosen to be used further on. Finally, the effect of the presence of ascorbic acid as previously recommended was evaluated and no difference in the presence or absence of ascorbic acid was observed, thus, the results thereof were omitted. The final derivatisation method as optimised to this point is described in section 5.7.2

5.6.3 LC-MS/MS method Optimisation

Before any other method optimisation steps were taken, the developed LC-MS method as described up to this point was applied separately on polar and non-polar metabolites, as a polar metabolite and non-polar metabolite analysis. This decision was based mainly on the results

obtained from the adjusted SPE method, where the more polar metabolites were collected in one fraction and the non-polar metabolites in another. Combining these fractions again, can increase the matrix effect for both polarities and since the sulphate and conjugated metabolites do not require derivatisation, the decision was made to keep the two fractions separate. Both fractions were dried after SPE elution (freeze drying for the washing step and nitrogen evaporation for the elution step). The polar fraction was then reconstituted and analysed on the LC-MS/MS system and the non-polar fraction was derivatised and then injected to be analysed. Keeping the fractions separate could allow the LC-MS/MS method to be more specific for each polarity and shorten the total method runtime. If both analyses were to be developed in such a way that the exact same mobile phases and column are used, they could also directly follow each other. The analysis for the sulphates and glucuronide conjugates could then be an all-negative ionising run and the non-polar and derivatised metabolites could then be analysed only in positive ionisation mode. The method as developed up until this point for ion polarities, was then initially just split in two, by keeping the gradient of the polar method as it was until all the metabolites eluted, increasing it to 100% organic solvent to ensure all matrix was washed from the column and then back to the initial composition. The method for the non-polar metabolites was adjusted by omitting low gradients, until the first metabolite of interest eluted; from there the whole method remained as it was. These final gradients, together with the rest of the optimised methods, can be seen in Table 5.5.

Additional internal standards were also included into the analyses after being obtained. These included 2 & 4-hydroxyestrone-d4 to be used for 2&4-hydroxyestrone, as well as 2 & 4-hydroxyestradiol. The other was 2-acetamidophenol, added as an internal standard for the polar conjugated metabolites. Until the division of the two analyses, estriol-d4 (with different properties, causing different SPE elution times) was used as stable isotope for these metabolites. The ideal would be to rather include stable isotopes for one or more of the conjugates, but due to availability and cost, a normal internal standard was added. These internal standards were built into the method as described in this section up until this point, following the exact same procedure as explained in Chapter 4.7 for all the other stable isotopes. The transitions and chromatographic conditions for these internal standards are included in Table 5.6a for 2-acetamidophenol, and Table 5.6b for 2-hydroxyestrone-d4 and 4-hydroxyestrone-d4.

The injection volumes were then evaluated again, where the injection volume was increased to evaluate the effect of it on the responses. A 100ppb standard mixture prepared from derivatising working standards, was used and 0.5 µl, 1 µl, 2.5 µl, 5 µl and 10 µl thereof was injected with the LC-MS method as described until this point. Although a 10 µl injection gave the best results for the underivatised method and also in the newly gathered data (Figure S34 supplementary), the

10 μ l injection caused an enormous amount of peak broadening, an indication that the Zorbax eclipse plus column had been overloaded. Injecting a large volume onto a column with a small diameter and very small particle sizes may cause an overloading of the column, leading to peak broadening and a loss of peak symmetry (Agilent-Technologies, 2016). Only volumes of 0.5 μ l to 5 μ l were, therefore, further considered. The results of the different injection volumes (Figure S36) showed that a 5 μ l injection volume still gave high response values (highest of the 0.5 μ l, 1 μ l, 2.5 μ l and 5 μ l volumes) and was then chosen as the injection volume for all further analyses. Although the binary pump flows of 0.3 ml/min and 0.4 ml/min were already evaluated, the effect of even higher flow rates was unknown. A rapid resolution high definition (RRHD) Zorbax eclipse column with all dimensions identical to those described before was used for higher flow rate evaluations. These high-resolution columns, with the exact same packing materials as the normal columns, are designed to withstand even higher binary pump pressures. While the normal Zorbax eclipse permitted a maximum pressure of 600bar, these columns can withstand up to 1200bar of pressure. The method as developed up to this point was evaluated for the RRHD column, and all responses and retention times were identical. These results were then compared to those of a higher flow rate (0.5 ml/min), which gave even sharper peaks, excellent peak shape, a decrease in the total chromatographic run time and still acceptable backpressure. This then led to the choice of a binary pump flow of 0.5 ml/min and to rather use the RRHD Zorbax eclipse plus C8 column to withstand the pressure associated with these higher flow rates. After all chromatographic conditions were optimised, and high sensitivity in the method was observed, segments were built into both of the analyses (polar and non-polar) to obtain even better specificity. The segments resulted in an increased MS dwell time on any specific metabolite as it is eluting from the column. The first time segment was switched to waste again, in both analyses, to result in elution of all the very polar metabolites. Table 5.4 (a&b) indicates the segments, time of segment, the transition of steroid hormone metabolites, and retention time for the polar and non-polar methods. Although, up until this point, DHEAS was still evaluated as part of the polar metabolite methods, very little useable responses were obtained from this metabolite. The DHEAS results were consequently omitted throughout Chapter 5, and the metabolite itself was no longer included in the developed method for optimisation or validation experiments.

Table 5.4 a: The segments created for more optimal detection of the sulphate and glucuronide metabolites.

Segment	Time (min)	Transition	Compound	Retention time (min)
1	0	To waste		
2	0.7	150>108	2-Acetamidophenol	2.33
		366>287	Estriol-3-Sulphate	2.60
		463>287	Estriol-16-Glucuronide	2.63
		447>271	Estradiol-3-Glucuronide	2.95
		444>113	Estrone-3-Glucuronide	3.27
3	3.6	351>97	Estradiol-17-Sulphate	3.87
		350>271	Estradiol-3-Sulphate	3.98
		348>269	Estrone-3-Sulphate	4.22

Table 5.4 b: The segments created for more optimal detection of the non-polar metabolites.

Segment	Time (min)	Transition	Compound	Retention time (min)
1	0	To waste		
2	1.5	287>109	Testosterone	2.5
		289>97	DHEA	2.82
		289>271	Androstenedione	2.93
		324>100	Progesterone-d9	4.54
		315>97.1	Progesterone	4.61
3	5	525>171	Estriol-d3	5.97
		522>171	Estriol	5.99
		520>171	16 α - Hydroxyestrone	7.61
		522>171	16-Epiestriol	7.89
		520>171	16-Ketoestradiol	7.90
		522>171	17-Epiestriol	8.40
4	10	536>171	2-Methoxyestradiol	11.71
		536>171	4-Methoxyestradiol	12.01
		506>171	17 β -Estradiol	12.18
		508>171	17 β -Estradiol-d4	12.18
		534>171	2-Hydroxyestrone-3-methyl ether	12.64
		506>171	17 α -Estradiol	13.84
		534>171	4-Methoxyestrone	13.96
		508>171	Estrone-d4	14.37
		504>171	Estrone	14.42
		534>171	2-Methoxyestrone	14.49
5	15.5	755>170	4-Hydroxyestradiol	17.02
		755>170	2-Hydroxyestradiol	17.63
		753>170	4-Hydroxyestrone	18.60
		757>170	4-Hydroxyestrone-d4	18.61
		753>171	2-Hydroxyestrone	19.00
		575>170	2-Hydroxyestrone-d4	19.02

5.7 Final optimised method before method validation

5.7.1 Sample preparation

Sample pre-treatment

Sample pre-treatment of biological samples is a step often included even before sample clean-up or extraction to prepare the sample for further clean-up. Usually during sample pre-treatment, biological samples are diluted with water or buffering solutions, internal standards or stable-isotopes are added and samples are cleared from possible sediment (Trontelj, 2012). As part of the sample pre-treatment, the urine samples (pH 7) were diluted 1:5 with water where 5 ml of water was added to a 1 ml urine sample. The reason for this higher dilution ratio is that SPE columns that have a 200 mg polymeric sorbent bed and a 3 ml capacity is used, and loading with 6 ml instead of only a 2 ml has been found to improve extraction. After dilution, an estrogen isotope and internal standard mixture with final concentrations of 100 ng/ml were added to the diluted urine sample. The diluted urine sample was then centrifuged at 2800 rpm for 10 minutes to allow any sediment to precipitate and form a pellet, and only supernatant was then used for further analysis.

Solid phase extraction

The Strata C18-E SPE columns were conditioned with 6 ml acetone, 6 ml methanol and 6 ml distilled water. The prepared urine sample supernatant (pH7) was then loaded onto the SPE columns. Each column was then washed with 6 ml 5% MeOH in water followed by a 6 ml 20% MeOH in water, from which the sulphate and glucuronide conjugates were collected. The SPE cartridge was then washed again with a 45% MeOH in water solution and dried under vacuum for 10 min. The sample was next eluted with 2 ml MeOH and 2 x 2 ml acetone. The SPE elutes were then evaporated to dryness by either freeze drying or under a gentle stream of nitrogen gas.

Derivatisation and resuspension

To the first, more polar eluent, now dried, 100 µl water and 100 µl MeOH was added to give a final volume of 200 µl. To the last (dried) eluent of non-polar metabolites, 50 µl Dansyl chloride (1 mg/ml) and 50 µl 1 M sodium bicarbonate buffer was added and incubated at 60°C for 5 min. After the incubation period, the samples were left to cool down before being diluted with 100 µl MeOH, to give a final volume of 200 µl. All resuspended samples were then filtered through 0.2 µm Nylon Spin-X filters for LC, where after the supernatant was transferred to inserts in LC analysis vials.

5.7.2 LC-MS/MS analysis

For both methods, 5 µl of the resuspended samples were injected onto the Agilent Technologies Zorbax eclipse plus RRHD C8-E chromatographic column at a controlled temperature of 40°C. Mobile phase A consisted of water with 5 mM ammonium formate (with 5% ACN) and mobile

phase B ACN. The gradient mobile phase changes for both methods are shown in Table 5.5. For the method of the more polar metabolites, the mobile phase gradient started at 0% B and increased to 5% in 0.5 min. From 5%, it was further increased to 10% in 0.5 min, 20% in a further 1 min and 25% in another 1 min. The percentage mobile phase B was then increased to a maximum of 100% in 2 min and kept isocratically for 2 min before it was reduced to 0% B in 1 min. The post run time was set at 3 min for a final run time of 11 min. The gas temperature of 250°C, gas flow of 8 ml/min, nebuliser at 50 psi, sheath gas temperature of 400°C, 12 ml/min flow, a capillary voltage of 4000 V for negative ionisation and a nozzle voltage of 1000V for negative ionisation were used. For the non-polar metabolites, the mobile phase gradient started at 20% B and increased to 40% in 0.5 min. From 40% it was further increased to 54% in 5.5 min, where it was kept isocratically on 54% for 1 min, then the percentage B was increased to 55% in 5 min and 69% in 4 min, where after it was again kept isocratic for 4 min. The percentage mobile phase B was then increased to a maximum of 100% in 1 min and kept isocratically for 1 min before it was reduced to 20% B in 1 min. The post run time is to be set at 5 min for a final run time of 28 min. The gas temperature of 300°C, gas flow of 8 ml/min, nebuliser at 25psi, sheath gas of 300°C and 8 ml/min flow, a capillary voltage of 2000 V for positive and negative ionisation, and a nozzle voltage of 500 V for positive ionisation were used. MRMs, as seen in Table 5.6, were conducted with specific transitions, collision and fragmentor energies, and retention times for each metabolite.

5.7.3 Data analysis

The LC-MS/MS data were then extracted to the MassHunter quantitative data analysis software for each of the metabolites, to be quantified relative to the stable isotopes added. The transitions and retention times for each of the metabolites in the final MRM method can be seen in Tables 5.6 (a & b) and all data analyses were also conducted from these parameters for each compound.

Table 5.5a: The table containing the mobile phase gradient changes with time for polar analyses

Time (min)	% mobile phase B
0.0	0.00
0.5	5.00
1.0	10.00
2.0	20.00
3.0	25.00
5.0	100.00
7.0	100.00
8.0	0.00

Table 5.5b: The table containing the mobile phase gradient changes with time for non-polar analyses

Time (min)	% mobile phase B
0.0	20.00
0.5	40.00
6.0	54.00
7.0	54.00
12.0	55.00
16.0	69.00
20.0	69.00
21.0	100.00
22.0	100.00
23.0	20.00

Table 5.6 a: The estrogen metabolites, transition, fragmentor and collision energies, and also retention times for the polar metabolites. This analysis was developed specifically for the detection of the sulphate and glucuronide metabolites

Compound Name	Polarity	Precursor ion (m/z)	Fragment or energy (V)	Product ion (m/z)	Collision energy (eV)	Retention time (min)
2-Acetamidophenol	-	150.1	50	108.1	8	2.33
Estradiol-3-sulphate	-	366.5	148	287.1	36	4.41
Estradiol-16 glucuronide	-	463.2	168	287.1	28	4.44
Estradiol-3-glucuronide	-	446.6	146	271.1	44	5.12
Estrone-3-glucuronide	-	444.6	131	113.1	16	5.59
Estradiol 17-sulphate	-	351.2	168	97	44	6.51
Estradiol-3-sulphate	-	350.5	146	271.1	32	6.78
Estrone-3-sulphate	-	348.2	143	269.1	28	7.60

Table 5.6 b: The estrogen metabolites, transition, fragmentor and collision energies and also retention times for the non-polar metabolites. This analysis was developed specifically for the detection of the more non-polar and derivatised metabolites, all analysed in positive ionisation.

Compound Name	Polarity	Precursor ion (m/z)	Fragmentor energy (V)	Product ion (m/z)	Collision energy (eV)	Retention time (min)
Testosterone	+	289.22	121	97.1	20	2.5
DHEA	+	289.22	120	213.2	20	2.82
Androstenedione	+	287.2	135	97.1	24	2.93
Progesterone	+	315.23	109	97.1	20	4.54
Progesterone-d9	+	324.3	141	100.1	24	4.61
Estriol-D3	+	525	170	171	36	5.97
Estriol	+	522	170	171	36	5.99
16 α -Hydroxyestrone	+	520.38	166	171.1	44	7.61
16-epiestriol	+	522	170	171	36	7.89
16-Ketoestradiol	+	520.38	166	171.1	44	7.90
17-Epiestrol	+	522	170	171	36	8.40
2-Methoxyestradiol	+	536.42	114	171.1	44	11.71
4-Methoxyestradiol	+	536.42	114	171.1	44	12.01
17 β - Estradiol	+	506.19	148	171.1	40	12.18
17 β - Estradiol-d4	+	510	210	171.1	40	12.18
2-Hydroxyestrone-3methyl ether	+	534.4	146	171.1	36	12.64
17 α - Estradiol	+	506.19	148	171.1	40	13.84
4-Methoxyestrone	+	534.4	146	171.1	36	13.96
Estrone-d4	+	508	200	171.1	36	14.37
Estrone	+	504.0	114	171.1	16	14.42
2-Methoxyestrone	+	534.4	146	171.1	36	14.49
4-Hydroxyestradiol	+	755.01	205	170.9	44	17.02
2-Hydroxyestradiol	+	755.01	205	170.9	44	17.63
4-Hydroxyestrone	+	753.37	173	170.1	36	18.60
4-Hydroxyestrone – d4	+	757.41	200	170.1	50	18.61
2-Hydroxyestrone	+	753.37	173	170.1	36	19.00
2-Hydroxyestrone – d4	+	757.41	150	170.1	40	19.02

5.8 Method validation

The validation of the two separate analyses was performed next, to ensure the repeatability of the method and that the results were accurate and quantifiable. Furthermore, the effect of the sample matrix was evaluated together with the capacity of the method to still be selective enough in the presence of matrix components, and whether the LC and chromatographic method caused

any carryover of metabolites (or sample) in between runs. The stability of the samples after derivatisation, under certain conditions that a sample may be subjected to or be stored at in between or during analyses, was evaluated next. The results of the analyses are shown in tables (in section 5.8), or in figures (included as part of the supplementary results) to support conclusions. Most validation analyses were performed on both standard solutions and matrix spiked samples to permit validation of the sample in its matrix form as well.

5.8.1 Calibration and linearity

Linearity and calibration evaluation analyses were done on calibration standards prepared as described in Chapter 3, for both standards in solvents and standards in matrix samples. These calibration standards, together with 100 ng/ml of the internal standard mixture (100 ppb) in each, were then dried and derivatised as described in section 5.7. Linearity was evaluated by using a total of 15 calibration standards, all analysed in triplicate, to construct the calibration curves by plotting response/IS response against the concentration of metabolite/concentration of the internal standard. The concentration ranges evaluated for each metabolite can be seen in Table 5.7 and visual illustrations of the linearity for some of the metabolites can be seen in Figure S37 (supplementary results). The r^2 values were calculated for all the metabolites (Table 5.7); an $r^2=1$ indicates a linear regression without any deviations from the regression line.

5.8.2 Relative response factor

Evaluating the response of the metabolite of interest (in matrix spiked before SPE) to its internal standard for each concentration, allowed for the calculation of a response factor value. This value could also be calculated from the analytical standard calibration or after SPE spiked matrix samples, but then the assumption would be made that: firstly, the matrix has no effect on the response factor and secondly, that the metabolite of interest and its internal standard would respond identically to each of the SPE washing and eluting steps, which is known to not be true. The response factor (RF) is per definition, the response of the metabolite per unit weight, while the relative response factor (RRF) is the ratio of the RF of the metabolite to that of its internal standard. This enables correction of differences in the response of the related but not identical substances (Chan *et al.*, 2004). If a linearity curve is then constructed by plotting the response versus the concentration, the relative RF can be calculated with Equation 5 from the curve slopes. The relative response factor values to its internal standard metabolite were then calculated as in table 5.7, which illustrates the IS used for each of the metabolites and its consequent relative response factor.

Equation 5: The relative response factor calculation equation

Relative response factor = Slope of metabolite of interest calibration curve *

*Response/IS response vs concentration/IS concentration

Table 5.7: Table to illustrate the specific IS used for each of the metabolites and consequently, the relative response factor calculated between the metabolite of interest and the IS with Equation 5.

Metabolite	Internal standard or stable isotope used for quantification	Relative response factor of IS to the metabolite of interest
16-Epiestriol	Estriol-d3	0.2
16-Ketoestradiol	Estriol-d3	2.3
16 α -Hydroxystrone	Estriol-d3	1.1
17-Epiestriol	Estriol-d3	1.0
17 α -Estradiol	17 β -Estradiol-d4	1.0
17 β -Estradiol	17 β -Estradiol-d4	0.5
2-Hydroxyestradiol	2-Hydroxyestrone-d4	0.1
2-Hydroxyestrone	2-Hydroxyestrone-d4	1.1
2-Methoxyestradiol	17 β -Estradiol-d4	1.0
2-Methoxyestrone	17 β -Estradiol-d4	0.1
4-Hydroxyestradiol	4-Hydroxyestrone-d4	0.2
4-Hydroxyestrone	4-Hydroxyestrone-d4	2.3
4-Methoxyestradiol	17 β -Estradiol-d4	0.4
4-Methoxyestrone	17 β -Estradiol-d4	0.3
Androstenedione	Progesterone-d9	0.7
DHEA	Progesterone-d9	0.1
Estradiol-17-Sulphate	2-Acetamidophenol	13.2
Estradiol-3-Sulphate	2-Acetamidophenol	9.3
Estradiol-3-Glucuronide	2-Acetamidophenol	1.1
Estriol	Estriol-d3	1.0
Estriol-16-Glucuronide	2-Acetamidophenol	1.12
Estriol-3-Sulphate	2-Acetamidophenol	11.1
Estrone	17 β -Estradiol-d4	1.0
Estrone-3-Glucuronide	2-Acetamidophenol	0.3
Estrone-2-hydroxy-3-methyl ether	17 β -Estradiol-d4	0.2
Estrone-3-Sulphate	2-Acetamidophenol	2.3
Progesterone	Progesterone-d9	0.5
Testosterone	Progesterone-d9	1.0

5.8.3 Limit of detection & quantification

The least square linear regression model was used for the following calculations. The equations used are as shown in Equation 1 & 2 in Chapters 2 & 4. For each metabolite, the concentration range evaluated, range with most linear results and r^2 values are presented in Table 5.8. Due to

detectable noise on the chromatograms, additional LOD and LOQ tests were performed to ensure that the noise does not affect the limits of detection and quantification. These tests included the calculation of a signal to noise ratio (S/N), in a biological matrix, which was spiked after sample clean up with five different concentrations (ranging from low to high), to evaluate what the effect of noise will be on the limit of detection and quantification. Several limitations were described by Westgard *et al.* (1999), using the latter method of LLOD and LLOQ evaluations; the calculation of these values by the use of the signal to noise ratio was only implemented to note any drastic differences in the LLOD or LLOQ caused by noise (Westgard *et al.*, 1999). The S/N ratio for each metabolite was then calculated using the peak to peak equation, where the ratio of the height of the highest noise peak to the height of the metabolite peak was calculated. LLOQ concentrations with response 10x S/N, are projected in Table 5.8.

The results, as seen in Table 5.8, indicate that all of the metabolites gave a very linear response $r^2 \geq 0.99$, being linear from very low to high concentrations. The LOD and LOQ values in the table, calculated by the slope regression model, showed that all LOD and LOQ values for metabolites were below 1 ng/ml except for metabolites estriol-3-glucuronide, with a LOD value of 3.403 ng/ml and LLOQ, a value of 11.346 ng/ml; androstenedione with a LOD value of 1.678 ng/ml and LLOQ a value of 5.599 ng/ml. Metabolites estrone-3-sulphate, estrone-3-glucuronide and estradiol-3-glucuronide also showed LLOQ values between 1 ng/ml – 2 ng/ml. The LLOQ, as calculated by the signal to noise ratio, showed comparable results for most metabolites' LLOQ, calculated using other methods, except for the hydroxyestrogens, which gave a higher LLOQ value when the S/N ratio was taken into account. The validity of the LOD and LOQ was evaluated when doing accuracy and precision experiments, as inaccurate quantification limits will lead to lower accuracy and precision values.

5.8.4 Accuracy

The accuracy of the developed method was evaluated by calculation of the interday and intraday accuracy of concentrations in the linear range, as indicated in Table 5.8. These values included the lower limit of quantification, lower, medium and high concentrations, the upper limit of quantification (ULOQ) and a dilution integrity sample. The accuracy analysis was performed again on standard and matrix spiked samples, in triplicate, for six consecutive days. Accuracy was then expressed as a percentage, as described in Chapter 2, where accuracy is the mean observed concentration, divided by the nominal concentration, multiplied by 100. For acceptable accuracy, deviation of the mean concentration should be below 15% for all values, except where the LLOQ of below 20% was accepted (González *et al.*, 2014). The accuracy results were again compared between the matrix spike and the standard solution, and the worst results (the matrix spike) of accuracy are shown in Table 5.9 & 5.10. The results for each individual metabolite can be seen in Table S3 for intraday variation and Table S4 for interday variation in the supplementary results.

Table 5.8: Table with the evaluated concentrations that gave a linear response, the r^2 values as well as the LOD and LOQ values of each metabolite as calculated by equations 1 & 2, previously described for both polar and non-polar metabolites. The results of the standard mixture were compared to that of the matrix spike, and because the matrix effect caused the matrix sample to give higher or equal LOD and LOQ values, the results of the matrix spikes are reported below.

Metabolite	Concentration range evaluated (ng/ml)	Concentration range linear (ng/ml)	r^2 value	LOD (ng/ml)	LLOQ (ng/ml)	LLOQ as per S/N calculated (ng/ml)
16-Epiestriol	0.015-1000	0.03-1000	0.992	0.014	0.047	0.015
16-Ketoestradiol	0.015-1000	0.03-1000	0.999	0.040	0.135	0.004
16 α -Hydroxystrone	0.015-1000	0.03-1000	0.996	0.035	0.117	0.004
17-Epiestriol	0.015-1000	0.03-1000	0.996	0.272	0.909	0.004
17 α -Estradiol	0.015-1000	0.03-1000	0.998	0.077	0.258	0.004
17 β -Estradiol	0.015-1000	0.03-1000	0.999	0.076	0.256	0.004
2-Hydroxyestradiol	0.015-1000	1.95-1000	0.985	0.024	0.080	7.813
2-Hydroxystrone	0.015-1000	0.24-1000	0.991	0.057	0.191	0.977
2-Methoxyestradiol	0.015-1000	0.03-1000	0.998	0.127	0.424	0.061
2-Methoxystrone	0.015-1000	0.03-1000	0.996	0.106	0.355	0.061
4-Hydroxyestradiol	0.015-1000	1.95-1000	0.991	0.023	0.079	7.813
4-Hydroxystrone	0.015-1000	0.24-1000	0.991	0.017	0.059	0.977
4-Methoxyestradiol	0.015-1000	0.03-1000	0.998	0.131	0.437	0.061
4-Methoxystrone	0.015-1000	0.03-1000	0.996	0.102	0.342	0.061
Androstenedione	0.015-1000	0.03-1000	0.998	1.678	5.599	7.812
DHEA	0.015-1000	7.8-1000	0.999	0.108	0.361	0.004
Estradiol-17-Sulphate	0.0019-500	0.122-500	0.991	0.199	0.664	0.244
Estradiol-3-Sulphate	0.0019-500	0.015-500	0.992	0.146	0.486	0.976
Estradiol-3-Glucuronide	0.0019-500	0.48-500	0.997	0.415	1.384	0.244
Estriol	0.015-1000	0.03-1000	0.995	0.074	0.247	0.004
Estriol-16-Glucuronide	0.0019-500	0.97-500	0.986	3.403	11.346	1.953
Estriol-3-Sulphate	0.0019-500	0.03-500	0.994	0.230	0.768	0.002
Estrone	0.015-1000	0.03-1000	0.999	0.109	0.364	0.061
Estrone-3-Glucuronide	0.0019-500	0.48-500	0.996	0.488	1.626	0.976
Estrone-2-hydroxy-3-methyl ether	0.015-1000	0.03-1000	0.992	0.088	0.294	0.061
Estrone-3-Sulphate	0.0019-500	0.015-500	0.989	0.303	1.012	0.030
Progesterone	0.015-1000	0.03-1000	0.999	0.162	0.541	0.003
Testosterone	0.015-1000	0.03-1000	0.998	0.150	0.501	0.004

5.8.5 Precision

Precision, as with the accuracy calculation, was done for interday and intraday variation, on concentrations in the linear range (lower limit of quantification, lower, medium and high concentrations, the upper limit of quantification and a dilution integrity sample). The calculation included the calculation of the relative standard deviation (RSD), by dividing the standard deviation of the observed concentration by the mean, multiplied by 100. This percentage of RSD should not exceed 15% for all values, except for the LLOQ, where an RSD percentage below 20% is acceptable (González *et al.*, 2014). These average results for the matrix spiked samples are shown in Table 5.9 and Table 5.10, together with the accuracy results. The results for each individual metabolite can be seen in Table S3 for intraday variation and Table S4 for interday variation in the supplementary results.

5.8.6 Dilution integrity

The dilution integrity analysis was performed together with all accuracy and precision samples. In some cases, especially in clinical environments, it does occur that a sample has a concentration that is very high (5-100 times the ULOQ) and this sample will then have to be diluted to be quantifiable. The dilution integrity results will be an indication of whether the dilution of the samples earlier in the sample preparation process, before injection, would affect the accuracy or the precision of that sample once quantified (Whitmire *et al.*, 2011). This is especially important to note for matrix samples. In this analysis, the dilution integrity sample was prepared at 5 times the ULOQ and then diluted 50 times, with either MeOH or urine matrix and analysed together with all other accuracy and precision samples. The average dilution integrity, accuracy and precision results can be seen in Table 5.9 and Table 5.10; the individual results are presented in Table S3, for intraday variation and Table S4, for interday variation in the supplementary results.

5.8.7 Stability

Stability was evaluated under conditions the samples may be exposed to during preparation, analysis and storage. This was done in triplicate on QC samples (low, medium and high) and standard samples. For storage, the stability was evaluated when: left on the bench top for 24 hours, left in the autosampler (4-6°C) for 3 days, kept in fridge storage (4°C) for 5 days and 7 days, and when frozen (-20°C) and thawed for five cycles. The responses of metabolites in each of these scenarios was compared with that of a freshly prepared sample or standard, and the stability was then calculated as a percentage of the freshly prepared sample (illustrated in Table 5.11). Again, any percentage below 75% and above 115% (deviation above 15%) was unacceptable and the method of storage was disregarded.

Table 5.9: The average intraday accuracy, and precision values for the LLOQ, low, medium and high QC, Dilution integrity QC and the ULOQ concentrations.

	LLOQ		Low QC		Medium QC		Dilution QC		High QC		ULOQ	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Average	11.09	100.42	5.04	99.87	4.73	94.44	4.35	101.00	7.12	94.81	6.18	100.73

Table 5.10: The average interday accuracy and precision values.

	LLOQ		Low QC		Medium QC		Dilution QC		High QC		ULOQ	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Average	15.45	97.61	8.13	98.33	9.10	97.50	8.42	96.82	7.90	96.13	6.45	95.35

Table 5.11: The stability of samples under certain storage conditions. These include fridge storage of 5 or seven days, autosampler storage for three days, benchtop storage for one day and 5 freeze thaw cycles.

Storage method	Low QC (% of the freshly prepared sample)	Medium QC (% of the freshly prepared sample)	High QC (% of the freshly prepared sample)
4°C for 7 days	102.93	126.11	116.68
4°C for 5 days	107.37	100.34	98.22
3 days in autosampler	89.25	101.69	93.85
5 freeze-thaw cycles	107.50	101.39	99.16
24h at room temperature	79.04	60.44	164.55

5.8.8 Selectivity

The selectivity of the analytical method is often improved by chromatographic separation, to cause certain compounds to be retained inside the column for a certain amount of time, resulting in less competition for ionisation inside the ionisation source. Unfortunately, the estrogen metabolite profile consists of a number of isomers, which are structurally similar and, therefore, selectivity of the method to differentiate between these compounds as well as between metabolites of interest and other compounds, in the presence of urine as biological matrix, needed to be evaluated. The selectivity of this method was assessed by evaluation of six blank human urine samples, pooled for the interference of the matrix at the estrogen retention times to be compared with the response of the analytes in a urine sample spiked with a concentration of the estrogen metabolites that represent the LLOQ. This was repeated on male and female urine samples due to the fact that some females might have high levels of these metabolites of interest present, which might be lower in a male urine sample. The selectivity analyses were performed in triplicate and the results are summarised in Figure S38 (supplementary). As seen in the results, there were no visible interferences observed in the blank urine at the retention times of the metabolites of interest. Although selectivity analyses are a necessity, in this case, the urine matrix blank also contained metabolites of interest and consequently, have the exact retention times as the metabolites spiked with.

5.8.9 Matrix effect

One very important validation step is to evaluate the effect of the biological matrix. The matrix effect may cause compounds to respond differently in a biological matrix than in a standard solution, and therefore, evaluation and validation processes should take these effects into account. The difference between a biological sample and a standard sample's response may be referred to as suppression or enhancement of the signal, depending on whether the biological matrix is magnified or diminished (Van Eeckhaut *et al.*, 2009). Matrix factor was calculated in order to determine what the matrix effect is, based on the equations as described in Chapter 2. By comparing the response of the metabolites to be investigated (in H₂O or MeOH), with the response found in human urine, the matrix factor was calculated for each compound. Six independent matrix samples, in triplicate, were used for these calculations. Each urine sample (1 ml) was prepared with the SPE method, as described in section 5.7 and then spiked with metabolites, with values equivalent to those in 200 µl of the standard mixtures of high and low QC values. Additionally, 1 ml of each urine sample (unspiked) was also prepared for analyses in triplicate. All samples (standards in MeOH, clean matrix samples and matrix spiked samples) were then dried, derivatised, and analysed using the LC-MS/MS method. The response of the endogenous estrogen metabolites were subtracted from the response of the human urine spike sample before calculations were performed. An MF=1 indicated that the matrix had no effect on

the response of the analytes, whereas $MF < 1$ indicated suppression and $MF > 1$ indicated enhancement of the response by the matrix (González *et al.*, 2014). These MF value deviations were expressed as a coefficient of variation (CV) value and only CV percentages below 15% were accepted for acceptable matrix effect. As a result, the permitted range for the matrix factor is $MF = 0.85$ to $MF = 1.15$. All MF values below 0.85 and above 1.15 are indicated in red in Table 5.12, which displays the average MF value for each metabolite at a low QC and at a high QC value.

5.8.10 Carry-over

The last validation parameter evaluated was carry-over, to ensure that no carry-over between injections occurred. This carry-over, if present, might be as a result of insufficient needle washing after injection. It could also be caused by some residual sample of previously injected samples being injected together with the newly injected sample, or that the amount of time the column is eluted with maximum (100%) organic mobile phase, is not enough, and some of the sample matrix or metabolites of the first injection are still retained in the column, only eluting during the next chromatographic run. Carry-over from any source would affect the quantification results, either by decreasing the accuracy of the quantified concentrations or by influencing the ionisation. The carry-over amount was calculated by evaluating the response of a blank methanol injection before and after the analysis of both a standard solution and a matrix spiked solution, to ensure that autosampler and column carryover, as well as adhering residue, does not affect the quantification of metabolites (Sargent, 2013). This was done for all QC concentrations. The results showed that no carry-over occurred throughout and during the analysis and all analyses were continued, assuming that no significant carryover occurred.

Table 5.12: The matrix factor value of each estrogen metabolite at low QC and high QC values where $0.85 \leq MF \leq 1.15$ are acceptable MF values

Metabolite	Matrix factor	
	Low QC	High QC
16-Epiestriol	1.00	1.10
16-Ketoestradiol	1.01	1.06
16 α -Hydroxystrone	0.94	0.99
17-Epiestriol	1.08	1.05
17 α -Estradiol	1.01	1.00
17 β -Estradiol	1.12	1.13
2-Hydroxyestradiol	0.85	1.04
2-Hydroxyestrone	1.01	0.90
2-Methoxyestradiol	1.11	1.11
2-Methoxyestrone	0.99	0.86
4-Hydroxyestradiol	0.87	1.01
4-Hydroxyestrone	0.99	0.86
4-Methoxyestradiol	0.87	0.90
4-Methoxyestrone	0.97	0.93
Androstenedione	1.07	1.00

Metabolite	Matrix factor	
DHEA	1.07	0.95
Estradiol-17-Sulphate	1.08	1.12
Estradiol-3-Sulphate	1.04	0.86
Estradiol-3-Glucuronide	0.76	0.78
Estriol	0.99	1.02
Estriol-16-Glucuronide	1.00	1.05
Estriol-3-Sulphate	0.98	1.07
Estrone	0.99	0.98
Estrone-3-Glucuronide	4.28	0.90
Estrone-2-hydroxy-3-methyl ether	1.12	0.91
Estrone-3-Sulphate	0.58	0.85
Progesterone	1.07	1.00
Testosterone	1.00	0.98

5.8.11 Discussion of validation results:

The overall validation results were positive, not only as an indication of the capacities (LLOQ, ULOQ and stability), but also as an indication of the limitations of the developed method. Some of these limitations include the effect of the matrix or the stability under certain storage conditions. Firstly, for the calibration and linearity, instead of focusing on the physiological concentrations as with the underivatized method, the approach this time was to rather evaluate a wide range of concentrations so that the results might be indicative of tendencies at very high or very low concentrations, which would be unknown if only a narrow range were evaluated. Therefore, a 20-point calibration was performed. The results showed that most metabolites were linear, over a wide range of concentrations with r^2 values ≥ 0.99 . For most of the metabolites, the lowest calibration data points were omitted because they were not in the linear response range of the method. Only 2&4- OHE2 and DHEA did not display linear responses from values below 1 ng/ml. Because all the metabolites display linear responses in the range of 10 ng/ml to 500 ng/ml, a concentration of 100 ng/ml was chosen for the internal standard concentration. The LOD and LOQ values were then calculated with linear regression and compared with the S/N model. Limits of quantification from as low as 59 pg/ml (4-OHE1) to 11.4 ng/ml (E3-16-G) were calculated, using the linear regression model and 1.9 pg/ml (E3I-3-S) to 7.8 ng/ml (2&4-OHE2, A4) using the S/N method. When the LLOQ were calculated as per S/N and linear regression model, some small variations in the LLOQ concentrations were observed, this was expected to a certain extent, as the principles that these methods are based upon, differed, being the reason for small variations. The LLOQ values were all below known normal physiological concentrations, although in pathophysiological conditions, where a much lower concentration is present, a value below the LLOQ might be obtained, and would then be reported as below quantification limit. The ULOQ was accepted as the highest concentration evaluated and in the case of samples containing values above this value, a dilution of the sample might be necessary. However, these values were assessed when the accuracy and precision of quantification was evaluated.

