PHYTOESTROGENS, BISPHENOL A AND STEROID HORMONES IN SELECTED HUMAN SAMPLES FROM MALAYSIA

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PHYTOESTROGENS, BISPHENOL A AND STEROID HORMONES IN SELECTED HUMAN SAMPLES FROM MALAYSIA

ABSTRACT

There are many endocrine disrupting chemicals (EDCs) that humans are exposed to, whether accidental or upon occupational exposure. EDCs disrupt the normal endocrine function and may cause endocrine related cancers such as prostate and breast cancer. Research has proven there are interaction effects between EDCs which can either be additive, synergistic or antagonistic. Therefore, with many possible EDCs present in one individual, the net effect could vary.

A smaller group of EDCs known as oestrogenic EDCs can originate from natural sources or it can be synthetic in nature. These EDCs mainly produce effects related to the actions of oestrogen. Though EDC levels detected may not reach toxicity level, its presence at low level and the occurrence of combination of EDCs may still cause unwarranted effects over time, or cause further damage due to its cumulative presence in the body.

This research is important in exploring the extent of oestrogenic EDC exposure and its effects on selected sex hormones in various adult populations. The choice of oestrogenic EDCs to be analysed is based on the oestrogenic EDCs which these populations are more commonly exposed to. They are soy isoflavones, a natural source of EDC and bisphenol A which is man-made and their common exposure route is via oral intake.

Method development and analysis of isoflavones (daidzein, genistein, equol, formononetin, biochanin A), bisphenol A, and sex hormones (oestrone, oestradiol, testosterone) was conducted using liquid chromatography tandem mass spectrometry (LCMSMS) for blood and breast tissue matrices. Biological samples were collected

from two separate populations. The first population involved 225 male volunteers who practiced vegetarian or non-vegetarian diet while the second population was among 252 female patients in a university hospital who were treated for breast pathology and non-breast pathologies.

It was found that among the male population, vegans had the highest percentage of equol producers. Chinese males who are non-vegetarians and consumed daidzein and biochanin A tend to have higher testosterone level than other groups. Male equol producers tend to be protected from an increased tendency of a high testosterone level even with consumptions of various isoflavones compared to non-equol producers. Non-equol producers however could have a lower testosterone level upon consumption of equol-rich foods. Among the female subjects, equol producers tend to have a lower oestradiol levels with higher consumption of all isoflavones. This supports the idea of equol playing a role in preventing adverse effects of EDCs. Higher concentration of isoflavones was present in the plasma than in the breast tissue. Highest concentration of EDC found in breast tissue was BPA which was 115 times higher than in plasma.

There was no meaningful association of EDC concentrations and hormone levels in the breast tissue except for oestrone which correlated positively with BPA in Malay subjects and with formononetin in menopausal subjects. Despite the lack of correlation with oestradiol, the extent of BPA bioaccumulation in the breast tissue which may have occurred over time and through countless unintentional exposure is still a major concern.

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ABSTRAK

Terdapat banyak bahan kimia yang boleh mengganggu sistem endokrin atau lebih dikenali sebagai EDC (endocrine disrupting chemicals) terdedah kepada manusia, sama ada sengaja atau tidak sengaja. EDC mengganggu fungsi normal endokrin dan boleh menyebabkan kanser berkaitan sistem endokrin seperti kanser prostat dan kanser payudara. Penyelidikan telah membuktikan terdapat kesan interaksi antara EDC yang mana boleh memberi kesan tambahan, sinergi atau berlawanan. Oleh itu, dengan banyaknya EDC yang mungkin terdapat dalam seseorang individu, kesan keseluruhannya mungkin tidak sama.

Sekumpulan kecil EDC dikenali sebagai "oestrogenic EDC" boleh berasal daripada sumber semula jadi atau dihasilkan oleh manusia (sintetik). *Oestrogenic EDC* ini terutamanya menghasilkan kesan berkaitan dengan tindakan estrogen. Walaupun tahap EDC dikesan mungkin tidak mencapai tahap keracunan, kehadirannya dalam kuantiti yang kecil dan kemungkinan berlakunya gabungan beberapa EDC masih boleh menyebabkan kesan tidak dikehendaki, atau menyebabkan akibat lebih teruk disebabkan oleh pengumpulan EDC tersebut di dalam badan.

Kajian ini penting dalam meneliti sejauh mana manusia terdedah kepada *oestrogenic EDC* dan kesannya terhadap hormon seks di dalam pelbagai populasi dewasa. Pemilihan jenis *oestrogenic EDC* untuk dianalisis adalah berdasarkan antara EDC yang kerap terdedah kepada manusia secara umumnya. EDC tersebut adalah isoflavon soya yang bersumber semula jadi dan bisphenol A yang merupakan buatan manusia dan kaedah pendedahan yang dialami adalah melalui pengambilan oral.

Penghasilan kaedah dan validasi bagi analisis isoflavon (daidzein, genistein, equol, formononetin, biochanin A), bisphenol A, dan hormon seks (oestrone, oestradiol, testosteron) menggunakan kromatografi cecair seiring spektrometri jisim (LCMSMS) untuk matriks darah dan tisu payudara. Sampel biologi diperolehi dari dua populasi berasingan. Populasi pertama melibatkan 225 sukarelawan lelaki pengamal diet vegetarian atau bukan vegetarian manakala populasi kedua adalah antara 252 pesakit wanita di hospital universiti yang dirawat kerana patologi payudara dan yang tidak mempunyai patologi payudara.

Kajian ini telah mendapati bahawa di kalangan lelaki, vegan mempunyai peratusan tertinggi pengeluar equol. Lelaki Cina bukan vegetarian, mengambil daidzein dan biochanin A cenderung mempunyai tahap testosteron yang lebih tinggi daripada kumpulan lain. Pengeluar equol lelaki cenderung dilindungi daripada kecenderungan peningkatan tahap testosteron yang tinggi walaupun dengan pengambilan pelbagai isoflavon berbanding dengan bukan pengeluar equol. Bukan pengeluar equol boleh mempunyai tahap testosteron yang rendah dengan mengambil makanan tinggi kandungan equol. Bagi wanita pula, pengeluar equol cenderung untuk mempunyai tahap estradiol yang lebih rendah dengan meningkatkan pengambilan semua isoflavon. Ini menyokong idea equol memainkan peranan dalam mencegah kesan buruk EDC. Didapati tahap isoflavon lebih tinggi di dalam plasma daripada dalam tisu payudara. Tahap tertinggi EDC di dalam tisu payudara adalah BPA iaitu 115 kali lebih tinggi daripada yang terdapat dalam plasma.

Tidak ada hubung-kait bermakna bagi tahap EDC dan tahap hormon di dalam tisu payudara kecuali estrone yang berkorelasi positif dengan BPA di kalangan pesakit Melayu dan dengan formononetin di kalangan pesakit menopos. Walaupun tidak banyak korelasi dengan estradiol, tahap pengumpulan BPA di dalam tisu payudara yang berlaku

dari masa ke masa melalui pendedahan yang tidak disengajakan dalam kekerapan yang tinggi masih menjadi kebimbangan utama.

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LIST OF SYMBOLS AND ABBREVIATIONS

APCI	:	Atmospheric pressure chemical ionisation
Bio A	:	Biochanin A
BPA	:	bisphenol A
CE	:	Collision energy
CXP	:	Collision exit potential
D	:	Daidzein
DBP	:	di-*n*-butyl phthalate
DDT	:	dichlorodiphenyltrichloroethane
DBT	:	dibutyltin
DEHP	:	di-ethyl(hexyl) phthalate
DNA	:	Deoxyribonucleic acid
DP	:	Declustering potential
E1	:	Oestrone
E2	:	Oestradiol
EDC	:	Endocrine disrupting chemical
ELISA	:	Enzyme linked immunosorbent assay
ER	:	Oestrogen receptor
Eq	:	Equol
ESI	:	Electrospray ionisation
F	:	Formononetin
G	:	Genistein
HPLC	:	High performance liquid chromatography
IQR	:	Interquartile range
GCMS	:	Gas chromatography mass spectrometry

GCMSMS	:	Gas chromatography tandem mass spectrometry
LC	:	Liquid chromatography
LCMS	:	Liquid chromatography mass spectrometry
LCMSMS	:	Liquid chromatography tandem mass spectrometry
LLE	:	Liquid-liquid extraction
LOD	:	Limit of detection
LOQ	:	Limit of quantification
LOV	:	lacto-ovo-vegetarians
LV	:	Lactovegetarians
MnBP	:	mono-*n*-butyl phthalate
MiBP	:	mono-isobutyl phthalate
MRM	:	Multiple reaction monitoring
µg/g	:	microgram/ gram
ND	:	Not detected
ng/g	:	nanogram/ gram
NP		4-*n*-nonylphenol
O-DMA	:	O-desmethylangolensin
OP	:	4-*t*-octylphenol
OV	:	Ovovegetarians
PAHs	:	polycyclic aromatic hydrocarbons
PCBs	:	polychlorinated biphenyls
PFCs	:	perfluorinated chemicals
pmol/g	:	picomole/gram
psi	:	pound force per square inch
r_s	:	Spearman's correlation coefficient
RIA	:	Radioimmunoassay

rpm	:	rotation per minute
SD	:	Standard deviation
SPE	:	Solid phase extraction
T	:	Testosterone
TNF-α	:	Tumour necrosis factor TNF-α
UMMC	:	University Malaya Medical Centre
V	:	vegans
WWTP	:	wastewater treatment plant

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Non-communicable diseases (NCDs) are the most frequent cause of deaths in regions such as Europe, Eastern Mediterranean, America and South East Asia (WHO, 2010). Among the NCDs which contribute to the rising healthcare cost is cancer. In the United States, the cost for cancer care is estimated to escalate by 27% in the span of 10 years from 2010 to 2020 (Mariotto *et al.*, 2011). The largest increases were especially in the care of prostate and female breast cancer patients (Mariotto *et al*., 2011). Cancer being the second cause of death in the US was predicted to cause 1620 deaths every day (ACS, 2015). Breast cancer is the leading cancer that affects women both in developed and developing countries (http://www.who.int/cancer/detection/breastcancer/en/ accessed on 19th Feb 2016). In Malaysia, the chance of one being diagnosed as breast cancer is 1 in 20 (Leong *et al.*, 2007). Cancer has multiple aetiologies but a strong determinant to be considered would be the effects of lifestyle. Cancers involving endocrine organs include breast and prostate cancers. Breast cancer accounts for 18% of all cancer cases in the Asia Pacific region in 2012 which also attributed to 9% of the cancer related deaths (Youlden *et al.*, 2014).

General advocates for health lifestyle which include healthy diet, maintaining a healthy weight and sustaining regular physical activity to prevent cancer showed that behaviour change is beneficial (Baade *et al.*, 2013). Modern age living, abundant with technologies to improve and ease our lives has inadvertently exposed us to all the manufactured products, canned and processed foods and fast foods. These products contain chemicals purportedly to increase the products' viability while at the same time posing health risks (Garcia-Jimenez *et al.*, 2007; Noorafshan *et al.*, 2014). These wide range of chemicals not only exist in foods we consume, but also exist in our surrounding environment such as the air we breathe, the water supply we use for

drinking and other uses, and in materials that we are in daily contact with such as office or medical supplies (Biedermann *et al.*, 2010; Jurek & Leitner, 2015; Rudel *et al.*, 2001; Santhi *et al.*, 2012; Tan & Mustafa, 2004).

Chemicals such as pesticides, though they are used in controlled amount for its dedicated purpose have been found to be hazardous causing unfavourable outcome such as preterm birth or other consequences in the environment even before reaching its toxic level (Tyagi *et al.*, 2015). Evidences of exposure to these chemicals associated with disorders in human have been well documented in various literatures (Chou *et al.*, 2011; Kalfa *et al.*, 2015; Stelmach *et al.*, 2015). Among these chemicals, those that disrupt our natural hormonal system are called endocrine disrupting chemicals or EDCs (Diamanti-Kandarakis *et al.*, 2009; Kiyama & Wada-Kiyama, 2015; Wuttke *et al.*, 2010). A more detailed definition of EDC is provided in Chapter 2.

Examples of EDCs include pharmaceuticals, dioxins, dioxin-like compounds, polychlorinated biphenyls, phytoestrogens, pesticides and plasticisers such as bisphenol A (BPA). Humans are constantly exposed to EDCs as these chemicals can be found in products that are used daily which include plastic bottles, metal food cans, personal care products, cosmetics, flame retardants, food, toys and pesticides (Antignac *et al.*, 2003; Bao *et al.*, 2015; Barr *et al.*, 2012; Szczepanska *et al.*, 2016; Legler, 2008; Rudel *et al.*, 2003; Sungur *et al.*, 2013; Wagner & Oehlmann, 2009).

This study aims at looking into the effects of selected exogenous oestrogenic compounds such as phytoestrogens and BPA on human body. These compounds are classed in the EDC group as they affect the endocrine system. Phytoestrogens are chosen as they form a major part of the diet in Malaysian population and are abundant. Phytoestrogens are natural plant based compounds that were reported to have activities affecting the normal physiology of the body associated with oestrogens (Lissin &

Cooke, 2000; Setchell & Cassidy, 1999). BPA is chosen as it acts similarly to oestrogens, but unlike phytoestrogens, it is synthetic in nature. BPA is an ubiquitous compound which can be found almost everywhere (Biedermann *et al*., 2010; Cao *et al.*, 2010; Sajiki *et al.*, 2007; Santhi *et al*., 2012) was previously regarded as harmless, but has recently posed some controversies among the related industry, as they were reported to have endocrine disruption effects (Costas *et al.*, 2015).

This research comprised of several aspects and areas of studies, including development of relevant analytical methods (liquid chromatography tandem mass spectrometry) and method validation which were carried out before applying the methods to real sample (blood and breast tissue) which represent the population of interest. Analytical methods to quantify natural sex hormones were also developed to enable comparison between EDCs and endogenous hormone levels. An overview of the whole research project is shown in Figure 1.1.

Despite the escalating interest in EDC, data on its effects and levels in some biological samples are still scarce in some populations. This research focuses on breast tissue and blood as samples to be investigated due to limited studies reporting on levels of EDCs in breast tissue which is probably due to difficulty in obtaining these samples. Although EDC studies in blood samples have been carried out in various populations globally, hardly any have compared the results in vegetarian population. The scarcity of the data from invasive biological samples (breast tissue) and from the vegetarian population makes this research a necessity as it will help in providing baseline information as well as create future directions for the research. This study is conducted to analyse the distributions of these compounds in the body because although both are oestrogenic compounds, phytoestrogen is from natural source; is more hydrophilic

which will be demonstrated in blood as compared to BPA which is a man-made chemical and lipophilic in nature which will be shown in breast tissue.

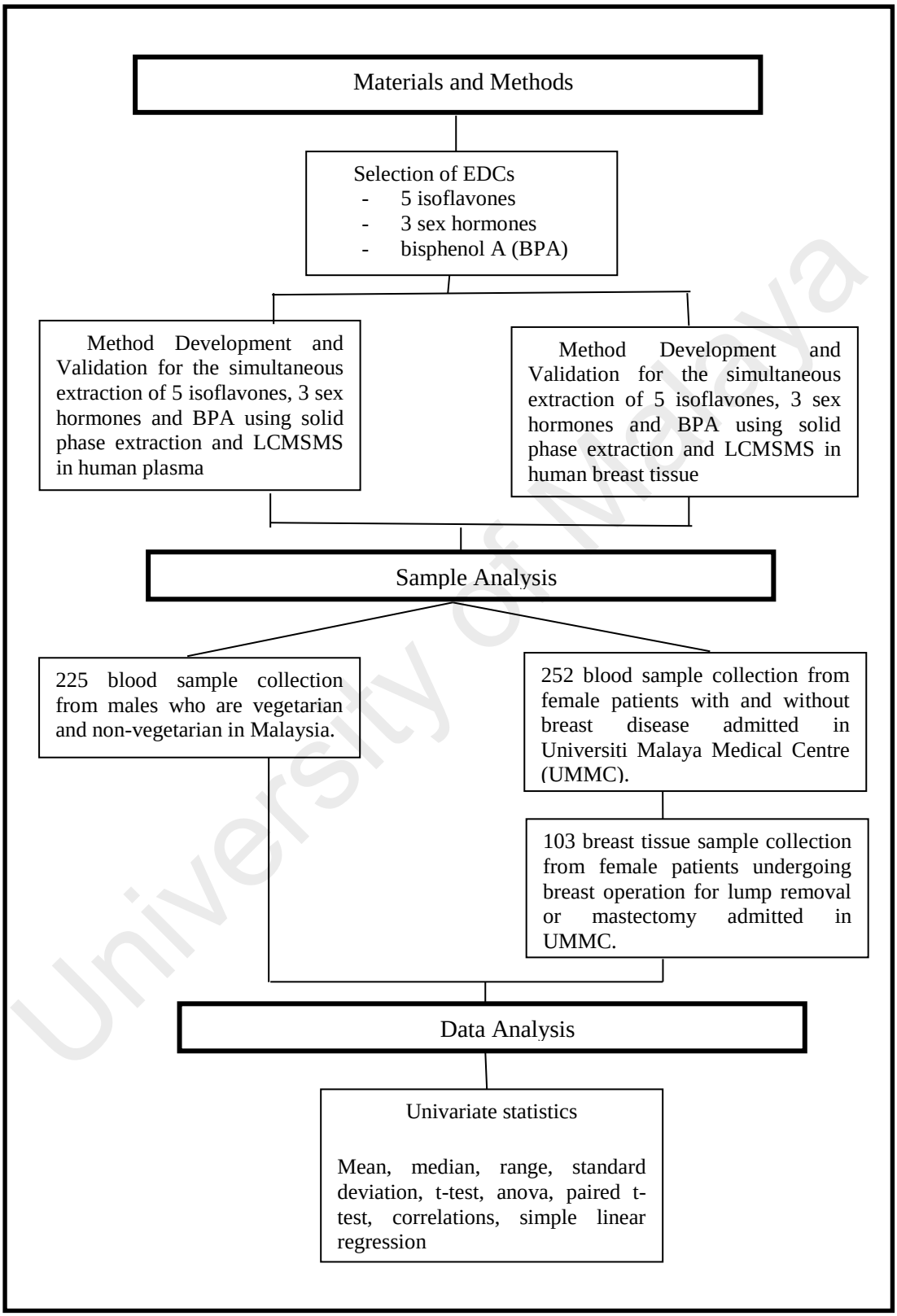

Figure 1.1: An overview of the research approach employed in this study

CHAPTER 2: LITERATURE REVIEW

EDCs possess the ability to mimic, antagonize or disrupt the effects of endogenous hormones in the body hence disrupting the normal endocrine functions to a certain extent. EDCs cover a broad range of chemicals including natural products, pharmaceuticals, industrial products and environmental pollutants

2.1 Definition of EDCs

The US Environmental Protection Agency (US EPA) defined EDC as an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction and developmental process (Kavlock *et al.*, 1996). The National Institute of Environmental Health Sciences (NIEHS) stated that EDCs are chemicals which can either be natural or man-made that may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological and immune effects in both humans and animals as suggested by several studies (Chalubinski & Kowalski, 2006; Huang *et al.*, 2014; Reijnders, 1986; Shiue, 2015; Tharp *et al.*, 2012).

Examples of EDCs include phytoestrogens, BPA, dioxin, phthalates, parabens and pesticide. Humans are frequently exposed to EDCs either intentionally or otherwise. For instance, farmers are regularly exposed to pesticides with endocrine disrupting properties causing adverse health effects such as prostate cancer (Settimi *et al.*, 2003).

Routes of exposure to EDCs can either be orally via diet intake, inhalation or skin contact. This is evidenced by reports on detected levels of EDCs such as phytoestrogens in food, industrial chemicals such as BPA and plasticisers such as phthalates from food

containers, can coatings of food products and thermal printer papers (Carmichael *et al.*, 2011; Wagner & Oehlmann, 2009; Cao *et al*., 2010; Biedermann *et al*., 2010).

Unintentional exposure is seen where urinary concentration of EDCs were detected in more than 90% of US children and exposure routes were either from diet, or use of household and personal care products (Wolff *et al.*, 2007). Even drinking water from mineral water bottles is not safe from EDC contamination as the chemical compounds from the plastic packaging are able to leach into the water (Wagner & Oehlmann, 2009).

2.2 Mechanisms of oestrogenic EDCs

In principles of endocrinology, hormones are generally secreted into the blood circulation where they travel to the targeted endocrine organs. Once the hormone binds to the specific receptors on the target cells of the endocrine organs, it will produce a series of physiological responses. Only specific target cells can respond to each hormone as the target cells have specific receptors for dedicated hormones, any compound/analyte which mimics the hormonal structure will be able to bind to the receptor.

2.2.1 Oestrogen receptors

Oestrogen has 2 known receptors. The first receptor, ER-α was first discovered by Elwood Jensen (Jensen, 1962) which was followed by the discovery of ER-β a few decades later (Kuiper *et al.*, 1996; Nilsson *et al.*, 2001). ER-α plays an important role in normal mammary development and is the more dominant receptor than ER-β (Hewitt *et al.*, 2005). ER-β is thought to oppose proliferative action of ER-α mammary cells (Strom *et al.*, 2004) however the role of ER-β is still being argued, hence requires further research.

Oestrogens induce and stimulate the cellular proliferation and differentiation of cells in target tissues, plays a role in body fat deposition, and prevent bone resorption in maintenance of bone structure (Nilsson *et al.*, 2001). Vasodilatory response for example, was observed to be mediated by ER-α in human umbilical vein endothelial cells (HUVEC) (Sobrino *et al*., 2010).

Oestrogen (Nilsson *et al*., 2001) has a large ligand cavity, allowing it to bind with various compounds (Brzozowski *et al.*, 1997). These compounds include xenoestrogens such as polycyclic aromatic hydrocarbons (PAHs), phthalates and pesticides (Bolger *et al.*, 1998). The effect of this binding can either be inhibitory, stopping the actual physiological responses of the target cell or it can result in agonistic effect, producing subsequent physiological response.

2.2.2 Effects of oestrogenic EDCs

An example of EDC with inhibitory effects is as demonstrated by a systemic fungicide, vinclozolin which was reported to inhibit androgen receptors in male off-springs (Moorman *et al.*, 2000). A single compound can also demonstrate multiple actions as shown with methoxyclor – a pesticide. Methoxyclor is an ER-α agonist, ER-β antagonist as well as an androgen receptor antagonist (Gaido *et al.*, 1999; Maness *et al.*, 1998).

It was shown in an *in vitro* assay that BPA though regarded as a weak xenoestrogen, is still able to compete with tamoxifen at high dose (Lewis *et al.*, 2000). Tamoxifen is a drug used in the treatment of hormone receptor positive breast cancer for more than 30 years as it can bind to oestrogen receptor, preventing oestrogen from binding (http://www.cancer.gov/types/breast/breast-hormone-therapy-fact-sheet).

It was found that the degree of oestrogenic and anti-androgenic activity was determined by the extent of hydroxylation and position of hydroxyl function of the EDCs. BPA and alkylphenol were among the tested chemicals found to have strong oestrogenic activity (Paris *et al.*, 2002).

Oestrogen plays an important role for cell proliferation in normal reproductive health in the mammary glands. It is for this same reason that oestrogen causes the development of breast cancer. Long duration of exposure to oestrogens in a woman's lifetime has long been identified as one of the risks for developing breast cancer.

As biological effects of oestrogens are too broad, involving many tissues and systems in the body, pharmaceutical industries are naturally more interested in developing drugs which can limit the unwanted side effects. These drugs are collectively known as selective oestrogen receptor modulators (SERMs). SERMs exhibit both antagonistic and agonistic activity depending on the target tissue; tissue selective. Examples of SERMs are tamoxifen and raloxifen which are approved by the US Food and Drug Administration (US FDA) to be used in reducing the risk for ER-positive breast cancer (Benetou *et al.*, 2015). Tamoxifen has been used in treating ER-positive breast cancer for decades (Swaby *et al.*, 2007) as tamoxifen acts antagonistically at ER-α receptors in mammary glands but an agonist in the endometrium. Therefore, this poses the risk of endometrial cancer for patients treated with tamoxifen. Many EDCs act like SERMs and one example is BPA (Wetherill *et al.*, 2007).

Several mechanism on how EDCs disrupt the normal hormonal function include affecting the expression of DNA methyltransferase enzyme which will induce cryptorchidism in off-spring, modulation of oestradiol synthesis via ER-α in the rat hippocampus, non-genomic ER signaling, affecting oestrogen biosynthesis and

menstrual cycle (Chen *et al.*, 2015; Kawato, 2004; Mense *et al.*, 2008; Watson *et al.*, 2005).

Different effects of EDCs have been reported in animals as well as in human population. Reports include *in vitro* and *in vivo* studies. Studies have found that EDCs may have addictive or synergistic effects in combinations with other EDCS or with endogenous hormones (Wolff, 2006). Some EDCs such as those contained in plasticisers and disinfectants act on sulphonyltransferase isoforms, which result in additive effects. Therefore, it is hypothesized that a combination of all EDCs rather than any single compound would result in EDC effects manifested in the individual/population (Waring *et al.*, 2008).

In vitro experiments have also proved that some environmental EDCs were also anti-androgens (Sohoni & Sumpter, 1998). Furthermore, studies looking at interaction effects of EDCs reported that a combination of nonylphenol and di-n-butyl phthalate (DBP) apparently gave additive effects which caused reduced cell viability of rat Sertoli cells (Li *et al.*, 2010). This is mainly due to induction of its nuclei apoptosis.

Fenvalerate which is used as an insecticide has also demonstrated harmful effects as it induces cell cycle progression in human uterine leiomyoma and myometrial cells which increased risk for developing uterine fibroids (Gao *et al.*, 2010).

Phthalates is also reported to give direct adverse effects on cardiomyocytes by affecting its calcium handling, hence causing negative chronotropic and inotropic effect on cardiomyocytes (Posnack *et al.*, 2015).

2.2.3 Quantification methods of EDCs

In the environment, EDCs were monitored in drinking water, treated and untreated water from water supply (Arditsoglou & Voutsa, 2008), air and dust while in animals and humans, biological samples such as blood (Sajiki, 2001), plasma, urine (Kawaguchi *et al.*, 2004), human amniotic fluid (Foster *et al.*, 2002), breast milk (Franke *et al.*, 1998), breast tissue (Maubach *et al.*, 2003), adipose tissue (Szafran-Urbaniak, 2008), prostate tissue (Uehar *et al.*, 2000) and saliva (Zimmerman-Downs *et al.*, 2010). Different analytical methods have been used to analyse the presence of EDCs as well as quantifying the levels in those matrices. Methods used include high performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GCMS), liquid chromatography mass spectrometry (LCMS), and liquid chromatography tandem mass spectrometry (LCMSMS) (Fukutake *et al.*, 1996; Griffith & Collison, 2001; Morton *et al.*, 1999; Vanderford *et al.*, 2003).

2.3 Xenoestrogen

Some of the EDCs will affect hormonal functions of the reproductive system more than any other system, in particular oestrogenic hormones (Barber *et al.*, 2015; S. V. Fernandez & Russo, 2010; Kiyama & Wada-Kiyama, 2015; Tyagi *et al*., 2015). Therefore, these chemicals are also termed xenoestrogens or environmental oestrogens. Xenoestrogens are compounds found in the environment which have oestrogenic properties (Degen & Bolt, 2000).

A single compound may demonstrate several different properties such as neurotoxic, oestrogenic and anti-androgenic (Sonnenschein & Soto, 1998). Additive oestrogenic effects were observed when multiple xenoestrogens such as o,p'-DDT, genistein, 4-nonylphenol and 4-n-octylphenol were combined (Payne *et al.*, 2000). Combined effects

of 8 weak xenoestrogens namely hydroxylated PCBs, benzophenones, parabens, BPA and genistein have also demonstrated additive outcome (Silva *et al.*, 2002).

As several studies have proven the additive effects of EDCs within the same class, Kortenkamp *et al* (2007) then pointed out the need for research to focus on combination effects of EDCs from different categories (Kortenkamp, 2007). Examples of xenoestrogens include plasticisers (e.g. phthalates, BPA), alkylphenols, pesticides and polychlorinated biphenyls (PCBs). Xenoestrogens can be found in industrial sealants, detergents, plastic manufacturing, electrical insulators and carbonless paper (Sonnenschein & Soto, 1998).

2.3.1 Distribution of xenoestrogen

Certain populations have increased tendency to be exposed to some EDCs. Farmers and agricultural workers are more exposed to pesticides and have increased risk to cancers such as prostate cancers (Band *et al.*, 2011). Industrial workers exposed to more chemicals will have higher EDC levels in blood, urine and other matrices. This is proven by a study confirming various chemicals such as phthalates, pesticides, PAHs and PCBs found in samples of dust and air collected from residential and workplace indicating human exposure to these substances (Rudel *et al*., 2001).

EDC has raised concerns on its health effects particularly in reproductive health and its role in carcinogenesis (Duty *et al.*, 2005; Main *et al.*, 2006; Prins *et al.*, 2008; Tsuda *et al.*, 2003). Among the major occurring EDCs are BPAs, phthalates, PCBs, PAHs, alkylphenols and perfluorinated chemicals (PFCs) (Basheer *et al.*, 2004; Rudel *et al*., 2003; Szafran-Urbaniak, 2008).

Other oestrogenic EDCs were also detected in various range of concentrations. Several studies have reported phthalate in urine at levels between 0.1 – 83.2 μg/L (Bai *et al.*, 2015; Wolff *et al*., 2007). A low level of mono-isobutyl phthalate (MiBP) was also detected in serum (Olsen *et al.*, 2012). Phthalate was detected in breast milk among the Danish-Finnish cohort with its highest phthalate concentration of mono-n-butyl phthalate (MnBP) 10,900 μg/L (Main *et al*., 2006). Similarly, phthalate was detected in breast milk of 89% Korean women although at a lower concentration of 2.1 μg/L (Kim *et al.*, 2015). MiBP and MnBP concentrations in the breast milk were found to be associated with consumption of whipped cream or purified water (Kim *et al*., 2015).

Alkylphenols, another group of oestrogenic EDCs were found in significant levels in various biological matrices such as human cord blood, urine, human breast milk and adipose tissue (Calafat *et al.*, 2005; Chen *et al.*, 2010; Ferrara *et al.*, 2011; Tan & Ali Mohd, 2003).

Levels were found to vary among different study populations, biological matrices as well as dietary habit as most of the exposure route is more likely from oral intake.

Chen *et al* (2010) detected alkylphenol as high as 4470 ng/g and 1290 ng/g of 4-*n*-nonylphenol (NP) and 4-*t*-octylphenol (OP) respectively, in human breast milk. The study also found that OP correlated with lower consumption of sea fish in Taiwan population. NP concentration was much lower (122 ng/g) in adipose tissue of Italians although samples measured were only from 16 subjects (Ferrara *et al*., 2011). Alkylphenol concentration in urine of 394 US adults was about 100 times higher than its level in human cord blood in Malaysia (Calafat *et al*., 2005; Tan & Ali Mohd, 2003). Calafat *et al* (2005) also reported that the detection rate for NP in urine was 51% of the samples examined.

Adipose and liver tissues from 16 subjects in Poland were found to contain 78 - 591 ng/g and 16 – 94 ng/g of PCBs respectively (Szafran-Urbaniak, 2008). Pesticides such as chlorpyrifos, *p,p'* – DDE and *p,p'*- DDD have been detected in biological matrices such as human cord blood and adipose tissue. Chlorpyrifos was detected in 18% of human cord blood samples ranging from 0 – 1.8 ng/ml (Tan & Ali Mohd, 2003) while measurement of adipose tissue recorded pesticide levels of 501 ng/g and 62.3 ng/g for *p,p'* – DDE and *p,p'*- DDD respectively (Fernandez *et al.*, 2004). The same study also reported a very high detection rate for *p,p'* – DDE (97.38%) in 458 adipose tissues of Spanish population. This further emphasised the fact that widespread exposure to various EDCs occurs consistently in many populations regardless of their geographical location.

Guo & Kannan (2013) has found that personal care products were also laden with EDCs especially parabens and phthalates. It was with great concern to note that the paraben levels via dermal intake found in infants and toddlers were higher than in adult females. Among the baby care products in this study were baby shampoo, lotion and oil, sunscreen, diaper cream and powder.

2.3.2 Effects of xenoestrogens in humans

Dioxins, one of the major environmental pollutants can cause adverse effects such as cardiovascular disease, cancer, endometriosis, reduced testosterone and thyroid hormones (White & Birnbaum, 2009). Dioxins are by-products of industrial processes and are persistent organic pollutants (POPs). They are highly toxic, causing reproductive and developmental irregularities, alter endocrine functions and impairs the immune system (White & Birnbaum, 2009).

Other EDCs were reported to interfere with physiological functions such as 4-octylphenol, alpha-zearanol, triclosan which are associated with disrupted sperm function (Schiffer *et al.,* 2014), pesticides are associated with increased risk of non-Hodgkin lymphoma (Hohenadel *et al.,* 2011), phthalates and alkylphenols are associated with an increased risk of hypospadias (Morales-Suarez-Varela *et al.,* 2011). Organochloride insecticides on the other hand were found to increase risk of prostate cancer in farmers (Settimi *et al.,* 2003) and dialkyl phosphate metabolites of organophosphate pesticides can probably cause hypospadias (Michalakis *et al.,* 2014).

Recently, EDC was also implicated as a new risk factor for the type 2 diabetes mellitus as scientists reported increasing evidence of this connection (Chevalier & Fenichel, 2015).

2.3.3 Effects of xenoestrogens on environment and animals

Some EDCs such as phthalate has a long half life. One such experiment was conducted in an attempt to find solution to reduce this bioavailability period for phthalates found in soil. It was found that soil with high organic content will produce a lower amount of phthalates in plant shoots grown (He *et al.,* 2015). In addition, it was also demonstrated that the combination of biochar in soil with low organic content has also significantly reduced the phthalate concentration in plant shoots. Findings such as these are extremely helpful in trying to control the burden of EDCs in the environment.

With EDCs' presence in the environment, they not only affect human populations but animals alike. As early as in the 1980s, several researchers have shown effects of EDCs on animals. Moccia *et al* (1986) reported of herring gulls at the Great Lakes of North America to have thyroid goitre exhibiting thyrotoxic effects and hypothesized the most

probable cause as polyhalogenated hydrocarbons (Moccia *et al.*, 1986). Reijnders (1986) found that the decreased production rate of Dutch common seals were most strongly associated with PCBs (Reijnders, 1986). Few years later, PCBs were reported to have strong association with higher incidences of egg death and deformities of hatchling in common snapping turtles (Bishop *et al.*, 1991).

EDCs such as insecticides which have been banned from use are still detected in the environment due to its persistent nature or illegal use. An example would be, organochloride pesticides. Organochloride pesticides were detected in marine species in coastal waters of Malaysia (Santhi *et al.*, 2011). In Punjab, a recent study showed the presence of several pesticides, predominantly chlorpyrifos, DDT and lindane (gamma-hexachlorocyclohexane) in cow's milk (Bedi *et al.*, 2015). Consuming contaminated cow's milk is one of the ways how biological fluids and tissues can contain various EDCs.

Effects by EDCs on animals are shown in several species. Vinclozolin, a systemic fungicide often used in farms was shown to exhibit anti-androgenic effects resulting in smaller weight gain during pubertal growth, and matured accessory glands that weigh less (Moorman *et al.*, 2000). Perinatal exposure to phthalates in rats reduced sexual behaviours after maturation suggesting permanent effects on the hypothalamus (Lee *et al.*, 2006).

Vinclozolin, a systemic fungicide often used in farms was shown to exhibit anti-androgenic effects resulting in smaller weight gain during pubertal growth, and lighter weight of matured accessory glands in peripubertal rabbits (Moorman *et al.*, 2000).

Other EDCs such as organophosphate pesticides, atrazine have been reported to affect glucose and fatty acid metabolism in the liver (Srinivas *et al.*, 1991; Debost-

Legrand *et al.*, 2016), disturb reproductive functions and cause intersex ovo-testis morphology changes (Zheng *et al.*, 2014), and development of obesity and diabetes (Jin *et al.*, 2014; Newbold, 2010). Numerous reports were published on imposex snails in Taiwan (Liu *et al.*, 2006), Japan (Horiguchi, 2006), the Mediterranean (Sole *et al.*, 1998) as a consequence of tributyltin (TBT) exposure. Imposex refers to a condition where female snails are able to grow male accessory sex organs. TBT and triphenyltin are chemicals from anti-fouling paints used on ships and fishing nets (Batley *et al.*, 1992; Horiguchi, 2006). Imposex leads to suppressed ovarian maturation and ovarian spermatogenesis which causes significant decline of species such as ivory shell which is of commercial value to the fishery industry (Horiguchi, 2006).

Another EDC, dibutyltin (DBT) in high concentration has been shown to decrease TNF-α secretion from T and NK (natural killer) cells while lower concentrations showed increased secretion (Hurt *et al.*, 2013). DBT is used as a stabiliser in polyvinyl chloride plastics and as a de-worming agent in poultry. TNF-α is a powerful inflammatory stimulus which regulate the innate immune responses.

2.4 Bisphenol A

Oestrogenicity refers to the ability of a compound to bind to oestrogen receptors either *in vivo* or *in vitro* (Sohoni & Sumpter, 1998). In 1995 it was reported that an assay for testing oestrogenicity of compounds was developed, called E-SCREEN assay (Soto *et al.*, 1995). It is developed based on oestrogen function which focuses on proliferative effects of oestrogen target cells as the end point.

Oestrogenicity of BPA : In recent years, more studies have been conducted using E-SCREEN to assess oestrogenicity of bottled mineral water (Wagner & Oehlmann, 2011), municipal wastewater (Limam *et al.*, 2007), fruits and vegetable (Schiliro *et al.*,

2011), industrial wastewater effluents (Schiliro *et al.*, 2012) and even underarm deodorants (Lange *et al.*, 2014).

Apart from the E-SCREEN assay, another alternative assay to screen potential xenoestrogens is by using MTT assay in which mitochondrial activity is assessed (Lewis *et al*., 2000). *In vitro* assay which tests the oestrogen receptor binding capacity shows that phytoestrogens and xenoestrogens bind to the receptors in intact cells, however, their ability to induce oestrogen end products differ from one another (Zava *et al.*, 1997).

2.4.1 Effects of BPA – *in vitro*, animals, human, epidemiological studies

A summary of *in vitro* studies on BPA and other oestrogenic EDCs that have been conducted more than 20 years ago until present is shown in Table 2.1. BPA was also observed to produce adverse effects on the resulting progeny cells (Calderon-Gierszal & Prins, 2015).

Table 2.1: *In vitro* studies of BPA and other xenoestrogens

EDCs	Cell type	Effects
BPA	human embryonic stem cells (hESC) (Calderon-Gierszal & Prins, 2015)	increase self-renewal of stem cells which lead to failure of differentiated progeny
Phenylphenols Biphenols BPA 4-tert-octylphenol	Various reporter cell lines (Paris *et al*., 2002)	exhibit oestrogenic and anti-androgenic responses
Fenvalerate	uterine leiomyoma (UtLM) cells and normal uterine smooth muscle (UtSMC) cells (Gao *et al*., 2010)	anti-apoptotic
o,p-DDT	human breast cancer cell (Zava *et al*., 1997)	oestrogen receptor binding capacity, induction of oestrogen end products, and activation of cell proliferation
Nonyl phenol DBP	rat testicular Sertoli cell (Li *et al*., 2010)	additive effect which decreases cell viability
DEHP	monolayers of gCAMP3 human embryonic stem cell-derived cardiomyocytes (Posnack *et al*., 2015)	affects calcium handling and intercellular connectivity of human stem cell-derived cardiomyocytes

BPA on the other hand showed activities such as reducing body weight gain in ovariectomised female rats (Nunez *et al.*, 2001), causing changes that might inhibit penile erection in white rabbits (Moon *et al.*, 2001), alters reproductive functions in mussels (Ortiz-Zarragoitia & Cajaraville, 2006), modulate the synthesis of oestradiol in the rat hippocampus (Kawato, 2004), alter morphology of the mammary gland with intraductal hyperplasia in mice (Vandenberg *et al.*, 2008), and increased risk of prostate carcinoma in rats (Prins *et al*., 2008).

2.4.2 Quantification methods for BPA

Scientists strive to develop methods which can determine multiple analytes simultaneously, to produce high throughput, reproducible, sensitive and specific analysis while at the same time requires non-exhaustive sample preparation with an acceptably simple steps. Available methods either measure BPA alone or BPA with other analytes.

More studies are using LCMSMS for an improved sensitivity and specificity in quantification of phytoestrogens (Anna KrAJčoVá 2010). Measurement of BPA in semen samples using ELISA and LCMS gave a range of non-detected to 12 ng/ml and non-detected to less than 0.5 ng/ml respectively (Inoue *et al.*, 2002). This comparison demonstrated that ELISA method is non-specific and can overestimate the actual BPA concentration.

In order to save time, cost and precious tissue sample, a method that can analyse most of the analytes in a single run was developed. One of the aims of this study is to develop and validate a LCMSMS method for simultaneous determination and quantification of isoflavones, BPA and sex hormones in human breast tissue.

2.4.3 BPA levels in foodstuff

BPA is among the most frequently studied EDCs as there are more than 3000 BPA related articles available on PubMed and other online journal database as compared to 629 and 141 phthalate and paraben respectively. Its presence is reported in numerous matrices including food products such as milk, tuna fish (Munguia-Lopez *et al.*, 2005), canned foods (Cao *et al*., 2010), canned beverages (Sungur *et al*., 2013) and bottled water (Cao & Corriveau, 2008). In Singapore, researchers found a significant level of BPA in canned seafood such as fish, squid, crab at levels of 65.6 ng/g, 118.9 ng/g and 213.1 ng/g respectively (Basheer *et al*., 2004).

One of the highest BPA level reported was in canned tuna fish from Mexico which reached 646.5 ng/g (Munguia-Lopez *et al*., 2005). In the same experiment, researchers were looking at factors that influence the magnitude of BPA migration such as type of can coatings (organosol, epoxy and its combination), processing conditions and heat involved, as well as storage times. The highest level of BPA migration was found in organosol coating of tuna fish cans using a fatty food stimulant with heat processing. Storage at 25^{o}C for over a year was also shown to result in BPA migration to the level of 138,400 ng/g for epoxy resin coated tuna cans (Munguia-Lopez *et al*., 2005). The same study also reported the highest migration levels were seen when these cans were subjected to 121^{o}C temperature during heat processing for 90 minutes.

BPA was also detected in foods such as eggs, milk (Shao, Han, Tu, *et al.*, 2007), canned foods and food in plastic containers (Sajiki *et al*., 2007). Levels in egg were between 0 – 10,450 ng/g while only one milk sample was contaminated at the level of 490 ng/g (Shao, Han, Tu, *et al*., 2007). In a study of six infant formula powder samples, BPA was detected in all but one sample at levels ranging between 45 – 113 ng/g (Kuo & Ding, 2004). Similarly, infant food formula was also found to be contaminated with

BPA at a wider range of 3 – 375 ng/g (Cirillo *et al.*, 2015). Low levels of BPA ranging from less than 0.5 – 1.4 μg/L was found in polycarbonated bottled water in Canada (Cao & Corriveau, 2008).

A study by Liao & Kannan (2013) reported concentration of BPA in various foodstuffs from the United States categorised as beverages, dairy products, fats and oils, fish and seafood, cereals and cereal products, meat and meat products, fruits, canned fruits, vegetables and canned vegetables. Highest mean BPA concentration at 8.99 ng/g was found in vegetables including canned vegetable products (Liao & Kannan, 2013). Canned foods contained higher levels of BPA than foods in other glass, paper or plastic containers.

Other xenoestrogens such as phthalates have also been contaminating the general population food supply. Basheer *et al* (2004) reported NP levels of 64.8 – 197 ng/g in canned seafood from Singapore supermarkets. In this study however, researchers did not take into account the type of can coating, nor the manufacturing process that resulted in the highest NP concentrations. Baby food puree was also contaminated with alkylphenol as high as 21 ng/g (Li *et al.*, 2008). Human breast milk was not spared from alkylphenol pollution as Chen *et al* (2010) reported levels ranging from 0.39 – 0.89 ng/g. Phthalate may even exceed BPA contamination levels as reported by Cirillo (2015) in which di-ethyl(hexyl) phthalate (DEHP) and DBP were detected as high as 5088 ng/g in infant food formula. Foodstuff purchased from New York was also reported to be contaminated with phthalates (Schecter *et al.*, 2013). Among the phthalate esters, DEHP was the most frequently detected at higher concentrations. Schecter *et al*. (2013) reported in this study, the highest mean level of 300 ng/g of DEHP was detected in pork. Meat and meat products, vegetable oils also recorded DEHP levels of more than 100 ng/g.

2.4.4 BPA levels in biological samples

Another oestrogenic EDC such as BPA was found in significant levels in various biological samples such as urine (Matsumoto *et al.*, 2003), blood (Sajiki, 2001), seminal fluid (Inoue *et al*., 2002), follicular fluids, amniotic fluids (Ikezuki *et al.*, 2002) and placental tissue (Schonfelder *et al.*, 2002). Schonfelder *et al*. (2002) reported BPA concentration as high as 18.9 ng/ml and 9.2 ng/ml in maternal and foetal plasma respectively. In the same study, it was reported that male foetuses had higher plasma BPA levels than female foetuses. Its highest concentration was 104.9 ng/g on the placental tissue. Another study also reported of males having higher amount of BPA than females in serum samples (Takeuchi & Tsutsumi, 2002). A study in elderly population of Sweden reported serum BPA levels of 0 – 27.3 ng/ml, however no difference was reported between male and female subjects (Olsen *et al*., 2012).

In other biological sample, such as urine, more than 94% of urine samples from American girls of multi ethnic groups was reported to contain high concentrations of BPA ranging from 1800 – 52,300 ng/ml (Wolff *et al*., 2007). The rate of BPA detection in urine was 70 – 95% indicating the wide spread exposure of BPA in humans (Calafat *et al*., 2005; Genuis *et al.*, 2012; Mendiola *et al.*, 2010). Sweat, a non-invasive biological sample was also investigated for BPA. Genuis *et al* (2012) analysed BPA in blood, urine and sweat in 20 subjects where they were subjected to exercise, steam or infrared sauna to obtain sweat sample. BPA was detected in sweat, however this study is not without its limitations. Apart from having a small sample size, researchers did not exclude the possibility of BPA's presence in sweat due to BPA contamination from the air of the sauna room or from water used to create the steam sauna. Hair is another example of non-invasive sample. Tzatzarakis *et al* (2015) reported a mean level of 64.1 ng/g and 40.3 ng/g of BPA in hair samples of 69 Greek volunteers in urban and rural areas respectively (Tzatzarakis *et al.*, 2015). In addition, this study also reported of a

higher detection rate for BPA among the urban population (41.2 %) than in rural (14.8 %).

BPA from 31 urine and 14 serum samples showed levels ranging from < LOQ – 18.7 and < LOQ - 0.59 ng/ml respectively (Liao & Kannan, 2012). In China, determination of BPA exposure to pregnant women and fetuses discovered mean levels of 3.58 ng/ml in blood of pregnant women and 0.58 ng/ml from the cord blood. It was also noted that women who went for intravenous drug administration has higher levels of BPA in their blood (Zhang *et al*., 2013).

2.5 Phytoestrogens

Phytoestrogens, are naturally occurring oestrogens, which originate from plants. They are non-steroidal compounds found in plants that mimic the effects of endogenous oestrogens. These chemicals are usually weaker hormone agonists than steroids, however their non-hormonal effects such as protective role of cardiovascular risk (Vanharanta *et al.*, 1999), osteoporosis (Tsuchida *et al.*, 1999) may be more potent.

Four classes of phytoestrogens are isoflavone, lignin, coumestrol and stilbenes (Sirtori *et al.*, 2005). Multiple classes of phytoestrogen have been reported to be present in a single plant (Usui, 2006). However, more studies are focused on isoflavone which can be found largely in soy products (Bingham *et al.*, 1998). The most frequently studied isoflavones are daidzein and genistein (Bing Shao, 2005; Kuhnle *et al.*, 2008; Anupongsanugool *et al.*, 2005; Cassidy *et al.*, 2006; Kurahashi *et al.*, 2007).

2.5.1 Isoflavones

Isoflavones demonstrate several biological properties which are important in human physiology. Apart from its oestrogenicity, other properties include inhibition of protein tyrosine kinase, modulation of enzyme activities as well as anti-oxidant properties (Wietrzyk *et al.*, 2005).

Daidzein and genistein are isoflavone phytoestrogen which are commonly found in soy foods such as soy milk, fermented soy or tempeh, miso soup, tofu (Arai *et al.*, 2000; Atkinson *et al.*, 2005; Haron *et al.*, 2009). It can also be found in mangifera fruits such as kuini, bambangan and bacang (Amin, 2008).

It was found that, these isoflavones are metabolized into equol and O-desmethylangolensin (O-DMA) by specific bacteria in the normal floral of the gut. Variations in gut microflora among individuals resulted in only 30-50% and 80-90% of the population who are able to produce equol and O-DMA respectively (Lampe, 2009).

2.5.2 Equol

Equol was first discovered in 1932 where it was isolated from urine of a pregnant horse (Marrian & Haslewood, 1932) and later in the plasma of sheep (Setchell & Clerici, 2010). It was found to be in high concentration in urine of adults consuming large amounts of soyfood (Setchell *et al.*, 1984).

The great interest in equol is due to the possibility that the efficacy of soy isoflavone is dependent upon the ability of the individual to produce equol (Setchell & Cole, 2006). Hence, Setchell *et al* (2002) highlighted that the inconsistent epidemiological findings of phytoestrogen studies might be due to the lack of emphasis on the equol producer status of the subjects in the population studied (Setchell, Brown, & Lydeking-Olsen, 2002). Up till 2009, the possibility of equol being the key to soy food efficacy was still

being established (Lampe, 2009). One study suggested that soybean isoflavones can prevent the onset of prostate cancer either through mechanisms directly involving equol or the transformation of daidzein to equol (Akaza *et al.*, 2004). Subsequently, it was thought that being an equol producer can be protective against prostate cancer. Apart from its potential role in cancer, various studies have also been conducted in attempts to establish the role of equol in health (Lampe, 2009; Magee *et al.*, 2014; Utian *et al.*, 2015; Kim *et al.*, 2014; Pusparini *et al.*, 2015).

The production of equol is dependent on intestinal microflora based on these evidences:

i. No urinary equol was detected upon feeding germ-free animals with soy diet (Axelson & Setchell, 1981).
ii. No equol was detected in plasma or urine of newborn infants whose gut lacks the normal flora (Setchell *et al.*, 1997).
iii. Reduction of equol production with administration of antibiotics (Blair *et al.*, 2003).

