Development of an analytical method to measure 17β-estradiol metabolite concentrations in MCF-7 and MCF-12A cell lines.

by

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This project is dedicated to my Lord God: Father, Son and Holy Spirit. For without His mercy, grace and continuous encouragement and strengthening, I would not have been able to complete what I have started

Numbers 23:19
God is not a man, that He should tell or act a lie, neither the son of man, that He should feel repentance or compunction [for what He has promised]. Has He said and shall He not do it? Or has He spoken and shall He not make it good?

Hebrews 10: 35-36
Do not, therefore, fling away your fearless confidence, for it carries a great and glorious compensation of reward. For you have need of steadfast patience and endurance, so that you may perform and fully accomplish the will of God, and thus receive and carry away [and enjoy to the full] what is promised.

Philippians 2: 14
Do all things without grumbling and faultfinding and complaining [against God] and questioning and doubting [among yourselves],

Philippians 4:13
I have strength for all things in Christ who empowers me [I am ready for anything and equal to anything through Him Who infuses inner strength into me; I am self-sufficient in Christ's sufficiency].

1 Corinthians 10:13
… But God is faithful [to His word and His compassionate nature], and He [can be trusted] not to let you be tempted and tried and assayed beyond your ability and strength of resistance and power to endure, but with the temptation He will [always] also provide the way out (the means of escape to a landing place), that you may be capable and strong and powerful to bear up under it patiently.

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Abstract

Breast cancer is one of the most common cancers affecting women. It remains the leading cause of death in American women from 30 – 70 years of age and approximately 10% of the women living in western countries will develop breast cancer during their life time. Estrogens are a family of female hormones involved in the reproductive function of the human body. Estradiol is the most abundant estrogen in premenopausal woman. Initially it was thought that estradiol itself was responsible for tumourigenesis, but it has since been discovered that the catechol metabolites of estradiol and other estrogens cause carcinogenesis. 4-Hydroxyestradiol ($4\text{-}HE_2$) is a potent cell proliferating estrogen whereas 2-methoxyestradiol ($2\text{-}ME_2$) is a potent inhibitor of cell proliferation through the activation of apoptosis. 2-Hydroxyestradiol ($2\text{-}HE_2$) also causes increased cell proliferation but it is not as potent as $4\text{-}HE_2$ and it is *O*-methylated rapidly to $2\text{-}ME_2$. Catechol metabolites of estradiol are also involved in producing reactive oxygen species through redox cycling. The reactive oxygen species cause DNA damage and mutations to occur which can lead to carcinogenesis. A significant ratio to consider as a biomarker for breast cancer risk therefore is the $4\text{-}HE_2/2\text{-}ME_2$ ratio. In this study, an analytical method was developed to measure the concentration levels of E_2, $2\text{-}ME_2$, $2\text{-}HE_2$ and $4\text{-}HE_2$ in cell culture medium. The analytical method made use of gas chromatography-mass spectrometry (GCMS) analysis, since the expected physiological concentrations of these metabolites were very low. Various extraction and derivatisation techniques were applied during the development of the method. The final method made use of protein precipitation with concentrated hydrochloric acid, liquid-liquid extraction using diethyl

ether and derivatisation with trimethylsilylimidazole (TMSI). During the validation of this method, it was found that the method did not produce accurate measurements and that it could only be used to determine trends. Since the precise *in vitro* concentration levels of the metabolites were still unknown, it was decided to proceed with experiments using this method, to provide preliminary results with which further course of action could be planned. Equal numbers (1×10^{6} cells/flask) of MCF-7 and MCF-12A cells were provided with 11 ml medium containing E_2/2-HE_2/4-HE_2/2-ME_2 (10^{-6} M) and medium containing E_2/2-HE_2/4-HE_2/2-ME_2 (10^{-8} M). Of each metabolite and each concentration two flasks were prepared. Each flask represented a specific time interval. At the appropriate time 10 ml of the medium was extracted. The time intervals used for each experiment were 0 hours, 8 hours and 24 hours for incubation with E_2, 0 hours, 1 hour and 8 hours for incubation with 2-ME_2, 0 hours, 10 minutes and 1 hour for incubation with 2-HE_2 and 4-HE_2. The time intervals used were according to the expected rate of metabolism. Each experiment was repeated three times. Differences in the metabolism of breast tumour cells and normal cells were found and the concentration of the metabolites present in the cell incubation medium had an influence on the metabolism of the cells. The need to investigate the intracellular concentrations of the metabolites has also been accentuated through the results obtained.

Opsomming

Borskanker is een van die mees algemene vorme van kanker wat by vrouens voorkom. Dit bly die vernaamste oorsaak van dood onder vrouens tussen 30 en 70 jaar en ongeveer 10% van die vrouens in westerse lande sal borskanker ontwikkel gedurende hulle leeftyd.   Estrogene is 'n familie van vroulike hormone wat betrokke is by die voortplantingsfunksie van die menslike liggaam. estradiol is die vollopste estrogeen in premenopousale vrouens. Oorspronklik is daar gedink dat estradiol self verantwoordelik was vir die tumorgenese maar intussen is vasgestel dat die katesjolmetaboliete van estradiol karsinogenese veroorsaak.   4-Hidroksie estradiol ($4\text{-}HE_2$) is 'n sterk selprolifererende estrogeen terwyl 2-metoksie estradiol ($2\text{-}ME_2$) 'n sterk inhibitor is van selproliferasie deur die aktivering van apoptose.   2-Hidroksie estradiol ($2\text{-}HE_2$) veroorsaak ook verhoogde selproliferasie, maar tot 'n mindere mate as $4\text{-}HE_2$ en dit word vinnig ge-*O*-metileer na $2\text{-}ME_2$. Die katesjolmetaboliete van estradiol is ook betrokke by die vorming van reaktiewe suurstofspesies deur die redokskringloop. Die reaktiewe suurstofspesies veroorsaak DNS skade en mutasies wat kan lei tot karsinogenese.   'n Vername verhouding om ingedagte te hou as 'n biomerker vir borskankerrisiko is die $4\text{-}HE_2/2\text{-}ME_2$ verhouding.   In hierdie studie is 'n analitiese metode ontwikkel om die konsentrasievlakke van E_2, $2\text{-}ME_2$, $2\text{-}HE_2$, en $4\text{-}HE_2$ in selkultuurmedium te meet.   Die metode maak gebruik van gaschromatografie-massaspektrometrie (GC-MS) analise omdat die verwagte fisiologiese konsentrasies van hierdie metaboliete baie laag is.   Verskeie ekstraksie- en derivatiseringstegnieke is beproef tydens die ontwikkeling van die metode. Die finale metode het gebruik gemaak

van proteïenpresipitasie met gekonsentreerde soutsuur, vloeistof-vloeistof ekstraksie met diëtieleter en derivatisering met trimetielsilielimidazool (TMSI).   Gedurende die validering van die metode is daar gevind dat die metode nie akkurate metings produseer nie en dat dit slegs gebruik kan word vir die bepaling van nygings. Omdat die presiese *in vitro* konsentrasievlakke van die metaboliete nog nie bekend is nie, is daar besluit om voort te gaan met eksperimente en gebruik te maak van hierdie analitiese metode om resultate te produseer waarmee die rigting vorentoe kan beplan word.   Gelyke hoeveelhede ($1x10^6$selle/fles) van MCF-7 en MCF-12A selle was voorsien van 11 ml medium wat E_2/2-HE_2/4-HE_2/2-ME_2 (10^{-6} M) en medium wat E_2/2-HE_2/4-HE_2/2-ME_2 (10^{-8} M) bevat. Vir elke metaboliet en elke konsentrasie is twee flesse voorberei. Elke fles het 'n spesifieke tydsverloop verteenwoordig.   Na afloop van die korrekte tydsinterval is 10 ml van die medium afgetrek. Die betrokke tydsintervalle was 0, 8 en 24 uur vir E_2, 0, 1 en 8 uur vir 2-ME_2, 0, 10 minute en 1uur vir 2-HE_2 en 4-HE_2. Die tydsintervalle was bepaal volgens die verwagte tempo van metabolisme.   Elke eksperiment was drie keer herhaal. Verskille in die metabolisme van borskankerselle in vergelyking met normale selle is gevind en die konsentrasies van die metaboliete teenwoordig in die selinkubasiemedium het 'n invloed op die metabolisme van die selle. Die behoefte aan verdere navorsing in hierdie gebied het ook na vore gekom uit die verkrygde resultate.

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Abbreviations

•OH	hydroxyl radical
16α-HE_1	16α-hydroxyestrone
16α-HE_2	16α-hydroxyestradiol
17β-HSD	17β-hydroxysteroid dehydrogenase
2-HE_2	2-hydroxyestradiol
2-ME_2	2-methoxyestradiol
4-HE_2	4-hydroxyestradiol
4-HE_2-d_5	deuterated 4-hydroxyestradiol
5β-C	5β-cholestane
B	longitudinal diffusional spreading
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
C4	carbon atom 4
CE	catechol estrogens
C_m	resistance to mass transfer in the mobile phase
COMT	catechol *O*-methyl transferase
C_s	resistance to mass transfer in the stationary phase
CV	coefficient of variation
CYP450	cytochrome P450
DEE	diethylether
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid

E_1	estrone
E_2	estradiol
E_3	estriol
ECD	electron capture detection
EI	electron impact
EIC	extracted ion chromatogram
FID	flame ionisation detection
FSH	follicle-stimulating hormone
GC	gas chromatography
GC-FID	gas chromatography with flame ionisation detection
GC-MS	gas chromatography – mass spectrometry
GSH-S-transferase	glutathione S-transferase
H	theoretical plate height
HCl	hydrochloric acid
HPLC	high pressure liquid chromatography
i.d.	internal diameter
kPa	kilopascal
LH	luteinising hormone
MCF-12A	Michigan Cancer Foundation cell line 12A
MCF-7	Michigan Cancer Foundation cell line 7
MEME	minimum essential medium eagle
MS	mass spectrometer
MTBSTFA	N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide

N	number of theoretical plates
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
$O_2^{\bullet-}$	superoxide radical
PBS	phosphate buffered saline
ppb	parts per billion
ppm	parts per million
ppt	parts per trillion
RIA	radioimmuno assay
RNA	ribonucleic acid
ROS	radical oxygen species
R_s	resolution
SCOT	support coated open tubular
SIM	single ion monitoring
t-BDMS	t-buthyldimethylsilyl
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDLU	terminal ductal lobular units
TIC	total ion chromatogram
TMS	trimethylsilyl
TMSI	trimethylsilylimidazole
WCOT	wall coated open tubular

1 Introduction

Cell replication is a process that is monitored and thoroughly regulated in multicellular organisms. In an organism that is still growing, cell growth exceeds cell death, so that the organism can increase in size. In an adult organism, cell death and cell replication are in equilibrium, which creates a steady state [1]. Occasionally the control mechanisms in cell multiplication break down, causing inappropriate cell growth and division [2]. The descendents of such cells inherit this capability to grow and divide uncontrolled. Subsequently a mass called a tumour forms [1, 2]. Tumour cells show certain characteristics.   These characteristics include the following: a) invasiveness and spreading; b) cell-to-cell interaction alterations; c) lack of normal control mechanisms [1, 2, 3].

a) Invasiveness and spreading

Tumours that are localized cause no threat to their host and are termed benign. An example of such a tumour is a wart [1]. Tumours become life threatening when they spread throughout the body. Tumours with this behaviour are termed malignant and they are the cause of cancer [2]. Benign tumours may become malignant [1].

Benign tumours closely resemble normal cells as they are well differentiated and the surface interactions hold the benign tumour localized to the tissue of origin [1, 2]. Normally a fibrous capsule surrounds the benign tumour [1]. Serious medical problems

may occur when the sheer bulk of a benign tumour interferes with normal tissue function, or when it secretes excess amounts of a certain substance, for example a hormone [2].

Malignant tumours are invasive and do not remain localized or encapsulated [1,2]. When these tumour cells establish secondary areas of growth at a site away from the original place, it is called metastasis [1,2]. Malignant cells are less differentiated than benign tumour cells, and they can lose most of their tissue specific functions [2]. Malignant tumours are classified as carcinomas if they derive from the endo- or ectoderm. Sarcomas are malignant tumours derived from the mesoderm [1].

Cancer cells have abnormal and unstable chromosome numbers [1]. They exhibit the characteristics of rapidly growing cells [2] and have a high nucleus-to-cytoplasm ratio, prominent nucleoli, high mitotic indices and little specialized structure [1].

b) Cell-cell interaction alterations

The normal restrictions by which specific cell types are localized to their specific organs are maintained by cell-cell interactions and physical barriers [1, 3]. The basal lamina is the primary physical barrier that keeps cells in place [2]. It underlies epithelial cells, as well as endothelial cells, of blood vessels [1]. Malignant cells contain elevated levels of receptors specific for the basal lamina proteins as well as enzymes that digest the proteins and collagen [1, 3, 4]. They can thereby breach this barrier and spread to surrounding and distant areas of the body.   Malignant cells can also elude the immune system through defence mechanisms, enabling them to render the immune system ineffective [1].

c) Lack of normal control mechanisms

Cell growth and cell division are highly regulated processes. In malignant cells these regulatory mechanisms are either lacking, or are rendered non-functional because of mutations; for example: mutations in the regulatory protein genes [1, 3, 4]. The cells also do not require all the growth factors necessary for normal cells to grow [3].

1.1 Breast Cancer

Breast cancer is one of the most common cancers affecting women [5]. It remains the leading cause of death in American women from 30 – 70 years of age and approximately 10% of the women living in western countries will develop breast cancer during their life time [6, 7].

1.1.1 Etiology of Breast Cancer

Carcinogenesis is considered to be a multi-step process, occurring often as a consequence of chronic exposure of cells to agents capable of inducing mutations by changing DNA base structures [8]. Between 5 - 10% of all breast cancer is hereditary [9, 10]. The majority of the cases, however, are sporadic.

The susceptibility of the breast to carcinogenesis is related to which developmental stage the breast is in when it is exposed to the mutagenic agents [10]. Immature breasts are especially susceptible to carcinogenesis [10]. The most undifferentiated structures are found in the breast of young nulliparous. These structures are referred to as terminal

ductal lobular units (TDLU) and they are the structures identified as the origin of ductal carcinomas [10]. The process of differentiation of the TDLU has a protective effect against breast cancer development and this effect is permanent [11].

The failure of eradicating breast cancer is mainly because:

- no single etiological agent has been identified

- uncertainty about the time of initiation exists

- the precise molecular mechanism responsible for cancer initiation and progression remains to be elucidated [12].

There is, however, substantial evidence implicating breast cancer risk factors. In addition to genetic factors, these risk factors mainly include environmental and hormonal components.

A number of environmental chemicals are suspected of contributing to breast cancer, but no single environmental chemical has been identified as a strong inducer of breast carcinogenesis [13]. The information derived from human studies, does not support evidence that environmental estrogen exposure and other chemicals in the air or drink water have a large impact on risk of breast cancer [14, 15]. Single large-dose ionising radiation (such as an atomic bomb), and repeated exposures from therapeutic regimes (*e.g.* treatment for certain cancers) are established causes for breast cancer [15]. There is also evidence from animal studies revealing a link between electromagnetic fields,

melatonin, and breast cancer *in vitro* and *in vivo*, although for humans there is little experimental evidence [15].

Estrogens are a family of female hormones involved in the reproductive function of the human body. Some estrogens have been associated with the induction and promotion of mammary tumours in certain circumstances. The estrogen levels in postmenopausal women correlate with body mass index [16, 17]. There is therefore a correlation between obesity and breast cancer risk in postmenopausal women [16, 18]. Increased breast cancer risk is also associated with increased central adiposity, (which is independently of relative body weight), in postmenopausal women - particularly if there is a family history of breast cancer [18].   However, in premenopausal women, there is an inverse relationship between body mass index and breast cancer risk, as well as no relation of breast cancer risk, with the adipose tissue distribution [17, 18].

1.1.1.1   Estrogen-related breast cancer

The more the breast epithelium is exposed to estrogen, the higher the chance for the formation of tumours. Factors that put one at risk for breast cancer (risk factors) can be seen as the level of estrogen that breast epithelium is exposed to over time [13, 17, 19, 20, 21, 22, 23]. Table 1 gives a summary of the established risk and protective factors for breast cancer.

Alcohol consumption shows a linear relationship to breast cancer incidence with women who consume 2-5 drinks per day. It is postulated that alcohol increases estrogen plasma

levels as well as insulin- like growth factor levels [19]. The main site for postmenopausal estrogen synthesis is in adipose tissue (conversion of androstenedione to estrogen). With increased postmenopausal obesity, estrogen production is increased because of the higher amount of adipose tissue, thereby increasing the risk of breast cancer [19, 21].

Table 1: Summary of the established risk and protective factors for breast cancer [25].

Risk factors	Protective factors
Early menarche	Young age at first full term pregnancy
Late menopause	Prolonged lactation
Alcohol consumption	Exercise
Postmenopausal obesity	Number of pregnancies
Hormone replacement therapy	

Above normal physical activity can delay menarche, thereby decreasing the number of ovulatory cycles the breast tissue is exposed to. It can also reduce the frequency of ovulatory cycles and circulating hormone levels [19]. During pregnancy, the high levels of circulating sex hormones cause differentiation of the TDLU thereby protecting it against tumourigenesis [11]. There also seems to be a protective effect of prolonged breast-feeding against breast cancer risk [24]

The main estrogens found in the human body are estrone (E_1), estradiol (E_2) and estriol (16α-hydroxyestradiol, E_3). Estradiol is the most abundant estrogen in premenopausal woman. Figure 1 provides a summary of the metabolism of 17β-estradiol [20, 21]. The reversible conversion between estradiol and estrone is catalysed in both directions by 17β-hydroxysteroid dehydrogenase (17β-HSD) [12, 20].   In the human uterus, the conversion favours the formation of estrone, whereas in breast tissue, the conversion

favours the formation of estradiol [20].   17β-HSD is a group of intracellular isozymes

that is widely distributed in human tissue.   Interconversion between the two hormones

has long been regarded as an important regulatory mechanism for the modulation of

estrogen action in tissues [20].   The reductive activity of 17β-HSD (formation of

estradiol) is increased in breast tumours compared to that in normal breast tissue.   This

can be one of the ways in which the tumour ensures a favourable environment for growth.

It is known that by ablation of estradiol, breast tumours become more sensitive to

estradiol. This is because of increased receptors as well as increased 17β-HSD activity in

the breast tumour [20, 25].
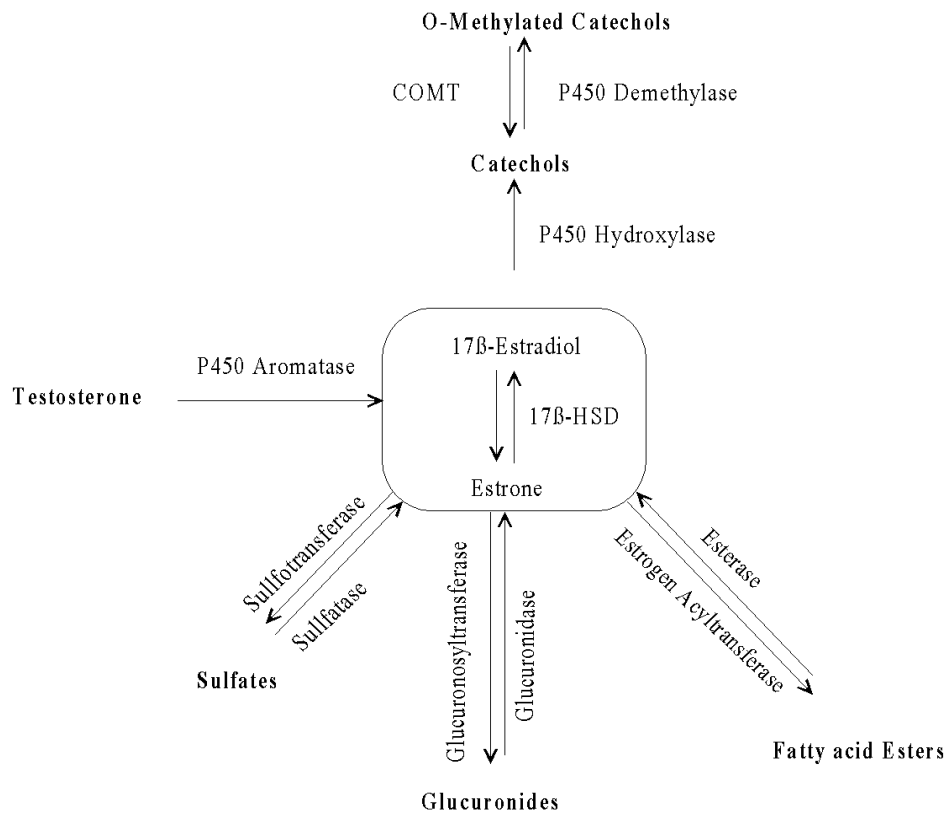
Figure 1: Summary of the metabolism of estradiol under discussion [20].

Estradiol (E_2) can mediate carcinogenesis via two distinct pathways [12, 13, 26, 27, 28]. These two pathways are not mutually exclusive, but can rather be seen as complimenting each other [26]. The first pathway involves the receptor-mediated stimulation of cell proliferation. Because of the enhanced cell proliferation, an opportunity is created for the accumulation of random errors in DNA replication. Mutations may occur more easily, giving rise to the malignant phenotype [12, 13, 19, 26, 27, 28].   The second pathway involves the formation of quinones and radical oxygen species (ROS), which can lead to DNA damage and mutation [12, 13, 26, 27, 28]. This pathway will be discussed in the section addressing 4-hydroxyestrogens (see section 1.1.2.2.2).

Initially it was thought that estradiol itself was responsible for tumourigenesis [29, 30]. In women younger than 45, an increased incidence of breast cancer was noted because of earlier onset of birth control usage. Estradiol levels were also found to be higher in malignant tissue than in normal tissue. It has since been discovered that the catechol metabolites (metabolites that are hydroxylated on the C2- or C4 position) of estradiol and other estrogens cause carcinogenesis   (carcinogens increase the amount of catechol estrogens that are formed) [31]. The pathways responsible by which these catechol estrogens (CE) can cause carcinogenesis are discussed in sections 1.1.2.2.1 and 1.1.2.2.2. It was believed that *O*-methylation of these CE was part of the breakdown and inactivation of active hormones, rendering "biologically inactive" metabolites. The catechol estrogens (CE) are very unstable and they are rapidly *O*-methylated or metabolised in other ways [20].

1.1.2 Estrogen Metabolism and Breast Cancer

There is a difference between the intracellular- and blood concentration of E_2 and its metabolites [32]. In post-menopausal women, the plasma level of estradiol is very low, but the intratumour levels can be up to 50 times higher than that of the circulating estradiol [28]. The levels of estradiol in tissue are close to the levels observed in plasma for premenopausal women [28, 33]. Malignant tissue showed to have a higher concentration of estrogen than normal tissue in pre- and postmenopausal woman [33]. (table 2) Estrogen biosynthesis in estrogen target tissues may contribute significantly to these levels [28].

Table 2: Summary of the E_2 concentrations in various tissue of pre- and pos-tmenopausal woman [33]

Menstrual Status	E_2 Concentration (pmol/ml)			
	Plasma		Normal Tissue	Malignant Tissue
Premenopausal	Follicular phase	0.62	0.25	0.40
	Luteal phase	0.87		
	Ovulatory phase	2.00		
Postmenopausal	0.12		0.25	0.50

1.1.2.1 Aromatisation of androgens

The aromatisation of androgens to estrogens is catalysed by aromatase (estrogen sythetase, CYP450 19) [20, 34]. The conversion of androgens to estrogens is the rate-limiting step in the production of endogenous estrogens. Aromatase activity is found in the ovary, placenta and several non-endocrine tissues such as brain, adipose tissue, liver, fibroblasts and mammary glandular cells [20]. The conversion of androstenedione to estrone shows a positive correlation with obesity and age [20]. This suggests that adipose tissue is a major site for estrogen biosynthesis in post-menopausal women and elderly

men. The estrogen production in adipose tissue is unlike that of the ovaries, as it is continuous and non-cyclic [20]. Aromatase activity is higher in the adipose tissue surrounding mammary carcinoma cells than in that surrounding normal breast tissue [20]. This suggests that the aromatisation of androgens is an important source of estrogen for mammary tumours [20]. Women who develop breast cancer in their premenopausal years tend to have subnormal androgen levels, whereas in women who develop breast cancer in their postmenopausal years the situation seems reversed [34].