Accuracy and precision were evaluated on the quantification of certain concentrations of samples on an interday and intraday basis. The average intraday RSD values were well below 7.5%, except for the LLOQ, which gave a deviation of 11.09%. However, when the RSD percentages of each metabolite were evaluated individually, metabolites DHEA and 16-epiestriol gave a percentage RSD above 20% for the LLOQ, and DHEA also gave a %RSD above 15% for all other concentrations. All other metabolites displayed RSD values well within 15% for intraday quantification. The precision for interday quantification gave an average 15.45% deviation for the LLOQ and below 9.5% deviation on all other concentrations. The individual metabolites with higher than acceptable interday variations on the LLOQ included E3 (27.28%), 16-OHE1 (20.72%), 16ketoE2 (26.15%), 17 β -E2 (33.35%), E1 (20.49%), E2-3-G (20.28%), E1-3-G (22.35%) and E1-3-S (20.24%). Other high %RSD values included E1-3-G with a low QC %RSD of 20.24% and DHEA with a ULOQ %RSD of 18.72%. The average intraday accuracy from 99.44% to 101.00% and interday accuracy of 95.35% to 98.33%, were all in the acceptable range. Individual metabolites, E-3-G gave accuracy for LLOQ and low QC values below the acceptable 85% (68.7% & 84.98%), while E2-17-S gave an accuracy percentage for the ULOQ of 74.83%. DHEA gave intraday accuracy values above 115% for the low QC concentration (122.20%) and accuracy percentage below 85% for the medium QC concentration (82.81%). Interday accuracy percentages for DHEA were again low for low concentrations, dilution integrity samples and high concentrations QC samples. The LLOQ interday accuracy of 2-OHE2 (120.51%), E2-3-G (77.41%), E1-3-G (77.56%) and E1-3-S (20.24%) were all out of the acceptable range. The low QC accuracy for E2-3-G and E1-3-G (116.65% & 116.87%) were above the allowed 115%. All other metabolites gave an accuracy percentage in the acceptable range. These results then led to a re-evaluation of the LLOQ for those metabolites and quantification of the DHEA metabolite as part of the developed method. Dilution integrity samples were included during all accuracy and precision analyses and gave inter- and intraday %RSD of below 15% for all metabolites, and accuracy values between 85% and 115% except for DHEA with an interday accuracy % of 119.33% for the dilution integrity sample. These results led to the conclusion that if a sample has a concentration of a metabolite above the ULOQ as indicated, that sample can be diluted and the quantification results could be treated as accurate and precise. The deviation that DHEA displayed might rather point to metabolite ionisation, response or detection than to the dilution integrity of the method.

The method stability, evaluated to ensure samples could be evaluated or re-evaluated after storage, also gave useful results. The percentage stability of the sample to a freshly prepared sample was 98.22% - 107.37%, after a 5-day fridge storage of 89.25% - 101.69% and 99.16% - 107.50% after 5 freeze-thaw cycles. However, when the medium QC was kept in the fridge for 7 consecutive days, the percentage of the freshly prepared sample increased to 126.11% and for

the high QC after 24 hours at room temperature, increased to 164.55%. The medium and low QCs reduced to 60.44% and 79.04%, respectively. This indicates that the samples were quite stable at a low temperature (below 4°C) for a short amount of time (1-5 days), but higher temperature or prolonged storage might cause inaccurate results. The effect of the urinary matrix and the capacity of the analytical method to distinguish between the matrix components and the metabolites of interest (selectivity) were also evaluated. Matrix factor values of 0.85 to 1.13 were calculated from the low QC concentrations for most of the metabolites, although metabolites E1-3-G and E2-3-G gave higher than expected MF values at low QC concentrations. This indicates that the matrix caused erroneous results at this low concentration. Estrone 3-sulphate, however, gave an MF value of 0.58 at low QC concentrations, indicative that the matrix causes ion suppression of this metabolite at low QC concentrations. At high QC concentrations, all the MF results were in the acceptable range, indicating that the urine matrix did not cause ion suppression nor enhancement for any of the metabolites in that higher concentration range. The selectivity results indicated whether any of the matrix components evaluated (with similar transitions), will elute from the chromatographic column at the exact same time (identical RT) as the metabolites of interest. For analysis, a blank urine sample was used, with the drawback to this being that the sample already contains some levels of the metabolites of interest. This was preferred over using synthetic or even a stripped urine sample, which may not give an accurate representation of the sample matrix. For stripped urine, all matrix components that might have had an effect are also removed, including other cholesterol backbone components, while synthetic urine only contains certain manually added metabolites and do not compensate for the individual variation. As expected, both male and female samples did contain some of the naturally occurring metabolites, although retention of the metabolites of interest did not seem to overlap with other matrix components. One of the major reasons for these results is that the derivatisation allows for an additional selectivity level by increasing the molecular weight area in which the metabolites are detected. As previously mentioned, not all metabolites are derivatised through the dansylation reaction and only metabolites with a naturally high-ionised mass and derivatised metabolites will benefit from this additional level of selectivity. Other validation results, however, did illustrate that matrix components might have an effect on the quantification of metabolites, but the selectivity results indicate that the matrix effect might be of concern only for very low concentrations.

The absence of carry-over, even in ULOQ concentrations, indicated that the method permits sufficient washing of the needle, injector valve and the column, between injections. Furthermore, this points to the fact that any results that are not within the permitted deviation, could not be explained by a carry-over phenomenon. All deviations from the expected validation results should then be explained by factors other than carry-over and contamination. Storage at high temperatures for long periods of time was the only condition affecting stability results. This

excluded stability as a parameter, which could contribute to poor validation results. Although linearity of the metabolite responses was observed at lower concentrations, the effect of the matrix compounds at these lower concentrations was often observed. These lower concentrations, including the LLOQ and sometimes even the low QC values, gave inaccurate and imprecise results for some metabolites. High matrix factor values were also observed for certain metabolites at lower concentrations. Considering and comparing the results of all validation parameters then led to alterations in the LLOQ values depicted in Table 5.8, provided that certain conditions are met. These conditions include storage (after sample preparation and derivatisation) only under lower temperatures (max 4°C) for short amounts of time (max 5 days). The adjusted LLOQ values are as shown in Table 5.13, which present only the LLOQ values of the metabolites where other validation results did not support the previously reported values.

Table 5.13: The adapted LLOQ values and reasons for alterations after evaluation of all the validations parameter results.

Metabolite	Adapted LOQ (ng/ml) *ULOQ	Reason for adjustment
16-Epiestriol	Low QC = 1.95	LLOQ Intraday %RSD >20%
16-Ketoestradiol	Low QC = 1.95	LLOQ Interday %RSD >20%
16 α -Hydroxystrone	Low QC = 1.95	LLOQ Interday %RSD >20%
17 β -Estradiol	Low QC = 1.95	LLOQ Interday %RSD >20%
2-Hydroxyestradiol	Low QC = 7.8	LLOQ Interday %accuracy >120%
DHEA	Completely omit from method	LLOQ Intraday %RSD >20% Low QC, medium QC, high QC intraday %RDS >20% ULOQ Interday %RSD >15%
Estradiol-17- Sulphate	*Dilute any sample above high QC concentration ULOQ =250	ULOQ intraday accuracy <85%
Estradiol-3-Glucuronide	Medium QC = 15.63	Low QC MF >1.15 Low QC Interday %accuracy >115%
Estriol	Low QC = 1.95	LLOQ Interday %RSD >20%
Estrone	Low QC = 1.95	LLOQ Interday %RSD >20%
Estrone-3-Glucuronide	Medium QC= 7.81	Low QC Interday %RSD >15% Low QC interday & intraday accuracy <85% or > 115% Low QC MF >1.15
Estrone-2-hydroxy-3-methyl ether	Medium QC= 7.81	Low QC Intraday %accuracy <85%
Estrone-3-Sulphate	Medium QC = 15.63	LLOQ Interday %RSD >20% LLOQ Interday %accuracy <80% Low QC MF <0.85

CHAPTER 6

ANALYSING SAMPLES USING THE DEVELOPED METHOD

6.1 Introduction

Studies that are performed on the metabolic fate and detoxification of drugs in living organisms are usually divided into different areas. First, the elucidation of the metabolic and biotransformation pathways; second, the pharmacokinetics of the parent drugs as well as the metabolites of the parent drugs and third, the identification of the reactive metabolites in order to determine the toxicity of the drugs (Roškar & Lušin, 2012). The metabolic fate of contraceptives containing estrogen has been previously investigated (Klipping & Marr, 2005), however, the second area dealing with how these drugs affect the estrogen-related metabolic and biotransformation pathways, still needs to be investigated.

In this study, a method was developed to enable the measurement of these estrogen metabolites and estrogen detoxification products. The developed method could now be used not only to investigate the effect of COCs on estrogen metabolism and estrogen detoxification for research purposes, but also to monitor the efficiency of these processes in a clinical setting. However, before this can be done, the ability of the developed method to detect the estrogen metabolites of interest and to quantify the concentrations thereof in the urine first needs to be determined. Therefore, as a proof of concept, this chapter will be focusing on using the developed method to quantify estrogen metabolites in a number of urine samples from different pre-menopausal women.

6.2 Sample collection

The urine samples of 10 pre-menopausal women, taking a certain formulation of combined oral contraceptives, were collected together with control urine samples from 10 pre-menopausal women not using any type of hormonal therapy or oral contraceptives. These urine samples were also taken on any day of their monthly cycle, as it would occur in a clinical setting. Although collection near day 20 would be ideal, most women might be unsure of the day of their cycle, and the biological variation over any of the 28 cycle days are analysed in the current study to avoid the limitation of sample collection on certain cycle days. The urine samples were then aliquoted into volumes of 1 ml each and stored at -80°C until analysis. These female participants were not only chosen based on their use or non-use of combined oral contraceptives but they also had to comply with the following inclusion criteria:

- Participants had to be of premenopausal age (18-35 years), preferably in their twenties.

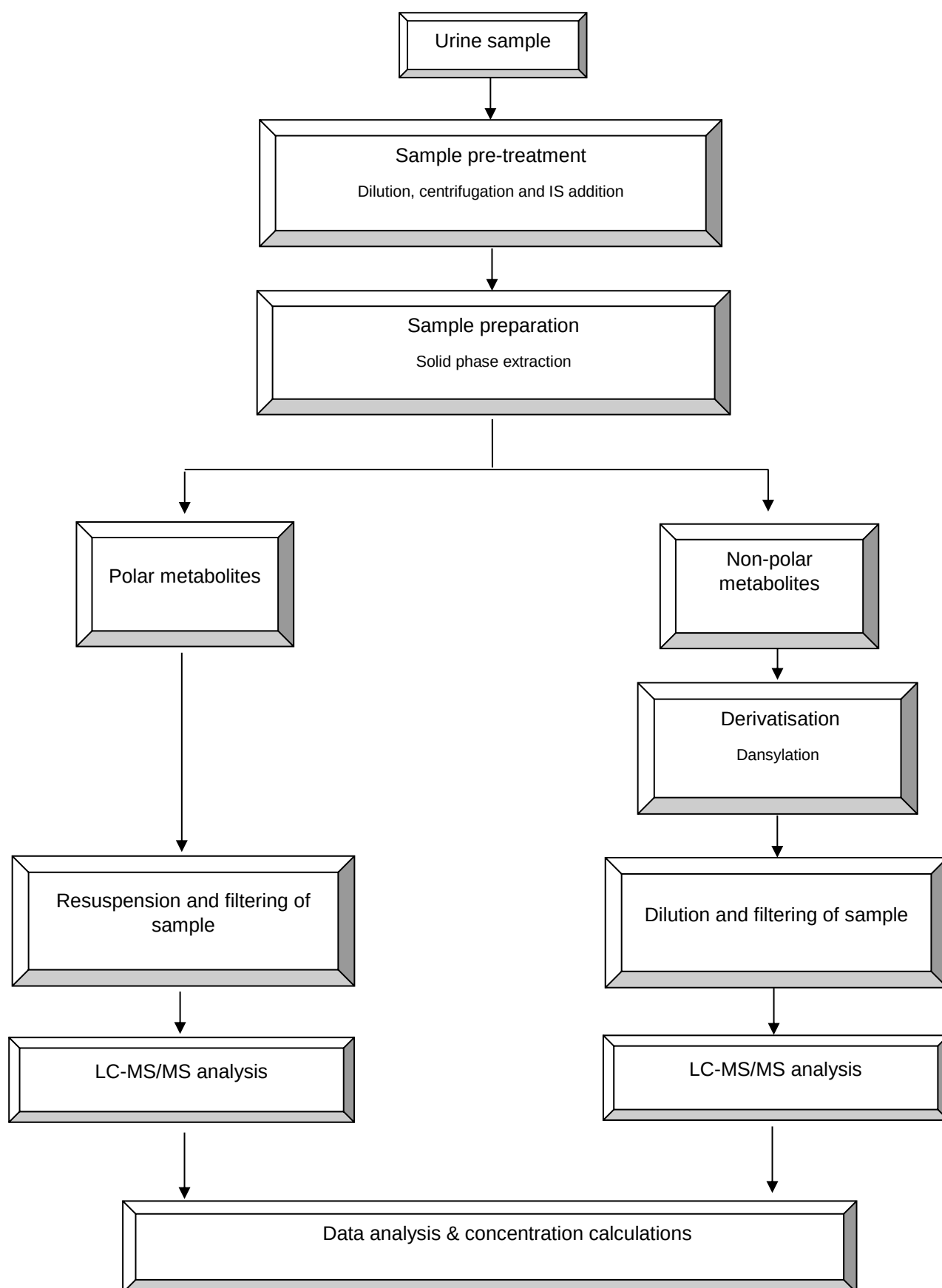


Figure 6.1: Schematic summary of the analyses used during quantification analysis of the estrogen and related steroid hormone metabolites. From the solid phase extraction, two different fractions of metabolites (polar & non-polar) were collected. All following steps are performed on each fraction individually.

- Participants should not have smoked or used alcohol excessively since this may alter the oxidative status of the participant as well as estrogen metabolism (Liska *et al.*, 2006; Seitz *et al.*, 2012)
- Participants should not have consumed tea, chocolate, stone fruits, and brassica vegetables excessively since these products are naturally rich in antioxidants and the biotransformation profile of estrogens will not reflect a true representation of the participant's ability to detoxify the estrogen metabolites (Galleano *et al.*, 2012).
- Participants had to be at a healthy weight with a body mass index between 18.5 and 29.9, which are the parameters of a healthy body mass index. Obesity is associated with oxidative stress, breast cancer (Mcdonnell *et al.*, 2014; Stefanović *et al.*, 2008) and changes in serum estrogen levels (Samavat & Kurzer, 2015).

All participants signed an informed consent to allow the use of their samples during analysis and ethical approval was obtained from the Ethics Committee of the North-West University in 2016, approval number NWU-00344-16-S1.

6.3 Creatinine analysis

All of the urine samples were thawed at room temperature. Creatinine values of all samples collected were measured at the Potchefstroom Laboratory for inborn errors of metabolism (PLIEM, North-West University, South Africa). The creatinine values of the urine samples were measured with the Thermo Scientific™ Konelab™ 20 Clinical Chemistry Analyser, and all results with a mmol/L concentration were used during data analysis.

6.4 Analytical materials and methods

For analysis of biological samples, the materials as described in Chapter 3 and methods explained in Chapter 5.7 were used exactly as previously explained. Due to evidence (obtained in Chapter 5) indicating increased sample instability over time or at temperatures above 4°C, care was taken to analyse samples immediately after derivatisation. The metabolites DHEA and DHEAS were also excluded from the analysis due to their poor method development and validation results. The flow of events during the sample analysis is illustrated in Figure 6.1. The samples were pre-treated, followed by sample preparation through SPE. The SPE fractions were then divided into a polar and non-polar fraction, of which only the non-polar fraction was derivatised. Each sample was analysed in triplicate on the LC-MS/MS instrumentation and the average of the three measurements was calculated for each sample. For the data analysis, the raw data was not divided into the different groups (controls and COC users). All samples were

pooled into one group as the aim of the analysis was not to identify differences or draw conclusions about the use of COCs, but rather to ensure that the metabolites identified could be successfully quantified, and to what extent these values correspond with those reported in the literature. Furthermore, that all metabolites present in the urine of COC and non-COC users (inside the detection limits) can be detected. Concentrations were calculated, using Equation 6, where the response of the metabolite to the response of the internal standard, together with the metabolite relative response factor as calculated in Chapter 5 and the internal standard concentration, were taken into account. All the concentrations of the metabolites were converted from nanogram per millilitre (ng/ml), to nanogram per milligram creatinine (ng/mg creatinine) values. This conversion gave more comparable results to the literature range and compensated for variations in the water content of urine samples. Outliers in concentrations were identified, using Tukey's method, which defines outliers as values falling outside the control limits ($1.5 \times \text{Inter quartile range [IQR]}$). Outliers were not excluded, but rather indicated in the data (x).

Equation 6: Calculation of metabolite concentration

$$\text{Concentration of metabolite} = \frac{\text{Response metabolite of interest}}{(\text{Response of IS} \times \text{RRF})} \times \text{IS concentration}$$

6.5 Results and discussion

A preliminary range of concentrations of the various estrogen metabolites was compiled from 10 different literature sources (Annexure A). Although this might give an idea of the concentrations to be expected, it should be noted that the groups in most of the discussed studies were not well-defined and varying methods on different analytical instrumentation on different participants or patients were used to generate the urine concentrations. The concentrations during the luteal phase of the menstrual cycle were mostly used as reference, since it would be the best part of the menstrual cycle for urine collection. However, it is important to note that the urine samples of the 20 participants were collected on a random basis of their monthly cycle and, therefore, some lower concentrations are expected. Furthermore, because samples from users as well as non-users were analysed together, some deviations might also occur in the form of outliers as the concentration ranges from the studies done in literature either did not include users or hormonal contraceptives, or did not specify that they had been included.

Table 6.1 contains the concentrations (in range format) as found in the 10 literature studies or resources; the range of concentration obtained from the current study from the 20 female urine samples for each estrogen and related metabolite and also the average concentration calculated

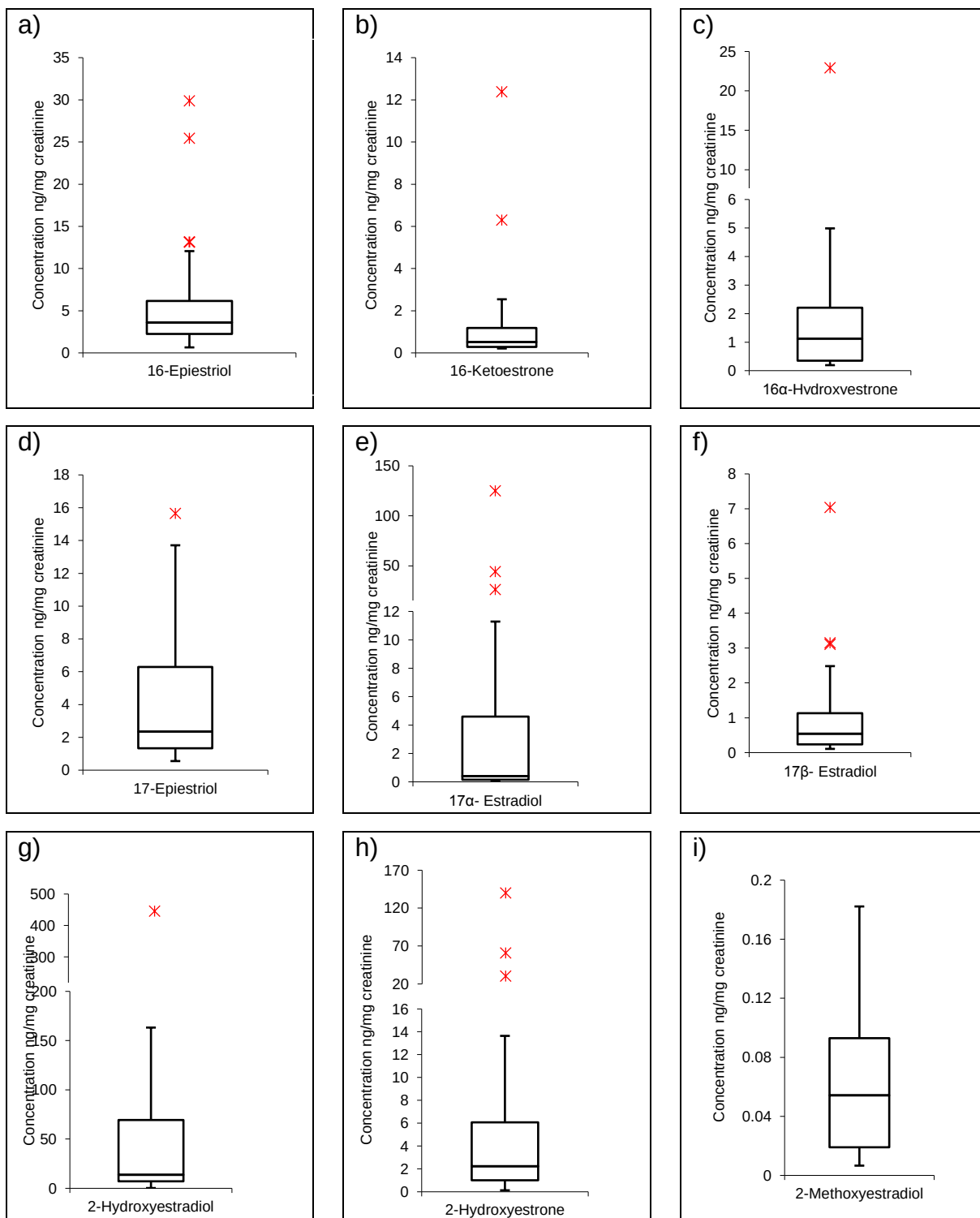
for the 20 participants. The included range consists of the lowest measured value as well as the highest measured value. For most of the metabolites, including 16-ketoestradiol, 16 α -hydroxyestrone, 17 α -estradiol, 4-methoxyestrone, androstenedione, estrone, estriol, 2-hydroxyestrone-3-methyl ether and estrone-3-sulfate, the concentrations calculated are well in and close around the range of previous studies, with very similar concentrations. For metabolites 16-epiestriol, 17-epiestriol and 2-hydroxyestrone, the concentrations were mostly at the upper end of the literature measured values and 17 β -estradiol, 2-methoxyestradiol, 4-methoxyestradiol, progesterone and testosterone were at the lower ends of the previously reported ranges. Most of the higher and lower values tested well within the literature reported ranges. For metabolites 2-hydroxyestradiol, 4-hydroxyestradiol and 4-hydroxyestrone, the calculated range was quite high in comparison to previously reported values, however, for most of these metabolites, an outlier is responsible for this tendency. For sulfate and glucuronide metabolites, not enough literature was available to conduct complete literature ranges and therefore comparisons are difficult. Analysing conjugates individually (without deconjugation), might also affect the concentrations of parent estrogens compared to those in literature that do use deconjugation methods.

Table 6.1: The table illustrating the measured metabolite concentrations from the developed method. The developed method was used on 20 female urine samples to measure their estrogen related metabolites and consequent concentration calculations. The literature range from 10 independent literature sources was used to conduct a range for comparison. The range measured in the urine and average concentration of the metabolite calculated from the results is included

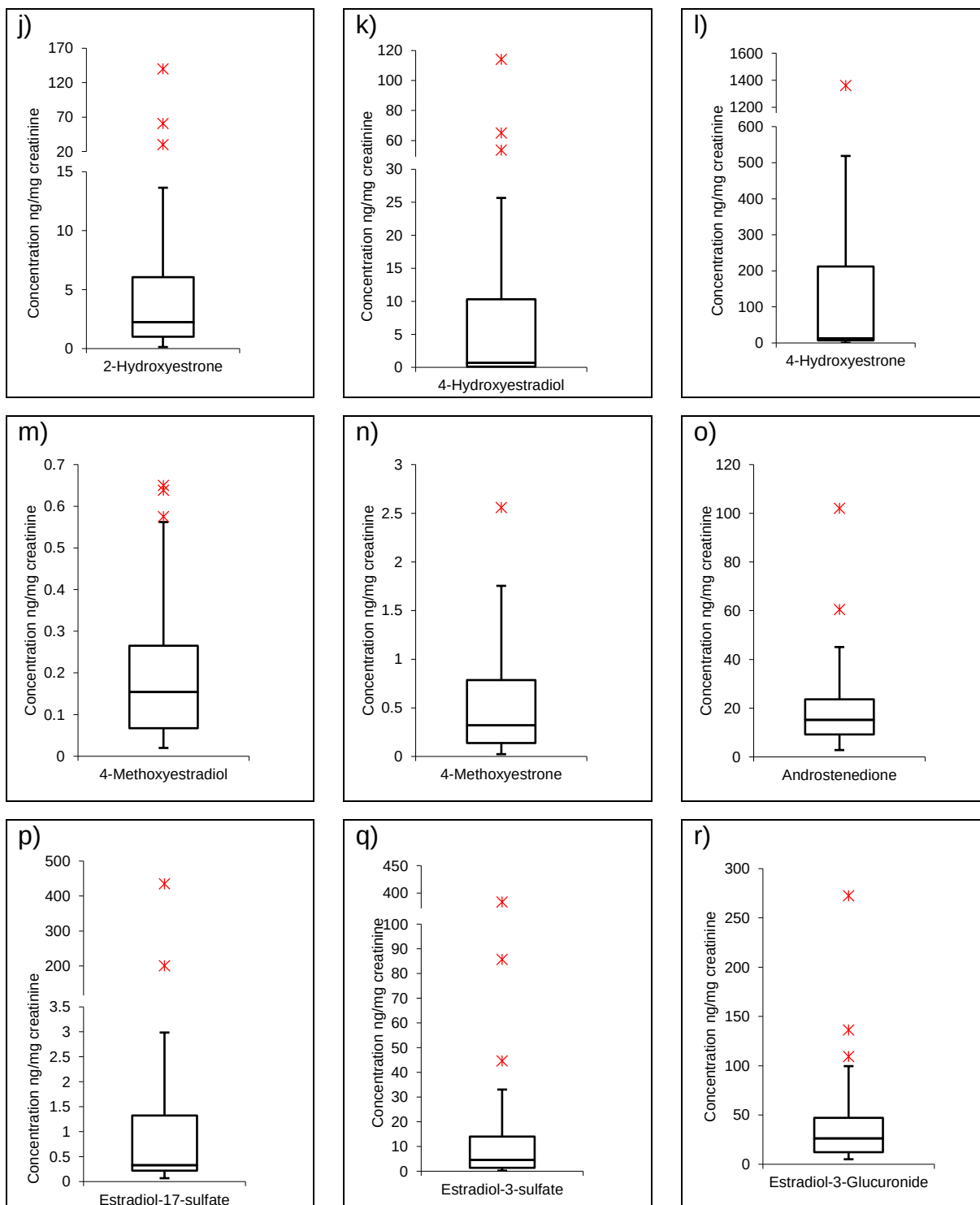
Metabolite	Literature range as per Annexure A (ng/mg creatinine)	Range measured (ng/mg creatinine)	Average concentration measured (ng/mg creatinine)
16-Epiestriol	0.25 - 7.87	0.65 - 29.86	6.29
16-Ketoestradiol	1.23 - 8.95	0.21 - 12.38	1.29
16 α -Hydroxyestrone	0.33 - 26.89	0.19 - 22.93	2.49
17-Epiestriol	0.04 - 3.70	0.55 - 15.66	3.84
17 α -Estradiol	0.05 - 172	0.05 - 125.12	10.93
17 β -Estradiol	0.05 - 172	0.11 - 7.03	1.15
2-Hydroxyestradiol	0 - 12.09	0.27 - 445.56	55.98
2-Hydroxyestrone	0.46 - 124.10	0.13 - 140.13	13.90

Metabolite	Literature range as per Annexure A (ng/mg creatinine)	Range measured (ng/mg creatinine)	Average concentration measured (ng/mg creatinine)
2-Methoxyestradiol	0.03 - 10.74	0.01 - 0.18	0.07
2-Methoxyestrone	0.22 - 10.66	0.28 - 6.29	1.58
4-Hydroxyestradiol	0.1 - 4.04	0.02 - 114.03	14.12
4-Hydroxyestrone	0.17 - 10.35	0.47 - 1361.83	165.06
4-Methoxyestradiol	0.01 - 2.51	0.02 - 0.65	0.22
4-Methoxyestrone	0.04 - 2.49	0.02 - 2.56	0.57
Androstenedione	0.2 - 227.86	2.81 - 102.01	21.46
Estradiol-17- Sulphate	39.47	0.07 - 435.21	32.38
Estradiol-3-Sulphate	22.32	0.22 - 383.68	31.00
Estradiol-3-Glucuronide		5.10 - 272.45	48.57
Estriol-16-Glucuronide		33.56 - 2297.28	326.50
Estriol	0.78 - 41.87	0.16 - 16.91	2.32
Estriol-3-Sulphate	174.15	0.45 - 370.33	25.89
Estrone	0.53 - 91.05	6.13 - 84.11	25.98
Estrone-3-Glucuronide		111.59 - 6467.51	772.96
Estrone-2-hydroxy-3-methyl ether	0.06 - 6.24	0.04-1.01	0.26
Estrone-3-Sulphate	1.16 - 295.59	0.00 - 286.74	24.25
Progesterone	5 - 1324	0.75 - 57.86	12.87
Testosterone	1.22 - 2728.16	0.24 - 5.38	1.40

The range of concentrations measured (lowest-highest), is in no way an indication of a new reference range, but is only an indication of how the concentration data for the 20 urine samples analysed are distributed. Furthermore, this concentration distribution is illustrated in Figure 6.2 a-aa, which shows boxplot diagrams for each of the metabolites. They were conducted by illustrating the minimum concentration, the median concentration and the highest concentration. The first quartile (Q1) and third quartile (Q3) ranges are also indicated, which represents the 25 and 75 percentile, respectively. Outliers are also indicated in these diagrams (ж), where a concentration point is considered an outlier if it is above $1.5 \times \text{IQR}$ or below $1.5 \times \text{IQR}$. The inter quartile range (IQR) was calculated as $Q3 - Q1$. The distribution of most of the samples, for example, 4-methoxyestradiol, estriol-16-glucuronide, estrone and testosterone were distributed quite evenly over the concentration range, if the outliers were to be omitted. For other metabolites (progesterone, estriol, 4-hydroxyestrone, 4-hydroxyestradiol, 17α -estradiol, 2-hydroxyestradiol and 16-ketoestradiol), most of the data are distributed in the lower concentration ranges as indicated by the median being closer to the 25% percentile than the upper 75% percentile. All of these diagrams include outliers, except for 2-methoxyestradiol for which no outlier values were calculated. It is, however, important to note that the number of samples analysed (20 samples) is fairly small and if a larger number were to be analysed the tendency might be different. The large number of outliers observed may not necessarily be indicative of the non-normal distribution of data, but the variations in the metabolite concentrations between different groups of patients. Due to the fact that no detailed knowledge on the urine samples were available, except for the fact that these patients were either users or non users, the reason for the occurrence of outliers cannot be confirmed. It is also important to note that the samples were collected on any day of the monthly female cycle, and these metabolite levels can fluctuate extensively over such a cycle period. Other variations in the estrogen metabolite concentration can ultimately be caused by inter individual variation. Enzyme reactions are mostly responsible for conversion, metabolism and detoxification of estrogen metabolites. Thus, enzyme expression, activity, co-factor availability and metabolite load may affect estrogen levels. Individual profiles of patients would, therefore, give a better indication if all the metabolites in a single sample would be increased/decreased, or if certain metabolite levels are higher due to build up, and other product metabolites lower due to lower conversion rates.



Key: ✖ Outliers



Key: ✖ Outliers

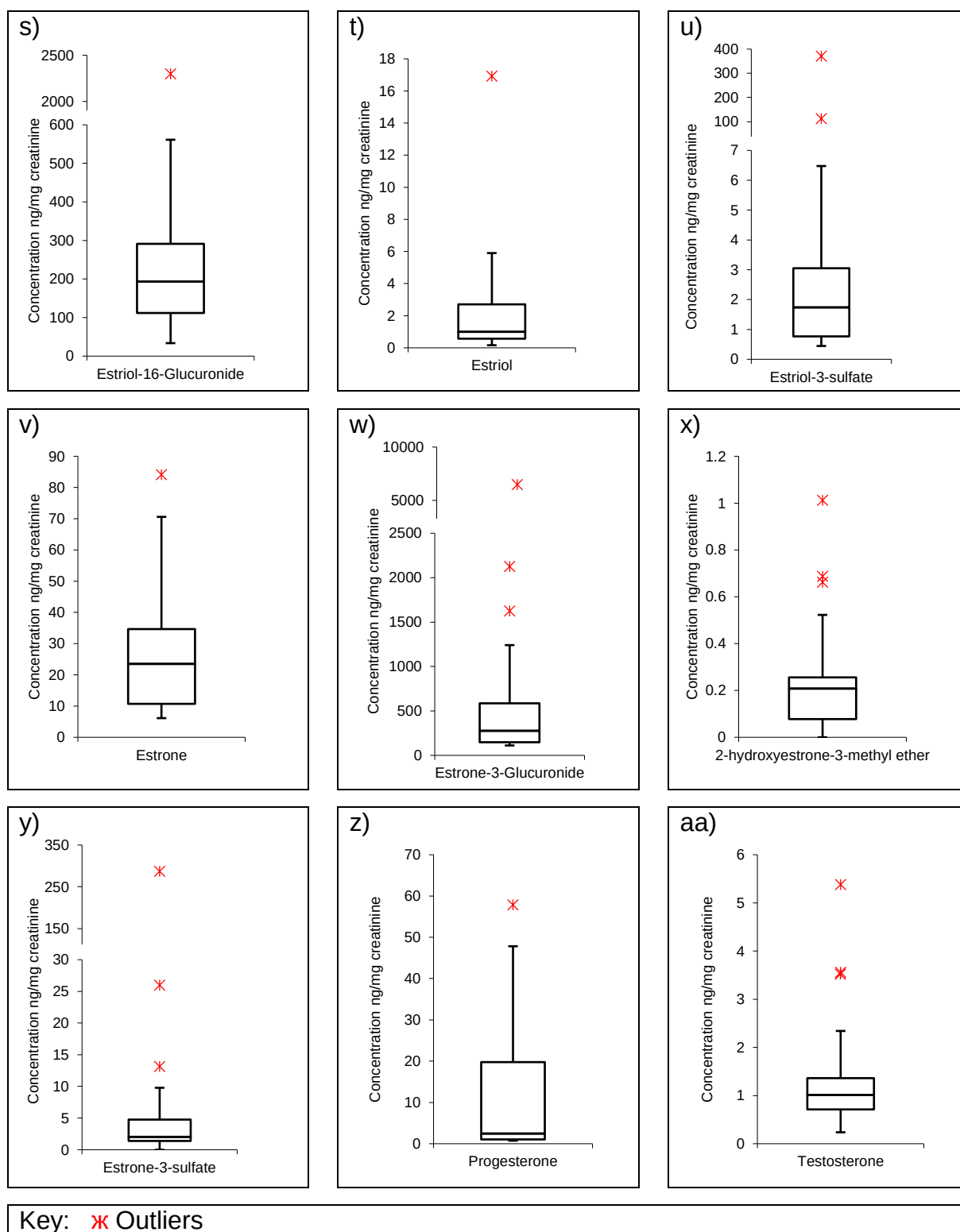


Figure 6.2: Visual illustrations of the concentration distributions of each of the estrogen metabolites. Box plots of the concentration data were plotted for all 20 samples, where the minimum, maximum, Q1, Q3, median and outliers are indicated.

6.6 Conclusion

Samples of twenty female participants (10 COC users and 10 non-users) were analysed on the method as developed in Chapter 5. This analysis indicated, firstly, whether all 27 metabolites were successfully detected in different samples with different matrixes. The results were positive with concentrations inside the detection limit of the analytical method, and consequent detection of the metabolites in all 20 samples. Furthermore, the ability to successfully quantify the concentrations of metabolites in the urine samples was achieved, where a concentration for each metabolite was calculated and reported. The concentration data was then visually illustrated to indicate the concentration distribution of the metabolites in the 20 urine samples. These data showed some variation concentrations in between different samples, as expected due to inter-individual variation. The concentration results were also compared to results from previous studies. This comparison was done only to see whether the concentrations calculated gave values within a realistic range. For most of the metabolites, this was the case. The ranges reported from 10 literature and clinical sources were also a relative range, as large variations in the concentration ranges were observed between these sources. Therefore, it was not expected that the measured ranges would fall exactly into reported ranges, and the 20 samples analysed were not aimed to set up our own reference range, as a larger sample volume would be necessary and only one group (non-users) would be used. The overall results were positive and the method was successfully applied on the twenty chosen samples.