Surprisingly, equol has higher oestrogenicity than its precursor daidzein, with an affinity to both oestrogen receptors (α and β) and its presence in only 30-50% of the population has led to the postulation that equol might have a role in hormone-dependent conditions as well (Setchell, Brown, & Lydeking-Olsen, 2002). This is supported by an earlier study which reported of equol's potential health benefits such as preventing the occurrence of breast cancer (Duncan *et al.*, 2000).

Various studies reported percentage of equol producers in the population. Setchell estimated about 30% of the human population are equol producers, however this percentage seems to vary in different populations (Setchell & Cole, 2006). Miyanaga *et*

al (2003) in his study reported 29% of prostate cancer patients and 45% of controls were equol producers (Miyanaga *et al.*, 2003).

a) Prevalence of equol producers

A comparison of equol producers percentage among prostate cancer patients and controls in three different countries were 29% and 46% in Japan, 30% and 59% in Korea and 17% and 14% in America respectively (Akaza *et al*., 2004). A study reported a prevalence of the equol producers among men (43%) and women (27%), however this difference is not significant (Lampe *et al.*, 1998). In France, 58-59% of the postmenopausal women were found to be equol producers after being fed on high dose isoflavones for 30-60 days (Vedrine *et al.*, 2006). On a cross sectional study, 35% of peri and post-menopausal women investigated were found to be equol producers (Newton *et al.*, 2015). However, it is important to note that, equol can also be detected in the blood of non-equol producers when they eat foods that are high in equol such as stinky tofu, cow milk, cheese, yoghurt and egg (Abiru *et al.*, 2012; Antignac *et al*., 2003; Kuhnle *et al*., 2008).

b) Possibility of converting equol producer status

Since being an equol producer is considered to have some added health benefits, researchers explored the possibility of converting one's status from a non-equol producer to an equol producer. Studies reported intake of probiotics for 2 months (Bonorden *et al.*, 2004) or consuming soy isoflavones for a month (Vedrine *et al*., 2006) did not succeed in converting the subjects to equol producers. Neither did feeding post-menopausal women who were non-equol producers with 100 mg isoflavones/day for a month (Vedrine *et al*., 2006) nor two months of probiotic capsule ingestion containing *Lactobacillus acidophilus* and *Bifidobacterium*

longum succeed in converting premenopausal women into equol producers (Bonorden *et al*., 2004).

However, one study reported ingestion of green tea (Miyanaga *et al*., 2003) and lactulose helped increase equol production (Zheng *et al.*, 2014). Conversion to equol producers was also made possible by administering 60 mg daily of soy supplements for 3 months (Tanaka *et al.*, 2009). However, this study involves a small sample size of 28 Japanese men and only 2 out of 10 non-equol producers converted to producers. Researchers agreed that maintaining a strict vegan or vegetarian diet will significantly change the microbiota without changing total cell numbers. However, the duration of dietary modification to cause such a change was not discussed (Zimmer *et al.*, 2012).

On other aspect, more than 400 species of bacteria were estimated to be present in the human gut's normal flora (Berg, 1996). However, barely more than 20 bacteria strains in animals and humans have been identified as possessing the ability to transform isoflavones to equol (Setchell & Clerici, 2010).

c) Determining criteria of equol producer status

The definition of an equol producer was not strictly consistent between one study and another. In one study, a person is regarded an equol producer when his equol plasma concentration was detected at 0.5 ng/ml or more (Miyanaga *et al*., 2003). Other studies used the limit of quantitation (LOQ) as the cut-off point. Setchell (2006) however, proposed a new way of determining whether one is an equol producer, by taking into consideration equol concentration both in plasma and urine by a given formula (Setchell & Cole, 2006). A more definitive way of determining the equol producer status is necessary as equol can also be present in food such as milk and tofu (Rui *et al.*, 2014). Therefore, those who are not equol producers but consumed food containing equol may still have significant levels

of equol circulating in the plasma and be mistakenly regarded as an equol producer.

2.5.3 Other metabolites of isoflavones

A lesser known metabolite of isoflavones; O-desmethylangolensin (O-DMA) is produced by a different group of normal flora (Frankenfeld, 2011). However, there are still limited studies correlating specific diseases and O-DMA producer status. Frankenfeld (2014) also reported that O-DMA producers are associated with obesity in adults (Frankenfeld *et al.*, 2014).

2.5.4 Oestrogenicity of phytoestrogens

Several EDCs demonstrated significant levels of oestrogenicity which is of concern. In vitro study which estimated relative oestrogenicity of phytoestrogens reported that in reference to oestradiol (oestrogenicity of 100), coumestrol, genistein, equol, daidzein, biochanin A and formononetin showed an oestrogenicity of 0.202, 0.084, 0.061, 0.013, less than 0.006 and less than 0.0006 respectively (Markiewicz *et al.*, 1993). In another study, equol demonstrated higher oestrogenicity than its precursor, daidzein and has a 13-fold higher affinity to oestrogen receptors β (ER-β) than ER-α (Muthyala *et al.*, 2004).

2.5.5 Mechanisms of oestrogenic EDCs

Genistein for example, effectively stimulates sex hormone binding globulin production in hepatocellular carcinoma cancer cells while suppressing the proliferation of these cells in culture (Mousavi & Adlercreutz, 1993). According to Patisaul & Jefferson (2010), the complexity of phytoestrogen mechanisms and its effects are

attributed to several factors which include: age at the time of exposure, dose of exposure, duration of exposure and equol producer status (Patisaul & Jefferson, 2010).

2.5.6 Pharmacokinetics of phytoestrogens

Plasma half-life of daidzein is 5.79 hours as compared to genistein which is 8.36 hours (Watanabe *et al.*, 1998). In another study, similarly daidzein has a shorter half life ($t_{½}$) of 5.2 hours than genistein, which is 6.6 hours while the $t_{½}$ of the glycoside counterparts are 4.59 (daidzin) and 7.0 hours (genistin) (Setchell *et al.*, 2001). In the same study, it was found that in adults, after ingestion of food containing daidzein and genistein, it takes 4 – 7 hours to achieve peak plasma concentration. However, it takes longer (about 8 -11 hours) when the glycoside form is ingested. Systemic bioavailability was also found to be higher for daidzin and genistin (the β-glycosides) than its aglycone form (daizein and genistein). These substances are absorbed in its aglycone form after hydrolysis of the sugar moiety of daidzin and genistin by the enzyme β–glycosidase occurs; an enzyme present in the human intestinal gut (Setchell *et al*., 2001). Bioavailability of isoflavones such as daidzein ranges between 13 to 35% depending upon types of bacteria in the gut (Xu *et al.*, 1995). Both daidzein and genistein are cleared from the body within 24 - 48 hours (Xu *et al*., 1995; Xu *et al.*, 1994). Excretion is via urine and faeces with 1 – 4.6 % being eliminated in faeces (Watanabe *et al*., 1998).

In a study by Setchell *et al* (2002), they predicted that frequent isoflavone intake is necessary to achieve a steady state plasma concentration rather than just a daily dose. This study has also shown that there is a non-linear relationship between bioavailability and dietary intake of isoflavones (Setchell, Brown, Zimmer-Nechemias, *et al.*, 2002).

Metabolites of daidzein include equol, O-desmethylangolensin (O-DMA), dihydrodaizein, tetrahydrodaidzein, 2-dehydro-O-desmethylangolensin (Kelly *et al.*, 1993). The metabolite equol however is only found in urine of 30 – 40% of those who consumed isoflavones; strongly indicating that certain normal flora are responsible for the conversion of daidzein into equol (Setchell *et al*, 2002). It is however more oestrogenic than its precursor (daidzein) and has higher antioxidant activity than other isoflavones (Setchell *et al*, 2002).

Glycitin, another isoflavone was found to have a t½ of 8.9 hours however, not much is known about glycitin (Setchell *et al*., 2001).

2.5.7 Dietary intake of phytoestrogen

Variations in dietary intake of phytoestrogens among populations showed that among the US adults, amount taken was 4 mg and 7 mg daily for daidzein and genistein respectively (Kirk *et al.*, 1999). Australian women aged 40 – 80 years old were reported to have a median isoflavone intake of 3.9 mg/d (Hanna *et al.*, 2010) while Europeans namely study subjects from Ireland, Italy, the Netherlands and the UK showed an even lower amount of less than 1 mg/day (van Erp-Baart *et al.*, 2003).

Easterners generally showed a higher amount of isoflavone intake, for example, daily isoflavone intake among Chinese in Hong Kong is 7.8 mg (Chan *et al.*, 2007), 14.88 mg/d among Koreans (Kim & Kwon, 2001), 20 – 50 mg daily among Japanese (Nagata, Takatsuka, Kurisu, *et al.*, 1998). Among the popular soy food consumed were tofu, soybean paste and soybean sprouts (Kim & Kwon, 2001). A recent study among Korean adults showed an increase to 23.1 mg daily (Lee *et al.*, 2007). An even higher isoflavone intake of 39.5 mg/d can be seen in the Japanese population (Kimira *et al.*, 1998). It was also reported that among the breast cancer survivors in Shanghai, their

daily isoflavone intake was 48.1 mg daily which surpassed the amount taken by the Japanese (Baglia *et al.*, 2015).

2.5.8 Effects of phytoestrogen – *in vitro*, human, epidemiological studies

a) Effects of phytoestrogen – in *in vitro* studies

Studies have also demonstrated the complexity of phytoestrogens mechanism of actions when effects on different tissues depend on the dose. For instance, Zierau *et al* (2006) reported an *in vitro* study that higher dose of isoflavones repress the activity in osteosarcoma cells while a lower dose stimulates activity in breast cancer cells (Zierau *et al.*, 2006). This poses a challenge to researchers in deciding the dose to administer when conducting a study.

Many researchers believed that phytoestrogens have some preventive properties against cancer (Adlercreutz, 1995; Goodman *et al.*, 1997; Higashi *et al.*, 2005; Peterson & Barnes, 1993). Opposing that belief, de Lemos (2001) reported that phytoestrogens such as daidzein and genistein may actually stimulate existing breast tumour growth as well as antagonise the effects of tamoxifen (de Lemos, 2001). This eventually raised the concern on how safe phytoestrogens are for existing breast cancer patients (de Lemos, 2002). Although phytoestrogens exhibit weak oestrogenic actions, a high dietary consumption can increase the concentration levels in the plasma many times higher than endogenous oestrogens (Adlercreutz *et al.*, 1993). Studies reported that high plasma phytoestrogen levels may likely prevent breast cancer (Lewis *et al*., 2000; Wood *et al.*, 2006). Phytoestrogens exhibit agonist and antagonist actions as well as possessing anti-oxidative activity (Mishra *et al.*, 2003). It is suggested that phytoestrogens may inhibit the start of carcinogenesis.

Past experiments have demonstrated biological properties in isoflavones. Among the properties affecting human physiology include oestrogenicity (Schmitt *et al.*, 2001) and anti-oxidant (Park *et al.*, 2010). Among the clinical effects of phytoestrogens on humans include improving bone mass (Setchell & Lydeking-Olsen, 2003), reducing hot flushes in post-menopausal women (D'Anna *et al.*, 2009), anti-diabetic effects, reduction in cholesterol levels (Mahalko *et al.*, 1984) and prevention of atherosclerosis (Dai *et al.*, 2004).

It was known that phytoestrogens may have additive or synergistic effects in combinations with other EDCs or with endogenous hormones (Wolff, 2006). Genistein was shown to suppress cancer cells proliferation (Mousavi & Adlercreutz, 1993) in human hepatocarcinoma cells (Hep-G2) by increasing SHBG production while equol and enterolactone were suggestive of breast cancer promoters as they stimulate growth of oestrogen dependent breast cancer cell lines (Welshons *et al.*, 1987).

b) Effects of phytoestrogen – in human and epidemiological studies

Phytoestrogen was shown to result in severe reproductive tract disorders and temporary infertility syndromes when given in high doses at critical stages of development in animals (Mitchell *et al.*, 2001).

Apart from its effects in animal kingdom, higher incidence rate of prostate cancer in Western population was reported which was attributed to the westernized lifestyle and diet intake of isoflavones (Akaza *et al.*, 2002). Past review published in 2005 compared 5 studies, however found no significant evidence of phytoestrogens as protective against prostate cancer (Ganry, 2005). Similarly, enterolactone was not found to be protective against prostate cancer among Swedish men (Stattin *et al.*, 2004).

Despite those evidences, several findings continue to suggest that dietary isoflavones, play a role in prostate carcinogenesis. It was reported that Japanese men who migrated to the US, increased the incidence of prostate cancer (Shimizu *et al.*, 1991). In addition, prostate cancer incidence also showed marked differences between Koreans, Korean Americans and Caucasian Americans which further supports the westernised lifestyle as one of the contributing factors to the increased incidence. However, one study reported that low fat local vegetarian food was probably protective against prostate cancer for thin men in Taiwan (Chen *et al.*, 2005). Though many pre-clinical data on *in vitro* experiments showed favourable results towards genistein as preventive against prostate cancers, no definitive clinical efficacy is available to strongly suggest its use for cancer therapy (Perabo *et al.*, 2008).

Role of genistein was also debated in breast cancer, on whether it is a promoter or protective agent against cancer (Bouker & Hilakivi-Clarke, 2000).

Phytoestrogen effects on cardiovascular system are mixed. While isoflavones and lignans were not shown to reduce cardiovascular disease risk, genistein and alpha-zearanol were found to have protective effects (Altavilla *et al.*, 2004; Dai *et al*., 2004).

Despite the mixed findings on health effects, soy isoflavones are widely marketed as health supplements. Dietary supplements and meal replacements are commercially available in the market, some of which provide information on the amount of isoflavone content. This information, however, is not always accurate as level determines independently have been found to differ from the labeled content (Chen *et al.*, 2005). Although phytoestrogens are considered to be safe, there is still a need to evaluate the potential health effects of phytoestrogens as various findings are still inconclusive.

2.5.9 Quantification methods for isoflavones

Isoflavones have been quantified in various biological matrices such as serum, plasma, urine and breast tissue. Daidzein, genistein and equol have been quantified in plasma, urine and breast tissue using HPLC-MS (Maubach *et al*., 2004). HPLC was also used to detect daidzein and genistein in mangoes (Amin, 2008). Taylor & Bingham (2005) used LCMSMS to detect isoflavones, enterodiol and enterolactone in urine and plasma while Saracino & Raggi (2010) used HPLC with coulometric detection for quantification of daidzein, genistein and glycitein.

2.5.10 Isoflavone levels in foodstuff

Scientists and researchers in various organisations and industries were monitoring EDCs in various matrices including food and drinks. New chemicals are also produced thus more potential new EDCs are possible. Fish species in streams with the greatest wastewater treatment plant (WWTP) effluent content have been shown to be contaminated with EDCs (Barber *et al*., 2015). Phytoestrogens has been shown at levels as high as 50 g/ kg in food such as milk, cereals and baby food (Antignac *et al.*, 2009). Soy infant formula was also reported to contain high daidzein and genistein which raises the concern of endocrine disrupting effects in neonates (Tuohy, 2003). Among the vegetables available in United Kingdom which was screened for phytoestrogen content, spring onion and leek showed significant levels of biochanin A, while only a few vegetables were found to contain formononetin at a much lower level than biochanin A (Kuhnle *et al.*, 2007). Examples are apples and cabbages. Enterolactone and enterodiols are lignan type phytoestrogens otherwise known as mammalian lignans. This is because they are being metabolized by gut bacteria from plant lignans which exist in barley, rye, wheat, seeds and nuts (Axelson & Setchell, 1981; Axelson *et al.*, 1982; Borriello *et al.*, 1985).

High amount of equols were reported in stinky tofu in Taiwan and China (Abiru *et al*., 2012; Rui *et al*., 2014). Equol was also reported at an average of 78 µg/L in bovine milk samples (Antignac *et al*., 2003). Equol was also detected in milk infant formulae and baby food which have traces of bovine milk (Antignac *et al*., 2009).

In this modern world, food is mostly packaged in cans, containers made of plastic, metal, aluminium and other processed materials which can prolong product's shelf life. Unmindful of the long term consequences, it is generally regarded that these materials are safe for use. Unknowingly, we have traded our health for convenience.

2.5.11 Isoflavone levels in biological samples

Phytoestrogen has been quantified in various biological matrices including plasma, serum, breast milk, amniotic fluid, cord blood, urine, breast tissue, prostatic fluid and prostate tissue (Adlercreutz *et al*., 1993; Adlercreutz *et al.*, 1999; Brossner *et al.*, 2004; Coward *et al.*, 1996; Maubach *et al*., 2003; Uehar *et al*., 2000; Morton *et al.*, 1997; Valentin-Blasini *et al.*, 2003). Tables 2.2 – 2.4 show distribution of daidzein, genistein and equol in various biological matrices. Phytoestrogen was even detected in as high as 96.2% of second trimester amniotic fluid samples with a mean range of 0 – 5.5 ng/ml and 0 – 6.5 ng/ml for daidzein and genistein respectively (Foster *et al*., 2002).

Table 2.2: Distribution of daidzein in human biological samples

Population	Matrix	Concentration Level (mean/median)
Subjects (n = 4) served with soy beverage for 14 days (Coward *et al*., 1996)	plasma	87.7 ng/ml (mean)
Healthy Japanese women (n = 111) Healthy Finnish women (n = 126) (Uehar *et al*., 2000)	plasma	30.1 ng/ml (mean) < 2.5 ng/ml (mean)
Women undergoing breast reduction (n = 3) (Maubach *et al*., 2003)	serum	76.3 ng/ml (mean)
Adults in US (n = 208) (Valentin-Blasini *et al*., 2003)	serum	3.9 ng/ml (mean)
Women undergoing breast reduction (n = 3) (Maubach *et al*., 2003)	urine	1271 ng/ml (mean)
Adults in US (n = 208) (Valentin-Blasini *et al*., 2003)	urine	317 ng/ml (mean)
Gestational women (15 - 23 weeks of gestation) (n = 59) (Foster *et al*., 2002)	amniotic fluid	1.4 ng/ml (mean)
Males in: UK Portugal Hong Kong Beijing (Morton *et al*., 1997)	prostatic fluids	 11.3 ng/ml (mean) 4.6 ng/ml (mean) 70.0 ng/ml (mean) 24.3 ng/ml (mean)
Women undergoing breast reduction (n = 4) (Maubach *et al*., 2003)	breast tissue	0 ng/ml

Table 2.3: Distribution of genistein in human biological samples

Population	Matrix	Concentration Level (mean/median)
Subjects (n = 4) served with soy beverage for 14 days (Coward *et al*., 1996)	plasma	150.3 ng/ml (mean)
Adults in US (n = 208) (Valentin-Blasini *et al*., 2003)	serum	4.7 ng/ml (mean)
Women undergoing breast reduction (n = 4) (Maubach *et al*., 2003)	serum	243.2 ng/ml (mean)
Adults in US (n = 208) (Valentin-Blasini *et al*., 2003)	urine	129 ng/ml (mean)
Women undergoing breast reduction (n = 4) (Maubach *et al*., 2003)	urine	2837 ng/ml (mean)
Women undergoing breast reduction (n = 4) (Maubach *et al*., 2003)	breast tissue	4160 ng/g (mean)
Gestational women (15 - 23 weeks of gestation) (n = 59) (Foster *et al*., 2002)	amniotic fluid	1.7 ng/ml (mean)
Men eating Western diet with benign prostate hyperplasia (n = 63) with prostate cancer (n = 31) (Brossner *et al*., 2004)	prostate tissue	 11.0 ng/g (median) 8.4 ng/g (median)

Table 2.4: Distribution of equol in human biological samples

Population	Matrix	Concentration Level (mean)
Women undergoing breast reduction (n = 4) (Maubach *et al*., 2003)	serum	36.3 ng/ml
Women undergoing breast reduction (n = 4) (Maubach *et al*., 2003)	urine	484.5 ng/ml
Males in: UK (n = 17) Portugal (n = 22) Hong Kong (n = 20) Beijing (n = 15) (Morton *et al*., 1997)	prostatic fluids	0.5 ng/ml 1.7 ng/ml 171.6 ng/ml 29.2 ng/ml
Women undergoing breast reduction (n = 4) (Maubach *et al*., 2003)	breast tissue	52,980 ng/g

Among European adults, isoflavone levels were 5 – 50 times higher in vegetarians than in non-vegetarian (Peeters *et al.,* 2007). This is due to the fact that vegans and vegetarians consume more vegetable and fibre diets daily compared to the rest of the population.

Setchell in 1998 showed that there is high variability in urinary isoflavone excretion among subjects who take soy food. A third of the subjects are unable to form equol, therefore higher levels of daidzein were seen in the urine of subjects. Urinary excretion increases with increased isoflavone intake but absorption is saturated at high doses (Setchell, 1998).

Phytoestrogen levels in biological samples that had been investigated in Malaysia include levels of daidzein (1.4 ng/ml), genistein (3.7 ng/ml) and coumestrol (3.3 ng/ml) in cord blood (Mustafa *et al.,* 2007). Higher levels of phytoestrogens in the blood were found to be distributed in rural subjects compared to urban.

It was shown in Table 2.1 that genistein level in plasma was high in subjects served with soy beverage for 14 days that it exceeded the genistein level found in Japanese women (Coward *et al*., 1996; Uehar *et al*., 2000). Isoflavone levels reported by Franke (1998) surpassed levels found in maternal plasma and cord plasma of Japanese population (Franke *et al*., 1998; Adlercreutz *et al*., 1999). Maubach (2003) studied isoflavones in three biological matrices and found that daizein and genistein concentrations were highest in urine than serum and breast tissue (Maubach *et al*., 2003). It was also observed from these studies that genistein levels are always higher than daidzein in serum/plasma and amniotic fluid which suggests high correlation between these 2 types of isoflavones (Coward *et al*., 1996; Foster *et al*., 2002; Maubach *et al*., 2003; Uehar *et al*., 2000). Maubach (2003) also discovered that in contrast to daidzein and genistein, a very high concentration of equol was present in breast tissue rather than in serum or urine. Equol was also reported by Setchell *et al* (2002) to have a higher bioavailability and slower clearance rate than daidzein and genistein (Setchell, Brown, & Lydeking-Olsen, 2002).

Comparing phytoestrogen levels in prostatic fluids between males in 4 separate geographical locations namely UK, Portugal, Hong Kong and Beijing, it was found that daidzein and equol levels were higher in Asian males (Morton *et al*., 1997). Morton *et al* (1997) also reported higher enterodiol and enterolactone concentrations in Portuguese males rather than Asians. This observation might be explained by the pattern of dietary intake of isoflavones and lignans in Asian and Western populations as reported in other studies. A higher intake of isoflavones and soy products in Asia for instance in Korea and China was reported at 14.9 mg/d and 97 g/d (Kim & Kwon, 2001; Tang *et al*., 2015) in contrast to 7 -9 mg/d of soy consumption in UK (Mulligan *et al*., 2007) and 0.8 mg/d in Finland (Valsta *et al*., 2003).

2.5.12 Enterolactone

The opposite is true for lignans, where intake levels among the Westerners were higher and probably a modest intake among Asians as there is scarce data on lignan intake among Asian. The amount of lignan taken daily in Spanish diet among those aged 0 – 24 years old was around 1 mg (Jose L. Penalvo, 2004), 0.7 mg among Spanish adults (Zamora-Ros *et al.*, 2013) and 0.9 – 1 mg in the Dutch population study (Milder *et al.*, 2007). A slightly higher lignan intake of 2.4 mg/d was reported among older Australian women (Hanna *et al*., 2010). One study reported plasma concentration of enterolactones in Finnish subjects was almost two times higher than in Japanese people (Uehar *et al*., 2000). In contrast, plasma daidzein and genistein in Japanese subjects were 67 and 33 times higher than Finnish subjects.

Distribution of enterolactone in human biological samples are as shown in Table 2.5. Male subjects from Portugal showed the highest enterodiol and enterolactone levels in prostatic fluids when compared against males in UK, Hong Kong and Beijing. This comparison however involved a small number of subjects.

Table 2.5: Distribution of enterolactone in human biological samples

Population	Matrix	Concentration Level (mean)
Adults in US (n = 208) (Valentin-Blasini *et al*., 2003)	serum	3.6 ng/ml
Adults in US (n = 208) (Valentin-Blasini *et al*., 2003)	urine	512 ng/ml
Males in: UK (n = 17) Portugal (n = 22) Hong Kong (n = 20) Beijing (n = 15) (Morton *et al*., 1997)	prostatic fluids	 20.3 ng/ml 162 ng/ml 31 ng/ml 32.9 ng/ml
Men eating Western diet with benign prostate hyperplasia (n = 63) with prostate cancer (n = 31) (Brossner *et al*., 2004)	prostate tissue	10.7 – 19.4 ng/g

2.6 Steroid hormones

2.6.1 Quantification methods for steroid hormones

Quantification of steroid hormones however is more challenging as physiological range for oestradiol is extremely low. Many assays were previously performed on automated platform such as immunoassays. It was found however, that overestimation and reproducibility are among the problems faced when using direct or indirect immunoassays as compared to GCMSMS (Lee *et al.*, 2006). With recent advances, measurement of steroid hormones with higher sensitivity and specificity is possible through LCMSMS methods (Yamashita *et al.*, 2007). Tables 2.6 – 2.7 list out the various quantification methods for sex steroids in biological matrices and water, reported in the literature.

Table 2.6: Quantification methods of sex steroids in biological matrices

Matrix	Method	Analyte
Plasma	Immunoassay	Testosterone, dihydrotestosterone (DHT), 3 alpha-androstanediol glucuronide (AAG), oestradiol, SHBG, and prolactin (Gann *et al.*, 1996)
	LCMSMS	Oestradiol, oestrone (Nelson *et al.*, 2004)
Serum	HPLC	Testosterone (Oka *et al.*, 1988)
	LCMSMS	Testosterone (Starcevic *et al.*, 2003)
	Isotope dilution LCMSMS	17- β -oestradiol (Tai & Welch, 2005)
	LC-ESI-MS	Oestrone, oestradiol (Yamashita *et al.*, 2007)
	LC-ESI-MS/MS	17-β-oestradiol (Xu & Spink, 2008)
Blood	LCMSMS	Oestradiol, oestrone, and testosterone (Jasuja *et al.*, 2013)
Bovine Blood	LCMSMS with APCI	17-β-oestradiol (Draisci *et al.*, 1998)
Urine	GC-NCI-MS	Oestrogens (Xiao & McCalley, 2000)
	LCMSMS	Oestrone, pregnandiol, testosterone (Hauser *et al.*, 2008)
Adipose tissue	RIA	Sex steroids (Szymczak *et al.*, 1998)
Breast adipocytes	RIA	Androstenedione, testosterone, oestrone, and oestradiol (Falk *et al.*, 2008)

Table 2.6, continued

Matrix	Method	Analyte
Breast cancer tissue	HPLC-RIA	Oestrone, oestradiol, oestrone sulphate (Geisler *et al.*, 2000)
Breast tissue, Urine	LCMSMS	Oestrogens and its metabolites (Taioli *et al.*, 2010)
Prostatic tissue	LCMSMS	Testosterone, 5α-dihydrotestosterone (Higashi *et al*., 2005)
Human hair	GCMS	Oestrone, 17- β oestradiol (Man Ho Choi, 2000)

Table 2.7: Quantification methods of sex steroids in water

Matrix	Method	Analyte
Water	LCMSMS	Oestrogen, progestogens (Isobe *et al.*, 2003)
Natural waters, drinking water treatment plant	LCMSMS	Oestradiol, oestrone, estriol, oestradiol-17-glucuronide, oestradiol diacetate, oestrone-3-sulfate, ethynyl oestradiol (Rodriguez-Mozaz *et al.*, 2004)
River water South Korea and several countries	GCMS	Oestrone, oestradiol (Duong *et al.*, 2010)

2.6.2 Sex hormone levels in biological samples

A summary of distribution of sex hormones is as shown in Tables 2.8 – 2.10. Higher concentration of oestrone was found in plasma than urine of premenopausal women (Aubertin-Leheudre *et al.*, 2008).

There was a slightly lower concentration of oestradiol than oestrone in plasma of premenopausal women (Aubertin-Leheudre *et al*., 2008).

Table 2.8: Distribution of oestrone in biological matrices

Matrix	Level
urine of premenopausal Boston women (Aubertin-Leheudre *et al*., 2008)	5.6 ng/ml/24 hours
urine of breast cancer female patients (Taioli *et al*., 2010)	20.5 ± 18.4 pg/ml
plasma of premenopausal Boston women (Aubertin-Leheudre *et al*., 2008)	71.4 pg/ml
serum of female (13 – 15 years old) male (13 – 15 years old) male (18 – 61 years old) postmenopausal women (41 – 63 years old) (Kushnir *et al.*, 2008)	 8.1 – 105 pg/ml 0.5 – 30 pg/ml 8.9 – 36 pg/ml 2.9 – 31.9 pg/ml
serum of 73 – 94 years old male (van den Beld *et al.*, 2000)	27.6 pg/ml
breast adipocytes of various pathological lesions Premenopausal Postmenopausal (Falk *et al*., 2008)	 889 pg/g 910 pg/g
breast cancer tissue (Taioli *et al*., 2010)	53.4 ± 19.0 pg/g
lymph node infiltrated by metastatic breast cancer (Blonder *et al*., 2008)	1.7 pg/tissue section

Serum oestradiol of men were 30 – 40% lower than plasma oestradiol in premenopausal women (Dolomie-Fagour *et al.*, 2008; van den Beld *et al*., 2000; Aubertin-Leheudre *et al*., 2008). A study involving more than 600 vegan, vegetarian and omnivore participants showed that vegans have 13% and 8% higher testosterone levels than omnivores and vegetarians respectively (Allen *et al.*, 2000). Higher testosterone, oestradiol levels were seen in breast adipocytes from pathological specimens of both premenopausal and post-menopausal women (Falk *et al*., 2008).

Table 2.9: Distribution of estradiol in biological matrices

Matrix	Level
urine of premenopausal Boston women (Aubertin-Leheudre *et al*., 2008)	2.9 ng/ml/24 hours
plasma of premenopausal Boston women (Aubertin-Leheudre *et al*., 2008)	60.2 pg/ml
serum (France) Male patients < 55 years old Male patients > 55 years old (Dolomie-Fagour *et al*., 2008)	 4.9 – 19.7 pg/ml 3.5 – 22.4 pg/ml
serum of 73 – 94 years old male (van den Beld *et al*., 2000)	26.7 pg/ml
serum of men less than 65 years old (Orwoll *et al.*, 2006)	0.5 pg/ml
serum female aged 13 – 15 years old male aged 13 – 15 years old male aged 18 – 61 years old postmenopausal women aged 41 – 63 years old (Kushnir *et al*., 2008)	 8.9 – 247.9 pg/ml 1.1 – 35.6 pg/ml 7.8 – 41.9 pg/ml 1.9 – 20.9 pg/ml
breast adipocytes of various pathological lesions Premenopausal Postmenopausal (Falk *et al*., 2008)	 211 pg/g 51 pg/g
breast cancer tissues (Vihma *et al*., 2016)	251 pg/g
lymph node infiltrated by metastatic breast cancer (Blonder *et al*., 2008)	6.271 pg/tissue section

Table 2.10: Distribution of testosterone in biological matrices

Matrix	Level
plasma of premenopausal Boston women (Aubertin-Leheudre *et al*., 2008)	0.4 ng/ml
serum of 73 – 94 years old male (van den Beld *et al*., 2000)	8.6 pg/ml
serum of men less than 65 years old (Orwoll *et al*., 2006)	83.6 pg/ml
serum (France) Male patients < 55 years old Male patients > 55 years old (Dolomie-Fagour *et al*., 2008)	 2.2 – 8.8 ng/ml 2.2 – 7.8 ng/ml
breast adipocytes of various pathological lesions Premenopausal Postmenopausal (Falk *et al*., 2008)	 393 pg/g 181 pg/g
lymph node infiltrated by metastatic breast cancer (Blonder *et al*., 2008)	1.992 pg/tissue section

2.7 Issues regarding EDCs

Several issues are frequently raised by international scientific experts regarding EDCs. First, there is still inconsistent data on the effects of EDCs on humans. Epidemiological data, *in vivo* and *in vitro* studies produce mixed results which is hard to conclude. For instance, increased or high soy consumption has been reported to be associated with reduced plasma/serum oestradiol concentrations in several studies (Nagata, Takatsuka, Inaba, *et al.*, 1998; Xu *et al.*, 1998), but remain unchanged in other studies (Cassidy *et al.*, 1995; Duncan *et al.*, 1999). There is even a report of increased oestradiol concentrations in one study (Cassidy, 1994).

2.7.1 Agonist and antagonist actions of EDCs

Second, the ability of some EDCs to demonstrate both agonist and antagonist actions results in ambiguous outcomes in studies carried out. Studies on genistein have highlighted the juxtaposition of its oestrogenic actions as well as anti-oestrogenic effects. On its oestrogenic properties, genistein was reported to stimulate growth on ER positive breast cancer cells (Hsieh *et al.*, 1998), to increase tissue proliferation in uterus of various rat species (Hertrampf *et al.*, 2006; Santell *et al.*, 1997) and to inhibit oestradiol degradation (Shertzer *et al.*, 1999). Contrary to those, genistein was also reported to inhibit proliferation in mammary and uterine tissues, to inhibit enzyme 17β-hydroxysteroid oxidoreductase Type 1 (HSOR-1) (Makela *et al.*, 1998) which is important for oestradiol secretion and to inhibit aromatase enzyme (Wang & Murphy, 1994). Therefore, the net effect of genistein and other EDCs which demonstrate both agonist and antagonist effects is unclear. While *in vitro* experiments for example, suggest that genistein can stimulate tumour cell proliferation. This is in contrast to meta analysis findings of epidemiological studies which showed an inverse relationship between genistein intake and breast cancer risk (Taylor *et al.*, 2009).

2.7.2 Non-linear dose response curve

Third, the dose response curve for most EDCs is not linear and therefore, poses a challenge to predict the outcome. This challenge is due to the occurrence of a non-monotonic dose response which refers to the effects at low doses that are not predicted by effects at higher doses (Vandenberg *et al.*, 2012). As reported in a study on rats, low doses of di-(2-ethylhexyl)-phthalate (DEHP) exposed *in utero* and lactation inhibited aromatase activity while high doses increased the activity resulting in a non-monotonic dose-response profile (Andrade *et al.*, 2006). In addition to the non-monotonic dose response, scientists are also faced with the problem of low-dose effects. The National Toxicology Program of the US Department of Health and Human Services defined low-dose effects as those that occur in the range of human exposures or effects observed at doses below those used for traditional toxicological studies. Vandenberg *et al*. (2012) reviewed the accumulating evidence of adverse health effects occurring even at low doses. An example is of BPA, which gives adverse effects on prostate development even at low doses (Chapin *et al.*, 2008).

2.7.3 Interactivity between EDCs

Fourth, the interactivity between the EDCs which can result in either additive, synergistic or antagonistic effect is also a factor which determines the outcome of its health effects. With lack of research on the interaction effects between EDCs, the resulting health effect observed is frequently assumed to be caused by the particular EDCs studied in the experiment when it can actually be due to the net effect of all the EDCs present in the subjects, including those EDCs which are not of interest in the study. Many studies are concentrating on investigating effects of an individual EDC when in real life, humans are subjected to multiple EDCs simultaneously.

2.7.4 Exposure time to EDCs

Fifth, most studies however agreed that the critical exposure time to EDCs is during the reproductive period, or critical developmental stages that include gestation, lactation, adolescence and senescence. Exposure to EDCs during critical developmental time window is the key to understanding how certain diseases including cancer can develop (Schug *et al.*, 2011).

2.7.5 Inconsistent classifications of dietary intake

Sixth, the use of inconsistent classification of soy intake makes it difficult for one study result to be compared to another. Among other factors which pose this challenge are inconsistent type and amount of soy products, dose frequency, diet recall and the various analytical methods to determine the phytoestrogen analytes either from urine, plasma or tissues (Wu *et al.*, 2004).

2.7.6 Inconclusive results from phytoestrogen studies

Inconclusive results are also due to the great diversity of phytoestrogens as was observed in about 105 clinical trials conducted to assess the potential health effects of phytoestrogens (Cornwell *et al.*, 2004). The results are also affected by non-oestrogenic analytes in phytoestrogen plant sources that may interact with phytoestrogen which subsequently will either potentiate or interfere with its activity and bioavailability.

In vivo and *in vitro* studies show inconsistent results due to factors such as the complexity of phytoestrogens and endogenous oestrogens and the status of equol and non-equol producers among humans that may greatly affect the outcome of dietary intake of phytoestrogens. A review on epidemiological evidence of soybean isoflavones effects revealed that isoflavones exert either protective effects or no effect against

various carcinomas such as prostate, stomach, colon, lung and breast cancer (Tsuda *et al*., 2003).

2.8 Aim of the present study

As there is still insufficient data in Malaysia, limited to the available data of BPA exposure in surface water, drinking water, edible marine biota (Santhi *et al*., 2011, 2012) this study is conducted to determine the baseline levels and distribution of phytoestrogens and BPA among vegetarians and non-vegetarians. BPA is added as a target analyte in this study as it is also a type of oestrogenic EDC which is present ubiquitously and acts on oestrogen receptors. Thus, the present study compared the distribution of selected phytoestrogens and BPA mong male vegetarians and non-vegetarians in Malaysia using validated LCMSMS method. It is also of great research interest to compare the plasma profiles in males between non-vegetarians and vegetarians.

The specific objectives of the study were:

a) To develop and validate the analytical method of selected oestrogenic EDCs from biological samples (plasma and breast tissue).
b) To quantify levels of selected endogenous sex hormones in the samples using validated method of two distinct populations;
    - male vegetarians and non-vegetarians
    - female patients with and without breast disease

The selected oestrogenic EDCs and sex steroids analysed in this study are daidzein, genistein, equol, formononetin, biochanin A, BPA, oestrone, oestradiol and testosterone.

The baseline data from the local population in different biological matrices obtained in this study shall contribute and complement the existing information of phytoestrogen exposure reported in cord blood and breast milk in Malaysia. This study also aims to clarify if there is any difference in EDC levels observed between the different groups studied and if the differences are associated with sex hormone levels.

2.9 Scope/outline of the study

This is a cross sectional study observing selected oestrogenic EDC levels in two different populations from human biological samples (blood and breast tissue). The first population studied is among males who are vegetarian and omnivores while the second population looked into female patients with and without breast disease. Two analytical methods for analysis of selected EDCs and sex steroids in plasma and breast tissue are developed. Following the method validations, analysis of the biological samples is carried out accordingly.

2.10 Possible outcomes of the study

This study has developed new analytical methods using liquid chromatography tandem mass spectrometry (LCMSMS) to simultaneously determine selected EDCs and endogenous steroid hormone levels in two human biological matrices; plasma and breast tissue. This study compares EDC levels among different groups of study population in each biological matrix. Comparisons were made on several characteristics of the subjects to obtain a possible significant association.

2.11 Knowledge gap

In Malaysia, there is insufficient data on EDC in the environment as well as in human biological samples. Even fewer data is available to be compared among special

interest groups such as vegetarians, and patients with breast cancers. With the natural EDC such as isoflavones, which is generally promoted as health food, there is a tendency to assume that levels present in the body are harmless. There is however, no definite study in Malaysia to provide data on these EDC baseline levels. EDC data on breast tissue samples is also lacking as obtaining the sample requires invasive procedure. Very few studies globally had reported EDC levels in breast tissue. Most of these studies involved only a small number of samples. There is also a need to know if the EDC levels in the special interest groups would be associated with sex hormone profile suggestive of endocrine cancers.

2.12 Research questions

This study aims to answer these research questions:

i. If there are differences in the distribution of the studied compounds between the blood and breast tissues
ii. Will the presence of these exogenous compounds affect the endogenous sex hormone levels?
iii. Will there be a difference in the distribution of these chemicals between vegetarian and non-vegetarian populations?
iv. Will there be a difference in the distribution of these chemicals between breast cancer and non-breast cancer patients?

CHAPTER 3: MATERIALS AND METHODS

This chapter will discuss the analytical procedures carried out throughout the study, which includes the procedures for the analysis of 2 types of biological samples (blood/plasma and breast tissue), sampling methods and statistical analysis used.

The aim of developing analytical methods for each type of sample is to have a method which is able to detect all analytes of interest in a single analytical run. The method has to be valid, reliable, cost-effective, efficient and fast. In this study, there were 9 analytes of which, 5 were isoflavones (daidzein, genistein, equol, formononetin, biochanin A), BPA, endogenous sex hormones (oestrone, oestradiol and testosterone).

3.1 Method development of 9 analytes in human plasma

From the literature review, different analytical methods have been used and published to analyse the presence of these 9 analytes as well as quantifying the levels in the matrices. Methods used include GCMS, HPLC, LCMS, GCMSMS and LCMSMS.

Phytoestrogens or the group of isoflavones may have similar chemical structure with the natural oestrogens, however, these phytoestrogens actually possess a hydroxyl group which makes them more hydrophilic as compared to the endogenous oestrogens which are more lipophilic. Therefore, running all the analytes with different polarities in a single run is challenging. Although this study is focused on detecting the phytoestrogens and BPA, at the same time it aims to compare the plasma/breast tissue levels of these compounds with the endogenous sex hormones which have very low circulating plasma levels. Therefore, a very sensitive method has to be developed. Figure 3.1 shows the molecular structure of the oestrogenic EDCs.

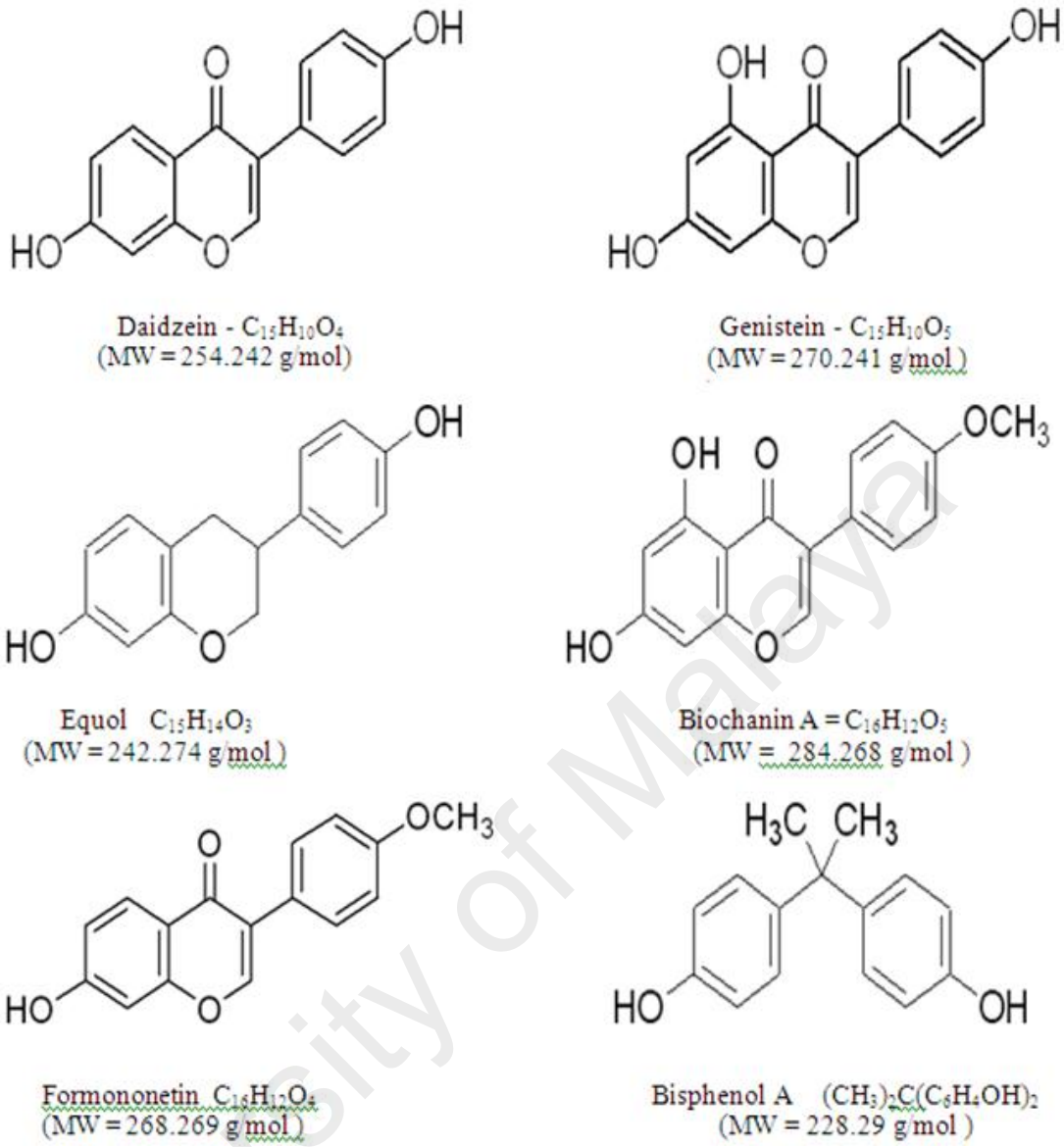

Figure 3.1: Molecular structure of oestrogenic EDCs

3.1.1 Experimental

3.1.1.1 Chemicals and reagents

Daidzein, genistein, formononetin, equol, biochanin A, oestrone, oestradiol were purchased from Sigma Aldrich, USA. Testosterone was purchased from Ridel-de-Haen, Germany and 4-hydroxybenzophenone as the internal standard was from Merck, Germany. Crude solution of β-glucuronidase of Type H-2, from *Helix pomatia* extract (Sigma Aldrich, United Kingdom) was diluted 10 times and used to hydrolyse isoflavones conjugates. HPLC grade methanol was purchased from Mallinckrodt, USA.

Acetone was from JT Baker, USA. Sodium acetate anhydrous 99% (Alfa Aesar, Lancaster) was used to prepare buffer solution.

All solvents were filtered using a vacuum filtration system with a 0.45 μm membrane filter. Ammonium hydroxide was from Fischer Scientific. All solvents and water used for LCMSMS were filtered with Millipore membrane filter of 0.45 μm pore size for solvent and 0.2 μm for water. Purified water was from PureLab Option-Q system (18.2 MΩ-cm). All samples were filtered with a 4 mm diameter syringe filter of regenerated cellulose by Phenomenex (0.2 μm pore size).

The solid phase extraction cartridges Strata-X 33μm with polymeric reversed phase (60 mg/3 ml) sorbent were supplied by Phenomenex, USA. The cartridges were fitted onto an IST Vacmaster manifold. The apparatus was connected to a vacuum pump. The cartridges were conditioned with 3 ml of 100% methanol followed by 3 ml of water. Stock standard solutions (1 mg/ml) were prepared every 2 months and stored at -20^{o}C. Working standard solutions of different concentrations were prepared daily by mixing aliquots of each individual stock solution and diluting it with methanol.

3.1.1.2 Preparation for Calibrators

Each chemical standard is approximately weighed 1 mg in a 2 ml amber vial. It is then dissolved in 1 ml of methanol resulting in 1000 ppm. With serial dilution each standard of 100 ppm is produced. A mixture of all 9 standards with 100 μl from each 100 ppm standards are mixed with 100 μl methanol to produce 10 ppm of mixed standards. Stock solutions are prepared by serial dilution of the actual concentrated solution. Stock solution of daidzein, genistein, formononetin, equol, biochanin A, oestrone, oestradiol and testosterone (10 ppm) and its appropriate dilutions were prepared in 100% methanol and stored at 20^{o}C in amber glass vials.

A volume of 200 µl of stock solution is then added to blank plasma to obtain 10 ml of stock plasma at final concentration of 200 ng/ml. Table 3.1 shows the following working standard solutions from the 200 ng/ml plasma stock solution prepared.

Table 3.1: Standard preparation for calibrators (plasma)

Mix standard concentration (ng/ml)	Stock plasma sample (ng/ml)	Volume of stock (µl)	Volume of blank plasma (µl)	Total (µl)
1	200	50	9950	10,000
3	200	150	9850	10,000
10	200	500	9500	10,000
15	200	750	9250	10,000
30	200	1500	8500	10,000
60	200	3000	7000	10,000
75	200	3750	6250	10,000

3.1.1.3 Preparation for Quality Control samples

Preparation for the respective concentrations of QC samples which were prepared separately from the calibrators samples are as shown in Table 3.2. Three concentrations of low (QC1 – 2 ng/ml), medium (QC2 – 35 ng/ml) and high (QC3 – 65 ng/ml) were chosen, these concentrations being within the range of calibration curve. The internal standard concentration was the same as that used for the calibration curve.

Table 3.2: Preparation for QC samples (plasma)

Mix standard concentration (ng/ml)	Stock plasma sample (ng/ml)	Volume of stock (µl)	Volume of blank plasma (µl)	Total (µl)
1 (LLOQ)	200	50	9950	10,000
2 (QC 1)	200	100	9900	10,000
35 (QC 2)	200	1750	8250	10,000
65 (QC 3)	200	3250	6750	10,000
75 (ULOQ)	200	3750	6250	10,000

3.1.1.4 Chromatography

Reversed phase chromatography (RPC) is used in this study, whereby, a relatively non-polar stationary phase is used with a polar mobile phase. The analytes have to be dissolved in a suitable solution.

In this study, analytes were dissolved in methanol. A Shimadzu LC system consisting of a binary pump (LC-20AD), an autosampler SIL-20AC (4^{o}C) a column oven (model CTO-20AC) at 40^{o}C and a system controller (CBM-20A) were used for separation of the analytes. Injection volume was 20.0 µl. Total flow for the binary pumps was set at 0.5 ml/min. needle rinsing volume was 2000 µl at 35 µl/second of rinsing speed done before and after aspiration of sample injection. Gradient conditions were as shown in Table 3.3. LCMSMS was performed on an API-3200 QTrap (Applied Biosystems, MD Sciex) connected to a Shimadzu LC-20ADXR system run in negative mode. The source temperature was 500^{o}C. The curtain gas, GS 1 and GS 2 was set to 10, 50 and 60 psi respectively. Nitrogen was used both as nebulising and drying gas. Nitrogen was generated by a nitrogen generator from Peak Scientific (MA, USA). Ion source voltage was -4500 V. In the mix mode, ion source used for the first period (0 – 4.0 min) is -4500 V, and in the second period (4.0 – 8.51 min), ion source was 5500 V

for positive mode. Analytes were detected by multiple reaction monitoring (MRM) mode. For each analyte, 2 MRM transitions were monitored. Mass spectral data were analysed using Analyst 1.4.2 software (Applied Biosystem, MDS Sciex). A seven point calibration curve was included with each assay, using calibrator concentrations from 1, 3, 10, 15, 30, 60 and 75 ng/ml.