1.1.2.2 Hydroxylation of estrogens

The hydroxylation of estradiol is catalysed by members of the cytochrome P450 (CYP450) family, which are nicotinamide adenine dinucleotide phosphate (NADPH)-dependent mono-oxygenases [13, 20]. At least nine different isoforms of CYP450 have been detected in the breast tissue of female rats [20]. They are situated in the membrane of microsomes that are found in the target organs [20, 21, 25]. The isozyme specific for hydroxylation on the C4 position is cytochrome P450 1B1. Estradiol has been found to induce tumours in those target organs where this enzyme is predominant. The isozymes specific for the 2-hydroxylation of estrogens are CYP450 1A1/1A2 [13, 35]. Target organs in which these enzymes predominate are not susceptible to estradiol-induced tumourigenesis. Another CYP450 isozyme, CYP450 3A, catalyses the formation of both 2- and 4-HE_2. It generates 85% 2-HE_2 and 15% 4-HE_2 and is situated mainly in the microsomes of the liver [35].

1.1.2.2.1 2-Hydroxylation of estrogens

2-HE$_2$ has little or no carcinogenic activity compared to 4-HE$_2$ [20], but it has more proliferating action than E$_2$ itself [36]. 2-Hydroxylation of estradiol is a major metabolic pathway in the liver as well as in some extra-hepatic tissues. The concentration of unconjugated 2-HE$_2$ metabolites is very low in the blood and in several other tissues. This is probably because of the instability of this hormone and its quick *O*-methylation by catechol *O*-methyltransferase (COMT) and subsequent urinary excretion [32]. The decreased carcinogenic activity of 2-HE$_2$ compared to 4-HE$_2$ can possibly be ascribed to the fact that the *O*-methylation of 2-HE$_2$ is faster than that of 4-HE$_2$, and it has a more rapid clearance [20, 21].

Several important consequences of locally produced 2-HE$_2$ and 2-HE$_1$ have been suggested. Both of these CE can bind to the classical estrogen receptor but they have a markedly reduced binding affinity compared to estradiol [20]. They also possess a much weaker hormonal potency for certain functions of estrogens [20]. 2-HE$_1$ partially antagonises the growth stimulatory effect of estradiol in MCF-7 breast cancer cells [20]. This effect may be due to the competitive nature of binding to the estrogen receptor or it can be due to the formation of free radicals, which are highly cytotoxic [20]. 2-HE$_2$ and 2-HE$_1$ generate free radicals when they undergo metabolic redox cycling, which may cause damage to DNA and other cell structures (figure 2) [20]. 2-HE$_2$ also serves as a metabolic co-oxidant, which strongly stimulates the metabolic oxidation of arachidonic acid to prostaglandins in the uterus during certain stages of pregnancy [20]. 2-HE$_2$ may thereby modulate the action of arachidonic acid and prostaglandins during pregnancy.

Because the hydroxy estradiols are O-methylated by the same enzyme as the catecholamines, the substrates for the enzyme compete for the active site (competitive inhibition) [20]. Thus 2-HE$_2$ inhibits the O-methylation of catecholamines. Of the two substrates, the catechol estrogens have the highest binding affinity to the enzyme and of the catechol estrogens, 2-HE$_2$ has the highest binding affinity [20]. The implication of this competitive inhibition is that 2-HE$_2$ may have a modulatory effect on the effects of catecholamines in the central nervous system and intracellular signal transduction [20]. 2-HE$_2$ alters the secretion of prolactin and luteinizing hormone/follicle-stimulating hormone (LH/FSH) by the anterior pituitary [20]. Both 2-HE$_2$ and 2-HE$_1$ modulate the affinity of dopamine for its receptor, thereby also exerting an effect on prolactin secretion [20]. 2-HE$_2$ is also found in the follicular fluid of humans and horses and an autocrine role for this hormone in follicles has been proposed [20].

1.1.2.2.2 4-Hydroxylation of estrogens

Recent studies showed that 4-hydroxylation is a dominant pathway in some extra-hepatic target tissue including breast and uterus [20]. Several important functions of 4-HE$_2$ are already known or have been suggested. 4-HE$_2$ is similar to E$_2$ in its binding ability and activation of the estrogen receptor. It also has a slower dissociation rate, creating a longer lasting effect, because of prolonged stimulation of estrogen receptors [20]. 4-HE$_2$ stimulates uterine growth when it is injected into animals, but it has lower uterotropic potency than estradiol itself. When 4-HE$_2$ is administered, the secretion of LH/FSH by the adenohypophysis is altered [20]. It also supports embryo implantation in mice. Like 2-HE$_2$, 4-HE$_2$ also serves a co-oxidation function in the metabolism of arachidonic acid to prostaglandins during certain stages of pregnancy [20]. 4-HE$_2$ is not O-methylated that

rapidly, which contributes to its prolonged effects [20, 27]. It also undergoes metabolic redox cycling, generating free radicals, which can cause DNA damage and initiate tumourigenesis (figure 2). $4-HE_2$ is a stronger carcinogen than $2-HE_1$ and $2-HE_2$ because it generates more free radicals and has a higher proliferating effect on cells [20, 26, 27, 28]. After estradiol has been metabolised to $4-HE_2$, it can undergo further oxidation if molecular oxygen is present. When this occurs, estradiol-3,4 quinone is formed [26, 27 28, 37]. These quinones react with purine bases causing DNA damage [26, 27, 28]. This is the second pathway which estrogen mediates induced carcinogenesis. The formation of quinones is a reversible reaction and the enzyme responsible for this reversal is a quinone reductase enzyme [27]. Oxido-reduction between catechol estrogens, their semiquinones and their quinines produces reactive oxygen species, which can damage DNA (figure 2) [26, 27, 28, 37].

Estradiol-3,4 quinone may react with DNA to form depurinating adducts, while estradiol-2,3 quinone reacts to form stable adducts [27]. DNA adducts form when metabolically activated compounds, such as the catechol estrogen quinones, with nucleophilic groups, react with the two purine bases, namely adenine and guanine (figure 2) [12, 27]. These adducts can either be stable or depurinating. Stable adducts remain covalently bound to DNA unless it is removed by the DNA repair systems. Depurinating adducts are spontaneously released from the DNA because of the destabilization of the glycosidic bond between the base and the sugar [27]. This type of adduct is formed when the metabolically activated catechol estrogen binds to the N-3 or N-7 position of adenine, or the N-7 or C-8 position of guanine (figure 2) [27]. Depurinating adducts cause the

purine base to be released from the DNA strand [27]. This causes faulty replication and

subsequent mutations.
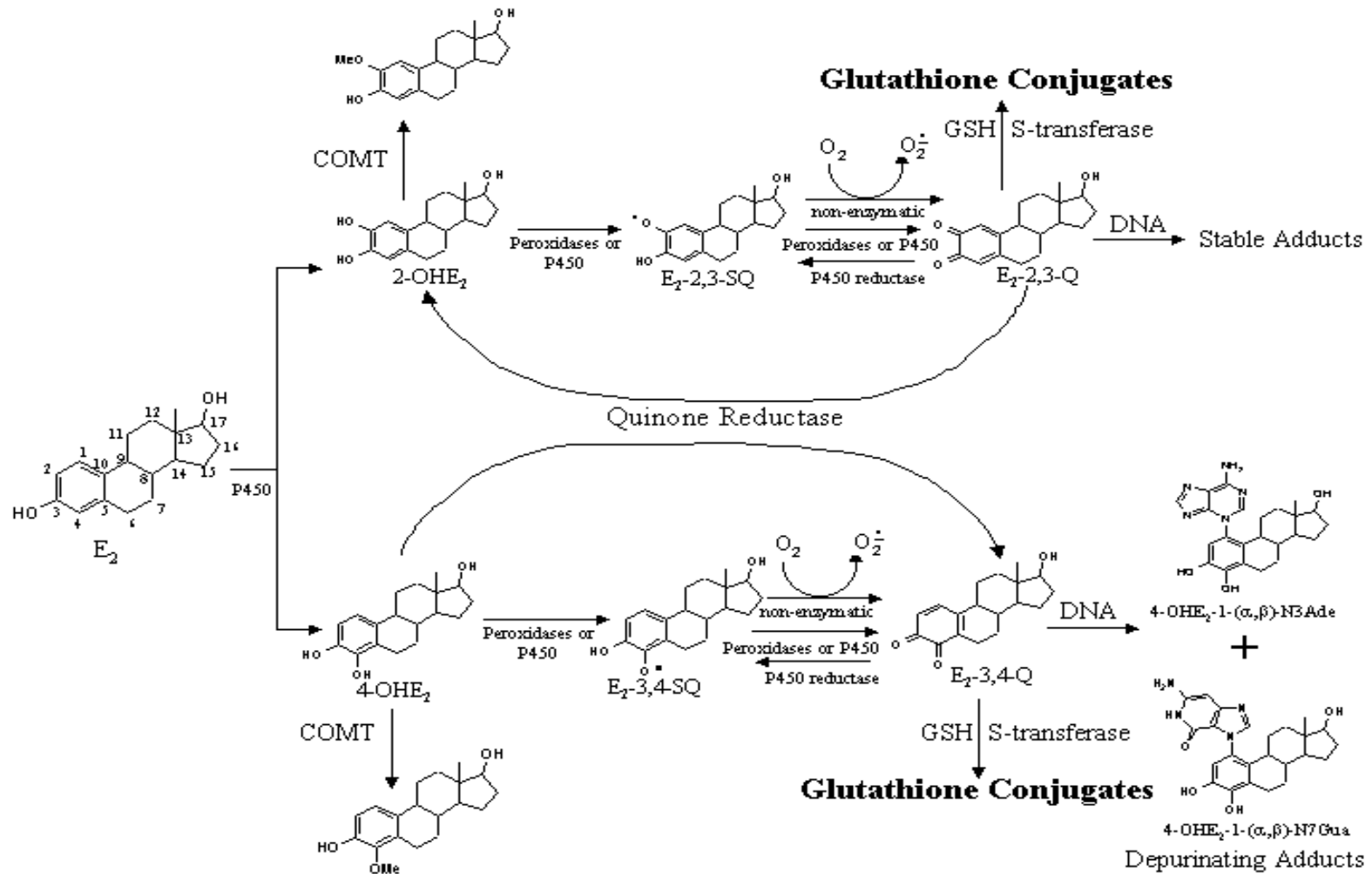
Figure 2: Activating and deactivating pathways of estradiol metabolism and the formation of DNA adducts [27].

Normal cellular processes continuously form and degrade oxidants. Cells have an extensive antioxidant defence system, but when ROS are formed when it is not called for, and in extensive amounts, the negative effects of these ROS become apparent [27]. The major type of damage caused by ROS are the oxidation of the genetic material [27]. Normally, oxidized bases are repaired through various repair enzymes. Interestingly, conditions leading to continuous elevation of oxidized bases are the same as those that induce tumour promoting processes [27].

2- and 4-HE_2 can be oxidized to semiquinones. In the presence of molecular oxygen, it is further oxidized to quinones (figure 2) [27]. This oxidation leads to the formation of superoxide anion radicals ($O_2^{\bullet}$). These $O_2^{\bullet}$ readily form H_2O_2. This can either happen spontaneously or enzymatically via superoxide dismutase [27]. In the presence of Fe^{2+} and Cu^{2+} ions, H_2O_2 is converted to the most powerful of oxidants, hydroxyl radicals ($\bullet OH$) [27]. H_2O_2 can readily cross the cell membrane because it is a neutral molecule. It can therefore cause damage to the DNA of neighbouring cells [27]. Quinones and semiquinones are capable of redox cycling as long as molecular oxygen is available. A small amount of E_2 may thus have substantial ROS production and subsequent cellular damage [26, 27].

Quinone reductase, an enzyme situated in the cells can reduce quinones back to catechols using NADH as a cofactor (figure 2). This enzyme therefore acts to counter the ROS formation [27]. COMT may prevent the oxidation of catechols by methylating the 2- or 4-hydroxyl groups. 4-HE_2 however, is not methylated that readily while 2-HE_2 is

methylated very rapidly. This results in the predominance of 4-HE$_2$ in redox cycling. Furthermore, 2-HE$_2$ inhibits the methylation of 4-HE$_2$ by COMT, which allows for the accumulation of 4-HE$_2$ [20, 27].

It has been found that 4-HE$_2$ levels are higher in tumours of the breast than in normal breast tissue [38]. Interestingly, target organs with a high level of CE are more susceptible to estrogen-induced carcinogenesis [20, 36]. It has also been shown that the differential formation of 2- and 4-HE$_2$ correlates with an organ's resistance or susceptibility to estrogen-induced carcinogenesis [34]. While 4-HE$_2$ shows increased expression in benign or malignant neoplastic tissue compared to normal tissue, 2-HE$_2$ expression remains fairly constant between tissues [21]. It is thus the relationship between 2-HE$_2$ and 4-HE$_2$ that should be considered as a marker for breast cancer risk. The ratios of 4-HE$_2$/2-HE$_2$ formation in breast adenocarcinoma and fibroadenoma were found to be 3.8 and 3.7 respectively, whereas in normal tissue the ratios were 1.3 and 0.7 respectively [31]. This shows an increased 4-HE$_2$ formation with respect to that of 2-HE$_2$, which gives an indication of tumourigenic activity in breast tissue.

1.1.2.3   16α-Hydroxylation of estrogens

In humans, CYP450 3A4 has strong 16α-hydroxylation catalytic activity with respect to estrones [20].   16α-Hydroxyestrogens possess unique properties and is not only hormonally active, but also chemically active [21]. 16α-HE$_1$ and 16α-HE$_2$, like 4-HE$_2$ have potent hormonal activity by binding to the estrogen receptor [20]. However, a covalent reaction takes place between the receptor and 16α-HE$_1$, leading to prolonged

stimulation of oncogene expression and growth [20]. The idea that 16α-HE$_1$ plays an important role in estrogen-induced mammary cancer is weakened by the low carcinogenic activity of this hormone compared to 4-HE$_2$ [20]. Thus, the emphasis has shifted from the absolute extent of 16α-hydroxylation to the ratio of 16α- to 2-hydroxylation as a possible biomarker for breast cancer risk [20]. The major reason why enhanced 2-hydroxylation of estradiol is associated with reduced breast cancer risk, is because of the rapid *O*-methylation of 2-hydroxylated estradiol to 2-ME$_2$. This can either be because 2-HE$_2$ is only available for short periods of time, or because of the characteristics of 2-ME$_2$ as discussed in the next section (1.1.2.4).

1.1.2.4   *O*-Methylation of estrogens

The *O*-methylation of the CE is catalysed by COMT, which is the same enzyme responsible for the methylation of catecholamines [13, 20, 21]. COMT is situated almost exclusively in the cytosol of target tissues, but some activity can be found in the membrane bound form [20]. *O*-Methylation of the hydroxyl group causes the previously hydroxylated carbon atom to become methoxylated, *e.g.* 2-HE$_2$ becomes 2-ME$_2$. *O*-Methylation of catechol estrogens inactivates their estrogenic potential and their ability to be further oxidized to their quinones and semiquinones [13]. Because of the rapid *O*-methylation of 2-HE$_2$, 2-ME$_2$ was found to be the most abundant estrogen in human plasma and urine.

2-ME$_2$ has little or no estrogen receptor binding affinity compared to estradiol. It had been regarded as a waste product with no biological activity until it was discovered that

2-ME$_2$ has very potent anti-tumour activity [13, 23, 29, 36, 39].   It exerts unique biological effects that cannot be contributed to estradiol, 2-HE$_2$, 4-HE$_2$ or any other *O*-methylated form of E$_2$.   However, it does not require the presence of the estrogen receptor to exert its effects. Thus, 2-ME$_2$ is the only endogenous estrogen metabolite that has potent non-specific anti-tumour activity *in vivo* [20, 21]. Cell lines that showed a decrease in cell proliferation after exposure to 2-ME$_2$ include MCF-7 (human breast adenocarcinoma), HeLa (human cervix carcinoma), fibroblasts, epithelial and smooth muscle cells [40]. In rodents, therapeutic doses of 2-ME$_2$ caused tumour regression, but not side effects normally associated with chemotherapy (*e.g.* weight loss, hair loss or loss of appetite) were observed [41].

There are three possibilities whereby 2-ME$_2$ can exert this anti-tumour activity. Firstly, 2-ME$_2$ binds to the colchicine-binding site on tubulin, thereby inhibiting microtubule polymerisation [20]. This leads to the disruption of spindle formation with incomplete spindles and fragmented spindle poles [20].   This causes incorrect chromosome distribution and subsequent mitotic catastrophe [20]. During the next round of mitosis, the presence of micro- and multi-nuclei causes the cells to undergo apoptosis [20, 21, 40, 41]. Secondly, 2-ME$_2$ causes direct induction of apoptosis via the extrinsic pathway (Death Receptor 5 - DR5) [40, 41]. Thirdly, it has anti-angiogenic activity *in vivo*. Fotsis *et al.* found in 1994 that 2-ME$_2$ showed anti-angiogenic activity in metastatic models in mice [40, 42]. Kothakota *et al.* showed in 1997 that 2-ME$_2$ controls metastatic invasion of cancer [40, 43]. In 2000, Pribluda *et al.* showed that 2-ME$_2$ regulates various steps in

the angiogenesis cascade [40, 44] and in 1999 Chauhan *et al.* showed it has anti-angiogenic activity in multiple myeloma cells [40, 45].

The high specificity with which 2-ME$_2$ exerts its effects suggests the existence of a specific receptor for 2-ME$_2$ [20, 21].   Such a receptor remains to be found. The equilibrium of the reaction catalysed by COMT favours the formation of 2-ME$_2$ from 2-HE$_2$ [39]. The plasma level of 2-ME$_2$ exceeds the levels of 2-HE$_2$ by one or two orders of magnitude [20, 39]. Lower levels of COMT in breast tumours may reflect higher concentrations of carcinogenic estrogens and a decrease in 2-ME$_2$ concentration. As indicated above, high levels of CE inhibit COMT and can, therefore, cause lower activity of COMT. The latter can be associated with a decrease in 2-ME$_2$ levels and particularly with an accumulation of 4-HE$_2$, increasing the risk of tumour [20]. Figure 3 shows a diagram of the beneficial effect of 2-hydroxylation of estrogens with subsequent *O*-methylation on estrogen-induced carcinogenesis.   Some studies also show that inducing 2-hydroxylation of estradiol may decrease spontaneous tumourigenesis in estrogen-sensitive tissues [20].
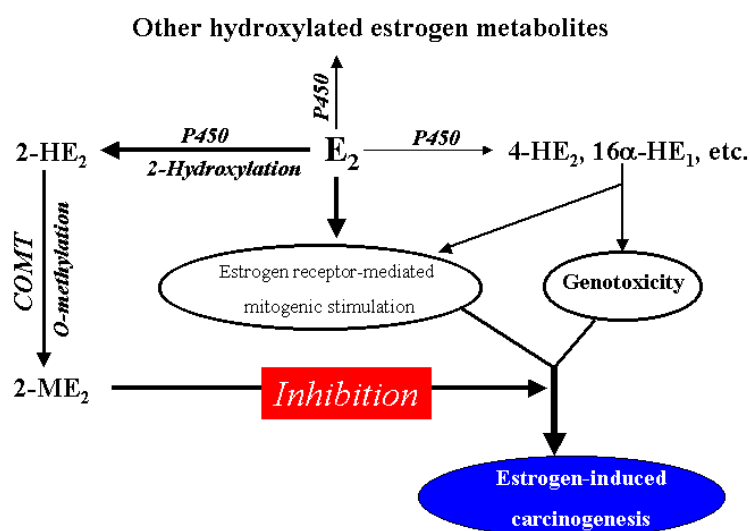
Figure 3: The effects of estradiol 2-hydroxylation with subsequent *O*-methylation on cancer [21].

It has been found that in certain instances COMT activity was higher in tumours than in normal tissue [21, 36, 46, 47]. An increased formation of 2-ME$_2$ by the breasts may be an important endogenous mechanism that attempts to suppress uncontrolled proliferation and thus acts as a defence mechanism against tumourigenesis.

The conversion between estradiol and estrone can also be found in the 2-hydroxylated forms of these hormones and can possibly be another pathway by which 2-ME$_2$ is formed. Interestingly, 17β-HSD activity is higher in mammary tumours than in normal breast tissue. This can confirm the statement that an increased formation of 2-ME$_2$ can be an endogenous defence mechanism against tumours in breast tissue [21].

There is an interesting correlation between breast cancer and stress that is worth mentioning. It lies in the fact that catecholamines such as adrenaline and noradrenaline, which are formed during stress, have an inhibitory effect on COMT. This is part of a negative feedback mechanism in which catecholamine levels are controlled, since COMT is also responsible for one of the reactions in their formation pathway. Thus the 2-ME$_2$ levels will decrease and the CE will accumulate in target organs causing an increased risk for cancer of these organs [20].

A single gene encodes human COMT with a wild type allele and a variant allele [21]. The variant allele is responsible for a lower activity form of COMT [13, 20, 23]. It is anticipated that individuals having the variant allele are more susceptible to estrogen-induced breast cancer, because of lower 2-ME$_2$ levels and accumulation of CE in target

cells [1, 20, 23]. There are marked person-to-person variations in COMT activity and it is necessary to correlate the level of COMT activity with the risk of estrogen-induced breast cancer [20].

1.1.2.5   Conjugation of estrogens

Conjugating enzymes in the liver and target cells cause the conjugation of estradiol and estrone to their glucuronides and sulphates. This in turn results in a decrease in their hormonal activity and marks them for excretion [20]. Sulphonated estrogens have almost no estrogen receptor binding affinity [20]. β-Glucuronidase catalyses the conversion of estrogen glucuronides to their parent hormones and β-glucuronidase inhibitors have been shown to suppress mammary tumour promotion [20].

1.2 Problem Statement

Of the metabolites discussed previously, 4-HE$_2$ is a potent cell proliferating estrogen. 2-ME$_2$ on the other hand is a potent inhibitor of cell proliferation. 2-HE$_2$ also causes increased cell proliferation, but it is not as potent as 4-HE$_2$, is labile and is rapidly *O*-methylated to 2-ME$_2$. Therefore a significant ratio to consider as a biomarker for breast cancer risk is the 4-HE$_2$/2-ME$_2$ ratio. The determination of this ratio can either be done through enzyme activity assays or by measuring the levels of the specific metabolites in question. Enzyme activity assays have to be performed on intracellular material and therefore requires surgical (invasive) intervention. If the ratio of 4-HE$_2$ and 2-ME$_2$ concentrations is constant in interstitial fluid, intracellular fluid and plasma, the determination of these compounds in plasma would be a non-invasive method to determine the risk for breast cancer.

The measurement of these metabolites requires the development of sensitive analytical methods able to measure sub µg/l (ppb) concentration levels, because the metabolites are present at very low concentrations extracellularly. The aim of this study is therefore to develop such methods in a simplified system, employing *in vitro* experimental conditions.

1.3 Analytical Procedures

Estradiol metabolites were initially quantitated with radioimmunoassays (RIA) and enzyme assays [47]. The RIA assays, however, can only be used when radioactively labelled antibodies specific for the metabolites are available. Enzyme assays are still being used, but specific concentrations of metabolites cannot be determined due to the nature of this assay. Enzyme activity is determined by adding substrate and measuring the tempo of product formation. Some assays make use of COMT to measure 2-HE_2 levels by measuring the 2-ME_2 formed. This is not practical in this scenario since 2-ME_2 is one of the metabolites of interest [47]. Later methods made use of gas chromatography (GC) with flame ionisation (FID) and electron capture detection (ECD). The advantage of using GC is that a number of metabolites can be analysed simultaneously. Currently the most powerful technique for the measurement of these metabolites is GC-MS due to its excellent sensitivity and selectivity. Mass spectra are "fingerprints" of the specific compounds, making identification far more accurate [48].