CHAPTER 7

CONCLUSION

7.1 Introduction

The interest in and importance of measuring various estrogen steroid hormones and their related metabolites, both in individuals (clinical settings) and within different study groups (research), is increasing. One of the main reasons for this is the mounting evidence that estrogen metabolites are involved in the initiation of cancers, especially breast cancer (Gaikwad *et al.*, 2008). Although estrogen is mainly believed to initiate cancer through a hormonal mechanism (Cavalieri, 2000), it is the non-hormonal effect of its metabolites that motivated this study. In the non-hormonal mechanism, reactive molecules can form and readily react with DNA to form depurating DNA adducts, which may be involved in the initiation of certain cancers (Cavalieri & Rogan, 2014; Cavalieri, 2000). Estrogen from various sources, including biosynthesis (reviewed in International Agency for Research on Cancer (1987) or exogenous exposure (Cavalieri, 2000), can initiate this estrogen-related cancerous effect, if these estrogens are not successfully detoxified. Many women are using estrogen-containing COCs from a young age already and this additional intake of estrogens might contribute to an imbalance of estrogen metabolism and detoxification (Collaborative Group on Hormonal Factors in Breast Cancer, 1996). Analysis of estrogens and estrogen metabolite profiles in all women, with an increased exposure to estrogens, for example those using COCs, will therefore, be of great value to evaluate how these exogenous estrogens affect estrogen metabolism and detoxification pathways. However, to our knowledge, little focus has been directed towards the role that additional estrogen intake in the form of COCs could have on estrogen metabolism and estrogen metabolic profiles. Previous studies concluded that there could be a relation between the urinary estrogen levels and estrogen-related breast-cancer risk (Ziegler *et al.*, 2015). The use of metabolic biomarkers in urine, rather than blood or serum, is less invasive, sample collection can be less expensive and could be useful in monitoring patients over time in clinical settings. Up to this point, however, no method for evaluation of estrogens and estrogen metabolites in urine has been developed at the Centre for Human Metabolomics (North-West University, Potchefstroom). Other groups that have developed and applied similar methods have used various techniques (non-derivatisation and derivatisation), and other analytical instrumentation, but very little, developed a method for such a vast combination of steroids and estrogenic metabolites.

Most of the previously developed methods for the evaluation of estrogen and related metabolites used more conventional and less specific methods and were mainly developed for other biological matrixes (serum or tissue) or may be incomplete profiling methods. The latter refers to methods that did not include different biotransformation products, but investigated only certain parts of the estrogen metabolism. Comprehensive evaluation of the estrogen metabolic products, together with the parent estrogens as developed by Gaikwad *et al.* (2008), would be beneficial. Most of the metabolites evaluated in this method were not commercially available and the authors

performed synthesis thereof. Other important related metabolites such as progesterone, estradiol precursors, sulphate and glucuronide conjugates, and metabolites from the 16-hydroxylation metabolic pathway of estrogens were also omitted in previous studies. Due to fairly large variations in the available infrastructure and analytical standards between the published methods and the current area of study, an in-house method with the currently available resources was developed, rather than implementation of previously developed methods. The available infrastructure and standards, however, are not identical to those in previously developed methods and, therefore, direct implementation of previously developed methods was not possible.

The aim of the study was, therefore, to develop and apply a sensitive and selective urine-based liquid chromatography-electrospray tandem mass spectrometry method to enable the evaluation of the estrogen metabolism in premenopausal women taking combined oral contraceptives. The method was validated to ensure precise and accurate quantification of metabolites when biological samples are analysed. The first developed method gave poor validation results, so a second method, where derivatisation was used to increase the sensitivity and selectivity of the method, was also developed. This second developed method yielded reliable validation results; therefore, it was used as a proof of concept analysis. For the latter analysis, the urine samples of 20 pre-menopausal women were analysed. These results showed that all the metabolites in the developed method could successfully be detected and quantified on different urine samples, and allowed a comparison of the measured concentrations to the expected ranges, as described in the literature.

7.2 General conclusion

In summary, three main conclusions could be made from the results obtained in this study. The first part of this study focused on optimising a LC-ESI-MS/MS method for the quantification of estrogen and related steroid hormones. The underivatized method was developed and the underlying parameters of the mass spectrometry conditions (ionisation and source conditions) were optimised to find the precursor ion and product ion that would be most successfully detected for each analyte. These parameters also gave optimal MRM conditions to produce the highest possible analyte signal. Different LC mobile phases and mobile phase modifiers were also evaluated and compared, together with an assessment of the ideal column for sufficient chromatographic separation between the relevant estrogen analytes. Different sample preparation methods (LLE, SPE) were evaluated and the results compared. Solid phase extraction as sample preparation was then chosen and different SPE parameters were compared and optimised. Various aspects and parameters of the LC-MS/MS method were optimised, including flow rates, temperatures, pH, peak shapes and injection volumes. The selectivity and

sensitivity of the analytic method, however, did not allow for the analysis of multiple urinary estrogen metabolites, and metabolism substrates and products in a single run. Although further validation in the form of accuracy and precision of the developed method were not evaluated, the linear range and repeatability in terms of the autosampler and ionisation were evaluated. The results obtained indicated higher than acceptable lower detection and quantification limits, and this would not allow the aim of a sensitive and selective method to be achieved. Hence, this method could not be employed to gather useful information regarding the metabolic load of the use of COC on the estrogen metabolism and detoxification of pre-menopausal women. Even though other groups obtained more promising results from underivatised methods, the in-house development and application of such a method was not satisfactory.

This led to the development of the second method in which derivatisation of the estrogenic metabolites was applied. This method gave results that are more satisfactory during the validation process. Again, mass spectrometry was optimised to give optimal MRM conditions to produce the highest possible derivatised analyte signal. Variations of other LC mobile phases and mobile phase modifiers were also evaluated and compared, together with an assessment of the ideal column for sufficient chromatographic separation between the relevant estrogen analytes. Some separation issues were encountered here, with the hydroxyestrogens that could only be solved by using a C8 column, which has (to the authors knowledge) never been reported to be used for estrogen separations before. The SPE as previously developed for sample preparation was optimised, leading to two fractions of estrogen metabolites. The LC-MS/MS method was then adapted accordingly and resulted in two analyses (polar and non-polar) that could easily be conducted directly following each other. The derivatisation and LC-MS/MS methods were then optimised and the final method was subjected to a series of method validation steps to ensure the accuracy and precision of the method before implementation in a clinical setting. This method validation resulted in the assessment of the analytical range and linearity in that range, LOD, LOQ, precision, accuracy, matrix effect, selectivity, stability and carry over. All these results gave useful information regarding the capacity as well as the limitations of method. The stability results indicated that after sample preparation, samples should not be stored at temperatures above 4°C, or for longer than 5 days prior to analysis. Furthermore, validation results indicated that the metabolites DHEAS and DHEA, do not give repeatable and accurate inter- and intraday results under the developed conditions of the method and were thus omitted from the analysis. The latter method was developed and optimised for the quantification of twenty-seven estrogen and estrogen-related metabolites (excluding IS) and validated for the implementation in a clinical laboratory. Therefore, the aim and first two objectives stated in Chapter 2, was achieved. The conclusion, based on the results in Chapter 6, provides final confirmation that the first and second objectives were achieved. To ensure that the developed method could ensure the detection and

quantification of the 27 metabolites in acceptable ranges, the evaluation of 20 urine samples from premenopausal women were done. Since the results obtained from this quantification were reasonably close to previously reported ranges, and reflected the normal biological variation, it is concluded that the final objective of the study was also met.

7.3 Final remarks

The need for an analytical method for investigation of the estrogen metabolite profiles was observed and motivated the current study. It is finally concluded that the developed method was successfully optimised for the detection and quantification of twenty-seven estrogen-related metabolites and was validated for the implementation into a clinical laboratory. The method was also successfully applied on different urine samples and therefore, the aim and objectives, stated in Chapter 2, was achieved. The results from this study will contribute to the larger context of biomonitoring estrogen-related drugs, relative breast cancer risk evaluation and studies on how these two factors could be connected. Assessment of estrogen detoxification will give the health professional more precise representations of an individual's capacity to detoxify endogenous and exogenous estrogens, to allow adaptation of the nutritional support or the levels of exposure to estrogens.

7.4 Recommendations for future studies

In hindsight, the following recommendations are made for future studies to adjust, improve and apply the method developed during the work done in this dissertation:

1. If made commercially available, the method could be further optimised by adding stable isotopes for all metabolites, or at least the metabolites that gave suboptimal validation results. Stable isotopes could improve the LLOQ and the accuracy of the quantification results.
2. It could be of value to add estrogen glutathione conjugates, their metabolic products and estrogen-related DNA adducts to the profile. The inclusion of all of these metabolites into the already developed method may allow the ratio as described by Gaikwad *et al.* (2008), to calculate the relative cancer risk in women based on their urinary estrogen profiles. These metabolites are currently not commercially available and synthesis, extraction and purification thereof might be necessary. Additional sulphate and glucuronide conjugates,

not available to us at time of method development, might also allow a more comprehensive profile.

3. Sufficient amounts of urine samples should be analysed for different groups (age & ethnicity) using the developed method, to result in in-house reference ranges to be established for each group of females.
4. The effect that the urine concentration (as result of the water content in the urine samples) has on the sample preparation and consequent method validation parameters (LLOQ, sensitivity, and matrix effect) should be investigated. The measurement of the creatinine values before method application will then be essential to confirm whether more concentrated or more diluted urine samples give the best results.
5. A supplementary method should be developed, specifically designed for the metabolites that had to be omitted from the current study, including DHEAS and DHEA. Although these values will not be indicative of estrogen biotransformation, such a method could be used when abnormal results are obtained, to confirm that estrogen production occurred, as it should.
6. In line, SPE might be a solution to numerous validation problems, and improving the sample preparation method may give lower LLOQ and more accurate and precise values. In line, SPE might increase the repeatability to allow better results.
7. Considering dansyl chloride derivatisation with isotopically labelled dansyl chloride as substrate, might be an alternative to consider. This will allow the addition of derivatised stable isotopes of all the metabolites that derivatised in a mixture allowing an increase in the concentration, but still giving separation, based on labelled masses.
8. The method should be implemented to analyse samples of COC users and non-COC users to evaluate whether the additional estrogens, in the form of COC, would disturb the estrogen biotransformation pathway.
9. Urine samples from women in different phases of their menstrual cycle should be analysed, in order to determine whether different metabolite levels could be detected across the entire menstrual cycle. This will allow women in a clinical setting to hand in samples on any given day of the month, which will further simplify the clinical use of the developed method.

10. Studies of the estrogen metabolites, ratios and consequent breast cancer risk on pregnant women should also be performed. Since the risk of developing breast cancer increase after a pregnancy, but multiple or later age pregnancies allows a decrease in breast cancer risk.
11. Breast cancer genes as well as the genes associated with the estrogen metabolising and detoxifying enzymes should be evaluated together with urine estrogen profiles. These genes may contribute to an increase in breast cancer risk and can therefore not be excluded when comparing users and non users of COCs.
12. In the long term the roles of phyto and myoestrogens on the urinary estrogen metabolite profiles and later on also the relative breast cancer risk might also be an important factor for evaluation. The exposure of estrogen from the environment are increasing and if an association thereof to the pathogenesis of breast cancer may allow precautions to be made.
13. Additionally to the urine DNA adducts, serum DNA metabolites such as the purine adducts may provide insight into the effect of the estrogen metabolism on the DNA and nucleotide biochemistry.

CHAPTER 8

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ANNEXURE A

Table A1: A summary of (literature obtained) estrogen and related metabolite concentrations in female urine samples. All concentrations are expressed as nanogram per milligrams of creatinine (ng/mg creatinine) values, and sources from which obtained include 1) Xu *et al.* (1999), 2) Oh *et al.* (2015), 3) Fuhrman *et al.* (2013), 4) Hartman *et al.* (2016), 5) Precision Analytical (2014), 6) Naldi *et al.* (2016), 7) Almeida and Nogueira (2006), 8) The Metabolomics Innovation Centre (2016), 9) ZRT Laboratory (2016), 10) Gaikwad *et al.* (2009).

Steroid hormone	1	2	3	4	5	6
16-Epiestriol	7.87	1.81	0.25	1.59		
16-Ketoestradiol	8.95	4.04	1.23	3.90		
16 α -Hydroxyestrone	24.23	3.29	0.33	3.12	1.30 - 4.60	
17-Epiestrol	3.70	0.43	0.04	0.49		
2-Hydroxyestradiol	12.09	1.53	0.19	1.70	0.00 - 1.80	
2-Hydroxyestrone	124.10	14.58	1.18	14.86	4.60 - 14.40	
2-Hydroxyestrone-3methyl ether		0.34	0.06	0.36		
2-Methoxyestradiol	2.53	0.22	0.03	0.22		
2-Methoxyestrone	27.83	2.53	0.22	2.37	2.90 - 5.90	
4-Hydroxyestradiol	4.04		0.29			
4-Hydroxyestrone	10.35	1.60	0.29	1.90		
4-Methoxyestradiol	0.70	0.02	0.01	0.02		
4-Methoxyestrone	1.85	0.04	0.04	0.04		
Androstenedione						
DHEA						
DHEAS						
Estradiol	16.56	3.73	0.56	3.35	2.00 - 4.90	0.05
Estradiol-17-sulphate						39.47
Estradiol-3-glucuronide						
Estradiol-3-sulphate						22.32
Estriol	36.88	8.71	1.56	7.90	5.60 - 23.00	41.87
Estriol-16 glucuronide						
Estriol-3-sulphate						174.15
Estrone	46.85	7.43	0.53	6.81	14.00 - 27.10	3.26
Estrone-3-glucuronide						
Estrone-3-sulphate						296.59
Progesterone					5.00 - 520.00	
Testosterone					5.50 - 17.80	

Table A1 Continues: A summary of (literature obtained) estrogen and related metabolite concentrations in female urine samples. All concentrations are expressed as nanogram per milligrams of creatinine (ng/mg creatinine) values, and sources from which obtained include 1) Xu *et al.* (1999), 2) Oh *et al.* (2015), 3) Fuhrman *et al.* (2013), 4) Hartman *et al.* (2016), 5) Precision Analytical (2014), 6) Naldi *et al.* (2016), 7) Almeida and Nogueira (2006), 8) The Metabolomics Innovation Centre (2016), 9) ZRT Laboratory (2016), 10) Gaikwad *et al.* (2009).

Steroid hormone	7	8	9	10	Range	Average ng/mg creatinine
16-Epiestriol					0.25-7.87	2.88
16-Ketoestradiol					1.23-8.95	4.53
16 α -Hydroxyestrone			0.35 - 1.07	26.89	0.33-26.89	7.24
17-Epiestrol					0.04-3.70	1.17
2-Hydroxyestradiol		4.08	0.17 - 0.70	4.30	0-12.09	2.66
2-Hydroxyestrone		3.80	0.70 - 2.54	0.46	0.46-124.10	18.12
2-Hydroxyestrone-3methyl ether		6.24			0.06-6.24	1.75
2-Methoxyestradiol		10.69	0.03 - 0.08	10.74	0.03-10.74	3.07
2-Methoxyestrone			0.26 - 0.68	10.66	0.22-10.66	5.93
4-Hydroxyestradiol			0.10 - 0.18		0.1-4.04	1.15
4-Hydroxyestrone			0.17 - 0.47		0.17-10.35	2.46
4-Methoxyestradiol			0 - 0.04	2.51	0.01-2.51	0.55
4-Methoxyestrone			0 - 0.04	2.49	0.04-2.49	0.75
Androstenedione		227.86	3.93 - 13.53	0.20	0.2-227.86	61.38
DHEA						
DHEAS						
Estradiol	172.00	1.44	0.78 - 1.79	1.47	0.05-172	17.39
Estradiol-17-sulphate						39.47
Estradiol-3-glucuronide						
Estradiol-3-sulphate						22.32
Estriol		2.55	0.78 - .198		0.78-41.87	13.08
Estriol-16 glucuronide						
Estriol-3-sulphate						174.15
Estrone	91.05	3.11	2.27 - 5.22	1.46	0.53-91.05	17.42
Estrone-3-glucuronide						
Estrone-3-sulphate				1.16	1.16-295.59	148.87
Progesterone	96.36		1324		5-1324	486.34
Testosterone		2728.16	1.22 - 3.97		1.22-2728.16	551.33

ANNEXURE B

SUPPLEMENTARY RESULTS

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Note to Reader

Data supplementary to figures and tables illustrated as part of the dissertation are herein displayed. These results are not necessarily to prove any statistical significance, but rather to allow visual comparisons of data obtained. Due to the large variations in response obtained for each metabolite, illustration on the same y-axis was a troubling factor, specifically for the figures. This was overcome by adapting the data in two separate ways. Firstly, by adjusting the response values of certain metabolites, so they would fit on the exact same axis. An example thereof is the responses multiplied by 10^{-2} for certain metabolites (A4, P4, T) in Figure S2. The other type of adaption made was to show the y-axis in a lower range, and again in higher range (Figure S5 for example), so the tendency in higher and lower responses is visible. It is important to note that adaptations were made for all the response values of a metabolite and, therefore, do not affect the tendencies at all, just makes it visible for more metabolites. All adaptations are, however, mentioned in the figure citations and clearly illustrated in the figures as well (* above bar or // on the y-axis). Some metabolites gave extremely low or zero response values that could not be adjusted for visual illustrations.

Table S1: A visual summary of the four modifiers used in the mobile phases, and the metabolites successfully ionising illustrated by an X. These modifiers included formic acid, ammonium acetate, ammonium fluoride and ammonium hydroxide. All product ions with abundances above 100 arbitrary units are indicated as obtained from the Optimizer program for the optimal fragmentor and collision energies after 1ppm standard injections.

Compound Name	Formic acid		Ammonium acetate		Ammonium fluoride		Ammonium hydroxide	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
16-Epiestriol								
16-Ketoestradiol						X		
16 α -Hydroxyestrone				X		X		X
17-Epiestriol				X				
17 α - Estradiol								
17 β - Estradiol B								
2-Hydroxyestradiol						X		
2-Hydroxyestrone		X		X		X		X
2-Hydroxyestrone-3methyl ether						X		X
2-Methoxyestrone				X		X		X
2-Metoxiestradiol						X		X
4-Hydroxyestradiol				X		X		
4-Hydroxyestrone		X	X	X		X	X	X
4-Methoxyestradiol						X		X
4-Methoxyestrone						X		X
Androstenedione	X		X		X		X	
Estradiol 17-sulfate		X				X		X
Estriol						X		
Estrone				X		X		X
Testosterone	X		X		X		X	

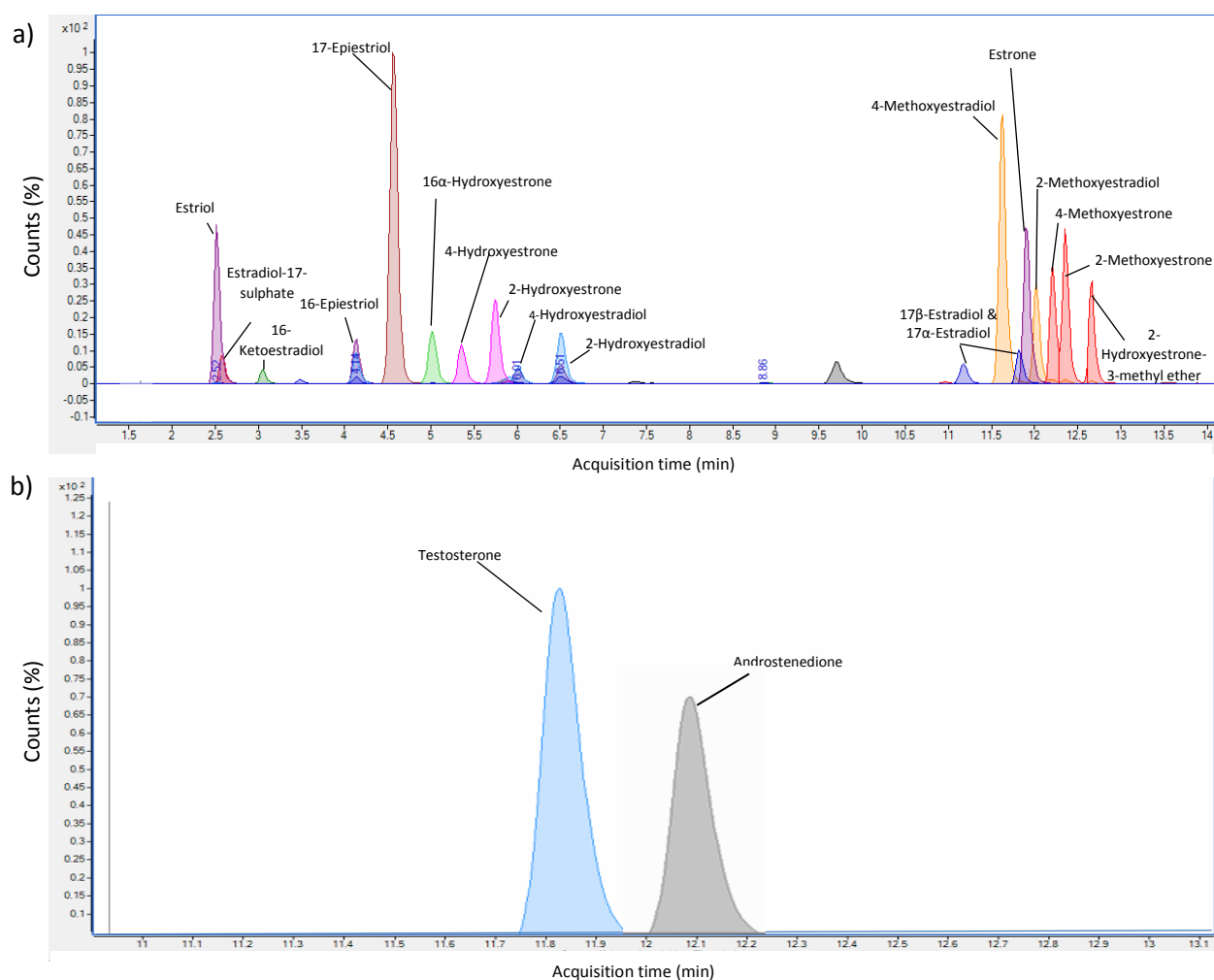


Figure S1 a & b: Visual illustration of the chromatograms following a) negative and b) positive ionisation. These results collected when injecting 1 μ l of 1 ppm on the Poroshell120 C18-EC allowed peak and retention time identification for each of the 20 metabolites.

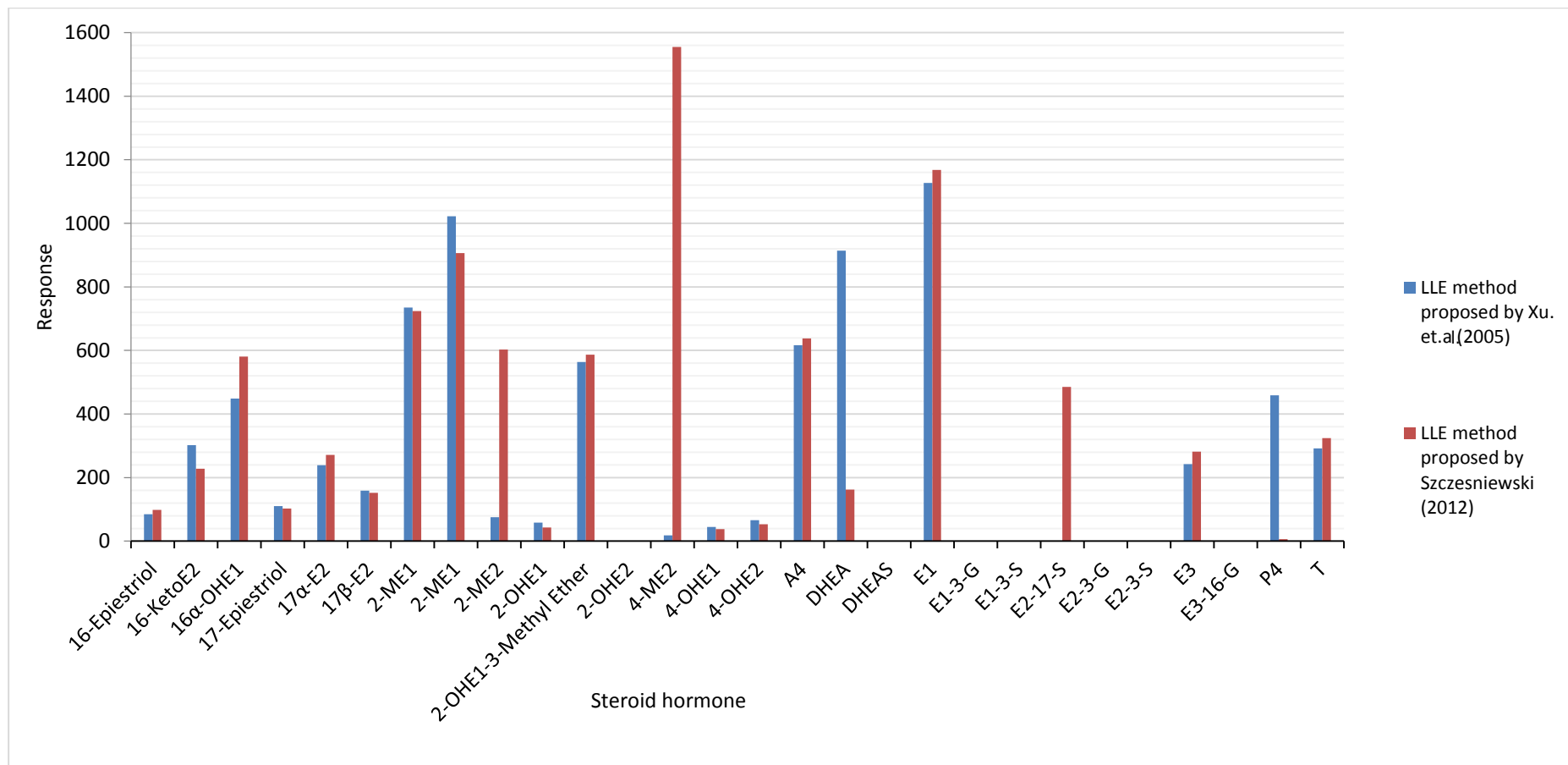


Figure S2: Visual illustration of the comparison of the relative responses on the mass spectrometer for each of the steroid hormones in a standard mixture after the two LLE sample preparation procedures. These LLE procedures include the one as published by Xu et.al (2005) and proposed by Agilent technologies, (Szczesniowski, 2012) in an application note. For metabolites 2OHE2, DHEAS, E1-3-G, E1-3-S, E2-3-G, E2-3-S and E3-16-G, very low to zero response was achieved after either sample preparation method.

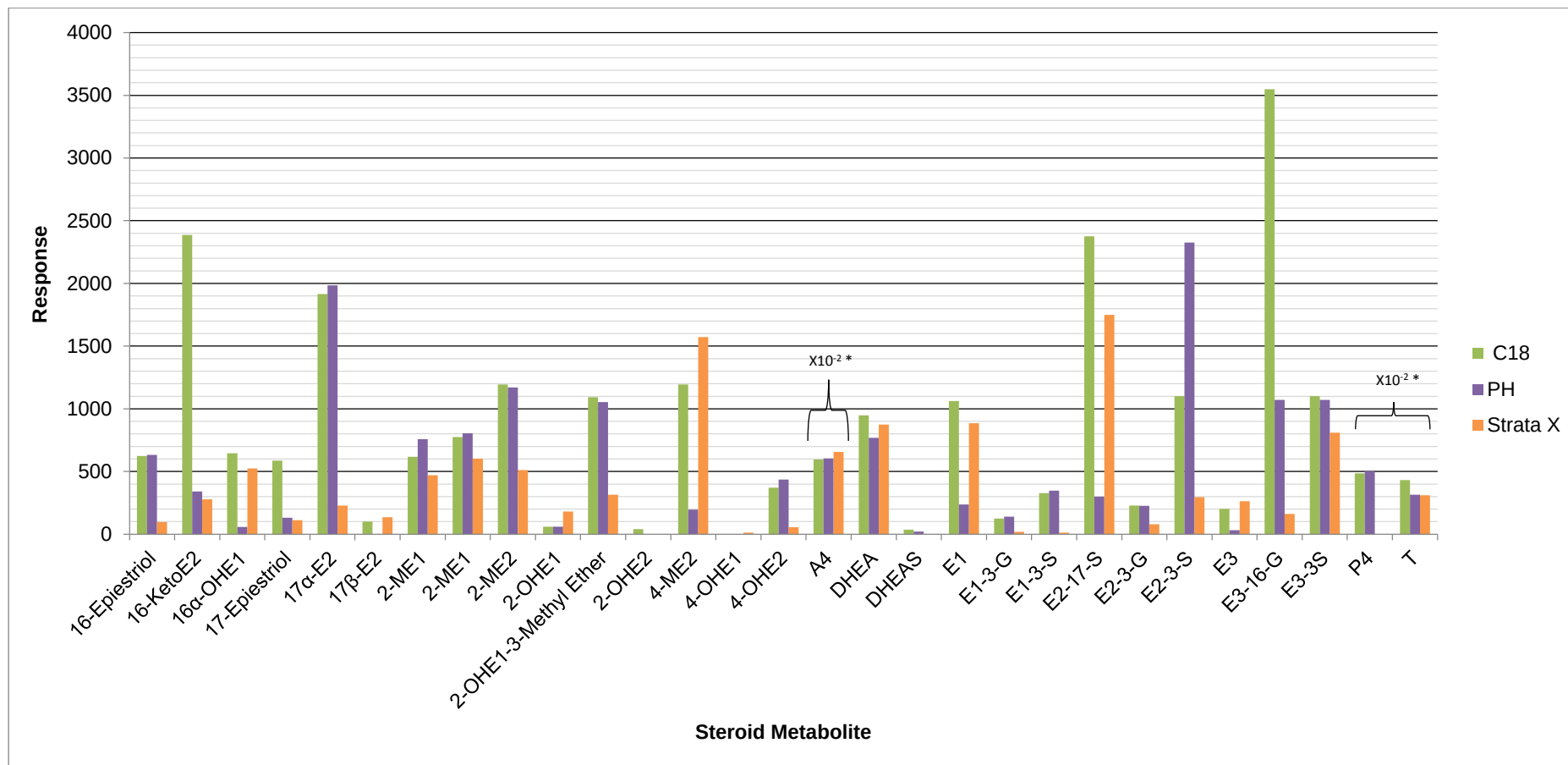


Figure S3: Visual illustration of the relative responses on the mass spectrometer of the steroid hormones in a standard mixture after the three SPE sample preparation cartridges were compared. These SPE cartridges included C18, Phenyl (PH) and Strata x Adsorbents. * These metabolite response values were adjusted by $\times 10^{-2}$ to allow visual illustration thereof on the same y-axis scale. For some of the metabolites (17 β -E2, 2-OHE2, 4OHE2, DHEAS) the response was extremely low or zero, making the visual illustration thereof not possible on the same y-axis.

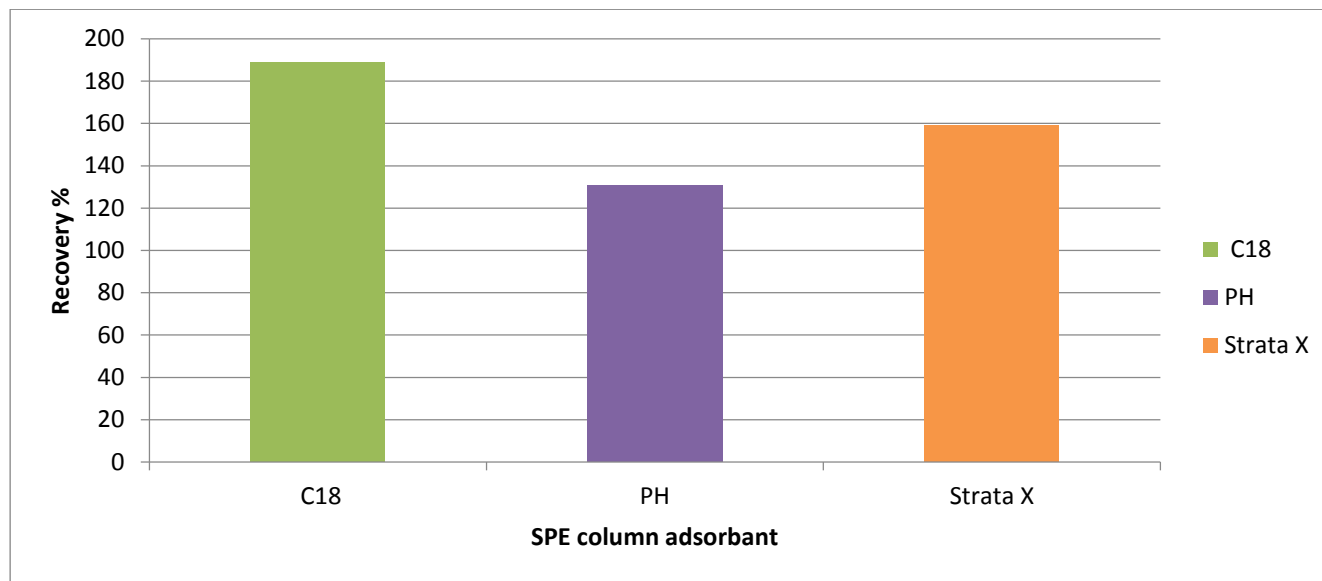


Figure S4: Summarised visual illustration of the average % recovery (response average for SPE cartridge/average response of standard injected without SPE x 100) of the steroid hormones in a standard mixture after the three SPE sample preparation cartridges were compared.

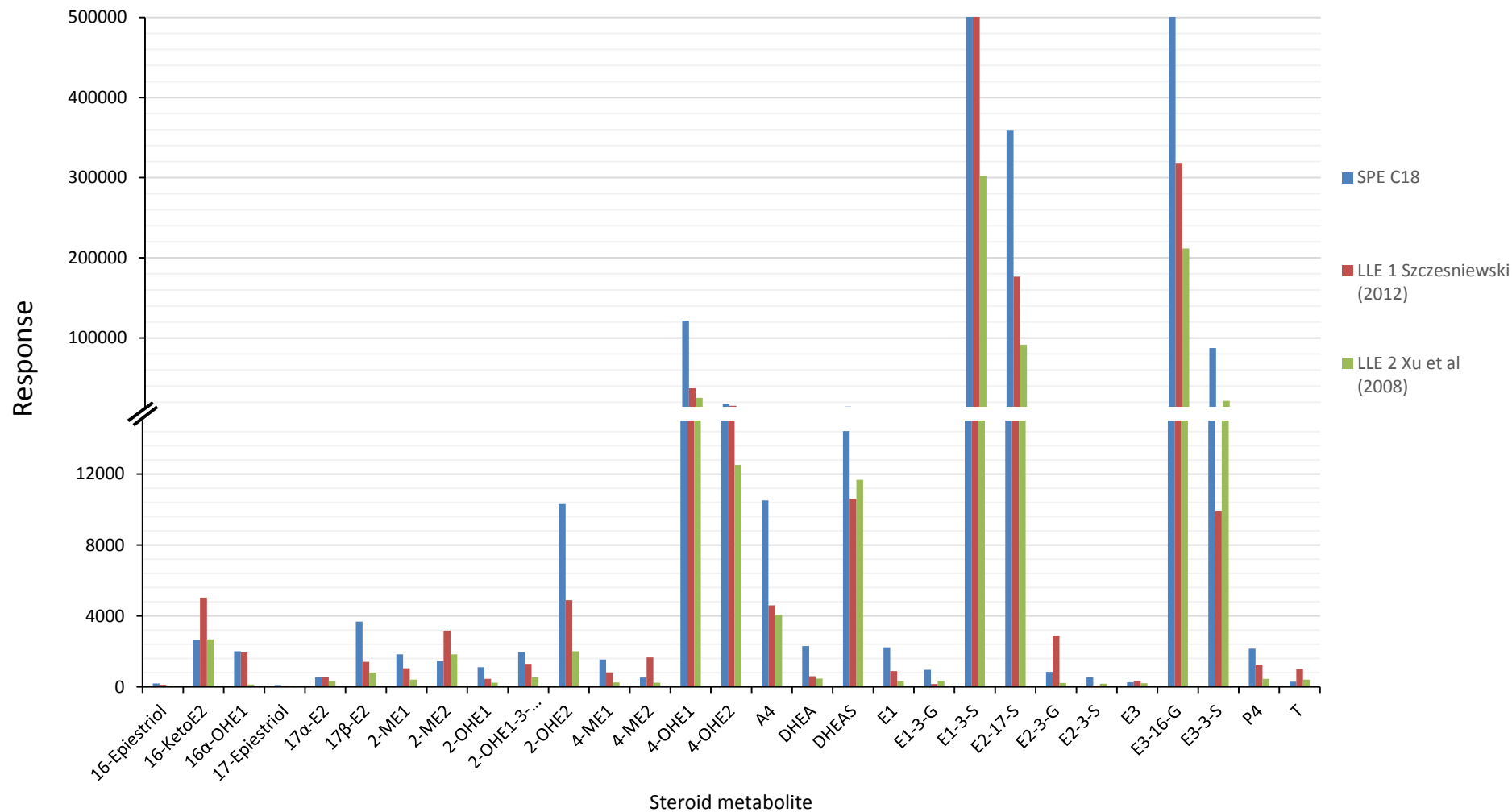


Figure S5: Visual illustration of the relative responses on the mass spectrometer of the steroid hormones in a standard mixture after the C18 SPE sample preparation and two different LLE methods were performed. // Gap in y-axis for better visual illustration, as the response for metabolites E1-3-S, E2-3-S, E1-3-G, E2-3-G and E3-16-G; were very low in comparison to very high responses obtained from positive ionising metabolites.