3.1.1.5 Solvent selection

In liquid-liquid partition chromatography, the mobile phase can be varied by the solvent strength. For a good separation of the analytes, adjusting the mobile phase is much easier to do than changing the LC columns. Through trial and error, an optimum percentage of the suitable solvent composition is obtained. Among the solvents attempted was ammonium acetate in methanol, acetonitrile (ACN), methanol with ammonium hydroxide. ACN gave good isoflavone peaks but has affected the ionization of sex hormones. Therefore, the best solvent for this analysis of all 9 analytes was made with addition of ammonium hydroxide to methanol.

3.1.1.6 LC Columns

Separation was tried on reversed phase Shim Pack C-18 column, C-18 XTerra column, 250 mm X 4.6 mm internal diameter with 5 μm packing. Most of the columns were able to separate several analytes well but produced an unsatisfactory chromatogram peaks for other analytes. The columns tried can only withstand a narrow range of pH of around pH 7. The addition of mobile phase with 0.01% ammonium hydroxide used in the gradient elution LC method increases the pH of the mobile phase. Therefore, a more versatile LC column that can withstand mobile phase of pH range of 2 – 11 was chosen. Separation was performed on reversed phase of C-18 Zorbax Extend column, 100 mm X 4.6 mm with 3.5 μm packing, which can withstand pH 2 – 11.

Analytical guard cartridge system with 3 mm ID cartridges (KJO-4282) from Phenomenex was also used as a guard column.

3.1.1.7 Gradient elution

A mobile phase gradient elution is employed to separate these analytes with different retention times. The separation was conducted under gradient system with mobile phase A consisting of water/methanol (98:2 by volume) and mobile phase B is 100 % methanol, both containing 0.01 % ammonium hydroxide. The flow rate was set to 0.5 ml/min. sample volume of 15 µl was injected.

Gradient conditions were as shown in Table 3.3 for both negative and mix mode. Mix mode consists of a negative mode for the first period of the run followed by a positive mode in the second period. The mobile phase for the mix mode however is without any ammonium hydroxide. All phytoestrogens, BPA and oestrogens were separated in one single gradient run in negative mode while testosterone was separated in a different run under a mix mode program.

Table 3.3: LC time program in gradient elution for plasma analysis (negative mode and mix mode)

Negative mode		Mix mode	
Time (min)	Mobile phase (solvent B)%	Time (min)	Mobile phase (solvent B)%
0.1	30	0.1	65
2.0	60	0.5	65
5.0	60	2.5	95
6.5	95	4.5	95
9.5	95	4.51	65
9.51	30	8.5	65
15.0	stop	8.51	stop

3.1.1.8 Internal standard (IS)

Characteristics of an appropriate IS include similarity of the internal standard's structure and properties with analyte of interest, detectable by the detector used and must not be present in the biological sample analysed. Based on literature review, 4-hydroxybenzophenone (4-HBPH) has been used as an internal standard in prior phytoestrogen analysis and fulfilled the criteria above (Bolca *et al.*, 2010; Maubach *et al*., 2003; Thomas *et al.*, 2001).

3.1.1.9 Sample preparation (plasma)

About 6 – 10 ml of blood samples were drawn from human subjects using BPA free 10 ml Terumo syringe with needle and a 10.0 ml BD vacutainer with lithium heparin. The vacutainer containing blood samples were placed in a cool box for transportation to the laboratory. All the blood samples were centrifuged at 4000 rpm for 10 minutes at room temperature within 3 hours of collection and plasma was separated. The plasma samples were stored at -80^{o}C until extraction.

Sample blanks are plasma matrices with no analyte of interest. To prepare sample blanks, a plasma pool (80 ml) obtained from the University Malaya Medical Centre (UMMC) blood bank was used. Only expired plasma was used to prepare sample blanks. Five grams of activated charcoal was added to the plasma pool in a 100 ml laboratory bottle.

Then, the plasma bottle was placed in an automated stirrer at room temperature for 4 hours. The bottle was then left overnight in 4^{o}C refrigerator. The plasma was then transferred into several BPA free centrifuge tubes and subjected to centrifugal separation at 4^{o}C, 5000 rpm for 10 min. the charcoal sediments would sink to the bottom and side of the tubes. The clear plasma was aspirated and the whole cycle starting from the addition of activated charcoal was repeated. This is carried out for 3

cycles. After 3 cycles, the clear aspirated plasma is stored in a clean glass bottle to be used as sample blanks. The whole process was done for 3 cycles to ensure the plasma is devoid of any analytes studied.

Sample extraction method for isoflavones was adapted from Chan *et al* (Chan *et al.*, 2006) but several steps were modified accordingly to include extraction of sex hormones. The modifications were the addition of a deproteinisation step, change of the incubation setting for the sample and usage of a different SPE cartridge to enable better extraction of the sex hormones. Sample extraction method for BPA was adapted from Volkel *et al* (2005) and Fernandez *et al* (2007).

Sample extraction was started by addition of 500 μl plasma and 3 ml of 50 mM sodium acetate buffer (pH 5) containing 10 μl of 50 ng/ml of 4-HBPH as internal standard into a glass tube. Deproteinisation was carried out by adding 4 ml of acetone to the shaken mixture. The tube was capped and vortexed for 30 seconds. After centrifugation at 4500 rpm for 10 minutes, sample was transferred into a clean tube. Following acetone evaporation under a steady flow of nitrogen stream into a 40^{o}C water bath, 250 μl of diluted β-glucuronidase enzyme was added. After gentle shaking, sample was incubated in a water bath at 45^{o}C for an hour. Sample was then subjected to solid phase extraction.

3.1.1.10 Solid phase extraction/SPE (plasma)

SPE is a technique used for sample clean up and concentration prior to chromatographic analysis. In this technique, organic functional groups are chemically bound to a solid surface, such as silica which is placed in a small cartridge. Sample is placed in the cartridge and forced through by negative pressure (using a vacuum). Trace organic molecules are extracted, pre-concentrated and remained on the column – separated from the sample matrix. It is further concentrated by evaporating the solvent.

The molecules then can be eluted with a solvent such as methanol and analysed by chromatography.

Conditioning steps are required in order to allow interaction with aqueous samples. Without conditioning steps, the SPE cartridge will be waterproof to aqueous samples. In this study, the SPE cartridge was conditioned with 3 ml of methanol and 3 ml of water. Then, plasma sample was loaded on the SPE cartridge. Washing step was done with 5 ml of water followed by a soft vacuum suction for 10 minutes before the final elution step with 4 ml of methanol. To further concentrate the analytes, the eluate was dried using nitrogen stream at 40^{o}C. Dried extract was stored in -80^{o}C freezer until analysis. The extract was reconstituted with 100 µl of 30 % methanol, vortexed and filtered with 0.2 µm syringe filter before injection into LCMSMS.

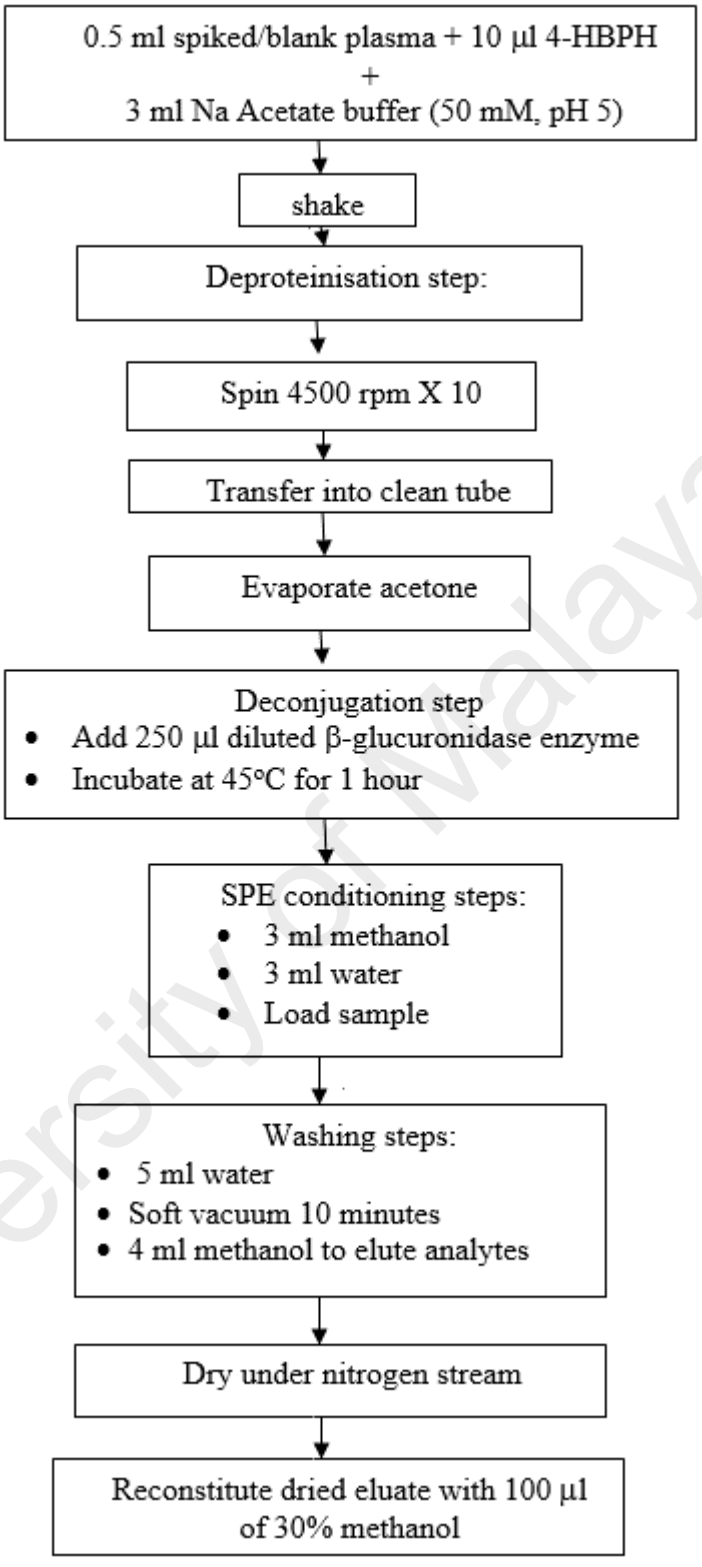

Figure 3.2: Extraction Step (plasma)

3.1.1.11 Matrix effects and recoveries (plasma)

Matrix effect is the direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample. Matrix effects will result in either enhanced or suppressed ionization of the analytes (Van Eeckhaut *et al.*, 2009). This could be due to co-eluting analytes in the sample extract that possess similar ions in the mass spectrometry, or a result of interaction between target analyses matrix components. Peak areas in the chromatogram of known amounts of standards (A) were compared with those measured in plasma extract, spiked after extraction with the same analyte amount (B). The ratio (B/A X 100) is defined as the absolute matrix effects (ME %). A value of > 100 % indicated signal enhancement and < 100 % means signal suppression while no matrix effect is indicated by 100 %. Recoveries were calculated by spiking the samples before extraction, and its peak areas (C) were compared with B. Recovery is defined as the ratio of (C/B X 100).

3.2 Method validation of 9 analytes in human plasma

Method validation is the process of defining an analytical requirement, and confirming that the method being developed is capable to consistently perform according to its application requirement (Guidance for Industry, Bioanalytical Method Validation, May 2001). Method validation includes the procedures required to demonstrate that a quantification method for analytes in a particular biological matrix is reliable. Two types of error may exist during the development of a method; random and systemic (Chan *et al.*, 2004). A random error may result from some imprecision associated with a measurement. Random error can be minimized but not eliminated. A systematic error is one that occurs when a result is consistently biased in one direction for example, the sample matrix which may suppress the instrument signal. For quality control measure, standards are run intermittently with samples.

Method validation runs were performed on three consecutive days, included a calibration curve and QC samples at five different concentrations in 5 replicates. Five QC samples concentration used were 1, 4, 35, 65 and 75 ng/ml for each LLOQ (lower limit of quantification), QC_{low}, QC_{mid}, QC_{high}, ULOQ (upper limit of quantification). Calibration curves were generated over the range of 1 – 75 ng/ml.

For method validation, parameters such as limit of detection (LOD), recoveries of spiked plasma at 3 different concentrations (4, 35 and 65 ng/ml), precision and accuracy of quality control (QC) samples were obtained and presented in Chapter 4 (Results). LOD refers to the lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise (Guidance for Industry, Bioanalytical Method Validation, May 2001). The LLOQ was determined by meeting the following two criteria: (1) signal to noise ration greater than 5 and (2) values for precision and accuracy less than 20%.

Specificity is the ability to assess an analyte in the presence of other components that may be present such as impurities, placebo ingredients and others (Chan *et al*., 2004). The specificity of the method was tested by visual inspection of chromatograms of extracted analytes.

3.2.1 Accuracy

Accuracy is the degree of agreement between a measured value and a true value (Westgard *et al.*, 1974). Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. It should be measured using a minimum of 5 replicates per concentration. A minimum of 3 different concentrations is recommended, however in this validation study – 5 concentrations were used as stated in the QC samples above.

3.2.2 Precision

Precision is the degree of agreement between replicate measurements of the same quantity and does not necessarily imply accuracy (Christian, 2004). As with accuracy, precision is also determined by a minimum of 5 replicates per concentration with a minimum of 3 different concentrations used. QC samples used for this study were 5 replicates of 5 different concentrations (1, 4, 35, 65 and 75 ng/ml).

3.2.3 Recovery

Recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure standard. Recovery looks at the extraction efficiency of an analytical method within the limits of variability. It is reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method. Recovery of the analyte need not be 100% but rather has to be consistent, precise and reproducible which also applies to the recovery of the IS. To ensure the above, recovery experiments were performed by comparing the analytical results for extracted samples at 3 concentrations (low, medium and high). In this study, the 3 concentrations used were 4, 35 and 65 ng/ml.

3.2.4 Calibration curve

A calibration curve is the relationship between instrument response and known concentration of the analyte. Each analyte has its own calibration curve which is prepared in the same biological matrix as the samples in the intended study.

Stock solutions of standards were prepared by dissolving accurately weighed reference standard of the analyte in methanol. Stock solutions of these were stored in glass vials at -20^{o}C and were found to be stable for 60 days. The stock solutions were

diluted further, on each day of analysis to prepare seven concentrations of calibration standards.

The plasma is spiked with known concentrations of the analyte. In this study, a 7 point calibration curve uses calibrator concentrations of 1, 3, 10, 15, 30, 60 and 75 ng/ml. The blank plasma is spiked with known concentrations of the analytes resulting in calibrator concentrations as shown in Table 3.1. Blank plasma refers to the matrix used (plasma) which does not contain any of the analytes studied.

3.3 Method development of oestrogenic EDCs and oestrogens in human breast tissue

Several analytical methods are available for determination and quantification of isoflavones, BPA and sex hormones in biological matrices such as urine (Mathey *et al.*, 2006; Nagata *et al.*, 2006), plasma (Mustafa *et al*., 2007; Nelson *et al*., 2004) and serum (Chan *et al*., 2006; Xu *et al.*, 2007). However, fewer methods have been developed for other matrix such as seminal fluid, placental tissue, prostatic tissue and breast tissue possibly due to the difficulty in obtaining the biological samples. The aim of this study is to develop and validate a LCMSMS method for the simultaneous determination and quantification of isoflavones, BPA and sex hormones in human breast tissue.

3.3.1 Experimental

Daidzein, genistein, formononetin, equol, biochanin A, oestrone, oestradiol were purchased from Sigma Aldrich, USA. The IS, 4-HBPH was from Merck, Germany. HPLC grade methanol was purchased from Mallinckrodt, USA. Hexane, ethyl acetate, hydrochloric acid and ammonium hydroxide were from Fisher Scientific. Solvent and water used for LCMSMS were filtered with Millipore membrane filter of 0.45 μm pore size for solvent and 0.2 μm for water. All samples were filtered with a 4 mm diameter

syringe filter of regenerated cellulose by Phenomenex (0.2 μm pore size). Stock standard solutions (1 mg/ml) were prepared every month and stored at -20°C. Working standard solutions of different concentrations were prepared daily by dilution of the stock solutions with methanol. A 2-year old collection of breast tissue was repeatedly thawed. It was tested to ensure there is no detectable level of analyte studied, before using it as blank matrix for preparation of calibration standards.

3.3.1.1 Chemicals and reagents

Description for this section is similar to 3.1.1.1.

3.3.1.2 Preparation for Calibrators

Stock standard solutions of mix standards are prepared in 100% methanol and stored at 20°C in amber glass vials as described in section 3.1.1.2. A volume of 200 μl of stock standard solution is then added to blank breast tissue which did not contain any of the analytes studied to obtain 10 mg of stock blank tissue at final concentration of 200 ng/g. Table 3.4 shows the following working standard solutions from the 200 ng/g stock breast tissue prepared.

Table 3.4: Standard preparation for calibrators (breast tissue)

Mix standard concentration (ng/g)	Stock breast tissue sample (ng/g)	Weight of stock (μg)	Weight of blank tissue (μg)	Total (μg)
0.5	200	25	9975	10,000
1	200	50	9950	10,000
5	200	250	9750	10,000
10	200	500	9500	10,000

Table 3.4, continued

Mix standard concentration (ng/g)	Stock breast tissue sample (ng/g)	Weight of stock (μg)	Weight of blank tissue (μg)	Total (μg)
20	200	1000	9000	10,000
30	200	1500	8500	10,000
50	200	2500	7500	10,000

3.3.1.3 Preparation for Quality Control samples

Preparation for the respective concentrations of QC samples which were prepared separately from the calibrators samples are as shown in Table 3.5. Three concentrations of low (QC1 – 0.75 ng/g), medium (QC2 – 25 ng/g) and high (QC3 – 38 ng/g) were chosen, these concentrations being within the range of calibration curve. The internal standard concentration was the same as that used for the calibration curve.

Table 3.5: Preparation for QC samples (breast tissue)

Mix standard concentration (ng/g)	Stock breast tissue (ng/g)	Weight of stock (μg)	Volume of blank plasma (μg)	Total (μg)
0.5 (LLOQ)	200	25	9975	10,000
0.75 (QC 1)	200	37.5	9962.5	10,000
25 (QC 2)	200	1250	8750	10,000
38 (QC 3)	200	1900	8100	10,000
50 (ULOQ)	200	2500	7500	10,000

3.3.1.4 Chromatography

A Shimadzu LC system consisting of a binary pump (LC-20ADXR), an autosampler SIL-20AXR, a column oven and a system controller (CBM-20A) were used for separation. All phytoestrogens, BPA and oestrogens were separated in one single gradient run in negative mode. Separation was performed on an Agilent reversed phase

C-18 Zorbax Extend column which can withstand pH 2 – 11, 100 mm X 4.6 mm with 3.5 μm packing. Analytical guard cartridge system with 3 mm ID cartridges (KJO-4282) from Phenomenex was used. Mobile phase A consists of water/methanol (98:2 by volume) and B: 100% methanol, both containing 0.01% ammonium hydroxide. The flow rate was set to 0.5 ml/min. Sample volume of 10 μl was injected. Gradient conditions were as shown in Table 3.2. LCMSMS was performed on an API-5500 QTrap (Applied Biosystems/ MD Sciex) connected to a Shimadzu LC-20ADXR system run in negative mode. The source temperature was 450^{o}C. The curtain gas, GS 1 and GS was set to 20, 40 and 40 psi respectively. Nitrogen was used both as nebulising and drying gas. Nitrogen was generated by a nitrogen generator from Peak Scientific (MA, USA). Ion source voltage was -4500 V. analytes were detected by multiple reaction monitoring (MRM) mode. For each analyte, 2 MRM transitions were monitored. Mass spectral data were analysed using Analyst 1.5.2 software (Applied Biosystem, MDS Sciex). A seven point calibration curve was included with each assay, using calibrator concentrations from 0.5, 1, 5, 10, 20, 30 and 50 ng/g.

3.3.1.5 Solvent selection

The analysis of breast tissue maintained the solvent methanol with ammonium hydroxide. The only difference is the biological matrix, which would depend more on sample preparation.

3.3.1.6 LC Columns

Similarly, with plasma analysis, separation of breast tissue sample analysis was performed on reversed phase of C-18 Zorbax Extend column, 100 mm X 4.6 mm with 3.5 μm packing, which can withstand pH 2 – 11. Analytical guard cartridge system with 3 mm ID cartridges (KJO-4282) from Phenomenex was also used as a guard column.

3.3.1.7 Gradient elution

A mobile phase gradient elution is employed to separate these analytes with different retention times. The separation was conducted under gradient system with mobile phase A consisting of water/methanol (98:2 by volume) and mobile phase B is 100 % methanol, both containing 0.01 % ammonium hydroxide. The flow rate was set to 0.5 ml/min. Sample volume of 15 µl was injected.

Gradient conditions were as shown in Table 3.6. All phytoestrogens, bisphenol A and oestrogens were separated in one single gradient run in negative mode.

Table 3.6: Liquid chromatography time program in gradient elution for breast tissue analysis (negative mode)

Time (min)	Mobile phase (solvent B)%
0.20	30
1.30	65
2.30	65
3.30	95
4.80	95 (Total flow 0.7 ml/min)
6.20	95 (Total flow 0.5 ml/min)
8.00	95
8.01	30
10.10	30 Stop run

A: water/methanol (98:2 by volume) with 0.01% ammonium hydroxide
B: 100% methanol with 0.01% ammonium hydroxide

3.3.1.8 Internal standard (IS)

This method maintained the use of 4-HBPH as the internal standard as it is also easily detectable with API-5500 QTrap LCMSMS.

3.3.1.9 Sample preparation (breast tissue)

Sample blanks used were breast tissue matrices with no analyte. To prepare sample blanks, a 2 year old collection of breast tissue samples were thawed at room temperature for 3 cycles. The sample blanks were ascertained not to contain any of the analytes

studied by subjecting the blanks to the method developed and analytical run which showed no peaks of the analytes of interest at the respective retention time.

About 2 – 5 g or the size of 20 cents coin of breast tissue was obtained in the operation theatre during the mastectomy, placed in a 20 ml glass jar and kept in a cold box for transportation to the laboratory within 6 hours. Tissue samples were then stored at 80^{o}C until extraction. Tissue extraction method for isoflavones was adapted from Bolca *et al* (2010) and modified accordingly to include extraction of sex hormones (Bolca *et al*., 2010).

About 500 mg breast tissue was finely chopped and minced into a disposable homogenizer tube. Ten µl of 100 ng/ml IS was added followed by homogenization with 3 ml ice cold 200 mmol/L hydrochloric acid in 90% aqueous methanol and 1 ml hexane/ethyl acetate (3:2 by volume) with an IKA Ultra Turrax Tube Drive and Disperser with BMT-20 G Ball-Mill Tube (IKA, Germany). Sample was homogenized for 120 seconds and transferred into a 50 ml Falcon centrifuge tube, capped and vortexed for 1 minute. Following 10 minutes sonication, tube was centrifuged at 14,000 rpm for 20 minutes at -4^{o}C.

After centrifugation, the yellowish supernatant fluid was collected in a separate clean tube (A). The excess methanol portion was poured into another clean tube (B) and dried under a steady flow of nitrogen stream. The pellet (C) was re-extracted with 1 ml ice cold 200 mmol/L hydrochloric acid in 90% aqueous methanol. It was then centrifuged at 14,000 rpm for 5 minutes. The yellowish supernatant fluid (A) was dried under nitrogen. Once dried and leaving a lipid layer at the bottom of the tube, 2 ml of methanol was added (A) and vortexed slowly.

The tube (A) was left standing and kept at -80^{o}C to freeze the lipid layer at the bottom. Once the bottom lipid layer was frozen, the upper methanol layer (A) was

added to (B) and dried under the nitrogen stream. The supernatant fluid from the pellet (C) was also added to (B) and dried under nitrogen stream.

Once dried, extract was reconstituted with 150 µl of 45% methanol or capped and stored at -80^{o}C until analysis. All extracts were filtered with regenerated cellulose 0.2 µm prior to LCMSMS injection.

3.4 Method validation of oestrogenic EDCs and oestrogens in human breast tissue

For method validation, parameters such as LOD, recoveries of spiked breast tissue matrix at 3 different concentrations (0.75, 25 and 38 ng/g), precision and accuracy of QC samples were obtained and presented in Chapter 4 (Results). Three QC sample concentrations used were 0.75, 25 and 38 ng/g for each QC_{low}, QC_{mid} QC_{high}. To obtain recovery, accuracy and precision data, the calibration curve (0.5 – 50 ng/g) and 3 QC samples in 5 replicates were run for 3 consecutive days.

3.4.1 Accuracy

Accuracy is determined by 5 replicate analysis of samples containing known amounts of the analyte at concentrations 0.75, 25 and 38 ng/g.

3.4.2 Precision

In this validation study, precision is determined by 5 replicates per concentration with 3 different concentrations. Therefore, QC samples consisted of 5 replicates of 0.75, 25 and 38 ng/g.

3.4.3 Recovery

To ensure the extraction efficiency, recovery experiments were performed by comparing the analytical results for extracted samples at 3 concentrations (low, medium and high). In this study, the concentrations used were 0.75, 25 and 38 ng/g.

3.4.4 Calibration curve

The breast tissue matrix is spiked with known concentrations of the analyte. In this study, a 7 point calibration curve in which the sample blanks were spiked with known concentrations of the analytes resulting in calibrator concentrations of 0.5, 1, 5, 10, 20, 30 and 50 ng/g. Sample blanks refer to the matrix used (breast tissue) which does not contain any of the analytes studied.

3.5 Study population of male vegetarian and non-vegetarian

The present study looks at the distribution of selected phytoestrogens and BPA in plasma of male vegetarians and non-vegetarians in Malaysia using a validated LCMSMS method. In addition, the concentration levels of phytoestrogens and BPA were compared with endogenous sex hormones in the plasma.

3.5.1 Study design

Healthy males aged 18 – 65 years old who practiced vegetarian diet recruited for this study were from various temples, religious associations and public advertisements in Malaysia. They were briefly interviewed to obtain consent and simple information and 10 ml of blood sample were taken. Consent and simple information obtained is as attached in Appendix F and G. Controls (non-vegetarian males) were randomly selected from the public who were at the blood donation venue after matching for age and race. Vegetarians are defined as those who do not take meat at all for more than 3 months.

Based on the information obtained about their type of diet, they are further classified as vegan, lactovegetarian (LV), ovovegetarian (OV) and lacto-ovo-vegetarian (LOV). Vegans are those who do not take meat, milk or eggs. Lactovegetarians are those who consume milk but do not take meat or eggs. Ovovegetarians consume eggs but not meat and milk while lacto-ovo-vegetarians consume both milk and eggs but not meat. The exclusion criteria were – any medical illness or on any form of antibiotics medication. Blood samples were analysed for daidzein, genistein, equol, formononetin, biochanin A, BPA, oestrone, oestradiol and testosterone. This study was approved by the Ethics Committee of the UMMC (Ethics No. 607.5) and informed consent was obtained from all subjects.

3.5.2 Sample collection

About 6 – 10 ml of blood was collected from each healthy subject using BPA free syringe. Blood sample collection is conducted within time range of 08:00 to 12:00 hours. Samples obtained were centrifuged within 3 hours at 4000 rpm for 10 minutes at room temperature. Sample was analysed using a validated LCMSMS method.

The isoflavones assayed were daidzein, genistein, equol, formononetin, BPA and three sex hormones; oestrone, oestradiol and testosterone were analysed. In this study, equol producers are those with equol plasma concentration of 0.5 ng/ml and above.

3.5.3 Statistical analysis

The statistical tests were performed using SPSS Statistics version 15.0 software. Statistical tests performed include Spearman's correlation coefficient, independent T-test and one way ANOVA test. A P value of < 0.05 was defined as representing a statistically significant difference.

Statistical analyses were performed using Spearman's correlation coefficient, Mann-Whitney, Kruskal-Wallis and paired t-test. A P value of < 0.05 was defined as representing a statistically significant difference.

With regards to plasma analyte concentrations, correlation study was carried out in all 225 subjects to obtain information on any relation of the isoflavone types and oestrogens. All subjects were divided into several different groups and subjected to correlation analysis to demonstrate any relation between exogenous EDCs (daidzein, genistein, equol, formononetin, biochanin A and BPA) and endogenous testosterone. The groups were : vegetarian (n = 103), non-vegetarian (n = 122), equol producers (n = 73), non-equol producers (n = 152), Chinese subjects (n = 63), Indian subjects (n = 132), other ethnic groups (n = 30), subjects with ≤ 2 types of isoflavones (n = 85), subjects with > 2 types of isoflavones (n = 140), equol producer vegetarians (n = 43), non-equol producer vegetarians (n = 60), Chinese vegetarians (n = 30), Indian vegetarians (n = 63), coffee drinker vegetarians (n = 79), equol producer non-vegetarians (n = 30), non-equol producer non-vegetarians (n = 92), Chinese non-vegetarians (n = 33), Indian non-vegetarians (n = 69), coffee drinker non-vegetarians (n = 89).

Further analysis compared mean plasma concentrations between several groups studied. The groups were: i) sub-groups of vegetarian status, ii) vegetarian (n = 103) and non-vegetarian (n = 122) iii) ethnic groups: Chinese (n = 63), Indian (n = 132), others (n = 30) iv) Chinese vegetarian (n = 30) and Chinese non-vegetarian (n = 33) v) Indian vegetarian (n = 63) and Indian non-vegetarian (n = 69) and vi) equol producers (n = 73) and non-equol producers (n = 152).

3.6 Study population of female patients with and without breast disease

This study looks at the distribution of 6 oestrogenic EDCs (daidzein, genistein, equol, formononetin, biochanin A and BPA) and compared the concentration to two oestrogen (oestrone, oestradiol) levels in plasma and breast tissue of female patients who were admitted to UMMC either with or without breast disease using a validated LCMSMS method.

A total of 252 female patients who agreed to participate in this study were recruited. They were female patients who were admitted to undergo a breast surgery (n = 109) and outpatients without any breast disease (n = 143). Ethical approval was granted by the Ethics Committee of UMMC (Ethics No. 607.6). The volunteers were explained about the purpose of the study and written consent was obtained.

3.6.1 Study design

The inclusion criteria are that all patients who were admitted to the breast surgery ward in UMMC from the period of December 2007 – January 2009 and female patients who visited the UMMC Outpatient Department. Exclusion criterion is: patients who were not scheduled for breast operation the next day. After explanation of the study and consent obtained, brief information was taken from the patient. Following that, blood collection was carried out.

3.6.2 Sample collection

For female outpatients without any breast disease, only blood samples were obtained at the outpatient clinic. For breast surgery patients, blood was collected on the day of admission in a 10 ml heparinised vacutainer, blood from the outpatients were taken at the outpatient clinic on their visit. Blood tubes were kept in a cold box and transported to the laboratory to be centrifuged within 4 hours. Plasma was obtained by

centrifugation (10 min at 4000 rpm, room temperature). Aliquots of plasma were stored at -80^{o}C freezer for storage until analysis. Breast tissue was obtained only from mastectomy operations, from large enough samples where the amount of sample taken will not compromise the diagnosis (complying with the conditions stated by the Ethics Committee). Tissue samples were taken at the most distal part of the affected lesion. In advanced statistical analysis, we excluded women who were diagnosed as having benign breast diseases and focused the results to those with malignant breast diagnosis to compare with those who do not have any breast disease. Both plasma and breast tissue samples were analysed using a validated experimental method with LCMSMS.

3.6.3 Statistical analysis

Statistical analyses were performed using Spearman's correlation coefficient, Mann-Whitney, Kruskal-Wallis and paired t-test. A P value of < 0.05 was defined as representing a statistically significant difference.

With regards to plasma analyte concentrations, correlation study was carried out in all 252 subjects to obtain information on any relation of the isoflavone types and oestrogens. All subjects were divided into several different groups and subjected to correlation analysis to demonstrate any relation between exogenous EDCs and endogenous oestrogens. The groups were : women with breast cancer (n = 79), women without breast cancer (n = 143), equol producers (n = 43), non-equol producers (n = 179), Malay patients (n = 55), Chinese patients (n = 130), Indian patients (n = 37), nulliparous patients (n = 51), non-nulliparous patients (n = 171), patients with history of lactation (n = 103), patients without history of lactation (n = 109), menopausal patients (n = 142), pre-menopausal patients (n = 76), patients with $\leq$ 2 types of isoflavones (n = 156), patients with > 2 types of isoflavones (n = 66). The number of

patients in each group is subject to the information obtained. For instance, there is some missing information for menopausal status for a few subjects.

Apart from correlation study, comparison of mean plasma concentration of analyte levels between subject groups were also conducted to discover any significant differences. Mean plasma concentrations were compared between groups : i) women with (n = 79) and without breast cancer (n = 143), ii) Malay (n = 55), Chinese (n = 130) and Indian (n = 37) patients, : iii) nulliparous (n = 51) and non-nulliparous patients (n = 171), iv) menopausal (n = 142) and pre-menopausal patients (n = 76), v) patients with ≤ 2 types of isoflavones (n = 156), patients with > 2 types of isoflavones (n = 66) and vi) equol producers (n = 43) and non-equol producers (n = 179).

With regards to breast tissue analyte concentrations, correlation study was carried out in 53 breast tissue samples. Further correlations to demonstrate relation between exogenous EDCs and endogenous oestrogens were carried out for these groups: i) equol producers (n = 13) and non-equol producers (n = 40), ii) Malay (n = 14), Chinese (n = 30) and Indian patients, iii) nulliparous (n = 14) and non-nulliparous (n = 39) patients, iv) patients with (n = 20) and without history of lactation (n = 33), iv) menopausal (n = 38) and pre-menopausal patients (n = 15) and v) patients with ≤ 2 types of isoflavones (n = 46).

Comparison of median concentration of analytes in breast tissue between subject groups were also conducted to demonstrate any significant differences. Groups involved were: i) menopausal (n = 38) and pre-menopausal patients (n = 15), ii) Malay (n = 14) Chinese (n = 30) Indian (n = 9), iii) nulliparous (n = 14) and non-nulliparous (n = 39) patients, and iv) equol producers (n = 13) and non-equol producers (n = 40).

All 53 breast tissue samples have accompanying plasma samples which allowed the comparison of mean analyte concentrations in 2 different matrices: plasma and breast tissue. This analysis was conducted by paired t-test.

CHAPTER 4: RESULTS

This chapter will cover experimental results for method development and validation of plasma and breast tissue samples. It will also cover results of the validated methods applied on real samples from the two distinct populations as described in Chapter 3.

4.1 Method development and validation of analytes in human plasma

An LCMSMS method which allows the simultaneous analysis of 8 analytes (daidzein, genistein, equol, formononetin, biochanin A, BPA, oestrone and oestradiol) from plasma in a single run was developed and validated. The method was run in negative mode and the running time is 15 minutes. This method gives an acceptable level of sensitivity without the need for derivatisation. Hence, allowing identification and quantification of some selected oestrogenic EDCs and sex hormones in human plasma.

4.1.1 Acquisition parameters

Each compound was monitored for two MRM-transitions. Acquisition parameters for all analytes are presented in Tables 4.1 and 4.2. Analysis was carried out in negative mode for all analytes except for testosterone. During method development, testosterone chromatogram was best in positive mode. Testosterone eluted at around 4 – 6 min of the positive mode run, a critical period where there were elutions of other analytes for the negative mode. Therefore, analysis of testosterone had to be carried out in a separate mix mode run maintaining the same internal standard which eluted in negative mode. Chromatogram of blank methanol is shown in Figure 4.1 revealed no interfering peaks. Chromatogram showing peaks of EDCs and sex hormones obtained in a single run

using LCMSMS in ESI negative mode is shown in Figure 4.2. Chromatogram showing peak of testosterone in mix mode is shown in Figure 4.3.

Table 4.1: Acquisition parameters of analytes quantitation in negative mode

Analytes	Retention Time (min)	MRM Transition (*m/z*)	Dwell time (ms)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
D	3.35	252.9→131.9	50	-65	-10.5	-16	-52	-4
		252.9→208.0	50	-65	-10.5	-16	-52	-4
G	3.72	268.9→133.0	50	-65	-4	-18	-40	-0.5
		268.9→62.9	50	-65	-4	-18	-50	-0.5
Eq	5.77	241.2→120.9	50	-45	-5.5	-16	-18	-2
		241.2→119.0	50	-45	-5.5	-16	-24	-2
F	4.79	267.0→222.8	50	-60	-7	-14	-28	-8
		267.0→194.9	50	-60	-7	-14	-42	-2
Bio A	5.18	283.3→238.9	50	-60	-8.5	-26	-34	-6
		283.3→167.1	50	-50	-8.5	-26	-42	-2
BPA	7.87	226.9→212.1	75	-50	-9	-22	-36	-2
		226.9→133.1	75	-50	-9	-22	-27.8	-2
E1	8.66	269.0→145.0	300	-82.3	-7	-13.06	-44	-1
		269.0→159.0	300	-82.3	-7	-13.06	-44	-4
E2	8.54	271.0→144.9	400	-70	-10	-30.19	-51	-0.10
		271.0→143.1	400	-70	-10	-30.19	-51	-0.10
* 4-HBPH	5.18	196.8→91.8	100	-55	-4	-14	-40	-2
		196.8→120.0	100	-55	-4	-14	-38	0

* Internal standard: 4-hydroxybenzophenone (4-HBPH)

i. DP = declustering potential EP = entrance potential CEP = collision cell entrance potential

ii. CE = collision energy CXP = collision exit potential

Table 4.2: Acquisition parameters of testosterone quantitation in positive mode

Analytes	Retention Time (min)	MRM Transition (*m/z*)	Dwell time (ms)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
T	4.51	289.2→109.1	150	61	8	18	31	4
		289.2→97.1	150	61	8	18	25	4

* Internal standard: 4-hydroxybenzophenone (4-HBPH)

i. DP = declustering potential EP = entrance potential CEP = collision cell entrance potential

ii. CE = collision energy CXP = collision exit potential

The mass spectrum of isoflavones, BPA and sex hormones showed a protonated molecular ion at *m/z* 252.9, 268.9, 241.2, 267.0, 283.3, 226.9, 269.0 and 271.0 for daidzein, genistein, equol, formononetin, biochanin A, BPA, oestrone and oestradiol respectively (Table 4.1). Further high collision energy fragmented the parent ions into several daughter ions. The major fragments of each ions were then selected for monitoring in the third quadrupole. Selectivity for the analysis is as shown in chromatogram of each analyte, and its daughter ion (Figures 4.4 – 4. 12). Selectivity refers to the ability of the bioanalytical method to measure and differentiate the analytes in the presence of other components that may be present such as metabolites, impurities, degradants or matrix components (Guidance for Industry, Bioanalytical Method Validation, May 2001). The chromatograms indicate the peak resolution with no interfering peak in the region of interest.

For chromatography, the earliest peak eluted at 3.35 min for daidzein, followed by genistein, formononetin, biochanin A and equol (retention time as shown in Table 4.1). Subsequently, BPA, oestradiol and oestrone eluted at 7.87, 8.54 and 8.66 respectively.

A separate mixed mode run was carried out for testosterone. It was run in a negative mode for the first 4 minutes for elution of internal standard (4-HBPH), and switched to positive mode allowing elution of testosterone at 4.51 min (Table 4.2). The whole run was completed after 8.51 min. The chromatogram for mix mode is as shown in Figure 4.2 showing peaks of 4-HBPH (internal standard) in negative mode, followed by testosterone which eluted at 4.51 min in positive mode.

Figures 4.2 and 4.3 showed the absence of any interfering peaks at the retention time of the peak of interest for the analytes.

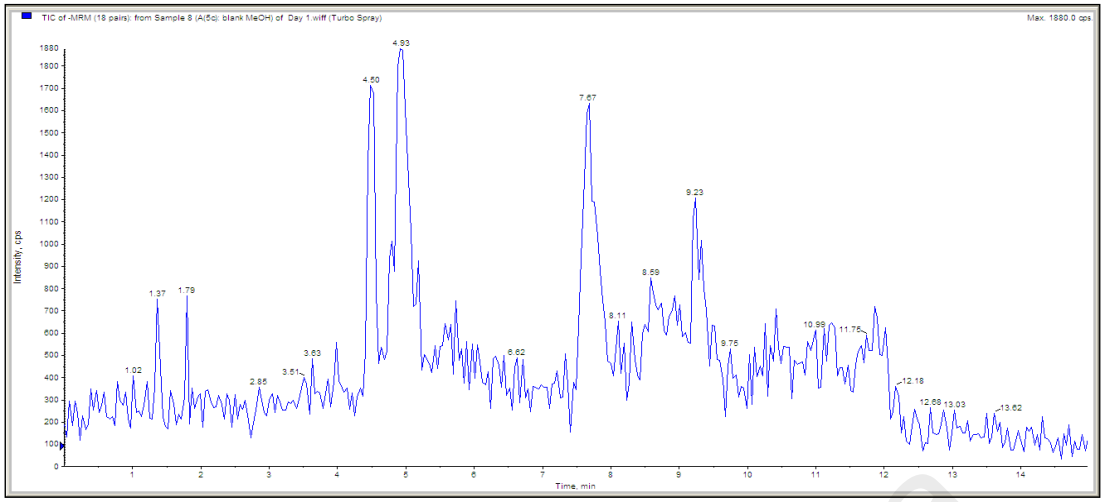

Figure 4.1: Chromatogram of blank methanol

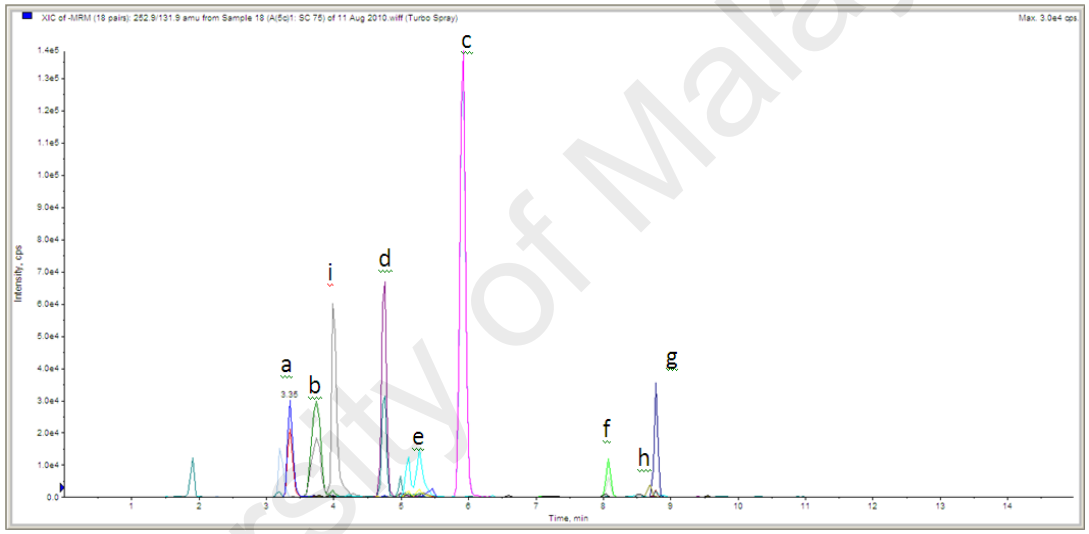

Figure 4.2: Total ion chromatogram in negative mode from a spiked plasma of 75 ng/ml of mixed standards

a = daidzein, b = genistein, c = equol d = formononetin e = bio A, f = BPA, g = E1, h = E2 i = int standard

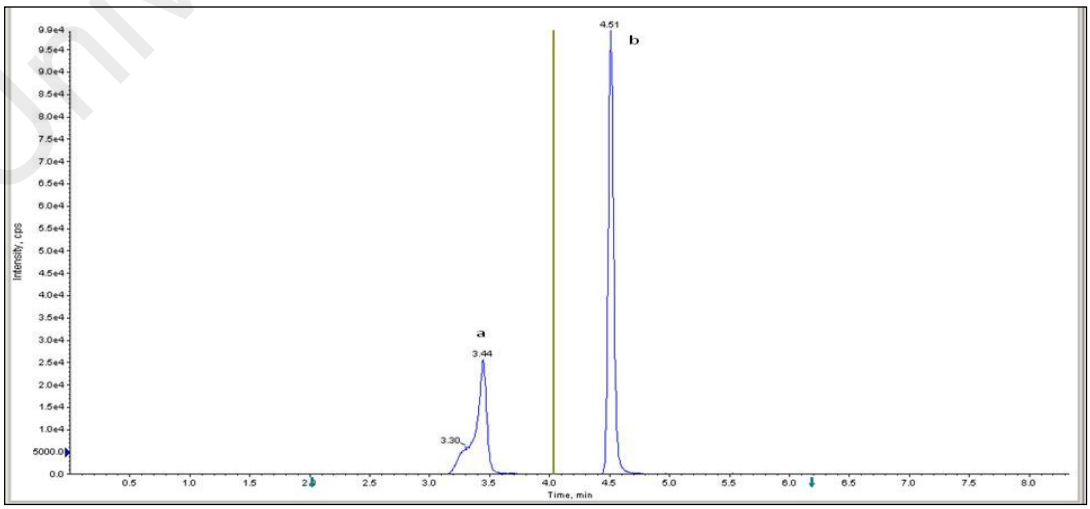

Figure 4.3: Total ion chromatogram in mix mode from a spiked plasma of 75 ng/ml of mixed standards

(negative mode from 0 – 4.0 min, positive mode from 4.0 – 8.51 min; a = 4-HBPH, b = testosterone)