1.3.1 Gas chromatography (GC)

The GC provides a simple, rapid and reproducible method for separation of volatile compounds therefore making the analysis of complex samples possible [48].

1.3.1.1   The Inlet System

An inert carrier gas (He, H or N) passes through the inlet system into the column at a constant flow rate. The samples are injected into the capillary column by a microlitre syringe, which is forced through a rubber septum at the injection port in the inlet system [48, 49, 50]. The inlet is heated so that the sample vaporises instantaneously and can be passed into the column by the carrier gas [48, 49, 50]. The introduction of the sample into the column is considered to be the most problematic part of GC. A few inlet systems have been developed, each with its advantages and disadvantages. In this study the split/splitless inlet system was used. It is a system that can change between split injection and splitless injection.

1.3.1.1.1 Split injection

Split injection is used to prevent the capillary columns from being overloaded. In this mode, only a part of the sample enters the column, while the rest is vented. This, however, affects quantitation negatively, as less of the sample is present in the column and the response will therefore be lower [51, 52]

1.3.1.1.2 Splitless injection

With splitless injection, the sample is not split initially - allowing more of the sample to enter the column [51, 52]. This, however, causes peak tailing, which reduces resolution. The split vent is opened 0.5-1min after injection, thereby "blowing" away the peak tails

[51].   Splitless injection provides better quantitative results, however, resolution is compromised [52].

1.3.1.2   The column

The analytical column is attached to the injection port or inlet system [49, 50]. The columns can either be packed with liquid coated particles, or the liquid phase is deposited directly on the walls of the long fused silica capillary tubing [49, 50, 52]. Packed columns are best for simple mixtures and have a short analysis time, while capillary columns are best for complex mixtures which require more resolution, but they have a longer analysis time [52].

The typical length of a packed column is 2 m and the inside diameter is 2 mm. The column is filled with fine particles of packing material that is coated with the liquid phase. This gives rise to a multitude of paths that the mobile phase can follow between the particles, which causes peak broadening and sample mixing if the length of the column is increased. This phenomenon is called the multiple path effect and sets an upper limit to the packed column length of about 3 to 4 m [52]. Packed columns however, are no longer commonly used because of the good results obtained with capillary columns.

Capillary columns have no packing material, the liquid phase is bonded directly onto the wall of the column (WCOT) or to a support material that is bonded to the column wall (SCOT). The multiple path effect is eliminated with these columns and due to the lower

pressure drop, their lengths can be increased.   Lengths of 25 to 50 m are the norm. The column efficiency is also increased due of the absence of the multiple path effect, as well as the fact that capillary columns are "open", and thus have a smaller pressure drop and better resolution.   The internal diameter must be small in order to assure effective interaction between the gas and liquid phases [52]. The smaller the internal diameter, the less space there is for diffusion in the gas phase. The typical internal diameter is 0.25 – 0.5mm and gas flow rates are therefore also low: 0.1 to 1 ml/min. Because of these low flow rates a connection can be made directly into the mass spectrometer without compromising the vacuum. [52].   Capillary columns are, however, limited in their sample and solvent capacity, and it is therefore often necessary to split the sample at the injector to prevent column damage.

Chromatography is based on the distribution of a specific compound between two phases: a stationary phase and a mobile phase [49].   The partition or distribution of the compounds between two phases is dependent on the compound's solubility in the stationary phase as well as vapour pressure [49, 50]. The compounds of a mixture are swept along the fixed stationary phase by the mobile phase and due to their different interaction with the stationary phase, they will move along the column at different rates, which results in their separation [49, 50]. Interaction with the stationary phase depends on the solubility and vapour pressure. The higher the interaction, the more a compound will be retained [49, 50].

1.3.1.2.1 Stationary phases

A wide variety of stationary phases are commercially available. They range from non-polar to polar. The type of stationary phase used is determined by the chemical characteristics of the compounds that are to be separated on the column. Non-polar compounds will dissolve in non-polar stationary phases and therefore be retained according to their sollubilities, whereas polar compounds will not dissolve readily in a non-polar stationary phase and will be retained the least and vice versa. Steroids are non-polar compounds. Non-polar columns will have the best selectivity towards steroids i.e. they will be retained on a non-polar stationary phase. The stationary phase used in this study was a methyl silicone phase.

$$\begin{array}{ccc} CH_3 & CH_3 \\ | & | \\ -Si-O-Si-O- \\ | & | \\ CH_3 & CH_3 \end{array}$$
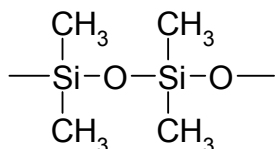
1.3.1.2.2 Variables affecting column performance

The variables that determine column performance or efficiency include column internal diameter, stationary film thickness, and selection of carrier gas [53]. Column efficiency is expressed in terms of theoretical plate number (N). The number of plates is the number of units in a column wherein equilibrium is established in each unit. Plate height (H) and resolution are also indicators of column performance. H is the theoretical indication of the length of column in which there is perfect equilibrium between the solutes in the mobile phase and the stationary phase [53]. Resolution is used to assess the separation of the chromatographic bands for two components [54]. Resolution is defined

as the ratio of peak resolution to the average peak width [54, 55]. For a certain column length, the shorter the plate height, the higher the plate number N, and increased resolution and separation is achieved [53,56].

H is also a measure of the band spreading during elusion and a function of carrier gas velocity [53]. The major sources for spreading of solutes in capillary columns are longitudinal diffusional spreading (B), resistance to mass transfer in the mobile phase (C_m) and resistance to mass transfer in the stationary phase (C_s). B increases if the diffusion coefficient in the mobile phase increases. C_m increases if the column radius is increased or if the diffusion coefficient in the mobile phase decreases. C_s increases if the film thickness of the stationary phase is increased or if the diffusion coefficient in the stationary phases decreases [53].

Separation is considered to be complete if two adjacent peaks are separated 95.4%. This gives a resolution of 1 [55]. Resolution is determined by the difference in retention time divided by the average peak width. Resolution is influenced by the number of theoretical plates as stated before as well as the separation factor (α) and the retention factor (k) [53].

$$R_s = \frac{\sqrt{N}}{4} \frac{\alpha - 1}{\alpha} \frac{k_2}{1 + k_2}$$

Thus, an increase in any of these factors will enhance the resolution. The retention factor (k) is the time the solute spends in the stationary phase, relative to the time it spends in

the mobile phase [55]. The retention factor can be increased by increasing the stationary phase film thickness, or by lowering the column temperature [55].

1.3.1.2.3 Mobile phase

In GC, the mobile phase is an inert gas also known as the carrier gas. The best carrier gas to use for GC would depend on the aim of the analysis. H_2 would be the good carrier gas to use if the speed of analysis needs to be fast [49]. In the case of a GC-MS however, H_2 cannot be used for safety reasons. He_2 gives a good balance between the speed of analysis and the resolution and is a safer gas to use because it is not so flammable.

1.3.1.2.4 Temperature

A programmed oven temperature program is used in this study. Raising the oven temperature during an analysis influences the diffusion coefficient of the solutes. If the elusion is started at a relatively low temperature, most of the compounds are soluble in the stationary phase and the solutes are retained at the inlet of the column. As the oven temperature is raised, the solubilities of the compounds will decrease and they will successively reach temperatures where they have a significant vapour pressure to elute - this provides a big advantage for compounds with different volatilities [54]. If an isothermal temperature chromatographic separation is performed on compounds that have a wide variety of boiling points, the compounds with the lowest boiling points will elute early with thin sharp peaks, and the compounds with higher boiling points will elute later and tend to have much broader and flatter peaks [54]. When a temperature programmed separation is used with the same compounds, the peak broadening of the compounds with

the higher boiling points is almost eliminated.   Increasing the temperature rate however, decreases column efficiency, but with capillary columns the resultant efficiency and resolution are normally still satisfactory [57].

1.3.1.3   Detectors

1.3.1.3.1 Flame Ionisation Detector (FID)

This detector is specific for all compounds that contain oxidizable carbon atoms [50]. It consists of a cube in which hydrogen and air can be mixed with the mobile phase that exits in the column [50]. These mixed gases are then burned in air, in an enclosure that is draught free [50]. Two electrodes are placed near or inside the flame. A constant potential difference is maintained over these two electrodes and the DC current between these electrodes is monitored [50]. The current is proportional to the amount of carbon that enters the flame [50]. This change in current is then presented as a chromatogram.

1.3.2 The GC-MS interface

Joining the gas chromatograph to the mass spectrometer is problematic since the outlet pressure of the GC is at atmospheric pressure, and the mass spectrometer inlet pressure must be at a vacuum of 10^{-5} Torr or less. The interface system must therefore reduce the carrier gas pressure about eight times while still delivering a useful quantity of the sample to the mass spectrometer [48]. The flow rates of capillary columns complement the coupling of a gas chromatograph to a mass spectrometer. The flow is not high enough

for the vacuum to be compromised. Only a transfer line from the GC to the MS is therefore necessary.

1.3.3 Mass Spectrometry (MS)

1.3.3.1   The ion source

The electron impact (EI) ion source was first used by A.J Dempster and was later further developed by A O Nier [58]. The electron impact ion source works in the following way: the vaporised sample flows at a reduced flow through an electron beam [48, 49, 52, 58]. A magnetic field is maintained over the electron beam to focus it. The gas molecules of the gaseous compound that enter this region interact with the electrons causing the ionisation or subsequent fragmentation of the molecules [48, 58]. The most commonly used voltage for the electrons are 70eV as the sensitivity is near its maximum and small changes in electron energy does not have an effect on the fragmentation of the molecules [48, 49, 52, 58]. Though this ion source is the first source developed, it remains the most popular because of its stability, ease of operation, control of beam intensity, lack of contamination problems; and sensitivity, and its lack of selectivity under electron impact conditions for a wide range of compounds [53]. There are, however, some drawbacks to this technique. One drawback is that some compounds fragment to such an extend that the molecular ion can be seen only weakly. In some cases it may even be totally absent [52].

The apparatus used for this study employs an electron impact ion source. There are other ion sources available, for example: chemical ionisation sources; but because only the electron impact source is used, it will be the only source discussed in the introduction.

1.3.3.2   The mass analyser

There are several forms of mass analysers available today.   All of these analysers separate ions according to their mass-to charge ratio [58]. These include magnetic sector analysers, quadroupole mass filters, time of flight analysers and ion cyclotron resonance instruments [58]. The apparatus used in this study makes use of a quadropole mass filter (figure 4). Quadropole instruments are popular because they are relatively inexpensive and need little experience to operate. Other advantages are that their data system control, ability to interface with a wide range of instruments and they are able to separate negative and positive ions without modification [52, 58]. The quadropole mass filter consists of four / five parallel rods arranged symmetrically [48, 52, 58].   The ions injected into the filter oscillate by means of an electric field created through applying a DC current, and a radio frequency component, to the rods. Different ions can move through the rods at different combinations of current and radio frequency. When a certain combination of current and frequency are produced, specific ions will oscillate with stable amplitudes through the rods, while the rest of the ions will be lost on the rod assembly. Only the ions with the stable oscillation amplitude are therefore able to reach the detector while the others do not; thus mass separation is achieved [48, 49, 52, 58].
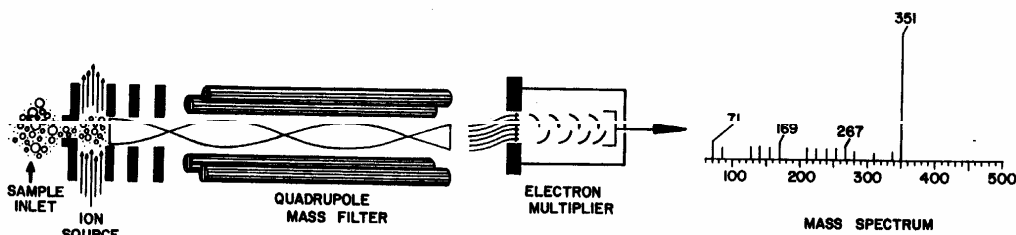
Figure 4: Schematic representation of the Quadropole MS system [49]

1.3.3.3 The ion detection system

The most widely used ion detection system in MS is the electron multiplier [48, 52, 58]. The ions that exit the mass analyser, bombard the metallic surface of the electron multiplier, causing electrons to be ejected from this surface. The ejected ions then cascade through the multiplier, causing the ejection of more ions. The end result is that the signal of each ion is multiplied about 10^5-10^7 times [48, 52, 58]. Both negative and positive ions can be detected this way.

1.3.3.4 Scanning modes

There are two modes, which can be used to acquire data on a MS: Scan, and Selected ion monitoring (SIM). In the scan mode, a range of masses is scanned over a few milliseconds. This gives rise to the conventional full mass spectrum of a compound (which, is like a fingerprint in that it is unique) and can be used for the identification of compounds. After the complete mass spectrum has been recorded, the most abundant and selective ions can be used to monitor the specific compound quantitatively [48]. This increases the sensitivity or detection limit of the instrument, as the background noise of

the chromatogram is reduced considerably. A higher signal to noise ratio, and a decreased detection limit, is therefore achieved.

1.3.3.5   Ion chromatograms

The chromatograms can either be total ion chromatograms (TIC) or extracted ion chromatograms. The sum of the total ionic abundances of the mass spectra is displayed over time in a total ion chromatogram (TIC). This chromatogram is similar in appearance to a chromatogram obtained using other detectors [48]. Even if the MS is running in SIM mode, the sum of the ions monitored is also represented on a TIC (this TIC, however, consists of the number of ions that are monitored). One specific ion can be extracted out of the total ion chromatogram, and its abundance over time can be represented on a chromatogram. This chromatogram is called an extracted ion chromatogram. More than one ion can be extracted at a time, and the chromatograms can be displayed separately or in a merged format. This is a very powerful tool for the identification of a specific peak.

1.3.3.6   Quantitation

The responses of different detectors to different compounds are not the same. There are also other factors that influence the detectors response *e.g.* temperature, flow rate of the carrier gas, gas composition etc. Peak area was used as an indication of the response. Peak height can also be used, but may vary more than peak area.

1.3.3.7   Method of quantitation

The internal standard method was used as a method of quantitation. An internal standard
is added at a known concentration before the sample preparation process is started. The
internal standard is used to compensate for sample losses that may occur in the sample
preparation, as well as for detector response differences. If some of the sample is spilt,
for example, some of the internal standard will also be lost, thereby correcting for percent
recovery and poor injection.   A chromatographic analytical internal standard should
possess chemical and chromatographic properties similar to that of the analytes [59]. The
ideal internal standard is an isotopic analogue of one of the analytes that will co-elute
with the analyte, but can be distinguished with the MS by mass [59]. The response of the
specific compounds relative to the response of the internal standard, is then used as an
indication of the abundance of the compound.

$$RRF = \frac{RF_A}{RF_{is}} = \frac{\dfrac{R_A}{C_A}}{\dfrac{R_{is}}{C_{is}}} = \frac{R_A}{C_A} \times \frac{C_{is}}{R_{is}}$$

$\dfrac{C_{is}}{R_{is}}$ and C_A however, is constant, thus $R_A = RRF \times C_A$

The response of different concentrations of a compound, relative to that of the internal
standard, is determined by drawing up a calibration curve, which shows the relationship
of the relative response over an increase in concentration.   An example of such a
calibration curve is given in figure 5. Ideally this should be a linear curve. Because ad-
and absorption are different over different concentrations, it is important to draw up the
calibration curve in the concentration range that the compound is expected.
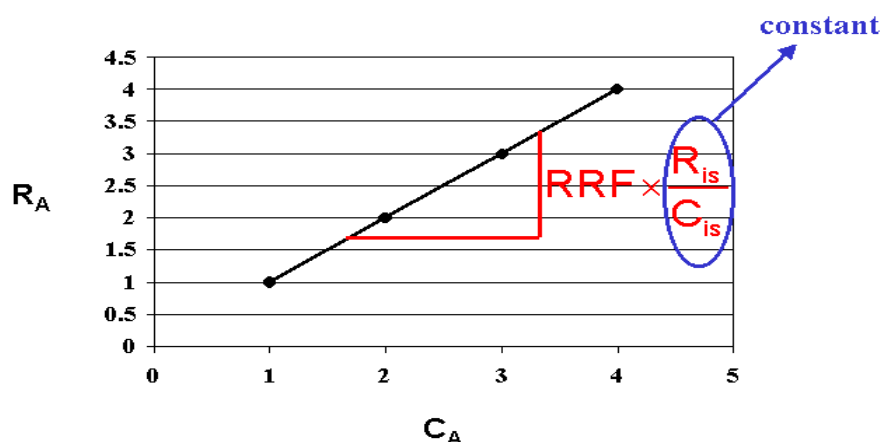
Figure 5: An example of a calibration curve used in internal standard quantitation

1.3.4 Analytical method validation

A new analytical method had to be developed and validated since the apparatus available to this study, and the implementation of cell culture, have not been described in the literature.

The definition of quantitation according to Krull and Swartz, is the ability to demonstrate exactly how much of a particular analyte is present in a particular sample [59].

When an analytical method is developed, method validation is essential to ensure quality of data. Method validation is the process of proving that an analytical method is acceptable for its intended purpose [60, 61]. It is therefore concerned with the identification of sources of, and the subsequent quantification of, errors in the method.

Three types of errors may come across one's path when analytical measurements are performed: gross, systematic and random.

Gross errors, owing to the degree and magnitude of the error, require the termination of the experiment [62]. Contamination, omission of critical reagents or instrumental failure are examples of this type of error. Systematic errors, cause all the results to be effected negatively in the same way [62]. This may be due to an error of the analyst, or the instrument, or one of the reagents. Random errors, are the result of uncontrollable variables in the measurement conditions that cause the results to fall on either side of the true mean [62].

When validating a method, the ultimate objectives and basic requirements must be kept in mind [62]. The most important aspects of validation are: stability, precision, accuracy, limit of detection, limit of quantitation, specificity, sensitivity and linearity within a specific range [60, 61, 62].

Accuracy, is the measurement of the exactness of an analytical method i.e. how close the measured value is to the true value [60, 61, 62].

Precision, is the measurement of the degree of repeatability of an analytical method under normal operation [61, 62]. According to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), precision should be performed at three levels: repeatability, intermediate precision and

reproducibility [62]. Repeatability, refers to the results obtained using the same method over a short interval of time and under the same conditions [62]. Intermediate precision, refers to the results obtained using the same method, but on different days for example [61, 62]. Reproducibility refers to the results obtained using the same method but in different laboratories [61].   In this study the last factor will not be determined.

Limit of detection is defined as the lowest concentration of a specific analyte that can be detected [60, 61, 62]. The general accepted detection limit for analytical methods is a signal-to-noise-ratio of 3:1. Limit of quantitation is defined as the lowest concentration that can be determined with acceptable precision and accuracy [60, 61, 62]. The general quantitation limit for analytical methods is a signal-to-noise level of at least ten to one with a coefficient of variance of less than 20% [62]. It is important to realise that both the limit of detection and quantitation are influenced by the chromatography. Higher and sharper peaks yield a better signal-to-noise level and thus a lower detection and quantitation limit.

Linearity is the ability of the method to produce results that are directly proportional to the analyte concentration within a given range [61, 62]. This is usually shown by setting up a calibration curve using a number of concentration levels (typically 5 standards). A correlation coefficient of >0.99 is acceptable, but depends strongly on the concentration level at which the analysis is performed.

1.3.5 Sample preparation

1.3.5.1   Protein precipitation

Protein precipitation or deproteinisation is needed when there are proteins in the matrix that may bind to the compounds of interest, and thereby inhibit their extraction. There are different methods of protein precipitation. Some make use of methanol and pH changes [63]. The aim of this sample preparation step is to denature the proteins. This will cause the proteins to precipitate and they can no longer fulfil a binding or carrier function. The compounds of interest can therefore be extracted.

1.3.5.2   Extraction techniques

1.3.5.2.1 Solid phase extraction

Solid phase extraction is a very powerful and simple extraction technique [64]. It is based on the principle of chromatography in that you have a solid phase, which retains solutes (extracts analytes out of a liquid phase), that is passed over the solid phase [64]. There are different types of solid phases. Some of them include:

- synthetic and foamed plastic resin

- silica and alumina

- cellulose

- activated carbon [64].

1.3.5.2.2 Liquid/liquid extraction

Liquid/liquid extraction is based on the principle that "like dissolves in like". Non-polar solvents will rather dissolve in non-polar solutes and polar solutes will rather dissolve in polar solvents. The polar and non-polar solvents do not mix, and the phase of interest can be decanted, and the other phase discarded [64].

1.3.5.3   Derivatisation

Gas chromatography is used as an analytical tool for compounds that are volatile. High boiling points can be due to intermolecular hydrogen bridge bonding [50]. Replacing the hydrogen atoms responsible for the hydrogen bridge bonds between the molecules (thus forming a suitable derivative) can increase compounds' volatility.   In this study silyl derivatives were used (figure 6).
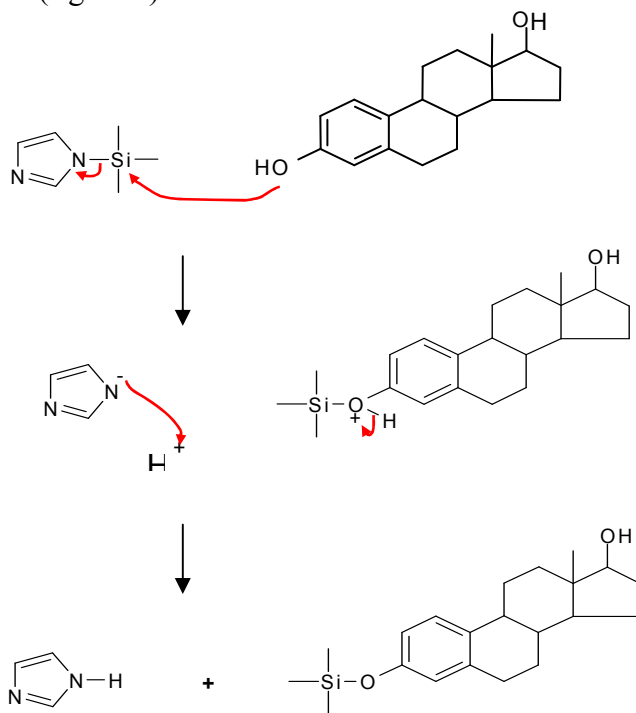
Figure 6: Derivatisation reaction: estradiol and trimethylsilylimidazole (TMSI). The arrows indicate the steps of the reaction

2   Experimental Procedures

2.1 Development of Analytical Method

2.1.1 Materials

- The following materials were obtained from *Merck*, Johannesburg, RSA:

    - Ethyl acetate of HPLC grade or higher

    - Ascorbic acid in salt form

    - Hydrochloric acid

    - Pyridine

    - Triethylamine

- The following materials were obtained from *Sigma-Aldrich,* Johannesburg, RSA

    - Diethyl ether of GC grade, it was kept on copper to remove epoxides.

    - BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide)

    - MTBSTFA (N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide)

    - 17β-estradiol

    - 5β-cholestane

    - 2-methoxyestradiol

    - 2-hydroxyestradiol

    - 4-hydroxyestradiol

    - 16 ml Kimax culture tubes with PTFE lined caps

    - Dichlorodimethylsilane

- The following materials were obtained from *Separations*, Johannesburg, RSA

  - TMSI (N-Trimethylsilylimidazole)

- The following materials were obtained from *C/D/N Isotopes Inc*, Quebec, Canada

  - 4-hydroxyestradiol-1,2,16,16,17-d5

- The following material were obtained from *Scientific Supply Services CC,* Johannesburg, RSA

  - 2ml wide mouthed Amber vials with PTFE lined solid caps

  - Glass inserts

2.1.2 Methods

2.1.2.1   Preparation of solvents

Because the catechol estrogens are oxidized so readily, ascorbic acid is added to the solvents used, thereby keeping these metabolites in their reduced form [65].   A 1% solution of ascorbic acid in methanol was prepared. This solution was then added to ethyl acetate in a 1:19 ratio [66].