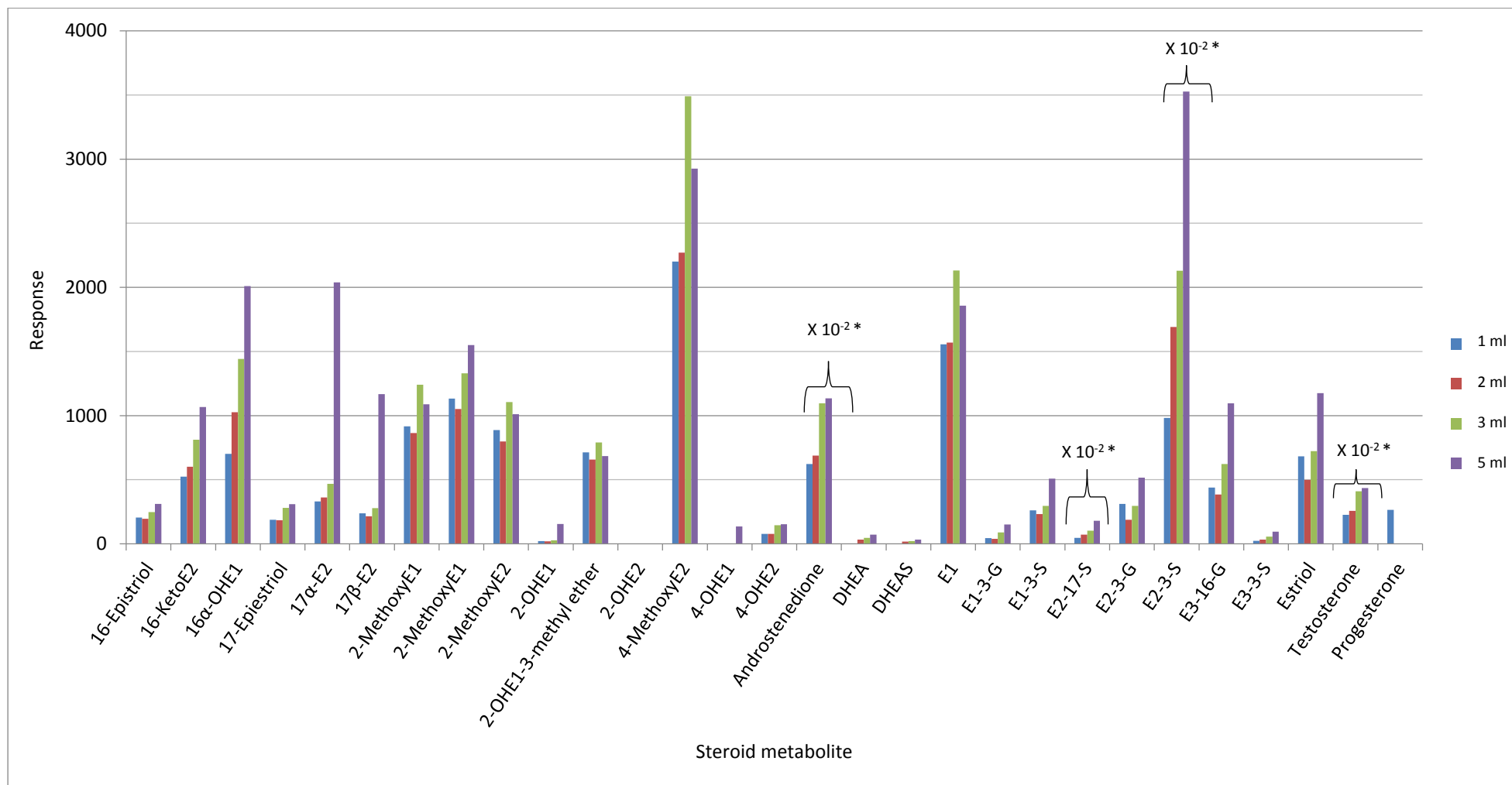
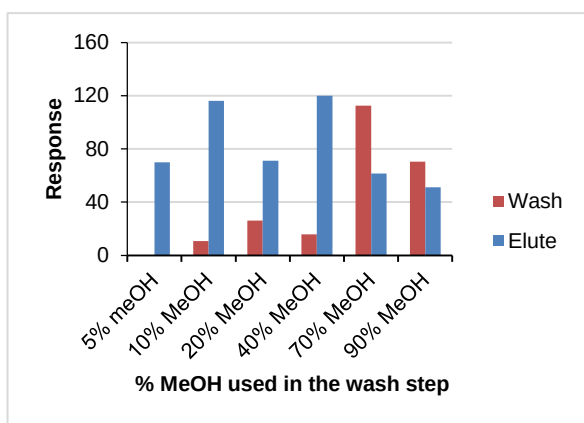
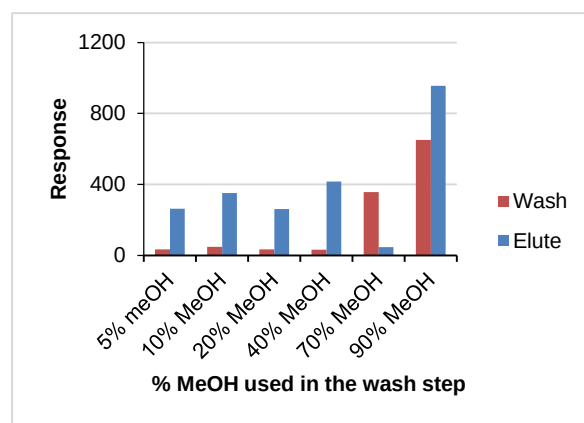


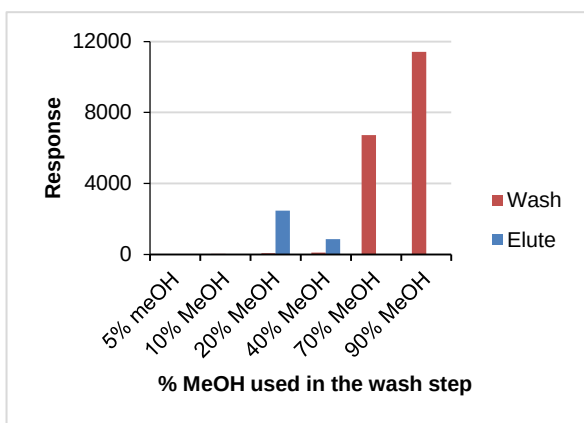
Figure S6: Visual summaries of relative mass spectrometric responses for urine volumes of 1 ml, 2 ml, 3 ml and 5 ml for each steroid hormone metabolite. These urine samples were spiked and cleaned through C18 SPE before being evaluated on LC-MS/MS. * These metabolite response values were adjusted by $\times 10^{-2}$ to allow visual illustration thereof on the same y-axis scale.



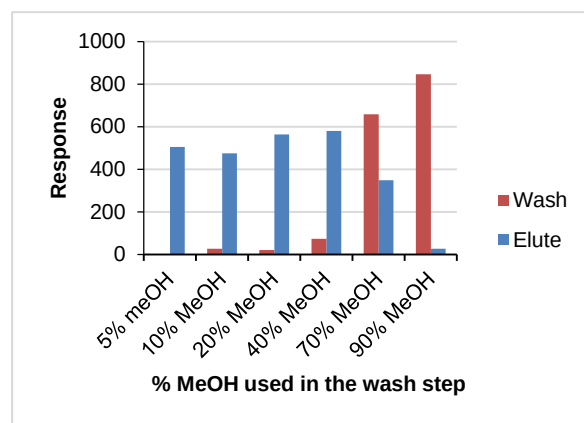
a) 16 α -Hydroxyestrone



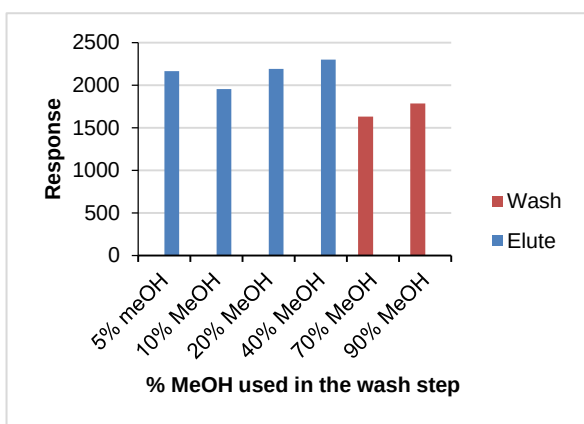
b) 16-Epiestriol



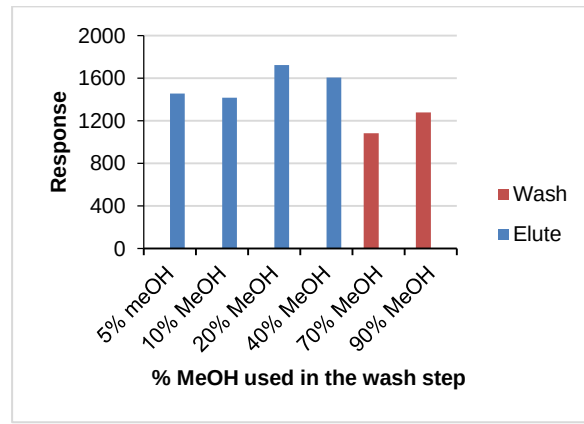
c) 16-Ketoestradiol



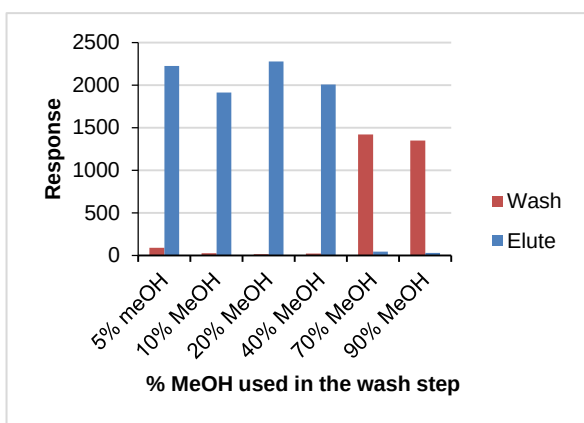
d) 17-Epiestriol



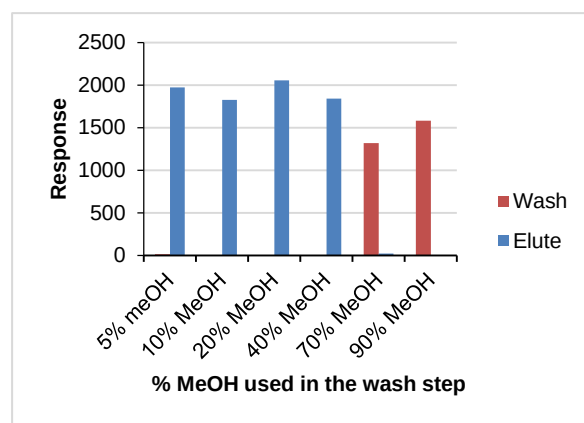
e) 17 α -Estradiol



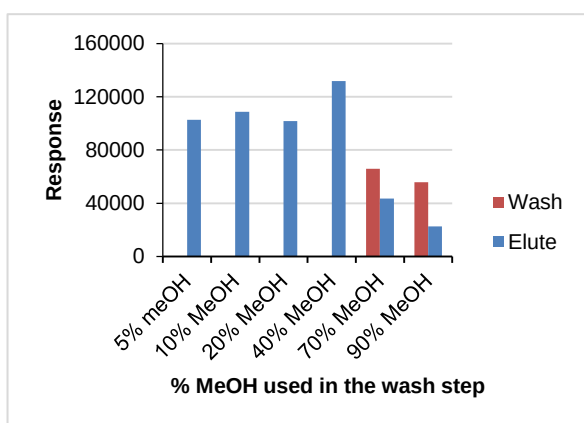
f) 17 β -Estradiol



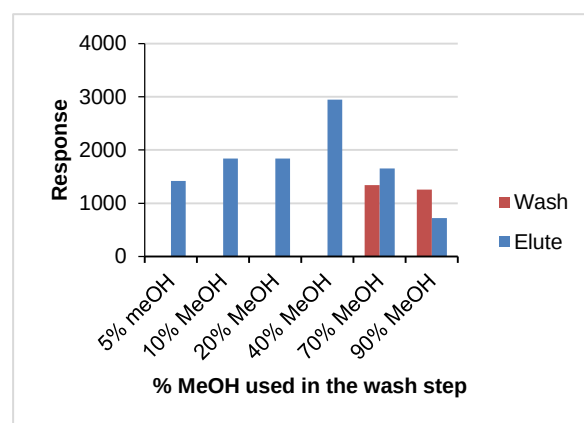
g) 2-Methoxyestrone



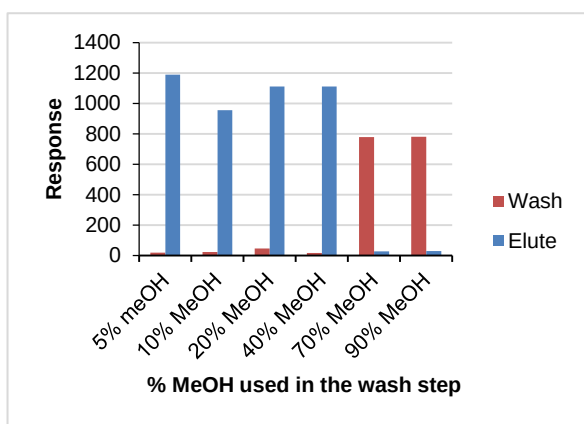
h) 2-Methoxyestradiol



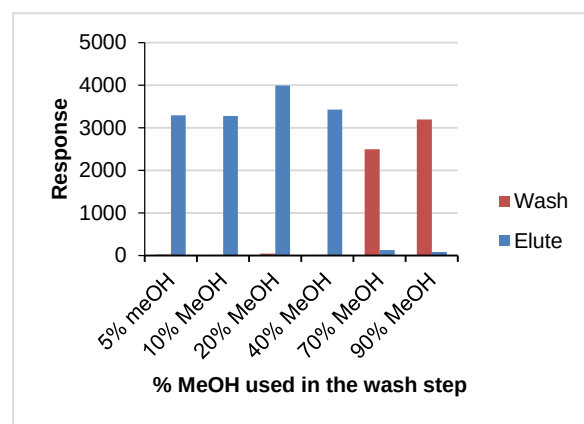
i) 2-Hydroxyestrone



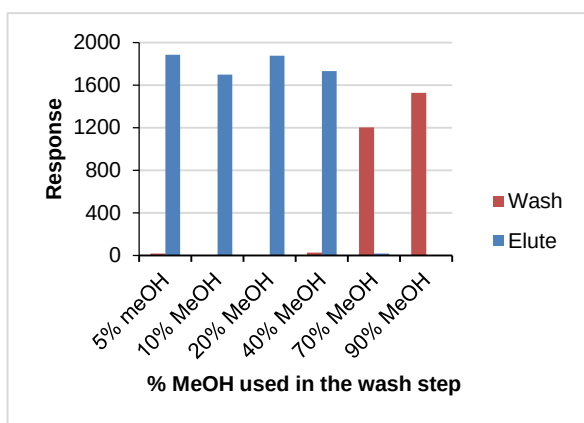
j) 2-Hydroxyestradiol



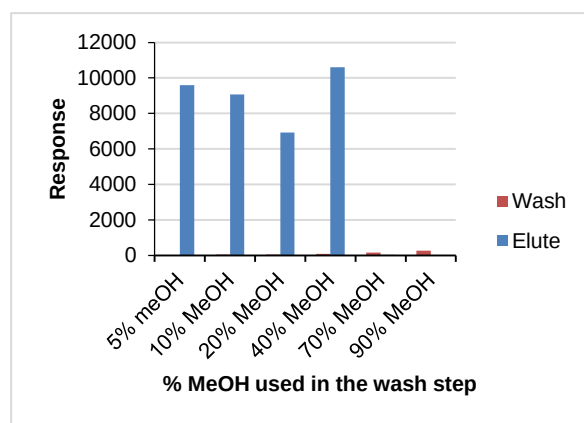
k) 2-Hydroxyestrone-3-Methyl Ether



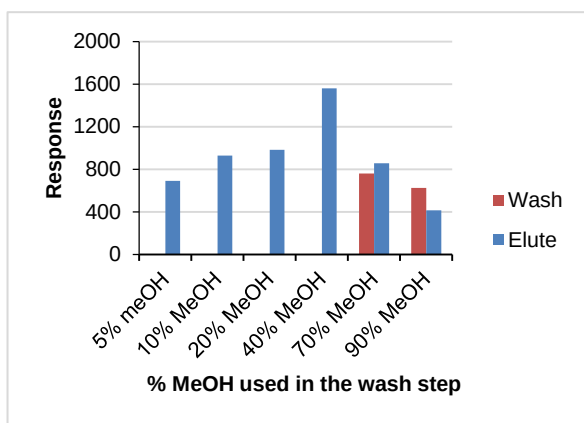
l) 4-Methoxyestrone



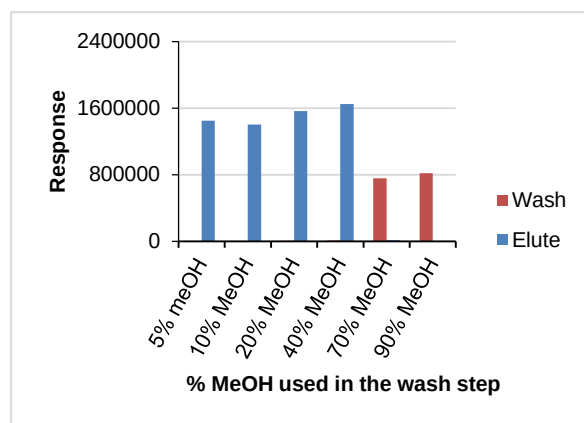
m) 4-Methoxyestradiol



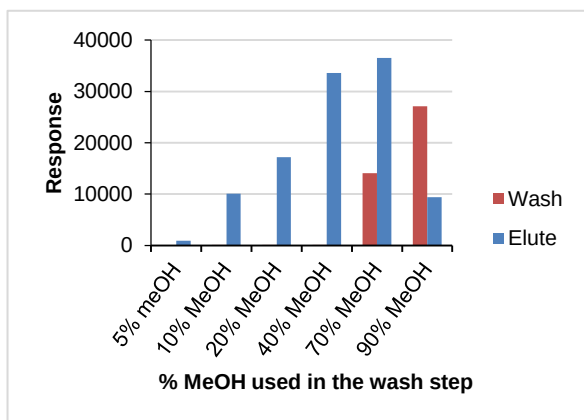
n) 4-Hydroxyestrone



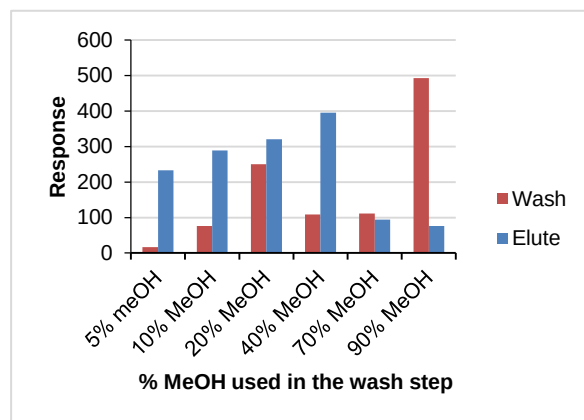
o) 4-Hydroxyestradiol



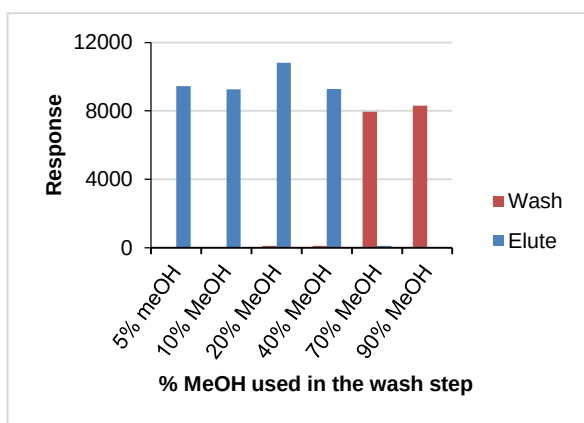
p) Androstenedione



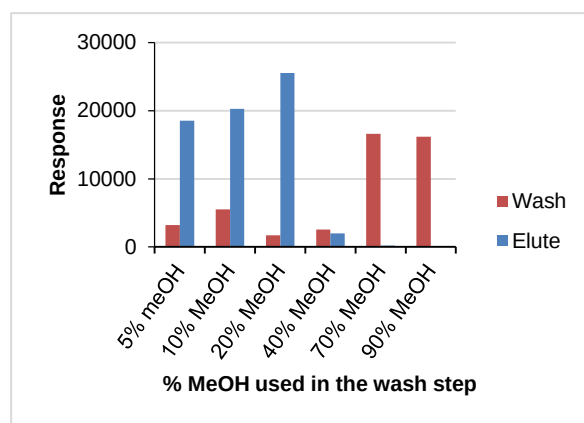
q) DHEA



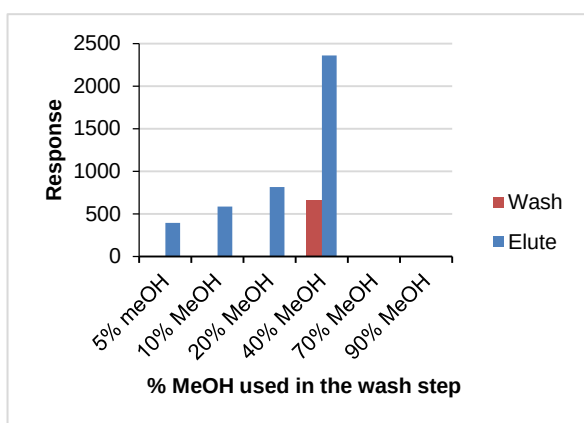
r) DHEAS



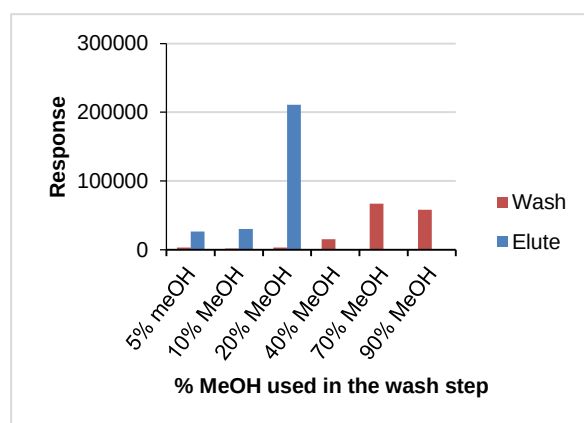
s) Estrone



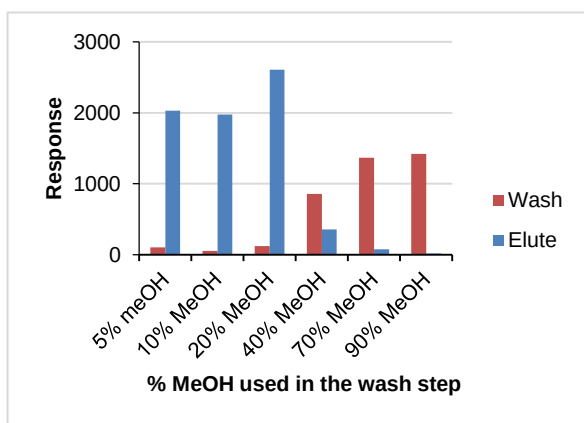
t) Estrone-3-Glucuronide



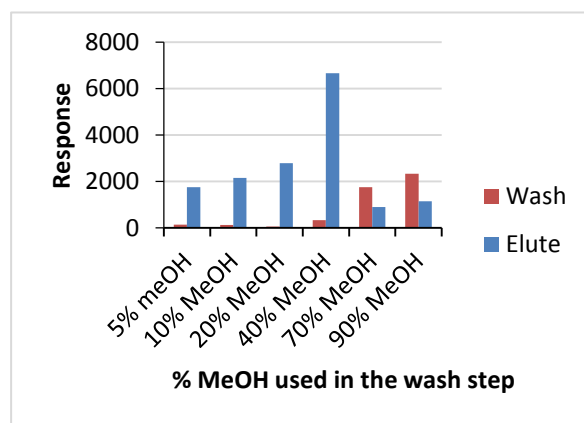
u) Estrone-3-Sulfate



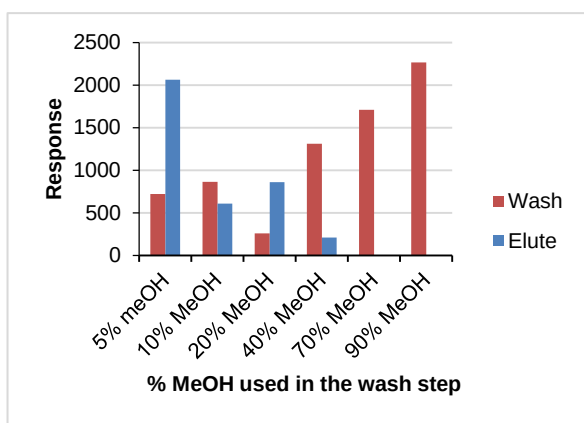
v) Estradiol-17-Sulphate



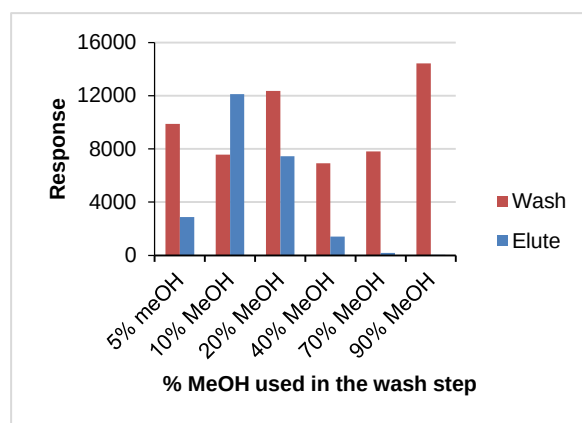
w) Estradiol-3-Glucuronide



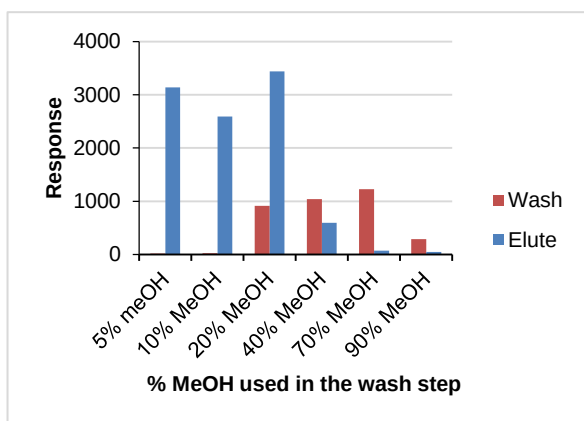
x) Estradiol-3-Sulphate



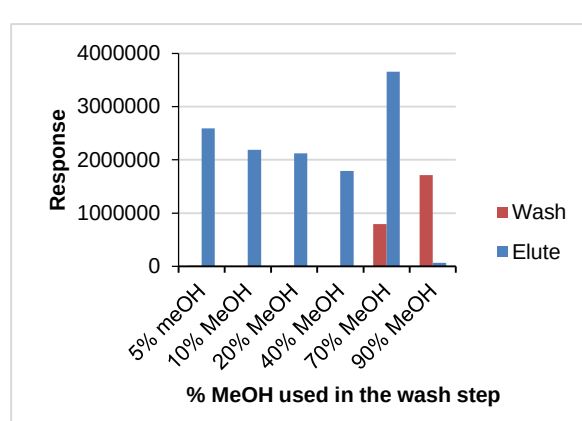
y) Estriol



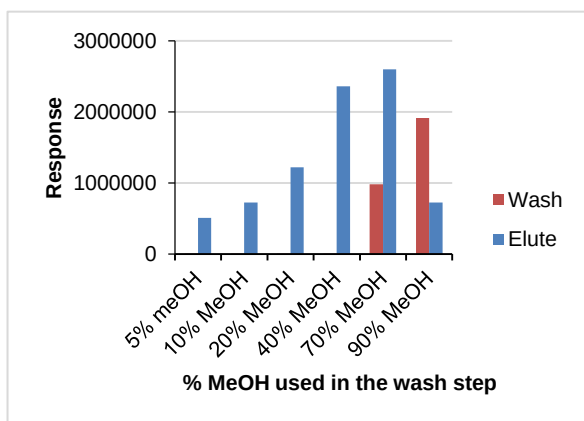
z) Estriol-16-Glucuronide



aa) Estriol-3-Sulphate



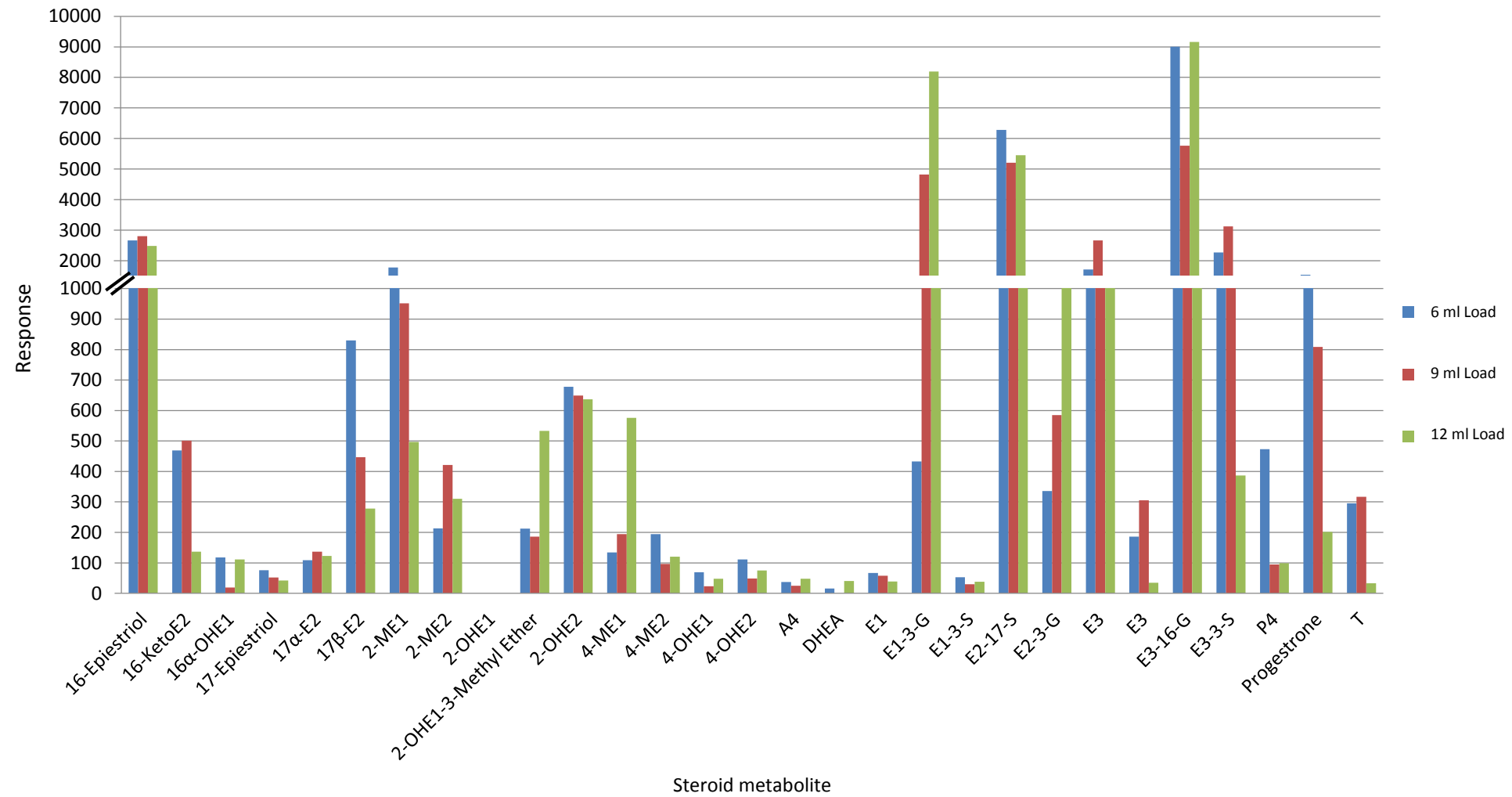
ab) Progesterone



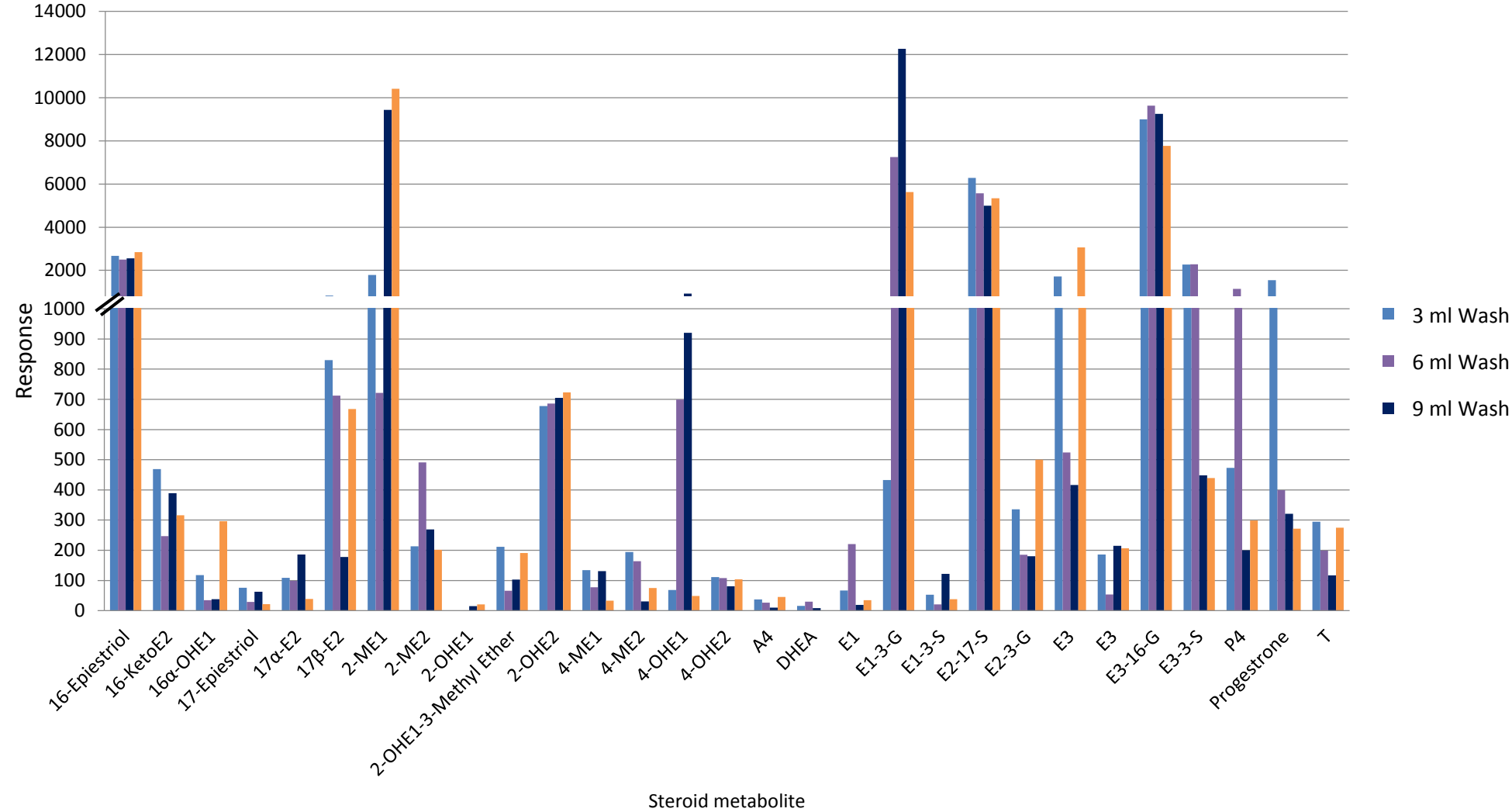
ac) Testosterone

Figure S7 (a-ac): Visual illustrations of the individual steroid hormone metabolite relative responses on the mass spectrometer of both the wash and elution steps after washing with different concentrations of methanol. These variations include 5%, 10%, 40%, 70% and 90% MeOH in the washing step, which was then analysed and in the elution steps following the washing. Names of the steroid metabolites are indicated directly below its corresponding figure.

a)



b)



c)

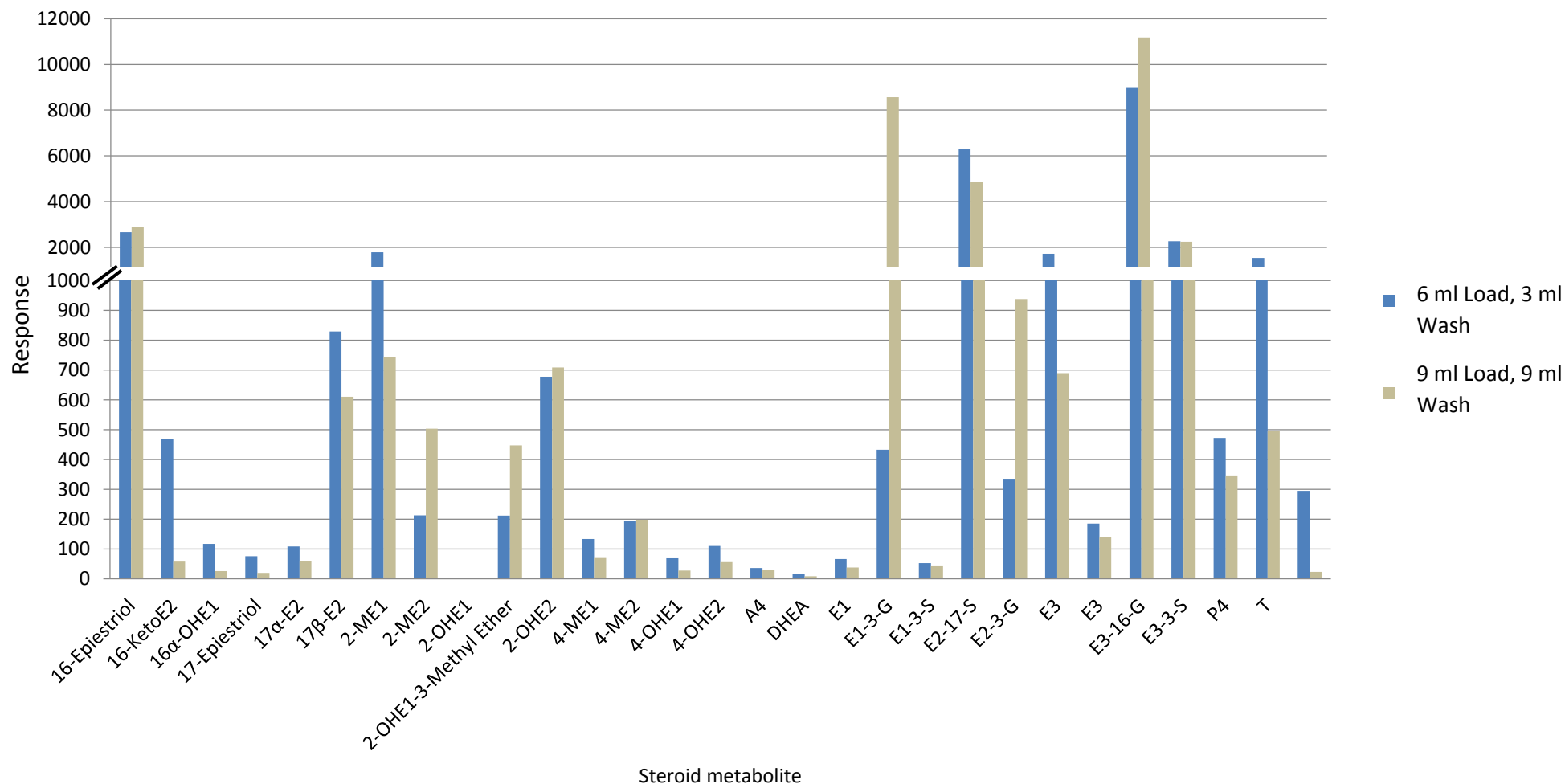


Figure S8: A figure illustrating the LC-MS/MS response for each steroid metabolite with different loading, washing and loading, and washing volumes.