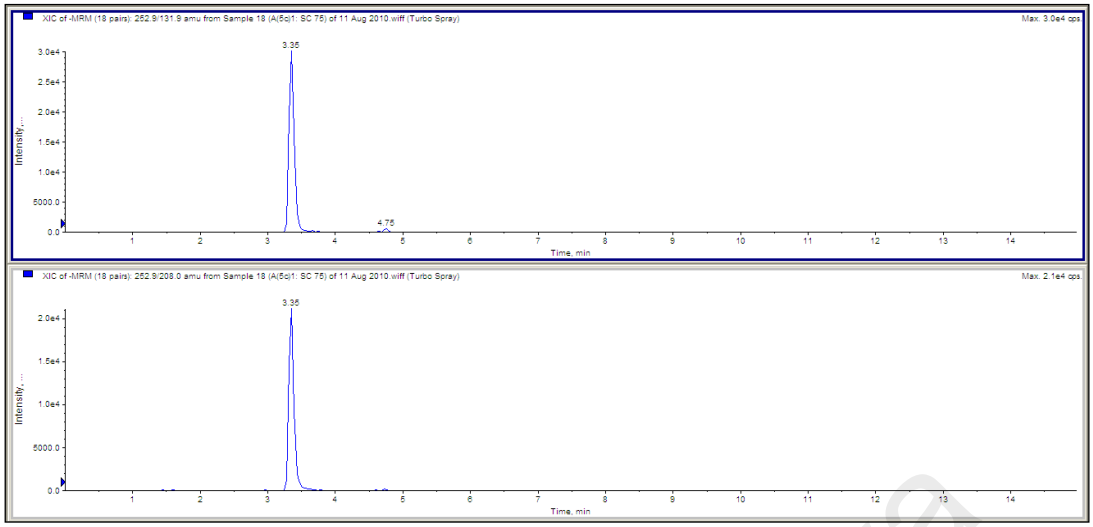

Figure 4.4: MRM chromatogram of daidzein

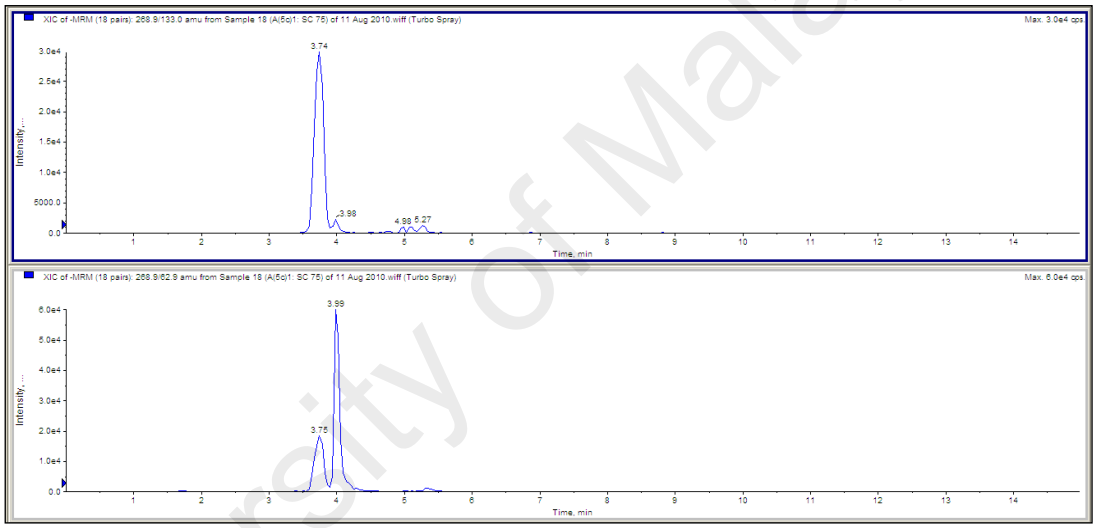

Figure 4.5: MRM chromatogram of genistein

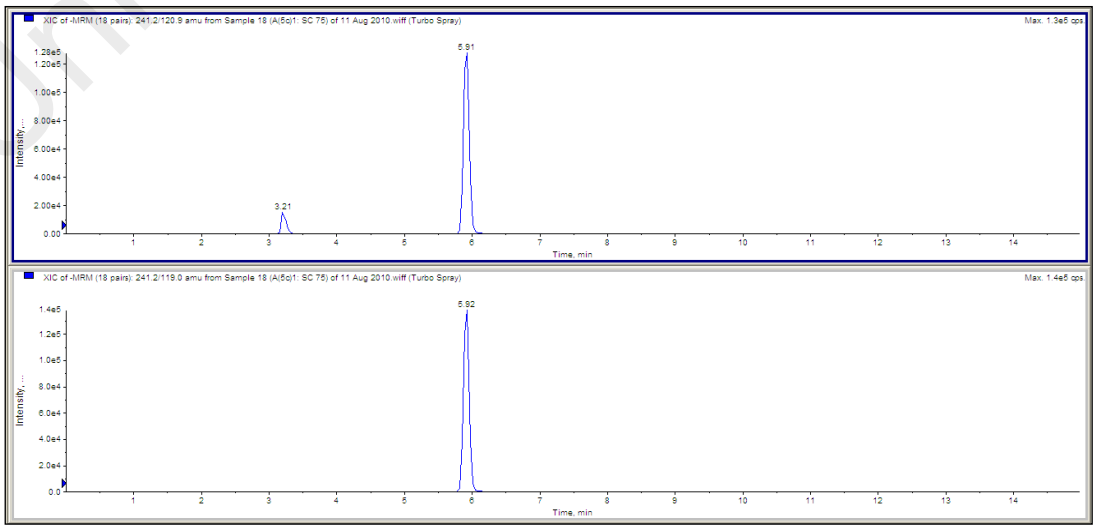

Figure 4.6: MRM chromatogram of equol

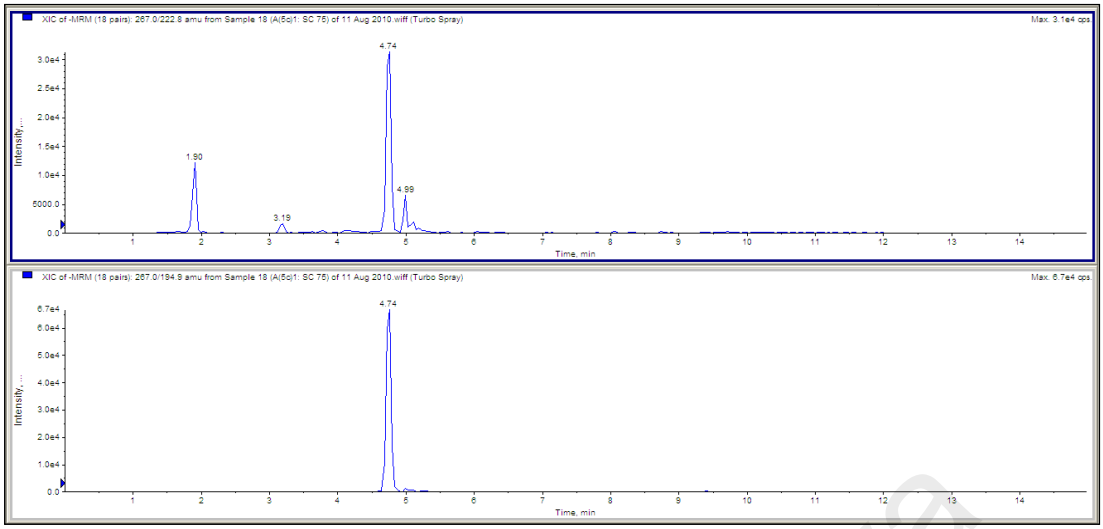

Figure 4.7: MRM chromatogram of formononetin

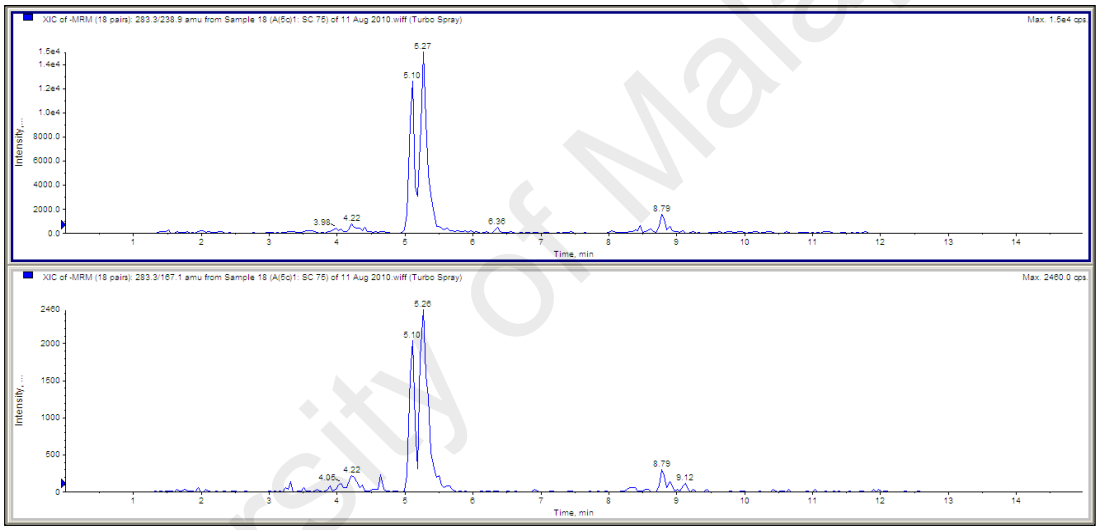

Figure 4.8: MRM chromatogram of biochanin A

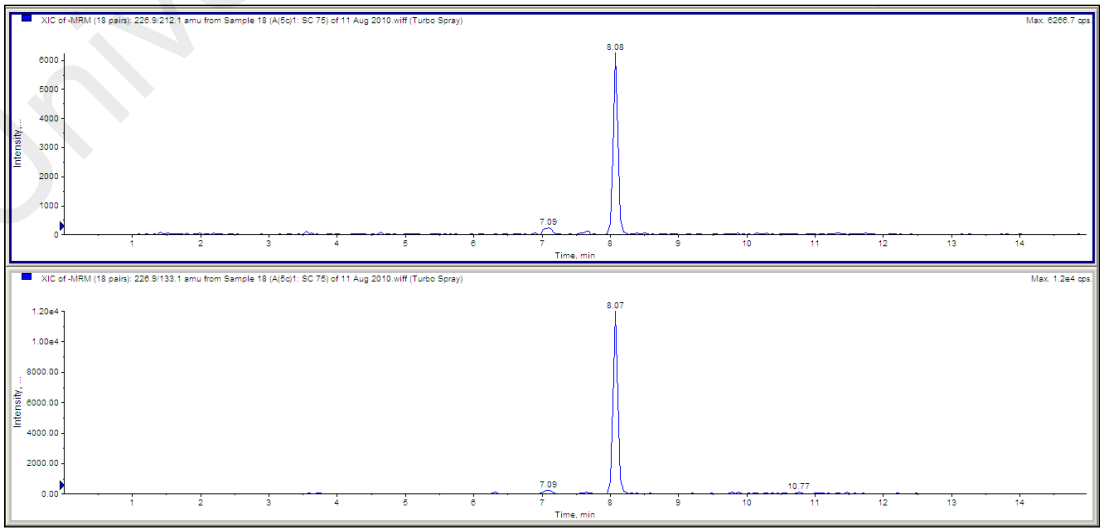

Figure 4.9: MRM chromatogram of BPA

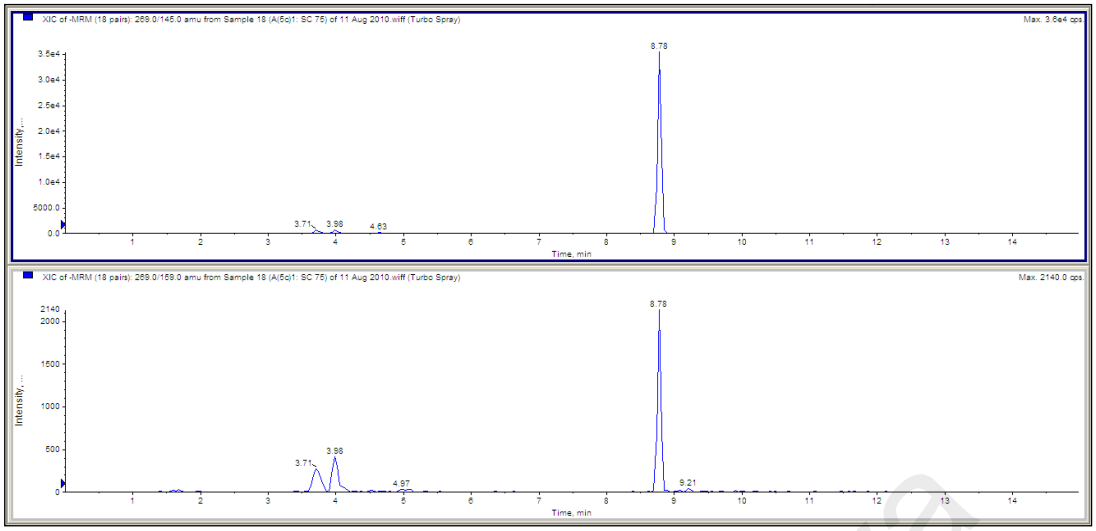

Figure 4.10: MRM chromatogram of oestrone

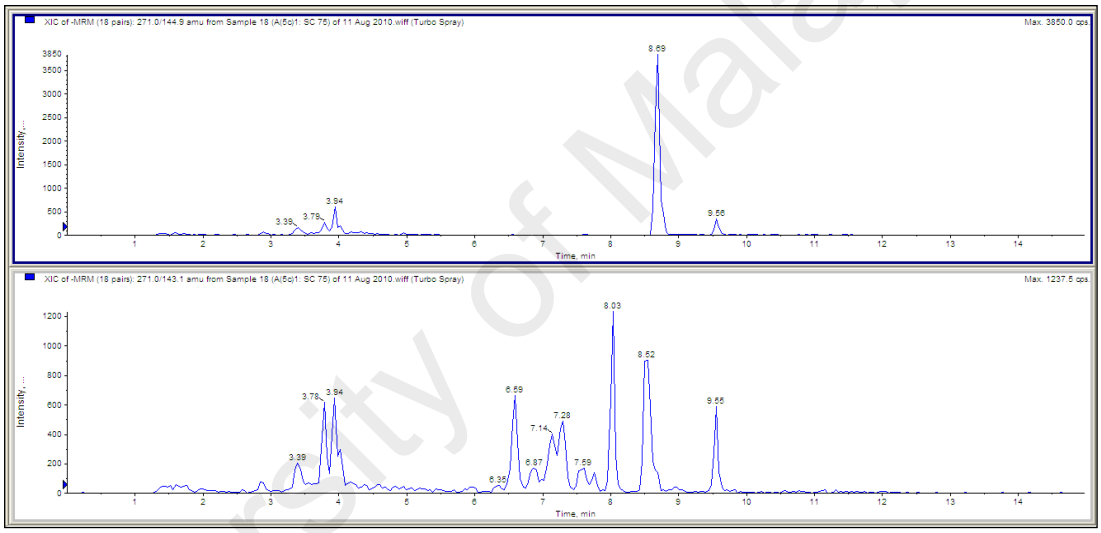

Figure 4.11: MRM chromatogram of oestradiol

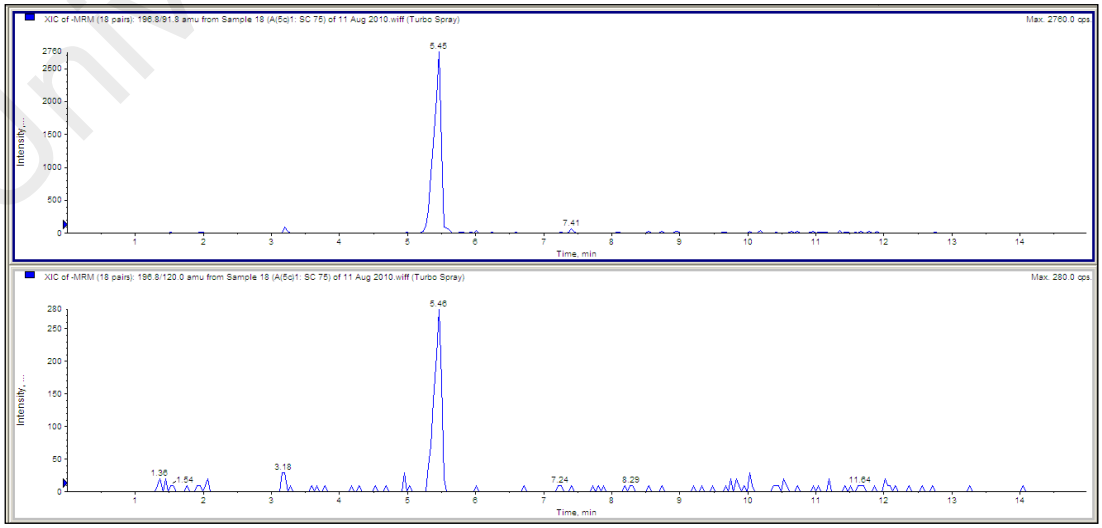

Figure 4.12: MRM chromatogram of 4-HBPH (internal standard)

4.1.2 Calibration curve

The calibration curves were constructed by plotting the peak area ratios (analyte/internal standard) versus the amounts of analyte.

Calibration curves of the analytes in charcoal-stripped human plasma over the range of 1 – 75 ng/ml were best fitted using a linear regression analysis. The correlation coefficients for the calibration curves of ≥ 0.99 were observed through out the validation.

Figures 4.13 – 4.21 represent the calibration curve for each analyte; daidzein, genistein, equol, formononetin, biochanin A, BPA, oestrone, oestradiol and testosterone.

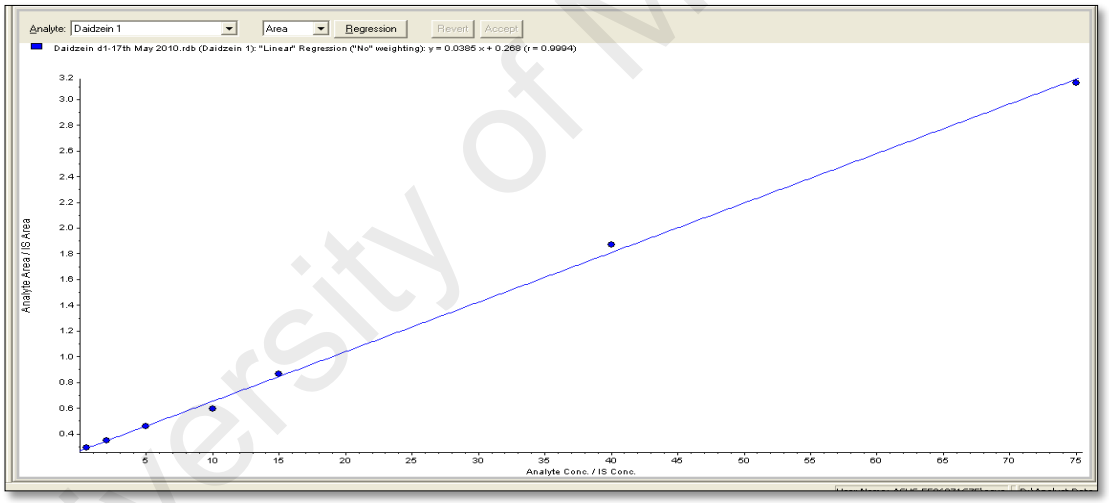

Figure 4.13: Calibration curve for daidzein

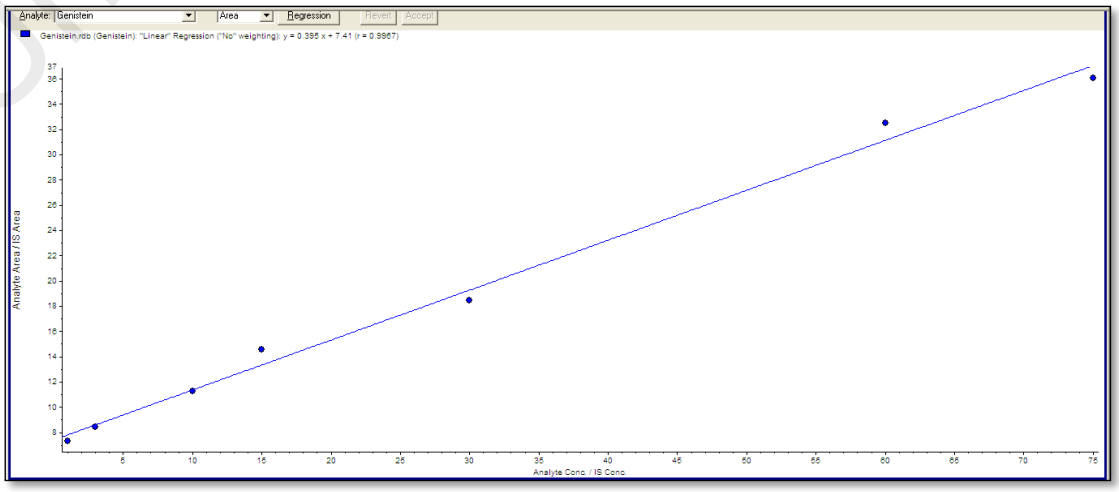

Figure 4.14: Calibration curve for genistein

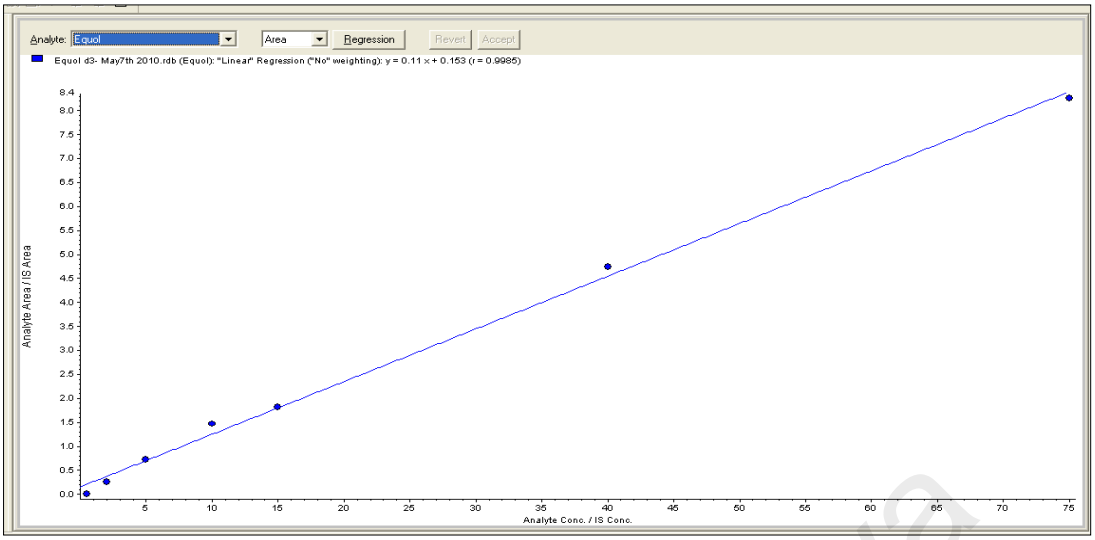

Figure 4.15: Calibration curve for equol

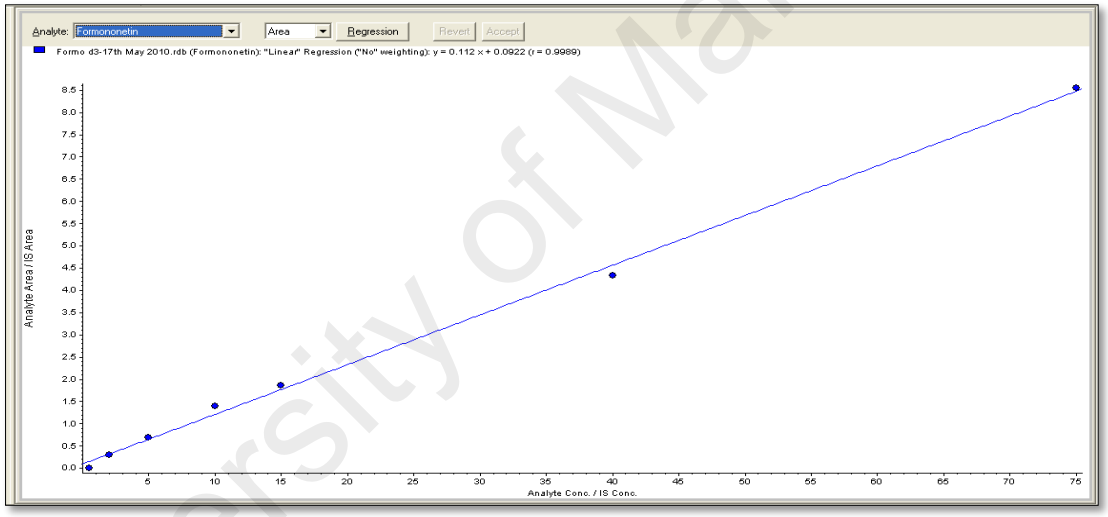

Figure 4.16: Calibration curve for formononetin

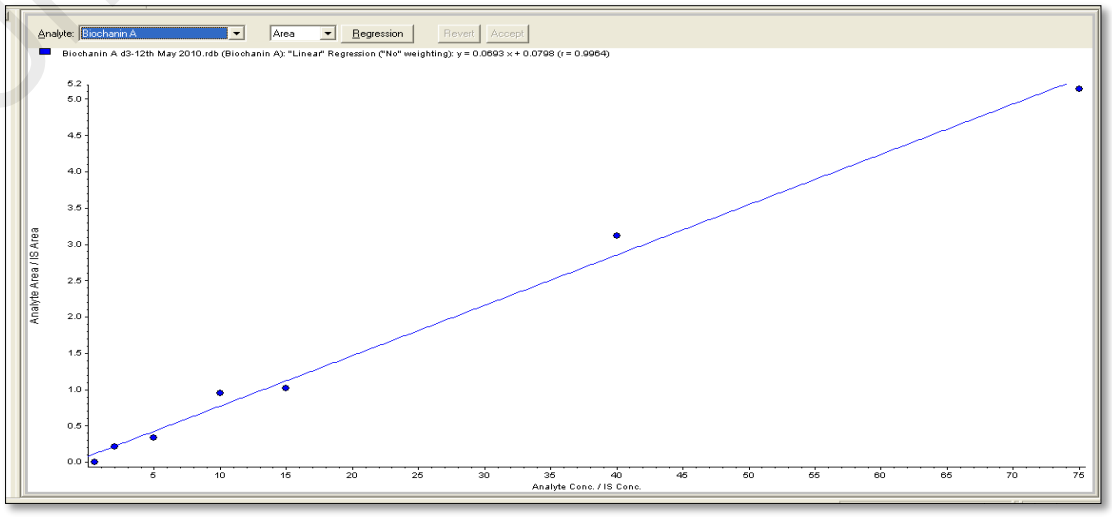

Figure 4.17: Calibration curve for biochanin A

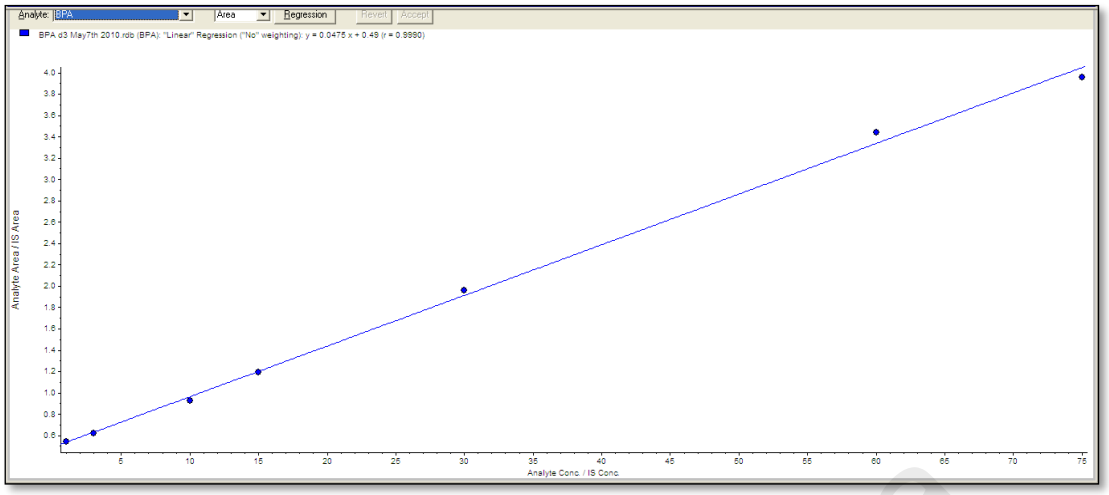

Figure 4.18: Calibration curve for BPA

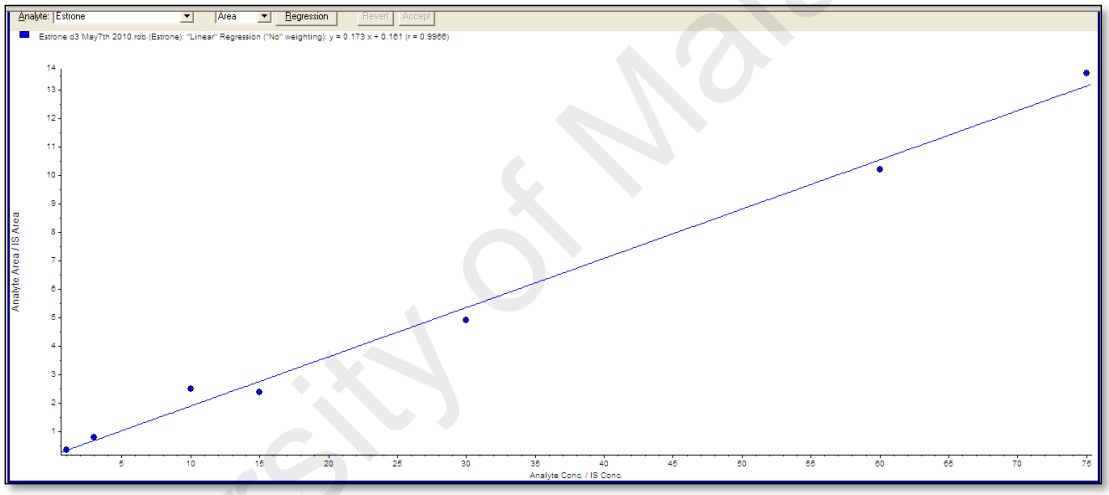

Figure 4.19: Calibration curve for oestrone

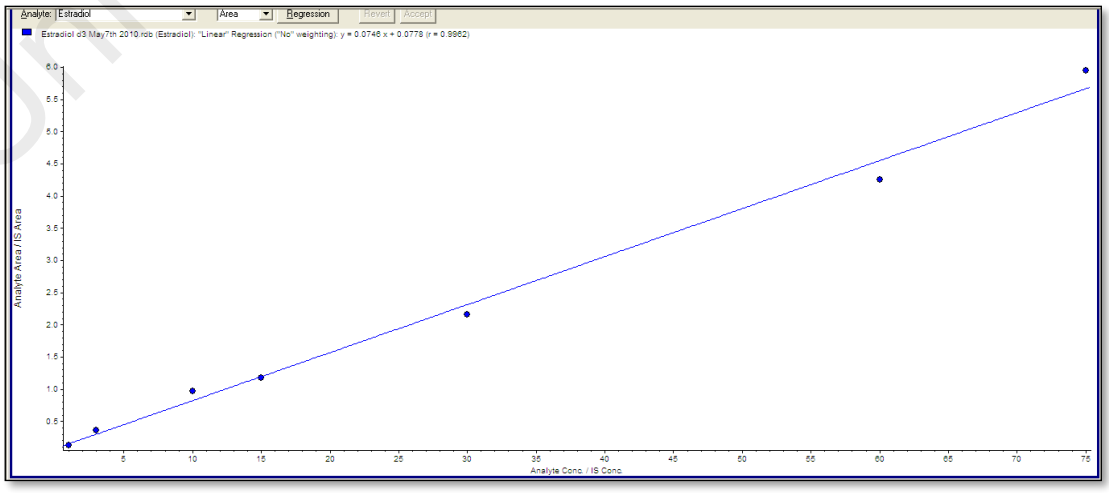

Figure 4.20: Calibration curve for oestradiol

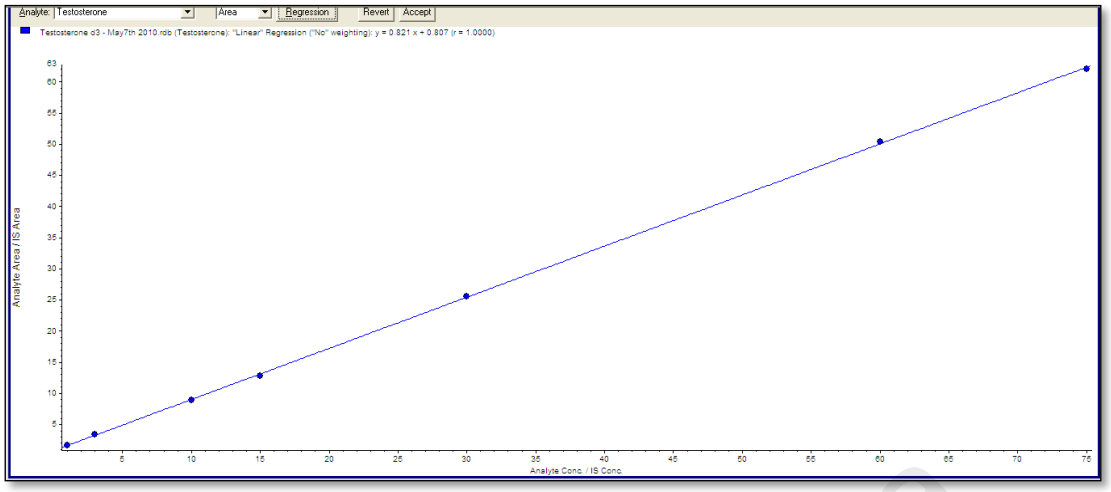

Figure 4.21: Calibration curve for testosterone

4.1.3 Precision and accuracy

The repeatability and intermediate precision of three different quality control (QC) samples, calculated in 5 replicates on three separate days for all analytes in human plasma as shown in Table 4.3, were < 15% over a wide range of isoflavones, BPA and sex hormone concentrations.

4.1.4 Limit of detection (plasma)

Limits of detection were 0.25 ng/ml for daidzein, genistein and testosterone; 0.30 ng/ml for equol, formononetin, biochanin A, BPA, oestrone and oestradiol.

Table 4.3: Precision and accuracy of quality control samples (plasma)

Analyte	Nominal Concentration (ng /ml)	Accuracy (%)			Precision (% CV)			Inter-day
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
D	1 (LLOQ)	87.9	98.4	102.2	4.3	14.7	14.1	10.4
	4 (QC1)	96.7	96.1	107.2	9.4	13.3	6.9	14.0
	35 (QC2)	104.2	109.7	103.6	6.8	6.7	8.9	7.1
	65 (QC3)	96.3	106.9	108.8	14.2	2.7	4.5	14.5
	75 (ULOQ)	95.9	98.9	110.3	14.0	10.2	4.6	10.4
G	1 (LLOQ)	90.9	91.3	115.4	5.9	6.1	12.5	11.3
	4 (QC1)	112.5	115.2	107.6	7.7	12.2	12.4	7.7
	35 (QC2)	106.3	103.1	107.3	5.5	8.4	3.4	4.7
	65 (QC3)	104.8	100.5	95.6	5.2	4.3	14.6	10.2
	75 (ULOQ)	116.9	108.4	94.8	10.4	13.9	4.6	10.5
Eq	1 (LLOQ)	96.7	98.1	102.8	14.1	4.4	2.5	14.3
	4 (QC1)	108.6	97.6	108.6	5.3	8.9	3.1	13.5
	35 (QC2)	96.9	98.4	98.8	7.5	5.2	4.9	2.4
	65 (QC3)	110.9	107.7	110.7	3.4	6.0	5.9	3.7
	75 (ULOQ)	97.9	105.2	113.8	4.3	14.1	14.7	10.6
F	1 (LLOQ)	89.9	91.4	105.4	7.6	12.3	12.5	7.8
	4 (QC1)	98.2	111.1	101.0	9.3	4.4	9.1	14.6
	35 (QC2)	110.8	107	106.8	5.3	8.4	5.0	4.7
	65 (QC3)	100.9	110.3	107.7	10.5	3.3	6.4	10.2
	75 (ULOQ)	103.9	98.3	105.7	10.7	6.7	5.5	13.3
Bio A	1 (LLOQ)	88.1	97.2	101.2	5.2	8.5	4.9	4.8
	4 (QC1)	85.1	92.6	96.5	10.7	8.2	4.6	14.2
	35 (QC2)	95.3	108.3	103.6	11.9	9.7	7.4	14.4
	65 (QC3)	90.9	98.6	94.3	4.6	3.03	5.9	9.1
	75 (ULOQ)	97.9	98.2	101.7	10.4	5.7	6.3	13.2
BPA	1 (LLOQ)	89.1	90.4	99.5	9.3	6.7	13.4	13.9
	4 (QC1)	106.1	94.8	98.2	9.4	14.2	3.9	13.0
	35 (QC2)	94.3	106.2	106.1	14.4	10.7	6.2	14.9
	65 (QC3)	97.9	110.5	103.4	10.2	6.9	5.3	13.6
	75 (ULOQ)	99.4	98.5	100.4	7.5	12.3	12.1	7.7
E1	1 (LLOQ)	98.6	97.6	109.3	4.3	5.3	14.5	10.1
	4 (QC1)	110.0	111.0	101.1	6.1	6.1	12.6	11.4
	35 (QC2)	110.6	114.4	106.6	11.9	11.7	14.3	7.9
	65 (QC3)	109.0	111.4	101.9	14.2	4.4	14.9	10.2
	75 (ULOQ)	112.1	101.8	104.9	14.3	4.5	14.6	10.7
E2	1 (LLOQ)	86.2	92.7	98.6	5.2	7.5	4.8	2.3
	4 (QC1)	112.2	98.7	108.6	14.8	9.1	13.2	14.7
	35 (QC2)	114.9	108.6	111.7	4.8	6.7	8.7	6.3
	65 (QC3)	115.0	111.8	112.7	11.2	7.5	8.0	3.3
	75 (ULOQ)	108.2	113.2	110.7	6.4	10.5	14.4	14.7
T	1 (LLOQ)	99.04	98.2	91.2	2.7	4.5	14.2	14.5
	4 (QC1)	92.1	100.7	89.0	4.8	10.7	3.3	14.4
	35 (QC2)	100.5	90.5	89.6	8.9	8.5	6.2	14.4
	65 (QC3)	102.2	93.0	91.2	11.3	9.3	6.6	13.8
	75 (ULOQ)	102.5	90.8	90.2	4.3	14.2	14.9	10.2

4.1.5 Matrix effects and recovery (plasma)

Recovery values were as shown in Table 4.4 with a mean percentage ranging from 80.3 – 119.7%. Mean recovery is highest for formononetin at 4 ng/ml while the lowest recovery is for genistein at 35 ng/ml.

Table 4.4: Recoveries of spiked plasma

Analyte	Mean recovery (%)		
	4 ng/ml	35 ng/ml	65 ng/ml
D	81.0	83.4	85.9
G	119.3	80.3	108.2
Eq	87.9	81.8	104.2
F	119.7	88.3	82.3
Bio A	81.3	84.7	83.6
BPA	96.7	88.1	90.5
E1	96.2	87.6	98.3
E2	87.8	99.6	111.9
T	89.9	81.6	99.3

The percentage of matrix effects varies with the concentration of analytes in plasma as shown in Table 4.5. Percentage of matrix effects range from 32.2 – 408.1 % for the isoflavones while BPA showed 78.7 – 93.3% of matrix effect. Oestrogens showed low percentage of matrix effect except for 30 ng/ml for oestradiol while testosterone showed a range of 81.4 – 113.1 % of matrix effect.

Table 4.5: Calculated percentage of matrix effects on the peak area response of analytes in plasma

Analyte	Matrix effects (%)		
	4 ng/ml	35 ng/ml	65 ng/ml
D	342.4	66.5	70.9
G	408.1	149.3	94.6
Eq	120.7	117.6	82.5
F	196.2	41.1	32.2
Bio A	210.9	88.1	69.4
BPA	81.6	93.3	78.7
E1	15.2	16.3	10.5
E2	9.1	79.8	25.6
T	113.1	104.8	81.4

4.2 Method development and validation of EDCs in human breast tissue

4.2.1 Acquisition parameters

The mass spectrum of the analytes with its related acquisition parameters are as shown in Table 4.6. while Figure 4.22 shows chromatogram of all analytes in negative mode run.

4.2.2 Calibration curve

Calibration curve was obtained using a series of concentration ranging from 0.5 – 50 ppb and 0.1 ng/ml internal standard 4-HBPH, showed good linearity.

Table 4.6: Acquisition parameters for the quantitations of analytes (breast tissue) - API-5500 QTrap

Analyte	Retention Time (min)	MRM transition (*m/z*)	Dwell (ms)	DP (V)	CE (V)	CXP (V)
D	2.52	252.974/223.800	100	-195	-36	-13
		252.974/132.100	100	-195	-52	-9
G	2.77	268.995/133.000	100	-175	-40	-7
		268.995/63.200	100	-175	-70	-5
Eq	4.10	240.981/121.000	100	-115	-20	-9
		240.981/119.200	100	-115	-24	-1
F	3.26	266.939/252.000	100	-170	-30	-13
		266.939/223.000	100	-170	-42	-9
Bio A	3.39	283.064/267.900	100	-190	-30	-7
		283.064/238.900	100	-190	-44	-17
BPA	4.66	226.936/211.900	100	-85	-24	-9
		226.936/133.100	100	-85	-34	-7
E1	4.96	269.044/145.100	100	-255	-46	-7
		269.044/159.300	100	-255	-46	-7
E2	4.90	271.039/145.200	100	-210	-52	-9
		271.039/143.200	100	-210	-64	-9
*4-HBPH	3.42	196.924/91.900	100	-160	-42	-15
		196.924/119.000	100	-160	-34	-9

* Internal standard
DP = declustering potential CE = collision energy CXP = collision exit potential

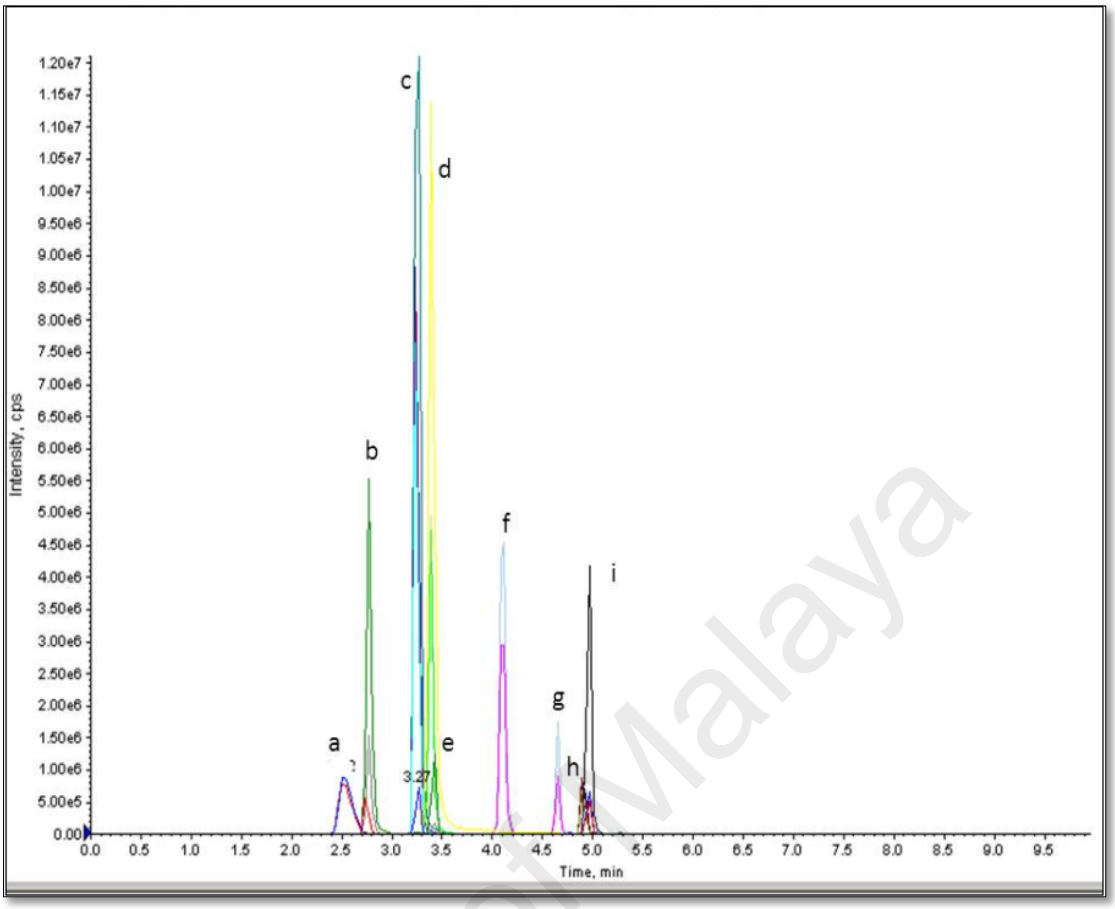

Figure 4.22: LCMSMS chromatogram in negative mode from a 100 ng/ml mix standard in solvent (API-5500 QTrap)

peaks of a = daidzein, b = genistein, c = formononetin, d = biochanin A, e = 4-HBPH, f = equol, g = BPA, h = oestradiol and i = oestrone

4.2.3 Precision and accuracy

Data on precision and accuracy are as shown in Table 4.7.

Table 4.7: Precision and accuracy of QC samples (breast tissue)

Analyte	Nominal Concentration (ng /g)	Accuracy (%)			Precision (% CV)			Inter-day
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
D	0.5 (LLOQ)	113.4	86.7	104.3	13.4	12.6	11.1	12.3
	0.75 (QC1)	115.8	96.1	107.8	14.4	14.7	14.2	14.8
	25 (QC2)	101.0	92.4	92.3	12.2	13.9	13.9	14.9
	38 (QC3)	82.7	86.7	84.0	14.8	14.7	13.4	14.7
	50 (ULOQ)	83.5	85.3	88.6	13.2	14.2	14.2	14.5
G	0.5 (LLOQ)	92.7	116.7	89.2	10.2	11.3	13.9	14.9
	0.75 (QC1)	98.7	112.1	96.3	9.1	14.7	12.8	14.9
	25 (QC2)	107.2	94.7	112.0	8.8	9.1	14.8	12.9

Table 4.7, continued

Analyte	Nominal Concentration (ng /g)	Accuracy (%)			Precision (% CV)			
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Inter-day
	38 (QC3)	94.9	88.9	102.5	9.5	9.1	4.3	9.4
	50 (ULOQ)	98.7	90.4	102.7	9.1	10.3	4.9	10.0
Eq	0.5 (LLOQ)	118.3	109.3	94.7	7.4	13.9	12.4	12.9
	0.75 (QC1)	112.4	106.9	114.2	6.3	14.8	11.8	12.9
	25 (QC2)	104.6	111.5	118.2	12.5	6.4	11.3	10.9
	38 (QC3)	98.1	95.1	90.4	7.3	8.3	12.5	9.5
	50 (ULOQ)	99.3	94.7	95.8	7.0	7.9	11.9	9.8
F	0.5 (LLOQ)	114.2	110.3	87.8	14.8	13.9	8.7	13.9
	0.75 (QC1)	112.7	86.7	85.8	14.7	14.3	7.5	14.6
	25 (QC2)	82.2	86.2	85.7	13.6	4.4	14.4	11.6
	38 (QC3)	89.3	113.3	86.1	9.9	5.9	6.6	14.8
	50 (ULOQ)	89.1	94.8	90.4	10.4	8.3	11.9	10.8
Bio A	0.5 (LLOQ)	114.3	105.4	111.3	8.8	6.9	12.5	12.3
	0.75 (QC1)	102.2	107.4	117.6	5.3	7.1	14.9	11.7
	25 (QC2)	93.8	94.0	89.0	14.3	10.5	13.7	14.9
	38 (QC3)	86.7	88.8	91.5	8.1	14.5	14.9	13.1
	50 (ULOQ)	88.3	89.6	92.6	4.2	3.3	7.6	5.9
BPA	0.5 (LLOQ)	115.3	108.3	101.5	6.4	7.1	14.7	11.7
	0.75 (QC1)	120.0	119.7	119.2	13.2	8.5	8.4	9.2
	25 (QC2)	84.8	85.2	85.2	9.1	4.2	5.6	6.3
	38 (QC3)	87.4	85.0	89.8	3.1	3.1	4.3	3.6
	50 (ULOQ)	98.1	86.1	100.5	13.9	10.2	9.7	13.5
E1	0.5 (LLOQ)	110.2	97.2	114.1	5.0	8.1	13.7	11.7
	0.75 (QC1)	80.2	96.9	114.2	13.8	14.6	14.8	14.2
	25 (QC2)	81.4	82.1	83.3	14.8	14.9	13.4	14.7
	38 (QC3)	84.9	85.5	85.2	14.9	12.2	9.9	12.0
	50 (ULOQ)	100.3	92.8	85.5	9.1	14.0	14.1	12.9
E2	0.5 (LLOQ)	99.3	98.4	108.4	7.5	10.1	12.8	12.9
	0.75 (QC1)	80.2	80.1	80.3	5.2	4.2	4.5	3.7
	25 (QC2)	80.6	85.2	80.5	13.2	11.4	11.1	14.6
	38 (QC3)	85.0	85.3	86.6	6.0	5.2	4.7	11.5
	50 (ULOQ)	96.5	87.3	103.7	9.4	13.8	14.1	12.7

4.2.4 Limit of detection (breast tissue)

Limit of detection (LOD) and recoveries of analytes in breast tissue samples are between 0.25 – 0.30 ng/g.

4.2.5 Matrix effects and recovery (breast tissue)

Table 4.8 showed the percentage of matrix effects at 3 different concentrations. Higher matrix effects were observed with isoflavones than the more lipophilic hormones. The highest matrix effect was observed for equol in its lower concentration, while the lowest effect was observed for formononetin at concentration 0.75 ng/g.

Table 4.8: Calculated percentage of matrix effects on the peak area response of analytes in breast tissue

Analyte	Matrix effects (%)		
	0.75 ng/g	25 ng/g	38 ng/g
D	36.7	10.0	40.0
G	38.6	3.8	7.9
Eq	60.6	0.9	6.2
F	0.9	5.4	9.6
Bio A	8.2	2.6	2.6
BPA	22.4	10.2	15.3
E1	31.2	14.7	17.5
E2	30.3	13.6	25.8

4.3 Study population of male vegetarian and non-vegetarian

A cross sectional study was carried out to investigate levels of selected oestrogenic EDCs and endogenous sex hormones among male vegetarians and non-vegetarians. A total of 225 subjects were randomly selected as described in sub-chapter 3.5.

4.3.1 Descriptive statistics

Samples were assayed for a total of 225 experimental subjects consisting of 122 male non-vegetarians and 103 male vegetarians. Characteristics of the subjects were surveyed and blood sample was taken for analysis using validated LCMSMS method described in

sub-chapter 3.2 and 4.1. Characteristics surveyed include age, ethnic groups, habits of either smoking, drinking coffee or alcohol. Table 4.9 shows the characteristics of the subjects from the 2 groups.

Table 4.9: Data observed from male vegetarians and non-vegetarians

Variable	Male non-vegetarian (n = 122)		Male vegetarian (n = 103)	
	Mean (S.D)	Frequency (%)	Mean (S.D)	Frequency (%)
Ethnic groups				
Chinese		33 (27.0)		30 (29.1)
Indian		69 (56.6)		63 (61.2)
Others		20 (16.4)		10 (9.7)
Age	38.10 (9.92)		39.03 (10.73)	
Smoking				
Yes		35 (28.7)		7 (6.8)
No		87 (71.3)		96 (93.2)
Drinks coffee				
Yes		89 (73.0)		79 (76.7)
No		33 (27.0)		24 (23.3)
Drinks alcohol				
Yes		40 (32.8)		14 (13.6)
No		82 (67.2)		89 (86.4)
Equol Producer		30 (24.6)		43 (41.7)
Number of isoflavone Types detected				
0		38 (31.1)		8 (7.8)
1		10 (8.2)		1 (2.9)
2		10 (8.2)		16 (15.5)
3		15 (12.3)		19 (18.4)
4		29 (23.8)		38 (36.9)
5		20 (16.4)		19 (18.4)

Figure 4.23 shows the number of isoflavone types found in non-vegetarians (NV) and sub-classes of vegetarian. Majority of vegans (V), lacto-ovo-vegetarians (LOV) and lactovegetarians (LV) had 4 types of isoflavones. Majority of vegans (91.6%) had more than 2 types of isoflavones as compared to only 53.5% in non-vegetarians.

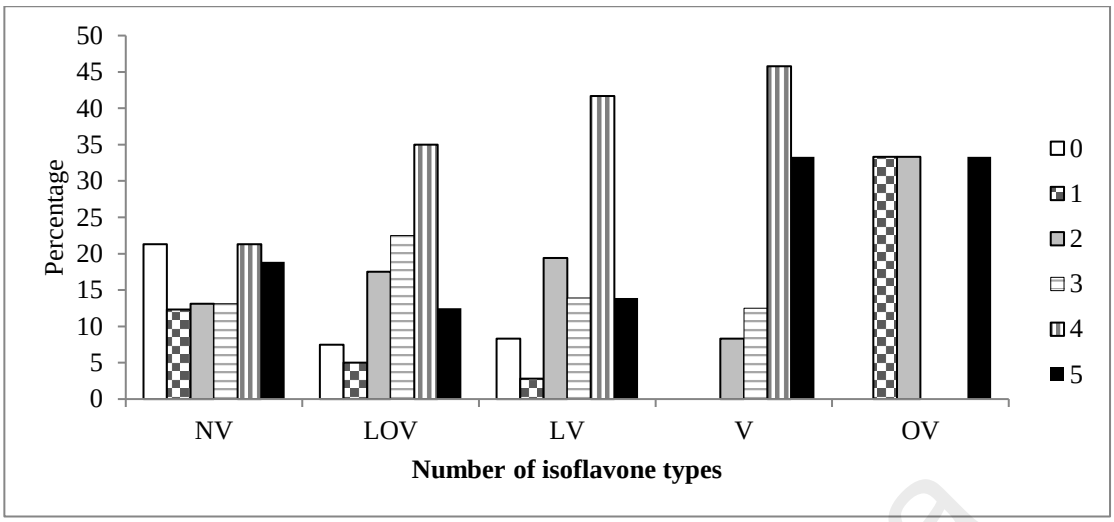

Figure 4.23: Number of isoflavone types in non-vegetarians and sub-classes of vegetarian

4.3.2 Percentage of analyte detection (plasma)

Figure 4.24 shows 100% detection of formononetin and testosterone in non-vegetarians while vegetarians showed 100% detection for daidzein, BPA and testosterone. All 103 vegetarians had daidzein, BPA and testosterone detected in their plasma samples. Percentage of detection of all analytes was about the same for both groups except for oestrone and oestradiol which was more frequently detected in vegetarians. Equol detection was around 60% for both non-vegetarian and vegetarian. BPA was present in almost all of the plasma samples.