2.1.2.2 Steroid extraction

2.1.2.2.1 Liquid-liquid extraction with diethyl ether

Cell culture medium samples (10 ml) were collected from MCF-7 cells and MCF-12A cells in 16 ml glass tubes that contained the internal standard. The final concentration of the internal standard was 200 pg/ml. The medium was then vortexed for one minute to ensure that the internal standard was dissolved.   Each sample was treated with concentrated hydrochloric acid (750μl) for the denaturation of serum proteins present in the cell culture medium. The samples were then vortexed for 0.5 minutes and heated for four minutes at 70°C. Samples were then left to cool down to room temperature for five minutes. 3ml diethyl ether was then added to each sample and the samples were vortexed further for 0.5 minutes (diethyl ether was stored on copper curls to remove the epoxides, which can destroy the catechol estrogens). The samples were subsequently centrifuged at 4°C for ten minutes at 4000rpm. The top layer of the samples were decanted 400μl at a time, into 2ml amber vials and dried under N_2 gas. The extraction step was repeated three times.

2.1.2.2.2 Solid phase extraction with Varian C18 columns

Cell culture medium samples (2ml) were collected from MCF-7 cells in 8ml glass tubes. –5kPa of vacuum was applied to Varian bond elute C18 columns. The columns were primed with methanol (10 ml) and phosphate buffered saline (PBS) (10 ml) (Care should be taken for the columns not to run dry.) The samples were applied to the columns and the columns left to dry under the vacuum. The steroids were eluted into 8 ml glass tubes

using ethyl acetate (EtOAc) (6 ml) containing ascorbic acid (1%) and dried under N_2 gas [67].

2.1.2.2.3 Extraction with ethyl acetate after freeze drying

2ml of cell culture medium samples were collected from MCF-7 cells. The samples were then frozen at -70°C and dried in a freeze dryer. 2 ml ethyl acetate containing 1% ascorbic acid was added to the samples and the samples vortexed for 10 minutes. The samples were left for five minutes until the ethyl acetate became clear again. The ethyl acetate was decanted and dried under N_2 gas.

2.1.2.3   Derivatisation

2.1.2.3.1 BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide)

BSTFA (50μl) was added to the dry samples in the 8ml glass tubes and heated for thirty minutes at 60°C. After derivatisation the samples were dried under N_2 and then redissolved in ethyl acetate containing 1% ascorbic acid. The derivatised samples were then transferred to 2ml amber vial containing an insert for injection into the GC [67].

2.1.2.3.2 MTBSTFA (N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide)

Pyridine (30μl), MTBSTFA (20μl) and triethylamine (2μl) were added to the dry sample in the 8ml glass tubes. The sample was then heated for thirty minutes at 75°C. After derivatisation the samples were dried under nitrogen and then redissolved in ethyl acetate

containing ascorbic acid (1%). Samples were then transferred to 2ml amber vials containing inserts for injection into the GC [68].

2.1.2.3.3 TMSI (N-Trimethylsilylimidazole)

TMSI (100μl) was added to the dry sample in the 2ml amber vials. The sample was then heated at 70°C for 30 minutes. Samples were then transferred to 2ml amber vials containing inserts and injected into the GC.

2.1.2.4   Deactivation of glassware

Glassware was cleaned by soaking it in concentrated chromic acid (which, must still be orange not coloured) for at least three hours. The glassware was then rinsed under running tap water for at least twenty minutes to remove all traces of chromium. Subsequently the glassware was rinsed with distilled water and placed in the oven at 110°C to dry. Glassware was soaked in a solution of dichlorodimethylsilane (10%) in anhydrous toluene for at least twenty minutes. The glass was then removed from the solution and immediately placed in methanol for fifteen minutes. It was then removed from the methanol and placed in the oven to dry.

2.1.2.5   Protein precipitation

Concentrated hydrochloric acid (750μl) was added and vortexed for 0.5 min.   The sample was then heated for 4 min at 70°C.

2.1.3 Instrumentation

The following instruments were used during the course of the study:

- HP 5890 GC-FID with an HP 7673A auto sampler

- HP 6890 GC – HP 5973 MS with an HP 7683 auto sampler

2.1.4 Procedures

2.1.4.1   GC-FID parameter settings

- Injection type and settings   1μl

    splitless, purge at 0.7 min

- Inlet temperature   200°C

- Column used   J&W DB-1 lenghth: 30m

    id: 250μm

    film thickness: 0.1μm

- Column flow   2 ml/min

- Initial oven temperature   215°C (3 min)

- Temperature gradient 1   1.2°C/min

- Final Temperature 1   234°C

- Temperature gradient 2   50°C/min

- Final temperature 2   300°C (6.85)

- Total analysis time   27 min

2.1.4.2   GC-MS column parameter settings

The use of three different columns were exploited to obtain optimum separation. The

GC-MS instrumental parameters for each column are reported below

2.1.4.2.1 J&W DB-1 Column

- Injection type and settings   1μl

  splitless, purge at 0.7 min (fast plunger speed)

- Inlet temperature   250°C

- Column parameters   length: 30m

  id: 250 μm

  film thickness: 0.1μm

- Column flow   2ml/min

- Initial oven temperature   215°C (3 min)

- Temperature gradient 1   1.2°C/min

- Final temperature 1   225°C

- Temperature gradient 2   50°C/min

- Final temperature 2   300°C (6.17 min)

- Total analysis time   19 min

- Solvent delay   3 min

- MS acquisition mode   SIM: 3 – 7.5 min   416

                 7.5 – 19 min 446, 504, 509

- Transfer line temp   280°C

2.1.4.2.2 J&W DB-5MS column

- Injection type and settings   1μl

  splitless, purge at 0.7 min (fast plunger speed)

- Inlet temperature   250°C

- Column parameters   length: 30m

  id: 250 μm

  film thickness: 0.25μm

- Column flow   2ml/min

- Initial oven temperature   215°C (3 min)

- Temperature gradient 1   4°C/min

- Final temperature 1   264°C
- Temperature gradient 2   100°C/min
- Final temperature 2   300°C (5 min)
- Total analysis time   21.61 min
- Solvent delay   10 min
- MS acquisition mode   SIM: 10 – 13 min   416

                                         13 – 15.5 min 446, 504, 509

- Transfer line temp   280°C

2.1.4.2.3 Zebron ZB-5 Column

- Injection type and settings   1μl

                                 splitless, purge at 0.7 min (fast plunger speed)

- Inlet temperature   250°C
- Column parameters   length: 30m

                                 id: 250 μm

                                 film thickness: 0.25μm

- Column flow   2ml/min
- Initial oven temperature   215°C (3 min)
- Temperature gradient 1   4°C/min
- Final temperature 1   269°C
- Temperature gradient 2   100°C/min
- Final temperature 2   300°C (5 min)
- Total analysis time   21.81 min
- Solvent delay   12 min
- MS acquisition mode   SIM: 12 – 14.5 min   416

                                         14.5 – 17 min 446, 504, 509

- Transfer line temp   280°C

2.1.4.3   Sample gathering

Samples were taken from cell culture media in which cells were grown at the cell culture laboratory of the Department of Physiology of the University of Pretoria.   The internal standard and ascorbic acid was added, and the samples were frozen and stored at -70°C until they could be analysed.

2.1.4.4   Statistics

Standard deviations, mean values, coefficients of variance, and 95% confidence intervals were used in the statistical validation of the method.

2.2 *In vitro* Application of Analytical Method

2.2.1 Aim

The aim of the experiment was to apply the developed method to determine whether it is sensitive enough to measure the low levels of metabolites present extracellularly and to investigate the rate of metabolism of the metabolites in question.   The experiments were designed to determine whether there is a difference in the rate of metabolism in normal *vs*. transformed cells and whether initial concentration influences this rate.

2.2.2 Materials

- Culture medium

    - Minimum essential medium eagle (MEME) with Earle's salts, L-glutamine and NaHCO3, Ham's F12 medium, Trypsin-EDTA, epidermal growth factor, cholera toxin, insulin and hydrocortisone were supplied by Sigma Chemical Co. (St. Louis, USA).

    - Heat-inactivated fetal calf serum, sterile cell culture flasks and plates were obtained through Sterilab Services (Johannesburg, SA).

    - Phosphate buffered saline (PBS) was purchased from Gibco BRL through Laboratory Specialist Services (Johannesburg, SA).

    - Dulbecco's modified Eagle's medium, penicillin, streptomycin and fungizone were obtained from Highveld Biological (Sandringham, SA). All other chemicals were of analytical grade and supplied by Sigma Chemical Co. (St. Louis, USA).

- MCF-7 cell line

    - MCF-7 cells were derived from a pleural effusion of human breast adenocarcinoma and were supplied by Highveld Biological (Sandringham, SA).

    - This cell line was chosen as it is a breast carcinoma cell line which shows the metabolism investigated in this study and therefore provides an ideal test subject and representative of tumourigenic tissue.

- MCF-12A cell line

    - The MCF-12A cell line is a non-tumorigenic epithelial cell line produced by long-term culture of normal mammary tissue. This cell line was a gift from Professor Parker (Division of Medical Biochemistry, University of Cape Town, Cape Town, SA).

    - This cell line was chosen as it is very close to a normal breast cell tissue and therefore provide an ideal control to this study as a representative of normal cells with normal metabolism.

2.2.3 Methods

2.2.3.1   Culturing of cell lines

Equal numbers of MCF-7 and MCF-12A cells (10^6 cells/flask) were grown as monolayers in Minimum Essential Medium, at 37°C in a humidified atmosphere containing 5% CO_2. Minimum Essential Medium Eagle (MEME) was used for the MCF-7 cancer cell line and supplemented with 10% heat-inactivated fetal calf serum, penicillin (100µg/l), streptomycin (100µg/l) and fungizone (250µg/l). MCF-12A media consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, epidermal growth factor (20ng/ml), cholera toxin (100ng/ml), insulin (10µg/ml) and hydrocortisone (500ng/ml). This cell line represents normal breast cells.

2.2.3.2 Determination of estrogen levels in cell culture medium

Equal numbers (1×10^6 cells/flask) of MCF-7 and MCF-12A cells were provided with 11 ml medium containing E_2/2-HE_2/4-HE_2/2-ME_2 (10^{-6} M) and medium containing E_2/2-HE_2/4-HE_2/2-ME_2 (10^{-8} M). Of each metabolite and each concentration two flasks were prepared. Each flask represented a specific time laps. At the appropriate time 10 ml of the medium was extracted. The time intervals used for each experiment were as follows:

- Incubation with E_2 – 0 hours, 8 hours and 24 hours

- Incubation with 2-ME_2 – 0 hours, 1 hour and 8 hours

- Incubation with 2-HE_2 and 4-HE_2 – 0 hours, 10 minutes and 1 hour

The time intervals used were according to the expected rate of metabolism. The rate of hydroxylation of E_2 does not occur as quickly as the methylation of 2-HE_2 and 4-HE_2 and therefore longer time intervals were chosen for the incubation with E_2 than with 2-ME_2 and the CE [36]. It is important to choose the time intervals in such a manner that an accurate view of metabolism can be seen.

Of each concentration and cell line, the following controls were prepared:

- Cell medium containing the analytes, but not administered to the cells (0 hours)

- Cell medium that was administered to the cells, but did not contain the analytes (blank)

Each experiment was repeated three times.

2.2.4 Statistics

Variation in results can be due to a) variation between subjects in a random sample, b) variation within subjects (biological variation) or c) variation in the analytical procedure. Comparing two cell lines is similar to comparing two subjects. The sample size (n) is therefore one in each category and thus too small to compare statistically.   Each experiment was repeated three times and the mean of the three sets of results were determined and presented. Due to the fact that the accuracy of the analytical method was not sufficient to produce reliable quantitative data, preliminary experiments were performed only. This would help with the future refinement of this method. The results were therefore only used to illustrate trends.

3 Results and Discussion

3.1 Method Development

In chromatography there are two characteristics in a chromatogram that are essential for the identification of compounds in later analyses. These two characteristics are retention time and eluting sequence. For a specific set of parameters, these characteristics remain constant.

3.1.1 Elution order and retention times (BSTFA)

The elution order was determined by dissolving the standards and the internal standard in EtOAc containing ascorbic acid (1%). The final concentration of the standard solutions were 30ng/ml. The samples were then dried, derivatised with BSTFA and subsequently analysed by GC-MS. All the compounds eluted between 10 and 20 min. The molecular masses of the derivatised metabolites were calculated (table 3) and used for identification of the chromatographic peaks. 2- and 4-HE_2 have the same molecular mass. There were therefore two peaks with the same mass spectra since they have such similar molecular structures and the same molecular mass. To confirm the identity of the peaks, each metabolite was spiked at a higher concentration selectively in turn and analysed. The structures of all the metabolites and internal standards used in this study are given in table 3. Table 4 shows the structures of the derivatised metabolites. The elution order was as follows: E_2, 5β-C (internal standard), 2-ME_2, 2-HE_2 and finally 4-HE_2. The 2-HE_2 and 4-HE_2 peaks showed some overlapping and the ascorbic acid peak could not be located.

A modified GC oven temperature program was used to improve resolution. This changed the retention time of the compounds since the equilibrium of the compounds between the stationary phase and the mobile phase was changed. The sequence of elution, however, did not change. The temperature program was started at a lower temperature. Improved separation was obtained and the ascorbic acid peak could also be identified.

Table 3: Molecular masses of the silyl derivatised metabolites.

Derivitised metabolite	Molecular mass (g/mole)
Ascorbic acid	464
5β-Cholestane	372
Estradiol	416
2M-Estradiol	446
2H-Estradiol	504
4H-Estradiol	504
4H-Estradiol-D$_5$	509

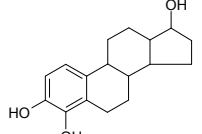Table 4: Structures of all the compounds applicable to this study.
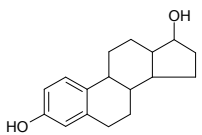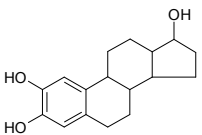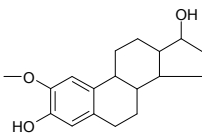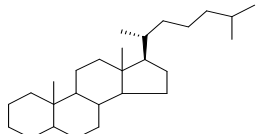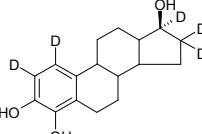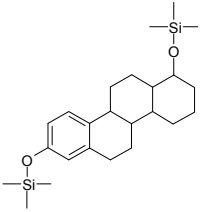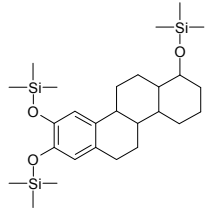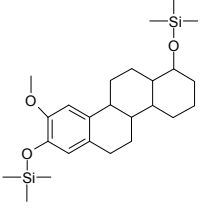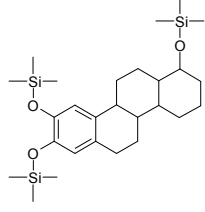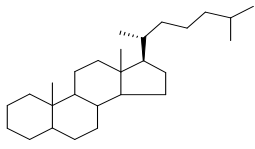
Metabolite	Structure	Metabolite	Structure
17β-estradiol		2-hydroxyestradiol	
2-methoxyestradiol		4-hydroxyestradiol	
5β-cholestane		4-hydroxyestradiol-d$_5$	

Table 5: Structures of the TMS (trimethylsilyl) derivatives formed by BSTFA derivatisation

Derivatised metabolite	Structure	Derivatised metabolite	Structure
17β-estradiol		2-hydroxyestradiol	
2-methoxyestradiol		4-hydroxyestradiol	
5β-cholestane			

3.1.2 Derivative stability (BSTFA)

The stability of the trimethylsilyl (TMS) derivatised metabolites over time, at room temperature, was determined. Derivatives must be stable over time, for samples to be run by an auto sampler. If the method were to be automated or used as a routine laboratory method, this would be a big advantage. A sample, with a final concentration of 1μg/ml, was prepared. The sample was then derivatised and analysed every hour for ten hours. The results of these analyses are given in figure 7. Response is given relative to that of the internal standard. All the metabolites showed a downward trend in relative response with 4-HE$_2$ showing the steepest gradient. The derivative is therefore hydrolysed over

time at room temperature. The internal standard (5β-C) does not have a hydroxyl group that can be derivatised, so the response will stay the same over time. The other metabolites that do have a derivatised group, showed a decrease in response over time. This internal standard is therefore not a good standard to compensate for the hydrolysis of derivatives on an autosampler. Another internal standard showing the same decrease over time would have been better. It was decided, however, that a different derivatising agent forming more stable derivatives should be used.
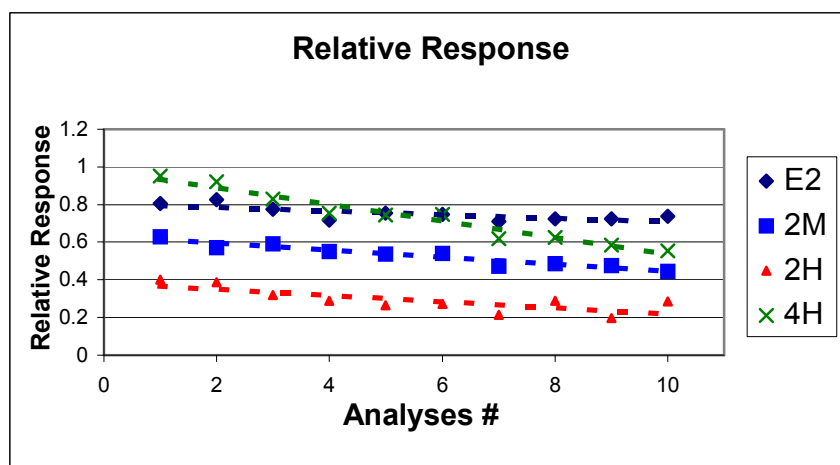
Figure 7: The results of the BSTFA derivative stability test

3.1.3 Elution order and retention time determination (MTBSTFA)

MTBSTFA was used as the new derivatising agent. MTBSTFA forms t-buthyldimethylsilyl (t-BDMS) derivatives. The molecular mass of the t-BDMS derivatives are larger than that of the TMS derivatives. The molecular masses of the metabolites derivatised with MTBSTFA are given in table 6 and their structures in table 7. The elution order had to be determined once again since the heavier derivatives behaved differently. GC-FID was used to determine the elution order. Using

gaschromatography with flame ionisation detection implied that there were no mass spectra to identify peak. Retention times therefore had to be determined by analysis of single component standards. The Figure 8 shows four superimposed chromatograms of the single component standards. The 4-HE$_2$ standard gave no peak when analysed. A new 4-HE$_2$ standard was ordered and the old standard discarded. Work was continued without 4-HE$_2$ until the new standard could be used. The elution order changed compared to that of the TMS derivatives due to the change in molecular masses.

Table 6: Molecular masses of MTBSTFA derivatised metabolites.

Derivatised metabolite	Molecular mass (g/mole)
5β-Cholestane	357
Estradiol	500
2-Methoxyestradiol	531
2-Hydroxyestradiol	630
4-Hydroxyestradiol	630
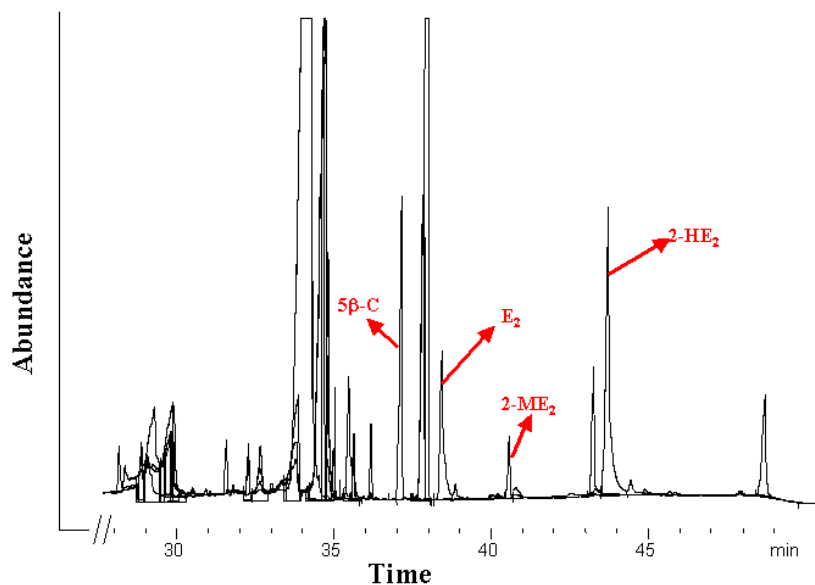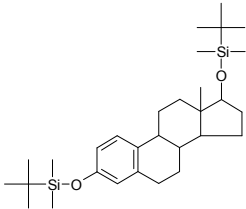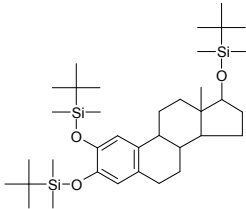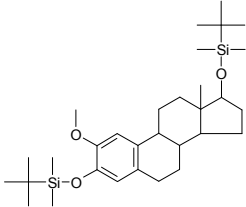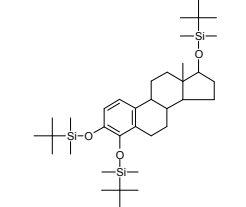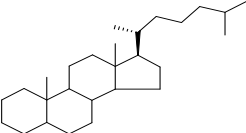
Figure 8: Elution order of MTBSTFA derivatised metabolites (5β-C = 5β-cholestane)

Table 7: Structures of the t-buthyldimethylsilyl (t-BDMS) derivatives formed by MTBSTFA derivatisation

Derivatised metabolite	Structure	Derivatised metabolite	Structure
17β-estradiol		2-hydroxyestradiol	
2-methoxyestradiol		4-hydroxyestradiol	
5β-cholestane			

5β-Cholestane, which has no hydroxyl group and is therefore not derivatised, has the same molecular mass as when BSTFA was used as a derivatising agent. The resultant eluting sequence is therefore 5β-cholestane, E_2, $2\text{-}ME_2$, $2\text{-}HE_2$ as displayed in figure 9.

3.1.4 Reproducibility (MTBSTFA)

The coefficient of variance (CV) for the sample preparation technique using MTBSTFA as derivative was determined. Seven samples containing a mixture of al the metabolites (except $4\text{-}HE_2$) were prepared at a concentration of 10µg/ml. The samples were then derivatised using MTBSTFA and subsequently analysed on the GC-FID. Figure 9 gives

the results of these analyses. CV was calculated using the following formula (Standard deviation/ mean) x 100. This value gives an indication of the amount of variation between the values. Normally a value of less than 5% is acceptable depending on the concentration level worked at. The CV for the specific metabolites is represented in table 7.
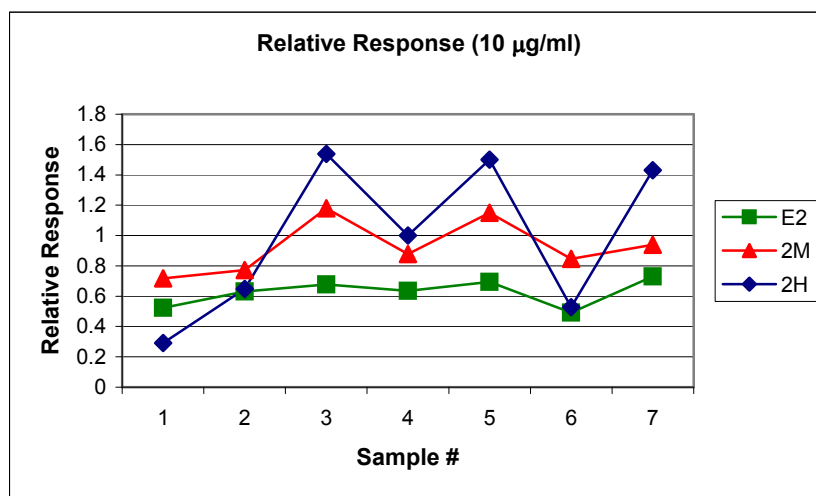
Figure 9: Relative response of samples analysed for determination of CV (MTBSTFA derivatised).