In Figure a) 6 ml, 9 ml and 12 ml loading volumes of sample diluted with water were compared, with little differences although lower load volumes show a tendency for some of the metabolites to be more ideal and decreases the time of SPE. In Figure b), different wash volumes of 3 ml, 6 ml, 9 ml and 12 ml, used as part of the washing step and the responses, was compared. For most of the metabolites, either the highest or a lowest volume for washing gave the best results. In Figure, c) the response of a low loading and washing volume compared to those of a high loading and washing volume. For the hydroxysterogens and positive ionising metabolites, lower volumes worked best and for some conjugates and methoxys, the higher volumes were more ideal. For all other metabolites, the responses were very much the same. // Gap in y-axis for better visual illustration.

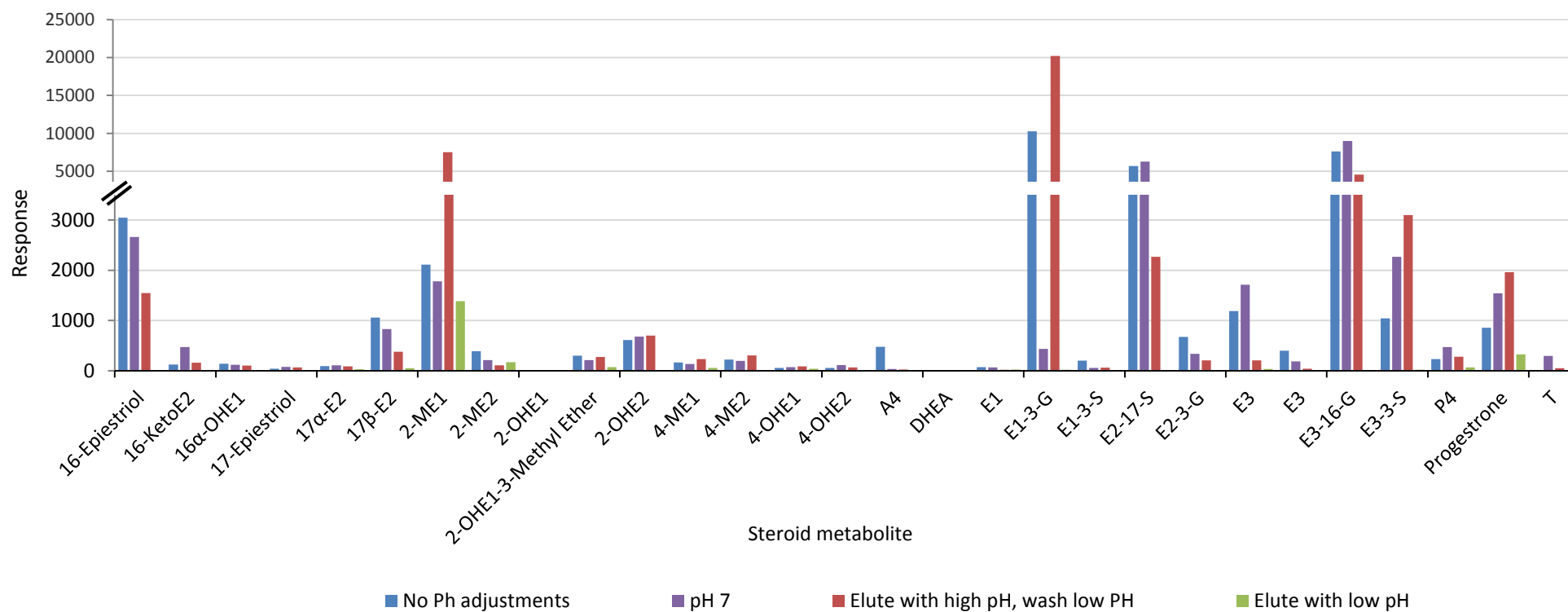
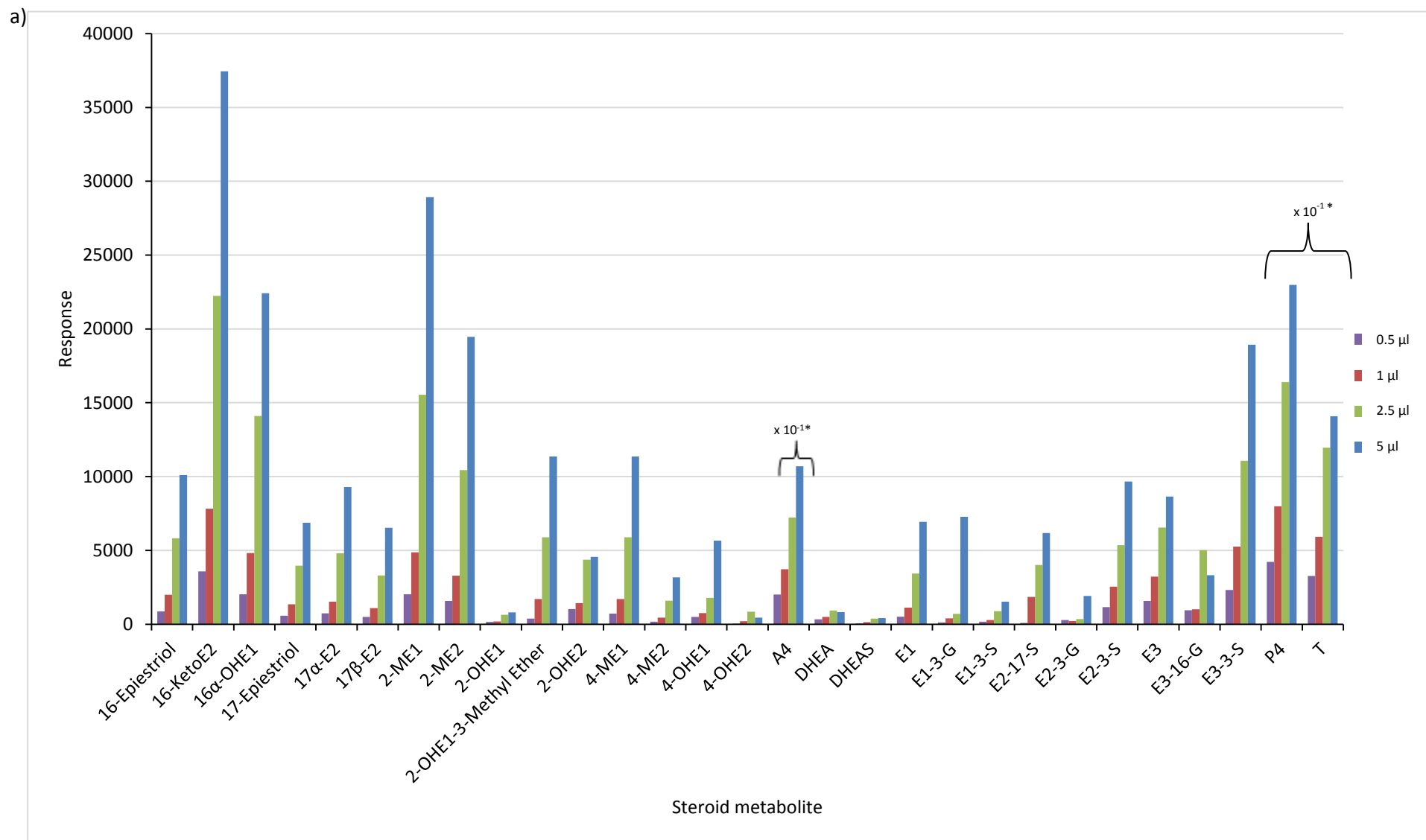


Figure S9: Summarised visual results of the effect of varying pH changes on the responses for each of the 29 estrogen metabolites. No pH adjustments (normal water and methanol) are indicated in blue, while setting the pH to 7 and using a buffer (ammonium formate) is indicated in purple. Red bars indicate a more acetic wash and alkaline elution, while green indicates elution with a lower pH (added acetic acid). // Gap in y-axis for better visual illustration.



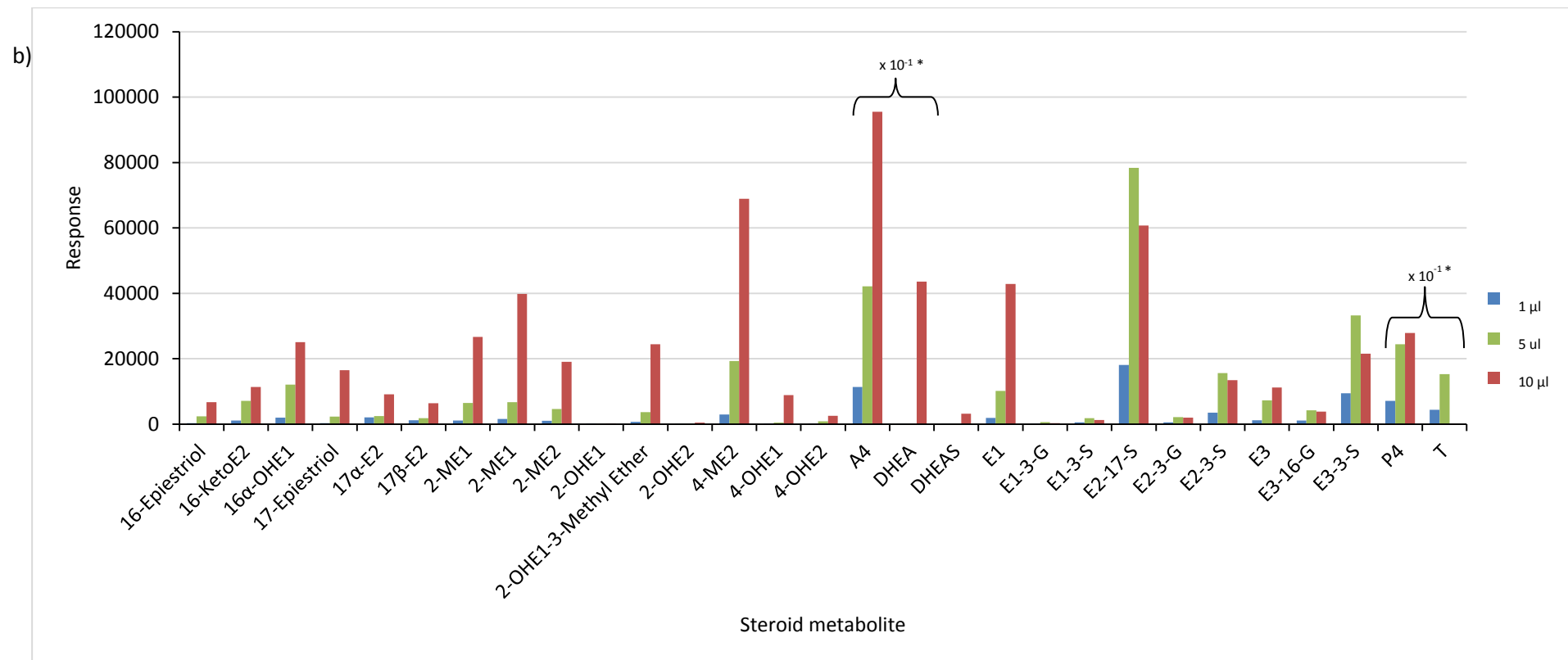


Figure S10 (a&b): Visual illustrations of the effect of different injection volumes from the sample in the autosampler. The relative responses on the mass spectrometer of each estrogen metabolite is shown. a) Injection volumes of 0.5 µl, 1 µl, 2.5 µl and 5 µl are shown, while b) compares injection volumes of 1 µl with 5 µl and 10 µl.

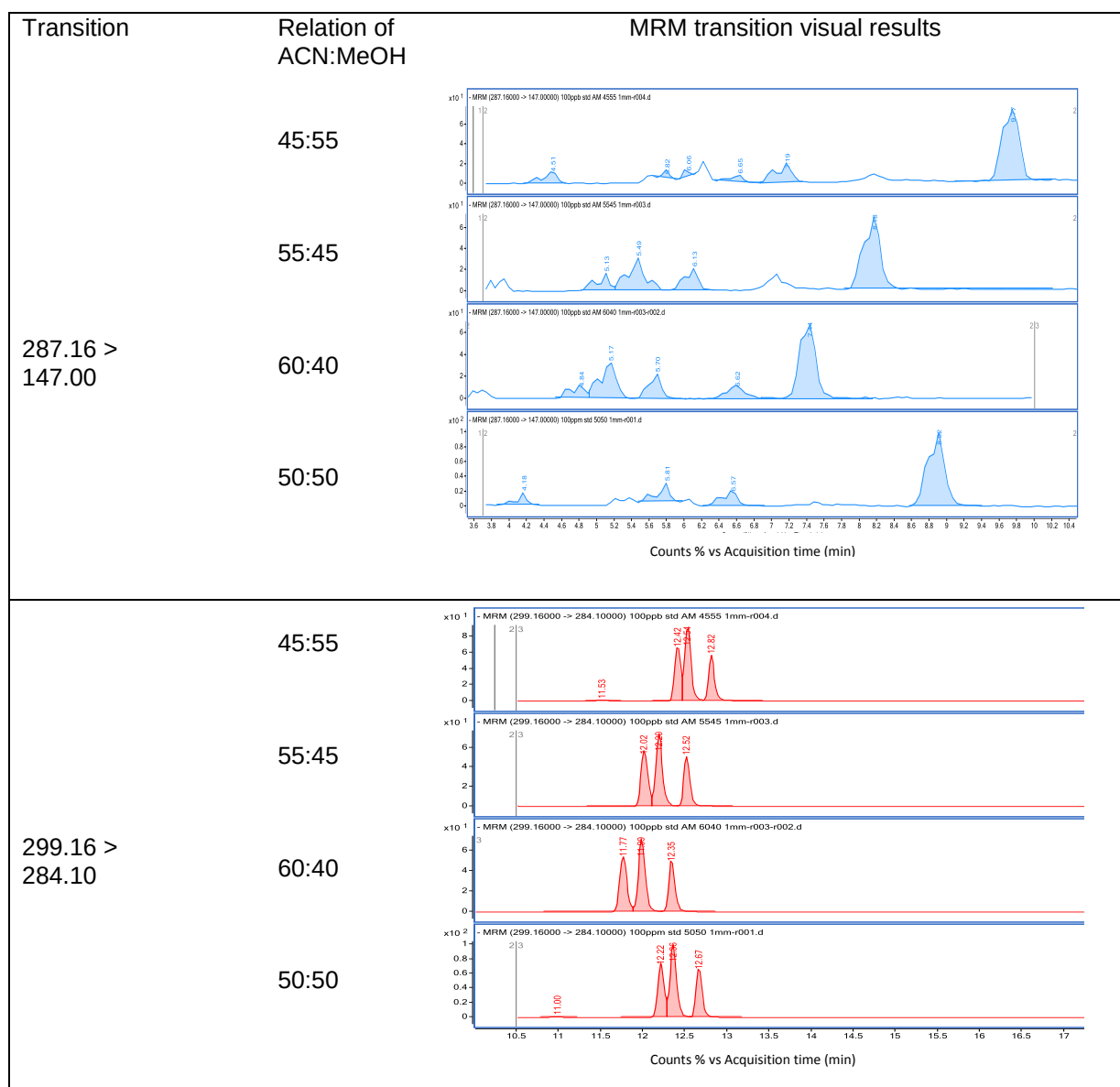


Figure S11: Visual representation of the effect of varying ratios of Acetonitrile: Methanol in mobile phase B on the chromatographic separations. The chromatographic transitions of 287>147 are indicated in blue and transition 299.16>284.10 are illustrated in red. The ratios of Acetonitrile:Methanol evaluated, included 45:55, 50:50, 55:45 and 60:40.

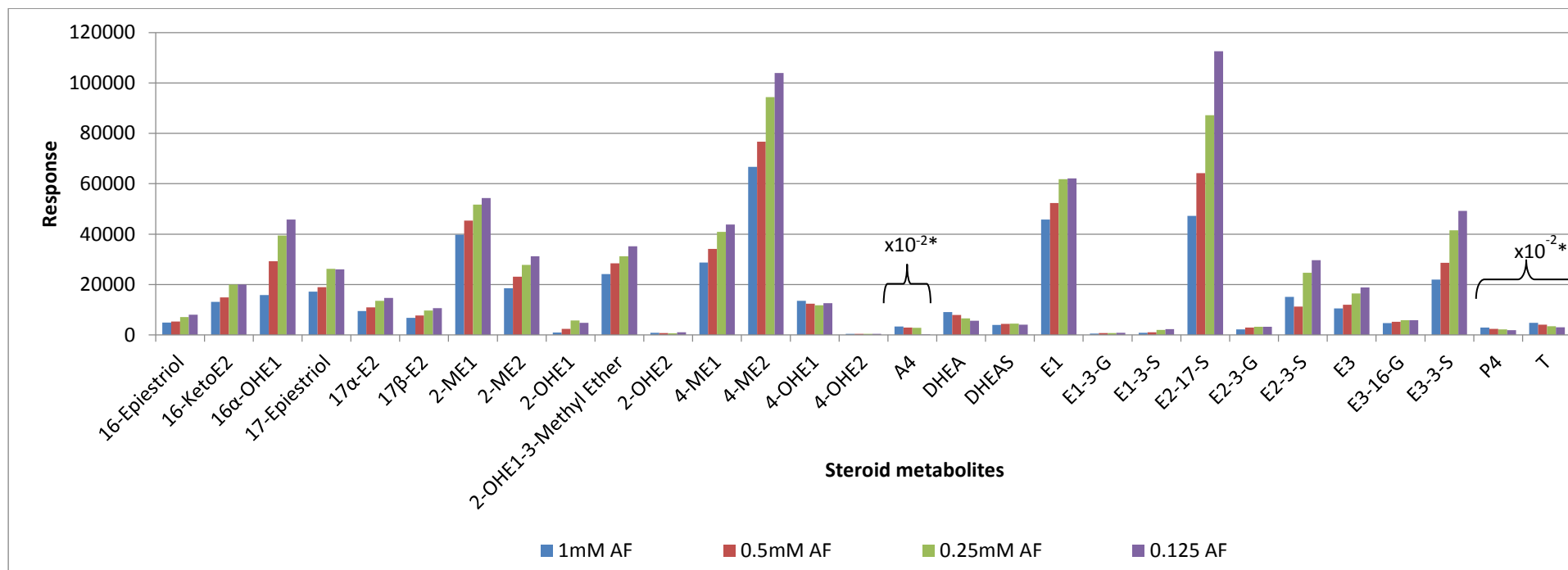
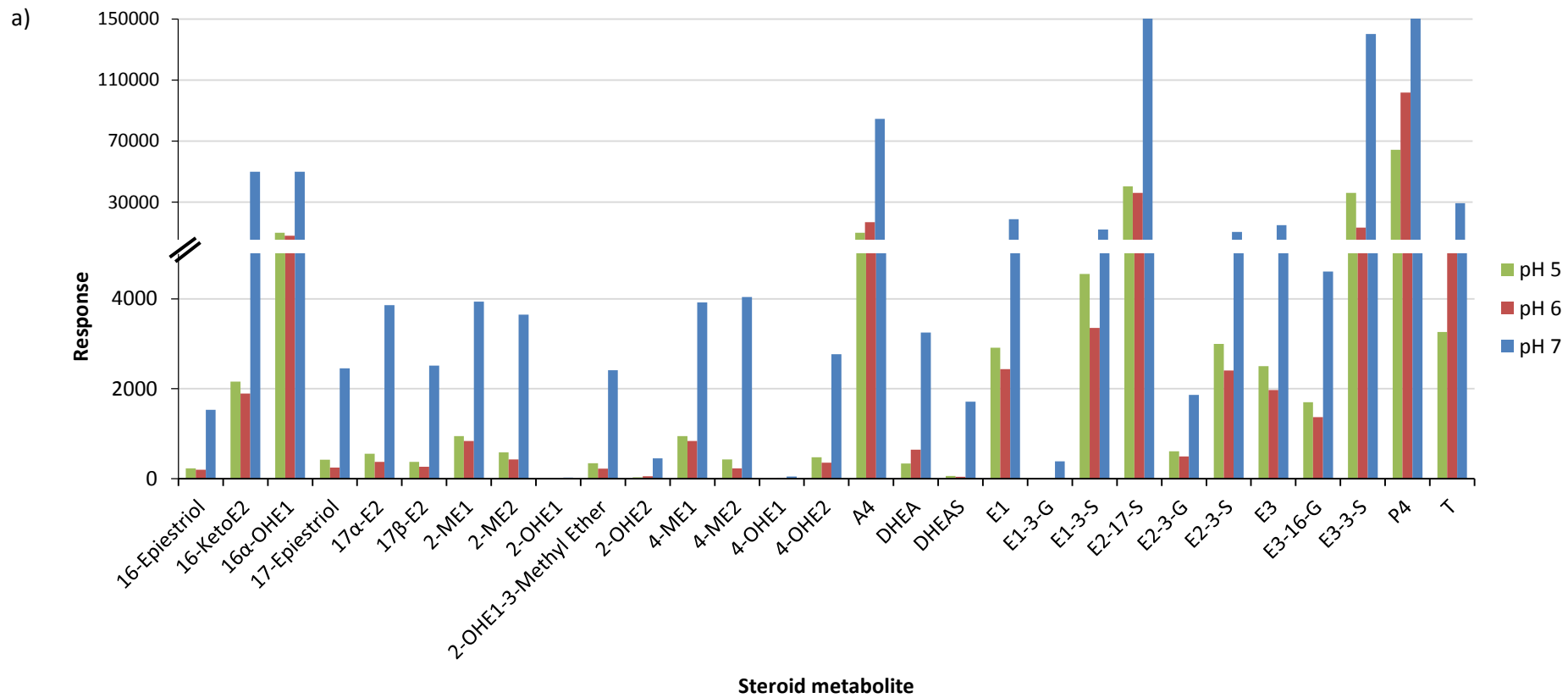


Figure S12: A schematic representation of processed results to show the effect of different concentrations of the ammonium fluoride modifier in the mobile phase A. Blue represents 1 mM ammonium fluoride, red 0.5 mM ammonium fluoride, green 0.25 mM ammonium fluoride and purple 0.125 mM ammonium fluoride. These data all represent the response obtained from each steroid hormone metabolite. * These metabolite response values were adjusted by $\times 10^{-2}$ to allow visual illustration thereof on the same y-axis scale. For some of the metabolites (2-OHE2, 4OHE2, E1-3-G, E1-3-S) the response was extremely low or zero, making the visual illustration thereof not possible on the same y-axis.



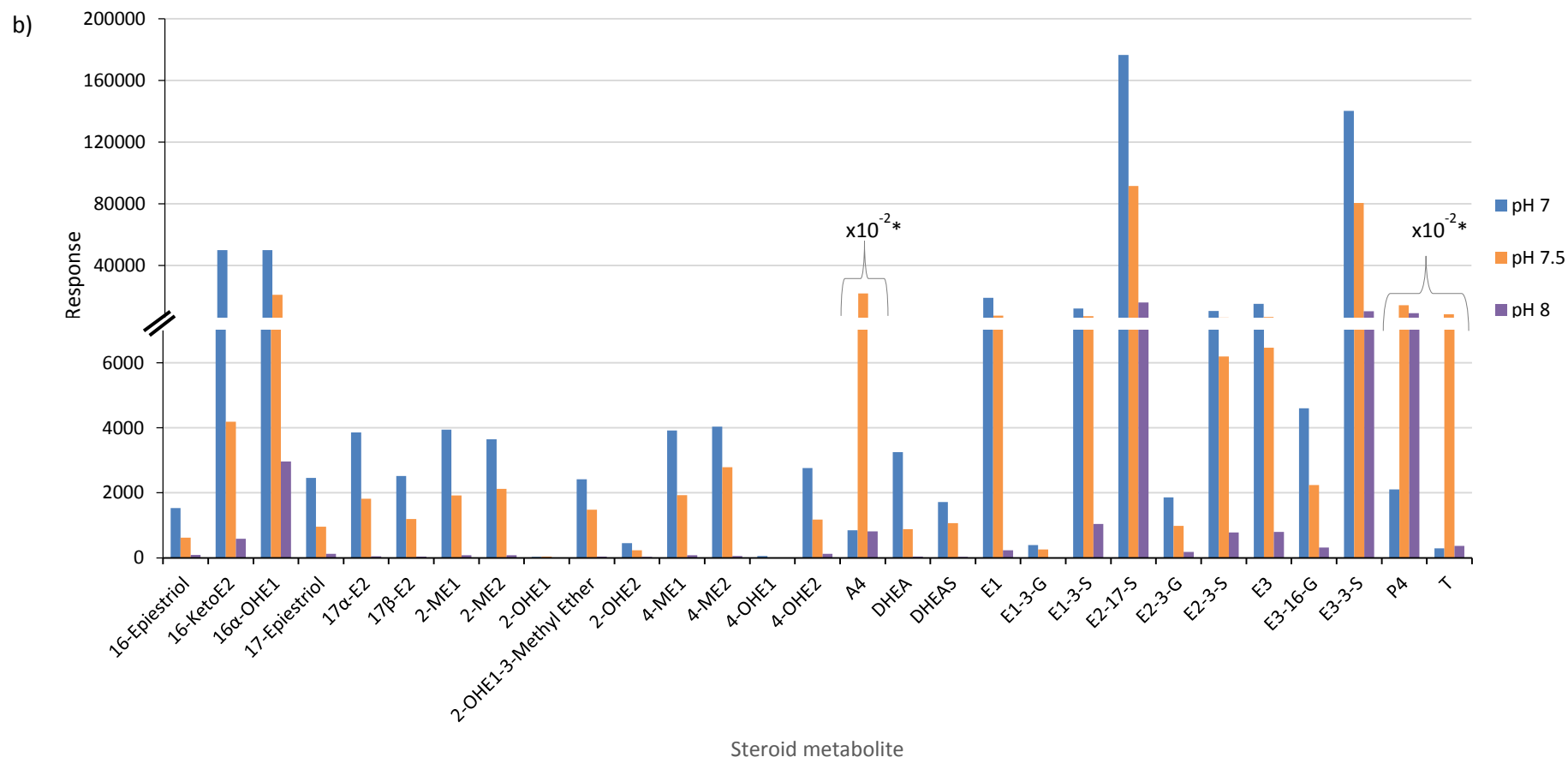


Figure S13 (a-b): Visual illustrations of the effect that the mobile phase pH has on the relative responses on the mass spectrometer of the steroid metabolites. a) pH of the normal working mobile phase (pH7) was reduced to pH5 and pH6 with HCl, and all the responses were compared. A pH value of 7 gave the best results for most compounds. b) The pH values of the normal mobile phase (pH7) were increased to 7.5 and 8 with NH₃, and responses were compared. A pH value of 7 still gave the best results for most compounds. * These metabolite response values were adjusted by $\times 10^{-2}$ to allow visual illustration thereof on the same y-axis scale. // Gap in y-axis for better visual illustration.

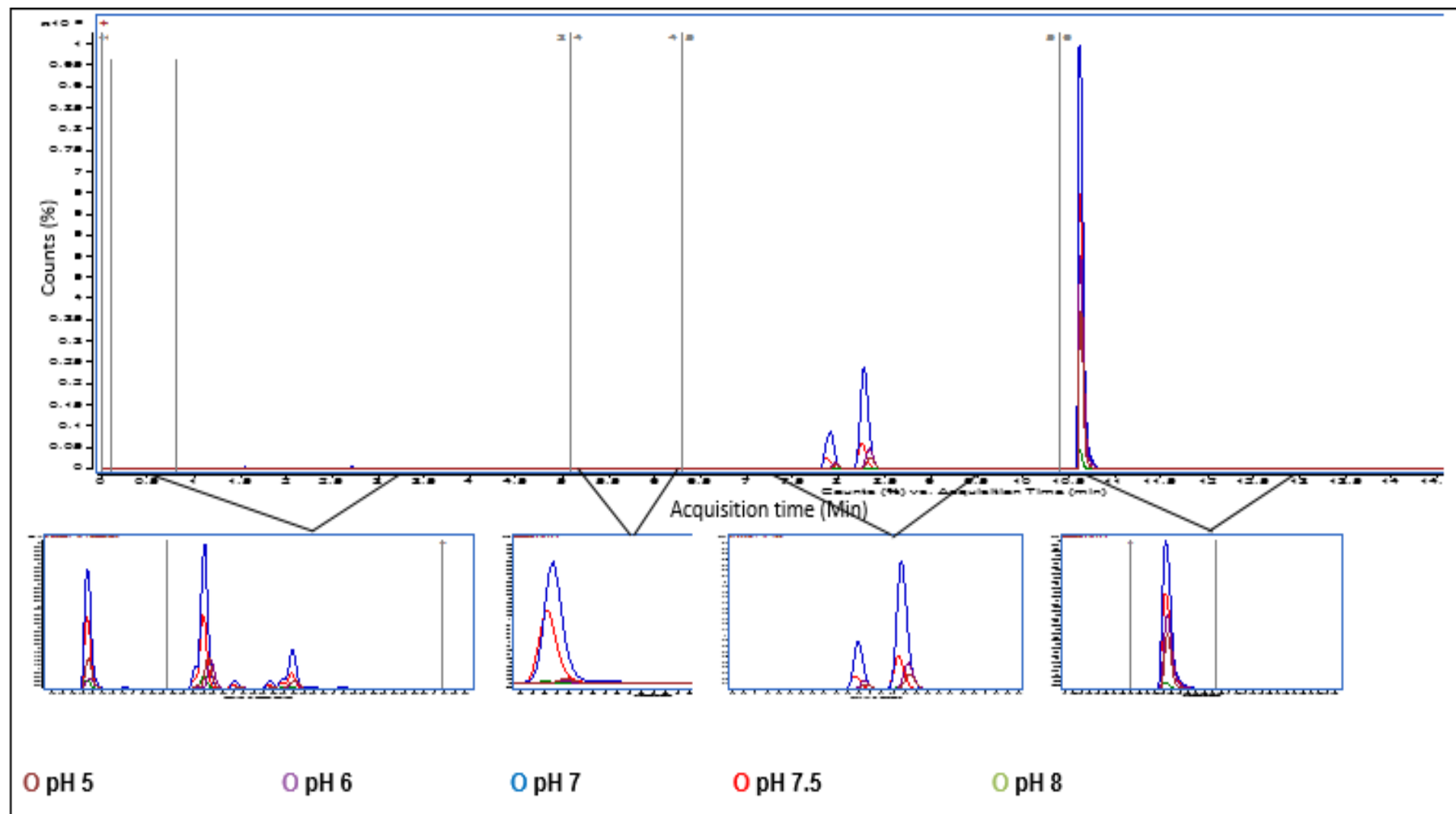


Figure S14: The effect of pH on mobile phase A, on the chromatographic peak abundances and peak shape. pH 5 is visually illustrated with magenta, pH 6 with purple, pH 7 with blue, red illustrated the peaks at pH 7.5 and green at pH 8.

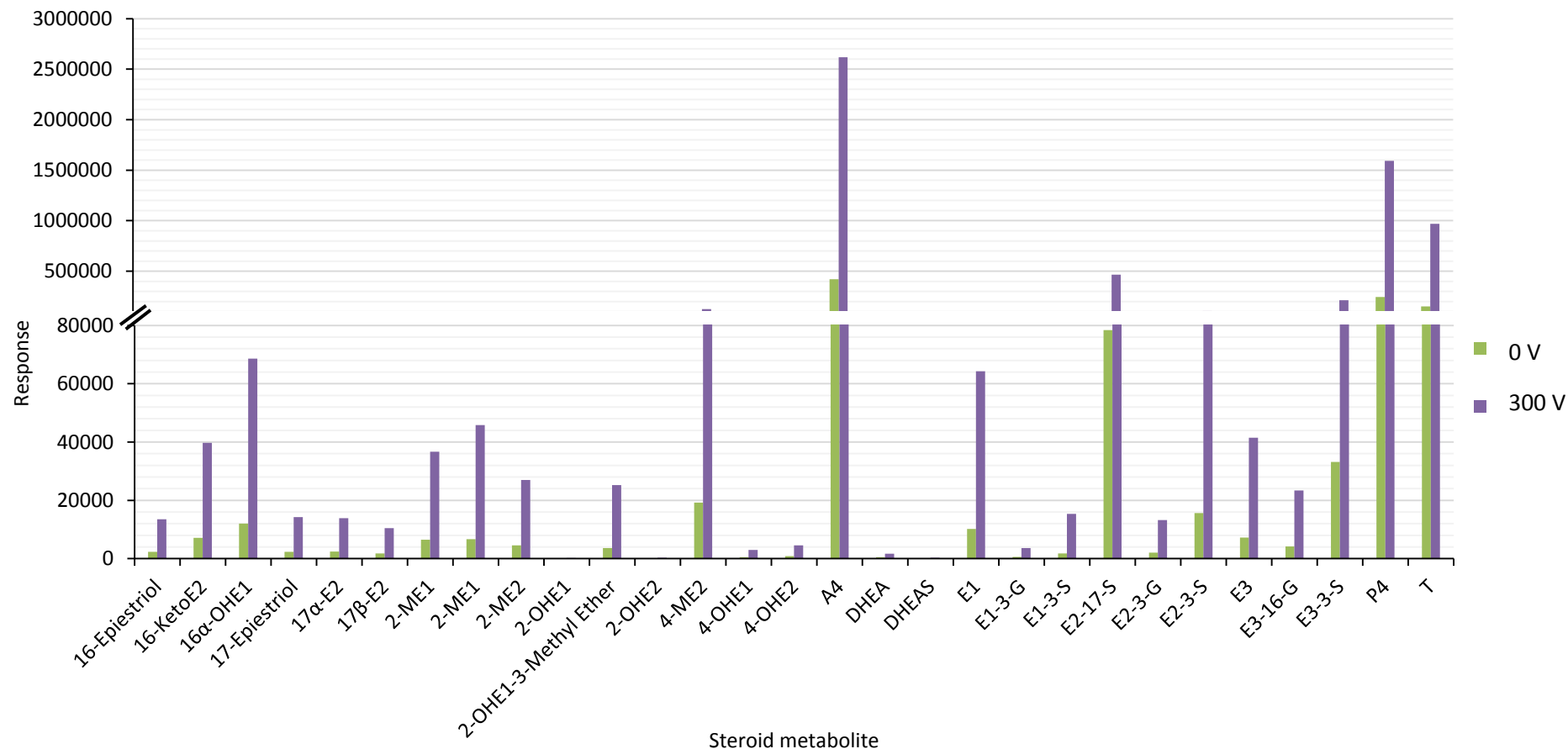


Figure S15: Visual comparison of the relative responses on the mass spectrometer for each metabolite when different EMV voltages were set in different chromatographic runs. For both runs, the exact same steroid metabolite standard was injected and all other parameters were kept identical. Green represents a 0V EMV and purple a 300 V EMV. The compared EMV voltages, indicates the default without any enhancement (0 V) and increased voltage inside the allowed range (300 V), without decreasing the multiplier lifespan as the highest voltage (500 V) would// Gap in y-axis for better visual illustration.