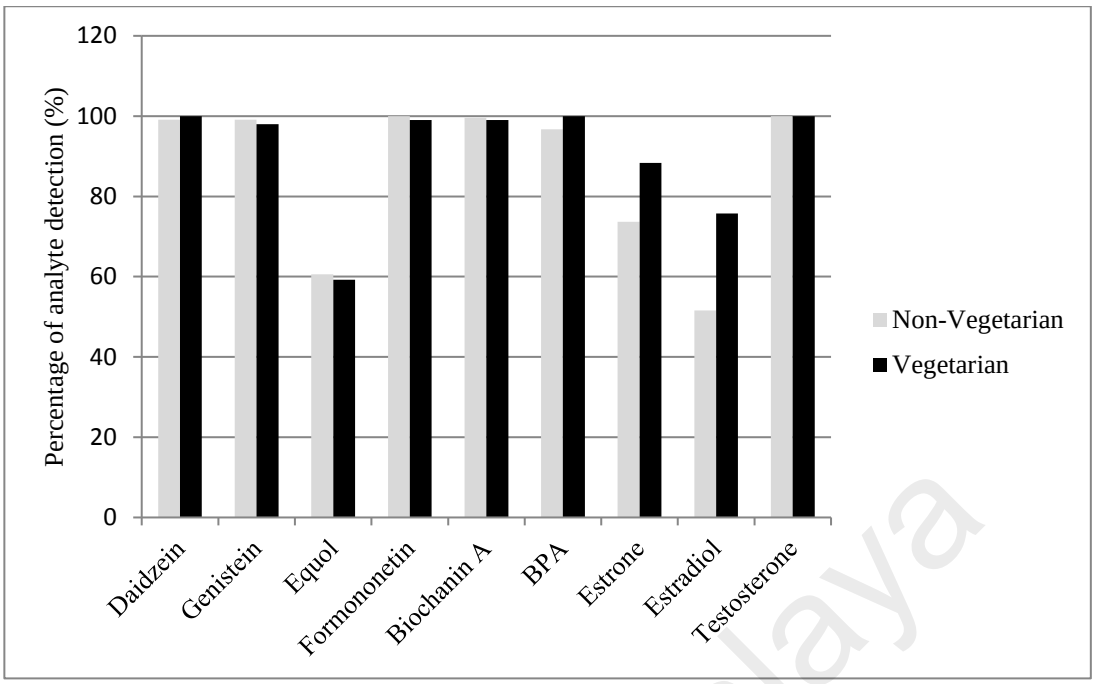

Figure 4.24: Percentage of analyte detection in plasma

Percentage of equol producers were compared between non-vegetarians and the sub-classes of vegetarians as shown in Figure 4.25. There were more equol producers among vegans (58.3%) and lacto-ovo-vegetarians (47.5%) as opposed to only 24% non-vegetarians who were equol producers (Figure 4.25).

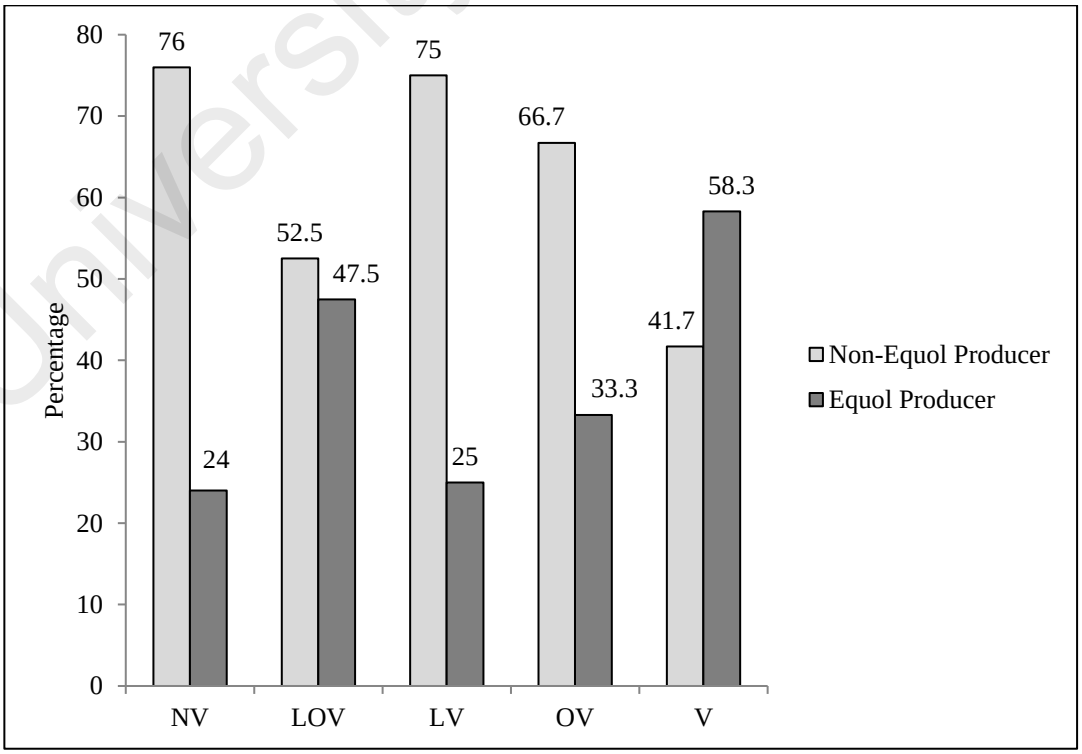

Figure 4.25: Percentage of equol producers in non-vegetarians and sub-classes of vegetarians

4.3.3 Statistical analyses

Statistical analysis was carried out using SPSS software version 15.0. Parametric tests for comparing means between groups (independent t-test, ANOVA and paired t-test) and Spearman's correlations were used in this analysis. Table 4.10 is used as a reference in all correlation analysis in this study when interpreting the strength of the correlation.

Table 4.10: Strength of correlation (Munro, 2000)

r value	Strength of correlation
0.00 – 0.25	Little or no correlation
0.26 – 0.49	Low
0.50 – 0.69	Moderate
0.70 – 0.89	High
0.90 – 1.00	Very high

4.3.4 Correlation of analytes (plasma)

There exists a wide range of correlation among the analytes. Almost all analytes showed positive relationship between them (Table 4.11). The highest significant correlation obtained was between biochanin A and formononetin ($r_s = 0.86$). This was followed by daidzein which showed significantly high correlation with genistein ($r_s = 0.76$). Daidzein had significant correlations with all analytes showing weaker correlations with BPA and sex hormones ($r_s < 0.3$). Similarly, for genistein, significant correlations were observed for all analytes except oestradiol. Equol showed significantly low correlation with daidzein, genistein, formononetin, biochanin A and BPA ($r_s < 0.3$). Equol was not correlated with any of the sex hormones. Formononetin showed significantly strong correlation with biochanin A; moderate correlation with BPA and oestrone while a significantly weaker correlation with oestradiol and testosterone. Biochanin A also showed significant, weak correlation with BPA and sex hormones. None of the analytes showed inverse relationship.

Table 4.11: Correlation between analytes in plasma (n = 225)

Variable	D	G	Eq	F	Bio A	BPA	E1	E2	T
D	1.00	**0.76***	**0.19***	**0.56***	**0.57***	**0.24***	**0.23***	**0.20***	**0.30***
G	-	1.00	**0.29***	**0.54***	**0.55***	**0.29***	**0.20***	0.12	**0.33***
Eq	-	-	1.00	**0.25***	**0.21***	**0.22***	0.12	0.12	0.11
F	-	-	-	1.00	**0.86***	**0.45***	**0.42***	**0.24***	**0.26***
Bio A	-	-	-	-	1.00	**0.31***	**0.24***	**0.24***	**0.23***
BPA	-	-	-	-	-	1.00	**0.44***	0.01	**0.27***
E1	-	-	-	-	-	-	1.00	**0.14***	**0.28***
E2	-	-	-	-	-	-	-	1.00	-0.07
T	-	-	-	-	-	-	-	-	1.00

*Spearman's correlation significant at 95% confidence interval

Relationship between plasma EDCs and sex hormones in vegetarians is expressed in Table 4.12 and non-vegetarians in Table 4.13. It was found that correlations of the analytes differ among vegetarians and non-vegetarians.

Table 4.12: Correlation of analytes (plasma) in vegetarian (n = 103)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	- 0.06	0.57	0.12	0.23	0.19	0.06
G	0.06	0.51	0.05	0.61	**0.24***	**0.01**
Eq	0.09	0.34	0.14	0.16	0.01	0.96
F	**0.36***	**< 0.001**	0.10	0.31	**0.22***	**0.02**
Bio A	**0.24***	**0.01**	0.13	0.18	0.17	0.09
BPA	**0.38***	**< 0.001**	0.02	0.81	**0.24***	**0.01**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

The highest significant correlation among analytes in vegetarians (Table 4.12) was between BPA and oestrone (r_s = 0.38). BPA was also correlated with testosterone but the association was weaker (r_s = 0.24).

Table 4.13: Correlation of analytes (plasma) in non-vegetarian (n = 122)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	**0.33***	**< 0.001**	**0.36***	**< 0.001**	**0.33***	**< 0.001**
G	**0.26***	**0.003**	**0.21***	**0.02**	**0.36***	**< 0.001**
Eq	0.06	0.52	0.09	0.30	0.16	0.07
F	**0.41***	**< 0.001**	**0.39***	**< 0.001**	**0.26***	**0.003**
Bio A	0.17	0.07	**0.35***	**< 0.001**	**0.31***	**0.001**
BPA	**0.29***	**0.001**	-0.04	0.69	**0.18***	**0.04**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

The most influential isoflavone among vegetarians was formononetin which correlated with oestrone (r_s = 0.36) and with testosterone (r_s = 0.22). Another isoflavone which correlated with oestrone was biochanin A (r_s = 0.24).

Relationship between plasma EDCs and sex hormones in non-vegetarians is expressed in Table 4.13. There were more positive correlations among the non-vegetarians. Daidzein, genistein and formononetin correlated significantly with all three sex hormones (r_s = 0.21 – 0.41). Equol however, did not correlate with any of the sex hormones. Biochanin A correlated significantly with oestradiol and testosterone (r_s = 0.35, r_s = 0.31) while BPA only correlated with oestrone and testosterone but not oestradiol.

Among the equol producers (Table 4.14), the only oestrogenic EDCs that correlated significantly were formononetin and BPA which correlated with oestrone (r_s = 0.29 and r_s = 0.30). Other EDCs did not show any significant correlation with the sex hormones.

Table 4.14: Correlation of analytes (plasma) in equol producers (n = 73)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	0.07	0.55	0.18	0.12	0.09	0.41
G	0.03	0.78	0.03	0.77	0.22	0.06
Eq	0.10	0.42	0.03	0.79	0.08	0.49
F	**0.29***	**0.01**	0.17	0.14	0.18	0.13
Bio A	0.15	0.21	0.22	0.07	0.04	0.73
BPA	**0.30***	**0.01**	-0.03	0.83	0.06	0.62

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

There were more significant correlations observed among the non-equol producers (Table 4.15). All oestrogenic EDCs significantly low correlation with the three sex hormones (r_s = 0.22 – 0.50). With exceptions for genistein and BPA, which only correlated with oestrone and testosterone. The strongest correlation among non-equol producers was for BPA and oestrone (r_s = 0.50).

Table 4.15: Correlation of analytes (plasma) in non-equol producers (n = 152)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	**0.30***	**< 0.001**	**0.22***	**0.005**	**0.38***	**< 0.001**
G	**0.29***	**< 0.001**	0.16	0.054	**0.36***	**< 0.001**
F	**0.48***	**< 0.001**	**0.28***	**< 0.001**	**0.26***	**0.001**
Bio A	**0.27***	**0.001**	**0.24***	**0.002**	**0.31***	**< 0.001**
BPA	**0.50***	**< 0.001**	0.01	0.901	**0.32***	**< 0.001**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In Tables 4.16 – 4.18, significant correlations were demonstrated for each ethnic group. Among the Chinese subjects (Table 4.16), there are little or low correlations between daidzein, genistein, equol and biochanin A. Moderate correlations are observed

between formononetin and oestrone (r_s = 0.63), as well as BPA and oestrone (r_s = 0.54). Of the oestrogenic EDCs in Chinese subjects, only daidzein and genistein had significant low correlation with testosterone.

Table 4.16: Correlation of analytes (plasma) in Chinese subjects (n = 63)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	**0.36***	**0.004**	**0.31***	**0.013**	**0.40***	**0.001**
G	**0.31***	**0.012**	0.22	0.088	**0.41***	**0.001**
Eq	**0.25***	**0.052**	**0.31***	**0.012**	0.07	0.584
F	**0.63***	**< 0.001**	0.19	0.135	0.12	0.340
Bio A	**0.37***	**0.003**	0.16	0.210	0.25	0.052
BPA	**0.54***	**< 0.001**	0.14	0.286	0.06	0.638

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In Table 4.17, Indian subjects showed significant correlations between formononetin, biochanin A and BPA in addition to daidzein and genistein to the sex hormones (r_s = 0.27 – 0.39). Unlike equol concentrations in Chinese subjects, equol levels in Indian subjects did not show any correlation to the sex hormones. Biochanin A is the only isoflavone that correlated with all three sex hormones in Indian subjects.

Table 4.17: Correlation of analytes (plasma) in Indian subjects (n = 132)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	**0.20***	**0.019**	0.13	0.126	**0.27***	**0.002**
G	**0.20***	**0.019**	0.06	0.509	**0.39***	**< 0.001**
Eq	0.08	0.356	-0.01	0.983	0.15	0.088
F	**0.36***	**< 0.001**	0.16	0.070	**0.34***	**< 0.001**
Bio A	**0.28***	**0.001**	**0.19***	**0.026**	**0.28***	**0.001**
BPA	**0.39***	**< 0.001**	-0.04	0.621	**0.38***	**< 0.001**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In other ethnic groups (Table 4.18), formononetin and biochanin A showed significantly low correlation with oestradiol.

Table 4.18: Correlation of analytes (plasma) in other ethnic group (n = 30)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	-0.26	0.163	0.19	0.324	0.28	0.128
G	-0.06	0.744	0.35	0.060	0.03	0.887
Eq	-0.11	0.577	0.16	0.406	0.22	0.238
F	-0.14	0.468	**0.41***	**0.023**	0.16	0.401
Bio A	**-0.44***	**0.014**	**0.37***	**0.042**	-0.01	0.974
BPA	0.17	0.357	-0.21	0.264	**0.45***	**0.013**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Surprisingly, biochanin A showed a significant inverse correlation with oestrone. BPA significantly correlated with testosterone ($r_s = 0.45$).

A difference in correlation results were also observed between groups who had more or less number of isoflavone types. In subjects with two and less than 2 types of isoflavones in their plasma (Table 4.19), correlation was significant between genistein and testosterone ($r_s = 0.23$); between equol and testosterone ($r_s = 0.26$). Formononetin correlated only with oestrone. BPA has significantly moderate correlation with oestrone ($r_s = 0.60$) and low correlation with testosterone ($r_s = 0.28$).

Table 4.19: Correlation of analytes (plasma) in subjects with ≤ 2 types of isoflavones (n = 85)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	-0.18	0.090	0.08	0.462	0.21	0.052
G	-0.09	0.386	0.14	0.195	**0.23***	**0.035**
Eq	0.20	0.061	0.17	0.114	**0.26***	**0.017**
F	**0.25***	**0.022**	-0.03	0.785	0.19	0.086
Bio A	-0.08	0.442	-0.05	0.632	0.06	0.573
BPA	**0.60***	**< 0.001**	0.12	0.278	**0.28***	**0.009**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In subjects with more than 2 isoflavone types (Table 4.20), there were correlations between daidzein, genistein and testosterone (r_s = 0.24, r_s = 0.31). There were significant correlations between formononetin and all three sex hormones (r_s = 0.22, r_s = 0.20, r_s = 0.22). Biochanin A showed significant low correlations with oestradiol and testosterone while BPA correlated with oestrone and testosterone.

Table 4.20: Correlation of analytes (plasma) in subjects with > 2 types of isoflavones (n = 140)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	0.11	0.194	0.13	0.131	**0.24***	**0.004**
G	0.04	0.664	-0.03	0.682	**0.31***	**< 0.001**
Eq	-0.06	0.481	0.01	0.862	-0.01	0.868
F	**0.22***	**0.011**	**0.20***	**0.018**	**0.22***	**0.007**
Bio A	0.01	0.878	**0.22***	**0.008**	**0.19***	**0.027**
BPA	**0.30***	**< 0.001**	-0.08	0.329	**0.20***	**0.017**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Among the vegetarians, there were 43 equol producers and 60 who were non-equol producers. Correlation among the equol producer vegetarians only showed significant correlation between formononetin and oestrone (Table 4.21).

Table 4.21: Correlation of analytes (plasma) in equol producer vegetarians (n = 43)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	-0.14	0.355	0.12	0.424	0.09	0.567
G	0.07	0.631	0.06	0.707	0.21	0.176
Eq	0.05	0.767	0.04	0.803	0.06	0.684
F	**0.33***	**0.029**	0.11	0.467	0.18	0.234
Bio A	0.20	0.190	0.17	0.263	-0.04	0.799
BPA	0.24	0.120	0.01	0.929	0.07	0.647

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Among non-equol producer vegetarians however, low correlation was observed between daidzein, genistein and testosterone (Table 4.22). Equol showed a significant inverse correlation with testosterone (r_s = -0.28). Formononetin significantly correlated with oestrone and testosterone (r_s = 0.38 and r_s = 0.25). BPA also showed low correlation with oestrone and testosterone r_s = 0.46 and r_s = 0.31).

Table 4.22: Correlation of analytes (plasma) in non-equol producer vegetarians (n = 60)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	0.01	0.936	0.14	0.293	**0.25***	**0.049**
G	0.05	0.698	0.07	0.605	**0.28***	**0.029**
Eq	-0.17	0.189	-0.06	0.639	**-0.28***	**0.028**
F	**0.38***	**0.003**	0.05	0.675	**0.25***	**0.049**
Bio A	**0.25***	**0.049**	0.08	0.519	**0.27***	**0.036**
BPA	**0.46***	**< 0.001**	-0.05	0.705	**0.31***	**0.016**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Among Chinese vegetarians (Table 4.23), only genistein correlated significantly with testosterone (r_s= 0.38). BPA showed low correlation with oestrone (r_s= 0.47). No other analytes showed significant correlation.

Table 4.23: Correlation of analytes (plasma) in Chinese vegetarians (n = 30)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	0.07	0.722	0.23	0.214	0.18	0.323
G	0.29	0.115	0.17	0.363	**0.38***	**0.034**
Eq	0.04	0.817	0.34	0.065	0.09	0.640
F	0.33	0.076	0.06	0.763	-0.22	0.252
Bio A	0.18	0.332	0.11	0.577	-0.24	0.194
BPA	**0.47***	**0.009**	0.14	0.462	0.14	0.463

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Among Indian vegetarians (Table 4.24), all oestrogenic EDCs except equol correlated significantly with testosterone (r_s= 0.26 – 0.41). Formononetin was also correlated with oestrone (r_s= 0.38). Biochanin A also had low correlation with oestrone (r_s= 0.26). BPA also showed low correlation with oestrone (r_s= 0.29).

Table 4.24: Correlation of analytes (plasma) in Indian vegetarians (n = 63)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	-0.07	0.608	0.07	0.567	**0.26***	**0.038**
G	-0.02	0.889	-0.01	0.921	**0.26***	**0.036**
Eq	0.06	0.625	-0.12	0.333	0.01	0.924
F	**0.38***	**0.002**	0.11	0.397	**0.41***	**0.001**
Bio A	**0.26***	**0.039**	0.18	0.154	**0.34***	**0.007**
BPA	**0.29***	**0.023**	-0.04	0.739	**0.27***	**0.030**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Among coffee drinker vegetarians (Table 4.25), all oestrogenic EDCs except equol correlated significantly with testosterone (r_s= 0.24 – 0.38). Formononetin was also correlated with oestrone (r_s= 0.33). Biochanin A also had low correlation with oestrone (r_s= 0.25). BPA also showed low correlation with oestrone (r_s= 0.39).

Table 4.25: Correlation of analytes (plasma) in coffee drinker vegetarians (n = 79)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	-0.05	0.637	0.14	0.233	**0.24***	**0.030**
G	0.10	0.358	0.02	0.830	**0.27***	**0.015**
Eq	0.01	0.984	0.11	0.350	-0.04	0.721
F	**0.33***	**0.003**	0.09	0.448	**0.30***	**0.006**
Bio A	**0.25***	**0.027**	0.12	0.289	**0.24***	**0.032**
BPA	**0.39***	**< 0.001**	-0.03	0.791	**0.38***	**< 0.001**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Correlation was further explored among non-vegetarian group. Table 4.26 showed no significant correlations of any analytes in equol producers who are not vegetarians.

Table 4.26: Correlation of analytes (plasma) in equol producers non-vegetarians (n = 30)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	0.10	0.595	0.27	0.149	0.22	0.242
G	0.02	0.918	0.03	0.882	0.27	0.153
Eq	0.04	0.840	-0.14	0.470	0.22	0.236
F	0.11	0.578	0.24	0.196	0.26	0.164
Bio A	-0.03	0.863	0.31	0.099	0.19	0.321
BPA	-0.05	0.783	-0.29	0.123	-0.08	0.670

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In non-equol producers non-vegetarians (Table 4.27), all oestrogenic EDCs showed positive correlation with testosterone (r_s = 0.26 – 0.39) except equol. Equol was inversely correlated with testosterone (r_s = -0.13). All of the natural EDCs correlated positively with oestradiol (r_s = 0.30 – 0.46). Except for equol, all oestrogenic EDCs were positively correlated with oestrone (r_s = 0.22 – 0.51).

Table 4.27: Correlation of analytes (plasma) in non-equol producers non-vegetarians (n = 92)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	**0.40***	**< 0.001**	**0.40***	**< 0.001**	**0.39***	**< 0.001**
G	**0.35***	**0.001**	**0.30***	**0.004**	**0.35***	**0.001**
Eq	0.08	0.459	**0.34***	**0.001**	**-0.13***	**0.224**
F	**0.51***	**< 0.001**	**0.46***	**< 0.001**	**0.26***	**0.012**
Bio A	**0.22***	**0.038**	**0.38***	**< 0.001**	**0.36***	**< 0.001**
BPA	**0.42***	**< 0.001**	0.06	0.543	**0.26***	**0.012**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In Chinese non-vegetarians (Table 4.28), daidzein had significantly high correlation with testosterone (r_s = 0.73). Biochanin A was moderately correlated with testosterone (r_s = 0.67) while genistein and formononetin had low correlation with testosterone (r_s = 0.43, r_s = 0.41). Genistein, formononetin and biochanin A had low correlation with oestrone (r_s = 0.39 – 0.53) and no analyte correlated with oestradiol.

Table 4.28: Correlation of analytes (plasma) in Chinese non-vegetarians (n = 33)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	0.30	0.090	0.28	0.110	**0.73***	**< 0.001**
G	**0.39***	**0.026**	0.28	0.114	**0.43***	**0.012**
Eq	0.18	0.308	-0.11	0.555	0.03	0.878
F	**0.53***	**0.001**	0.25	0.165	**0.41***	**0.016**
Bio A	**0.41***	**0.016**	0.24	0.165	**0.67***	**< 0.001**
BPA	0.30	0.087	-0.13	0.473	-0.06	0.734

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In Indian non-vegetarians (Table 4.29), genistein, equol and BPA had low correlation with testosterone (r_s = 0.30 – 0.38). BPA was also correlated with oestrone (r_s = 0.32).

Table 4.29: Correlation of analytes (plasma) in Indian non-vegetarians (n = 69)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	0.16	0.188	**0.26***	0.031	0.09	0.465
G	0.22	0.073	0.16	0.178	**0.38***	**0.001**
Eq	0.11	0.347	0.13	0.271	**0.30***	**0.014**
F	0.10	0.426	0.20	0.066	0.17	0.170
Bio A	0.13	0.284	0.23	0.059	0.16	0.177
BPA	**0.32***	**0.007**	-0.12	0.330	**0.38***	**0.001**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In coffee drinker non-vegetarians (Table 4.30), all isoflavone except for equol were significantly correlated with oestrone, oestradiol and testosterone (r_s = 0.24 – 0.50). BPA was correlated only to oestrone (r_s = 0.34).

Table 4.30: Correlation of analytes (plasma) in coffee drinker non-vegetarians (n = 89)

Variable	E1		E2		T	
	r_s	*p*	r_s	*p*	r_s	*p*
D	**0.42***	**< 0.001**	**0.36***	**< 0.001**	**0.36***	**< 0.001**
G	**0.33***	**0.001**	**0.25***	**0.019**	**0.43***	**< 0.001**
Eq	0.01	0.953	0.12	0.244	0.15	0.169
F	**0.50***	**< 0.001**	**0.43***	**< 0.001**	**0.27***	**0.011**
Bio A	**0.24***	**0.023**	**0.36***	**0.001**	**0.31***	**0.003**
BPA	**0.34***	**0.001**	0.04	0.713	0.20	0.055

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In summary, correlation results suggest that oestrogenic EDCs tend to affect levels of sex hormones in various groups. A summary of significant correlations is presented in Table 4.31. All significant correlations were consistently positive except for 3 pairs. The first negative correlation was between biochanin A and oestrone in ethnic groups other than Chinese and Indian. The second negative correlation was between equol and testosterone in non-equol producer among vegetarians. The third negative correlation was again between equol and testosterone in non-equol producer among non-vegetarians. The negative correlation results suggest that in non-equol producers, with higher equol levels tend to lower testosterone levels. Results also suggest equol producers have fewer significant correlations to the sex hormones than non-equol producers or other groups. Result also indicates that Chinese non-vegetarian tend to get higher testosterone level with consumption of daidzein and biochanin A compared to other groups.

Table 4.31: Summary of significant correlations

		D	G	Eq	F	BioA	BPA
Vegetarian	E1				0.36	0.24	0.38
	E2						
(n = 103)	T		0.24		0.22		0.24
Non Vegetarian (n = 122)	E1	0.33	0.26		0.41		0.29
	E2	0.36	0.21		0.39	0.35	
	T	0.33	0.36		0.26	0.31	0.18
Equol producer (n = 73)	E1				0.29		0.30
	E2						
	T						
Non Equol producer	E1	0.30	0.29		0.48	0.27	0.50
	E2	0.22			0.28	0.24	
(n = 152)	T	0.38	0.36		0.26	0.31	0.32
Chinese	E1	0.36	0.31	0.25	0.63	0.37	0.54
	E2	0.31		0.31			
(n = 63)	T	0.40	0.41				
Indian	E1	0.20	0.20		0.36	0.28	0.39
	E2					0.19	
(n = 132)	T	0.27	0.39		0.34	0.28	0.38
Others	E1					-0.44	
	E2				0.41	0.37	
(n = 30)	T						0.45
≤ 2 isoflavone	E1				0.25		0.60
(n = 85)	E2						
	T		0.23	0.26			0.28
> 2 isoflavone	E1				0.22		0.30
(n = 140)	E2				0.20	0.22	
	T	0.24	0.31		0.22	0.19	0.20
Equol producer vegetarians	E1				0.33		
(n = 43)	E2						
	T						
Non-equol producer vege-	E1				0.38	0.25	0.46
tarians	E2						
(n = 60)	T	0.25	0.28	-0.28	0.25	0.27	0.31
	E2						
	T	0.73	0.43		0.41	0.67	
Chinese Vegetarian	E1						
(n = 30)	E2						
	T		0.38				0.47
Indian vegetarian	E1				0.38	0.26	
(n = 63)	E2						
	T	0.26	0.26		0.41	0.34	0.27
Coffee-drinker	E1				0.33	0.25	0.39
Vegetarian	E2						
(n = 79)	T	0.24	0.27		0.30	0.24	0.38
Equol producer non-	E1						
vegetarian	E2						
(n = 30)	T						
Non-equol producer non-	E1	0.40	0.35		0.51	0.22	0.42
vegetarian	E2	0.40	0.30	0.34	0.46	0.38	
(n = 92)	T	0.39	0.35	-0.13	0.26	0.36	0.26
Chinese non-vegetarian	E1		0.39		0.53	0.41	
(n = 33)	E2						
	T	0.73	0.43		0.41	0.67	
Indian non-vegetarian	E1						0.32
(n = 69)	E2						
	T		0.38	0.30			0.38
Coffee drinker non vegetarian	E1	0.42	0.33		0.50	0.24	0.34
(n = 89)	E2	0.36	0.25		0.43	0.36	
	T	0.36	0.43		0.27	0.31	

4.3.5 Comparison between subject groups (plasma)

Table 4.32 shows the level of plasma analytes among non-vegetarians and sub-classes of vegetarian (lacto-ovovegetarian, lactovegetarian, vegan and ovovegetarian) Vegans gaves the highest mean level for daidzein, genistein, equol, formononetin, biochanin A and total isoflavone. Ovovegetarians displayed only a slight increase than non-vegetarians for equol and total isoflavone levels. Except for daidzein, ovovegetarians showed lower levels than non-vegetarians for all isoflavones. Mean level for BPA among ovovegetarians was 2-4 times higher than in other groups.

Table 4.32: Comparison of analyte concentration in plasma of non-vegetarians and sub-classes of vegetarian

Analyte	Plasma concentration (ng/ml) Mean (SD)				
	Non-vegetarian (n = 122)	Lacto-ovovegetarian (n = 40)	Lactovegetarian (n = 36)	Vegan (n = 24)	Ovovegetarian (n = 3)
D	9.8 (33.7)	35.4 (66.7)	33.7 (63.6)	42.5 (49.5)	31.5 (51.7)
G	32.6 (88.4)	72.6 (143.6)	71.5 (125.3)	120.8 (155.2)	29.8 (49.6)
Eq	1.6 (6.9)	8.8 (37.9)	4.2 (14.6)	10.7 (22.3)	2.6 (4.5)
F	7.2 (17.9)	7.9 (11.8)	12.9 (17.5)	14.4 (17.3)	4.8 (8.3)
Bio A	4.9 (7.1)	5.5 (8.9)	8.9 (11.5)	10.8 (12.8)	1.3 (2.2)
BPA	0.7 (2.3)	1.7 (3.5)	1.1 (2.1)	1.4 (2.3)	3.1 (5.4)
E1	0.2 (0.5)	0.4 (0.6)	0.7 (0.8)	0.6 (0.6)	ND
E2	0.1 (0.4)	0.6 (2.4)	1.9 (10.5)	0.3 (0.9)	ND
T	1.8 (1.4)	2.6 (2.3)	3.6 (4.6)	2.6 (2.7)	3.1 (2.4)
Total isoflavone	56.2 (134.3)	130.2 (238.2)	131.4 (184.9)	199.4 (220.1)	69.6 (94.6)

SD = standard deviation ND = not detected

Table 4.33 shows the level of plasma analytes among non-vegetarians in comparison with vegetarians. Significant differences were observed for all analytes except for formononetin, BPA and oestradiol. Vegetarians had significantly higher daidzein, genistein, equol, biochanin A, BPA, oestrone and testosterone levels than non-vegetarians.

Table 4.33: Comparison of plasma analyte concentration in vegetarians and non-vegetarians

Analyte	Plasma concentration in ng/ml		Mean difference (95% CI)	*t* stats (df[a])	*P* value[a]
	Vegetarian (n = 103) mean (SD)	Non-vegetarian (n = 122) mean (SD)			
D	**36.3 (60.9)**	**9.4 (34.1)**	26.9 (13.57, 40.23)	3.99 (155)	< **0.001**
G	**82.2 (138.8)**	**32.7 (89.3)**	49.5 (18.15, 80.87)	3.12 (168)	**0.002**
Eq	**7.4 (27.3)**	**1.6 (7.0)**	5.8 (0.38, 11.32)	2.12 (113)	**0.036**
F	11.0 (15.4)	7.2 (17.9)	3.8 (-0.65, 8.24)	1.68 (223)	0.094
Bio A	**7.2 (10.4)**	**4.0 (6.3)**	3.2 (0.87, 5.50)	2.72 (161)	**0.007**
BPA	2.2 (4.4)	1.5 (8.4)	0.71 (-1.10, 2.53)	0.78 (223)	0.438
E1	**0.6 (0.7)**	**0.2 (0.5)**	0.38 (0.22, 0.56)	4.49 (184)	< **0.001**
E2	1.0 (6.4)	0.1 (0.4)	0.88 (-0.37, 2.13)	1.39 (103)	0.167
T	**3.0 (3.3)**	**1.6 (1.5)**	1.34 (0.64, 2.04)	3.79 (135)	< **0.001**

[a] independent t-test. SD = standard deviation

Level of oestrone, oestradiol and testosterone was 3, 10 and 1.8 times higher in vegetarians than non-vegetarians. However, this finding was significant only for oestrone and testosterone. Analyte with the highest mean concentration detected was genistein for both vegetarians and non-vegetarians.

Disregarding the vegetarian status, comparison between analyte concentrations were made between different ethnic groups (Table 4.34). Significant differences between the 3 ethnic groups were evident for BPA and oestrone. BPA was significantly higher among the Chinese subjects in this study and Indians were with the lowest mean plasma BPA concentration. Though highest mean of daidzein and genistein concentration was among the Chinese, this difference was not significant. There was also no significant difference in testosterone levels among the ethnic groups.

Table 4.34: Comparison of plasma analyte concentration in different ethnic groups

Analytes	Plasma concentration in ng/ml			*F* stats[a] (df)	*P* value[a]
	Chinese (n = 63)	Indian (n = 132)	Others (n = 30)		
	mean (SD)	mean (SD)	mean (SD)		
D	29.5 (57.5)	20.9 (50.8)	10.8 (12.3)	1.50 (222)	0.224
G	77.2 (144.6)	53.1 (112.7)	19.4 (25.1)	2.58 (222)	0.078
Eq	4.1 (13.9)	5.2 (23.3)	0.8 (1.9)	0.63 (222)	0.533
F	7.5 (9.8)	9.5 (20.5)	9.9 (9.4)	0.342 (222)	0.711
Bio A	6.4 (8.6)	5.9 (9.5)	7.5 (8.9)	0.363 (222)	0.696
BPA	**1.7 (3.4)**	**0.6 (1.9)**	**1.4 (3.0)**	4.03 (222)	**0.019**
E1	**0.5 (0.8)**	**0.2 (0.6)**	**0.5 (0.6)**	3.55 (222)	**0.030**
E2	0.5 (2.0)	0.6 (5.5)	0.4 (0.7)	0.02 (222)	0.982
T	1.9 (1.7)	2.5 (2.9)	2.3 (2.1)	1.15 (222)	0.318

[a] One-way ANOVA test SD = standard deviation

Comparison was also made between non-vegetarian and vegetarian within the same ethnic groups in Table 4.35 and Table 4.36 for Chinese and Indian subjects respectively. The Chinese vegetarian were found to have higher concentrations of isoflavone and BPA, however the difference was only significant for formononetin. Plasma concentration of formononetin among Chinese non-vegetarian was twice as high in vegetarian Chinese (Table 4.35).

Table 4.35: Comparison of plasma analyte concentration between vegetarians and non-vegetarians among Chinese subjects

Analyte	Plasma concentration in ng/ml		Mean difference (95% CI)	*t* stats (df[a])	*P* value[a]
	Vegetarian (n = 30) mean (SD)	Non-vegetarian (n = 33) mean (SD)			
D	36.8 (52.5)	22.8 (61.7)	13.9 (-15.00 , 42.98)	0.96 (61)	0.338
G	101.3 (173.0)	55.2 (111.1)	46.1 (-26.49, 118.72)	1.27 (61)	0.209
Eq	7.6 (19.7)	0.9 (1.9)	6.7 (-0.13, 13.64)	1.87 (29)	0.071
F	**10.2 (9.3)**	**5.1 (9.8)**	5.1 (0.29, 9.94)	2.12 (61)	**0.038**
Bio A	7.6 (8.9)	5.2 (8.3)	2.4 (-1.98, 6.69)	1.08 (61)	0.281
BPA	2.1 (3.5)	1.4 (3.3)	0.7 (-0.95, 2.49)	0.89 (61)	0.374
E1	**0.8 (0.8)**	**0.2 (0.7)**	0.6 (0.24, 0.99)	3.30 (59)	**0.002**
E2	1.0 (2.8)	0.1 (0.3)	0.9 (-0.13, 2.01)	1.80 (29)	0.081
T	1.9 (1.8)	2.0 (1.6)	-0.08 (-0.95, 0.78)	-0.19 (61)	0.853

[a] independent t-test. SD = standard deviation

It was found among the Indian subjects (Table 4.36), comparison of mean between the non-vegetarian and vegetarian using independent t-test yields significant differences between the two groups. Daidzein, genistein, biochanin A and BPA were significantly higher in vegetarian by 13, 3.5, 2.6, 3.7 magnitude respectively. Though, equol was found to be higher in Indian vegetarians, this difference was not significant. Oestrone and testosterone were also significantly higher in vegetarians by 4 and 2 magnitudes.

Table 4.36: Comparisons of plasma analyte concentration between vegetarians and non-vegetarians among Indian subjects

Analyte	Plasma concentration in ng/ml		Mean difference (95% CI)	*t* stats (df[a])	*P* value[a]
	Vegetarian (n = 63) mean (SD)	Non-vegetarian (n = 69) mean (SD)			
D	**40.4 (68.1)**	**3.1 (8.0)**	37.3 (20.98, 53.66)	4.32 (64)	**< 0.001**
G	**84.7 (128.8)**	**24.2 (86.9)**	60.4 (22.18, 98.74)	3.13(107)	**0.002**
Eq	8.3 (32.2)	2.3 (9.1)	5.9 (-2.40, 14.34)	1.42 (71)	0.159
F	12.6 (18.2)	6.6 (22.1)	6.1 (-0.95, 13.08)	1.71 (130)	0.090
Bio A	**8.7 (12.2)**	**3.4 (5.0)**	5.3 (2.01, 8.57)	3.21 (81)	**0.002**
BPA	**1.1 (2.4)**	**0.3 (1.1)**	0.8 (0.15, 1.47)	2.44 (86)	**0.017**
E1	**0.4 (0.7)**	**0.1 (0.2)**	0.4 (0.17, 0.56)	3.84 (73)	**< 0.001**
E2	1.1 (7.9)	0.1 (0.2)	1.1 (-0.89, 3.11)	1.10 (62)	0.272
T	**3.4 (3.9)**	**1.7 (1.2)**	1.7 (0.72, 2.73)	3.42 (72)	**0.001**

[a] independent t-test. SD = standard deviation

4.3.6 Comparison between equol producer status (plasma)

All 225 subjects were divided into two groups, based on their ability to produce equol. Independent t-test comparing the means between the groups as shown in Table 4.37 found that there is significant difference for genistein, equol, BPA. Equol producers have higher levels for genistein, equol and BPA than non-equol producers by 1.8, 132 and 2.7 magnitude. No significant difference was found between the groups for any of the sex hormones.

Table 4.37: Comparison of plasma analyte concentration in equol producers and non-equol producers

Analyte	Plasma concentration in ng/ml		Mean difference (95% CI)	*t* stats (df[a])	*P* value[a]
	Equol producer (n = 73) mean (SD)	Non-Equol producer (n = 152) mean (SD)			
D	26.6 (52.8)	19.8 (48.2)	6.8 (-7.14, 20.78)	0.96 (223)	0.337
G	**79.0 (129.6)**	**43.9 (108.5)**	35.1 (0.36, 69.78)	2.00 (122)	**0.048**
Eq	**13.2 (32.3)**	**0.1 (0.1)**	13.2 (5.63, 20.70)	3.48 (72)	**0.001**
F	11.8 (23.1)	7.6 (12.7)	4.1 (-0.56, 8.87)	1.74 (223)	0.084
Bio A	6.5 (8.5)	6.1 (9.5)	0.4 (-2.20, 2.94)	0.28 (223)	0.777
BPA	**1.9 (3.3)**	**0.7 (2.1)**	1.23 (0.40, 2.06)	2.94 (101)	**0.004**
E1	0.4 (0.6)	0.3 (0.7)	0.1 (-0.08, 0.28)	1.01 (223)	0.310
E2	0.5 (1.9)	0.6 (5.1)	-0.1 (-1.33, 1.11)	-0.18 (223)	0.859
T	2.5 (2.3)	2.3 (2.6)	0.3 (-0.43, 0.99)	0.79 (223)	0.429

[a] independent t-test. SD = standard deviation

4.4 Study population of female patients with and without breast disease (plasma)

4.4.1 Descriptive statistics

The characteristics of the study population are described in Table 4.38. There were 109 patients with breast disease while another 143 subjects were those without breast disease. Both groups were homogenous in terms of age, menarche and menopause age. There were slightly more nulliparous subjects in patients with breast disease (32.3%) compared with those without breast disease (22.5%). Higher percentage of patients without breast disease (36.4%) were found to have more than 2 types of isoflavone detected in their blood compared to those with breast disease (17.4%).

Table 4.38: Characteristics of female patients with and without breast disease (n = 252)

Variable	Female patients with breast disease (n = 109)		Female patients without breast disease (n = 143)	
	Mean (S.D.)	Frequency (%)	Mean (S.D.)	Frequency (%)
Ethnic groups				
Malay		28 (25.7)		36 (25.2)
Chinese		56 (51.4)		84 (58.7)
Indian & others		25 (22.9)		23 (16.1)
Age	52.0 (12.9)		52.3 (10.9)	
Parity		n = 96		n = 142
Nulliparous		31 (32.3)		32 (22.5)
1 - 3		41 (42.7)		72 (50.7)
≥ 4		24 (25.0)		38 (26.8)
Lactation		n = 94		n = 142
Yes		38 (40.4)		73 (51.4)
No		56 (59.6)		69 (48.6)
Previous history of breast disease		n = 95		n = 142
Yes		10 (10.5)		6 (4.2)
No		85 (89.5)		136 (95.8)
Family history of breast disease		n = 95		n = 142
Yes		12 (12.6)		11 (7.7)
No		83 (87.4)		131 (92.3)
Menarche	n = 93 12.9 (1.4)		n = 140 12.9 (1.5)	
Age at 1st Birth	n=59 26.5 (5.8)		n = 108 26.1 (5.2)	
Menopause		n = 102		n = 143
Yes		60 (58.8)		90 (62.9)
No		42 (41.2)		53 (37.1)
Menopause age	n = 49 49.7 (4.5)		n = 86 49.7 (4.6)	
Smoking		n = 96		n = 14
Yes		3 (3.1)		0 (0)
No		93 (96.9)		142 (100)
Alcohol Intake		n = 96		n = 142
Yes		2 (2.1)		4 (2.8)
No		94 (97.9)		138 (97.2)
HRT		n = 96		n = 142
Yes		2 (2.1)		11 (7.7)
No		94 (97.9)		131 (92.3)
Antibiotics last 3 months		n = 96		n = 142
Yes		14 (14.6)		33 (23.2)
No		82 (85.4)		109 (76.8)
Soy intake last 24 hours		n = 96		n = 142
Yes		19 (19.8)		28 (19.7)
No		77 (80.2)		114 (80.3)
Equol Producer		27 (24.8)		22 (15.4)
Types of isoflavones detected in plasma				
None		39 (35.8)		11 (7.7)
1		41 (37.6)		14 (9.8)
2		10 (9.2)		66 (46.2)
3		11 (10.1)		16 (11.2)
4		7 (6.4)		21 (14.7)
5		1 (0.9)		15 (10.5)

Equol producers were categorized as those with ≥ 0.5 ng/ml of plasma equol concentration. In this population of female patients, 19.4% of 252 subjects were equol producers.

Among the different ethnic groups, percentages of equol producers were 9.4%, 27.9% and 8.3% for Malay, Chinese and Indian patients. It was found that 26.6% of patients with breast cancer were equol producers as opposed to 15.4% in those without breast cancer. Among the premenopausal subjects, only 13.2% were equol producers and a slightly higher percentage of 23.3% among the menopause females. Majority of the breast tumours were of ductal (66%, n = 72) origin while the remaining were of lobular histology, unspecified carcinoma and other benign diagnoses (Table 4.39).

4.4.2 Percentage of analyte detection (plasma)

Figure 4.26 compares percentage of analyte detection in plasma samples of women with breast disease (n = 109) and those without breast disease (n = 143). All analytes were detected in plasma of women with breast disease with detection ranging from 11 – 94.5%.

Table 4.39: HPE diagnosis of breast tissue samples (n = 109)

Diagnosis	Frequency	Percent (%)
Intraductal carcinoma (IDC)	59	54.1
Ductal Carcinoma in situ (DCIS)	13	11.9
Intralobular carcinoma (ILC)	3	2.8
Mixture of IDC & DCIS	2	1.8
Carcinoma, metaplastic cancer, metastatic breast cancer, mixed carcinoma, neuroendocrine carcinoma	5	4.6
Fibroadenoma	10	9.2
Fibrocystic changes	6	5.5
Other diagnosis : accessory breast, chronic inflammation, intraductal papilloma, no malignancy, no residual tumour	11	10.1

The most commonly detected analyte was oestrone. Isoflavone and BPA detection percentage among women with breast disease was below 35%. This was in contrast with the other group. Women without breast disease had daidzein, genistein, formononetin and biochanin A more frequently detected in their plasma ranging from 21.1 to 84.6% of detection. There was higher percentage of detection for oestrogens in women with breast disease compared to those without.

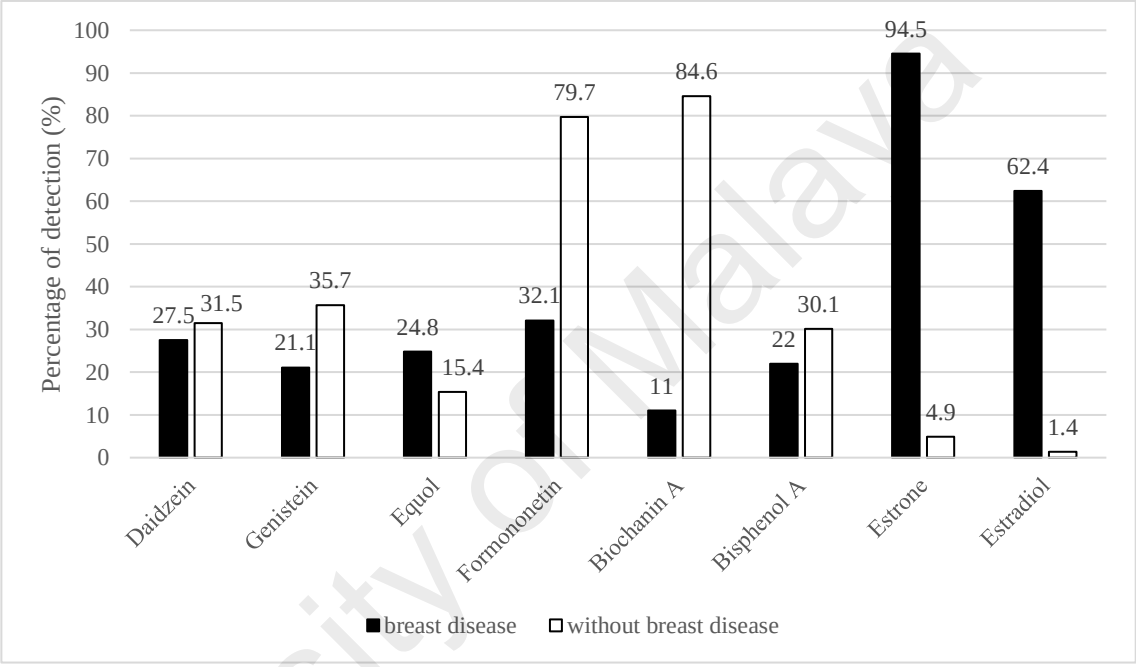

Figure 4.26: Percentage of analyte detection (plasma)

4.4.3 Statistical analyses

Statistical analysis was carried out using SPSS software version 15.0. Parametric tests for comparing means between groups (independent t-test, ANOVA and paired t-test) and Spearman's correlations were used in this analysis. Table 4.10 is used as a reference in all correlation analysis in this study when interpreting the strength of the correlation.

4.4.4 Correlation of analytes (plasma)

Spearman's correlation was run to assess the relationship between oestrogenic EDCs and sex hormones in the plasma using a sample of female patients with and without breast disease. There exists a wide range of correlation among the analytes. All significant relationships were positively correlated (Table 4.40) except for BPA which was inversely correlated with biochanin A (r_s = -0.17). BPA however, correlated positively with formononetin. The highest significant correlation obtained was between daidzein and genistein (r_s = 0.87). Other than genistein, daidzein had significant correlations with equol, formononetin and biochanin A.

Table 4.40: Correlation coefficients between plasma analytes (n = 252)

Variable	D	G	Eq	F	Bio A	BPA	E1	E2
D	1.00	**0.87***	**0.25***	**0.38***	**0.19***	-0.08	0.01	0.04
G	-	1.00	**0.26***	**0.40***	**0.27***	-0.11	0.06	0.11
Eq	-	-	1.00	-0.08	-0.01	-0.05	-0.07	-0.12
F	-	-	-	1.00	**0.46***	**0.14***	**0.19***	**0.31***
Bio A	-	-	-	-	1.00	**-0.17***	**0.27***	**0.45***
BPA	-	-	-	-	-	1.00	**0.21***	**0.14***
E1	-	-	-	-	-	-	1.00	**0.54***
E2	-	-	-	-	-	-	-	1.00

r_s = Spearman's rho p = p value
* Spearman's correlation significant at 95% confidence interval

In Table 4.41 – 4.55, correlation was conducted to assess the relationship between exogenous oestrogenic EDCs and endogenous oestrogens. Table 4.41 showed that plasma concentrations of daidzein, formononetin and biochanin A correlated positively with oestrone among women with breast cancer. Equol showed a negative correlation with oestradiol (r_s = -0.34). Biochanin A and BPA had low correlation with oestradiol.

Table 4.41: Correlation of analytes (plasma) among women with breast cancer (n = 79)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	**0.25***	**0.025**	-0.06	0.582
G	0.16	0.16	-0.12	0.308
Eq	0.08	0.475	**-0.34***	**0.002**
F	**0.30***	**0.008**	-0.13	0.253
Bio A	**0.23***	**0.039**	**0.27***	**0.017**
BPA	-0.05	0.672	**0.44***	**< 0.001**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In Table 4.42, no significant correlation was observed among women without breast disease.

Table 4.42: Correlation of analytes (plasma) among women without breast cancer (n = 143)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	0.15	0.081	0.06	0.481
G	0.11	0.187	0.03	0.749
Eq	0.01	0.926	-0.05	0.549
F	0.09	0.280	0.01	0.889
Bio A	-0.09	0.293	-0.13	0.112
BPA	-0.15	0.083	-0.08	0.364

r_s = Spearman's rho *p* = p value
*Spearman's correlation significant at 95% confidence interval

Table 4.43 shows, with the exception of correlation between BPA and oestradiol, women who were equol producers, had significant negative correlations (r_s = -0.69 to -0.35) between exogenous and endogenous analytes.

Table 4.43: Correlation of analytes (plasma) in equol producers (n = 43)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	**-0.39***	**0.009**	**-0.39***	**0.009**
G	**-0.56***	**<0.001**	**-0.41***	**0.007**
Eq	**-0.50***	**0.001**	**-0.36***	**0.019**
F	**-0.58***	**< 0.001**	**-0.38***	**0.012**
Bio A	**-0.69***	**<0.001**	**-0.35***	**0.021**
BPA	**-0.58***	**<0.001**	-0.24	0.113

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Among women who were not equol producers, there were both positive and negative correlations (Table 4.44). There was positive correlation between daidzein and equol (r_s = 0.16) as well as between BPA and oestradiol (r_s = 0.21). Formononetin correlated negatively with oestrone and oestradiol (r_s = -0.16, r_s = 0.37). Biochanin A was also inversely correlated with oestrone and oestradiol (r_s = -0.52, r_s = -0.44).