Table 8: Coefficient of variance for the different metabolites using MTBSTFA.

Metabolite	Coefficient of variance (CV)
Estradiol	14.05%
2-Methoxyestradiol	19.25%
2-Hydroxyestradiol	50.70%

The CV values determined were too high. The experiment was repeated using deactivated glassware. The resultant values did not decrease satisfactory. The derivatising agent was therefore changed again as an attempt to improve the accuracy of the analytical method with more stable derivatives.

3.1.5 Elution order (TMSI)

TMSI was used as a derivatising agent. The same derivatives as with BSTFA derivatisation are formed (TMS derivatives). The elution order and masses were therefore the same. The difference with TMSI as a derivatising agent is that no drying step is required after derivatisation. The sample can be injected into the GC using TMSI as a solvent. This could not be done with BSTFA seeing that it can harm the column. Another advantage of this is that the metabolites stay in an environment with an excess of derivatising agent. This may then have a stabilising effect on the derivatives. Samples were stored at -70ºC to enhance stability. After injection it was again frozen at -70°C. This also has a stabilising effect on the derivatives, reducing their hydrolysis.

3.1.6 Reproducibility (TMSI)

The CV was determined as described previously. TMSI, however, was used as derivatisation agent. Deactivated glassware were also used. Results are given in figure 10 and table 9. The CV values obtained were more acceptable. The mass spectra of the TMS derivatised metabolites are given in section 5.

Table 9: CV of different metabolites using TMSI.

Metabolite	CV (5β-Cholestane)
Estradiol	9.58 %
2-ME$_2$	8.27 %
2-HE$_2$	13.35 %
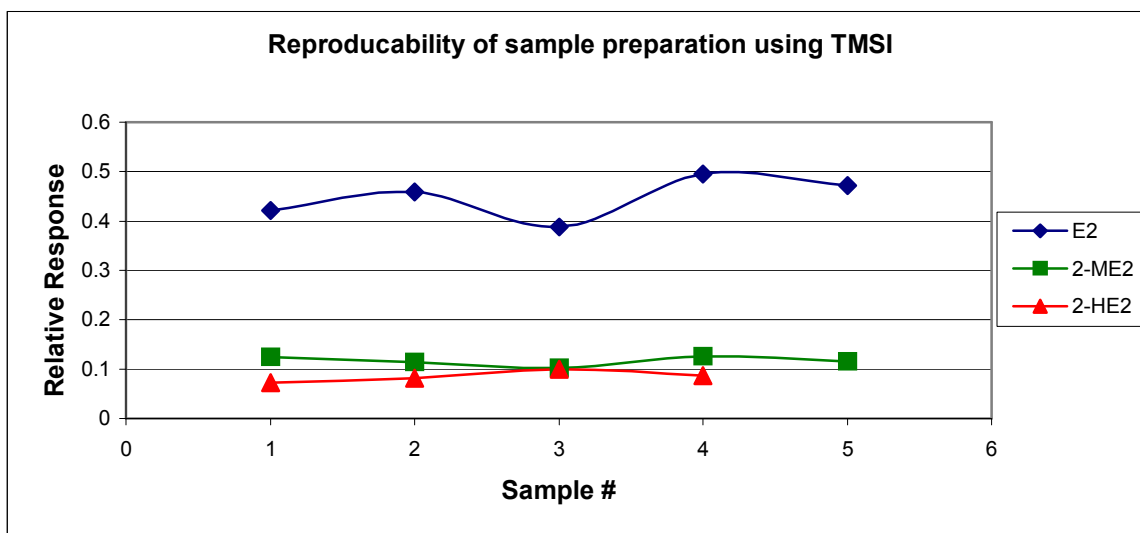
Figure 10: Relative response of samples analysed for determination of CV (TMSI).

3.1.7 Optimisation of steroid extraction from physiological

matrix

MCF-7 cell culture medium was spiked with a metabolite mixture. The samples were then freeze dried. The steroids were extracted using ethyl acetate. The results obtained, however, were not satisfactory. The pH was lowered to 2 to ensure that the steroids were in their hydroxylated form. Peaks could be observed but the recovery of the metabolites was poor. Medium containing no serum was used to determine whether the steroids were being lost because of interactions with serum proteins. The results showed a high increase in steroid recovery when no proteins were present in the matrix (figure 11). It was, however, not as high when compared to the standards alone. The normal function of serum proteins in blood is to serve as carriers. The metabolites, being steroids, will therefore bind to serum proteins in the cell culture medium. After a protein precipitation step was included in the sample work-up process, an increase in recovery was observed.
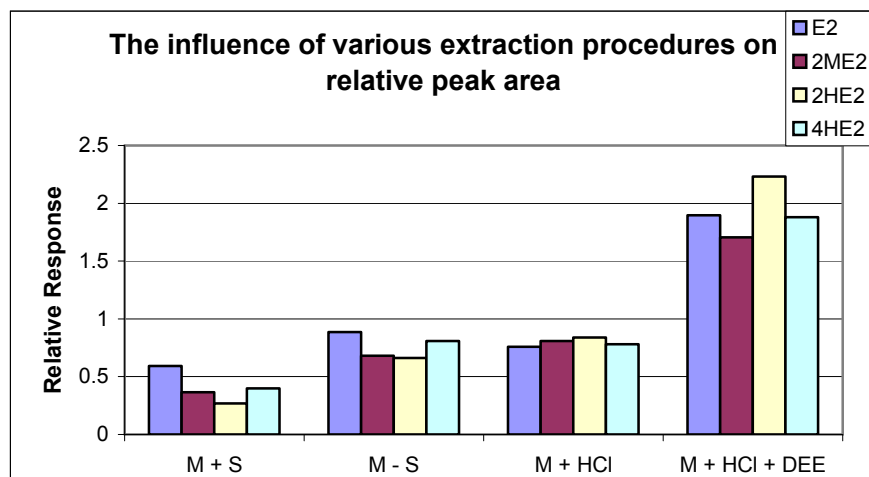
Figure 11: Diagram showing the effect of serum and the extraction procedure on relative peak area (DEE = diethyl ether)

After the samples were freeze-dried, protein precipitation by addition of HCl produced the same results as when the medium containing no serum was used. The end result of the sample preparation, however, was a very thick reddish liquid. This could block the inlet of the GC and therefore the method had to be refined. Aqueous samples, which were not dried, were then used. Concentrated hydrochloric acid was added to the aqueous samples and they were subsequently heated. Both steps aimed to denature the serum proteins. The steroids were then extracted by liquid-liquid extraction making use of diethyl ether as the non-polar solvent. The top layer (diethyl ether) containing the steroids was then decanted, dried under N_2 and derivatised with TMSI. The results showed an increase in steroid recovery (figure 11). The increase in relative response may, however, also have been due to the fact that the internal standard, 5β-cholestane dissolved less in the diethyl ether than the rest of the metabolites concerned. The extraction with diethyl ether was performed only once. The response of the metabolites relative to that of the internal standard will therefore be increased. The ratios at which

they dissolve, however, remains constant. The relative response for each metabolite at a specific concentration will therefore remain constant.

3.1.8 Calibration

The GC-FID was used to conduct the initial calibration. Higher concentrations were used at first to ensure that all the metabolites could be observed. A calibration curve consisting of the following concentration was prepared: 0.5µg/ml, 2.5µg/ml, 5µg/ml, 7.5µg/ml and 10µg/ml. 5µg/ml was used to determine the CV. The CV of all the metabolites were about 5% and below (table 10) and the correlation coefficient (R^2), which shows the linearity (linear $R^2 = 1$) of the calibration curves, were all approximately 0,99.

Table 10: CV (concentration) of metabolites in calibration curve (0.5-10µg/ml)

Metabolites	CV (%)
17β-estradiol	5.12
2-metoxyestradiol	5.03
2-hydroxyestradiol	4.46
4-hydroxyestradiol	4.68

The concentration series was then decreased to 5ng/ml, 25ng/ml, 50ng/ml, 250ng/ml, 1000ng/ml and another calibration curve was prepared.   50ng/ml was used to determine the CV. The CV for each metabolite is given in table 11. The limit of quantitation using the GC-FID was found to be 5ng/ml .

The R^2 values for the calibration curves lay between 0.95 and 0.99. This shows linearity at low concentrations.   The limit of quantitation however, is above the expected

physiological concentrations.   The samples prepared for the1-1000 ng/ml calibration curve were analysed on the GC-MS in the SIM mode.   The results are represented in figures 12 to 15 and table 12.

Table 11: CV (concentration) of metabolites in calibration curve (5-1000ng/ml)

Metabolites	CV (%)
17β-estradiol	14.22
2-metoxyestradiol	14.21
2-hydroxyestradiol	15.77
4-hydroxyestradiol	23.54
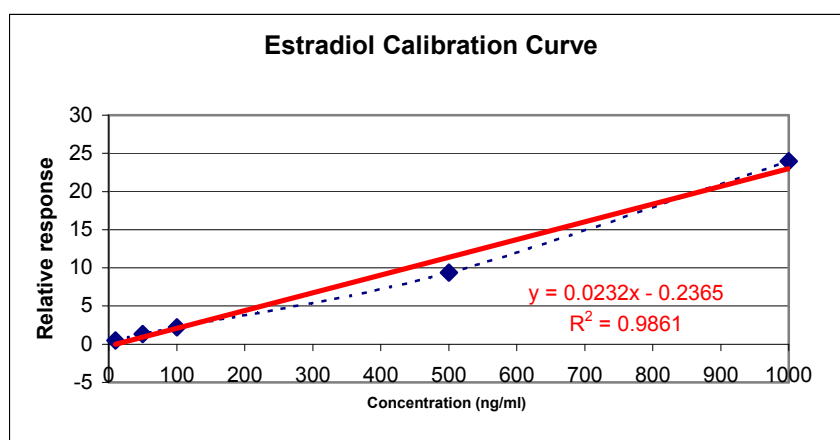
Figure 12: Estradiol calibration curve on the GC-MS (5-1000ng/ml)
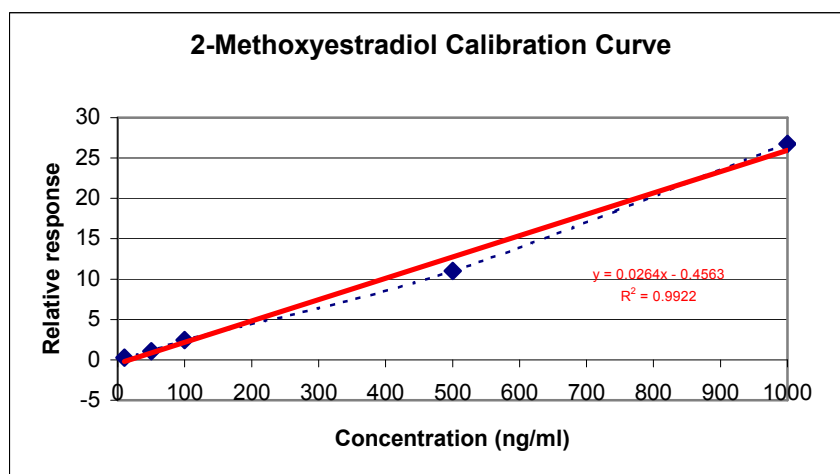
Figure 13: 2-Methoxyestradiol calibration curve on the GC-MS (5-1000ng/ml)

Table 12: CV(concentration) of metabolites in calibration curve on GC-MS (5-1000ng/ml)

Metabolites	CV (%)
17β-estradiol	15.50
2-metoxyestradiol	15.93
2-hydroxyestradiol	13.00
4-hydroxyestradiol	12.10

2-Hydroxyestradiol Calibration Curve

$y = 0.0128x - 0.298$
$R^2 = 0.9882$
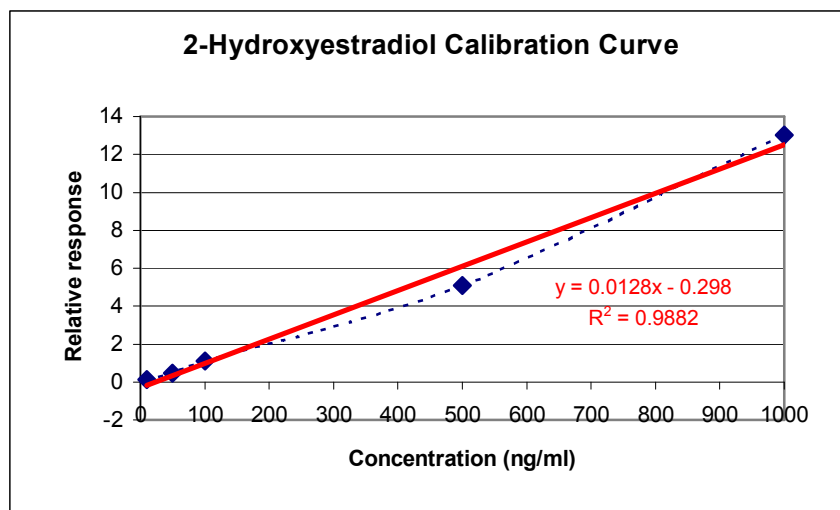
Figure 14: 2-Hydroxyestradiol calibration curve on the GC-MS (5-1000ng/ml)

4-Hydroxyestradiol Calibration Curve
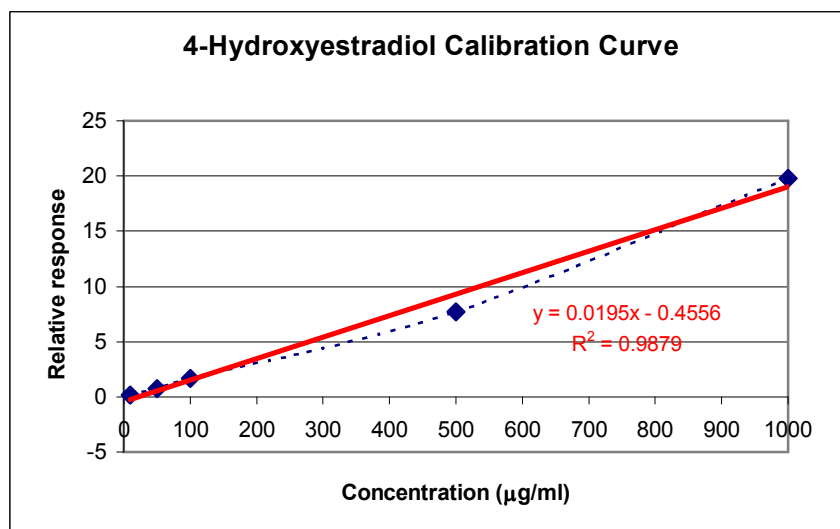
$y = 0.0195x - 0.4556$
$R^2 = 0.9879$

Figure 15: 4-Hydroxyestradiol calibration curve on the GC-MS (5-1000ng/ml)

The CV of the metabolites were between 12 and 16% which was an improvement on the

results obtained on the GC-FID. The linearity of the calibration curves was better than

the results of the GC-FID, with R^2 values between 0.98 and 0.99.

Medium taken from growing cell culture were analysed to make sure that the cells do not manufacture compounds with the same retention time and molecular masses as the compounds of interest. No peaks with the same retention time and mass spectra as that of the metabolites of interest were found as can be seen in figure 16.

As stated before, 5β-C does not have a hydroxyl group. The compound is therefore not derivitised if TMSI is used as derivatising agent. It is therefore not that good an internal standard since it does not compensate for hydrolysis of derivatives. An isotope of 4-HE$_2$ was used in the final calibration rather than 5β-cholestane. Using an isotope as internal standard is very reliable. It does however limit analyses to the GC-MS. The isotope will elute at the same time as 4-HE$_2$, but can be distinguished by the heavier molecular ion. Chemically it will react precisely the same as the normal compound. It is the ideal internal standard.

Calibration in the correct concentration range is of vital importance. Preliminary experiments with cells were performed to establish this range. To improve the limit of quantitation and sensitivity, the steroid extraction method was refined further. 10 ml of medium was taken from the cells instead of two and extraction with diethyl ether was increased to three times per sample, thereby increasing the steroid recovery. The internal standard was also changed to the deuterated isotope of 4-HE$_2$. MCF-7 cells were exposed to 1μM E$_2$. 10 ml medium was extracted after 8 hours of exposure and 24 hours of exposure. Results are given in figure 17 to 20. The internal standard was spiked at a

concentration of 50ng/ml.   4-HE$_2$ had the lowest peak area relative to the internal standard.   Because 4-HE$_2$ and its isotope will have approximately the same detector response the ratio of the obtained peak area of 4-HE$_2$ to that of its isotope was used to get an estimate of the concentration level of the metabolites.   A calibration curve was prepared in this estimated concentration range (40-1000 pg/ml).   Calibration was also done over the 0.5-50 ng/ml and 50-300 ng/ml concentration range.   The internal standards for al these curves was at a concentration of 200 pg/ml.

Figure 16: Extracted Ion Chromatogram (EIC) (A), TIC (B), and mass spectrum of peak at 8.16 min (C) of medium in which cells had grown. (416 = E_2, 446 = 2-ME_2, 504 = 2-HE_2 & 4-HE_2, 357 = 5β-cholestane)
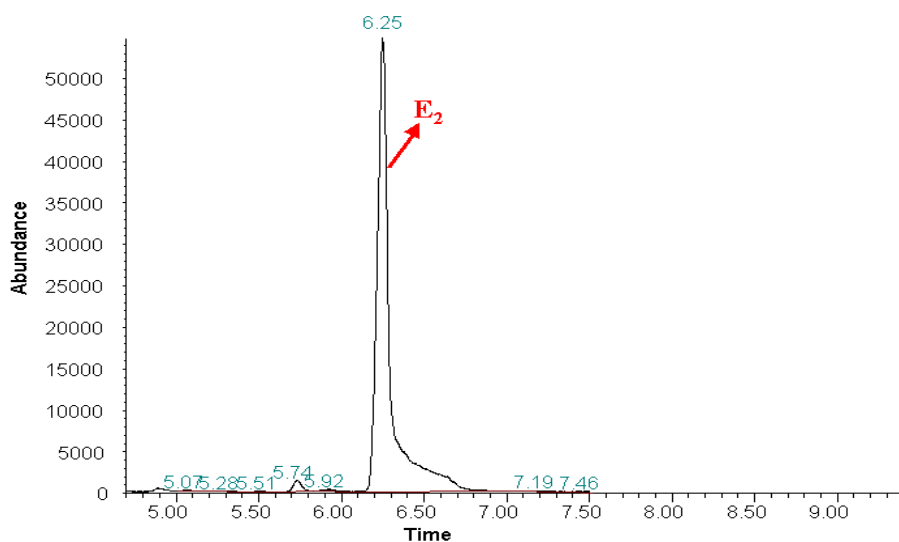
Figure 17: The Extracted Ion Chromatogram (EIC) of the ion used to identify E$_2$ (416) after 8 hours of exposure of cells to E$_2$.
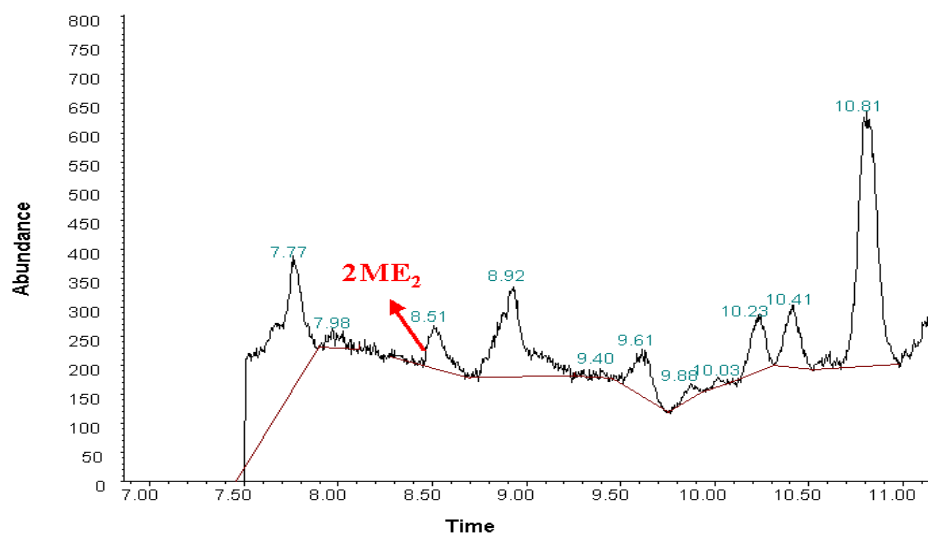
Figure 18: The EIC of the ion used to identify 2-ME$_2$ (446) after 8 hours of exposure of cells to E$_2$.
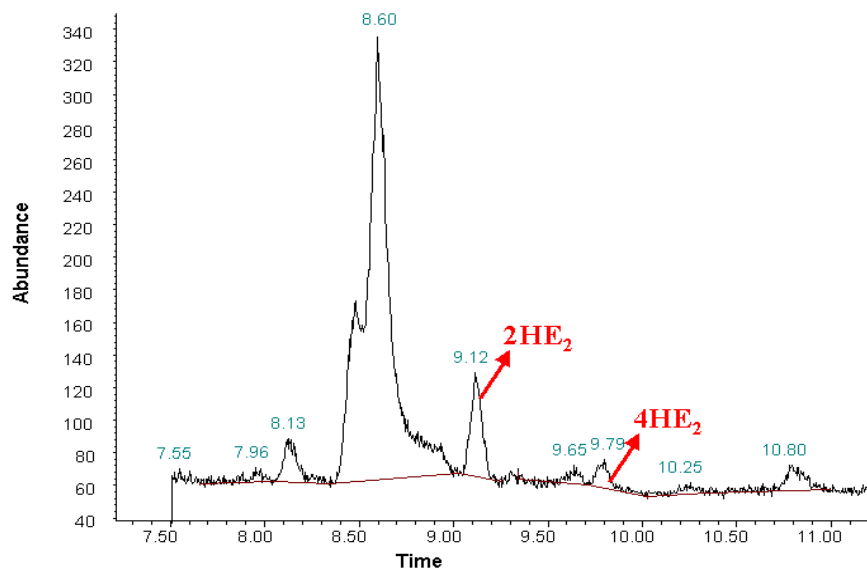
Figure 19: The EIC of the ion used to identify 2-HE$_2$ and 4-HE$_2$ (504) after 8 hours exposure of cells to E$_2$.
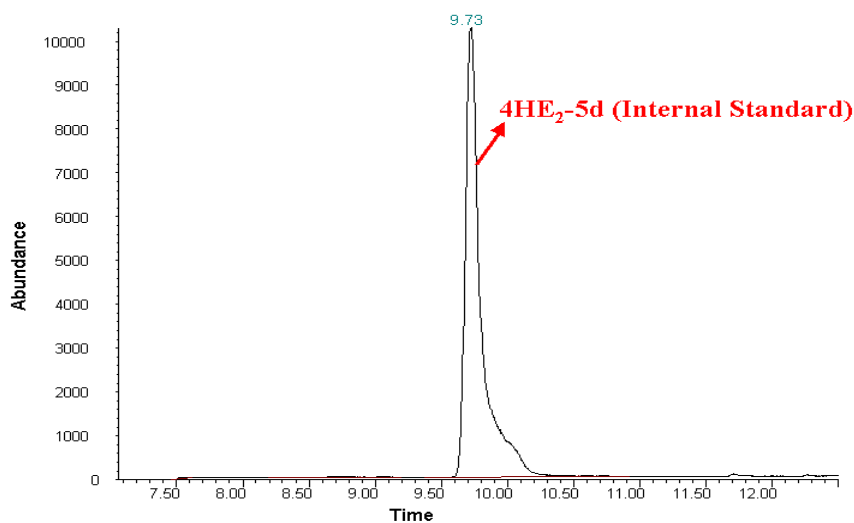
Figure 20: The EIC of the ion used to identify 4-HE$_2$-d5 (509) 8 hours exposure of cells to E$_2$.