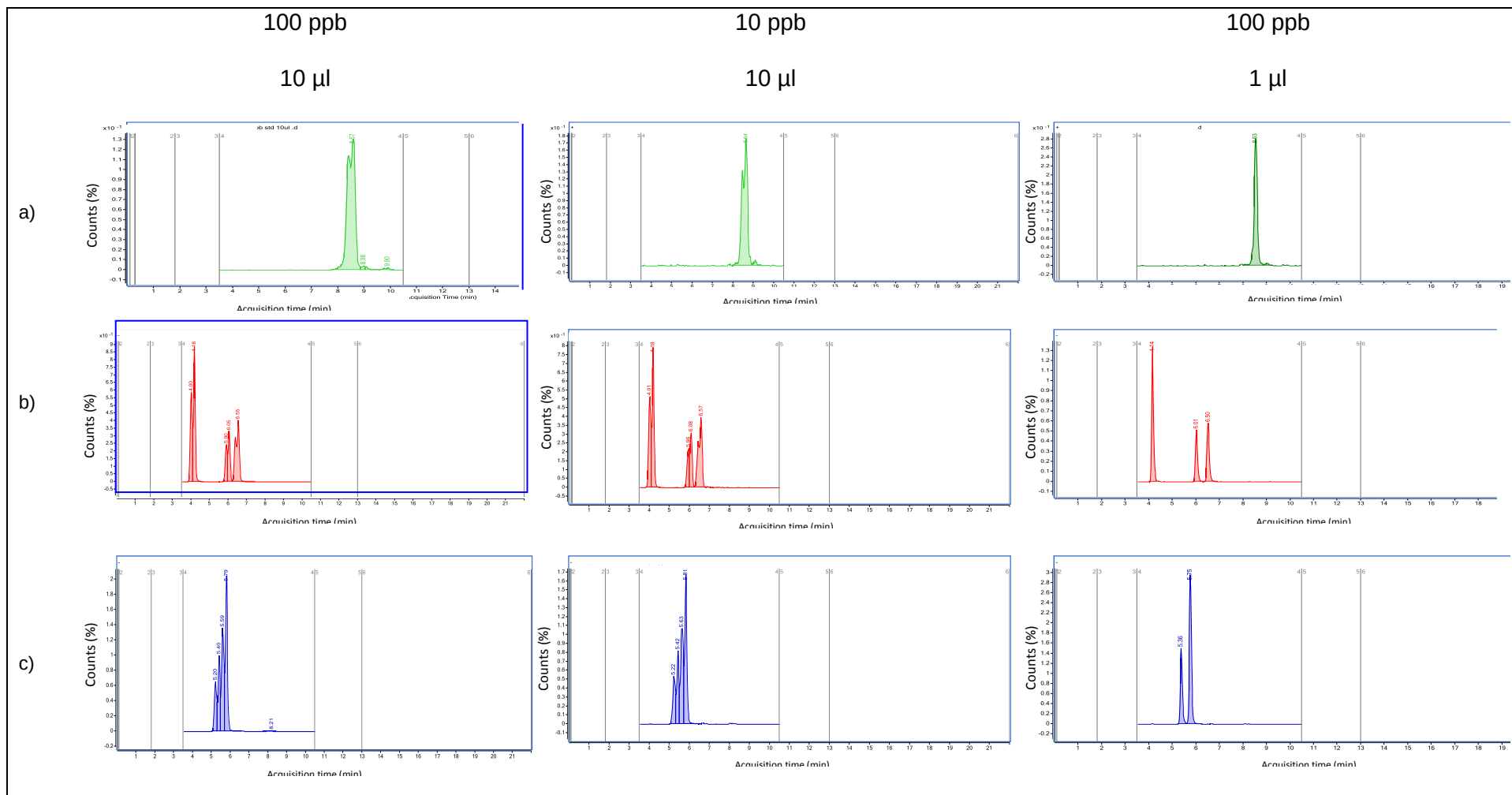


Table S2: The cell accelerator voltage (CAV) that gave the most abundant peaks and highest response when compared to other CAVs. As part of the optimisation, the CAV values of only the very weak ionising metabolites were investigated. The CAV voltages investigated included 1 V, 3 V and 7 V.

Metabolite	Optimal Cell Accelerator Voltage (V)
DHEAS	7
4-Hydroxyestadiol	3
4-Hydroxyestrone	1
2-Hydroxyestradiol	1
2-Hydroxyestrone	1

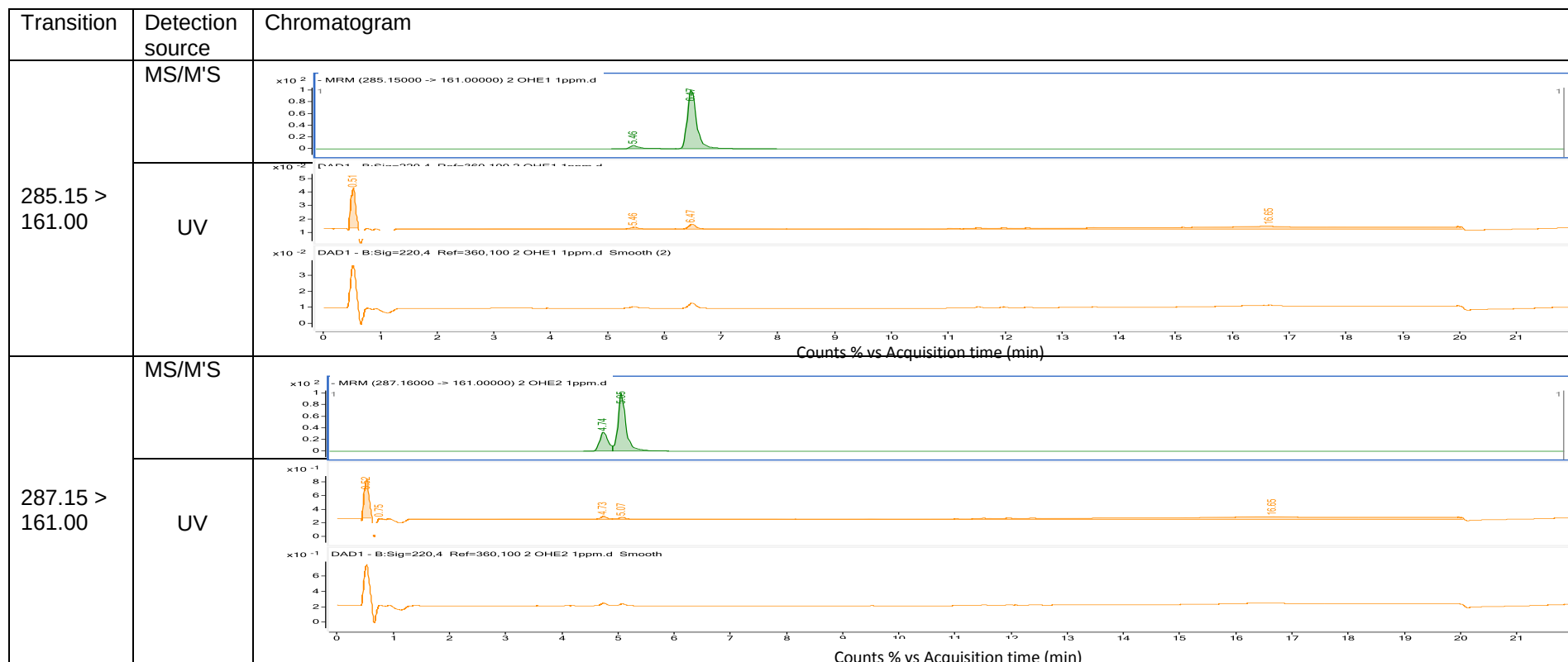


Figure S17: A figure to visually depict the difference in chromatograms of the UV and MS/MS detection sources, where green reflects the MS/MS chromatographs and orange, the UV spectrum. The results for the hydroxysterogens in the form of two transitions (285.15> 161.00 & 287.15> 161.00) are illustrated.

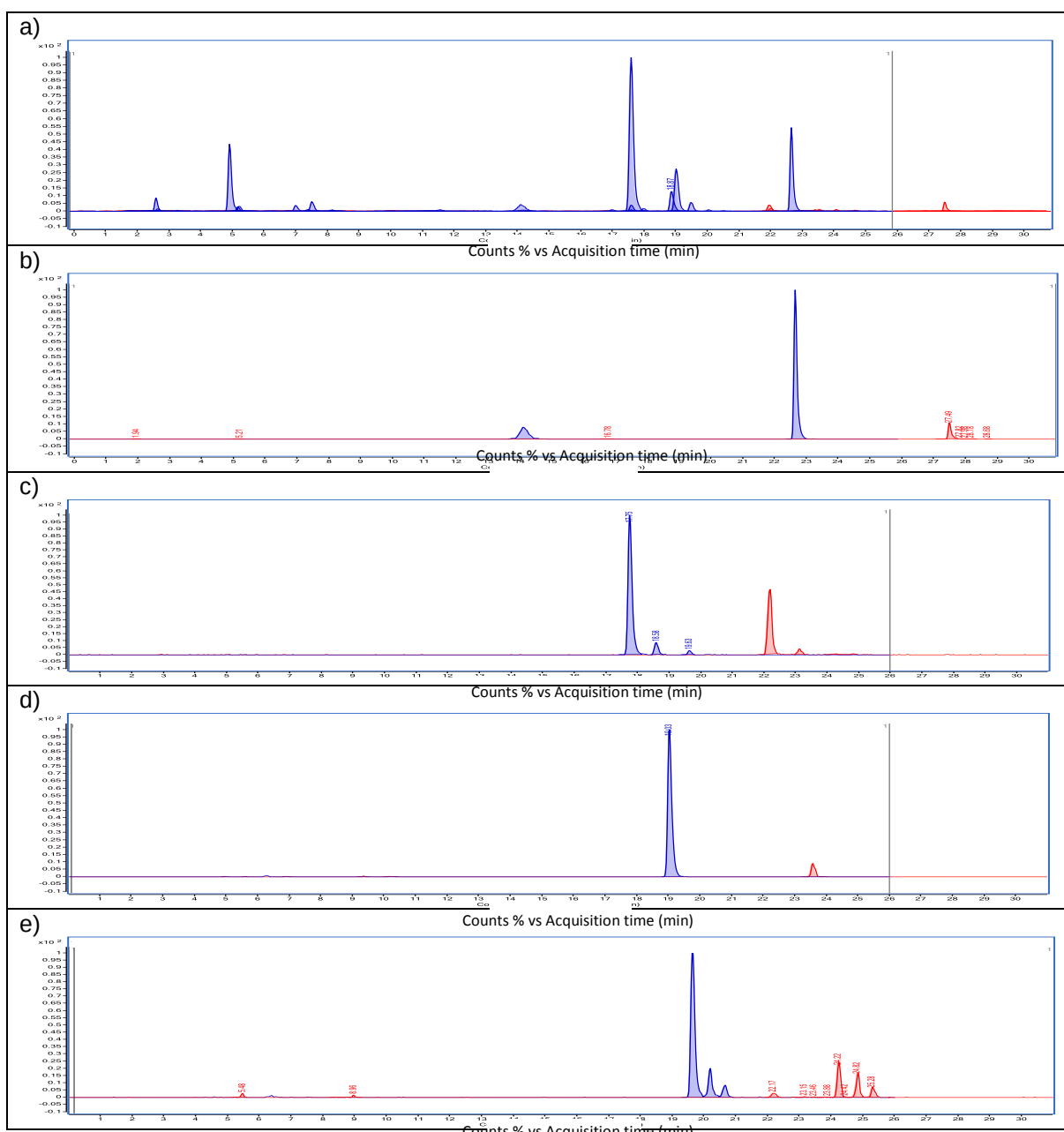


Figure S18: Visual illustrations of the APCI ionisation versus the ESI ionisation methods performed on the same concentration standard mixture. Blue indicates the ESI ionisation chromatograms and red, the APCI ionised metabolite chromatograms. The retention times differ slightly, not due to the change in ionisation but rather a reduced flow rate and thus an adjusted gradient to ensure that the APCI ionisation source will have sufficient time for ionisation. a) TIC, b)progesterone, c) 2 & 4 methoxyestradiol, d) estrone and e) 2&4 methoxyestrone and 2hydroxyestrone-3-methyl-ether.

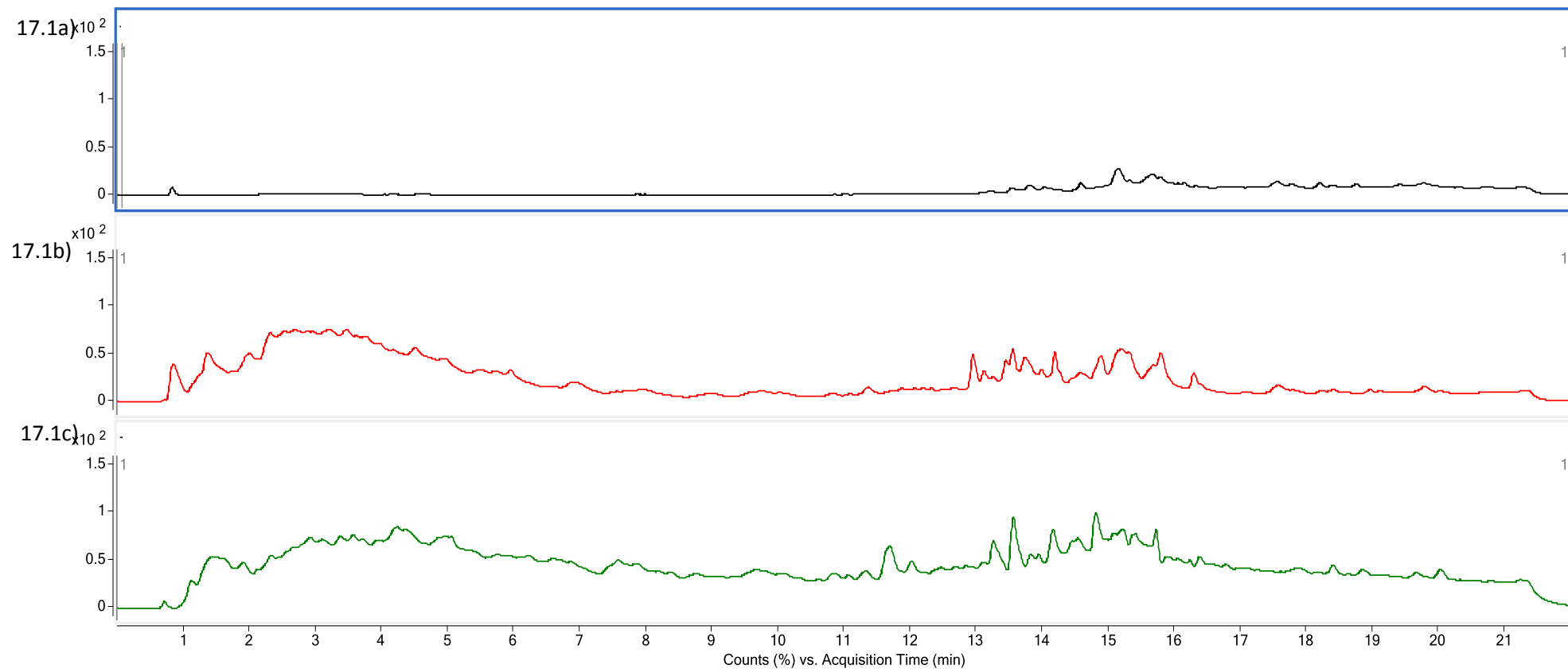


Figure S19.1: Schematic representation of the scan results shown in chromatographic formats to show matrix interferences that might occur. a) The chromatographic results from the LC-MS in the blank scan, b) negative scan and c) positive scan.

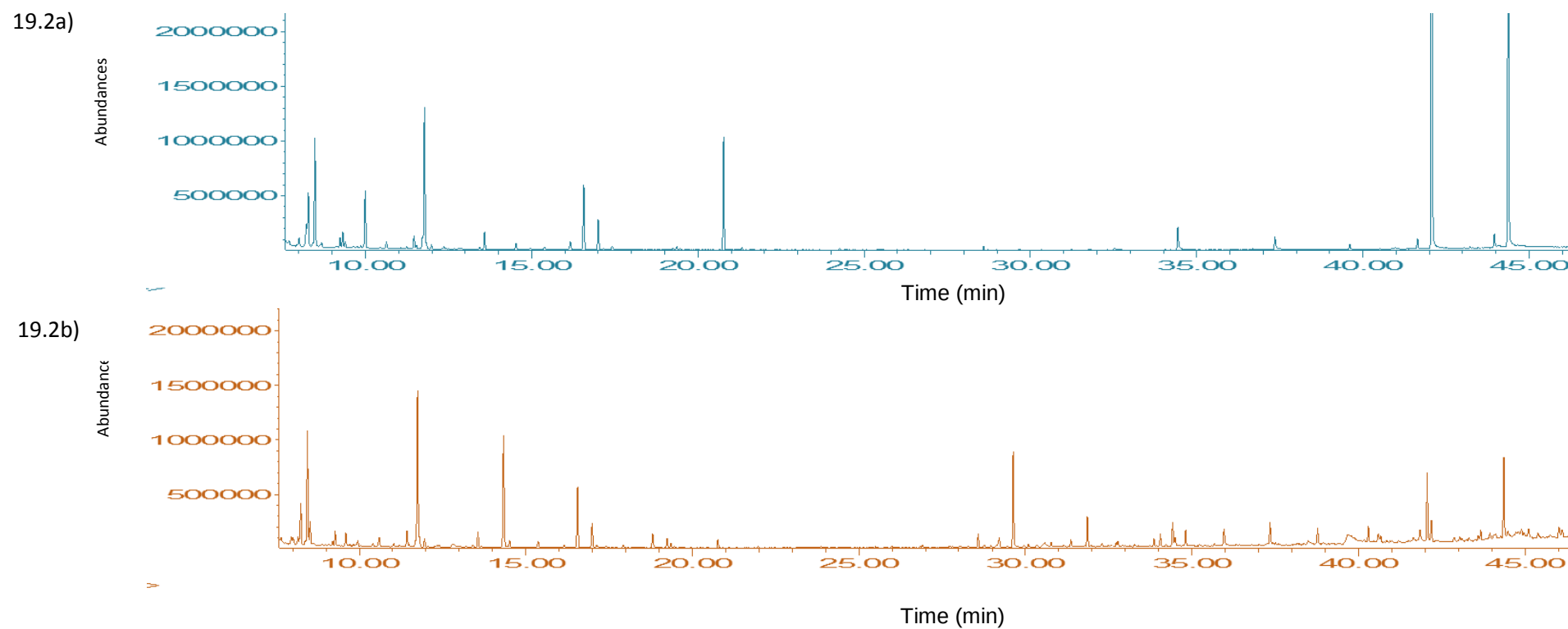


Figure S19.2: Visual illustrations of the GC chromatogram scan results of a) a blank water sample derivatised and evaluated and b) a control human urine sample derivatised after SPE sample preparation.

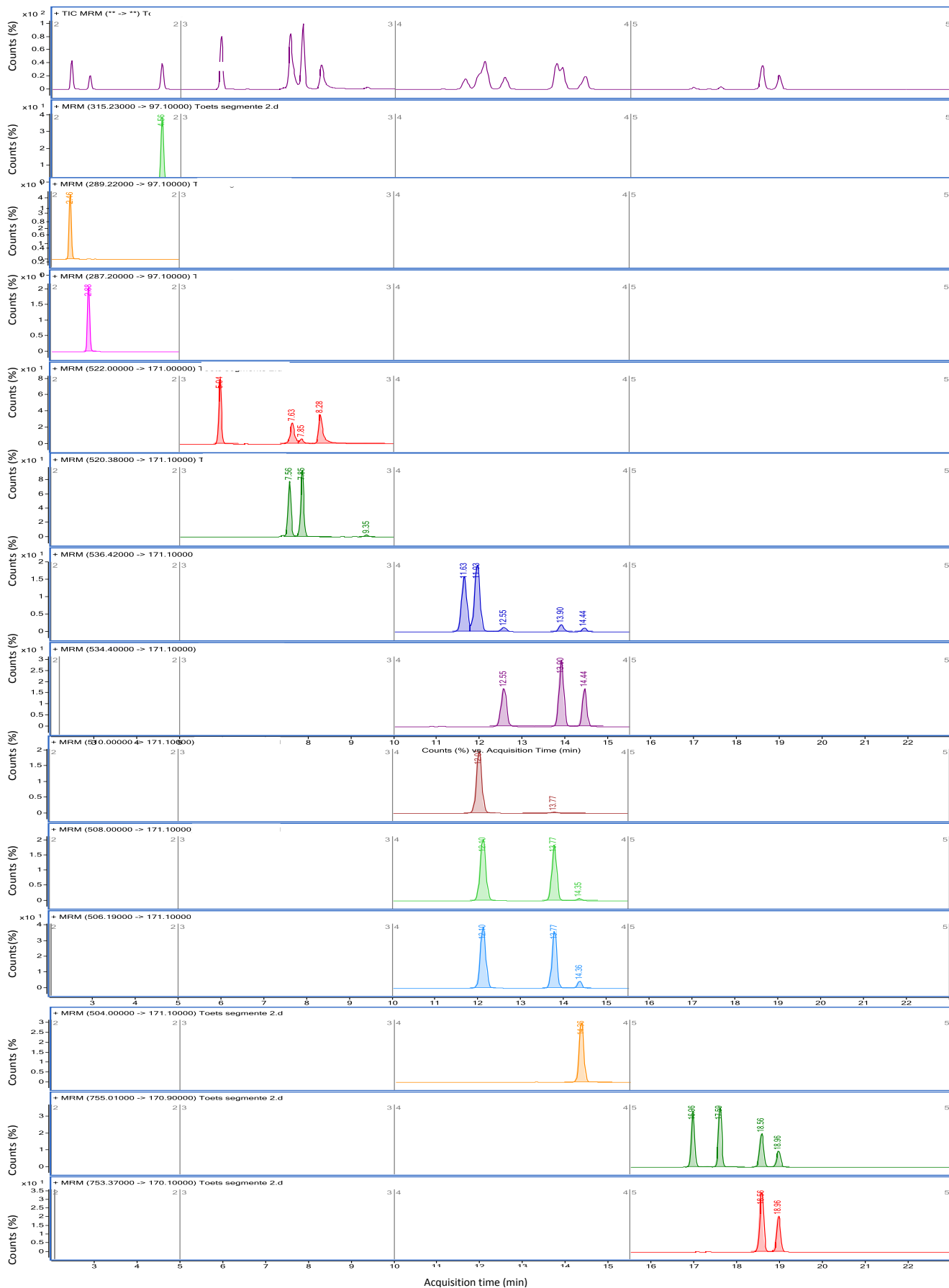


Figure S20: Visual illustration of the chromatographic separation obtained on the Zorbax eclipse C8 column for some of the metabolite transitions. The Transition (in top left corner), separation and retention times (directly above each peak) are indicated as well as time segments 2-5 built into the method in Chapter 5.7.

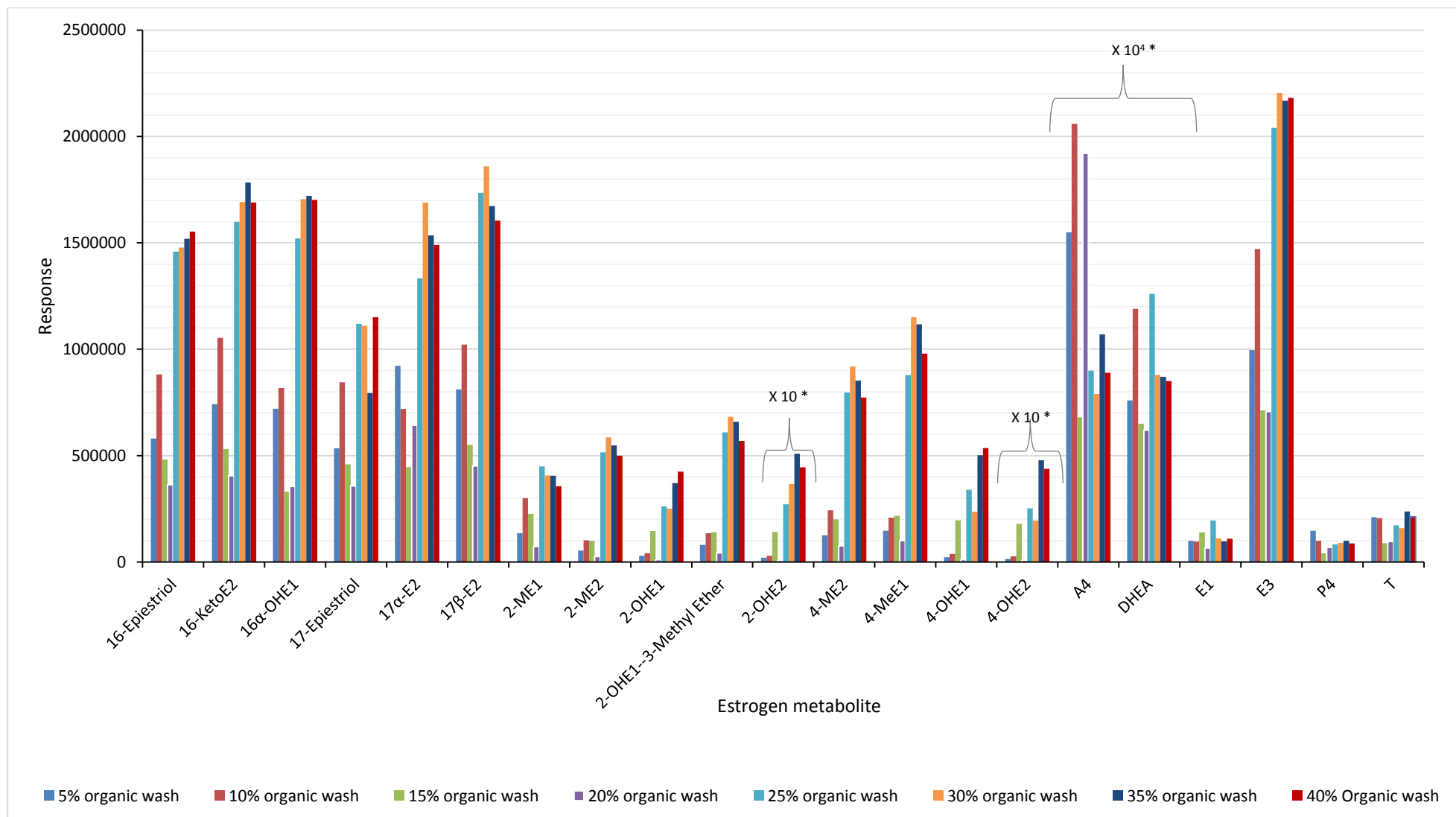


Figure S21: A visual illustration of the relative responses on the mass spectrometer of the non-polar metabolites eluted from SPE, with acetone and methanol. Elution followed washing with 5%, 10%, 15%, 20%, 25%, 30%, 35% and 40% MeOH in the washing solution. * These metabolite response values were adjusted by x10 or x10⁴ to allow visual illustration thereof on the same y-axis scale.

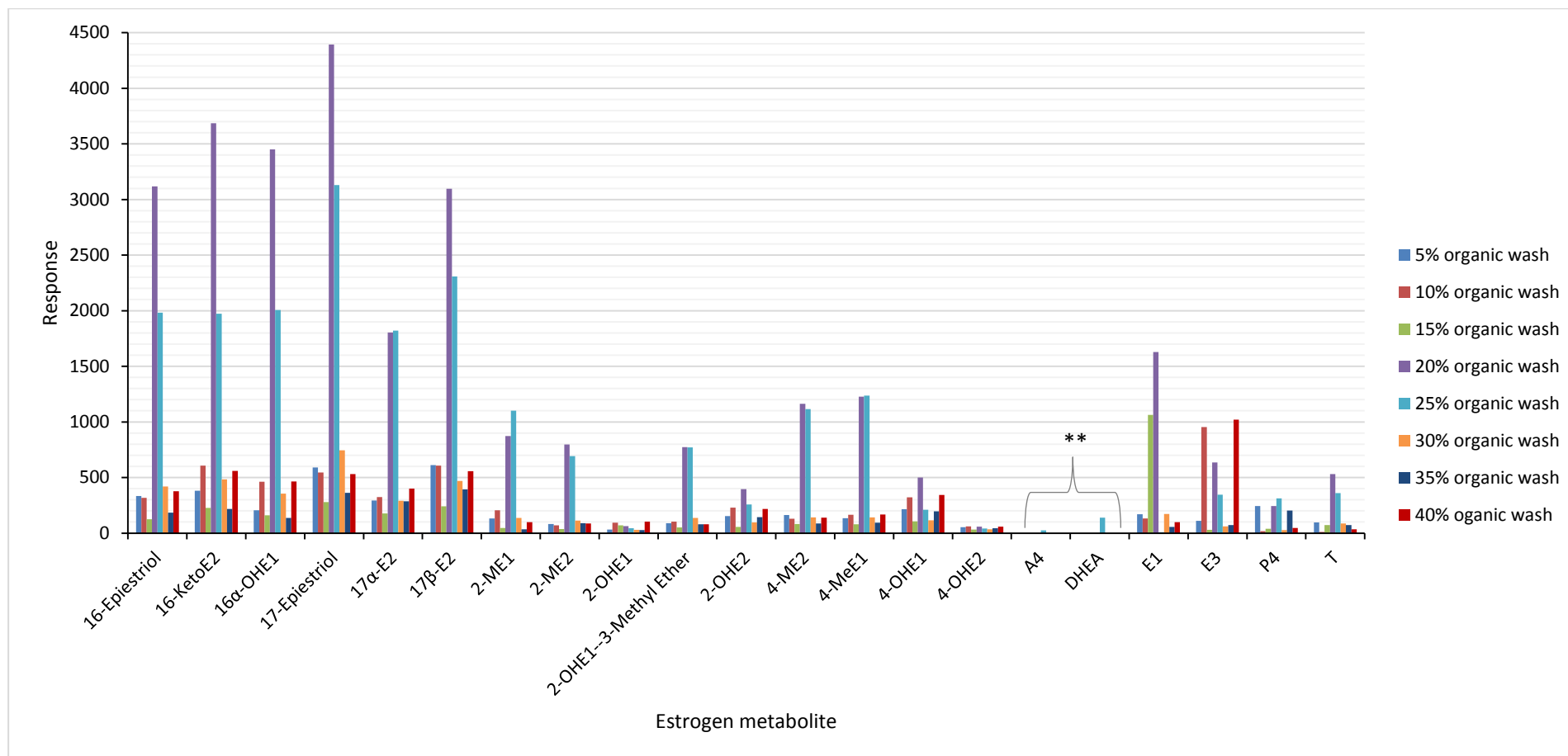


Figure S22: A visual illustration of the relative responses on the mass spectrometer of the non-polar metabolites washed from SPE with the 5-40% methanol in water, respectively. The tendency for most metabolites shows an increase in metabolites washed out as the organic solvent percentage increases to 20-25%, while the response of metabolites washed off after 25%, is not that much. ** These metabolite gave no response values except those indicated in the figure (no adjustments could be made for better visual illustration).

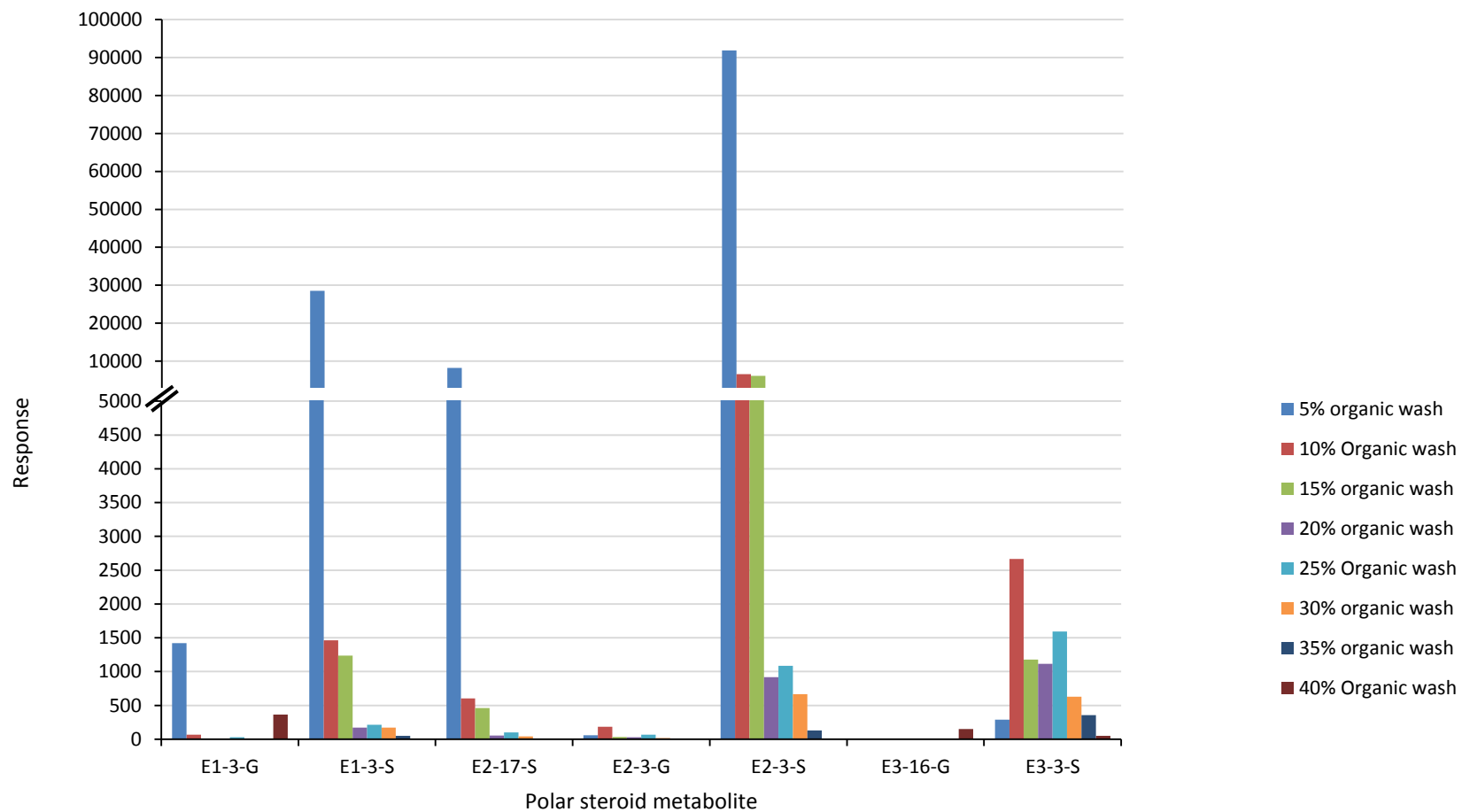


Figure S23: A visual illustration of the relative responses on the mass spectrometer of the polar metabolites washed from SPE with the 5%-40% methanol in water, respectively. // Gap in y-axis for better visual illustration.