Table 4.44: Correlation of analytes (plasma) in non-equol producers (n = 179)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	**0.16***	**0.032**	0.03	0.720
G	0.06	0.430	-0.05	0.505
Eq	**-0.16***	**0.039**	**-0.37***	**< 0.001**
F	**-0.52***	**< 0.001**	**-0.44***	**< 0.001**
Bio A	0.04	0.591	**0.21***	**0.006**

r_s = Spearman's rho *p* = p value
*Spearman's correlation significant at 95% confidence interval

Correlation among the three different ethnic groups are shown in Tables 4.45 – 4.47. Among Malay subjects, all significant correlations showed an inverse relationship. Formononetin was inversely correlated with oestradiol (r_s = -0.47). Biochanin A was inversely correlated with oestrone (r_s = -0.49) and oestradiol (r_s = -0.42). Similarly,

Chinese subjects showed several significant negative correlations in Table 4.46. Genistein was negatively correlated with oestradiol (r_s = -0.19).

Table 4.45: Correlation of analytes (plasma) in Malay subjects (n = 55)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	0.12	0.382	-0.07	0.592
G	0.01	0.924	-0.24	0.072
Eq	-0.25	0.066	**-0.47***	**< 0.001**
F	**-0.49***	**< 0.001**	**-0.42***	**0.001**
Bio A	-0.03	0.820	0.18	0.187

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Formononetin was negatively correlated to both oestrone and oestradiol (r_s = -0.30, r_s = -0.30). Biochanin A gave a higher negative correlation to oestrone and oestradiol (r_s = -0.62, r_s = -0.43). BPA was also negatively correlated to oestrone (r_s = -0.20).

Table 4.46: Correlation of analytes (plasma) in Chinese subjects (n = 130)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	-0.04	0.610	-0.15	0.087
G	-0.16	0.062	**-0.19***	**0.028**
Eq	0.11	0.207	-0.13	0.126
F	**-0.30***	**0.001**	**-0.30***	**< 0.001**
Bio A	**-0.62***	**< 0.001**	**-0.43***	**0.001**
BPA	**-0.20***	**0.025**	0.06	0.527

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Among Indian subjects, daidzein was positively correlated with oestrone (r_s = 0.36) in Table 4.47. Formononetin showed slightly stronger negative correlation to oestradiol than equol (r_s = -0.36). BPA however, correlated positively with oestradiol (r_s = 0.41).

Table 4.47: Correlation of analytes (plasma) in Indian subjects (n = 37)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	**0.36***	**0.031**	0.31	0.064
G	0.25	0.133	0.22	0.186
Eq	0.21	0.221	**-0.10***	**0.557**
F	-0.14	0.399	**-0.36***	**0.030**
Bio A	**-0.40***	**0.014**	**-0.33***	**0.045**
BPA	0.26	0.117	**0.41***	**0.013**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Correlation in nulliparous subjects (Table 4.48) showed all 4 pairs of significant negative correlations. Formononetin was negatively correlated with oestrone (r_s = -0.31) and oestradiol (r_s = -0.29). Likewise, Biochanin A showed a stronger negative correlation to oestrone (r_s = -0.70) and oestradiol (r_s = -0.44).

Table 4.48: Correlation of analytes (plasma) in nulliparous subjects (n = 51)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	0.01	0.988	0.13	0.371
G	-0.14	0.311	0.03	0.838
Eq	0.07	0.606	-0.15	0.287
F	**-0.31***	**0.026**	**-0.29***	**0.036**
Bio A	**-0.70***	**< 0.001**	**-0.44***	**0.001**
BPA	-0.26	0.060	0.09	0.504

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In non-nulliparous subjects (Table 4.49), genistein was negatively correlated with oestradiol (r_s = -0.18). Formononetin was negatively correlated with oestrone (r_s = -0.24) and oestradiol (r_s = -0.38). Again, biochanin A gave a stronger negative correlation to oestrone (r_s = -0.52) and oestradiol (r_s = -0.40) than formononetin.

Table 4.49: Correlation of analytes (plasma) in non-nulliparous subjects (n = 171)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	0.06	0.396	-0.12	0.122
G	-0.03	0.725	**-0.18***	**0.017**
Eq	0.11	0.138	-0.09	0.221
F	**-0.24***	**0.002**	**-0.38***	**< 0.001**
Bio A	**-0.52***	**< 0.001**	**-0.40***	**< 0.001**
BPA	-0.03	0.646	**0.16***	**0.036**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

In subjects with history of lactation (Table 4.50), formononetin and biochanin A was negatively correlated with oestrone (r_s = -0.25, r_s = -0.51) and oestradiol (r_s = -0.39, r_s = -0.40) but BPA was positively correlated with oestradiol (r_s = 0.28).

Table 4.50: Correlation of analytes (plasma) in subjects with history of lactation (n = 103)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	0.13	0.205	-0.01	0.897
G	0.09	0.374	-0.04	0.658
Eq	0.17	0.081	-0.01	0.932
F	**-0.25***	**0.010**	**-0.39***	**< 0.001**
Bio A	**-0.51***	**< 0.001**	**-0.40***	**< 0.001**
BPA	0.08	0.440	**0.28***	**0.004**

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

For those without any history of lactation (Table 4.51), formononetin and biochanin A showed negative correlation with oestrone (r_s = -0.25, r_s = -0.58) and oestradiol (r_s = -0.33, r_s = -0.39) but BPA was positively correlated with oestrone (r_s = 0.25) instead of oestradiol.

Table 4.51: Correlation of analytes (plasma) in subjects without history of lactation (n = 109)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	-0.03	0.753	-0.08	0.389
G	-0.17	0.083	-0.16	0.101
Eq	0.04	0.641	-0.13	0.186
F	**-0.25***	**0.009**	**-0.33***	**< 0.001**
Bio A	**-0.58***	**< 0.001**	**-0.39***	**< 0.001**
BPA	**0.25***	**0.009**	0.01	0.886

r_s = Spearman's rho *p* = p value
*Spearman's correlation significant at 95% confidence interval

Among menopausal subjects (Table 4.52), daidzein and genistein had equally negative correlation with oestradiol (r_s = -0.17, r_s = -0.17). Formononetin showed a stronger negative correlation to oestrone (r_s = -0.27) and oestradiol (r_s = -0.37). Biochanin A showed the strongest negative correlation to oestrone (r_s = -0.65) and oestradiol (r_s = -0.41).

Table 4.52: Correlation of analytes (plasma) in menopausal subjects (n = 142)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	-0.04	0.670	**-0.17***	**0.048**
G	-0.13	0.132	**-0.17***	**0.038**
Eq	0.17	0.170	-0.09	0.252
F	**-0.27***	**0.001**	**-0.37***	**< 0.001**
Bio A	**-0.65***	**< 0.001**	**-0.41***	**< 0.001**
BPA	-0.12	0.169	0.12	0.146

r_s = Spearman's rho *p* = p value
*Spearman's correlation significant at 95% confidence interval

For premenopausal subjects (Table 4.53), formononetin only correlated with oestradiol (r_s = -0.34) and not oestrone. Biochanin A showed slightly stronger correlation to oestrone (r_s = -0.39) and oestradiol (r_s = -0.38).

Table 4.53: Correlation of analytes (plasma) in pre-menopausal subjects (n = 76)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	0.20	0.080	0.12	0.295
G	0.08	0.494	-0.04	0.697
Eq	0.08	0.480	-0.11	0.360
F	-0.22	0.051	**-0.34***	**0.002**
Bio A	**-0.39***	**< 0.001**	**-0.38***	**0.001**
BPA	-0.05	0.641	0.15	0.197

r_s = Spearman's rho *p* = p value
*Spearman's correlation significant at 95% confidence interval

In Table 4.54, subjects with isoflavone types of two and less were analysed. A positive correlation was seen between daidzein and oestrone (r_s = 0.20), as well as between equol and oestrone (r_s = 0.39). Formononetin correlated negatively with oestradiol (r_s = -0.38) and oestrone (r_s = -0.39). Biochanin A showed a stronger negative correlation to oestrone (r_s = -0.62) and oestradiol (r_s = -0.49).

Table 4.54: Correlation of analytes (plasma) in subjects with ≤ 2 types of isoflavone (n = 156)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	**0.20***	**0.013**	0.15	0.053
G	0.15	0.055	0.15	0.054
Eq	**0.39***	**< 0.001**	0.03	0.699
F	**-0.38***	**< 0.001**	**-0.39***	**< 0.001**
Bio A	**-0.62***	**< 0.001**	**-0.49***	**< 0.001**
BPA	-0.08	0.296	0.13	0.104

r_s = Spearman's rho *p* = p value
*Spearman's correlation significant at 95% confidence interval

In Table 4.55, subjects with isoflavone types of two and less were analysed. A positive correlation was seen between daidzein and oestrone (r_s = 0.29). In contrast, biochanin A was inversely correlated with oestrone (r_s = -0.44).

Table 4.55: Correlation of analytes (plasma) in subjects with > 2 types of isoflavone (n = 66)

Variable	E1		E2	
	r_s	*p*	r_s	*p*
D	**0.29***	**0.016**	0.22	0.076
G	0.04	0.772	0.07	0.570
Eq	-0.14	0.249	-0.23	0.062
F	0.14	0.271	0.01	0.948
Bio A	**-0.44***	**< 0.001**	-0.11	0.356
BPA	-0.12	0.340	0.18	0.141

r_s = Spearman's rho *p* = p value
*Spearman's correlation significant at 95% confidence interval

In summary, Table 4.56 showed most of the significant correlations found were inversely related between oestrogenic EDCs and oestrogens. No significant correlation was found in 143 subjects without breast disease as compared to breast cancer. The most frequent significant correlations were found in the equol producer group. All of the oestrogenic EDCs were negatively correlated with oestrone and oestradiol except for a non-significant correlation between BPA and oestradiol. BPA was commonly in positive correlation with oestradiol as seen in breast cancer subjects, non-equol producers, Indian subjects, non-nulliparous and those with history of lactation.

4.4.5 Comparison between subject groups (plasma)

Female patients were grouped into women with and without breast cancer. It was found that women with breast cancer had lower mean concentrations for all oestrogenic EDCs as well as oestrogens when compared to those without breast cancer (Table 4.57).

Table 4.56: Summary of significant correlations in plasma

		D	G	Eq	F	BioA	BPA
Breast Cancer (n = 79)	E1	0.25			0.30	0.23	
	E2			**-0.34**		0.27	0.44
Equol producers (n = 43)	E1	**-0.39**	**-0.56**	**-0.50**	**-0.58**	**-0.69**	**-0.58**
	E2	**-0.39**	**-0.41**	**-0.36**	**-0.38**	**-0.35**	
Non- equol producers (n = 179)	E1	0.16			**-0.16**	**-0.52**	
	E2				**-0.37**	**-0.44**	0.21
Malay subjects (n = 55)	E1					**-0.49**	
	E2				**-0.47**	**-0.42**	
Chinese subjects (n = 130)	E1				**-0.30**	**-0.62**	**-0.20**
	E2		**-0.19**		**-0.30**	**-0.43**	
Indian subjects (n = 37)	E1	0.36				**-0.40**	
	E2			**-0.10**	**-0.36**	**-0.33**	0.41
Nulliparous (n = 51)	E1				**-0.31**	**-0.70**	
	E2				**-0.29**	**-0.44**	
Non-nulliparous (n = 171)	E1				**-0.24**	**-0.52**	
	E2		**-0.18**		**-0.38**	**-0.40**	0.16
History of lactation (n = 103)	E1				**-0.25**	**-0.51**	
	E2				**-0.39**	**-0.40**	0.28
No history of lactation (n = 109)	E1				**-0.25**	**-0.58**	0.25
	E2				**-0.33**	**-0.39**	
Menopausal (n = 142)	E1				**-0.27**	**-0.65**	
	E2	**-0.17**	**-0.17**		**-0.37**	**-0.41**	
Pre-menopausal (n = 76)	E1					**-0.39**	
	E2				**-0.34**	**-0.38**	
≤ 2 isoflavone (n = 156)	E1	0.20		0.39	**-0.38**	**-0.62**	
	E2				**-0.39**	**-0.49**	
> 2 isoflavone (n = 66)	E1	0.29				**-0.44**	
	E2						

E1 = oestrone E2 = oestradiol T = testosterone, D = daidzein, G = genistein, Eq = equol, F = formononetin, BioA = biochanin A, BPA = bisphenol A. Negative correlation is typed in **bold**

However, the differences were significant only for formononetin and biochanin A which were higher by almost 2 and 18 magnitude respectively, in women without breast cancer.

Table 4.57: Comparison of oestrogenic EDC and oestrogen levels in plasma of women with and without breast cancer (n = 222)

Analyte	Plasma concentration in ng/ml Women with breast cancer (n = 79) mean (SD)	Women without breast cancer (n = 143) mean (SD)	Mean difference (95% CI)	*t* stats (df[a])	*P* value[a]
Daidzein	4.9 (15.6)	6.1 (15.9)	-1.1 (-5.48 , 3.23)	-0.51 (220)	0.611
Genistein	7.0 (31.9)	11.9 (35.4)	-4.8 (-14.26 , 4.61)	-1.00 (220)	0.315
Equol	0.4 (1.0)	1.6 (7.2)	-1.1 (-2.35 , 0.08)	-1.85 (152)	0.067
Formononetin	**7.5 (18.3)**	**14.4 (18.5)**	-6.9 (-12.03 , -1.85)	-2.69 (220)	**0.008**
Biochanin A	**0.7 (3.2)**	**13.0 (19.6)**	-12.2 (-15.56 , -8.94)	-7.31 (156)	**< 0.001**
BPA	0.5 (0.9)	0.6 (1.2)	-0.2 (-0.47 , 0.13)	-1.1 (220)	0.265
Oestrone	0.1 (0.1)	0.1 (0.6)	0.0 (-0.09 , 0.17)	0.59 (220)	0.558
Oestradiol	0.1 (0.5)	0.1 (0.4)	0.0 (-0.07 , 0.17)	0.82 (220)	0.415

[a] independent t-test SD = standard deviation

The mean plasma concentrations were also compared between three ethnic groups (Table 4.58). Chinese subjects had the highest mean plasma concentration for daidzein, equol, biochanin A and BPA. The difference was only significant for BPA. After conducting a post-hoc test, the only significant difference of BPA mean concentrations between the three ethnic groups was only for Malay-Chinese subjects. Therefore, Chinese subjects had significantly higher mean plasma concentration of BPA by 3.5 magnitude than Malay subjects.

Table 4.58: Comparison of plasma analyte concentration between 3 ethnic groups

Analytes	Plasma concentration in ng/ml Malay (n = 55) mean (SD)	Chinese (n =130) mean (SD)	Indian (n = 37) mean (SD)	*F* stats[a] (df)	*P* value[a]
Daidzein	3.6 (12.9)	6.6 (15.1)	5.4 (21.0)	0.70 (2, 219)	0.498
Genistein	8.1 (41.3)	10.7 (26.5)	11.2 (45.7)	0.13 (2, 219)	0.881
Equol	0.1 (0.4)	1.9 (7.6)	0.1 (0.2)	2.76 (2, 219)	0.065
Formononetin	13.6 (18.2)	11.8 (19.4)	9.8 (16.7)	0.47 (2, 219)	0.626
Biochanin A	6.7 (8.0)	9.9 (20.8)	7.0 (9.0)	0.89 (2, 219)	0.409
BPA	**0.2 (0.6)**	**0.7 (1.2)**	**0.5 (1.0)**	4.22 (2, 219)	**0.016***
Oestrone	0.1 (0.3)	0.1 (0.6)	0.1 (0.2)	0.17 (2, 219)	0.840
Oestradiol	0.1 (0.6)	0.1 (0.4)	< 0.1 (0.05)	0.31 (2, 219)	0.732

[a] One-way ANOVA test SD = standard deviation
* Only Malay-Chinese pair of mean scores is significantly different by post-hoc test (Scheffe's procedure).

Comparison was made on the parity variable. It was found that non-nulliparous women had higher concentrations of daidzein, genistein, equol, formononetin, biochanin A and oestradiol than nulliparous females (Table 4.59). However, these were significant only for formononetin and oestradiol (p = 0.012, p = 0.043). Therefore, non-nulliparous women had significantly higher concentrations of formononetin and oestradiol than nulliparous females by 1.8 and 10 magnitude respectively.

Table 4.59: Comparison of plasma analyte concentration between nulliparous and non-nulliparous women

Analyte	Plasma concentration in ng/ml Nulliparous women (n = 51) mean (SD)	Women of parity ≥ 1 (n = 171) mean (SD)	Mean difference (95% CI)	*t* stats (df[a])	*P* value[a]
Daidzein	4.5 (14.4)	6.0 (16.2)	-1.5 (-6.48 , 3.44)	-0.60 (220)	0.546
Genistein	8.9 (31.4)	10.5 (35.0)	-1.6 (-12.38 , 9.15)	-0.29 (220)	0.768
Equol	1.1 (3.2)	1.2 (6.4)	-0.1 (-1.94 , 1.75)	-0.09 (220)	0.922
Formononetin	**7.3 (13.0)**	**13.3 (19.9)**	-6.1 (-10.76 , -1.37)	-2.56 (126)	**0.012**
Biochanin A	8.0 (8.5)	8.8 (18.7)	-0.8 (-6.07 , 4.56)	-0.28 (220)	0.779
BPA	0.8 (1.2)	0.5 (1.0)	0.3 (-0.02 , 0.65)	1.86 (220)	0.064
Oestrone	0.1 (0.2)	0.1 (0.5)	-0.1 (-0.23 , 0.07)	-1.04 (220)	0.298
Oestradiol	**<0.1 (0.02)**	**0.1 (0.5)**	-0.1 (-0.15 , -0.01)	-2.04 (171)	**0.043**

[a] independent t-test. SD = standard deviation

The mean plasma concentration between menopausal and premenopausal women are not significantly different ($P > 0.05$) as shown in Table 4.60. Therefore there is no significant association between plasma concentration of oestrogenic EDCs and menopausal status. The mean plasma concentration between those with 2 or less isoflavone types and those with more isoflavone types were compared in Table 4.61.

The mean concentration for all isoflavones are significantly different ($P < 0.05$). It is observed that those with more than 2 types of isoflavone had significantly higher levels of daidzein, genistein, equol, formononetin and BPA.

Table 4.60: Comparison of plasma analyte concentration between menopausal and premenopausal women

Analyte	Plasma concentration in ng/ml Menopause women (n = 143) mean (SD)	Premenopausal women (n = 79) mean (SD)	Mean difference (95% CI)	*t* stats (df[a])	*P* value[a]
Daidzein	5.6 (16.1)	5.7 (15.3)	-0.1 (-4.52, 4.20)	-0.07 (220)	0.943
Genistein	10.5 (37.1)	9.6 (28.3)	0.9 (-8.56, 10.36)	0.19 (220)	0.852
Equol	0.9 (2.8)	1.6 (9.1)	-0.7 (-2.35, 0.88)	-0.90 (220)	0.371
Formononetin	11.0 (17.3)	13.6 (21.0)	-2.7 (-8.15, 2.81)	-0.96 (136)	0.337
Biochanin A	9.1 (20.0)	7.8 (8.6)	1.2 (-3.43, 5.90)	0.52 (220)	0.603
BPA	0.6 (1.2)	0.6 (0.9)	-0.1 (-0.29, 0.29)	-0.01 (220)	0.990
Oestrone	0.1 (0.4)	0.1 (0.5)	-0.1 (-0.19, 0.08)	-0.81 (220)	0.418
Oestradiol	0.1 (0.5)	< 0.1 (0.07)	0.1 (-0.05, 0.19)	1.16 (220)	0.246

[a] independent t-test. SD = standard deviation

However, no significant difference for BPA was observed. Therefore, there is no significant association between plasma BPA concentration and number of isoflavone types. Having more than 2 types of isoflavone was not associated with plasma levels of oestrone or oestradiol.

Table 4.61: Comparison of plasma analyte concentration between number of isoflavone types

Analyte	Plasma concentration in ng/ml Isoflavone type ≤ 2 (n = 156) mean (SD)	Isoflavone type > 2 (n = 66) mean (SD)	Mean difference (95% CI)	*t* stats (df[a])	*P* value[a]
Daidzein	**0.2 (1.2)**	**18.5 (24.6)**	-18.2 (-24.27, -12.19)	-6.03 (65)	< **0.001**
Genistein	**0.3 (2.3)**	**33.5 (56.3)**	-33.3 (-47.11, -19.42)	-4.80 (65)	< **0.001**
Equol	**0.2 (0.5)**	**3.5 (10.4)**	-3.4 (-5.94, -0.83)	-2.65 (65)	**0.010**
Formononetin	**5.7 (8.4)**	**26.6 (26.6)**	-20.9 (-27.60, -14.28)	-6.27 (70)	< **0.001**
Biochanin A	**7.1 (18.7)**	**12.4 (10.8)**	-5.3 (-10.13 , -0.45)	-2.15 (220)	**0.032**
BPA	0.6 (1.1)	0.6 (1.1)	-0.0 (-0.34 , 0.28)	-0.18 (220)	0.856
Oestrone	0.1 (0.4)	0.2 (0.6)	-0.1 (-0.21, 0.08)	-0.93 (220)	0.353
Oestradiol	0.1 (0.5)	< 0.1 (0.3)	0.0 (-0.09, 0.15)	0.46 (220)	0.648

[a] independent t-test. SD = standard deviation

4.4.6 Comparison between equol producer status (plasma)

The mean plasma concentrations for neither oestrogenic EDCs nor the oestrogens between the two groups are significantly different ($P > 0.05$) in Table 4.62. Therefore, there is no significant association between analyte concentrations and equol producer status.

Table 4.62: Comparing plasma analyte concentrations between equol producers and non-equol producers

Analyte	Plasma concentration in ng/ml Equol producers (n = 43) mean (SD)	Non-equol producers (n = 179) mean (SD)	Mean difference (95% CI)	*t* stats (df[a])	*P* value[a]
Daidzein	10.0 (20.0)	4.6 (14.4)	-5.4 (-11.94, 1.02)	-1.69 (53)	0.097
Genistein	19.9 (49.0)	7.8 (29.2)	-12.1 (-27.78, 3.49)	-1.56 (49)	0.125
Formononetin	10.2 (18.7)	12.3 (18.7)	2.1 (-4.14, 8.38)	0.67 (220)	0.506
Biochanin A	7.9 (9.5)	8.8 (18.2)	0.9 (-4.71, 6.60)	0.33 (220)	0.742
BPA	0.6 (1.2)	0.6 (1.0)	-0.1 (-0.38, 0.34)	-0.13 (220)	0.895
Oestrone	0.2 (0.7)	0.1 (0.4)	-0.1 (-0.23, 0.09)	-0.82 (220)	0.416
Oestradiol	< 0.1 (0.01)	0.1 (0.5)	0.1 (-0.07, 0.22)	1.04 (220)	0.300

[a] independent t-test. SD = standard deviation

4.5 Study population of female patients with breast disease (breast tissue)

4.5.1 Descriptive statistics

For further breast tissue analysis, non-malignant diagnoses and ethnic groups other than Malay, Chinese and Indian were excluded leaving only 53 pairs (breast tissue samples and plasma) for subsequent statistical analysis. Table 4.63 described the characteristics of the subjects in this study in which each subject has contributed to both breast tissue and plasma samples. Majority of them were Chinese, menopause and only 24.5% were equol producers. Table 4.64 showed mean concentration of analytes in the breast tissue. BPA gave the highest mean of 69.3 ng/g.

Table 4.63: Characteristics of female patients with malignant breast tissue and plasma samples (n = 53)

Variable	Mean (S.D.)	Frequency (%)
Ethnic groups		
Malay		14 (26.4)
Chinese		30 (56.6)
Indian		9 (17.0)
Age	55 (11.6)	
Parity		
Nulliparous		14 (26.4)
1 - 3		26 (49.1)
≥ 4		13 (24.5)
Lactation		
Yes		20 (37.7)
No		33 (62.3)
Previous history of breast disease		
Yes		5 (9.4)
No		48 (90.6)
Family history of breast disease		
Yes		3 (5.7)
No		50 (94.3)
Menarche	n = 48 13.2 (1.4)	
Age at 1st Birth	n = 31 26.6 (6.1)	
Menopause		
Yes		38 (71.7)
No		15 (28.3)
Menopause age	n = 34 48.4 (9.7)	
Smoking		
Yes		2 (3.8)
No		51 (96.2)
Alcohol Intake		
Yes		0 (0)
No		53 (100.0)

Table 4.63, continued

Variable	Mean (S.D.)	Frequency (%)
HRT		
Yes		1 (2.0)
No		52 (98.0)
Antibiotics last 3 months		n = 49
Yes		9 (18.4)
No		40 (81.6)
Soy intake last 24 hours		
Yes		9 (17.0)
No		44 (83.0)
Equol Producer		13 (24.5)
Types of isoflavones detected in plasma		
0		18 (34.0)
1		23 (43.4)
2		5 (9.4)
3		3 (5.7)
4		3 (5.7)
5		1 (1.9)

Table 4.64: Mean concentration of analytes in breast tissue (n = 52)

Analyte in breast tissue	Relative Molecular Mass	Analyte concentration (ng/g) Mean (S.D).	SI Units equivalent (nmol/L) Mean (S.D)
Daidzein	254.24	0.6 (2.6)	2.36 (10.4)
Genistein	270.24	0.01 (0.1)	0.037 (0.4)
Equol	242.27	0.01 (0.1)	0.041 (0.5)
Formononetin	268.27	3.5 (23.2)	13.04 (86.4)
Biochanin A	284.26	0.7 (1.2)	2.46 (4.1)
BPA	228.28	69.3 (43.2)	303.57 (189.4)
Oestrone	270.37	3.3 (3.6)	12.20 (13.3)
Oestradiol	272.38	0.9 (2.2)	3.30 (8.1)

4.5.2 Percentage of analyte detection (breast tissue)

Figure 4.27 shows the percentage of analyte detection in 53 breast tissue samples analysed. These were divided into menopausal (n = 38) and premenopausal group (n =

15). Menopausal women had all analytes detected in the breast tissue samples ranging from 2.6 – 100%. Most commonly detected isoflavones in breast tissue of the menopausal group was biochanin A. All of the menopausal women had BPA detected in their samples.

Among the premenopausal women, the most commonly detected isoflavone in the breast tissue was daidzein. However, its metabolite, equol was not found in any of the premenopausal breast tissues. Neither was genistein detected. As was with the menopausal group, BPA was again the most commonly detected analyte in the breast tissue samples of premenopausal women. Oestrogens were more frequently detected in samples of the menopausal group than in premenopausal.

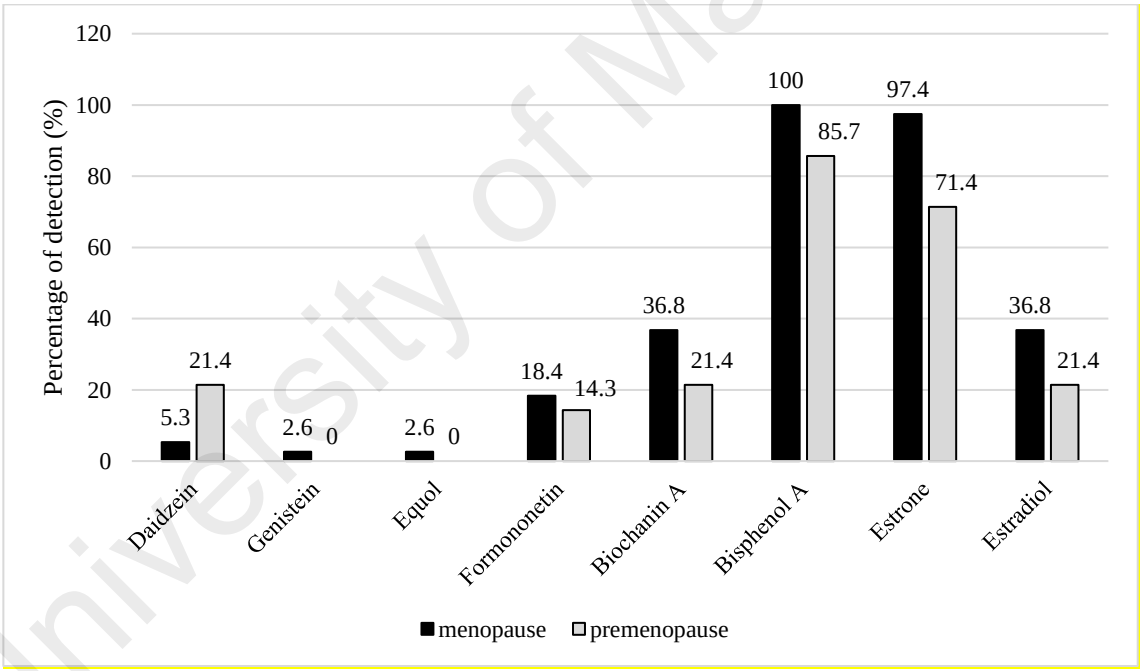

Figure 4.27: Percentage of analyte detection (breast tissue)

4.5.3 Statistical analyses

Statistical analysis was carried out using SPSS software version 15.0. Parametric tests for comparing means between groups (independent t-test, ANOVA and paired t-test) and Spearman's correlations were used in this analysis. Table 4.10 is used as a

reference in all correlation analysis in this study when interpreting the strength of the correlation.

4.5.4 Correlation of analytes (breast tissue)

Spearman's correlation was performed to assess the relationship between oestrogenic EDCs and sex hormones in the 53 samples of breast tissue (Table 4.65). There were only 2 correlations which were significant. Biochanin A was significantly in negative correlation with BPA (r_s = -0.31) in the breast tissue while oestrone was positively correlated with oestradiol (r_s = 0.46).

Table 4.65: Correlation of analytes in breast tissue (n = 53)

Variable	D	G	Eq	F	Bio A	BPA	E1	E2
Daidzein	1.00	-0.04	-0.04	-0.01	0.01	-0.26	0.02	0.19
Genistein	-	1.00	-0.02	-0.08	-0.17	-0.01	-0.19	-0.09
Equol	-	-	1.00	0.19	-0.17	0.09	0.15	-0.09
Formono-netin	-	-	-	1.00	0.26	-0.09	0.10	0.01
Biochanin A	-	-	-	-	1.00	**-0.31***	0.07	0.08
BPA	-	-	-	-	-	1.00	0.16	-0.05
Oestrone	-	-	-	-	-	-	1.00	**0.46***
Oestradiol	-	-	-	-	-	-	-	1.00

*Significant correlation at 95% confidence interval

There was no significant correlation for these analytes in the breast tissue of equol producers, non-equol producers, in Malay and Chinese subjects, nulliparous, non-nulliparous subjects, those with or without history of lactation, premenopausal, menopausal subjects, and those with less than 2 types of isoflavone (Tables 4.66 – 4.76). Therefore, there was no significant correlation of oestrogenic EDCs and oestrogens in breast tissue in any of the groups.

Table 4.66: Correlation of analytes (breast tissue) in equol producers (n = 13)

Variable	Oestrone		Oestradiol	
	r_s	*p*	r_s	*p*
Daidzein	0.15	0.615	0.53	0.064
Genistein	n.a	n.a	n.a	n.a
Equol	n.a	n.a	n.a	n.a
Formononetin	-0.20	0.517	-0.12	0.690
Biochanin A	-0.18	0.566	-0.40	0.170
BPA	-0.10	0.734	-0.18	0.546

n.a. = not applicable (analyte not detected in breast tissue)
r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Table 4.67: Correlation of analytes (breast tissue) in non- equol producers (n = 40)

Variable	Oestrone		Oestradiol	
	r_s	*p*	r_s	*p*
Daidzein	0.01	0.935	0.11	0.482
Genistein	-0.21	0.194	-0.11	0.506
Equol	0.17	0.281	-0.11	0.506
Formononetin	0.11	0.480	0.04	0.824
Biochanin A	0.14	0.384	0.26	0.110
BPA	0.19	0.223	-0.02	0.883

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Table 4.68: Correlation of analytes (breast tissue) in Malay subjects (n = 14)

Variable	Oestrone		Oestradiol	
	r_s	*p*	r_s	*p*
Daidzein	-0.32	0.270	-0.23	0.432
Genistein	n.a.	n.a.	n.a.	n.a.
Equol	0.32	0.270	-0.23	0.432
Formononetin	-0.03	0.910	-0.33	0.241
Biochanin A	-0.08	0.793	0.09	0.766
BPA	**0.57***	**0.034**	0.42	0.136

n.a. = not applicable (analyte not detected in breast tissue)
r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Table 4.69: Correlation of analytes (breast tissue) in Chinese subjects (n = 30)

Variable	Oestrone		Oestradiol	
	r_s	*p*	r_s	*p*
Daidzein	0.02	0.909	0.25	0.186
Genistein	-0.27	0.151	-0.13	0.501
Equol	n.a.	n.a.	n.a.	n.a.
Formononetin	0.17	0.360	0.27	0.154
Biochanin A	0.15	0.433	0.04	0.821
BPA	0.01	0.973	-0.18	0.348

n.a. = not applicable (analyte not detected in breast tissue)
r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Table 4.70: Correlation of analytes (breast tissue) in nulliparous subjects (n = 14)

Variable	Oestrone		Oestradiol	
	r_s	*p*	r_s	*p*
Daidzein	-0.04	0.890	0.24	0.411
Genistein	n.a.	n.a.	n.a.	n.a.
Equol	n.a.	n.a.	n.a.	n.a.
Formononetin	0.23	0.432	0.47	0.087
Biochanin A	0.17	0.552	0.48	0.082
BPA	0.22	0.444	-0.26	0.362

n.a. = not applicable (analyte not detected in breast tissue)
r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Table 4.71: Correlation of analytes (breast tissue) in non-nulliparous subjects (n = 39)

Variable	Oestrone		Oestradiol	
	r_s	*p*	r_s	*p*
Daidzein	0.14	0.395	0.15	0.364
Genistein	-0.24	0.144	-0.11	0.500
Equol	0.18	0.252.	-0.11	0.499
Formononetin	0.08	0.616	-0.17	0.304
Biochanin A	0.08	0.612	-0.09	0.574
BPA	0.09	0.574	0.03	0.832

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Table 4.72: Correlation of analytes (breast tissue) in subjects with history of lactation (n = 20)

Variable	Oestrone		Oestradiol	
	r_s	*p*	r_s	*p*
Daidzein	-0.02	0.934	-0.09	0.687
Genistein	n.a.	n.a.	n.a.	n.a.
Equol	0.26	0.270	-0.09	0.687
Formononetin	0.33	0.161	-0.04	0.851
Biochanin A	0.04	0.857	0.04	0.869
BPA	0.32	0.167	0.12	0.604

n.a. = not applicable (analyte not detected in breast tissue)
r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Table 4.73: Correlation of analytes (breast tissue) in subjects without history of lactation (n = 33)

Variable	Oestrone		Oestradiol	
	r_s	*p*	r_s	*p*
Daidzein	0.04	0.827	0.25	0.157
Genistein	-0.20	0.190	-0.15	0.398
Equol	n.a.	n.a.	n.a.	n.a.
Formononetin	-0.03	0.846	0.07	0.688
Biochanin A	0.08	0.673	0.06	0.722
BPA	0.09	0.630	-0.08	0.674

n.a. = not applicable (analyte not detected in breast tissue)
r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Table 4.74: Correlation of analytes (breast tissue) in menopausal subjects (n = 38)

Variable	Oestrone		Oestradiol	
	r_s	*p*	r_s	*p*
Daidzein	0.04	0.806	0.27	0.099
Genistein	-0.23	0.159	-0.13	0.446
Equol	0.20	0.222	-0.13	0.446
Formononetin	**0.33***	**0.041**	0.14	0.390
Biochanin A	0.08	0.628	0.07	0.668
BPA	0.01	0.942	-0.20	0.226

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Table 4.75: Correlation of analytes (breast tissue) in premenopausal subjects (n = 15)

Variable	Oestrone		Oestradiol	
	r_s	*p*	r_s	*p*
Daidzein	-0.01	0.975	0.16	0.559
Genistein	n.a.	n.a.	n.a.	n.a.
Equol	n.a.	n.a.	n.a.	n.a.
Formononetin	-0.36	0.187	-0.29	0.286
Biochanin A	-0.08	0.785	0.02	0.941
BPA	0.45	0.092	0.19	0.482

n.a. = not applicable (analyte not detected in breast tissue)
r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

Table 4.76: Correlation of analytes (breast tissue) in subjects with ≤ 2 types of isoflavones (n = 46)

Variable	Oestrone		Oestradiol	
	r_s	*p*	r_s	*p*
Daidzein	0.04	0.772	0.19	0.209
Genistein	-0.20	0.176	-0.11	0.466
Equol	0.16	0.277	-0.11	0.466
Formononetin	0.17	0.267	0.08	0.604
Biochanin A	0.09	0.565	0.14	0.350
BPA	0.06	0.684	-0.06	0.685

r_s = Spearman's rho *p* = p value
* Spearman's correlation significant at 95% confidence interval

4.5.5 Comparison between subject groups (breast tissue)

Comparison was made between cancerous breast tissue samples from menopause and premenopausal women (Table 4.77). There was almost no isoflavones detected in the breast tissue samples of either group but BPA was found in very high amount in both groups. It was found that menopause women had higher concentration of BPA and oestrone than premenopausal women, however this difference was not significant.

Table 4.77: Comparison of oestrogenic EDC and oestrogen levels in breast tissue of menopausal and premenopausal women (n = 53)

Analyte Concentration in breast tissue (ng/g)	Menopause women (n = 38) median (IQR)	Premenopause women (n = 15) median (IQR)	*Z* stats	*P* value[a]
Daidzein	< 0.01(0.00)	< 0.01(0.00)	-1.60	0.110
Genistein	< 0.01(0.00)	< 0.01(0.00)	-0.63	0.530
Equol	< 0.01(0.00)	< 0.01(0.00)	-0.63	0.530
Formononetin	< 0.01(0.1)	< 0.01(0.2)	-0.15	0.879
Biochanin A	0.3(0.9)	0.4(0.7)	-0.33	0.740
BPA	73.3(63.6)	63.4(74.1)	-0.74	0.459
Oestrone	2.1(4.8)	1.7(6.4)	-0.47	0.641
Oestradiol	< 0.01(1.2)	< 0.01(0.00)	-1.01	0.314

[a] Mann-Whitney IQR = inter-quartile range

Table 4.78: Comparison of analyte concentration in breast tissue of different ethnic groups (n = 53)

Analytes	Breast tissue concentration in ng/g Malay (n = 14) median (IQR)	Chinese (n =30) median (IQR)	Indian (n = 9) median (IQR)	X^2 stats (df)[a]	*P* value[a]
Daidzein	< 0.01(0.00)	< 0.01(0.00)	< 0.01(0.00)	0.10 (2)	0.951
Genistein	< 0.01(0.00)	< 0.01(0.00)	< 0.01(0.00)	0.77 (2)	0.682
Equol	< 0.01(0.00)	< 0.01(0.00)	< 0.01(0.00)	2.79 (2)	0.248
Formononetin	< 0.01(0.00)	< 0.01(0.3)	< 0.01(0.08)	1.56 (2)	0.459
Biochanin A	< 0.01(0.9)	0.5 (0.9)	0.4 (0.5)	2.83 (2)	0.243
BPA	**73.6 (79.6)**	**55.7 (52.9)**	**114.0 (59.1)**	6.61 (2)	**0.037**
Oestrone	0.8 (5.5)	2.1 (4.2)	4.3 (10.5)	2.45 (2)	0.294
Oestradiol	< 0.01(1.9)	< 0.01(0.75)	< 0.01(0.63)	1.23 (2)	0.542

[a] Kruskal-Wallis test IQR = inter-quartile range

A Kruskal-Wallis test (Table 4.78) revealed that there was a significant effect of ethnic groups on BPA breast tissue concentration. Inspection of the group median suggests that compared to Malays and Chinese, breast tissue samples from Indian subjects were significantly increased ($p = 0.037$).

In Table 4.79, concentration of oestrogenic EDCs and oestrogens in breast tissue of nulliparous and non-nulliparous women showed no difference. Therefore, this suggests there is no effect of parity of the subjects on concentration found in the breast tissue.

Table 4.79: Comparison of oestrogenic EDC and oestrogen levels in breast tissue of nulliparous and non-nulliparous women (n = 53)

Analyte Concentration in breast tissue (ng/g)	Nulliparous women (n = 14) median (IQR)	Non- nulliparous women (n = 39) median (IQR)	*Z* stats	*P* value[a]
Daidzein	< 0.01(0.6)	< 0.01(0.00)	-1.85	0.064
Genistein	< 0.01(0.00)	< 0.01(0.00)	-0.60	0.549
Equol	< 0.01(0.00)	< 0.01(0.00)	-0.60	0.549
Formononetin	< 0.01(0.2)	< 0.01(0.1)	-0.34	0.735
Biochanin A	0.6 (0.7)	0.2 (0.9)	-0.82	0.412
BPA	73.3 (64.5)	67.6 (64.5)	-0.58	0.558
Oestrone	1.6 (5.1)	2.0 (4.9)	-0.86	0.389
Oestradiol	< 0.01 (1.7)	< 0.01 (1.0)	-0.33	0.738

[a] Mann-Whitney test IQR = inter-quartile range

4.5.6 Comparison between equol producer status (breast tissue)

In Table 4.80, concentration of formononetin, biochanin A, BPA and oestrone were higher in equol producers than non-equol producers. However, significant difference was only observed for formononetin. This suggests that being an equol producer could affect the concentration of formononetin in breast tissue.

Table 4.80: Comparing breast tissue analytes between equol producers and non-equol producers (n = 53)

Analyte Concentration in breast tissue (ng/g)	Equol producer (n = 13) median (IQR)	Non-equol producer (n = 40) median (IQR)	*Z* stats	*P* value[a]
Daidzein	< 0.01(0.00)	< 0.01(0.00)	-0.16	0.870
Genistein	< 0.01(0.00)	< 0.01(0.00)	-0.57	0.569
Equol	< 0.01(0.00)	< 0.01(0.00)	-0.57	0.569
Formononetin	**0.2 (1.9)**	**< 0.01(0.00)**	**-3.30**	**0.001**
Biochanin A	0.5(1.3)	0.3(0.8)	-0.78	0.437
BPA	73.3(62.4)	68.6(66.4)	-0.04	0.967
Oestrone	3.3(3.7)	1.3(4.9)	-1.44	0.149
Oestradiol	< 0.01(1.1)	< 0.01(1.00)	-0.29	0.769

[a] Mann-Whitney test IQR = inter-quartile range

4.5.7 Distribution of analytes in plasma and breast tissue

There were 53 paired samples available for analysis. The paired samples were plasma and breast tissue samples which came from the same individual. Analyte concentrations were compared statistically by paired t-test for any significant differences between concentrations of analytes in the plasma and in the breast tissue. In Table 4.81, significant differences can be seen between plasma concentration and breast tissue concentration for daidzein, genistein, equol, BPA and oestradiol. Concentrations of daidzein, genistein and equol were significantly higher in plasma than breast tissue. BPA and oestradiol however, were found to be higher in breast tissue than plasma. BPA was extremely high in breast tissue by 115 magnitude as compared to plasma. Oestradiol concentration was also significantly 10 times higher in breast tissue than in plasma.

Table 4.81: Comparison of analyte concentrations in plasma and breast tissue within the same subjects (n = 53)

Analyte	Plasma concentration (n = 53) mean (SD) ppb (ng/ml)	Breast tissue concentration (n = 53) mean (SD) ppb (ng/g)	Mean of Score difference (95% C.I)	*t* stats (df[a])	*P* value[a]
Daidzein	**3.5 (8.3)**	**0.6 (2.6)**	2.9 (0.5, 5.2)	2.4 (52)	**0.018**
Genistein	**3.5 (9.9)**	**< 0.1 (0.1)**	3.5 (0.7, 6.2)	2.5 (52)	**0.014**
Equol	**0.5 (1.2)**	**< 0.1 (0.1)**	0.5 (0.1, 0.8)	2.8 (52)	**0.008**
Formononetin	6.8 (16.7)	3.5 (23.2)	3.2 (-4.9, 11.3)	0.8 (52)	0.427
Biochanin A	0.6 (2.5)	0.7 (1.2)	-0.01 (-0.8, 0.7)	-0.1 (52)	0.910
BPA	**0.6 (0.9)**	**69.3 (43.2)**	-68.8 (-80.7, 56.9)	-11.6 (52)	**< 0.001**
Oestrone	0.2 (0.2)	5.8 (1.9)	-5.6 (-24.9, 13.8)	-3.7 (52)	0.170
Oestradiol	**0.1 (0.6)**	**0.96 (2.2)**	-0.84 (-1.5, 0.2)	-2.7 (52)	**0.009**

[a] Paired t-test C.I = confidence interval

CHAPTER 5: DISCUSSION

5.1 Method development and validation of analytes in human plasma

In this study, biological samples chosen for analysis were plasma and breast tissue. Urine was not considered in this study as research subjects are adults and warded hospital patients, therefore obtaining these samples will not be much of a problem. Furthermore, urine concentration would not give the true picture of the extent of circulating EDCs in the blood that would go to the cells of the body and affect the endocrine system. Vandenberg *et al*. (2014) advocates toxicokinetic studies to address real-world exposure scenarios as multiple sources of exposure would influence levels detected.

This method analyses 9 chemical analytes in human plasma which are daidzein, genistein, equol, formononetin, biochanin A, BPA, oestrone, oestradiol and testosterone. These are endogenous and exogenous an alytes which have relatively similar chemical structure.

It was found that of the mobile phases, daidzein, genistein, formononetin and biochanin A gave higher peaks in methanol/water alone. Equol, BPA, oestrone and estradioil peaks were best in mobile phase with ammonium hydroxide addition and methanol/water without any addition was best for testosterone.

During development of a single run method for both endocrine disruptors and sex hormones, it was finally decided to use methanol/water as the mobile phase with addition of 0.01% ammonium hydroxide for the negative mode run and without ammonium hydroxide for testosterone analysis in mix mode run.

Eight of the analytes were analysed in a single run while testosterone has to be run in a separate mix mode as the elution peak in the positive mode were too close to elution peaks of other analytes in the negative mode making it impossible to analyse

testosterone with the rest of the 8 analytes. The challenge is in extracting and separating the elution time of each compound. This will be discussed further in sub-topics below.

5.1.1 Ionisation

Ionisation problems such as ion suppression can be due to the presence of less volatile analytes which can reduce the efficiency of droplet formation, subsequently affecting the amount of charged ion in the gas phase that reaches the detector (Annesley, 2003)

LCMSMS method was used in this study following a recommendation which supported the use of tandem mass spectrometry for steroid analysis over GCMS as it has less matrix interference, allowing detection limit beyond 2 ng/ml as well as requires no derivatisation step as in GCMS (Joos & Van Ryckeghem, 1999). As found by Guo *et al* (2008), this study observed that electrospray ionization in the negative mode yields best results for oestradiol and oestrone (Guo *et al.*, 2008). Guo *et al* used C-8 analytical columns for chromatography and the sample was run on API-5000 tandem mass spectrometry giving a very low LOD of 1 – 2 pg/ml. Those conditions may work very well on the endogenous oestrogens alone. This study however, has other analytes which will not bear well with those conditions. As the aim is to develop a method for determination of several analytes in a single run, various parameters have to be adjusted to obtain good peaks for all analytes.

Immunoassay is one of many methods used for measurement of oestradiol and oestrone (Dorgan *et al.*, 2002). These assays whether direct or indirect are mostly targeted at younger female population which have higher levels of hormones and are not suitable for use in menopausal group or male population.

Moreover, these assays can only measure one analyte per assay and possible cross-reactivity can result in overestimation of the actual values. A more practical, sensitive

and accurate method needs to be developed for practical assessment of sex hormones in biological matrices.

Comparison of methods to measure low serum oestradiol levels in postmenopausal women show that indirect and direct assays were overestimated and mean oestradiol levels obtained by the assays were higher by 14 – 68 % than what was obtained from GCMSMS (Lee *et al*., 2006).

Measurements by GCMS have been considered as one of the most accurate methodology however, they are less sensitive and have a longer run time per sample. LCMSMS is better than GCMS in terms of sensitivity and productivity. Existing LCMSMS methods provide adequate sensitivities for oestrogens measurement in premenopausal women. However, a higher sensitivity is required as physiological levels of oestradiol are reported to be < 50 ng/L in postmenopausal women (Lee *et al*., 2006).

In consideration of increasing the sensitivity for oestradiol, a study by Nelson *et al* (2004) reported how an LOQ of 11.9 ng/ L and 6.3 ng/ L were obtained for oestrone and oestradiol respectively, by incorporating derivatisation step with dansyl chloride. His study used similar equipment as in this study but utilised the atmospheric pressure chemical ionization instead of ESI (Nelson *et al*., 2004).

In this study however, when APCI and ESI were evaluated for determination of the sex hormones in both positive and negative modes, it was observed that the ESI negative mode was most effective for oestrogens which is similar to another study's findings (Mitani *et al.*, 2005).

In comparison with the above study which conducted an analysis of 5 oestrogens including oestradiol and oestrone, peaks obtained were not as sharp as the ones obtained in this study.

Another study which incorporated derivatisation as one of the steps, obtained LOQ of 1 pg/ ml and 0.5 pg/ ml for oestrone and oestradiol respectively (Yamashita *et al*.,

2007). The derivatising agent used was picolinoyl chloride. Although derivatisation may help in improving sensitivity, several disadvantages exist such as decreased ionisation efficiency due to formation of an adduct ion and a longer sample preparation time is taken to incorporate derivatisation.

The use of dansyl chloride was attempted. However, trial experiments showed that this compromised isoflavone peaks and therefore this option was abandoned. Analysing the isoflavones together with oestrogens in a single run limits the options to further enhance sensitivity for the oestrogens as oestrogens are more hydrophobic while isoflavones are more hydrophilic.

Testosterone ionisation however was better in the ESI positive mode. To ascertain the optimal composition, different mobile phases were tested. This method aims for short retention time of each analyte while maintaining good peaks and resolution and minimizing the matrix effects. Due to the more hydrophobic nature of oestrogens as compared to the isoflavones, a higher proportion of organic solvent was required to co-elute oestrone and oestradiol (Table 3.2). Though the last analyte to elute was oestrone at 8.66 min (Table 4.1), the LC run was extended to include a column wash program for about 5 min, reducing the possibility of analyte carryover in the subsequent run.