The calibration curves showed good linearity over all the concentration ranges that were covered. The detection limit of this method was lower than 40 pg/ml (40 ppt) and the limit of quantification was 80 ng/ml (80 ppt). The results of calibration are given in figures 21-24 and are summerised in table 13.
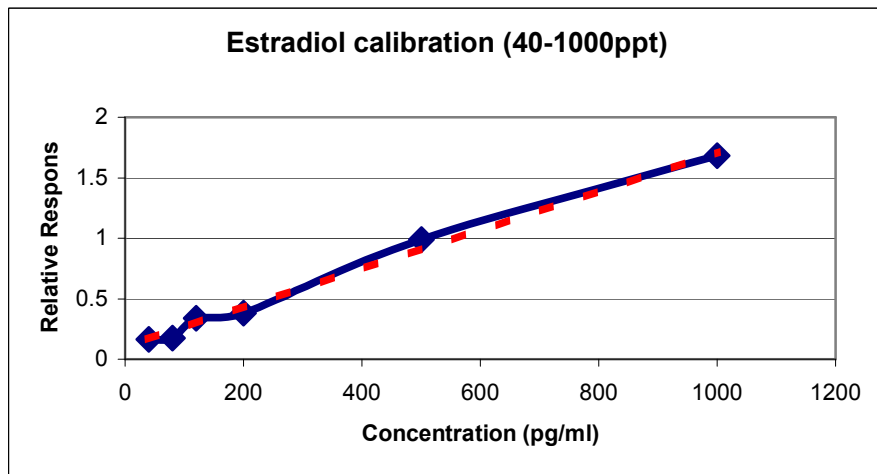
Figure 21: Estradiol calibration over the 40-1000 pg/ml range.
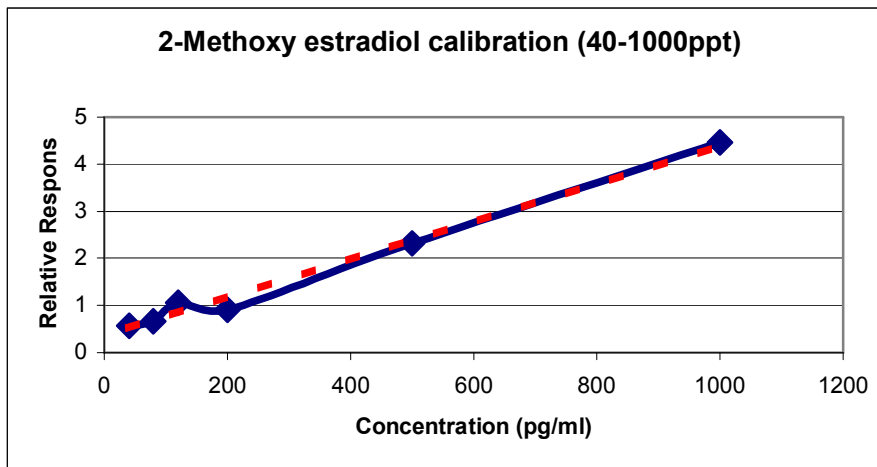
Figure 22: 2-Methoxy estradiol calibration over the 40-1000 pg/ml range.
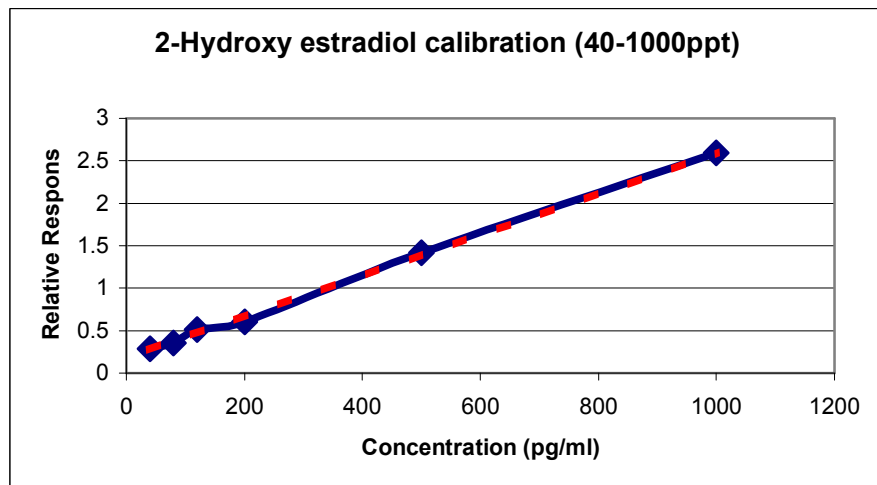
Figure 23: 2-Hydroxy estradiol calibration over the 40-1000 pg/ml range.
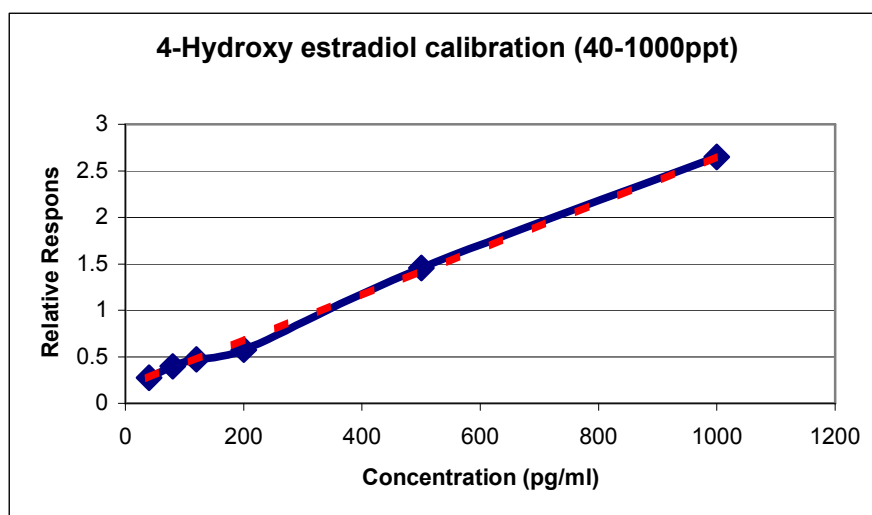
Figure 24: 4-Hydroxy estradiol calibration over the 40-1000 pg/ml range.

Table 13: Summary of the linear statistics of the calibration curves.

Metabolite	40-1000pg/ml		0.5-50ng/ml	
	R^2	Formula	R^2	Formula
E_2	0.9917	y = 0.0016x + 0.0991	0.9972	y = 1.1163x + 1.3059
$2\text{-}ME_2$	0.9891	y = 0.0041x + 0.3505	0.9948	y = 2.1052x + 3.7525
$2\text{-}HE_2$	0.9984	y = 0.0024x + 0.1771	0.9951	y = 1.6142x + 2.356
$4\text{-}HE_2$	0.9973	y = 0.0025x + 0.168	0.9935	y = 1.5734x + 2.6538

When the samples of the calibration over the 50-300ng/ml were analysed an interesting phenomenon was observed. As shown in figure 25, a non-linear relation exists between the response of 4-hydroxy estradiol and that of the internal standard from 100ng/ml onwards. The average response of the internal standard increased by about 30 000 units for each 50ng/ml increase in concentration. This may have been because a deuterated 4-hydroxy estradiol standard was used as internal standard at a concentration of 200 pg/ml. The difference in concentration is so vast that the natural occurring C^{13}-isotope range of 4-hydroxyestradiol may start to contribute substantially to the internal standard at this stage. An experiment was set up to investigate whether that is the case.
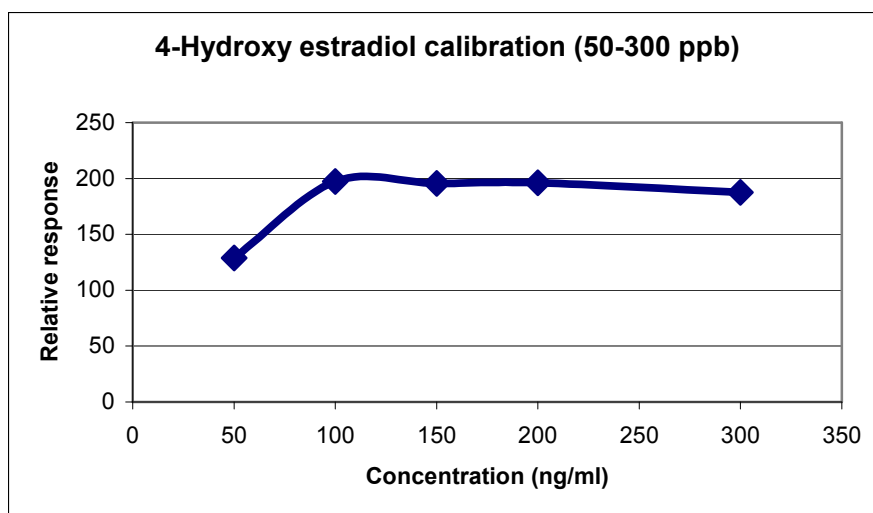
Figure 25: 4-Hydroxyestradiol shows a non-linear relationship with the internal standard at a concentration higher than 100 ng/ml.

The experiment consisted of samples prepared to contain the 4-HE$_2$ standard at different concentration levels starting at 40 pg/ml (ppt) and going up to 300 ng/ml (ppb); the internal standard at a concentration of 200 pg/ml (ppt); and a series of samples containing only the internal standard at a concentration of 200 pg/ml (ppt). The absolute response of the internal standard obtained in the series, that only contained the internal standard, was used to determine the standard deviation of the internal standard. Then the absolute response of the internal standard of the series containing both the standards as described previously was plotted over the concentration of 4-HE$_2$ (figure 26). The 2σ values were indicated by two black dashed lines on the graph. At a 4-HE$_2$ concentration of about 140 ppb, the internal standard shows an absolute response outside the mean+2σ interval. The natural isotopes of 4-HE$_2$ therefore begin to make a contribution to the internal standard at a concentration higher than 140 ppb. Calibration is therefore not accurate with this internal standard if 4-HE$_2$ is added at a concentration higher than 140 ppb and the internal standard is at a concentration of 200 ppt.
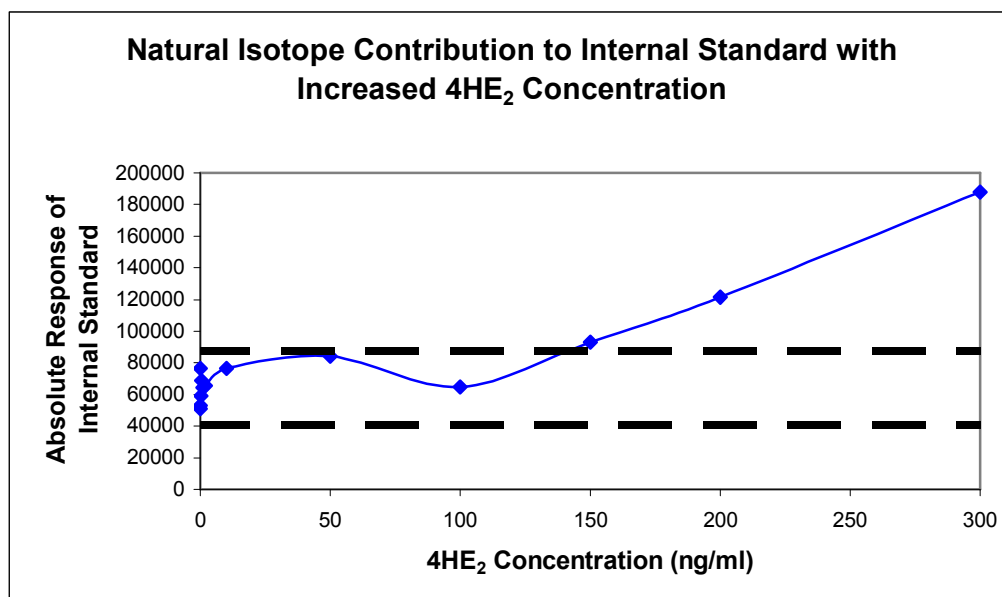
Figure 26: The Natural isotope contribution of 4-HE$_2$ to the internal standard with increased 4-HE$_2$ concentration. (The two black dashed lines indicate mean $\pm$ 2σ)

3.1.9 Characterisation of controls by Standard Addition

Two controls were prepared, one at 80ppt and one at 400 ppt. The internal standard was at 200 ppt. 1 Litre of each control was made up and 10 ml aliquots were made and frozen away at −70°C.   Four aliquots were defrosted and standard addition was done in the following manner: one aliquot was analysed as is, 100 ppt of each metabolite was added to the next aliquot, 200 pt to the next and 300 ppt to the last aliquot. This was repeated five times. The results of the standard addition are given in table 14.

The 95% confidence interval obtained during this experiment was much too wide and indicated that this method is not accurate and since the standard deviation is too wide, the precision is also not good. The method as is, can only be used to determine trends and not to quantify accurately.   Inter experimental comparisons can also not be done.

Table 14: Statistical characterisation of the two controls

Metabolite	80 pg/ml control			
	σ	CV (%)	Mean	95% CI (pg/ml)
E_2	19.10	43.85	43.55	17.19-73.92
2-ME_2	25.76	64.96	41.58	-22.38-105.53
2-HE_2	12.80	29.87	42.87	11.08-74.66
4-HE_2	31.29	17.23	181.63	131.88-231.38
Metabolite	400 pg/ml control			
	σ	CV (%)	Mean	95% CI (pg/ml)
E_2	45.73	25.92	176.43	103.72-249.15
2-ME_2	43.12	24.99	172.56	118.95-226.17
2-HE_2	272.84	58.05	470.02	36.2-903.84
4-HE_2	67.31	20.92	321.75	238.07-405.43

3.2 Application of Analytical Method

3.2.1 Results

Two breast tissue cell lines (MCF-7 and MCF-12A) were exposed to two different concentrations of each metabolite for different lengths of time. The two concentrations used were 1×10^{-8} M and 1×10^{-6} M. The experiment was repeated three times and the representative data is given in Figures 27 – 31.

The two cell lines showed a difference in the metabolism of each of the metabolites they were exposed to. Within a specific cell line, the different concentrations also influenced the metabolism of the cells.

3.2.1.1 Estrogen levels in cell culture medium after incubation with E_2

When the MCF-7 cells were exposed to medium containing 1×10^{-8} M E_2, the E_2 concentration showed a decrease over a period of 24 hours whereas the same was not observed for 1×10^{-6} M (figure 27A). In this case it showed an increase of about 150% at 8 hours and then returned to about the same level as the initial level after 24 hours. The MCF-12A cell line did not show the same trend. When the cells were exposed to the medium containing 1×10^{-8} E_2, the concentration stayed fairly stable, whereas cells exposed to 1×10^{-6} M E_2 showed an increase in the E_2 medium concentration over time (figure 27A). At 24 hours the E_2 concentration in the medium was more than 200% of

the initial concentration. 2-ME$_2$, 2-HE$_2$ and 4HE$_2$ were not detectable above the quantification limit (figure 28A-D).