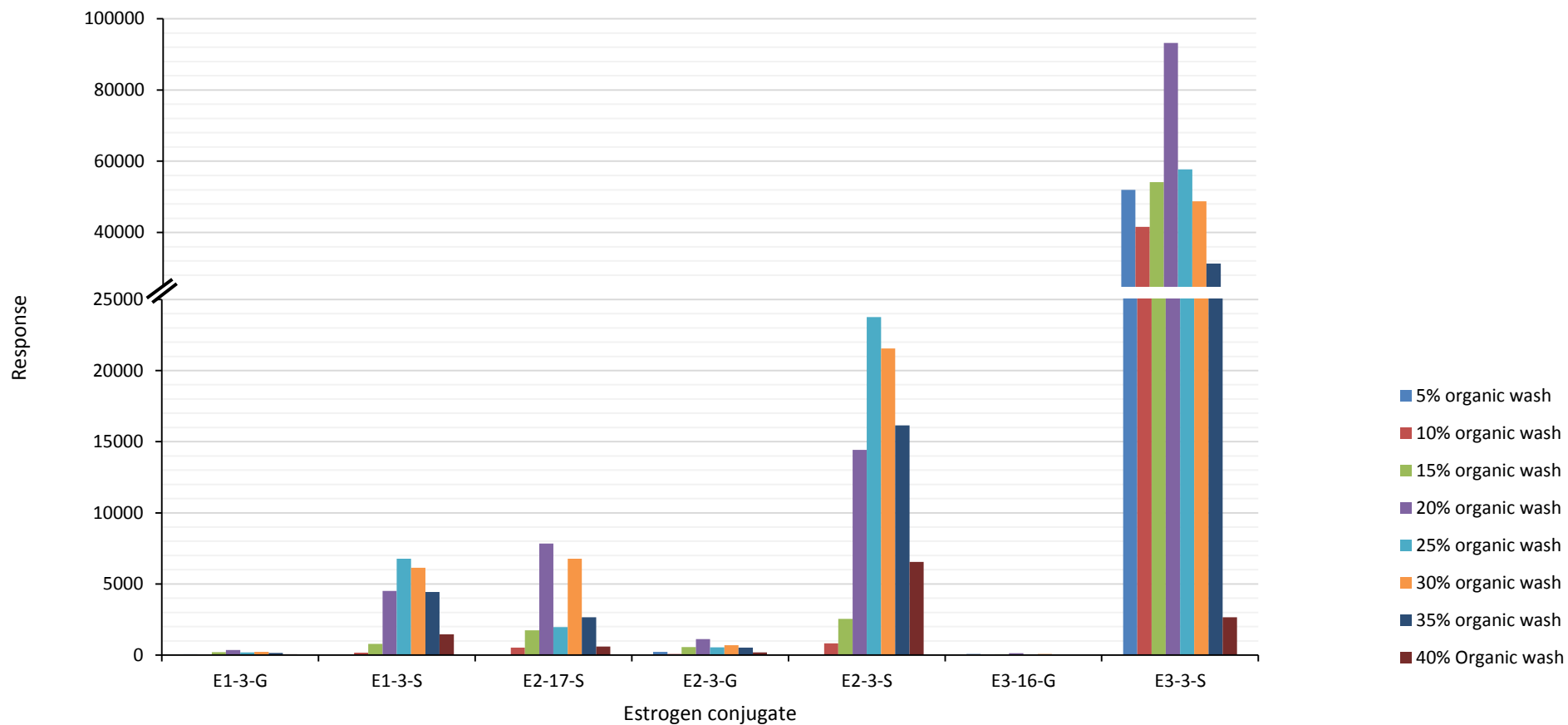


Figure S24: A visual illustration of the relative responses on the mass spectrometer of the polar metabolites eluted from SPE with acetone and methanol after washing with 5-40% MeOH in the wash step. // Gap in y-axis for better visual illustration.

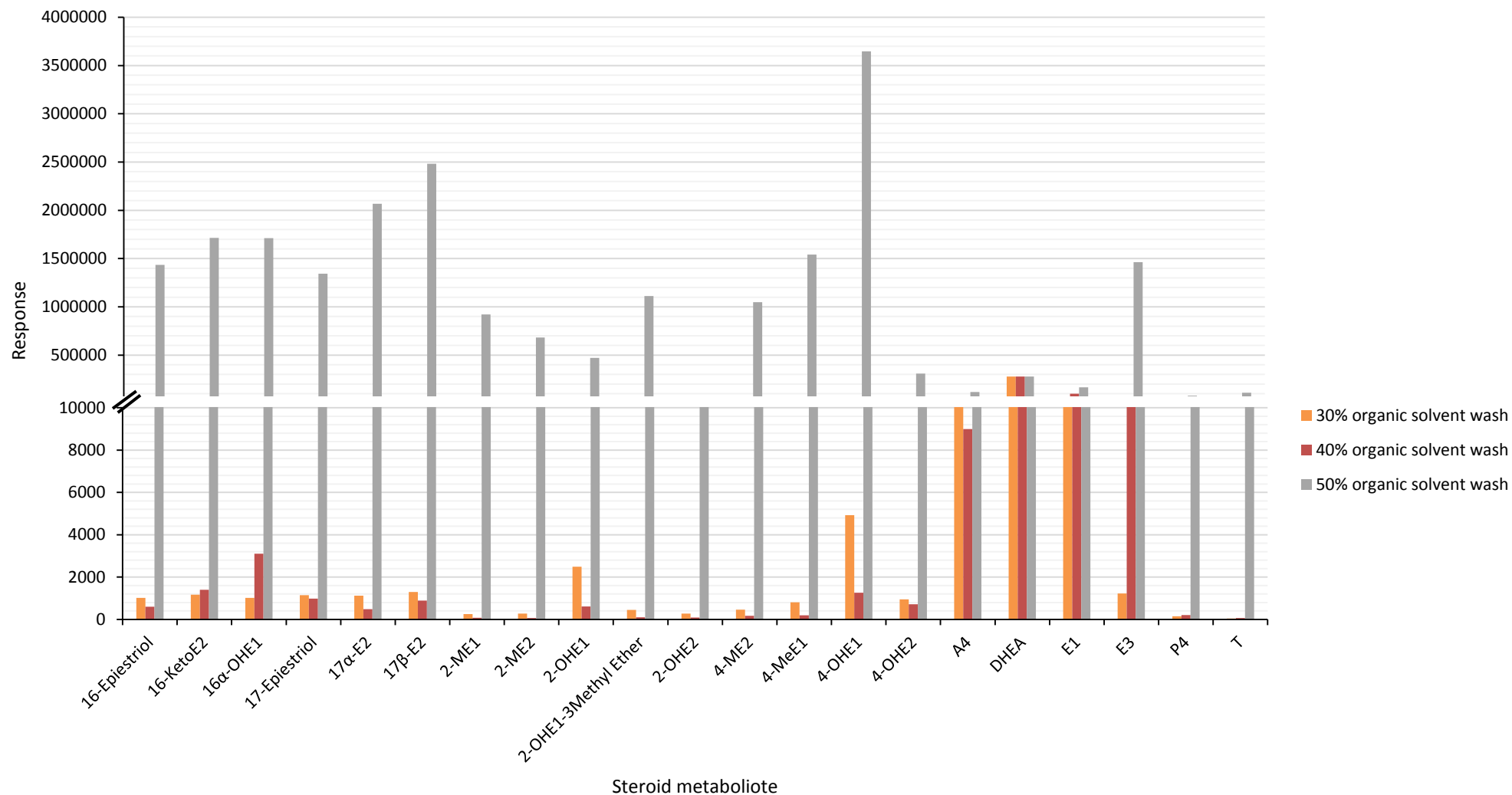


Figure S25: A visual illustration of the relative responses on the mass spectrometer of the non-polar metabolites washed from SPE with the 30%-50% methanol in water, respectively. The tendency for all the metabolites showed an increase in metabolites washed out as the organic solvent percentage increases to 50%. Almost double the amount of metabolites washed out with a 30-40% wash, were washed out with a 50% organic solvent wash. // Gap in y-axis for better visual illustration.

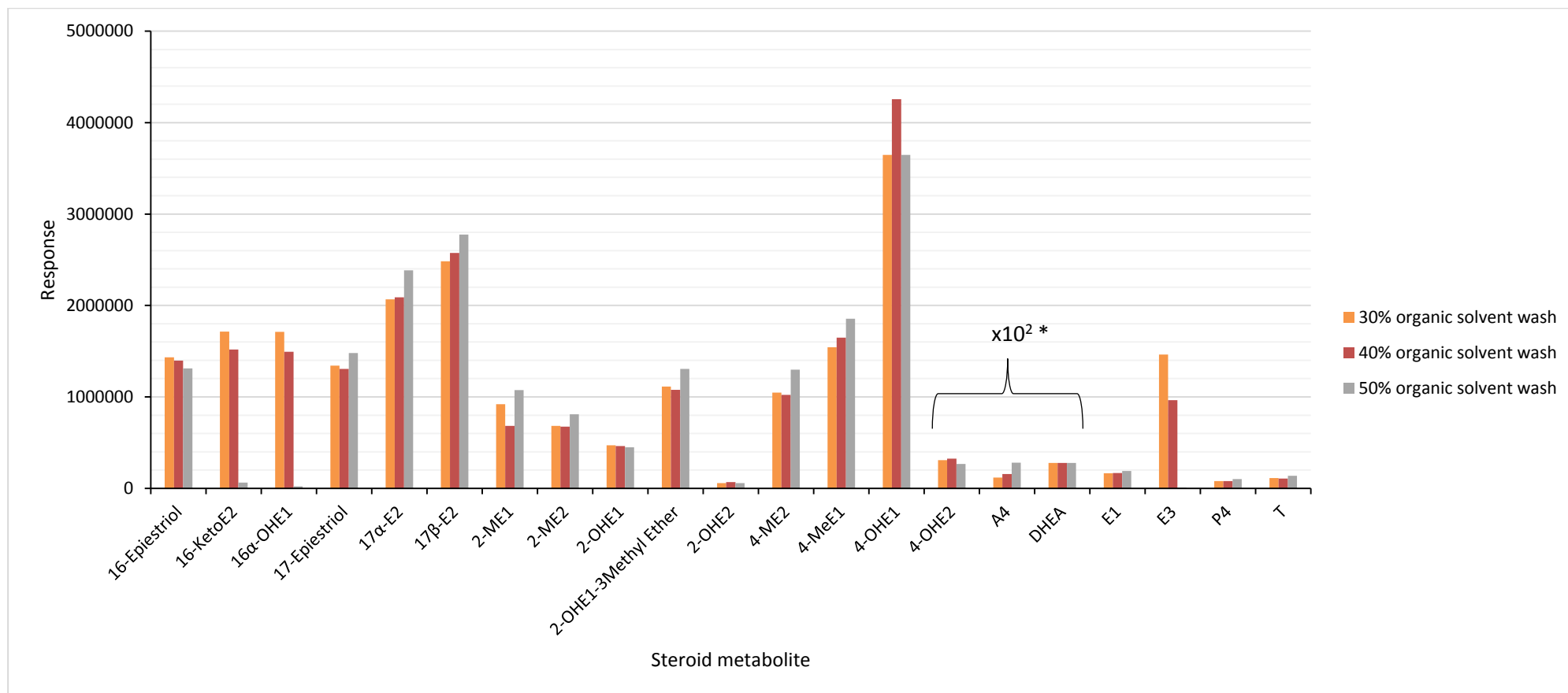


Figure S26: A visual illustration of the relative responses on the mass spectrometer of the non-polar metabolites eluted from SPE with acetone and methanol. For organic solvent percentage 30-50%, there are almost no differences in the response of the metabolites eluted from the SPE column. * These metabolite response values were adjusted by $\times 10^2$ to allow visual illustration thereof on the same y-axis scale.

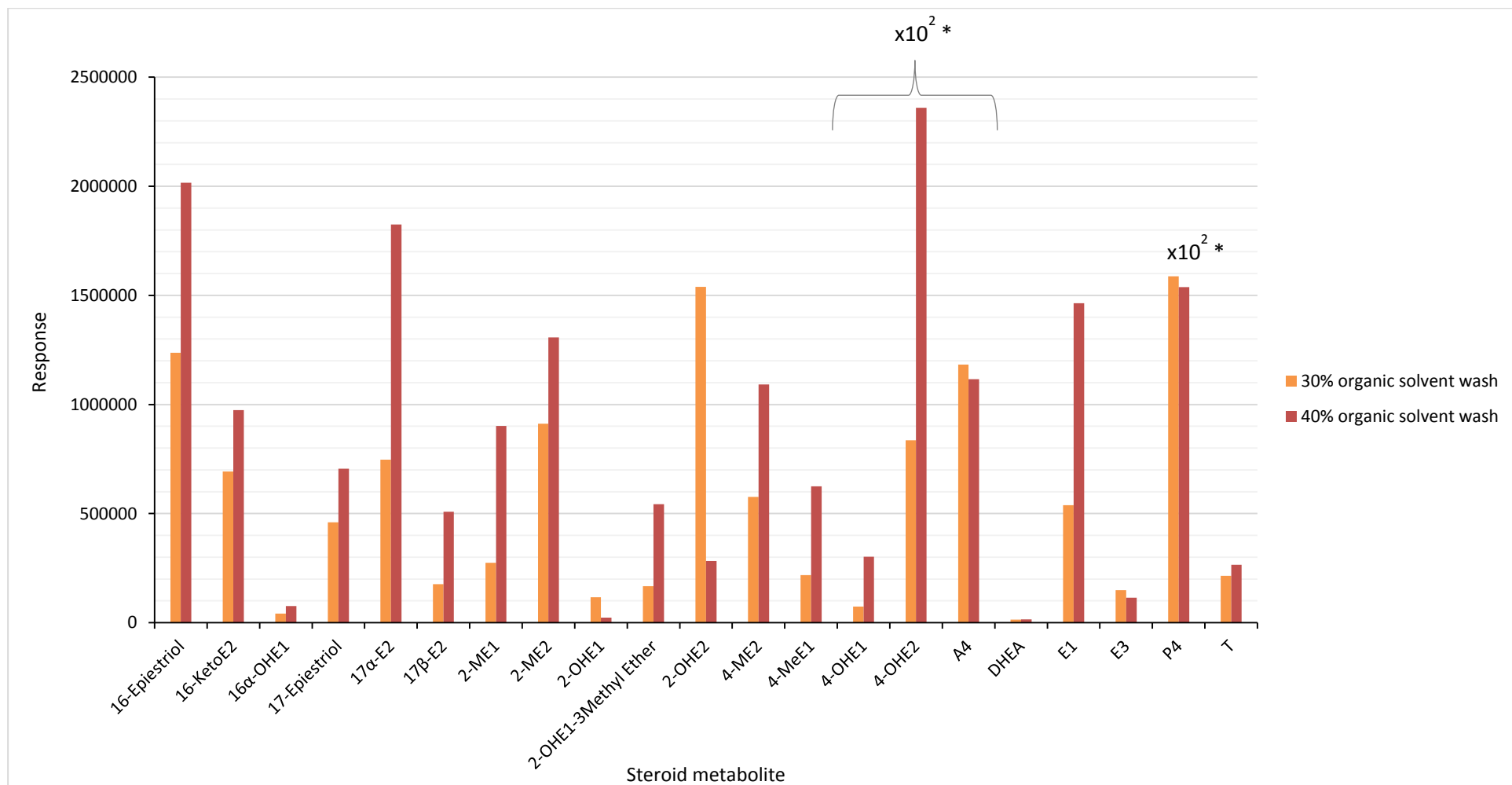


Figure S27: Visual comparison of the relative responses on the mass spectrometer between the eluents of a 30% and a 40% MeOH solution in the SPE washing step. Only 2-Hydroxyestrone and 2-Hydroxyestradiol shows higher responses with a 30% organic solvent wash, all other metabolites gave higher responses for higher (40%) organic solvent in the washing step. * These metabolite response values were adjusted by $\times 10^2$ to allow visual illustration thereof on the same y-axis scale.

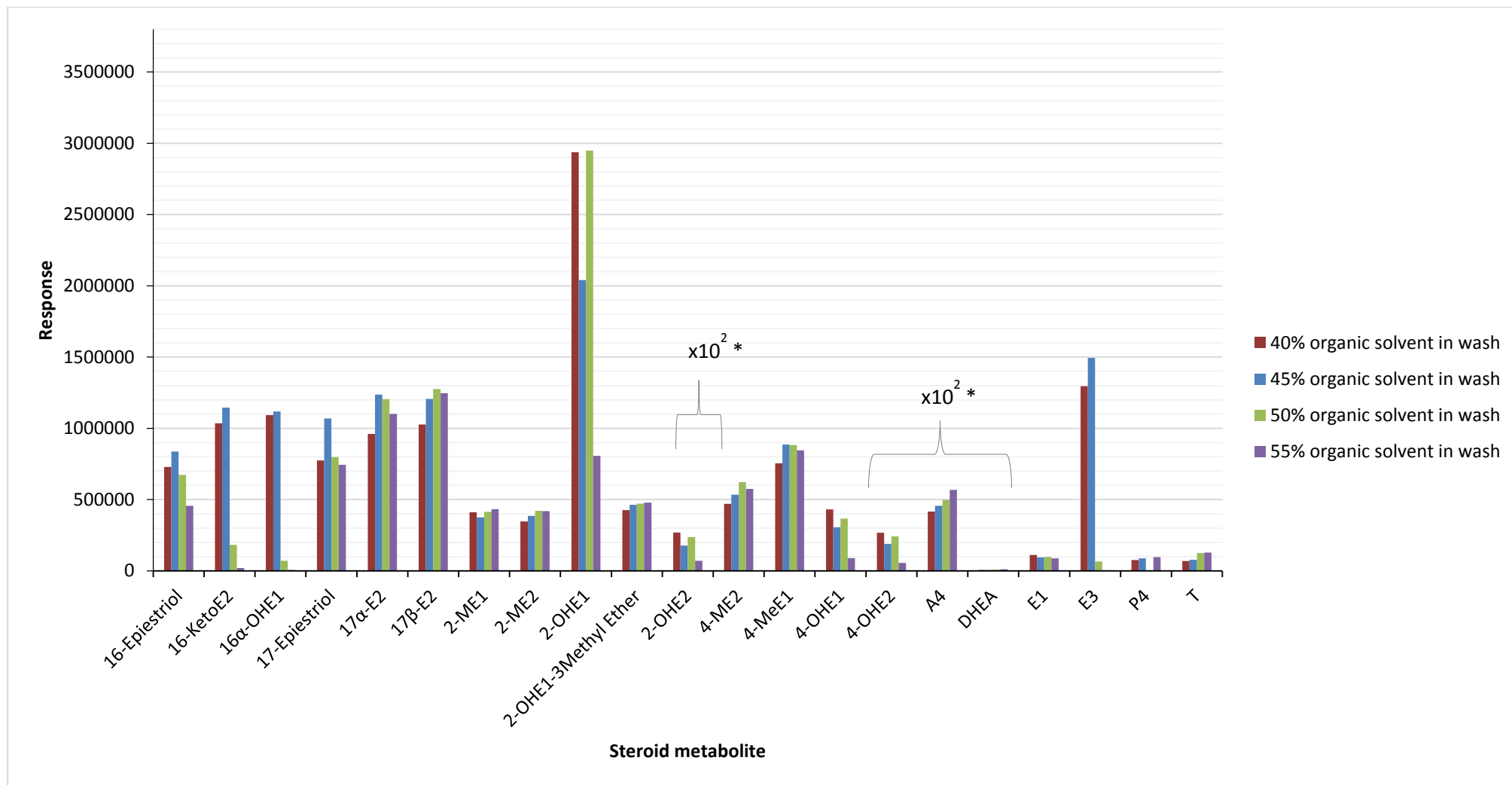
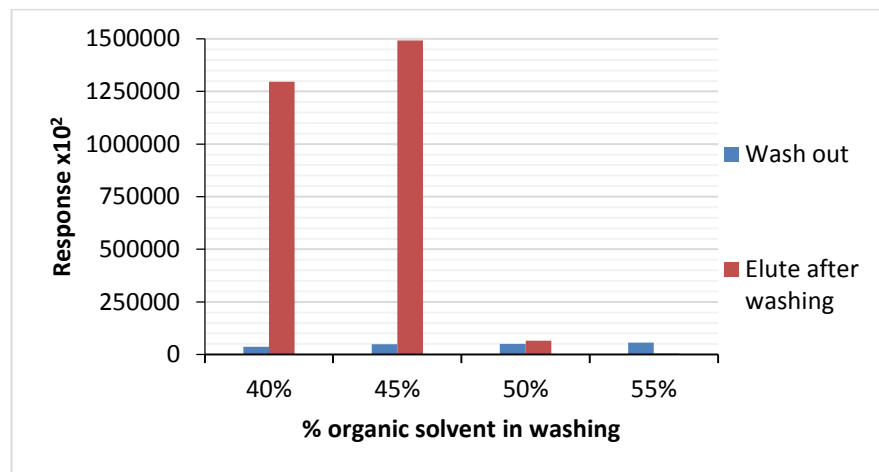
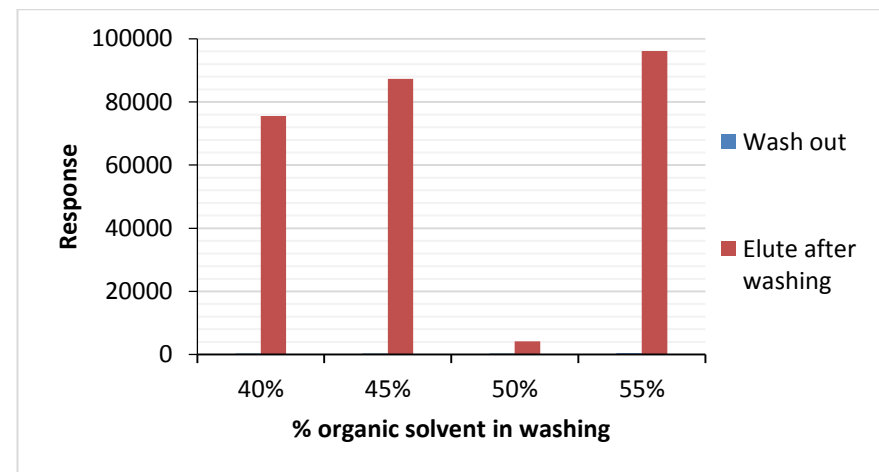


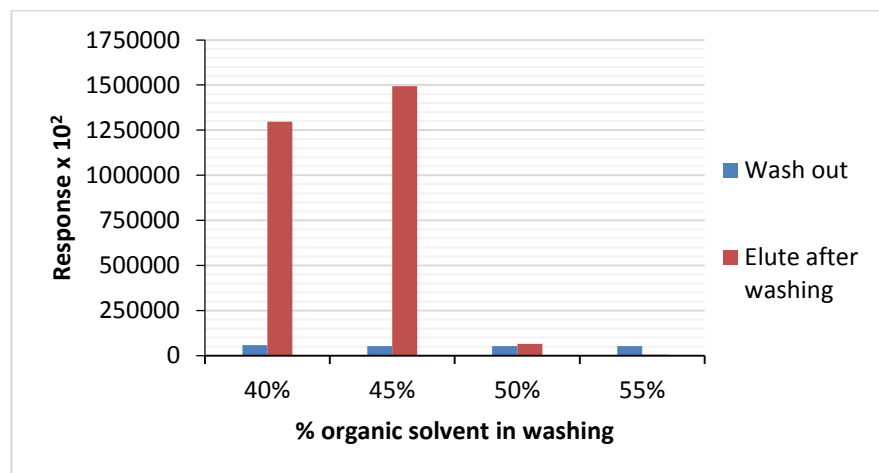
Figure S28: The relative responses on the mass spectrometer values of the SPE eluate after a 40%, 45%, 50% and 55% MeOH in the SPE washing solution for each of the steroid hormone metabolites. * These metabolite response values were adjusted by $\times 10^2$ to allow visual illustration thereof on the same y-axis scale.



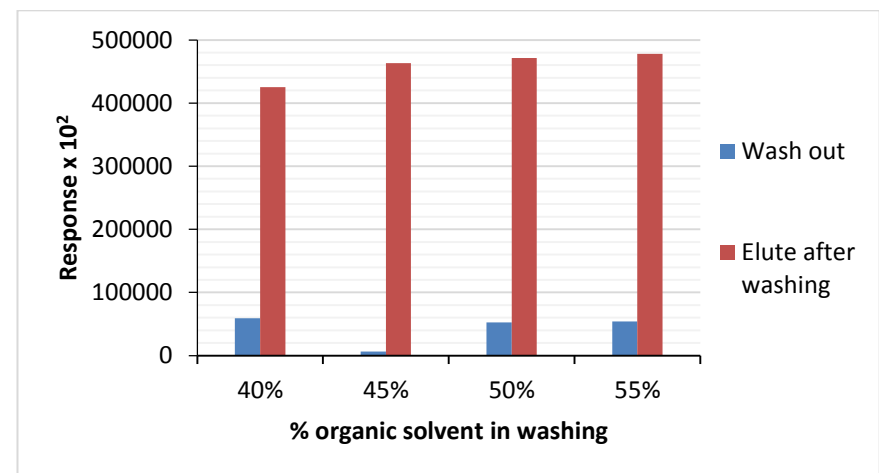
a) 2-Hydroxyestrone



b) 4-Hydroxyestrone



c) 2-Hydroxyestradiol



d) 4-Hydroxyestradiol

Figure S29 (a-d): Visual illustrations of the relative responses on the mass spectrometer (or response x10² if adapted in figure S26) of each of the four hydroxyestrogen metabolites, a) 2-hydroxyestrone, b) 4-hydroxyestrone, c) 2-hydroxyestradiol and d) 4-hydroxyestradiol, by comparing the response of the amount of the metabolite that washed out during the SPE wash step for each percentage of organic solvent in the washing solution, with the response obtained from the eluent after washing with the different percentages. These percentages of organic solvent included 40%, 45%, 50% and 55%.

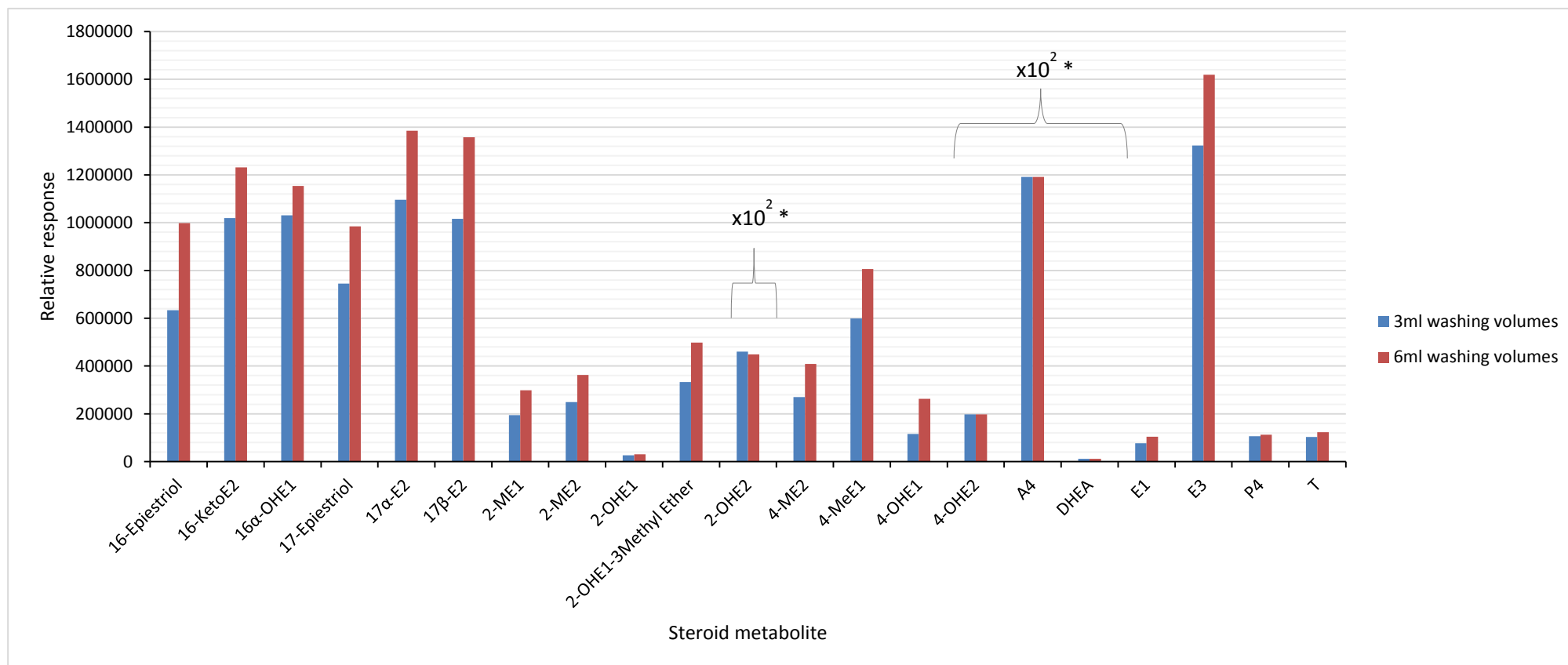


Figure S30: Comparison figures of the effect of washing with 3 ml and 6 ml washing volumes on the standard mixture for the non-polar metabolites. These washing steps include the 5% wash, 20% wash step (for washing out polar metabolites) and the 45% wash. The relative responses of each metabolite, which indicates the major effect of the washing volumes on the hydroxyestrogens. * These metabolite response values were adjusted by $\times 10^2$ to allow visual illustration thereof on the same y-axis scale.

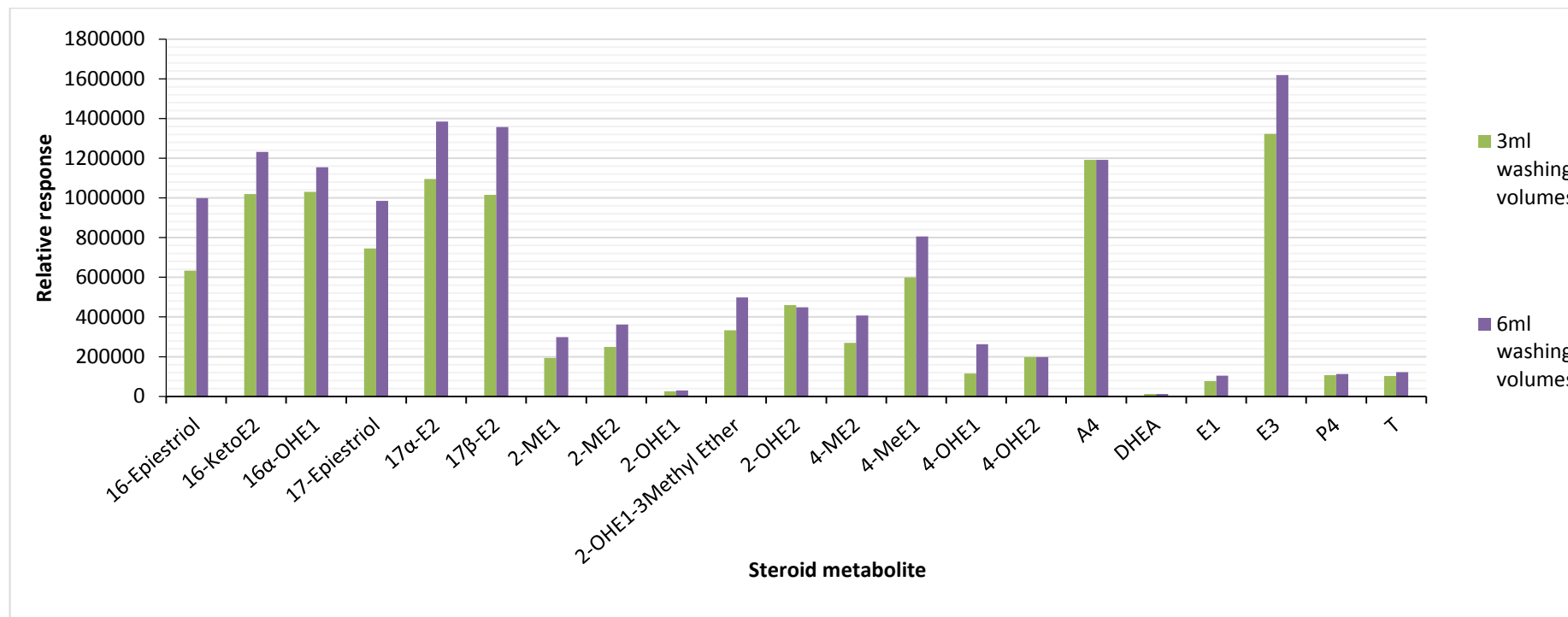
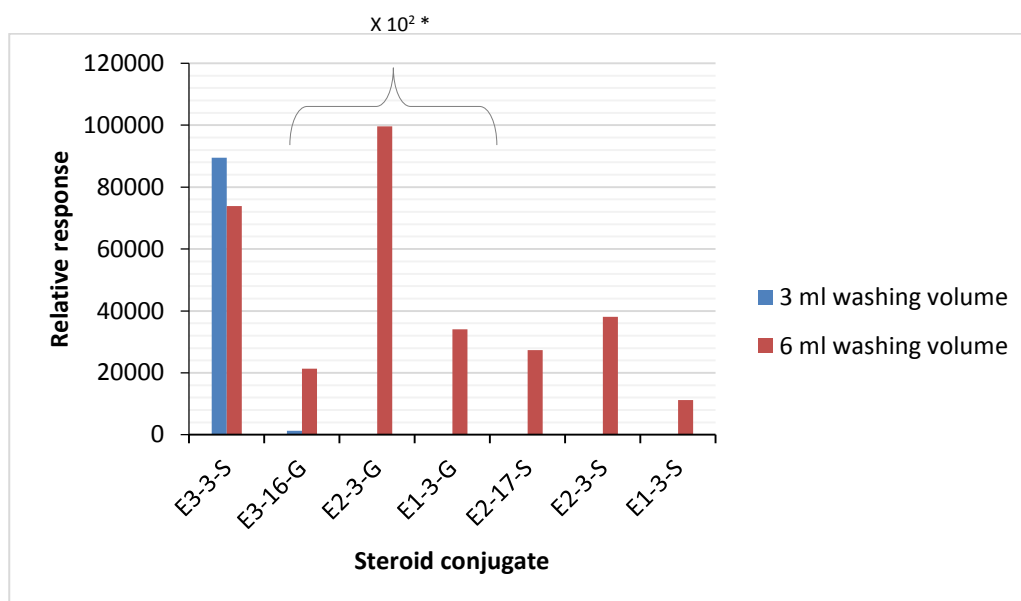
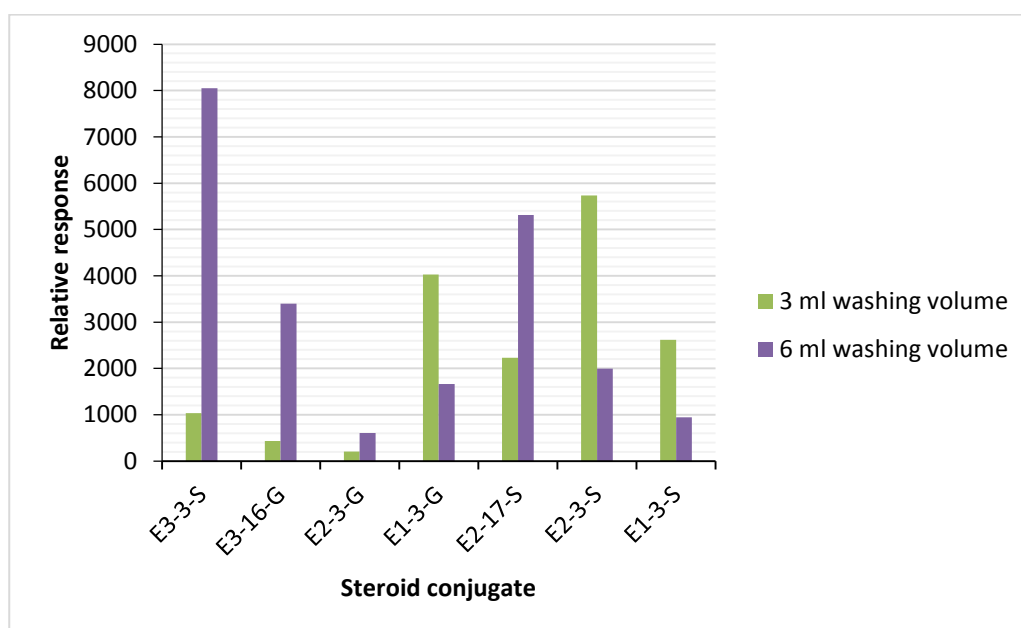


Figure S31: Comparison figures of the effect of washing with 3 ml and 6 ml washing volumes on the matrix spiked mixture for the non-polar metabolites. These washing steps include the 5% wash, 20% elution step and the 45% wash: the relative responses of each metabolite, which indicates the major effect of the washing volumes on the hydroxyestrogens.



a) Standard solution



b) Matrix spike

Figure S32: Comparison figures of the effect of washing with 3 ml and 6 ml washing volumes on the a) standard mixture & b) matrix spiked mixture for the polar metabolites. These washing steps include the 5% wash, 20% elution step and the 45% wash. * These metabolite response values were adjusted by $\times 10^2$ to allow visual illustration thereof on the same y-axis scale.