Endogenous steroid hormones were previously analysed using GCMS, however these procedures are taxing and time-consuming as it requires the derivatisation step to increase its sensitivity. Such studies which employ derivatisation methods in analysing oestrogens in human urine (Xiao & McCalley, 2000) and water (Yang *et al.,* 2006), were conducted with GCMS rather than LCMS. Employing methods which do not require derivatisation steps will increase productivity as it is less time-consuming to produce analytical results, there more studies aimed to develop methods using LCMSMS instead. Derivatisation with LCMSMS have been reported however these analysis only involved simultaneous determination of steroid hormones without other

EDCs such as phytoestrogens which were more hydrophilic (Anari *et al.*, 2002; Higashi *et al*., 2005).

5.1.2 Choice of internal standard

Internal standard is a test compound which is added to both calibration standards and samples at known and constant concentration to facilitate quantification of the target analytes (Guidance for Industry, Bioanalytical Method Validation, May 2001). An external/internal standard is usually used in any analytical method. For an internal standard, some considerations in choosing a certain internal standard are: the similarity in its physical and chemical structure with the analytes studied as well as it being absent from the matrix analysed. Internal standards are added before sample pre-treatment steps. This is to ensure that any losses of the analyte during sample preparation can be paralleled by losses of the internal standard.

A common belief is that using a stable isotopically labeled (SIL) internal standards would resolve a lot of problems concerning variability in chemical derivatisation, sample extraction and LCMSMS analysis. However, these SIL are not always available and are usually quite expensive.

Furthermore, there is evidence that showed these SIL can demonstrate unexpected peaks (Wang *et al.*, 2007). Therefore, 4-HBPH is used as the internal standard due to the similarity of the structure to most of our target analytes.

5.1.3 Sample preparation

Plasma sample preparation in this study involves an incubation step (Figure 3.2) with β-glucuronidase enzyme for deconjugation of isoflavones at 45^{o}C instead of 37^{o}C or other temperatures. This is based on reports of hydrolysis rates which increased with raised temperature to 45^{o}C (Taylor *et al.*, 2005). This study used pH 5 for hydrolysis

conditions as Taylor *et al*. reported that pH 6 is unfavourable to phytoestrogen conjugates.

For injection volume, this study decided to use 20.0 µl after considering advantages and disadvantages of large volume injection (LVI) (Trenholm *et al.*, 2006). LVI can improve LOD and sensitivity but at the same time it can induce signal suppression and peak broadening. Trenholm *et al*., (2006) also recommended offline solid phase extraction for complex matrix however, this is a time consuming step.

5.1.4 Choice of column and mobile phase

In previous experiments, various columns were tried. In the early phase of this study, an XTerra C-18 column 4.6 X 250 mm (5 µm packing) by Waters was used but elution time of the steroid hormones was too long. Similarly an Altima C-18 column was tried and achieved good isoflavone peaks but elution time was too long.

It is important to obtain a high sensitivity for oestrone and oestradiol as the physiological level present in the blood is extremely low. Upon the use of mobile phase acetonitrile/water or methanol/water, there was very poor ionization for oestrone and oestradiol. The addition of 0.05% formic acid did not improve the ionization either. However, a study reported on how addition of ammonium hydroxide helped improve ionization of the steroid analytes (Reddy *et al.*, 2005). This had successfully improved the detection of analytes in this study, but inevitably changed the pH of the mobile phase to alkaline. Since most LC columns are made of silica, which can only endure a narrow range of pH, this study chose the Zorbax Extend C-18 column 4.6 X 110 mm (3.5 µm packing) by Agilent instead, which can withstand a pH of 2 -11 for further analysis.

Comparing the peaks eluted with three different mobile phase of methanol-water, with addition of formic acid and with addition of ammonium hydroxide, it was found

that most isoflavone peaks were best in mobile phase of methanol-water without any addition while equol, BPA, oestrone, oestradiol and testosterone were giving sharper peaks in methanol-water with addition of 0.01 % ammonium hydroxide. With that finding, the mobile phase A was set at 90 % plain water-methanol and mobile phase B of 100 % methanol with 0.01 % ammonium hydroxide. The LC program was adjusted to elute most isoflavone peaks with mobile phase A followed by increasing mobile phase B for elution of the more hydrophobic analytes.

Some researchers used the same Zorbax Extend column but with a smaller internal diameter (ID) of 2.1 mm (Reddy *et al*., 2005) which can give sharper peaks. However, a smaller ID can also lead to a higher column backpressure. Considering that the mobile phase used in this study was methanol instead of acetonitrile, which tends to result in an increase in the column pressure as the viscosity of the mobile phase increased, a wider ID of 4.6 mm was preferred and used in this study.

The addition of ammonium hydroxide, apart from turning the mobile phase to more alkaline was also worsening the increase of column pressure. Hence, to maintain a longer lasting LC column, a guard column was used and the flow rate was set at 0.5 ml/ min instead of the recommended 1 ml/min for a column with an ID of 4.6 mm. using these parameters, the separation and peak heights obtained were found to be adequate for quantification.

5.1.5 Limits of detection

Limits of detection (LOD) and limits of quantification (LOQ) were in the range of 0.20 – 0.5 ng/ml. As the sensitivity varies from one analyte to another, running all analytes simultaneously in a single run inevitably compromised LOD of certain analytes.

5.1.6 Matrix effects and recovery

Signal enhancement is evident for the isoflavones at low concentrations while BPA and oestrogens in contrast, showed signal suppression (Table 4.5). The oestrogens as discussed, showed extreme signal suppression ranging from 9.1 – 79.8% while testosterone showed very little matrix effect and almost no matrix effect was observed at concentration of 30 ng/ ml.

In handling matrix effects, literature shows one of the common approaches is post-column infusion of the target analyte in the effluent of the chromatographic column (Annesley, 2003). Other approaches that have been reported in the literature were analyte-specific extraction, use of stable isotope labeled standards and standard addition.

As experienced by other researchers, the problem of matrix effect is not easy to solve (Kushnir *et al.*, 2011). In this study, the direct comparison approach was used where the signal intensity of the analytes measured were compared with and without the sample matrix, as this is more applicable to our experiments which involved hundreds of samples.

Recovery of oestradiol and testosterone for concentration of 4, 35 and 65 ng/ ml in this study was relatively better than recovery rates reported in human testicular fluid (60.6 – 82.9 %) at 0.06 – 1.5 ng/ ml (Zhao *et al.*, 2004).

5.2 Method development and validation of oestrogenic EDCs and oestrogens in human breast tissue

One of the biggest challenges in this analysis is in measuring oestrogens. It is a widely known that ionisation was very poor for oestrone and oestradiol. This study found that no difficulty was faced in obtaining good peaks for the phytoestrogens using

methanol/water or acetonitrile/water as the mobile phase. However, even with API-5000, obtaining a peak with oestrogens was a big challenge.

Some studies reported the use of tributyl amine (Al-Odaini *et al.*, 2010), trimethylamine, tri-n-butylamine and tetrabutylammonium acetate (Gao *et al.*, 2005) as the ion-pairing agent in the negative detection mode, but when added to the mobile phase in this study, no increase in peak height was observed. Therefore, as what was performed in plasma analysis, the addition of 0.01% ammonium hydroxide to both mobile phase was still the best option.

Comparing the mobile phases of methanol/water with and without ammonium hydroxide addition, it was found that daidzein, genistein, formononetin, biochanin A gave higher peaks in methanol/water alone. While equol, BPA, oestrone and oestradiol peaks were best in mobile phase with ammonium hydroxide addition. As the analysis is more challenging with the steroid hormones, the better peaks obtained for daidzein, genistein, formononetin, biochanin A has to be compromised. Therefore, this study opted for the addition of 0.01% ammonium hydroxide to both mobile phases and analysis was conducted in negative mode.

5.2.1 Limits of detection (breast tissue)

This study achieved LODs between 0.25 – 0.30 ng/g for all analytes which is acceptable. Although an HPLC-RIA method used for measurement of oestrogens in breast tissue obtained detection limits as low as 4.3 fmol/g and 19.8 fmol/g for oestrone and oestradiol respectively (Geisler *et al*., 2000), this method is very costly and involves labour intensive sample preparation (Kushnir *et al*., 2011).

5.2.2 Matrix effects and recovery

Matrix effects of the isoflavones show a great degree of suppression of the ion signals (0.97 – 60.56 %). Matrix effect is even more pronounced on steroid analyses due to greater interferences from high amount of endogenous isomers or isobars (Kushnir *et al*., 2011).

The method used in this study showed comparable recovery rates as used in another study which used 100 – 200 mg of breast tissue, resulting in a 50 % recovery rate when measured by nano LC-ESI-MSMS (Zhang *et al.*, 2008).

The recovery rate (48.9 – 52.6 %) for oestrone and oestradiol obtained from this study showed a more consistent and higher levels than a study using 150 mg of breast tissue sample which only gave 25 – 50 % recovery, in which their method involved ether extraction followed by purification step with HPLC before measuring levels using radioimmunoassay (Geisler *et al*., 2000).

5.2.3 Sample preparation (breast tissue)

One of the major tasks faced is getting the best extraction method for all analytes in a complex biological matrix such as breast tissue. In a previous study, 150 mg of breast tissue sample was used in oestrogen analysis which involved ether extraction followed by purification step with HPLC before measuring the levels using radioimmunoassay (Geisler *et al*., 2000). In another study, 100 – 200 mg of breast tissue was measured for oestrogen metabolite using nano LC-ESI-MSMS (Zhang *et al*., 2008) while this study used 500 mg of breast tissue. Among the organic solvents used for LLE in steroid analyses are: hexane, ethyl acetate (Chetrite *et al.*, 2000; Dighe & Sluss, 2004), methylene chloride (Nelson *et al*., 2004; X. Xu *et al*., 2007), methyl-tert-butyl ether (Kushnir *et al*., 2004).

5.3 Study population of male vegetarian and non-vegetarian

5.3.1 Analyte detection

High percentage (more than 95 %) of the exogenous EDCs detection in the plasma (Figure 4.22) suggests that these analytes can be found ubiquitously in the environment. BPA was detected in all of vegetarian subjects and almost all in non-vegetarian subjects. This finding supports other studies which reported of extensive exposure of humans to BPA which were detected in 50 – 80% of the subjects' urine samples (Calafat *et al*., 2005; He *et al.*, 2009; Matsumoto *et al*., 2003; Ye *et al.*, 2005). Most of the food products which reported on positive BPA detection, were related to non-vegetarian diets. This include crab, squid, fish and blood cockle which were reported to contain BPA levels ranging from 56.5 ng/g to 213.3 ng/g (Basheer *et al*., 2004).

BPA was also reported in milk (0.49 μg/kg), mutton (0.54 μg/kg), chicken (0.73 μg/kg), fish (1.01 μg/kg), pork (7.08 μg/kg) and eggs (10.45 μg/kg) (Shao, Han, Li, *et al.*, 2007; Shao, Han, Tu, *et al*., 2007). In the present study, mean plasma BPA concentration was not significantly different between vegetarian and non-vegetarians (Table 4.33). However, regardless of their vegetarian status, mean plasma BPA of Chinese males were higher compared to Indian and other ethnic groups (Table 4.34). BPA was also found to be higher in vegetarian Indians compared to non-vegetarian Indians (Table 4.36). A possible explanation for higher BPA levels in vegetarian Indians might be due to other food sources and other exposure routes to BPA which are more relevant to their lifestyle. Other routes include river water, drinking water (Rodriguez-Mozaz *et al*., 2004), coastal waters (Basheer *et al*., 2004), residential air and dust (Rudel *et al*., 2001) which all had BPA levels detected in them. Coastal waters might involve seaweed which can be consumed by these vegetarians though data on BPA in seaweed is not available.

One study had reported on BPA amounts which can be transferred to the skin from thermal receipts paper (Biedermann *et al*., 2010). However, the experiment did not prove if any BPA was absorbed through the skin. Other study that supported the inevitable BPA exposure was the reported 17% of the Chinese population with no occupational exposure to BPA had levels of BPA detected in their serum (He *et al*., 2009).

The lower percentage of detection for endogenous sex hormones (oestrone and oestradiol) was due to the physiologically low levels of these hormones in the blood of males. Method in this study was only able to detect oestrone and oestradiol levels of 0.3 ng/ml and more.

5.3.2 Correlation of analytes

The high correlations between daidzein and genistein in both vegetarian and non-vegetarian suggests that both compounds were most likely from the same food source (Table 4.12). This is as what has been reported in local food source of isoflavone, which found fruits such as kuini, bambangan and bacang contained daidzein and genistein (Khoo & Ismail, 2008). Tempeh, a fermented soy product and readily available in local markets had total isoflavone content as high as 205 mg/100 g (Haron *et al*., 2009). Similarly for biochanin A and formononetin which were also highly correlated to one another. Less is known about the distribution of biochanin A and formononetin in edible sources in Malaysia.

In answering one of the research questions on whether presence of these exogenous oestrogenic EDCs affect the endogenous sex hormone levels, correlation study was carried out, bearing in mind that correlation does not indicate causality.

It was believed that EDCs play a role in preventing some reproductive cancers. Reproductive cancers are usually associated with an elevated level of endogenous sex

hormone. Therefore, this study analysed and looked for any possible relationship between EDCs and sex hormones by using correlation tables.

In this study among male vegetarians and non-vegetarians, it was found that there were many significant correlations between oestrogenic EDCs and endogenous sex hormones which proved there was an association between them.

Among the oestrogenic EDCs, formononetin had the most significant correlation pairs with the endogenous hormone. Formononetin, biochanin A, BPA, genistein and daidzein each had 32, 28, 25, 24 and 22 pair of correlations. Formononetin was mostly correlated with oestrone (15 correlation pairs) while BPA was mostly correlated with testosterone (13 correlation pairs). Of the three sex hormones, testosterone had the highest correlation pairs with daidzein (11 pairs), genistein (14 pairs), formononetin (11 pairs), biochanin A (10 pairs) and BPA (13 pairs). Testosterone had the highest number of correlation pairs with analytes in various groups (Table 4.33). There were 52, 23 and 61 significant correlations for oestrone, oestradiol and testosterone respectively. Of these, 3 correlations were in inverse relationship. Though most of these significant correlations were weak to low in its strength, there were also 8 of moderate positive correlations and 1 strong positive correlation.

The strong positive correlations found in Chinese non-vegetarians between daidzein and testosterone suggesting higher intake of daidzein tend to have a higher plasma level of testosterone. The same subject group also had a moderate correlation between biochanin A and testosterone (Table 4.32). As this study is a cross-sectional study, correlation result should not be interpreted as causality. Therefore, this suggests that non-vegetarian Chinese males tend to get higher plasma testosterone levels with consumption of daidzein and biochanin A.

It is interesting to note how a distinct pattern can be seen in correlations for vegetarians and non-vegetarians. Vegetarians were seen to have fewer significant

correlations than non-vegetarians. Only 3 analytes were correlated positively with testosterone in vegetarians (genistein, formononetin and BPA) while non-vegetarians had all but equol correlating positively with testosterone. Therefore, being vegetarians – they only need to be concerned of only three exogenous EDCs that can affect the endogenous testosterone levels. For non-vegetarians however, they need to be concerned with all exogenous EDCs except for equol, as high levels of exogenous EDCs are more likely to increase the natural testosterone levels in non-vegetarians. There was however, no significant correlation between equol and testosterone in non-vegetarians. This suggests that while vegetarians tend to have higher testosterone plasma levels with higher levels of genistein, formononetin and BPA, non-vegetarians tend to have higher testosterone plasma levels with higher levels of daidzein, genistein, formononetin, biochanin A and BPA. Therefore, vegetarian status alone, does not provide sufficient `protection' against endocrine related cancers as there are still variables that are associated with high plasma testosterone.

A similar pattern can be appreciated for correlations among equol producers and non-equol producers (Table 4.32). Equol producer subjects had no significant correlation except for 2 positive correlations of low strength which are formononetin-oestrone and BPA-oestrone. This was supported by findings in another study which supplemented 40 mg daily of isoflavone for 2 months in healthy men and resulted in no significant effect on gonadotrophin, sex hormone levels although the mean plasma concentration of genistein and daidzein reached 270.24 ng/ml and 127.12 ng/ml, respectively (Mitchell *et al*., 2001). In that study however, equol producer status of subjects involved was not reported.

Non-equol producers on the other hand, displayed several significant positive correlations of low and moderate strength. Considering correlation findings in both groups of equol producer and non-equol producers, this study suggests that an equol

producer could have a high isoflavone intake with high subsequent plasma levels and yet this would not significantly increase or decrease the level of endogenous testosterone.

An almost exact pattern can be seen with equol producers and non-equol producers among non-vegetarians (Table 4.32). In equol producers from non-vegetarians, there was no significant correlation at all. In contrast to that, non-equol producers from non-vegetarians, had all significant positive correlations except for correlation between equol and testosterone (r_s = -0.13). Though they are non-equol producers, there could still be significant amount of equol in their plasma had they taken food high in equol content such as dairy products, stinky tofu (Antignac *et al*., 2003; Abiru *et al*., 2012). This means that, for non-equol producers of non-vegetarians, it is more likely to have a lower testosterone level with higher equol levels. As one study reported, one's ability to produce equol is not associated with age, body mass index, consumption of yoghurt, dairy, fruit or American fast food (Hedlund *et al.*, 2005). This further supports suggestion from this present study that having plasma equol concentration is more likely to be beneficial. Even if one is a non-equol producer, rather than finding ways to convert an individual into becoming an equol producer, a faster and more practical way would be to just consume more of equol-rich foods. This finding further supports the notion that equol is protective against cancer as it is associated with a lower plasma testosterone in non-equol producers. Therefore, this result suggests that non-equol producers in non-vegetarians could benefit more by taking diet with high equol content and lowering food intake of high daidzein, genistein, formononetin and biochanin A content. Therefore, consumption of equol-rich food in non-vegetarians alone and consumption of equol-rich food in non-equol producers alone would not make a difference to the plasma testosterone level of these two subject groups. Instead,

consumption of equol-rich food will only be beneficial if the non-equol producers were among non-vegetarians, where it tends to result in a lower plasma testosterone level.

Another negative correlation between equol and testosterone was in non-equol producers among vegetarians. This suggests that this group, as with non-equol producers among non-vegetarians would also benefit by increasing consumption of equol-rich foods as this tends to be associated with a lower plasma testosterone level.

In one cross sectional study, BPA was shown to significantly decrease the free testosterone levels and increased the sex hormone binding globulins in men (Zhou *et al.*, 2013). Contrary to that study, no negative correlation was found between BPA and testosterone in this present study. BPA frequently correlated positively at moderate strength with oestrone (r_s = 0.50, 0.54, 0.60) in non-equol producers, Chinese subjects and those with less than 2 types of isoflavone. BPA also correlated positively with testosterone at weak to low strength in several groups which include non-vegetarians, non-equol producers, Indians, in those with less than 2 types of isoflavone, non-equol producers among vegetarians, Chinese vegetarians, Indian vegetarians, coffee drinking vegetarians, non-equol non-vegetarians and Indian non-vegetarians. Therefore, from this correlation study, it is concluded that plasma phytoestrogen levels do affect the level of sex hormones in adult males.

5.3.3 Comparison between subject groups

This study found ovovegetarians had 2 – 4 times higher level of BPA when compared to other sub-class of vegetarians (Table 4.33). However, this may not be significant since ovovegetarians were only represented by 3 subjects in this study. Plasma daidzein, genistein and equol levels in vegans of this study were also noted to be higher than levels detected among Japanese women: 25, 94.1 and 9.6 ng/ml respectively (Zhao *et al.*, 2006). The testosterone level of lactovegetarians in this study was almost

similar to levels detected among males in France (Dolomie-Fagour *et al*., 2008). In contrast to findings in this study, vegans in another study were reported not to have a higher level of free and total testosterone but a higher plasma level of sex hormone binding globulin (SHBG) instead when compared to omnivores (Key *et al.*, 1990). In another study, no difference was detected in free testosterone levels between meat eaters, vegetarians and vegans (Allen *et al*., 2000). Another study was also in contrast with findings in this study, reported of a decreased plasma concentration of sex hormones in vegetarians (Howie & Shultz, 1985). Howie & Shultz (1985) however, studied on only 12 vegetarians and 18 non-vegetarians. Although there are concerns about the diurnal variation of testosterone that might affect levels among subjects in this study, (Crawford *et al*, (2007) who studied 3006 male subjects on the influence of time at blood sampling provided evidence to assure that it is not a major factor that can contribute to the high variability of testosterone levels in men. Crawford *et al*, (2007) reported that testosterone levels of older men are stable from morning till early afternoon followed by a modest decline. Furthermore, standard recommendations of obtaining early morning blood samples for testosterone levels are based on reports from studies of fewer than 20 male subjects (Tenover & Bremner, 1991), (Diver *et al*., 2003).

This study found that the mean plasma concentration of all analytes showed higher values in vegetarians (Table 4.33). The differences were significant for all analytes, except formononetin, BPA and oestradiol. Vegetarian subjects in this study showed a higher concentration of isoflavones (36.3 ng/ml: daidzein and 82.2 ng/ml: genistein) than European vegetarians in another study which reported a mean of 20 ng/ml and 40 ng/ml for daidzein and genistein respectively (Peeters *et al*., 2007; Settimi *et al*., 2003). Although the levels in this study were higher than vegetarians among European populations, the mean concentrations of daidzein and genistein among vegetarians in this study were still 50 – 60 % lower than concentrations reported in Japanese people

(Morton *et al.*, 2002). Testosterone levels in this study for both vegetarian and non-vegetarians (equivalent to 10.4 nmol/L and 5.2 nmol/L) were 36 times higher than in elderly American men (Orwoll *et al*., 2006) and 104 times higher than adult Japanese males in their 30s (Nagata *et al.*, 2001) and comparable to French males whose range was 7.5 – 30.5 nmol/L as shown in Table 2.2 (Dolomie-Fagour *et al*., 2008).

Daidzein, genistein, equol, biochanin A were significantly higher by 1.6 to 4.6 magnitudes in vegetarians than non-vegetarians. Higher level of isoflavone among vegetarians is expected as it was reported in one study that 31 out of 35 vegetarian diets contained isoflavone (Clarke, 2003). The vegetarian diets consumed locally include tempeh, tofu and soymilk which contains 31 – 35 mg/100 g (Haron *et al*., 2009), 16.2 – 31.2 mg/100 g, 7.6 – 19.9 mg/100g of isoflavone (Molamma *et al.*, 2005), respectively.

Similarly, another study reported the total amount of daidzein and genistein in tofu and soymilk as 22.6 and 11.3 mg/100 g, respectively (Horn-Ross *et al.*, 2000). The daidzein content (9.4 – 10.5 mg/100 g) in local fruits of mangifera species such as kuini (*Mangifera odorata*), was found to be comparable to the amount found in miso soup and tofu (Khoo & Ismail, 2008). Miso soup, tofu and soymilk contain more than 10 mg/100 g of total daidzein and genistein with the highest content detected in tofu (Horn-Ross *et al*., 2000). Daidzein was also detected between 0.4 – 0.8 mg/100 g in other magifera species, such as bambangan (*Mangifera pajang*) and bacang (*Mangifera foetida*) (Khoo & Ismail, 2008).

Though in this study, no diet recall history was taken from the subjects, there is every possibility that among the diet intake of the subjects with high isoflavone levels would include tofu, soymilk, and various local fruits including kuini as these foods are easily obtained from the market, sold as cooked dishes in restaurants. Apart from soy-based products, a study has also reported traces of daidzein, genistein and biochanin A in beer (Clarke *et al.*, 2004). Vegetables and fruits such as grapefruit, cooked leeks and spring

onion which are frequently available in the local urban supermarkets have been reported to contain biochanin A of 99 – 163 μg/100 g while daidzein and genistein levels in French beans were 124 μg/100 g and 381 μg/100 g respectively (Kuhnle *et al.*, 2007). The availability of isoflavones in a variety of fresh foods from the market explains the reason for vegetarians and non-vegetarians in this present study to have a significant amount of plasma isoflavone levels.

The levels found in the food samples however, may only serve as a guide, as studies have shown there is a large variation of isoflavone levels between and within soy-based products (Hutabarat *et al.*, 2001) in which isoflavone composition, can be influenced by the crop year and growth location of the soybeans (Wang & Murphy, 1994).

Comparison of plasma concentrations among Chinese, Indians and other ethnic groups revealed significant differences for BPA and oestrone only. Similarly, there was no evidence of affected semen quality on low or high levels of soy isoflavone intake among healthy adult males (Beaton *et al.*, 2010).

5.3.4 Comparison between equol producer status

The ability to produce equol is considered a significant factor that distinguishes the various reports of isoflavone efficacy to health. Findings from several studies suggest that maximal clinical response to soy isoflavones are observed in good equol producers (Setchell, Brown, & Lydeking-Olsen, 2002). Setchell & Cole (2006) proposed a standardized method of defining one as an equol producer. His method requires measurement of urine and serum concentration of equol rather than just equol concentration in serum or in urine alone (Setchell & Cole, 2006). In this study however, equol concentration in urine was not measured.

Most studies did not differentitate the effects of isoflavone diet among equol producers and non-producers which Setchell *et al.*, (2002) hypothesized as a possible

cause of mixed results in phytoestrogen research. The new paradigm proposed by Setchell on the association between the ability to produce equol and the effectiveness of soy protein has led to comparison of results from this study against equol producer status of the subjects.

Following what has been done by Akaza *et al*., (2002) the LOQ of equol is taken as the cut-off point in defining whether one is an equol producer. For the purpose of this study, those with equol plasma concentration ≥ 0.5 ng/ml were regarded as equol producers. It was found that percentage of equol producers among female patients with breast disease in this study (Table 4.38) was almost similar to those found in prostate cancer patients in Japan - 29%, Korea - 30% and non-vegetarian adults (Setchell & Cole, 2006).

There was 41.7% and 24.6% of equol producers among vegetarian and non-vegetarian males respectively. This percentage was higher than the percentage of equol producers in American males reported to be less than 20% by Akaza *et al*., (2004). There were more equol producers in the vegetarian group especially vegans with 58.3% of them were able to produce equol (Figure 4.23). This strongly suggests the possibility that eating more vegetables and fibres increase the likelihood of having normal flora bacteria related to conversion of daidzein to equol.

Other studies which reported high percentage of equol producers were among vegetarians in Sydney (59%) and adult males in Korea (Akaza *et al*., 2004; Setchell & Cole, 2006). In addition to high fibre, a high intake of polyunsaturated fatty acids was also observed among young Swedish vegans (Larsson & Johansson, 2002). This is consistent with our findings that vegans have the highest percentage of equol producers as they are more likely to have a higher intake of polyunsaturated fatty acids and fibre.

The variable percentage of equol producers attributed to the kind of diet consumed by the different populations would eventually affect the diversity of the normal gut flora

in which some of the bacteria were responsible for the metabolism of daidzein into equol. More than 400 species of bacteria were estimated to be present in the human gut's normal flora (Berg, 1996). However, barely more than 20 bacteria strains in animals and humans have been identified as possessing the ability to transform isoflavones to equol (Setchell & Clerici, 2010).

Though a significant difference was observed among vegetarian and non-vegetarians, with vegetarians having a higher testosterone level, similar observation were not found for equol producer and non-equol producers (Table 4.37). There was no significant difference of testosterone levels nor oestrone and oestradiol between equol producers and non-equol producers. Equol producers however, had significantly higher genistein, equol and BPA levels than non-equol producers. Higher BPA levels among equol producers could possibly be due to BPA contamination from food and water intake of these equol producers.

Prostate cancer risk is increased with high levels of testosterone and low levels of sex hormone binding globulin (Gann *et al.*, 1996). As with one study which failed to demonstrate significant differences between equol producers and non-producers in cardiovascular disease outcomes (Vafeiadou *et al.*, 2006), this present study also failed to observe any significant difference in terms of testosterone levels between the two groups. Therefore, this study did not observe a sex hormone profile suggestive of being protective against prostate cancer risk in equol producers.

5.4 Study population of female patients with and without breast disease (plasma)

There were 252 plasma samples obtained from 109 female patients with breast disease and 143 from female patients without breast disease (Table 4.38). Majority of the subjects were Chinese, menopause and non-equol producers. There were only 24.8%

and 15.4% of equol producers from female patients with breast disease and female patients without breast disease respectively.

5.4.1 Analyte detection (plasma)

Percentage of analyte detection in the plasma of women with and without breast disease ranges 11 – 94.5% (Figure 4.24). Isoflavones were more frequently detected in women without breast disease while estrogens were more often detected in plasma of women with breast disease. Unlike the male population of vegetarians and non-vegetarians (Figure 4.22), percentage of BPA detection in plasma of female patients was much lower. Only 22% of patients with breast disease and 30.1% of patients without breast disease had BPA detected in their plasma sample.

5.4.2 Correlation of analytes (plasma)

There were more inverse relationships between oestrogenic EDCs and oestrogens in female population (Table 4.56) as compared to male population in vegetarians and non-vegetarians (Table 4.32). Breast cancer female with a higher plasma equol levels whether produced from daidzein metabolism or ingested from other foods suggests that having a high plasma equol level is beneficial as it tends to be associated with a lower plasma oestradiol levels. However, a higher plasma level of formononetin and biochanin A were in positive relationship with oestrogens rather than inverse. An increase in biochanin A or increase in BPA levels is more likely to be associated with a high oestradiol level (Table 4.41). Higher plasma equol however, is associated with a lower oestradiol level. Therefore, this suggests that breast cancer patients should avoid biochanin A and BPA, but increase intake in equol containing foods. However, the findings of the current study do not support two previous research which reported the absence of any relationship between dietary isoflavone and plasma oestradiol (Low *et*

al., 2005) as well as a study on postmenopausal women which failed to show any reduction of oestrogen levels after being subjected to 2 months of soyfood and very low fat diet (Wu, 2005).

Female equol producers had the most inverse relationship between oestrogenic EDCs and oestrogens. Female equol producers were more likely to have lower oestrogen levels with a higher intake of isoflavones. This suggests that being an equol producer has some protective effect as the endogenous oestrogen levels were lower with an increase in isoflavone intake and even BPA.

All three ethnic groups showed inverse relationships between oestrogenic EDCs and oestrogens. These inverse relationships were seen more commonly with formononetin and biochanin A rather than daidzein and genistein. In addition to formononetin and biochanin A, a higher plasma genistein concentration was also associated with a lower estradiol levels in Chinese females. Indian females however had a tendency for a higher oestradiol plasma concentration with an increase in plasma BPA level and a higher oestrone levels with an increase of plasma daidzein levels.

Among subsequent groups such as nulliparous women, non-nulliparous, those who had a history of lactation and those who did not, menopause and premenopause women; all showed an inverse relationship between biochanin A, formononetin and oestrogens ranging from low to moderate correlations.

5.4.3 Comparison between subject groups

Population of female patients who participated in this study showed very low mean plasma concentration levels for daidzein, genistein and equol (less than 7, 12 and 2 ng/ml) as compared to the population of female farmers in Japan with 4, 8 and 6 magnitude higher (Zhao *et al*., 2006). Participants in this study however had higher levels of daidzein and genistein than in cord blood samples from Malaysian mothers of

rural and urban populations (Mustafa *et al*., 2007). Low levels of daidzein and genistein (less than 5 ng/ml) were also reported among male and females in the UK population (Morton *et al*., 2002).

The difference in phytoestrogen levels is most probably due to the diverse dietary intake among these populations which differ among cultures and geographical locations. Among the reported dietary isoflavone intakes are 154 μg/d among American women (de Kleijn *et al.*, 2001), 4.7 mg/d in Singaporean women (Seow *et al.*, 1998), 33.4 mg/d in Chinese women (Chen *et al.*, 1999) and highest in Japanese women 46.5 mg/d (Arai *et al*., 2000).

Chan *et al*., (2007) reported a daily isoflavone intake of 7.8 mg among Hong Kong Chinese women aged 50 – 61 years old (Chan *et al*., 2007) which is slightly higher than Singaporean women. More than 30% of women without breast disease had 3 or more types of isoflavone detected in the plasma (Table 4.38). Majority of the patients without breast disease had 2 types of isoflavone while most of the patients with breast disease had just 1 type.

Comparison of plasma analyte concentrations were made between various subject groups: those with and without breast cancer, ethnic groups, nulliparous and non-nuliparous, premenopausal and menopausal groups as well as groups based on the number of isoflavone types of more or less than 2.

This study found that there was no difference in levels of oestrogenic EDCs and oestrogens between women with and without breast cancer, except for formononetin and biochanin A. Women without breast cancer showed a higher level of formononetin and biochanin A. This might be due to the wider selection of food intake in women who do not have breast cancer. For women with breast cancer, they probably have a generally lower food intake due to the constitutional symptoms they had such as loss of appetite as well as possibly being depressed which further worsens the appetite.

Among the three ethnic groups, this study found no difference for all analytes except for BPA which was higher in Chinese than Malay females. Therefore, Chinese females had more exposure to BPA than Malays, which came from various possible sources. One of the sources of BPA contamination would be canned foods, drinking water as other studies have proven the occurrence of BPA migration from the food container into the food itself.

This study involving Chinese, Malay and Indian patients does not suggest ethnic group as one of the factors affecting plasma phytoestrogen levels. This was in contrast with one study between Hispanics and non-Hispanics which found dietary phytoestrogen intake was higher in Hispanics than non-Hispanics (Carmichael *et al*., 2011).

Considering steps taken during analysis of samples to prevent contamination of BPA, levels obtained in our sample was considered high since BPA is not supposed to be in our natural diet. However, BPA can be found ubiquitously contaminating our food source. Various levels of BPA were reported in different matrices including infant milk powders (Kuo & Ding, 2004), river water (Rodriguez-Mozaz *et al*., 2004), aquatic creatures (Santhi *et al*., 2011) and aquicolous animals (Shao *et al*., 2007). Even babies were not spared from the harmful effects of BPA as evidence of BPA leaching from babies feeding teats into 37ºC water have been reported as high as 22.86 ng/g (Tan & Mustafa, 2003).

Non-nulliparous women had higher formononetin and estradiol than their nulliparous counterpart.

There was no significant difference of any analyte concentrations between menopause and premenopausal women in this study. Plasma BPA concentration for both menopause and premenopausal women in this study was 0.6 ng/ml which were almost similar with control group for a study on women with polycystic ovarian

syndrome (Takeuchi & Tsutsumi, 2002). Women in this study had lower plasma BPA concentrations than postmenopausal women with endometrial cancer which had serum BPA concentration of 1.4 ng/ml (Hiroi *et al.*, 2004), polycystic ovarian syndrome women with 1.04 ng/ml (Takeuchi & Tsutsumi, 2002), pregnant women with 2.5 ng/ml of BPA in the blood concentration and BPA serum of 2.5 ng/ml in healthy premenopausal Japanese women (Hiroi *et al*., 2004). Finding from this study of no significant difference in phytoestrogen levels between premenopausal and postmenopausal was consistent with another study by (Grace *et al.*, 2004) which reported mean oestradiol plasma level in post-menopausal women as 0.006 ng/ml. This study did not find any difference in plasma concentration of oestradiol nor oestrone between those with and without breast cancers (Table 4.57). The findings of this study do not support the previous research which which reported 29% higher plasma oestradiol concentration than controls in postmenopausal women with breast cancer (Thomas *et al.*, 1997). That research however had taken plasma oestradiol concentration in subjects 7-8 years prior to diagnosis while in the current study, plasma samples were taken after the diagnosis of a malignancy. One prospective study among Japanese women also supported the hypothesis of higher breast cancer risk for women with high level of biologically available oestradiol (Kabuto *et al.*, 2000; Nagata, Takatsuka, Inaba, *et al*., 1998), attempted to prove that soy consumption can reduce endogenous oestrogen levels. However, results of their study could not prove any significant findings mainly due to the small sample size used – 29 and 31 subjects for controls and cases respectively.

This study showed females who had more than 2 types of isoflavones detected had significantly higher mean plasma concentrations of daidzein, genistein, equol, formononetin and biochanin A than those who had 2 or less isoflavone types. The two groups however did not show any difference in mean concentrations of oestrogens.

5.4.4 Comparison between equol producer status

Some investigators had suggested a lowered breast cancer risk among equol producers, whom reflect a better hormonal profile (Duncan *et al*., 2000). Thus, determining the status of equol producer among study subjects have gained even more importance since.

In this study among the female patients, a higher percentage of equol producers were among patients with breast disease rather than those without breast disease (Table 4.38). Only 15.4% of patients without breast disease were equol producers as opposed to 24.8% among patients with breast disease. Percentage of equol producers among patients without breast disease was almost similar to the reported 14% of American control subjects were equol producers (Akaza *et al*., 2004). This lower percentage among patients without breast disease could partially be explained by the fact that a higher percentage (23.1%) of patients without breast disease had taken antibiotics in the past 3 months as compared to only 11.4% in patients with breast disease.

Factors affecting the ability to produce equol are not clearly established. However, type of intestinal bacteria, host genetics and diet are among factors frequently reported to play some role (Lampe, 2009). Equol is a metabolite of daidzein by intestinal bacteria (Atkinson *et al*., 2005). Therefore, any form of antibiotic treatment will affect the distribution of intestinal bacteria, consequently affect the ability of the individual to produce equol.

Another probable cause could be due to patients without breast disease whom might also be among patients with prolonged and chronic metabolic diseases with poor diet intake, on several medications which might affect the normal gut flora hence, influencing the ability to produce equol.

This study reported of 39 % of equol producers among postmenopausal women. Several associated factors were also observed among equol producers in

postmenopausal women which include the high intake of polyunsaturated fatty acids and alcohol (Bolca *et al.*, 2007). It is interesting to note that, although this study found oestrogenic EDCs were significantly in inverse correlation with oestrogens in equol producers, mean plasma concentrations of all analytes did not show any significant difference between equol producers and non-equol producers. This is probably due to the the correlations strength which were not strong enough, even so – correlation does not imply causation.

5.5 Study population of female patients with breast disease (breast tissue)

There were 53 samples with both biological samples of plasma and breast tissue available for statistical analysis. Majority of the subjects were Chinese, menopause and non-equol producers. Only 24.5% were equol producers. To ease discussion, Table 5.1 is provided to compare results of breast tissue concentrations (n = 53) of this study to other reported studies in the literature.

Mean concentration of daidzein from the malignant breast tissues in this study was 9 – 326 times lower than what was reported in normal breast tissues by (Bolca *et al*., 2010). Similarly for daidzein in breast tissue which was given 3 times of isoflavone tablets prior to obtaining breast tissue samples reported by Maubach *et al*., (2004). Breast tissue concentrations of genistein and equol were also very low as compared to other studies (Bolca *et al*., 2010; Maubach *et al*., 2004).

There was no available report of formononetin or biochanin A concentration in breast tissue samples at present to be compared to. BPA concentration in breast tissue samples from this study however were extremely high (almost 22 times higher) as compared to what was reported in female adipose tissue by Fernandez *et al*. (2007). In that research, adipose tissue was obtained from 20 women aged 24 – 81 years old from

Southeast Spain in the course of surgical treatment of malignant and benign breast disease.

Table 5.1: Comparison of current study results with other research

Analyte in breast tissue	This study (ng/g)	This study (pmol/g)	other studies
	Mean (S.D).	Mean (S.D).	Mean concentration /range
Daidzein	0.6 (2.64)	2.36 (10.38)	22.15 - 770.80 (pmol/g) (Bolca *et al*., 2010) 7.03 nmol/L (Maubach *et al*., 2004) 35.1 µg/g (Maubach *et al*., 2003)
Genistein	0.01 (0.103)	0.037 (0.38)	92.33 – 493.80 (pmol/g) (Bolca *et al*., 2010) 16.0 µg/g (Maubach *et al*., 2003)
Equol	0.01 (0.12)	0.041 (0.49)	681.7 µg/g (Maubach *et al*., 2003) 2.44 nmol/L (Maubach *et al*., 2004)
Formononetin	3.5 (23.18)	13.04 (86.40)	-
Biochanin A	0.7 (1.18)	2.46 (4.15)	-
BPA	69.3 (43.23)	303.57 (189.37)	3.16 (4.11)* (Fernandez *et al*., 2007) ND - 20.9* (Wang *et al*., 2015) 0.54 - 50.5** (Nahar *et al*., 2015) 1.36[#] (Nahar *et al*., 2015)
Oestrone	3.3 (3.59)	12.20 (13.27)	0.897 ng/g (Falk *et al*., 2008) 0.31 ng/g (Bonney *et al*., 1983) 0.047 ng/g (Geisler, 2003)
Oestradiol	0.9 (2.21)	3.30 (8.11)	0.114 ng/g (Falk *et al*., 2008) 0.48 ng/g (Bonney *et al*., 1983) 0.059 ng/g (Geisler, 2003)

* Adipose tissue ** Foetal liver [#]placenta

The concentrations of oestrone and oestradiol in the samples from this study were higher by 4 – 70, 2 – 15 times respectively than what was reported by (Falk *et al*., 2008; Bonney *et al*., 1983; Geisler, 2003). One possible reason for a high level of oestradiol was the local synthesis of oestradiol via the aromatase enzyme in the breast tissue itself (Geisler, 2003). Other possible reasons for the high oestrogen levels may be related to the type of diagnosis of the malignancy, stage of cancer and age of the subjects.

5.5.1 Analyte detection (breast tissue)

Breast tissue samples were divided into samples from premenopausal and menopausal women. In menopausal women, all analytes were detected in the breast tissue sample, with biochanin A being the most commonly detected (Figure 4.25). This is probably due to its lipophilic nature as biochanin A is the most lipophilic analyte among daidzein, genistein, equol and formononetin. All of the menopausal breast tissues had BPA detected while in premenopausal breast tissue, only 85.7% were positive for BPA.

5.5.2 Correlation of analytes (breast tissue)

Correlation of analytes (Table 4.64) indicates that when biochanin A concentration is high, BPA level is more likely to be lower. This suggests that biochanin A and BPA does not come from the same food source. Biochanin A can be found in food such as red clover, soy, alfalfa sprouts, peanuts, and chickpea (Medjakovic & Jungbauer, 2008). BPA however is an industrial chemical which is used to make polycarbonate. It is also found in epoxy resins which form the protective lining in metal-based cans for food and beverages (http://www.fda.gov/NewsEvents/PublicHealthFocus/ucm064437.htm).

Correlation of the breast tissue analytes were investigated in a few types of subject grouping such as ethnic groups, nulliparous and non-nulliparous, those with and without history of lactation, menopausal status as well as those with less or more than 2 types of isoflavone (Tables 4.67 – 4.77). However, no significant association was found between oestrogenic EDCs and oestrogens in those groups.

5.5.3 Comparison between subject groups

Median of analyte concentrations in the breast tissue were also compared in various groups as above (5.5.2). However, the findings of the current study do not support the

previous research. This study reported no significant difference in analyte concentration including oestrogen levels for breast tissue samples between menopause and premenopausal women. Previous research had found high levels of oestrogens in breast tumour samples in postmenopausal women (Yue *et al.*, 1998; Masamura *et al.*, 1997). Investigators explained that such finding could be due to factors such as uptake of oestrogens from the circulation, local oestrogen synthesis intratumour and oestrogen metabolism (Geisler, 2003).

Comparison among the three ethnic groups of Malay, Chinese and Indian showed a significant difference of the median concentration for BPA and no other analytes showed significant difference. Median BPA concentration in the breast tissue among the Indian subjects was higher than Chinese and Malays. This suggests there was a higher BPA exposure among the Indian subjects which could probably be related to their diet intake. The diet intake in Indians may have a higher BPA contamination than Malays and Chinese. This result may be explained by the fact that Indians in this study could have consumed more food that comes in packaging rather than fresh foods. Food packaging contains BPA and other industrial chemicals such as phthalates of which can leach into the food. A study reported that among families whose diet came from only fresh foods instead of packaged foods had a drop of 66% in the urinary BPA concentration (Rudel *et al.*, 2011). BPA was also found in low but significant amount (40 -100 mg/kg) in PVC stretch films used for food packaging (Lopez-Cervantes & Paseiro-Losada, 2003). Findings of this study could not conclude that ethnic group plays a role in endogenous oestrogens though other reviewed evidences are of the opinion that sex hormones level may be affected by environmental factors as well as by the different ethnic groups (Kendall *et al.*, 2007).

In this study, being nulliparous or non-nulliparous was found not to affect the median concentration of any analytes.

5.5.4 Comparison between equol producer status

This study found that equol producers had significantly higher concentration of formononetin in the breast tissue than non-equol producers. Formononetin like all phytoestrogens originate from plants. It can be found in leguminous plants, green beans and soy (Medjakovic & Jungbauer, 2008). Formononetin can also be found in black cohosh, a natural alternative to hormone replacement therapy for menopausal symptoms (Kennelly *et al.*, 2002). Unfortunately, this study did not have any information on the use of black cohosh among the 53 subjects involved.

5.5.5 Distribution of analytes in plasma and breast tissue

This study found that there were significantly higher plasma concentrations of daidzein, genistein and equol than in breast tissue. BPA and oestradiol concentrations on the other hand were significantly higher in breast tissue than plasma. This supports other studies which reported higher BPA levels in tissues with high fat content.

In this study, concentration of BPA in the breast tissue was 115 times higher than in plasma which strongly indicated the bioaccumulation nature of BPA. It is probable that continuous exposure through environment and dietary factors could pose a constant risk to these breast cancer patients despite some views which consoled that BPA exposure levels are low and negligible.

The highest reported BPA level in biological samples was 104.9 ng/g in placental tissue (Schonfelder *et al*., 2002) which exceeded the mean BPA level of breast tissue samples in the current study (69.3 ng/g). The BPA concentration in the breast tissue of this study were 16 times higher than mean concentrations found in umbilical cord tissue reported as 4.4 ng/g (Todaka & Mori, 2002).

BPA plasma concentration among the 53 subjects whose breast tissue were analysed was 0.6 ng/ml were very low compared to maternal plasma (18.9 ng/ml) and foetal plasma (9.2 ng/ml) reported by Schonfelder *et al*. (2002).

CHAPTER 6: CONCLUSION

This study sets out to determine if there are differences in the distribution of the oestrogenic EDCs and sex hormones between the blood and breast tissues, if the presence of exogenous compounds affect the endogenous sex hormones, if difference in analyte concentration exists between vegetarian and non-vegetarian subjects as well as among breast cancer and non-breast cancer patients. Analytical chemistry methods involving LCMSMS was developed and validated to determine concentration of analyte levels in plasma and breast tissue samples.

It was demonstrated that the single run of LCMSMS methods of analysing oestrogenic EDCs and oestrogens in both plasma and breast tissue samples allow sensitivity and detection limits without compromising chromatographic peak shape and resolution. This study provides a reliable analytical method suitable for high throughput simultaneous analysis of oestrogenic EDCs and oestrogens in plasma and breast tissue.

This study confirms there are differences in the distribution of oestrogenic EDCs and oestrogens between blood and breast tissue with BPA being one of the main findings showing an extremely high concentrated levels in breast tissue as compared to plasma. It also concludes that exogenous EDCs were significantly correlated with endogenous sex hormones with majority of the correlations of low and weak strength in both male and female populations in this study. It is also recommended that Chinese non-vegetarian males to reduce or avoid daidzein consumption as it is strongly correlated with high levels of testosterone. There were also significant differences of the analytes found in vegetarians and non-vegetarians, as well as in female breast cancer patients and non-breast cancer patients. Male vegetarians had higher level of testosterone than non-vegetarians, while male equol producers showed no difference in testosterone levels compared to non-equol producer males. Non-breast cancer females had higher levels of

formononetin and biochanin A than those with breast cancer. It was also observed that formononetin and biochanin A were more frequently in inverse correlation with endogenous oestrogens in female subjects than male subjects. Therefore, perhaps a more thorough study and special focus on formononetin and biochanin A could possibly provide new perspectives.

The most important limitation lies in the fact that not all EDCs present in the subjects were analysed in this study. The EDCs not studied could probably affect the resulting sex hormone levels measured, in a similar way as the EDCs studied or even in ways that are unexpected. It is however, impossible to analyse all EDCs that could possibly be present in the biological samples as humans are exposed to countless numbers of EDCs everyday. In addition, an increasing number of EDCs are being identified.

Another important limitation is the absence of a normal breast tissue sample as control samples. It is often difficult to obtain normal breast tissue samples here in Malaysia as compared to western countries. Most researchers obtained normal breast tissue samples from patients going for breast reduction surgery. In Malaysia, it is uncommon for such operations to be carried out. Any such operation will most probably be carried out in private hospitals.

As this study is a cross-sectional design, correlation findings are not to be regarded as causality to the outcome of the levels measured. Thus, a more definitive and different research design is more appropriate to answer subsequent advanced research questions from this study. This research finding however, will serve as a basis for future studies and its current findings shall add substantially to our understanding of EDCs and how it affected endogenous hormones in vari ous subject groups.

From this study, important recommendations to reduce the risk of having high EDC levels in the blood would be to implement some lifestyle changes. This include using BPA-free utensils, plastic containers, avoiding/reducing canned foods especially oily

food. Taking more of vegetables and dairy products that are more likely to contain equol.

Future recommendations for research would be to investigate on local foods that contain high equol, formononetin and biochanin A and to see its effects on the endogenous sex hormones prospectively. Another suggestion is to consider if being an equol producer or having high equol levels in the body could reduce the chances of BPA bioaccumulation.

REFERENCES

Abiru, Y., Kumemura, M., Ueno, T., Uchiyama, S., & Masaki, K. (2012). Discovery of an S-equol rich food stinky tofu, a traditional fermented soy product in Taiwan. *Int J Food Sci Nutr, 63*(8), 964-970.

Adlercreutz, H. (1995). Phytoestrogens: epidemiology and a possible role in cancer protection. *Environ Health Perspect, 103 Suppl 7*, 103-112.

Adlercreutz, H., Markkanen, H., & Watanabe, S. (1993). Plasma concentrations of phyto-oestrogens in Japanese men. *Lancet, 342*(8881), 1209-1210.

Adlercreutz, H., Yamada, T., Wahala, K., & Watanabe, S. (1999). Maternal and neonatal phytoestrogens in Japanese women during birth. *Am J Obstet Gynecol, 180*(3 Pt 1), 737-743.

Aedin Cassidy, S. B., and Kenneth DR Setchell. (1994). Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *The American Journal of Clinical Nutrition, 60*, 333-340.

Akaza, H., Miyanaga, N., Takashima, N., Naito, S., Hirao, Y., Tsukamoto, T., . . . Pantuck, A. J. (2004). Comparisons of percent equol producers between prostate cancer patients and controls: case-controlled studies of isoflavones in Japanese, Korean and American residents. *Jpn J Clin Oncol, 34*(2), 86-89.

Akaza, H., Miyanaga, N., Takashima, N., Naito, S., Hirao, Y., Tsukamoto, T., & Mori, M. (2002). Is daidzein non-metabolizer a high risk for prostate cancer? A case-controlled study of serum soybean isoflavone concentration. *Jpn J Clin Oncol, 32*(8), 296-300.