3.2.1.2 Estrogen levels in cell culture medium after incubation with 2-ME$_2$

MCF-7 cells exposed to the medium containing 1×10^{-8} M of 2-ME$_2$ showed a steady decrease in medium concentration of 2-ME$_2$ over 8 hours (figure 27B). After 1 hour, all the other metabolites were present and after 8 hours only a slight decrease of E$_2$, 2-ME$_2$, and 2-HE$_2$ was detected (figure 29A). However, cells exposed to the medium containing 1×10^{-6} M 2-ME$_2$ didn't react in the same way. After 1 hour all the metabolites were present, but in comparison to 2-ME$_2$, their levels were much lower (figure 29B). 4-HE$_2$ was just above the quantification limit and after 8 hours no 4-HE$_2$ could be detected (figure 29B). Demethylation also took place to a larger extent with 1×10^{-6} M than with 1×10^{-8} M 2-ME$_2$. MCF-12A cells exposed to medium containing 1×10^{-8} M 2-ME$_2$ did not show a decrease in 2-ME$_2$ over 1 hour, but in addition, E$_2$ was formed (figure 29C). After 8 hours the 2-ME$_2$ level increased by 10%, but the E$_2$ level remained constant (figure 27B). None of the other metabolites were formed. MCF-12A cells exposed to 1×10^{-6} M 2-ME$_2$ showed a decrease in 2-ME$_2$ in the medium after 1 hour (figure 29D). No change of concentration levels took place after 8 hours except for the 2-ME$_2$ levels that increased by 20% to that of the initial level (figure 27B).
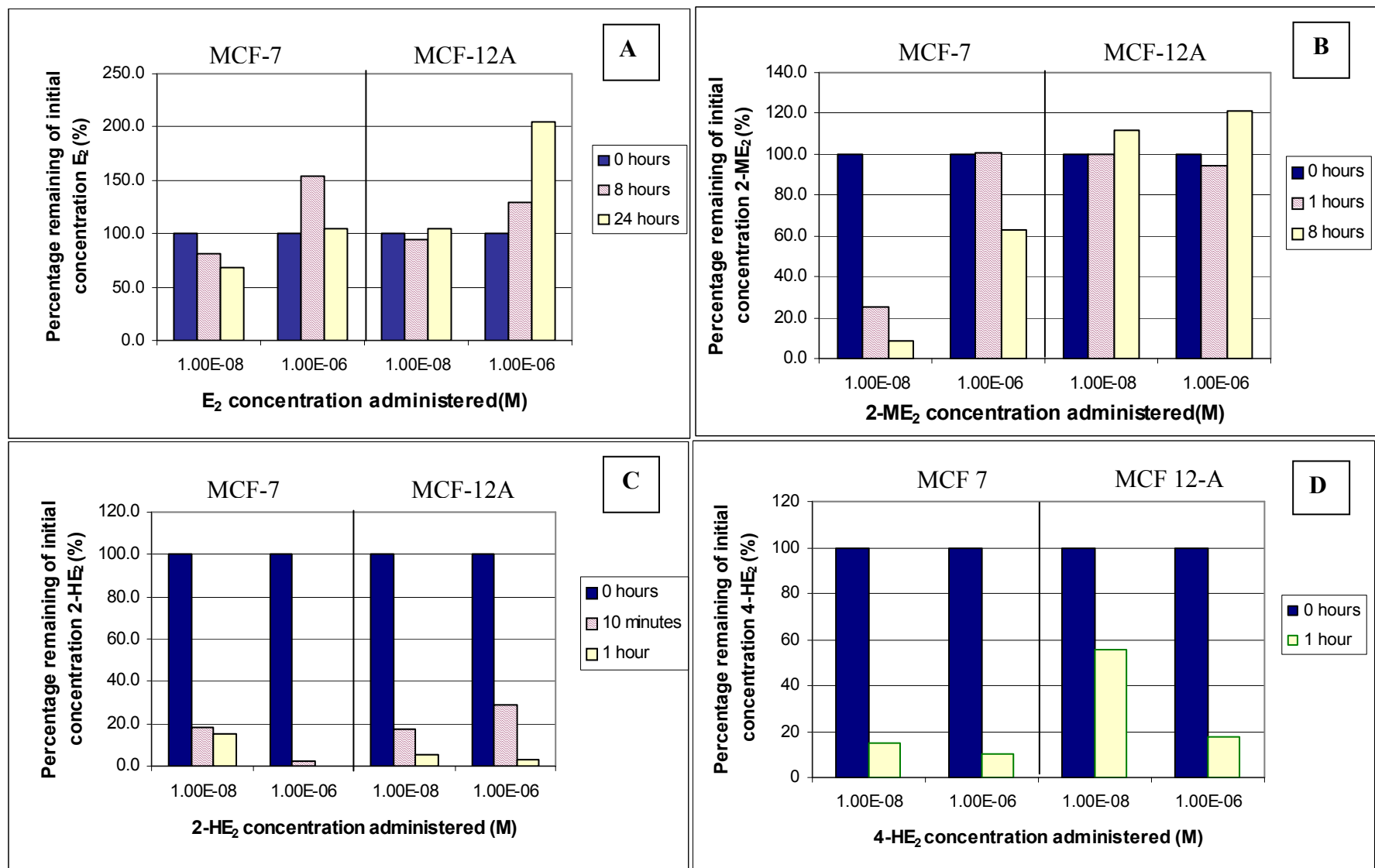
Figure 27: E_2 (A), 2-ME_2 (B), 2-HE_2 (C), 4-HE_2 (D) remaining in the medium of MCF-7 cells and MCF-12A cells after 0, 8, and 24 hours of incubation. Medium concentration is presented as a persentage of the initial concentration of the metabolite the celss were exposed to.
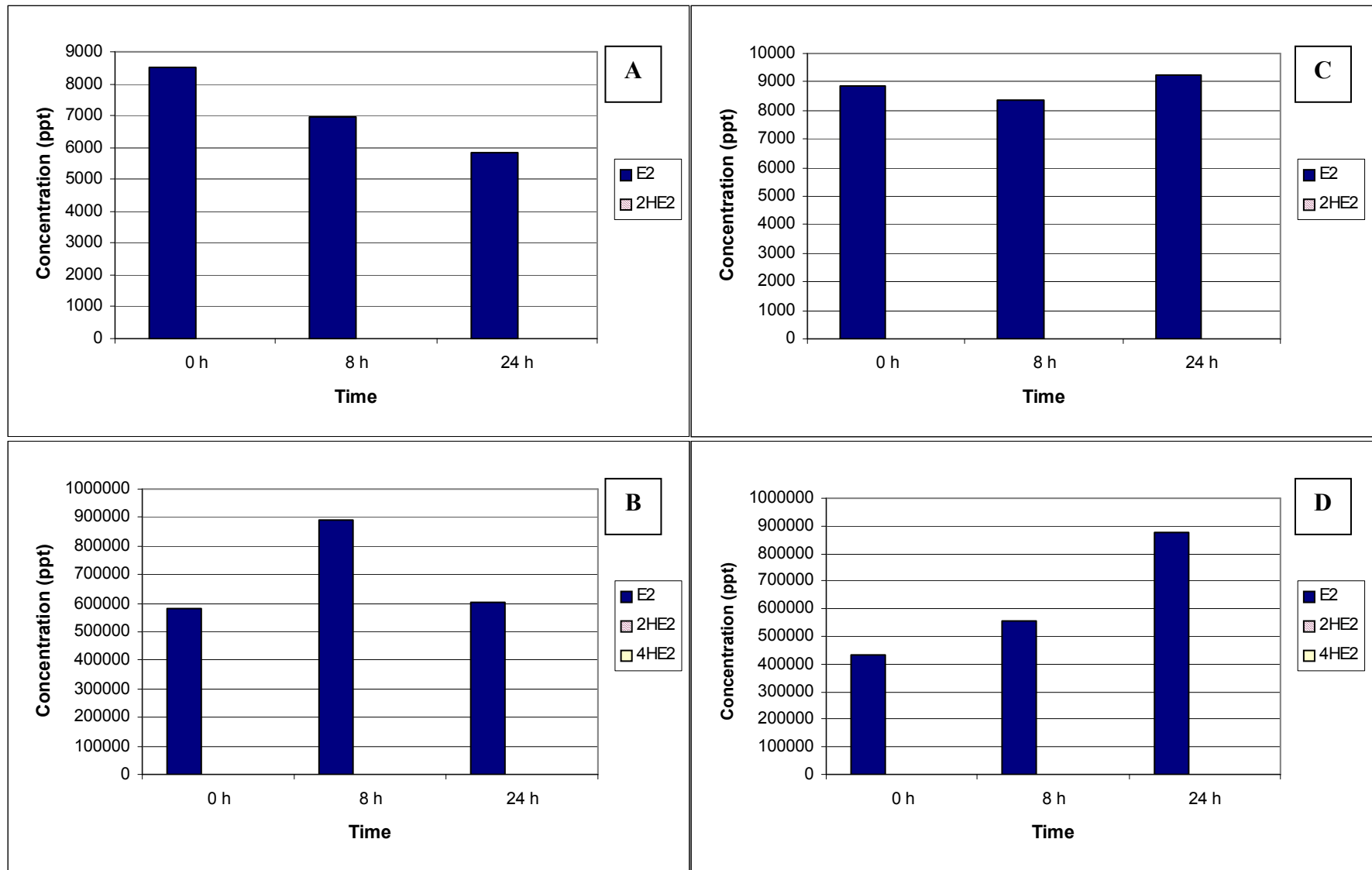
Figure 28: Concentration of metabolites in medium of MCF-7 cells exposed to 1 x 10^{-8} M E$_2$ (A), 1 x 10^{-6} M E$_2$ (B), and in medium of MCF-12A cells exposed to 1 x 10^{-8} M E$_2$ (C), as well as 1 x 10^{-6} M E$_2$ (D) measured at different intervals including 0, 8 and 24 hours.
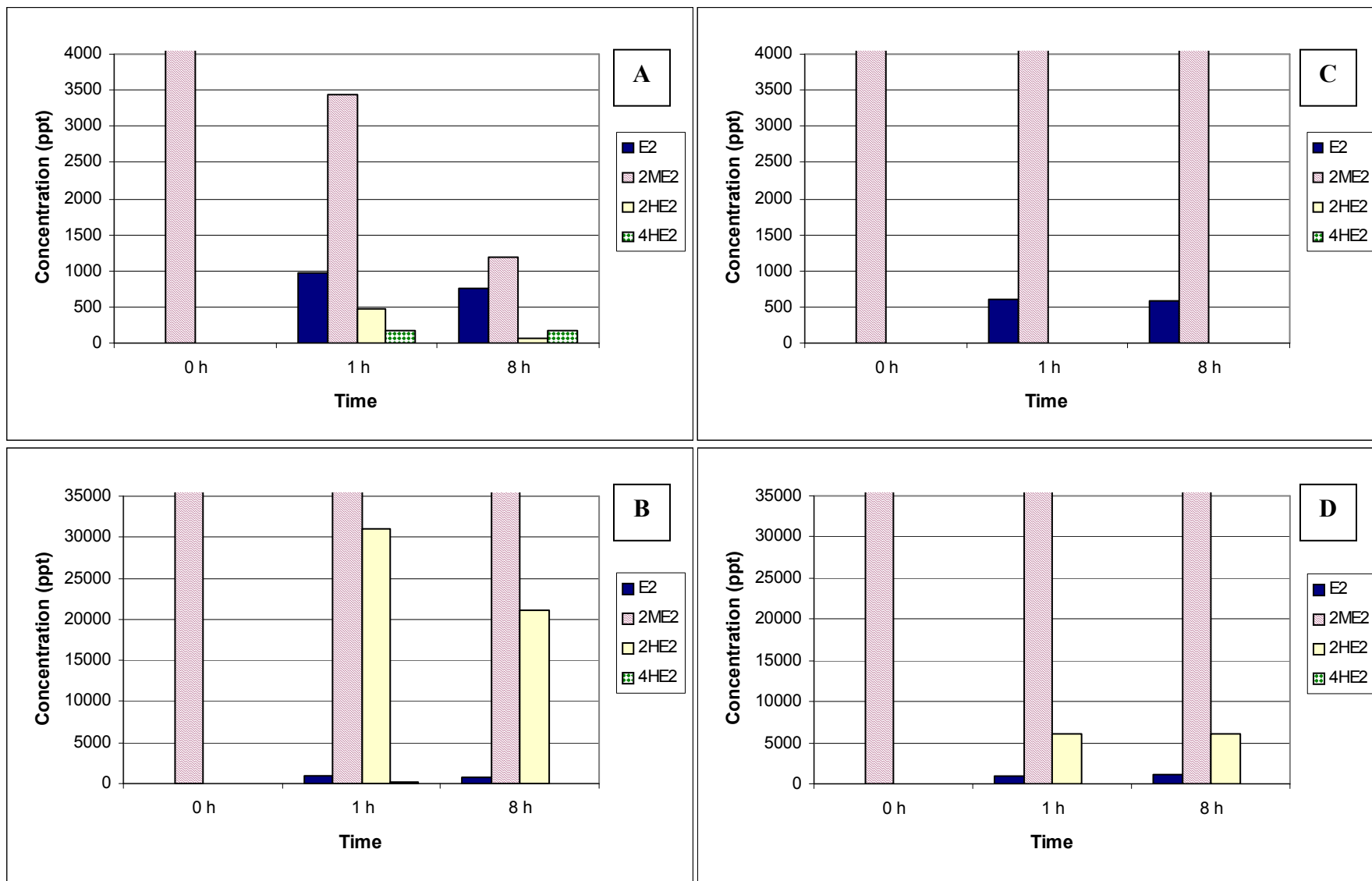
Figure 29: Concentration of metabolites in medium of MCF-7 cells exposed to 1 x 10^{-8} M 2-ME_2 (A), 1 x 10^{-6} M 2-ME_2 (B), and in medium of MCF-12A cells exposed to 1 x 10^{-8} M 2-ME_2 (C), as well as 1 x 10^{-6} M 2-ME_2 (D) measured at different intervals including 0, 1 and 8 hours.
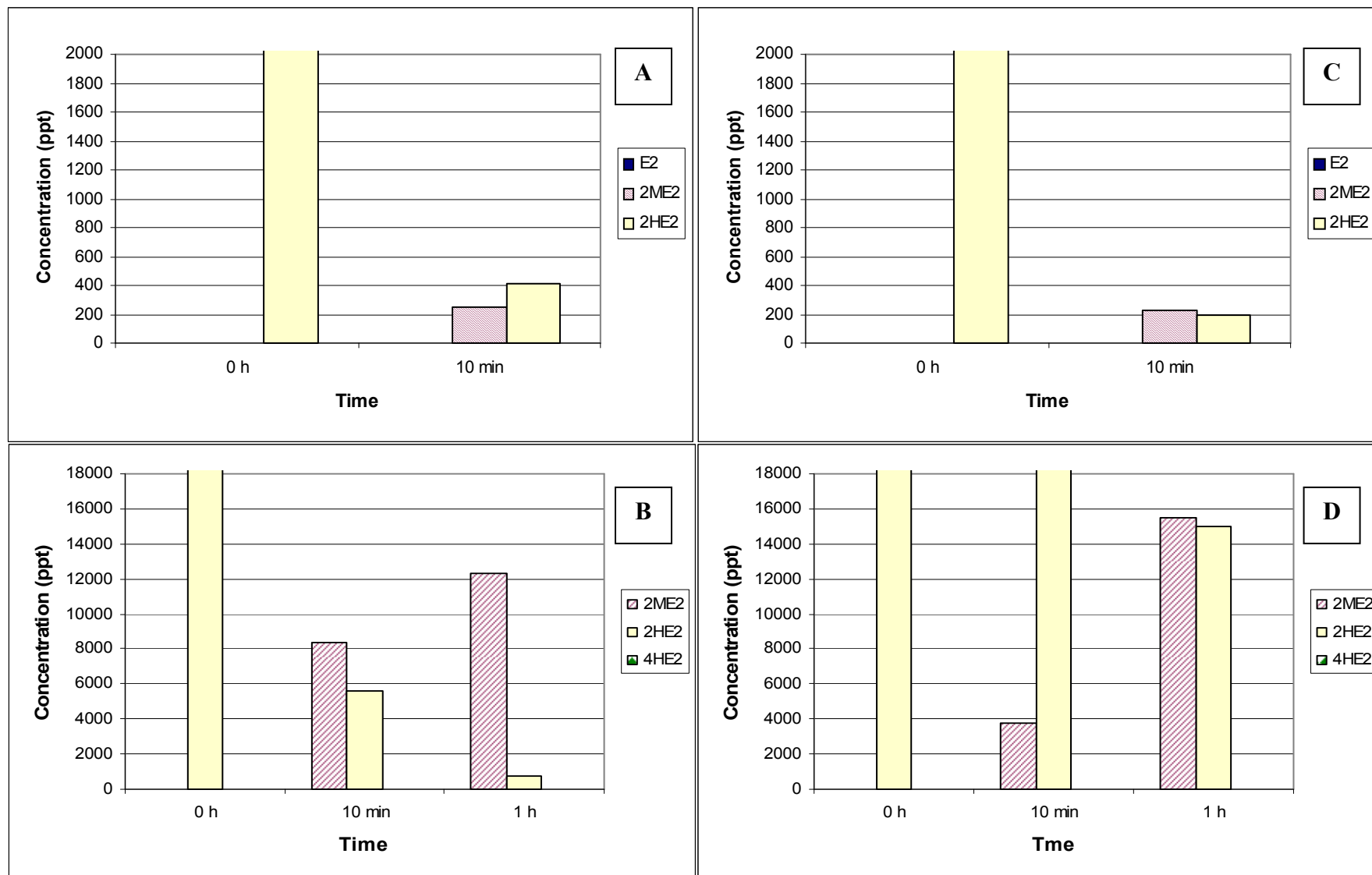
Figure 30: Concentration of metabolites in medium of MCF-7 cells exposed to 1 x 10^{-8} M 2-HE$_2$ (A), 1 x 10^{-6} M 2-HE$_2$ (B), and in medium of MCF-12A cells exposed to 1 x 10^{-8} M 2-HE$_2$ (C), as well as 1 x 10^{-6} M 2-HE$_2$ (D) measured at different intervals including 0, 10 minutes and 1hour.
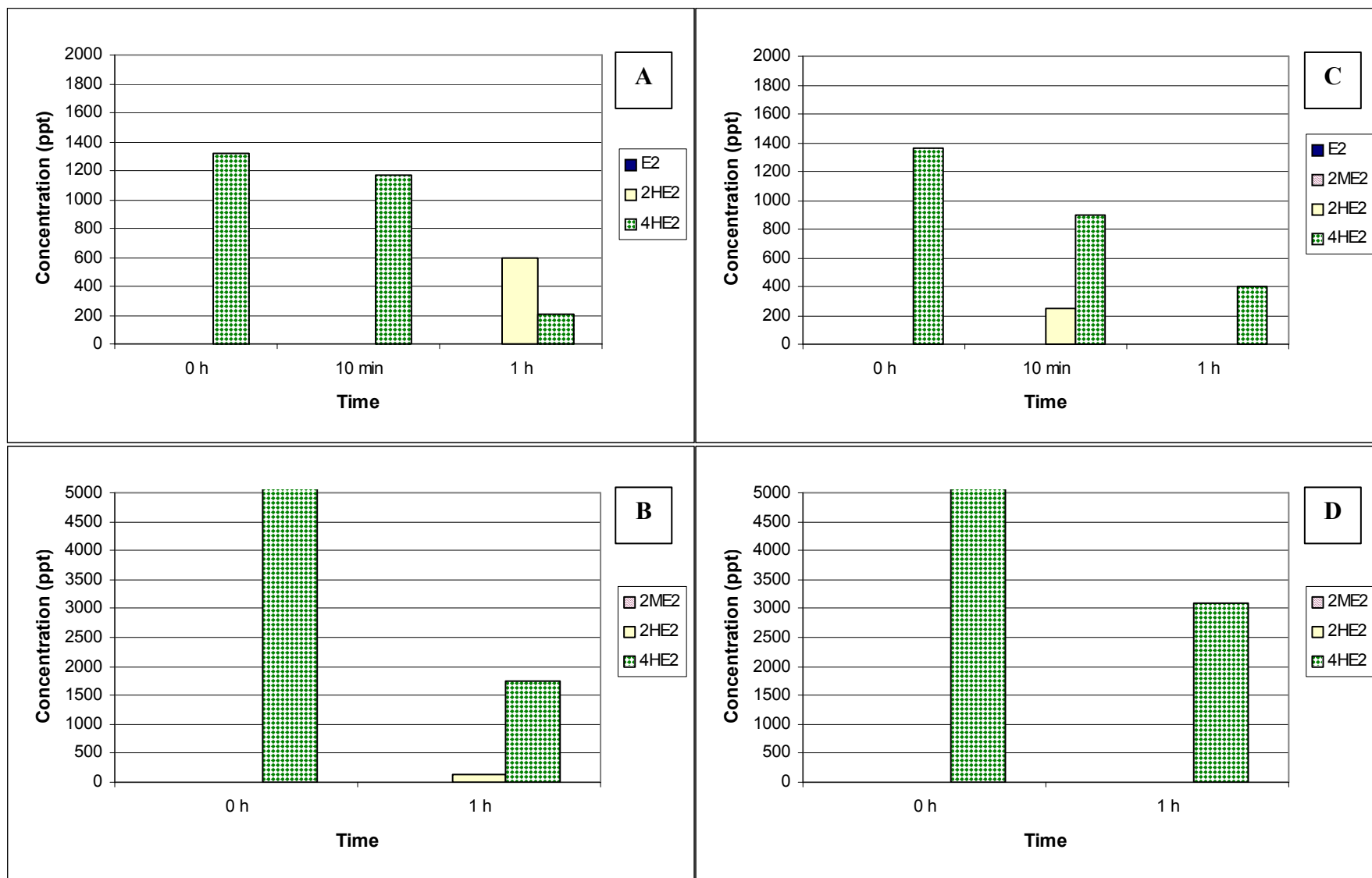
Figure 31: Concentration of metabolites in medium of MCF-7 cells exposed to 1 x 10^{-8} M 4-HE$_2$ (A), 1 x 10^{-6} M 4-HE$_2$ (B), and in medium of MCF-12A cells exposed to 1 x 10^{-8} M 4-HE$_2$ (C), as well as 1 x 10^{-6} M 4-HE$_2$ (D) measured at different intervals including 0, 10 minutes and 1 hours.

3.2.1.3   Estrogen levels in cell culture medium after incubation with 2-HE$_2$

Both the MCF-7 and MCF-12A cell lines showed a dramatic decrease of about 80% in the medium levels of 2-HE$_2$, following exposure to the medium containing 1 x 10^{-8} M 2-HE$_2$ after 10 minutes (figure 27C). After 1 hour the level decreased further by about 2% for the MCF-7 cells and with 10% for the MCF-12A cells (figure 27C). Similar results were observed in the medium of cells exposed to 1x10^{-6} M 2-HE$_2$, but the decrease in 2-HE$_2$ concentration in the medium of the MCF-7 cells was 98% after 10 minutes (figure 27C). The medium given to the MCF-12A cells showed a decrease of 70% after 10 minutes, and a further decrease of 28% after 1 hour (figure 27C). *O*-Methylation of 2-HE$_2$ took place in all instances, but more pronounced with 1x10^{-6} M 2-HE$_2$ than with 1x10^{-8} M 2-HE$_2$ (figure 30D). After 10 minutes the medium given to the MCF-7 cells showed more *O*-methylation than the MCF-12A cell medium, but after 1 hour the situation was reversed (figure 30B, D). The MCF-12A medium showed a higher level of 2-ME$_2$ than the MCF-7 medium. Although the MCF-7 cells showed faster initial *O*-methylation, after 1 hour the situation was reversed.

3.2.1.4   Estrogen levels in cell culture medium after incubation with 4-HE$_2$

Both cell lines' medium containing 1 x 10^{-8} M 4-HE$_2$ initially showed a decrease in the 4-HE$_2$ level (figure 27D). This decrease was more pronounced in the MCF-12A medium after 10 minutes (figure 31A, C). However, after 1 hour the MCF-7 medium showed the lowest level of the two cell lines (figure 27D). After 10 minutes some 2-HE$_2$ could be detected in the MCF-12A medium but not in the MCF-7 medium (figure 31A, C). The

latter showed a higher over all level of 2-HE$_2$ than the MCF-12A medium (figure 31A, C). In general, it seems that a lower percentage of the 4-HE$_2$ present in the medium disappeared over an hour's time in the MCF-12A medium, than in the MCF-7 medium.

3.2.2 Discussion

In the etiology of breast cancer, estradiol (E$_2$) and its catechol metabolites play a major part. A significant ratio to consider as a biomarker for breast cancer risk is the 4-HE$_2$/2-ME$_2$ ratio. In this study, an analytical method was developed to measure the concentration levels of E$_2$, 2-ME$_2$, 2-HE$_2$ and 4-HE$_2$ in cell culture medium making use of a GC-MS. The analytical method was then applied to observe trends in the metabolism of E$_2$ and its metabolites in cell culture medium.

When considering the results obtained and the possible fate of the administered metabolites, it must be kept in mind that metabolite levels were determined in medium and may have been influenced by the following factors:

- metabolites could have been taken up by the cells

- metabolites could have been broken down by the cells

- metabolites could have been changed metabolically by the cells

- metabolites could have been produced and secreted by the cells

3.2.2.1   Estrogen levels in cell culture medium after incubation with E_2

After cells were exposed to medium containing E_2 for 8 hours and 24 hours, none of the other metabolites could be detected. This could be ascribed to two possibilities: either the cells did not metabolise the E_2 into the specific metabolites detectable by this method, or the levels produced are under the detection limit of this method. Lavigne *et al.* stated that MCF-7 breast tumour epithelial cells do not express the enzyme responsible for catalysing the formation of the catechol estrogens unless induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [69]. However, the literature referred to, showed that TCDD increases this metabolic pathway dramatically due to the induction of CYP1A1 and CYP1B1, but that there is no evidence that TCDD is necessary for its activation. Metabolism takes place in the absence of TCDD as well [70]. The fact that the catechol estrogens are being metabolised when administered to the cells, shows that metabolism in these directions does occur (figures 31 - 32). The second probability suggested, is therefore the more plausible one.

Spink *et al.* investigated the effect of TCDD on E_2 metabolism in MCF-7 cells under slightly different culturing conditions than those of our study, making use of a GC-MS [67]. Cells were propagated in Dulbecco's modified Eagle's medium that were supplemented with 5% foetal calf serum and non-essential amino acids and containing 10 ng of insulin, 100 units of penicillin, and 100 μg of streptomycin per ml. Phenol red was omitted [67]. Results obtained concerning the depletion of E_2 in MCF-7 culture not treated with TCDD over time did not correlate exactly with the results obtained during our study. The addition of 1×10^{-8} M E_2 to the medium of MCF-7 cells led to a decrease

of about 60% in the medium E_2 concentration after only 12 hours. We observed a decrease of about 40% after 24 hours.   We decided not to omit phenol red in the medium, because the metabolism we were interested in did not involve the estrogen receptor and phenol red would therefore not have had an influence on our experiments [71]. Any unknown effects that phenol red could have had on the experiments were compensated for by the control. In our study 10% foetal calf serum was used, that should have had an enhancing effect on the metabolism of the cells because of an increased concentration of growth factors present [72, 73, 74, 75]. This inconsistency would need further exploration.

MCF-7 cells exposed to medium containing 1 x 10^{-8} M E_2 showed a decrease in E_2 medium concentration over time, while MCF-12A cells did not produce the same results.   This indicates that E_2 metabolism in transformed cells differs from E_2 metabolism in normal cells. It could also indicate that the transformed cells show a higher uptake of E_2 into the cells than the normal cell line. The fact that it differs for the two cell lines is important and merits further investigation.

Castagnetta *et al.* [65] devised a method of GC-MS analysis for the identification of CE derivatives after their measurement in benign and malignant breast tissues by reverse-phase, high-performance liquid chromatography and electrochemical detection.   Their results indicated that of the metabolites investigated in this study, 4-HE$_2$ showed the highest level in breast tumour extracts. This finding, together with the fact that we found no 4-HE$_2$ in the medium of cells exposed to E_2, raises the question of whether the

intracellular concentration differs from the extra cellular concentration (in the medium). This is definitely an avenue of exploration that must be considered in the future.

An unexpected result was the increase of E_2 levels in the medium when the cells were exposed to a concentration of 1 x 10^{-6} M E_2. This shows that the cells either form E_2, or that the cells release it into the medium when exposed to such a high concentration. The MCF-12A cell line especially showed this increase in E_2 concentration in the medium over time. After 24 hours the concentration in the medium doubled that of the initial value. This specific metabolism of E_2, however, was not observed in the MCF-7 medium. Although a 50% increase could be seen after 8 hours, the E_2 level returned to the initial level after 24 hours (figure 27). The levels of the other metabolites (if any), even with the increase in E_2 concentration over time, were still lower than the detection limit (figure 28). The difference in metabolism observed within a cell line because of different concentrations of E_2 the cells were incubated with, together with the difference in metabolism observed between the cell lines, shows that estrogen metabolism is concentration sensitive. It also shows that a change in metabolic pathways accompany cell transformation. The impact of this change in metabolism can only be fully understood if the specific concentration changes that took place can be determined. Because of the detection limit not being low enough, this could not be determined.

3.2.2.2   Estrogen levels in cell culture medium after incubation with 2-ME$_2$

The results obtained from this experiment provided very significant information. The metabolism of 2-ME$_2$ given to cells at a concentration of 1 x 10^{-8} M is different for the

two cell lines. The MCF-7 medium showed all four metabolites of interest after 1 hour, where the MCF-12A medium only showed the presence of E_2 (figure 29). This however, does not mean that they may not be present within the cells. $4\text{-}HE_2$ is formed or released by the breast tumour cells but not by the normal cells. As explained previously these two metabolites ($2\text{-}ME_2$ and $4\text{-}HE_2$) have antagonistic effects on cell number homeostasis. The fact that the MCF-7 cells form/release the hormone known to enhance carcinogenesis from $2\text{-}ME_2$ shows that there is a shift in the metabolic pathway equilibrium to favouring the formation of $4\text{-}HE_2$ in carcinoma cells with respect to normal cells and that the $4\text{-}HE_2/2\text{-}ME_2$ ratio is an important aspect to consider in the determination of breast cancer risk. This may also indicate that although COMT activity has been found to be higher in malignant tumours than in benign tumours or normal breast tissue [21], demethylation and the formation of $4\text{-}HE_2$ is also taking place in transformed cells and could therefore counteract the higher COMT activity. It may also be that the increased COMT activity is the way in which the cells try to counteract the formation of $4\text{-}HE_2$ from $2\text{-}ME_2$.

3.2.2.3   Estrogen levels in cell culture medium after incubation with $2\text{-}HE_2$

The decrease in the $2\text{-}HE_2$ level in MCF-7 medium was more severe in the medium containing 1×10^{-6} M $2\text{-}HE_2$ than in medium containing the 1×10^{-8} M $2\text{-}HE_2$ (figure 28). The implication of this result is that in this specific cell line, the increased $2\text{-}HE_2$ concentration causes a shift in the equilibrium of the metabolic pathways to favour the removal of $2\text{-}HE_2$ even more. The formation of $2\text{-}ME_2$ was also higher in relation with the 1×10^{-6} M than with the 1×10^{-8} M, which strengthens the previous statement (figure

30). In the cells incubated in medium containing 1×10^{-6} M, the initial O-methylation was slower in the MCF-12A cell line than in the MCF-7 cell line, but after 1 hour more 2-ME_2 was formed in the MCF-12A medium than in the MCF-7 medium (figure 30). The 2-HE_2 level after 1 hour of incubation decreased less in the MCF-12A medium than in the MCF-7 medium. This is consistent with the results that we expected in that the normal cells showed a higher formation of 2-ME_2 and a slower breakdown of 2-HE_2 than the breast tumour cells. 2-HE_2 causes less cell proliferation and DNA damage than 4-HE_2 and 2-ME_2 causes apoptosis in tumourogenic cells. It is therefore of benefit to have more of these two metabolites than of 4-HE_2. However, this is only the case for the 1×10^{-6} M concentration, which is above physiological concentrations. When the cells were incubated with medium containing a concentration of 1×10^{-8} M 2-HE_2, the same may well have happened, but the detection limit of our analytical method was not low enough to detect it.