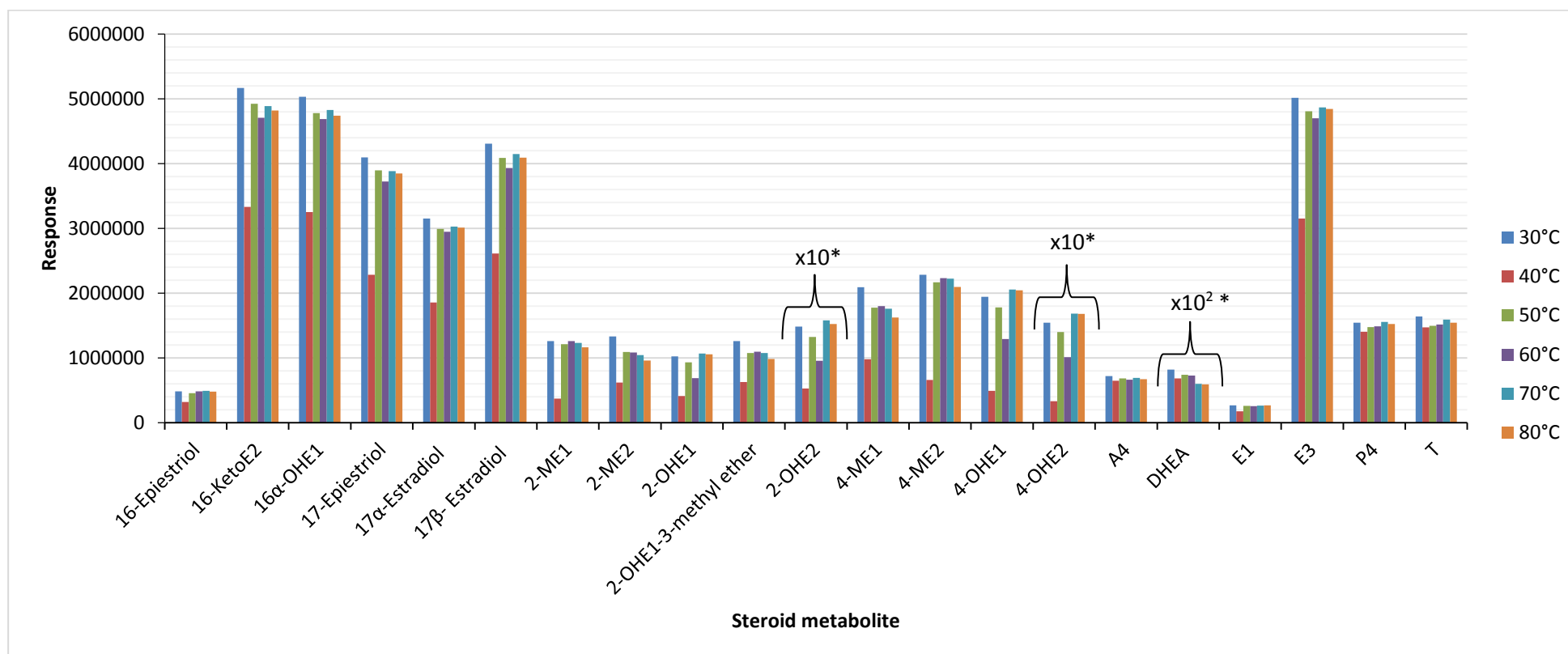


Figure S33: Comparison between the relative responses on the mass spectrometer values for various incubation temperatures during the derivatisation process for each of the non-polar metabolites. These temperatures include 30°C-80°C in 10°C intervals. Non-derivatising metabolites such as testosterone and androstenedione were included to evaluate the effect of the derivatisation procedure on these metabolite responses. * These metabolite response values were adjusted by x10 or x10² to allow visual illustration thereof on the same y-axis scale.

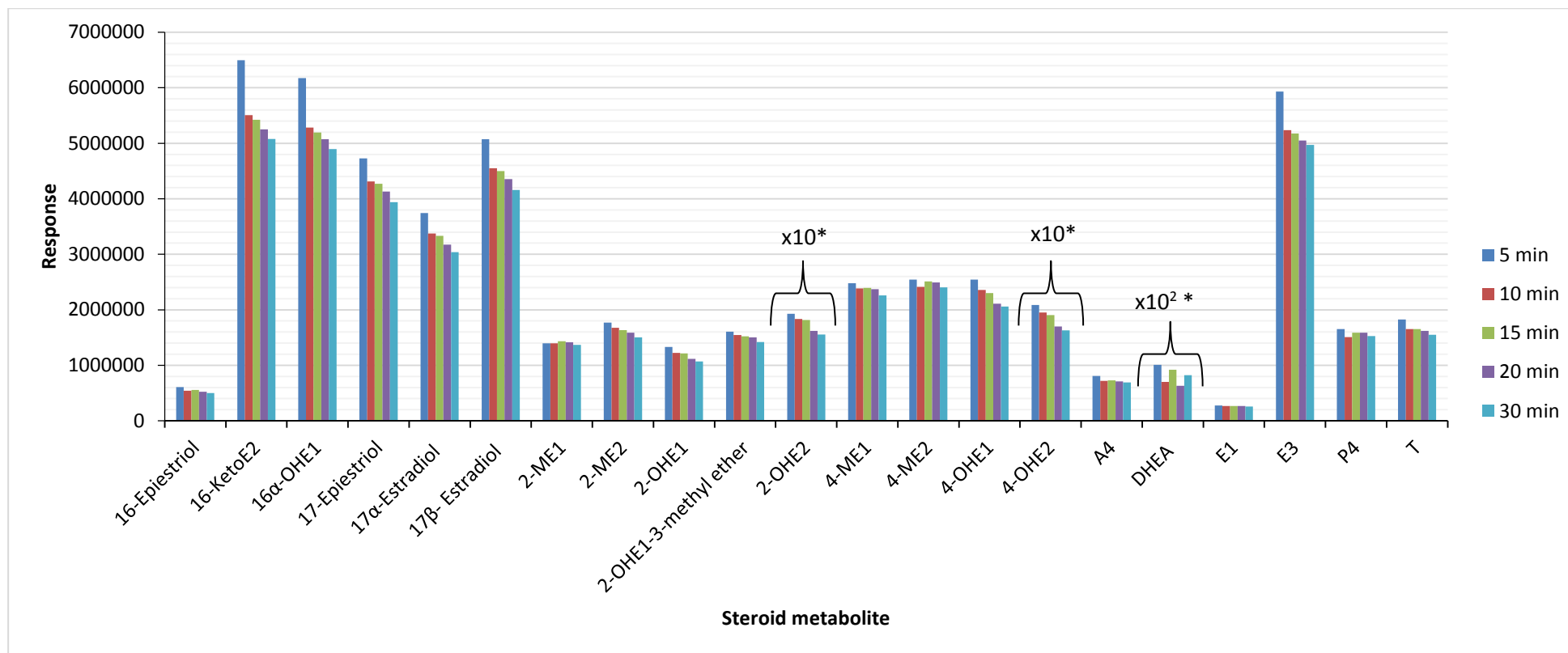


Figure S34: Comparison between the relative responses on the mass spectrometer values for various incubation times to allow the derivatisation process for each of the non-polar metabolites. These times include 5 min, 10 min, 15 min, 20 min and 30 min incubation intervals. * These metabolite response values were adjusted by $\times 10$ or $\times 10^2$ to allow visual illustration thereof on the same y-axis scale

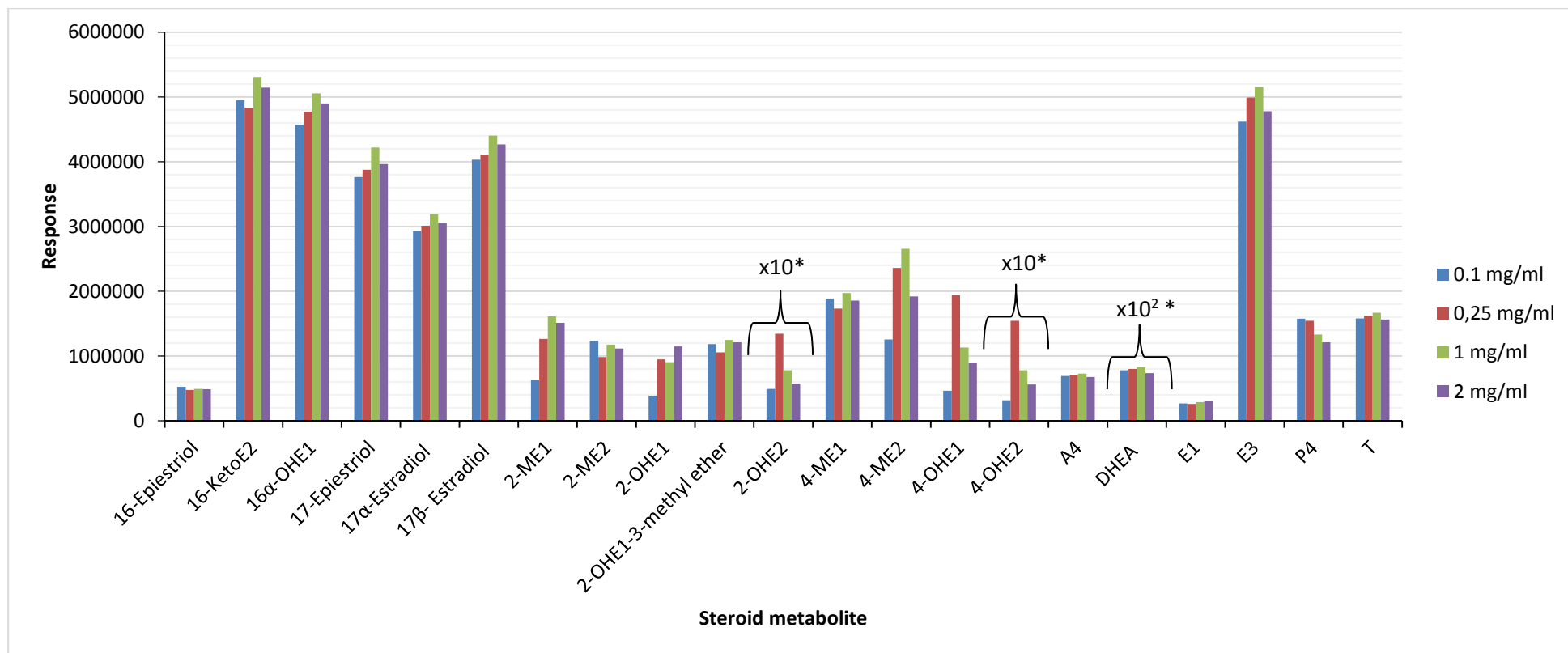


Figure S35: Comparison between the relative responses on the mass spectrometer values for various dansyl chloride concentrations to allow the derivatisation process for each of the non-polar metabolites. These concentrations include 0.1 mg/ml, 0.25 mg/ml, 1 mg/ml, 2 mg/ml in the final 200 μ l volume. * These metabolite response values were adjusted by $\times 10$ or $\times 10^2$ to allow visual illustration thereof on the same y-axis scale.

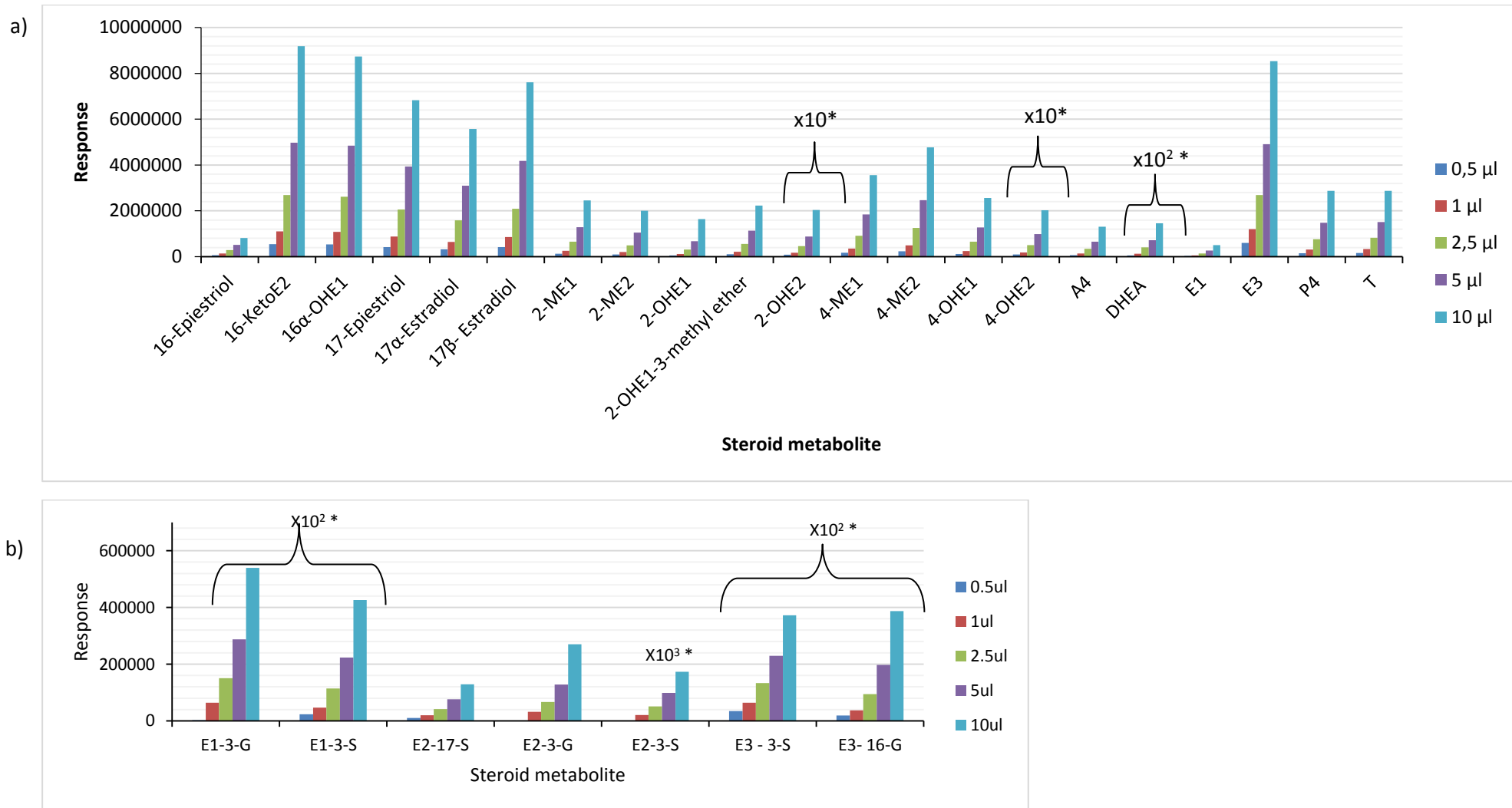
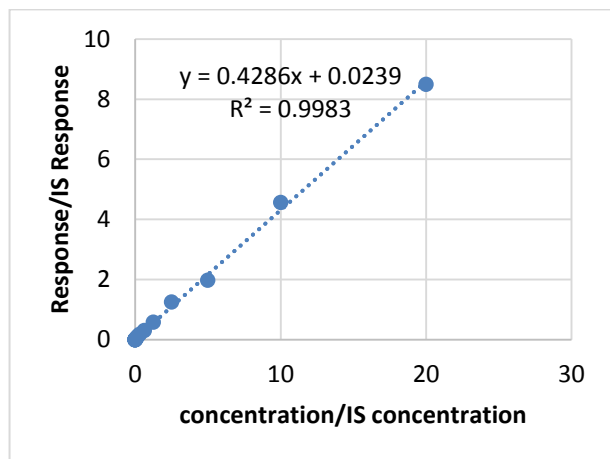
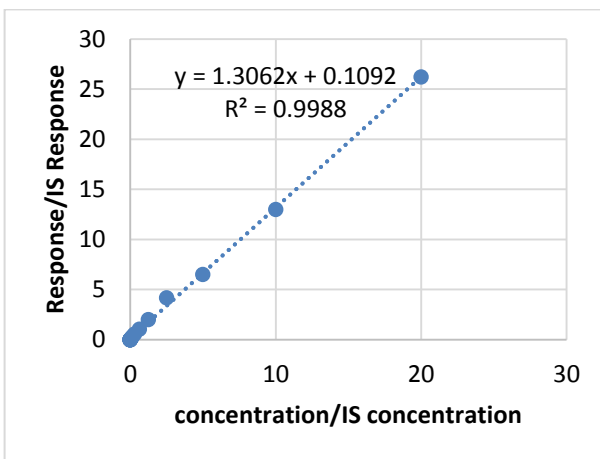


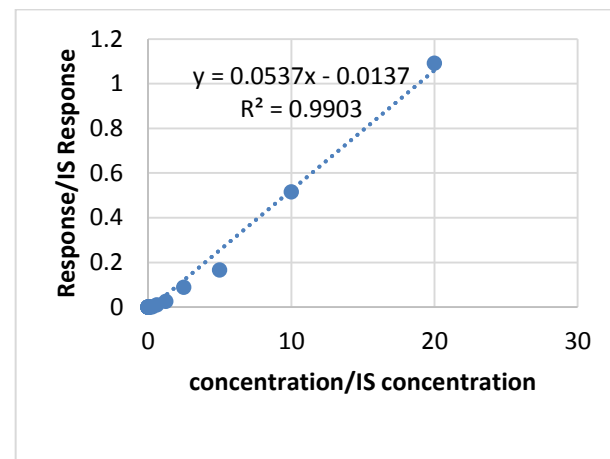
Figure S36: Comparison between the relative responses on the mass spectrometer values for different injection volumes onto the LC-MS system, for each of the a) non-polar metabolites and b) polar metabolites. These injection volumes include 0.5ul, 1ul, 2.5ul, 5ul and 10ul of the final suspended sample *. These metabolite response values were adjusted by $\times 10$, $\times 10^2$ or $\times 10^3$ to allow visual illustration thereof on the same y-axis scale.



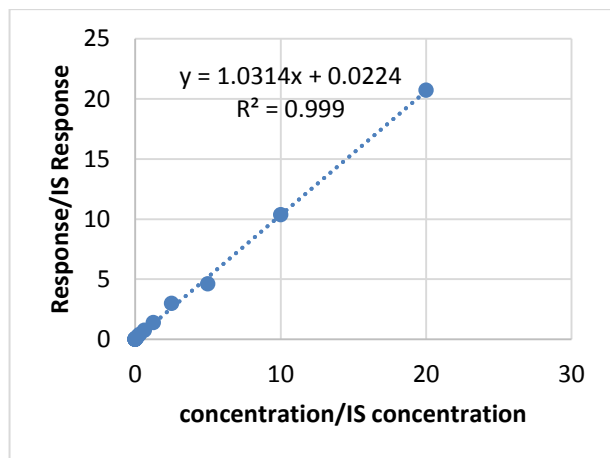
a) Testosterone



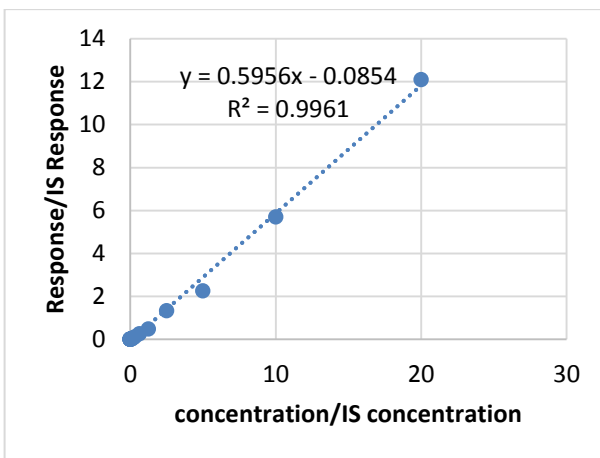
b) 16 α -Hydroxyestrone



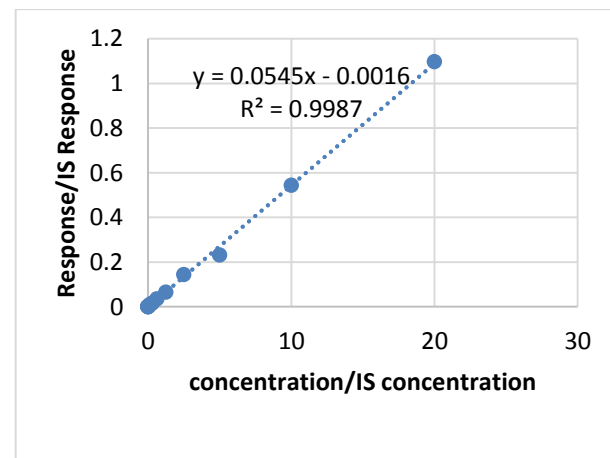
c) 4-Hydroxyestradiol



d) 17 β -Estradiol



e) 2-Methoxyestrone



f) Estrone

Figure S37: Illustration of linearity curves during validation of derivatised method for metabolites a) testosterone, b) 16 α -hydroxyestrone, c) 4-hydroxyestradiol, d) 17 β -estradiol, e) 2-methoxyestrone and f) estrone.

Table S3: The table with all the intraday RSD% and accuracy % values for each of the estrogen metabolites, for the LLOQ, low QC, medium QC, high QC, ULOQ and dilution integrity sample.

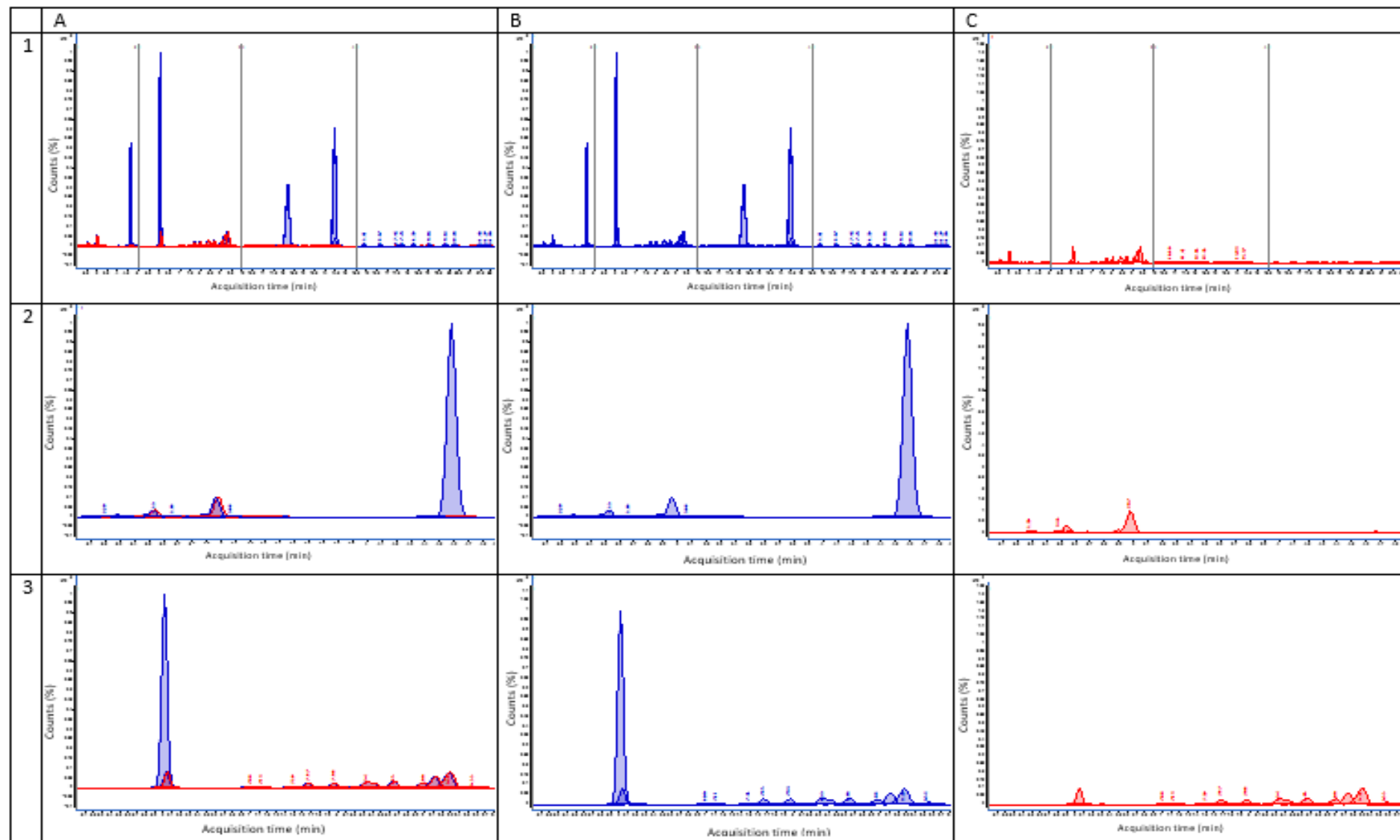
Metabolite	LLOQ		Low QC		Medium QC		Dilution QC		High QC		ULOQ	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Testosterone	12.94	99.46	1.37	101.43	1.18	98.35	1.15	101.37	1.54	95.99	2.16	105.72
DHEA	23.71	105.27	35.81	122.20	33.99	82.81	26.93	119.33	76.23	85.00	43.58	107.04
Androstenedione	12.15	99.87	1.16	92.17	1.04	95.73	1.01	102.07	1.36	85.072	1.29	89.85
Progesterone	11.67	113.00	1.46	91.81	0.74	91.81	0.92	96.62	0.94	88.69	1.42	107.01
Estriol	2.80	105.112	0.76	101.18	0.30	94.29	0.36	100.45	0.85	101.25	0.45	96.21
16-Hydroxystrone	5.2	94.37	2.48	94.23	2.41	91.20	2.35	96.45	2.89	98.49	2.60	99.01
16-Epiestriol	20.60	92.35	13.91	107.11	4.43	112.94	1.93	88.96	12.84	100.92	14.12	105.37
16-Ketoestradiol	4.77	95.67	2.56	87.95	2.55	87.38	2.27	92.54	2.94	81.97	4.12	103.60
17-Epiestriol	11.70	111.49	1.38	91.07	2.24	92.68	1.74	105.87	2.75	95.66	3.23	106.54
2-Methoxyestradiol	10.71	101.32	3.41	101.39	3.26	103.40	3.77	111.97	4.20	107.28	3.39	88.27
4-Methoxyestradiol	9.11	92.21	1.68	96.85	1.07	90.98	1.27	100.77	1.53	102.40	1.56	99.92
Estradiol Beta	6.79	97.73	1.94	88.26	2.55	102.37	2.77	98.52	2.93	97.06	3.34	97.34
Estrone-2-hydroxy-3-methyl ether	10.19	110.3	2.90	114.06	3.54	85.5	3.87	107.42	4.19	94.17	2.24	96.11
Estradiol Alpha	9.01	107.14	2.01	105.48	2.37	86.48	2.41	107.67	2.79	95.74	3.34	112.98
4-Methoxyestrone	11.45	94.46	2.41	98.80	3.01	99.68	3.40	94.63	4.57	88.46	3.01	93.70

Estrone	7.89	99.34	3.49	92.92	6.36	99.54	1.29	98.74	3.55	96.53	5.13	107.50
2-Methoxyestrone	13.92	108.85	3.10	97.76	2.96	87.43	4.26	93.66	4.71	93.50	5.02	96.26
4-Hydroxyestradiol	12.89	94.96	5.36	97.60	4.29	102.38	5.17	101.61	3.56	105.77	5.59	99.15
2-Hydroxyestradiol	13.43	108.77	6.04	100.69	4.13	108.71	3.03	95.42	3.59	99.31	5.25	97.59
4-Hydroxyestrone	10.48	95.07	3.47	96.235	2.97	98.97	3.96	95.51	4.93	96.81	7.51	99.21
2-Hydroxyestrone	12.84	116.2	2.37	113.00	3.07	112.65	3.90	87.88	4.95	108.96	6.88	99.95
Estriol-3-Sulphate	7.38	89.86	8.53	90.42	7.00	97.65	2.78	95.35	7.15	85.86	5.62	108.78
Estriol-16-Glucuronide	7.68	86.64	0.13	103.52	7.80	113.09	13.06	110.34	8.24	103.37	6.12	113.05
Estradiol-3-Glucuronide	11.52	118.03	4.43	115.90	5.05	95.11	6.07	97.86	5.51	105.38	11.50	88.73
Estrone-3-Glucuronide	13.78	68.70	10.75	84.98	5.69	85.64	7.21	82.45	10.35	87.87	13.00	90.78
Estradiol-17-Sulphate	11.99	104.69	8.19	98.99	6.40	112.50	3.76	95.17	4.48	92.11	3.86	74.83
Estradiol-3-Sulphate	10.37	88.35	4.93	87.92	6.69	94.13	3.80	91.60	6.47	92.34	3.49	90.28
Estrone-3-Sulphate	13.61	98.42	5.191	99.24	5.296	100.74	7.33	96.50	9.25	101.98	4.14	99.50
Average	11.09	100.42	5.04	99.87	4.73	94.44	4.35	101.00	7.12	94.81	6.18	100.73

Table S4: The table with all the interday RSD% and accuracy % values for each of the estrogen metabolites, for the LLOQ, low QC, medium QC, high QC, ULOQ and dilution integrity sample.

Metabolite	LLOQ		Low QC		Medium QC		Dilution QC		High QC		ULOQ	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Testosterone	14.41	102.82	6.31	107.97	3.40	106.87	3.34	97.69	5.19	102.47	6.00	95.61
DHEA	13.57	91.62	3.35	82.52	2.27	90.64	3.8	83.96	6.33	84.61	18.72	107.04
Androstenedione	17.90	99.13	5.94	91.18	3.15	98.30	2.82	89.11	4.00	96.22	5.94	90.33
Progesterone	12.76	113.74	5.11	109.23	3.07	109.00	1.41	109.07	2.59	106.52	12.54	88.91
Estriol	27.28	91.63	3.72	95.37	3.70	103.64	2.27	98.11	1.34	100.66	3.10	102.11
16-Hydroxystrone	20.72	109.03	5.23	90.09	10.68	95.82	13.64	99.20	8.70	93.43	6.24	91.78
16-Epiestriol	16.52	86.23	12.59	97.36	14.87	92.50	11.30	89.93	13.16	95.94	0.50	88.94
16-Ketoestradiol	26.15	93.59	5.69	87.08	9.79	91.49	13.01	91.08	11.09	95.36	10.23	98.95
17-Epiestriol	10.53	93.27	7.05	89.25	4.95	88.48	4.65	92.72	2.87	88.69	3.25	96.06
2-Methoxyestradiol	9.24	90.26	13.84	101.39	14.90	89.85	14.56	86.51	6.80	86.10	5.01	99.41
4-Methoxyestradiol	11.28	97.31	13.32	91.14	11.20	93.88	12.30	100.51	5.56	93.72	2.74	99.91
Estradiol Beta	33.35	91.74	5.29	90.15	4.58	100.35	3.19	100.73	2.91	91.06	6.23	96.41
Estrone-2-hydroxy-3-methyl ether	15.43	110.34	12.71	100.69	12.31	85.49	12.20	94.54	5.92	88.93	3.45	98.75
Estradiol Alpha	14.71	98.96	5.66	96.20	4.74	91.41	3.09	91.83	3.34	93.16	6.23	97.92
4-Methoxyestrone	2.48	99.46	8.58	98.76	14.32	95.39	7.56	92.45	6.33	102.47	3.77	84.99
Estrone	20.49	86.66	6.70	98.95	13.94	94.78	10.24	101.97	11.55	107.37	11.78	101.37

2-Methoxyestrone	8.43	87.73	13.69	86.68	5.65	87.42	12.08	91.26	7.06	93.46	12.30	92.24
4-Hydroxyestradiol	4.93	103.33	3.28	105.02	12.78	102.57	11.42	104.43	12.72	103.44	8.98	101.81
2-Hydroxyestradiol	17.26	120.51	1.94	108.78	8.70	104.7	10.76	90.57	14.88	97.37	6.69	97.59
4-Hydroxyestrone	3.31	97.76	11.48	112.35	11.55	115.01	6.61	114.74	9.39	99.15	9.00	85.05
2-Hydroxyestrone	11.86	84.67	4.036	88.20	10.16	109.95	9.84	112.83	10.13	98.53	7.05	90.59
Estriol-3-Sulphate	16.80	102.48	13.26	99.53	13.74	86.43	12.84	114.03	11.66	100.22	7.54	97.45
Estriol-16-Glucuronide	10.65	94.40	5.03	94.99	13.69	99.96	2.39	105.04	2.51	104.52	13.78	103.84
Estradiol-3-Glucuronide	20.28	77.41	10.41	116.65	10.31	105.94	14.12	97.02	6.36	89.02	0.44	91.94
Estrone-3-Glucuronide	22.35	77.56	20.24	116.87	14.32	105.57	9.77	85.84	8.72	87.87	4.33	97.86
Estradiol-17-Sulphate	16.61	101.84	4.41	97.93	3.83	97.17	1.70	104.90	13.56	104.51	3.12	99.93
Estradiol -3-Sulphate	13.06	96.87	9.43	103.12	6.19	85.66	10.76	111.21	13.56	91.97	0.57	108.96
Estrone-3- Sulphate	20.24	68.38	9.34	106.26	12.14	93.90	14.02	108.80	13.03	99.33	1.08	88.48
Average	15.45	97.61	8.13	98.33	9.10	97.50	8.42	96.82	7.90	96.13	6.45	95.35



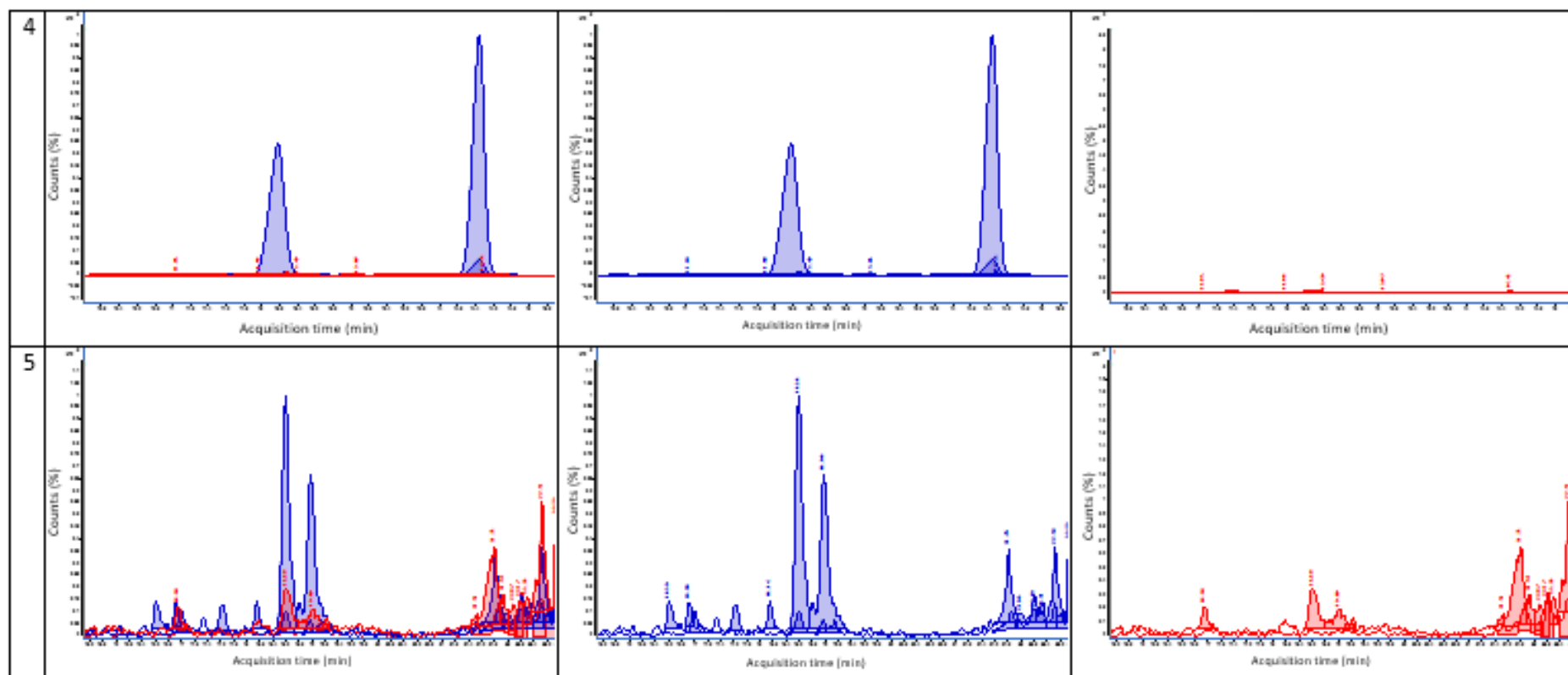


Figure S38: The figure illustrating that the retention times of the metabolites of interest are not affected by any matrix compounds eluting at the exact same time. In the figure, in column A, both the urine matrix and matrix spike is illustrated whereas column B shows only the LLOQ spike in matrix results and in column C clean matrix results. Although selectivity analysis is a necessity, the urine matrix bank, in this case, also contains metabolites of interest and thus has the exact same retention times as the spiked LLOQ metabolites, as visually illustrated. Row 1 illustrates the total ion chromatogram, while rows 2-5 are illustrative of segments 2-5 in the chromatographic method.

Supplementary results references

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