3.2.2.4   Estrogen levels in cell culture medium after incubation with 4-HE_2

Roy *et al.* [76] stated that the metabolic clearance rate of 4-HE_2 is considerably lower than that of 2-HE_2 because of the inhibition of the O-methylation of 4-HE_2 by 2-HE_2. Emons *et al.* [77] and Ball *et al.* [78] showed that 4-HE_2 has a lower metabolic clearance rate than 2-HE_2 in human males and in rats. We have found that MCF-12A cells incubated in medium containing 1×10^{-8} M 4-HE_2 showed a decrease of about 40% after 1 hour of incubation whereas a decrease of about 90% was observed in the medium of cells incubated with 1×10^{-8} M 2-HE_2 (figure 28). This shows that even without the presence of 2-HE_2, 4-HE_2 is metabolised or absorbed slower than 2-HE_2. This was also true for

MCF-12A cells incubated in medium containing 1×10^{-6} M the metabolites, but to a lesser extent (figure 28). The MCF-7 cell line produced different results in this respect than the MCF-12A cell line. The medium of cells incubated in medium containing 1×10^{-8} M of the metabolites showed similar decreases over 1 hour, but the cells incubated in medium containing 1×10^{-6} M showed a slightly lower decrease in $4\text{-}HE_2$ medium levels than $2\text{-}HE_2$ levels (figure 28). The fact that $4\text{-}HE_2$ decreases at a slower rate in the normal cell line than in the tumourogenic cell line is not consistent with what we expected to find. The expected results were just the opposite of what occurred. Once again it should be kept in mind that the medium levels of metabolites can be influenced not only by metabolism, but also by the fact that the metabolites may be taken up or released by the cells. It therefore becomes evident that the intracellular concentration of the metabolites should also be determined to further unravel this metabolic puzzle.

An interesting phenomenon observed in this experiment is the formation of $2\text{-}HE_2$ from $4\text{-}HE_2$ when cells were incubated in medium containing 1×10^{-8} M of $4\text{-}HE_2$ in both cell lines. The MCF-7 cell line showed the presence of $2\text{-}HE_2$ only after 1 hour, whereas the MCF-12A cell line already showed the presence of $2\text{-}HE_2$ after 10 minutes. After 1 hour there were, however, no traces of $2\text{-}HE_2$ left in the medium of the MCF-12A cell line. The formation of $2\text{-}HE_2$ from $4\text{-}HE_2$ will need some further investigation to fully determine to what extent this plays a role in the metabolism of E_2 and its metabolites.

4 Conclusion

Breast cancer is one of the most common cancers affecting women [5]. It remains the leading cause of death in American women from 30 – 70 years of age and approximately 10% of the women living in western countries will develop breast cancer during their life time [6, 7]. Estradiol is the most abundant estrogen in premenopausal woman [20, 21]. Initially it was thought that estradiol itself was responsible for tumourigenesis [29, 30]. It has since been discovered that the catechol metabolites of estradiol and other estrogens cause carcinogenesis [31]. In this study, an analytical method was developed to measure the concentration levels of E_2, $2\text{-}ME_2$, $2\text{-}HE_2$ and $4\text{-}HE_2$ in cell culture medium. The analytical method made use of GC-MS analysis, since the expected physiological concentrations of these metabolites were very low.

During the validation of this technique, it was found that the method did not produce accurate measurements and that it could only be used to determine trends. For quantitative work and more accurate results, the accuracy of this method will have to be improved. The GC-MS remains the most powerful analytical tool, so continued use of this instrument is at the heart of determining the concentrations of these metabolites *in vitro* as well as *in vivo*.

 Since uncertainty still existed concerning what would be found during *in vitro* experiments in terms of concentration levels and metabolism, it was decided to proceed with the experiments using the method as is, so that we could get an idea of how the method should still be refined and what the concentration levels were.

The results from the physiological experiments showed that further refinement with respect to the quantification limit is necessary. The levels of the metabolites may have been below the limit of detection. Currently the method only injects 1μl of the sample onto the column making use of splitless injection. By increasing the injection volume to 2μl and injecting pulsed splitless, the detection limit can be lowered because more of the analytes are injected into the column. The extraction method could also be refined in order to achieve better extraction, thereby also lowering the detection limit. Stir Bar Sorptive Extraction (SBSE) should be explored as an extraction procedure instead of liquid-liquid extraction, or the solid phase extraction technique could be re-implemented and refined [79, 80]. SBSE and solid phase extraction generally have a higher percentage in sample recovery than liquid-liquid extraction because they are based on the principle of chromatography in that you have a solid phase, which retains solutes (extracts analytes out of a liquid phase), that is passed over the solid phase [64]. Liquid-liquid extraction is based on the principle that like dissolves in like. SBSE and solid phase extraction therefore may increase the sensitivity of the method [80]. With a lowered detection limit, it would be possible to detect the metabolites that may have been present in concentration levels lower than the detection limit.

Although the aim of this study was not achieved fully, the results obtained show the need for continued research in this area. Some of the results obtained were consistent with what was expected, *e.g.*increased 2-ME$_2$ levels in the medium of MCF-12A cells *vs*. that of the MCF-7 cells, suggesting a possible protective function against tumourigenesis.

There were, however, also inconsistencies from what was expected, *e.g.* the slower decrease observed in 4-HE$_2$ levels in MCF-12A cell medium than in MCF-7 cell medium. With a refined analytical method that shows enhanced sensitivity and accuracy, some of the unanswered questions may be addressed.   Knowledge of the intracellular concentrations of the metabolites will also contribute greatly to this effect.

It is clear that a difference in concentration of the metabolites causes a difference in its metabolism. In addition, estrogen metabolites are differentially metabolised in normal and transformed breast cell lines. It is therefore essential to continue researching this area. If an accurate, reproducible and sensitive detection method can be developed, the applications and benefits are numerous. It will provide a way of determining the normal levels of these metabolites *in vivo*. The complex metabolic pathways can be unravelled further and a better understanding of the mechanism of carcinogenesis can be obtained. There are also diagnostic applications through the 4-HE$_2$/2-ME$_2$ ratio, which can be monitored and a potential shift towards carcinogenesis picked much earlier.

5 Mass spectra of TMS derivatives
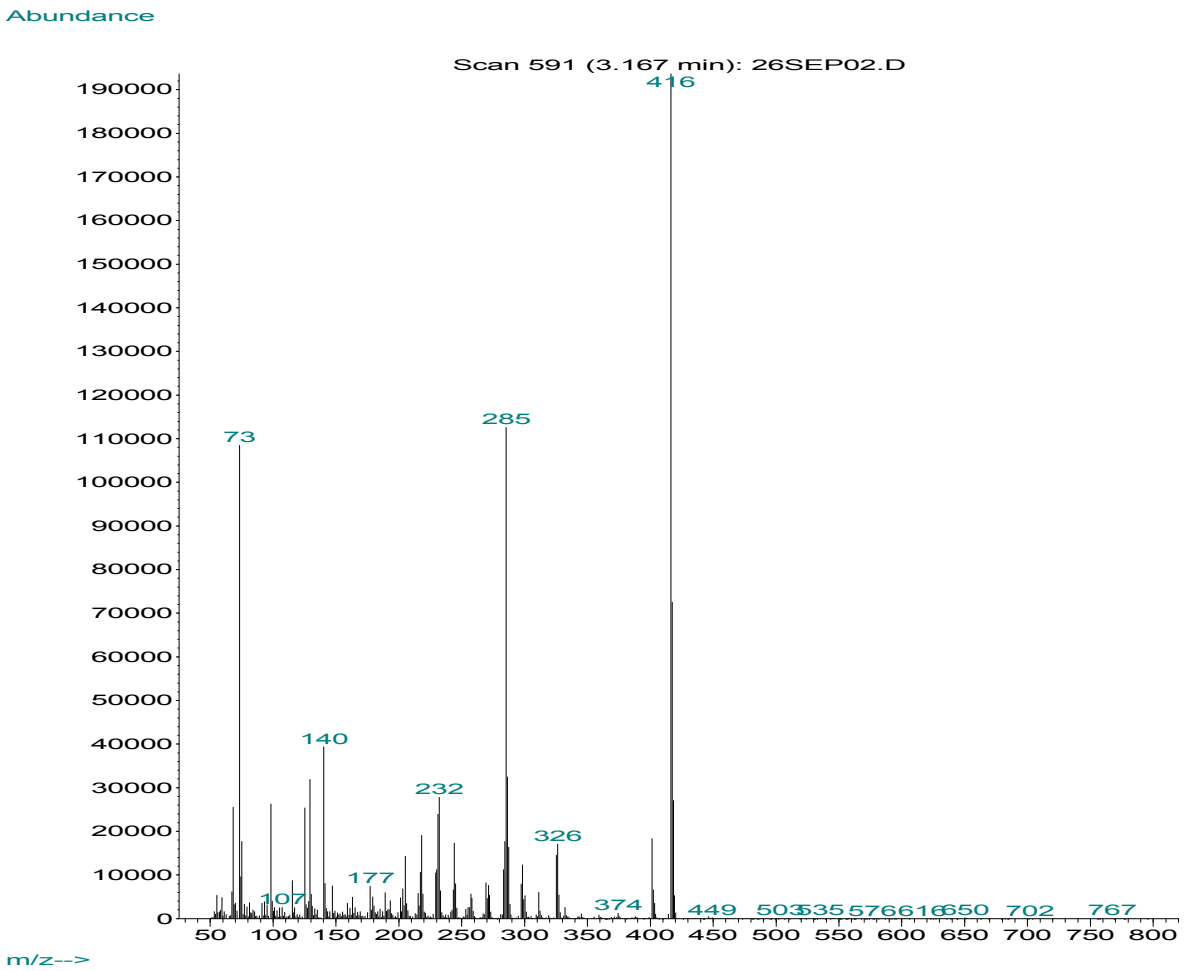
5.1 Mass spectrum of TMS derivative of E₂

Figure 32: Mass spectrum of TMS derivative of E₂.

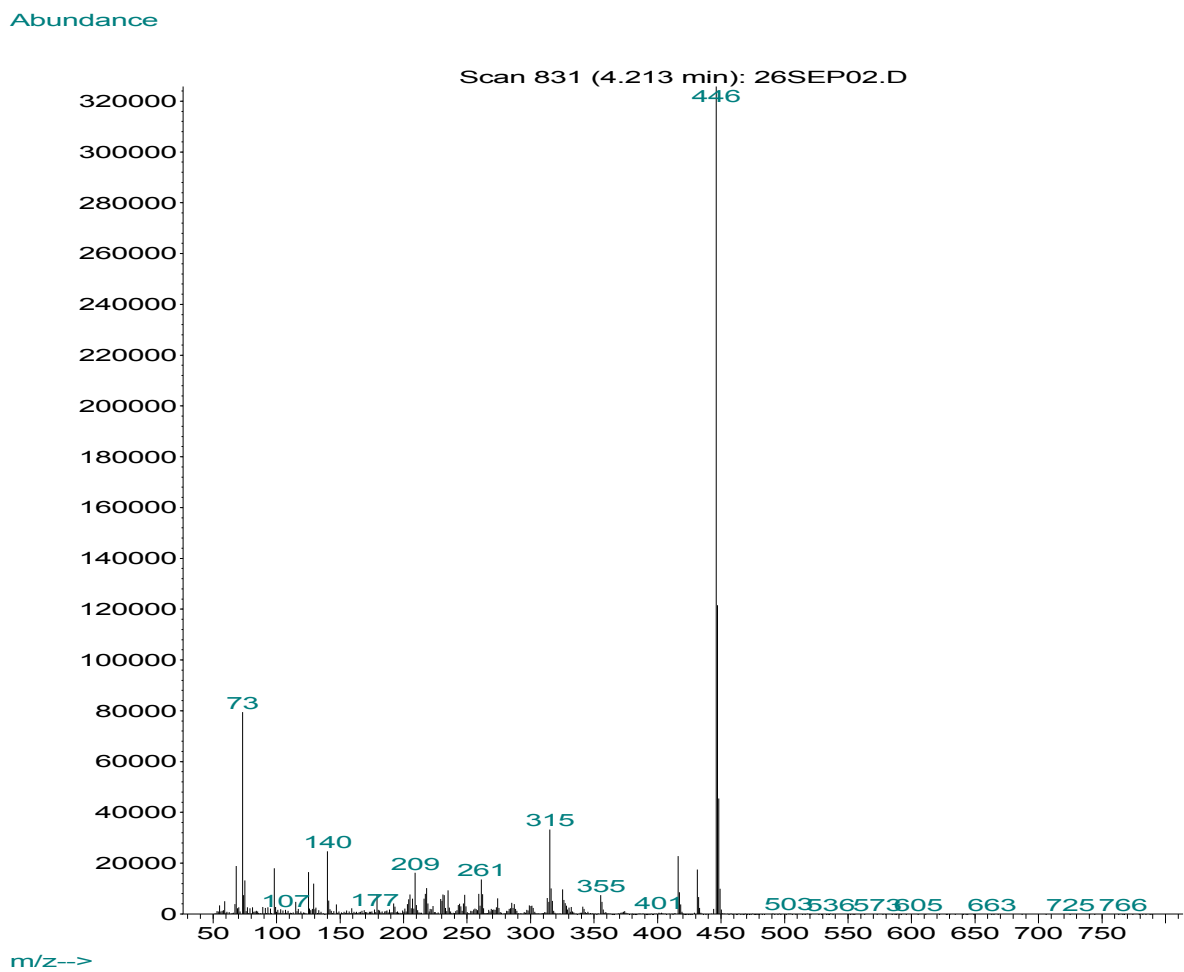**Figure 33: Mass spectrum of TMS derivative of 2-ME₂.**

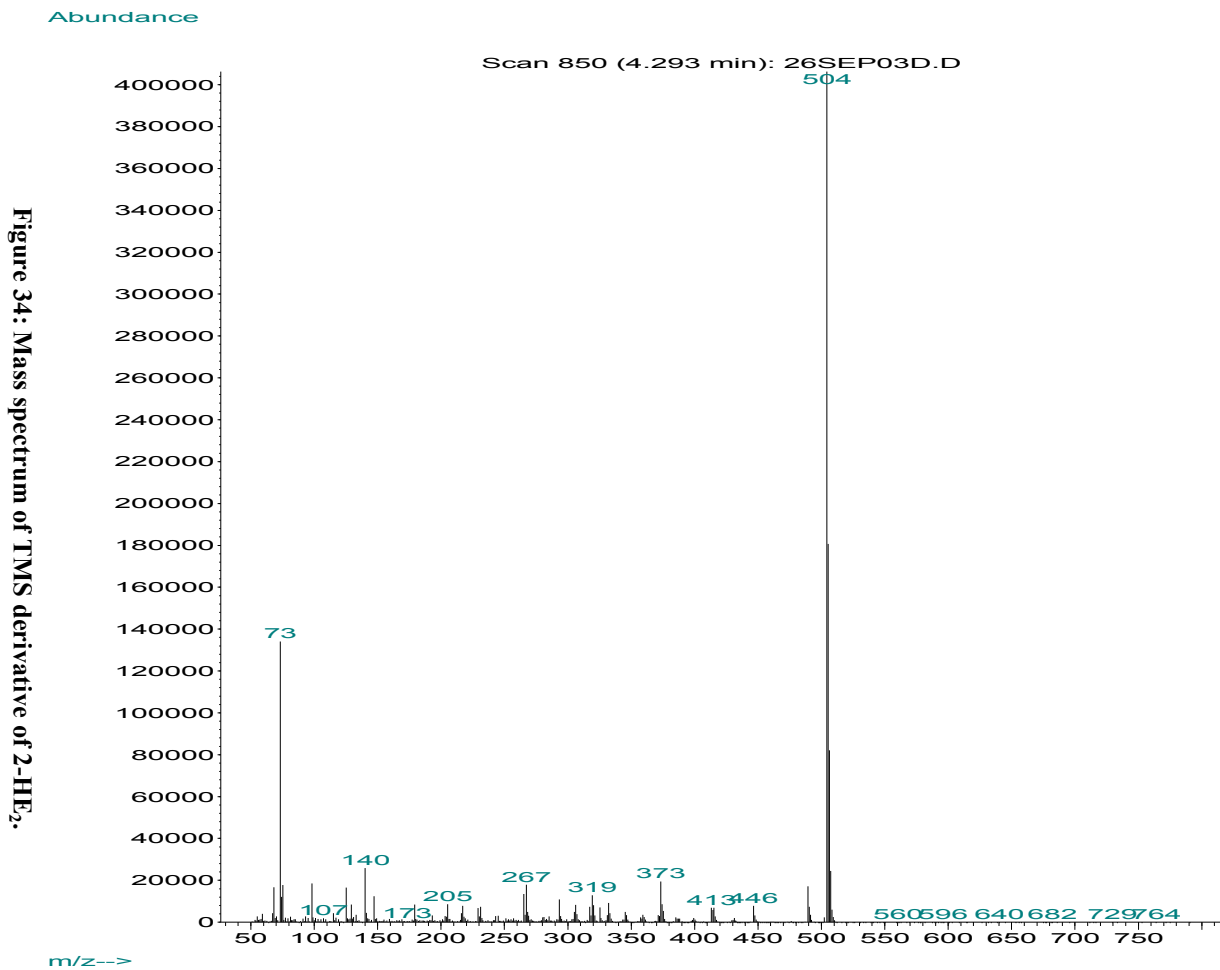Figure 34: Mass spectrum of TMS derivative of 2-HE$_2$.

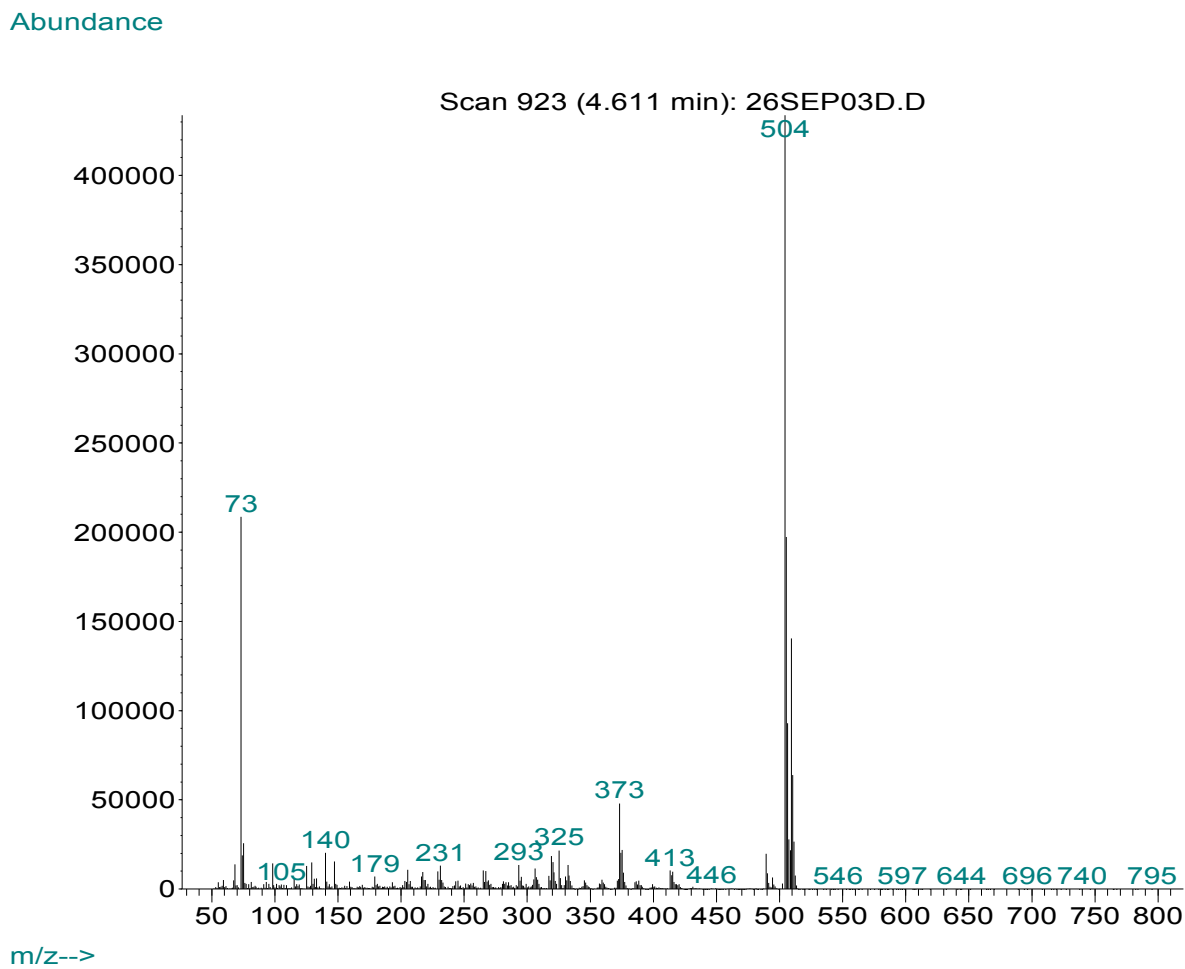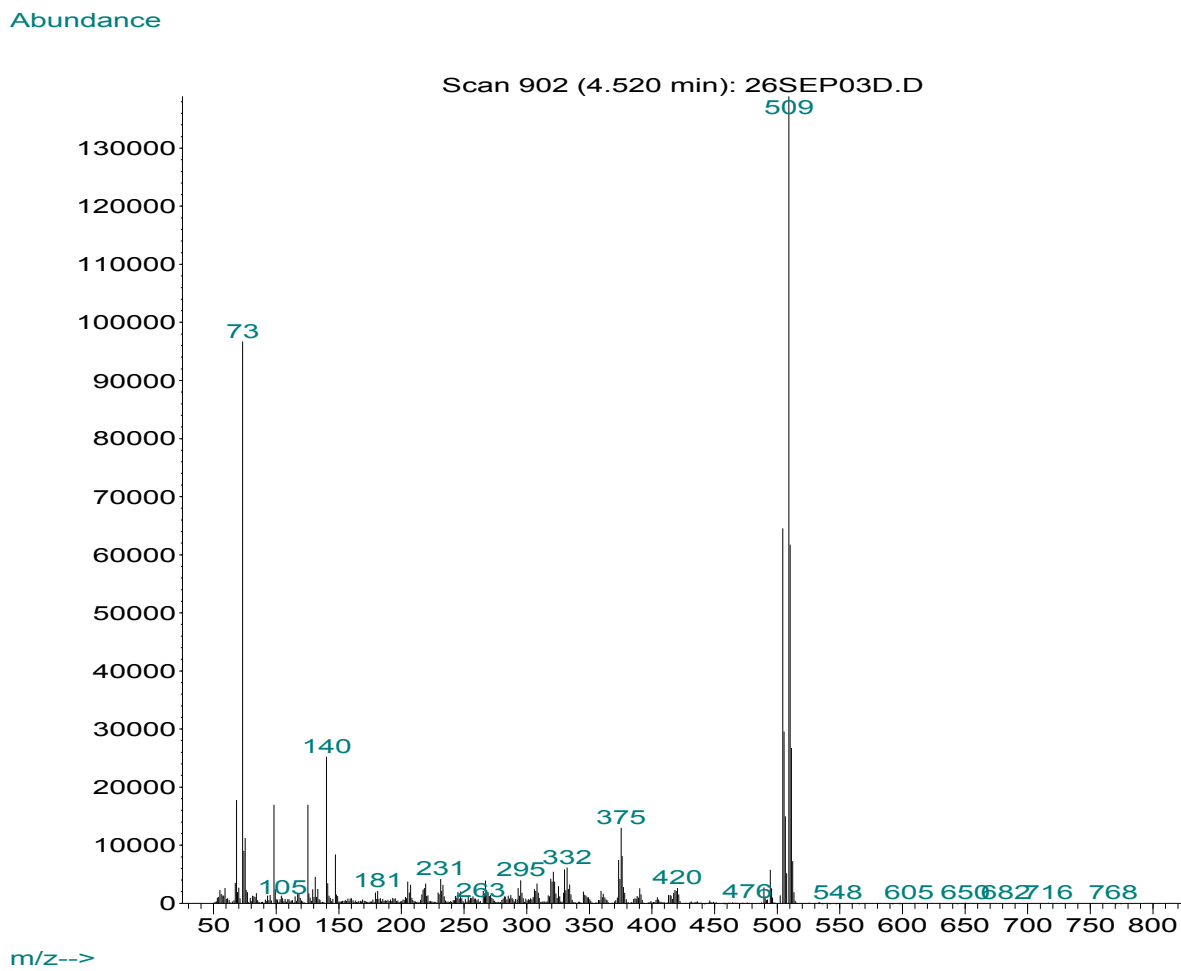Figure 35: Mass spectrum of TMS derivative of 4-HE$_2$.

Figure 36: Mass spectrum of TMS derivative of 4-HE$_2$-5d.

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