Al-Odaini, N. A., Zakaria, M. P., Yaziz, M. I., & Surif, S. (2010). Multi-residue analytical method for human pharmaceuticals and synthetic hormones in river water and sewage effluents by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *J Chromatogr A, 1217*(44), 6791-6806.

Allen, N. E., Appleby, P. N., Davey, G. K., & Key, T. J. (2000). Hormones and diet: low insulin-like growth factor-I but normal bioavailable androgens in vegan men. *Br J Cancer, 83*(1), 95-97.

Altavilla, D., Crisafulli, A., Marini, H., Esposito, M., D'Anna, R., Corrado, F., . . . Squadrito, F. (2004). Cardiovascular effects of the phytoestrogen genistein. *Curr Med Chem Cardiovasc Hematol Agents, 2*(2), 179-186.

Amin, K. H. I. (2008). Determination of daidzein and genistein contents in mangifera fruit. *Mal J Nutr, 14*(2), 189 - 198.

Anari, M. R., Bakhtiar, R., Zhu, B., Huskey, S., Franklin, R. B., & Evans, D. C. (2002). Derivatization of ethinylestradiol with dansyl chloride to enhance electrospray ionization: application in trace analysis of ethinylestradiol in rhesus monkey plasma. *Anal Chem, 74*(16), 4136-4144.

Andrade, A. J., Grande, S. W., Talsness, C. E., Grote, K., & Chahoud, I. (2006). A dose-response study following in utero and lactational exposure to di-(2-ethylhexyl)-phthalate (DEHP): non-monotonic dose-response and low dose effects on rat brain aromatase activity. *Toxicology, 227*(3), 185-192.

Angamuthu, M., & Ramalingam, A. (2011). Balanced key tree management for multi-privileged groups using (N, T) policy. *Security and Communication Networks*.

Anna H Wu, F. Z. S., Carmen Martinez, Chiu-Chen Tseng, Suzanne Hendrich, Patricia Murphy, Sukanya Chaikittisilpa, Daniel O Stram, and Malcolm C Pike. (2005). A controlled 2-mo dietary fat reduction and soy food supplementation study in postmenopausal women. *Am J Clin Nutr, 81*, 1133-1141.

Anna KrAJčoVá , V. S., Jaromír loJzA, ludmila KřížoVá, Jana hAJšloVá. (2010). Phytoestrogens in Bovine Plasma and Milk – LC-MS/MS Analysis. *Czech J. Food Sci., 28*, 264–274.

Annesley, T. M. (2003). Ion suppression in mass spectrometry. *Clin Chem, 49*(7), 1041-1044.

Antignac, J. P., Cariou, R., Le Bizec, B., Cravedi, J. P., & Andre, F. (2003). Identification of phytoestrogens in bovine milk using liquid chromatography/electrospray tandem mass spectrometry. *Rapid Commun Mass Spectrom, 17*(12), 1256-1264.

Antignac, J. P., Gaudin-Hirret, I., Naegeli, H., Cariou, R., Elliott, C., & Le Bizec, B. (2009). Multi-functional sample preparation procedure for measuring phytoestrogens in milk, cereals, and baby-food by liquid-chromatography tandem mass spectrometry with subsequent determination of their estrogenic activity using transcriptomic assay. *Anal Chim Acta, 637*(1-2), 55-63.

Anupongsanugool, E., Teekachunhatean, S., Rojanasthien, N., Pongsatha, S., & Sangdee, C. (2005). Pharmacokinetics of isoflavones, daidzein and genistein, after ingestion of soy beverage compared with soy extract capsules in postmenopausal Thai women. *BMC Clin Pharmacol, 5*(1), 2.

Arai, Y., Uehara, M., Sato, Y., Kimira, M., Eboshida, A., Adlercreutz, H., & Watanabe, S. (2000). Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. *J Epidemiol, 10*(2), 127-135.

Arditsoglou, A., & Voutsa, D. (2008). Determination of phenolic and steroid endocrine disrupting compounds in environmental matrices. *Environ Sci Pollut Res Int, 15*(3), 228-236.

Asimakopoulos, A.G., Wang, L., Thomaidis, N. S. & Kannan, K. (2014). A multiclass bioanalytical methodology for the determination of bisphenol A diglycidyl ethers, *p*-hydroxybenzoic acid esters, benzophenone-type ultraviolet filters, triclosan, and triclocarban in human urine by liquid chromatography tandem mass spectrometry. *J Chromatogr A, 15*(3), 1324, 141 - 148.

Atkinson, C., Frankenfeld, C. L., & Lampe, J. W. (2005). Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. *Exp Biol Med (Maywood), 230*(3), 155-170.

Aubertin-Leheudre, M., Gorbach, S., Woods, M., Dwyer, J. T., Goldin, B., & Adlercreutz, H. (2008). Fat/fiber intakes and sex hormones in healthy premenopausal women in USA. *J Steroid Biochem Mol Biol, 112*(1-3), 32-39.

Axelson, M., & Setchell, K. D. (1981). The excretion of lignans in rats -- evidence for an intestinal bacterial source for this new group of compounds. *FEBS Lett, 123*(2), 337-342.

Axelson, M., Sjovall, J., Gustafsson, B. E., & Setchell, K. D. (1982). Origin of lignans in mammals and identification of a precursor from plants. *Nature, 298*(5875), 659-660.

Baade, P. D., Youlden, D. R., Cramb, S. M., Dunn, J., & Gardiner, R. A. (2013). Epidemiology of prostate cancer in the Asia-Pacific region. *Prostate Int, 1*(2), 47-58.

Baglia, M. L., Gu, K., Zhang, X., Zheng, Y., Peng, P., Cai, H., . . . Shu, X. O. (2015). Soy isoflavone intake and bone mineral density in breast cancer survivors. *Cancer Causes Control, 26*(4), 571-580.

Bai, P. Y., Wittert, G. A., Taylor, A. W., Martin, S. A., Milne, R. W., & Shi, Z. (2015). The association of socio-demographic status, lifestyle factors and dietary patterns with total urinary phthalates in Australian men. *PLoS One, 10*(4), e0122140.

Band, P. R., Abanto, Z., Bert, J., Lang, B., Fang, R., Gallagher, R. P., & Le, N. D. (2011). Prostate cancer risk and exposure to pesticides in British Columbia farmers. *Prostate, 71*(2), 168-183.

Bao, J., Wang, M., Ning, X., Zhou, Y., He, Y., Yang, J., . . . Chen, B. (2015). Phthalate concentrations in personal care products and the cumulative exposure to female adults and infants in Shanghai. *J Toxicol Environ Health A, 78*(5), 325-341.

Barber, L. B., Loyo-Rosales, J. E., Rice, C. P., Minarik, T. A., & Oskouie, A. K. (2015). Endocrine disrupting alkylphenolic chemicals and other contaminants in wastewater treatment plant effluents, urban streams, and fish in the Great Lakes and Upper Mississippi River Regions. *Sci Total Environ, 517*, 195-206.

Barr, L., Metaxas, G., Harbach, C. A., Savoy, L. A., & Darbre, P. D. (2012). Measurement of paraben concentrations in human breast tissue at serial locations across the breast from axilla to sternum. *J Appl Toxicol*.

Basheer, C., Lee, H. K., & Tan, K. S. (2004). Endocrine disrupting alkylphenols and bisphenol-A in coastal waters and supermarket seafood from Singapore. *Mar Pollut Bull, 48*(11-12), 1161-1167.

Batley, G. E., Scammell, M. S., & Brockbank, C. I. (1992). The impact of the banning of tributyltin-based antifouling paints on the Sydney rock oyster, Saccostrea commercialis. *Sci Total Environ, 122*(3), 301-314.

Beaton, L. K., McVeigh, B. L., Dillingham, B. L., Lampe, J. W., & Duncan, A. M. (2010). Soy protein isolates of varying isoflavone content do not adversely affect semen quality in healthy young men. *Fertil Steril, 94*(5), 1717-1722.

Bedi, J. S., Gill, J. P., Aulakh, R. S., & Kaur, P. (2015). Pesticide Residues in Bovine Milk in Punjab, India: Spatial Variation and Risk Assessment to Human Health. *Arch Environ Contam Toxicol*.

Benetou, V., Lagiou, A., & Lagiou, P. (2015). Chemoprevention of cancer: current evidence and future prospects. *F1000Res, 4*(F1000 Faculty Rev), 916.

Berg, R. D. (1996). The indigenous gastrointestinal microflora. *Trends Microbiol, 4*(11), 430-435.

Biedermann, S., Tschudin, P., & Grob, K. (2010). Transfer of bisphenol A from thermal printer paper to the skin. *Anal Bioanal Chem, 398*(1), 571-576.

Bing Shao, R. Z., Juan Meng, Ying Xue, Guohua Wu, Jianying Hu, Xiaoming Tu. (2005). Simultaneous determination of residual hormonal chemicals in meat, kidney, liver tissues and milk by liquid chromatography–tandem mass spectrometry. *Analytica Chimica Acta, 548*, 41-50.

Bingham, S. A., Atkinson, C., Liggins, J., Bluck, L., & Coward, A. (1998). Phyto-oestrogens: where are we now? *Br J Nutr, 79*(5), 393-406.

Bishop, C. A., Brooks, R. J., Carey, J. H., Ng, P., Norstrom, R. J., & Lean, D. R. (1991). The case for a cause-effect linkage between environmental contamination and development in eggs of the common snapping turtle (Chelydra S.serpentina) from Ontario, Canada. *J Toxicol Environ Health, 33*(4), 521-547.

Blair, R. M., Appt, S. E., Franke, A. A., & Clarkson, T. B. (2003). Treatment with antibiotics reduces plasma equol concentration in cynomolgus monkeys (Macaca fascicularis). *J Nutr, 133*(7), 2262-2267.

Blonder, J., Johann, D. J., Veenstra, T. D., Xiao, Z., Emmert-Buck, M. R., Ziegler, R. G., Xu, X. (2008). Quantitation of steroid hormones in thin fresh frozen tissue sections. *Anal Chem, 80*(22), 8845-8852.

Bolca, S., Possemiers, S., Herregat, A., Huybrechts, I., Heyerick, A., De Vriese, S., . . . Van de Wiele, T. (2007). Microbial and dietary factors are associated with the equol producer phenotype in healthy postmenopausal women. *J Nutr, 137*(10), 2242-2246.

Bolca, S., Urpi-Sarda, M., Blondeel, P., Roche, N., Vanhaecke, L., Possemiers, S., . . . Depypere, H. (2010). Disposition of soy isoflavones in normal human breast tissue. *Am J Clin Nutr, 91*(4), 976-984.

Bolger, R., Wiese, T. E., Ervin, K., Nestich, S., & Checovich, W. (1998). Rapid screening of environmental chemicals for estrogen receptor binding capacity. *Environ Health Perspect, 106*(9), 551-557.

Bonney, R. C., Reed, M. J., Davidson, K., Beranek, P. A., & James, V. H. (1983). The relationship between 17 beta-hydroxysteroid dehydrogenase activity and oestrogen concentrations in human breast tumours and in normal breast tissue. *Clin Endocrinol (Oxf), 19*(6), 727-739.

Bonorden, M. J., Greany, K. A., Wangen, K. E., Phipps, W. R., Feirtag, J., Adlercreutz, H., & Kurzer, M. S. (2004). Consumption of Lactobacillus acidophilus and Bifidobacterium longum do not alter urinary equol excretion and plasma reproductive hormones in premenopausal women. *Eur J Clin Nutr, 58*(12), 1635-1642.

Borriello, S. P., Setchell, K. D., Axelson, M., & Lawson, A. M. (1985). Production and metabolism of lignans by the human faecal flora. *J Appl Bacteriol, 58*(1), 37-43.

Bouker, K. B., & Hilakivi-Clarke, L. (2000). Genistein: does it prevent or promote breast cancer? *Environ Health Perspect, 108*(8), 701-708.

Brossner, C., Petritsch, K., Fink, K., Auprich, M., Madersbacher, S., Adlercreutz, H., . . . Petritsch, P. (2004). Phytoestrogen tissue levels in benign prostatic hyperplasia and prostate cancer and their association with prostatic diseases. *Urology, 64*(4), 707-711.

Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., . . . Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature, 389*(6652), 753-758.

Calafat, A. M., Kuklenyik, Z., Reidy, J. A., Caudill, S. P., Ekong, J., & Needham, L. L. (2005). Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. *Environ Health Perspect, 113*(4), 391-395.

Calderon-Gierszal, E. L., & Prins, G. S. (2015). Directed Differentiation of Human Embryonic Stem Cells into Prostate Organoids In Vitro and its Perturbation by Low-Dose Bisphenol A Exposure. *PLoS One, 10*(7), e0133238.

Cao, X. L., & Corriveau, J. (2008). Survey of bisphenol A in bottled water products in Canada. *Food Addit Contam Part B Surveill, 1*(2), 161-164.

Cao, X. L., Corriveau, J., & Popovic, S. (2010). Bisphenol a in canned food products from canadian markets. *J Food Prot, 73*(6), 1085-1089.

Carmichael, S. L., Gonzalez-Feliciano, A. G., Ma, C., Shaw, G. M., & Cogswell, M. E. (2011). Estimated dietary phytoestrogen intake and major food sources among women during the year before pregnancy. *Nutr J, 10*, 105.

Cassidy, A., Bingham, S., & Setchell, K. (1995). Biological effects of isoflavones in young women: importance of the chemical composition of soyabean products. *Br J Nutr, 74*(4), 587-601.

Cassidy, A., Brown, J. E., Hawdon, A., Faughnan, M. S., King, L. J., Millward, J., . . . Setchell, K. D. (2006). Factors affecting the bioavailability of soy isoflavones in humans after ingestion of physiologically relevant levels from different soy foods. *J Nutr, 136*(1), 45-51.

Chalubinski, M., & Kowalski, M. L. (2006). Endocrine disrupters--potential modulators of the immune system and allergic response. *Allergy, 61*(11), 1326-1335.

Chan, C. C., Lam, H., Lee, Y. C., & Zhang, X. M. (2004). *Analytical method validation and instrument performance verification* USA: John Wiley & Sons, Inc. .

Chan, S. A., Lin, S. W., Yu, K. J., Liu, T. Y., & Fuh, M. R. (2006). Quantitative analysis of isoflavone aglycones in human serum by solid phase extraction and liquid chromatography-tandem mass spectrometry. *Talanta, 69*(4), 952-956.

Chan, S. G., Ho, S. C., Kreiger, N., Darlington, G., So, K. F., & Chong, P. Y. (2007). Dietary sources and determinants of soy isoflavone intake among midlife Chinese Women in Hong Kong. *J Nutr, 137*(11), 2451-2455.

Chapin, R. E., Adams, J., Boekelheide, K., Gray, L. E., Jr., Hayward, S. W., Lees, P. S., Woskie, S. R. (2008). NTP-CERHR expert panel report on the reproductive and developmental toxicity of bisphenol A. *Birth Defects Res B Dev Reprod Toxicol, 83*(3), 157-395.

Chen, G. W., Ding, W. H., Ku, H. Y., Chao, H. R., Chen, H. Y., Huang, M. C., & Wang, S. L. (2010). Alkylphenols in human milk and their relations to dietary habits in central Taiwan. *Food Chem Toxicol, 48*(7), 1939-1944.

Chen, J., Wu, S., Wen, S., Shen, L., Peng, J., Yan, C., . . . Wei, G. (2015). The Mechanism of Environmental Endocrine Disruptors (DEHP) Induces Epigenetic Transgenerational Inheritance of Cryptorchidism. *PLoS One, 10*(6), e0126403.

Chen, L. J., Zhao, X., Plummer, S., Tang, J., & Games, D. E. (2005). Quantitative determination and structural characterization of isoflavones in nutrition supplements by liquid chromatography-mass spectrometry. *J Chromatogr A, 1082*(1), 60-70.

Chen, Y. C., Chiang, C. I., Lin, R. S., Pu, Y. S., Lai, M. K., & Sung, F. C. (2005). Diet, vegetarian food and prostate carcinoma among men in Taiwan. *Br J Cancer, 93*(9), 1057-1061.

Chen, Z., Zheng, W., Custer, L. J., Dai, Q., Shu, X. O., Jin, F., & Franke, A. A. (1999). Usual dietary consumption of soy foods and its correlation with the excretion rate of isoflavonoids in overnight urine samples among Chinese women in Shanghai. *Nutr Cancer, 33*(1), 82-87.

Chetrite, G. S., Cortes-Prieto, J., Philippe, J. C., Wright, F., & Pasqualini, J. R. (2000). Comparison of estrogen concentrations, estrone sulfatase and aromatase activities in normal, and in cancerous, human breast tissues. *J Steroid Biochem Mol Biol, 72*(1-2), 23-27.

Chevalier, N., & Fenichel, P. (2015). Endocrine disruptors: new players in the pathophysiology of type 2 diabetes? *Diabetes Metab, 41*(2), 107-115.

Chou, W. C., Chen, J. L., Lin, C. F., Chen, Y. C., Shih, F. C., & Chuang, C. Y. (2011). Biomonitoring of bisphenol A concentrations in maternal and umbilical cord blood in regard to birth outcomes and adipokine expression: a birth cohort study in Taiwan. *Environ Health, 10*, 94.

Cirillo, T., Latini, G., Castaldi, M. A., Dipaola, L., Fasano, E., Esposito, F., . . . Cobellis, L. (2015). Exposure to di-2-ethylhexyl phthalate, di-n-butyl phthalate and bisphenol A through infant formulas. *J Agric Food Chem, 63*(12), 3303-3310.

Clarke, D. B., Barnes, K. A., & Lloyd, A. S. (2004). Determination of unusual soya and non-soya phytoestrogen sources in beer, fish products and other foods. *Food Addit Contam, 21*(10), 949-962.

Cornwell, T., Cohick, W., & Raskin, I. (2004). Dietary phytoestrogens and health. *Phytochemistry, 65*(8), 995-1016.

Costas, L., Infante-Rivard, C., Zock, J. P., Van Tongeren, M., Boffetta, P., Cusson, A., . . . de Sanjose, S. (2015). Occupational exposure to endocrine disruptors and lymphoma risk in a multi-centric European study. *Br J Cancer, 112*(7), 1251-1256.

Coward, L., Kirk, M., Albin, N., & Barnes, S. (1996). Analysis of plasma isoflavones by reversed-phase HPLC-multiple reaction ion monitoring-mass spectrometry. *Clin Chim Acta, 247*(1-2), 121-142.

Crawford, E. D., Barqawi, A. B., O' Donnell, C. & Morgentaler, A. (2007). The association of time of day and serum testosterone concentration in a large screening population. *BJU Int, 100*(3), 509-513.

D'Anna, R., Cannata, M. L., Marini, H., Atteritano, M., Cancellieri, F., Corrado, F., . . . Squadrito, F. (2009). Effects of the phytoestrogen genistein on hot flushes, endometrium, and vaginal epithelium in postmenopausal women: a 2-year randomized, double-blind, placebo-controlled study. *Menopause, 16*(2), 301-306.

Dai, S., Duan, J., Lu, Y., Cheng, J., Ren, J., Deng, W., & Wu, Y. (2004). Alpha-Zearalanol, a phytoestrogen for cardiovascular therapy. *Endocrine, 25*(2), 117-119.

de Kleijn, M. J., van der Schouw, Y. T., Wilson, P. W., Adlercreutz, H., Mazur, W., Grobbee, D. E., & Jacques, P. F. (2001). Intake of dietary phytoestrogens is low in postmenopausal women in the United States: the Framingham study(1-4). *J Nutr, 131*(6), 1826-1832.

de Lemos, M. (2002). Safety issues of soy phytoestrogens in breast cancer patients. *J Clin Oncol, 20*(13), 3040-3041; author reply 3041-3042.

de Lemos, M. L. (2001). Effects of soy phytoestrogens genistein and daidzein on breast cancer growth. *Ann Pharmacother, 35*(9), 1118-1121.

Debost-Legrand, A., Warembourg, C., Massart, C., Chevrier, C., Bonvallot, N., Monfort, C., Cordier, S. (2016). Prenatal exposure to persistent organic pollutants and organophosphate pesticides, and markers of glucose metabolism at birth. *Environ Res, 146*, 207-217.

Degen, G. H., & Bolt, H. M. (2000). Endocrine disruptors: update on xenoestrogens. *Int Arch Occup Environ Health, 73*(7), 433-441.

Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., . . . Gore, A. C. (2009). Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr Rev, 30*(4), 293-342.

Dighe, A. S., & Sluss, P. M. (2004). Improved detection of serum estradiol after sample extraction procedure. *Clin Chem, 50*(4), 764-766.

Diver, M. J., Imtiaz, K. E., Ahmad, A. M., Vora, J. P. & Fraser, W. D. (2003). Diurnal rhythms of serum total, free and bioavailable testosterone and of SHBG in middle-aged men compared with those in young men. *Clin Endocrinol (Oxf), 58*, 710-717

Dolomie-Fagour, L., Gatta, B., Nguyen, T. D., & Corcuff, J. B. (2008). Bioavailable estradiol in man: relationship with age and testosterone. *Clin Chim Acta, 398*(1-2), 145-147.

Don B. Clarke, K. A. B., Laurence Castle, Martin Rose, Lesley A. Wilson, Malcolm J. Baxter, Keith R. Price, M. Susan DuPont. (2003). Levels of phytoestrogens, inorganic trace-elements, natural toxicants and nitrate in vegetarian duplicate diets. *Food Chemistry, 81*, 287-300.

Dorgan, J. F., Fears, T. R., McMahon, R. P., Aronson Friedman, L., Patterson, B. H., & Greenhut, S. F. (2002). Measurement of steroid sex hormones in serum: a comparison of radioimmunoassay and mass spectrometry. *Steroids, 67*(3-4), 151-158.

Draisci, R., Palleschi, L., Ferretti, E., Marchiafava, C., Lucentini, L., & Cammarata, P. (1998). Quantification of 17 beta-estradiol residues in bovine serum by liquid chromatography-tandem mass spectrometry with atmospheric pressure chemical ionization. *Analyst, 123*(12), 2605-2609.

Duncan, A. M., Merz-Demlow, B. E., Xu, X., Phipps, W. R., & Kurzer, M. S. (2000). Premenopausal equol excretors show plasma hormone profiles associated with lowered risk of breast cancer. *Cancer Epidemiol Biomarkers Prev, 9*(6), 581-586.

Duncan, A. M., Underhill, K. E., Xu, X., Lavalleur, J., Phipps, W. R., & Kurzer, M. S. (1999). Modest hormonal effects of soy isoflavones in postmenopausal women. *J Clin Endocrinol Metab, 84*(10), 3479-3484.

Duong, C. N., Ra, J. S., Cho, J., Kim, S. D., Choi, H. K., Park, J. H., . . . Inam, E. (2010). Estrogenic chemicals and estrogenicity in river waters of South Korea and seven Asian countries. *Chemosphere, 78*(3), 286-293.

Duty, S. M., Calafat, A. M., Silva, M. J., Ryan, L., & Hauser, R. (2005). Phthalate exposure and reproductive hormones in adult men. *Hum Reprod, 20*(3), 604-610.

Falk, R. T., Gentzschein, E., Stanczyk, F. Z., Brinton, L. A., Garcia-Closas, M., Ioffe, O. B., & Sherman, M. E. (2008). Measurement of sex steroid hormones in breast adipocytes: methods and implications. *Cancer Epidemiol Biomarkers Prev, 17*(8), 1891-1895.

Fernandez, M. F., Arrebola, J. P., Taoufiki, J., Navalon, A., Ballesteros, O., Pulgar, R., . . . Olea, N. (2007). Bisphenol-A and chlorinated derivatives in adipose tissue of women. *Reprod Toxicol, 24*(2), 259-264.

Fernandez, M. F., Rivas, A., Olea-Serrano, F., Cerrillo, I., Molina-Molina, J. M., Araque, P., . . . Olea, N. (2004). Assessment of total effective xenoestrogen burden in adipose tissue and identification of chemicals responsible for the combined estrogenic effect. *Anal Bioanal Chem, 379*(1), 163-170.

Fernandez, S. V., & Russo, J. (2010). Estrogen and xenoestrogens in breast cancer. *Toxicol Pathol, 38*(1), 110-122.

Ferrara, F., Ademollo, N., Orru, M. A., Silvestroni, L., & Funari, E. (2011). Alkylphenols in adipose tissues of Italian population. *Chemosphere, 82*(7), 1044-1049. doi: 10.1016/j.chemosphere.2010.10.064

Foster, W. G., Chan, S., Platt, L., & Hughes, C. L., Jr. (2002). Detection of phytoestrogens in samples of second trimester human amniotic fluid. *Toxicol Lett, 129*(3), 199-205.

Franke, A. A., Custer, L. J., & Tanaka, Y. (1998). Isoflavones in human breast milk and other biological fluids. *Am J Clin Nutr, 68*(6 Suppl), 1466S-1473S.

Frankenfeld, C. L. (2011). O-desmethylangolensin: the importance of equol's lesser known cousin to human health. *Adv Nutr, 2*(4), 317-324.

Frankenfeld, C. L., Atkinson, C., Wahala, K., & Lampe, J. W. (2014). Obesity prevalence in relation to gut microbial environments capable of producing equol or O-desmethylangolensin from the isoflavone daidzein. *Eur J Clin Nutr, 68*(4), 526-530.

Fukutake, M., Takahashi, M., Ishida, K., Kawamura, H., Sugimura, T., & Wakabayashi, K. (1996). Quantification of genistein and genistin in soybeans and soybean products. *Food Chem Toxicol, 34*(5), 457-461.

Gaido, K. W., Leonard, L. S., Maness, S. C., Hall, J. M., McDonnell, D. P., Saville, B., & Safe, S. (1999). Differential interaction of the methoxychlor metabolite 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane with estrogen receptors alpha and beta. *Endocrinology, 140*(12), 5746-5753.

Gann, P. H., Hennekens, C. H., Ma, J., Longcope, C., & Stampfer, M. J. (1996). Prospective study of sex hormone levels and risk of prostate cancer. *J Natl Cancer Inst, 88*(16), 1118-1126.

Ganry, O. (2005). Phytoestrogens and prostate cancer risk. *Prev Med, 41*(1), 1-6.

Gao, S., Zhang, Z. P., & Karnes, H. T. (2005). Sensitivity enhancement in liquid chromatography/atmospheric pressure ionization mass spectrometry using derivatization and mobile phase additives. *J Chromatogr B Analyt Technol Biomed Life Sci, 825*(2), 98-110.

Gao, X., Yu, L., Castro, L., Moore, A. B., Hermon, T., Bortner, C., . . . Dixon, D. (2010). An endocrine-disrupting chemical, fenvalerate, induces cell cycle progression and collagen type I expression in human uterine leiomyoma and myometrial cells. *Toxicol Lett, 196*(3), 133-141.

Garcia-Jimenez, J. F., Valencia, M. C., & Capitan-Vallvey, L. F. (2007). Simultaneous determination of antioxidants, preservatives and sweetener additives in food and cosmetics by flow injection analysis coupled to a monolithic column. *Anal Chim Acta, 594*(2), 226-233.

Geisler, J. (2003). Breast cancer tissue estrogens and their manipulation with aromatase inhibitors and inactivators. *J Steroid Biochem Mol Biol, 86*(3-5), 245-253.

Geisler, J., Berntsen, H., & Lonning, P. E. (2000). A novel HPLC-RIA method for the simultaneous detection of estrone, estradiol and estrone sulphate levels in breast cancer tissue. *J Steroid Biochem Mol Biol, 72*(5), 259-264.

Genuis, S. J., Beesoon, S., Birkholz, D., & Lobo, R. A. (2012). Human excretion of bisphenol A: blood, urine, and sweat (BUS) study. *J Environ Public Health, 2012*, 185731.

Goodman, M. T., Wilkens, L. R., Hankin, J. H., Lyu, L. C., Wu, A. H., & Kolonel, L. N. (1997). Association of soy and fiber consumption with the risk of endometrial cancer. *Am J Epidemiol, 146*(4), 294-306.

Grace, P. B., Taylor, J. I., Low, Y. L., Luben, R. N., Mulligan, A. A., Botting, N. P., Bingham, S. A. (2004). Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European prospective investigation of cancer and nutrition-norfolk. *Cancer Epidemiol Biomarkers Prev, 13*(5), 698-708.

Griffith, A. P., & Collison, M. W. (2001). Improved methods for the extraction and analysis of isoflavones from soy-containing foods and nutritional supplements by reversed-phase high-performance liquid chromatography and liquid chromatography-mass spectrometry. *J Chromatogr A, 913*(1-2), 397-413.

Guo, T., Gu, J., Soldin, O. P., Singh, R. J., & Soldin, S. J. (2008). Rapid measurement of estrogens and their metabolites in human serum by liquid chromatography-tandem mass spectrometry without derivatization. *Clin Biochem, 41*(9), 736-741.

Guo, Y. & Kannan, K. (2012). Challenges encountered in the analysis of phthalate esters in foodstuffs and other biological matrices. *Anal Bioanal Chem, 404*, 2539-2554.

Guo, Y. & Kannan, K. (2013). A survey of phthalates and parabens in personal care products from the United States and its implications for human exposure. *Enviro Sci Technol, 47*, 14442-14449.

Guo, Y. & Kannan, K. (2014). Urinary concentrations of phthalates in couples planning pregnancy and its association with 8-hydroxy-2'-deoxyguanosine, a biomarker of oxidativec stress : longitudinal investigation of fertility and the environment study. *Enviro Sci Technol, 48*, 9804-9811.

Hanna, K. L., O'Neill, S., & Lyons-Wall, P. M. (2010). Intake of isoflavone and lignan phytoestrogens and associated demographic and lifestyle factors in older Australian women. *Asia Pac J Clin Nutr, 19*(4), 540-549.

Haron, H., Ismail, A., Azlan, A., Shahar, S., & Loh, S. P. (2009). Daidzein and genestein contents in tempeh and selected soy products. *Food Chem, 115*, 1350-1356.

Hauser, B., Deschner, T., & Boesch, C. (2008). Development of a liquid chromatography-tandem mass spectrometry method for the determination of 23 endogenous steroids in small quantities of primate urine. *J Chromatogr B Analyt Technol Biomed Life Sci, 862*(1-2), 100-112.

He, L., Fan, S., Muller, K., Hu, G., Huang, H., Zhang, X., . . . Wang, H. (2015). Biochar reduces the bioavailability of di-(2-ethylhexyl) phthalate in soil. *Chemosphere*.

He, Y., Miao, M., Herrinton, L. J., Wu, C., Yuan, W., Zhou, Z., & Li, D. K. (2009). Bisphenol A levels in blood and urine in a Chinese population and the personal factors affecting the levels. *Environ Res, 109*(5), 629-633.

Hedlund, T. E., Maroni, P. D., Ferucci, P. G., Dayton, R., Barnes, S., Jones, K., . . . Gray, K. J. (2005). Long-term dietary habits affect soy isoflavone metabolism and accumulation in prostatic fluid in caucasian men. *J Nutr, 135*(6), 1400-1406.

Hertrampf, T., Schmidt, S., Seibel, J., Laudenbach-Leschowsky, U., Degen, G. H., & Diel, P. (2006). Effects of genistein on the mammary gland proliferation of adult ovariectomised Wistar rats. *Planta Med, 72*(4), 304-310.

Hewitt, S. C., Harrell, J. C., & Korach, K. S. (2005). Lessons in estrogen biology from knockout and transgenic animals. *Annu Rev Physiol, 67*, 285-308.

Higashi, T., Yamauchi, A., Shimada, K., Koh, E., Mizokami, A., & Namiki, M. (2005). Determination of prostatic androgens in 10 mg of tissue using liquid chromatography-tandem mass spectrometry with charged derivatization. *Anal Bioanal Chem, 382*(4), 1035-1043.

Hiroi, H., Tsutsumi, O., Takeuchi, T., Momoeda, M., Ikezuki, Y., Okamura, A., . . . Taketani, Y. (2004). Differences in serum bisphenol a concentrations in premenopausal normal women and women with endometrial hyperplasia. *Endocr J, 51*(6), 595-600.

Hohenadel, K., Harris, S. A., McLaughlin, J. R., Spinelli, J. J., Pahwa, P., Dosman, J. A., Blair, A. (2011). Exposure to multiple pesticides and risk of non-Hodgkin lymphoma in men from six Canadian provinces. *Int J Environ Res Public Health, 8*(6), 2320-2330.

Horiguchi, T. (2006). Masculinization of female gastropod mollusks induced by organotin compounds, focusing on mechanism of actions of tributyltin and triphenyltin for development of imposex. *Environ Sci, 13*(2), 77-87.

Horn-Ross, P. L., Barnes, S., Lee, M., Coward, L., Mandel, J. E., Koo, J., . . . Smith, M. (2000). Assessing phytoestrogen exposure in epidemiologic studies: development of a database (United States). *Cancer Causes Control, 11*(4), 289-298.

Howie, B. J., & Shultz, T. D. (1985). Dietary and hormonal interrelationships among vegetarian Seventh-Day Adventists and nonvegetarian men. *Am J Clin Nutr, 42*(1), 127-134.

Hsieh, C. Y., Santell, R. C., Haslam, S. Z., & Helferich, W. G. (1998). Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells in vitro and in vivo. *Cancer Res, 58*(17), 3833-3838.

Huang, Y., Wang, X. L., Zhang, J. W., & Wu, K. S. (2014). Impact of endocrine-disrupting chemicals on reproductive function in zebrafish (Danio rerio). *Reprod Domest Anim, 50*(1), 1-6.

Hurt, K., Hurd-Brown, T., & Whalen, M. (2013). Tributyltin and dibutyltin alter secretion of tumor necrosis factor alpha from human natural killer cells and a mixture of T cells and natural killer cells. *J Appl Toxicol, 33*(6), 503-510.

Hutabarat, L. S., Greenfield, H., & Mulholland, M. (2001). Isoflavones and coumestrol in soybeans and soybean products from Australia and Indonesia. *J Food Composition and Analysis, 14*, 43-58.

Ikezuki, Y., Tsutsumi, O., Takai, Y., Kamei, Y., & Taketani, Y. (2002). Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. *Hum Reprod, 17*(11), 2839-2841.

Inoue, K., Wada, M., Higuchi, T., Oshio, S., Umeda, T., Yoshimura, Y., & Nakazawa, H. (2002). Application of liquid chromatography-mass spectrometry to the quantification of bisphenol A in human semen. *J Chromatogr B Analyt Technol Biomed Life Sci, 773*(2), 97-102.

Isobe, T., Shiraishi, H., Yasuda, M., Shinoda, A., Suzuki, H., & Morita, M. (2003). Determination of estrogens and their conjugates in water using solid-phase extraction followed by liquid chromatography-tandem mass spectrometry. *J Chromatogr A, 984*(2), 195-202.

Jasuja, G. K., Travison, T. G., Davda, M., Rose, A. J., Zhang, A., Kushnir, M. M., . . . Bhasin, S. (2013). Circulating estrone levels are associated prospectively with diabetes risk in men of the Framingham Heart Study. *Diabetes Care, 36*(9), 2591-2596.

Jensen, E. V. (1962). On the mechanism of estrogen action. *Perspect Biol Med, 6*, 47-59.

Jin, Y., Lin, X., Miao, W., Wang, L., Wu, Y., & Fu, Z. (2014). Oral exposure of pubertal male mice to endocrine-disrupting chemicals alters fat metabolism in adult livers. *Environ Toxicol*.

Joos, P. E., & Van Ryckeghem, M. (1999). Liquid chromatography-tandem mass spectrometry of some anabolic steroids. *Anal Chem, 71*(20), 4701-4710.

Jose L. Penalvo, T. N., Herman Adlercreutz. (2004). A simplified HPLC method for total isoflavones in soy products. *Food Chemistry, 87*, 297-305.

Jurek, A., & Leitner, E. (2015). Comparing different gas chromatographic methods for the quantification of bisphenol A (BPA) trace levels in paper and cardboard products from the market. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 32*(8), 1331-1342.

Kabuto, M., Akiba, S., Stevens, R. G., Neriishi, K., & Land, C. E. (2000). A prospective study of estradiol and breast cancer in Japanese women. *Cancer Epidemiol Biomarkers Prev, 9*(6), 575-579.

Kalfa, N., Paris, F., Philibert, P., Orsini, M., Broussous, S., Fauconnet-Servant, N., Sultan, C. (2015). Is Hypospadias Associated with Prenatal Exposure to Endocrine Disruptors? A French Collaborative Controlled Study of a Cohort of 300 Consecutive Children Without Genetic Defect. *Eur Urol*.

Kavlock, R. J., Daston, G. P., DeRosa, C., Fenner-Crisp, P., Gray, L. E., Kaattari, S., . . . Tilson, H. A. (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. *Environ Health Perspect, 104 Suppl 4*, 715-740.

Kawaguchi, M., Inoue, K., Yoshimura, M., Ito, R., Sakui, N., Okanouchi, N., & Nakazawa, H. (2004). Determination of bisphenol A in river water and body fluid samples by stir bar sorptive extraction with in situ derivatization and thermal desorption-gas chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci, 805*(1), 41-48.

Kawato, S. (2004). Endocrine disrupters as disrupters of brain function: a neurosteroid viewpoint. *Environ Sci, 11*(1), 1-14.

Kelly, G. E., Nelson, C., Waring, M. A., Joannou, G. E., & Reeder, A. Y. (1993). Metabolites of dietary (soya) isoflavones in human urine. *Clin Chim Acta, 223*(1-2), 9-22.

Kendall, A., Folkerd, E. J., & Dowsett, M. (2007). Influences on circulating oestrogens in postmenopausal women: relationship with breast cancer. *J Steroid Biochem Mol Biol, 103*(2), 99-109.

Kennelly, E. J., Baggett, S., Nuntanakorn, P., Ososki, A. L., Mori, S. A., Duke, J., . . . Kronenberg, F. (2002). Analysis of thirteen populations of black cohosh for formononetin. *Phytomedicine, 9*(5), 461-467.

Key, T. J., Roe, L., Thorogood, M., Moore, J. W., Clark, G. M., & Wang, D. Y. (1990). Testosterone, sex hormone-binding globulin, calculated free testosterone, and oestradiol in male vegans and omnivores. *Br J Nutr, 64*(1), 111-119.

Khoo, H. E., Jr., & Ismail, A. (2008). Determination of daidzein and genistein contents in mangifera fruit. *Malays J Nutr, 14*(2), 189-198.

Kim, E. Y., Shin, J. Y., Park, Y. J., & Kim, A. K. (2014). Equol induces mitochondria-mediated apoptosis of human cervical cancer cells. *Anticancer Res, 34*(9), 4985-4992.

Kim, J., & Kwon, C. (2001). Estimated dietary isoflavone intake of Korean population based on National Nutrition Survey. *Nutr Res, 21*(7), 947-953.

Kim, S., Lee, J., Park, J., Kim, H. J., Cho, G., Kim, G. H., . . . Choi, K. (2015). Concentrations of phthalate metabolites in breast milk in Korea: estimating exposure to phthalates and potential risks among breast-fed infants. *Sci Total Environ, 508*, 13-19.

Kimira, M., Arai, Y., Shimoi, K., & Watanabe, S. (1998). Japanese intake of flavonoids and isoflavonoids from foods. *J Epidemiol, 8*(3), 168-175.

Kirk, P., Patterson, R. E., & Lampe, J. (1999). Development of a soy food frequency questionnaire to estimate isoflavone consumption in US adults. *J Am Diet Assoc, 99*(5), 558-563.

Kiyama, R., & Wada-Kiyama, Y. (2015). Estrogenic endocrine disruptors: Molecular mechanisms of action. *Environ Int, 83*, 11-40.

Kortenkamp, A. (2007). Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. *Environ Health Perspect, 115 Suppl 1*, 98-105.

Kuhnle, G. G., Dell'Aquila, C., Aspinall, S. M., Runswick, S. A., Mulligan, A. A., & Bingham, S. A. (2008). Phytoestrogen content of foods of animal origin: dairy products, eggs, meat, fish, and seafood. *J Agric Food Chem, 56*(21), 10099-10104.

Kuhnle, G. G., Dell'aquila, C., Low, Y. L., Kussmaul, M., & Bingham, S. A. (2007). Extraction and quantification of phytoestrogens in foods using automated solid-phase extraction and LC/MS/MS. *Anal Chem, 79*(23), 9234-9239.

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., & Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A, 93*(12), 5925-5930.

Kuo, H. W., & Ding, W. H. (2004). Trace determination of bisphenol A and phytoestrogens in infant formula powders by gas chromatography-mass spectrometry. *J Chromatogr A, 1027*(1-2), 67-74.

Kurahashi, N., Iwasaki, M., Sasazuki, S., Otani, T., Inoue, M., & Tsugane, S. (2007). Soy product and isoflavone consumption in relation to prostate cancer in Japanese men. *Cancer Epidemiol Biomarkers Prev, 16*(3), 538-545.

Kushnir, M. M., Neilson, R., Roberts, W. L., & Rockwood, A. L. (2004). Cortisol and cortisone analysis in serum and plasma by atmospheric pressure photoionization tandem mass spectrometry. *Clin Biochem, 37*(5), 357-362.

Kushnir, M. M., Rockwood, A. L., Bergquist, J., Varshavsky, M., Roberts, W. L., Yue, B., . . . Meikle, A. W. (2008). High-sensitivity tandem mass spectrometry assay for serum estrone and estradiol. *Am J Clin Pathol, 129*(4), 530-539.

Kushnir, M. M., Rockwood, A. L., Roberts, W. L., Yue, B., Bergquist, J., & Meikle, A. W. (2011). Liquid chromatography tandem mass spectrometry for analysis of steroids in clinical laboratories. *Clin Biochem, 44*(1), 77-88.

Lampe, J. W. (2009). Is equol the key to the efficacy of soy foods? *Am J Clin Nutr, 89*(5), 1664S-1667S.

Lampe, J. W., Karr, S. C., Hutchins, A. M., & Slavin, J. L. (1998). Urinary equol excretion with a soy challenge: influence of habitual diet. *Proc Soc Exp Biol Med, 217*(3), 335-339.

Lange, C., Kuch, B., & Metzger, J. W. (2014). Estrogenic activity of constituents of underarm deodorants determined by E-Screen assay. *Chemosphere, 108*, 101-106.

Larsson, C. L., & Johansson, G. K. (2002). Dietary intake and nutritional status of young vegans and omnivores in Sweden. *Am J Clin Nutr, 76*(1), 100-106.

Lee, D. H., Steffes, M., & Jacobs, D. R. (2007). Positive associations of serum concentration of polychlorinated biphenyls or organochlorine pesticides with self-reported arthritis, especially rheumatoid type, in women. *Environ Health Perspect, 115*(6), 883-888.

Lee, H., Yamanouchi, K., & Nishihara, M. (2006). Effects of perinatal exposure to phthalate/adipate esters on hypothalamic gene expression and sexual behavior in rats. *J Reprod Dev, 52*(3), 343-352.

Lee, J. S., Ettinger, B., Stanczyk, F. Z., Vittinghoff, E., Hanes, V., Cauley, J. A., . . . Cummings, S. R. (2006). Comparison of methods to measure low serum estradiol levels in postmenopausal women. *J Clin Endocrinol Metab, 91*(10), 3791-3797.

Legler, J. (2008). New insights into the endocrine disrupting effects of brominated flame retardants. *Chemosphere, 73*(2), 216-222.

Leong, B. D., Chuah, J. A., Kumar, V. M., & Yip, C. H. (2007). Breast cancer in Sabah, Malaysia: a two year prospective study. *Asian Pac J Cancer Prev, 8*(4), 525-529.

Lewis, J. B., Lapp, C. A., Schafer, T. E., Wataha, J. C., Randol, T. M., & Schuster, G. S. (2000). 4-Hydroxytamoxifen-induced cytotoxicity and bisphenol A: competition for estrogen receptors in human breast cancer cell lines. *In Vitro Cell Dev Biol Anim, 36*(5), 320-326.

Li, C. T., Cheng, C. Y., & Ding, W. H. (2008). Determination of alkylphenol residues in baby-food purees by steam distillation extraction and gas chromatography-mass spectrometry. *Food Chem Toxicol, 46*(2), 803-807.

Li, D., Hu, Y., Shen, X., Dai, X., & Han, X. (2010). Combined effects of two environmental endocrine disruptors nonyl phenol and di-n-butyl phthalate on rat Sertoli cells in vitro. *Reprod Toxicol, 30*(3), 438-445.

Liao, C., & Kannan, K. (2012). Determination of free and conjugated forms of bisphenol A in human urine and serum by liquid chromatography tandem mass spectrometry. *Enviro Sci Technol, 46*, 5003-5009.

Liao, C., & Kannan, K. (2013). Concentrations and profiles of bisphenol A and other bisphenol analogues in foodstuffs from the United States and their implications for human exposure. *J Agric Food Chem, 61*, 4655-4662.

Limam, A., Talorete, T. P., Ali, M. B., Kawano, M., Jenhani, A. B., Abe, Y., . . . Isoda, H. (2007). Assessment of estrogenic activity in Tunisian water and wastewater by E-screen assay. *Environ Sci, 14 Suppl*, 43-52.

Lissin, L. W., & Cooke, J. P. (2000). Phytoestrogens and cardiovascular health. *J Am Coll Cardiol, 35*(6), 1403-1410.

Liu, W. H., Chiu, Y. W., Huang, D. J., Liu, M. Y., Lee, C. C., & Liu, L. L. (2006). Imposex in the golden apple snail Pomacea canaliculata in Taiwan. *Sci Total Environ, 371*(1-3), 138-143.

Lopez-Cervantes, J., & Paseiro-Losada, P. (2003). Determination of bisphenol A in, and its migration from, PVC stretch film used for food packaging. *Food Addit Contam, 20*(6), 596-606.

Low, Y. L., Taylor, J. I., Grace, P. B., Dowsett, M., Scollen, S., Dunning, A. M., . . . Bingham, S. A. (2005). Phytoestrogen exposure correlation with plasma estradiol in postmenopausal women in European Prospective Investigation of Cancer and Nutrition-Norfolk may involve diet-gene interactions. *Cancer Epidemiol Biomarkers Prev, 14*(1), 213-220.

Magee, P. J., Allsopp, P., Samaletdin, A., & Rowland, I. R. (2014). Daidzein, R-(+)equol and S-(-)equol inhibit the invasion of MDA-MB-231 breast cancer

cells potentially via the down-regulation of matrix metalloproteinase-2. *Eur J Nutr, 53*(1), 345-350.

Mahalko, J. R., Sandstead, H. H., Johnson, L. K., Inman, L. F., Milne, D. B., Warner, R. C., & Haunz, E. A. (1984). Effect of consuming fiber from corn bran, soy hulls, or apple powder on glucose tolerance and plasma lipids in type II diabetes. *Am J Clin Nutr, 39*(1), 25-34.

Main, K. M., Mortensen, G. K., Kaleva, M. M., Boisen, K. A., Damgaard, I. N., Chellakooty, M., Skakkebaek, N. E. (2006). Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. *Environ Health Perspect, 114*(2), 270-276.

Makela, S., Poutanen, M., Kostian, M. L., Lehtimaki, N., Strauss, L., Santti, R., & Vihko, R. (1998). Inhibition of 17beta-hydroxysteroid oxidoreductase by flavonoids in breast and prostate cancer cells. *Proc Soc Exp Biol Med, 217*(3), 310-316.

Man Ho Choi, K. R. K. a. B. C. C. (2000). Determination of estrone and 17b-estradiol in human hair by gas chromatography–mass spectrometry. *Analyst, 125*, 711–714.

Maness, S. C., McDonnell, D. P., & Gaido, K. W. (1998). Inhibition of androgen receptor-dependent transcriptional activity by DDT isomers and methoxychlor in HepG2 human hepatoma cells. *Toxicol Appl Pharmacol, 151*(1), 135-142.

Mariotto, A. B., Yabroff, K. R., Shao, Y., Feuer, E. J., & Brown, M. L. (2011). Projections of the cost of cancer care in the United States: 2010-2020. *J Natl Cancer Inst, 103*(2), 117-128.

Markiewicz, L., Garey, J., Adlercreutz, H., & Gurpide, E. (1993). In vitro bioassays of non-steroidal phytoestrogens. *J Steroid Biochem Mol Biol, 45*(5), 399-405.

Marrian, G. F., & Haslewood, G. A. (1932). Equol, a new inactive phenol isolated from the ketohydroxyoestrin fraction of mares' urine. *Biochem J, 26*(4), 1227-1232.

Masamura, S., Santner, S. J., Gimotty, P., George, J., & Santen, R. J. (1997). Mechanism for maintenance of high breast tumor estradiol concentrations in the absence of ovarian function: role of very high affinity tissue uptake. *Breast Cancer Res Treat, 42*(3), 215-226.

Mathey, J., Lamothe, V., Coxam, V., Potier, M., Sauvant, P., & Bennetau-Pelissero, C. (2006). Concentrations of isoflavones in plasma and urine of post-menopausal women chronically ingesting high quantities of soy isoflavones. *J Pharm Biomed Anal, 41*(3), 957-965.

Matsumoto, A., Kunugita, N., Kitagawa, K., Isse, T., Oyama, T., Foureman, G. L., . . . Kawamoto, T. (2003). Bisphenol A levels in human urine. *Environ Health Perspect, 111*(1), 101-104.

Maubach, J., Bracke, M. E., Heyerick, A., Depypere, H. T., Serreyn, R. F., Mareel, M. M., & De Keukeleire, D. (2003). Quantitation of soy-derived phytoestrogens in

human breast tissue and biological fluids by high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci, 784*(1), 137-144.

Maubach, J., Depypere, H. T., Goeman, J., Van der Eycken, J., Heyerick, A., Bracke, M. E., De Keukeleire, D. (2004). Distribution of soy-derived phytoestrogens in human breast tissue and biological fluids. *Obstet Gynecol, 103*(5 Pt 1), 892-898.

Medjakovic, S., & Jungbauer, A. (2008). Red clover isoflavones biochanin A and formononetin are potent ligands of the human aryl hydrocarbon receptor. *J Steroid Biochem Mol Biol, 108*(1-2), 171-177.

Mendiola, J., Jorgensen, N., Andersson, A. M., Calafat, A. M., Ye, X., Redmon, J. B., . . . Swan, S. H. (2010). Are environmental levels of bisphenol a associated with reproductive function in fertile men? *Environ Health Perspect, 118*(9), 1286-1291.

Mense, S. M., Hei, T. K., Ganju, R. K., & Bhat, H. K. (2008). Phytoestrogens and breast cancer prevention: possible mechanisms of action. *Environ Health Perspect, 116*(4), 426-433.

Michalakis, M., Tzatzarakis, M. N., Kovatsi, L., Alegakis, A. K., Tsakalof, A. K., Heretis, I., & Tsatsakis, A. (2014). Hypospadias in offspring is associated with chronic exposure of parents to organophosphate and organochlorine pesticides. *Toxicol Lett, 230*(2), 139-145.

Milder, I. E., Kuijsten, A., Arts, I. C., Feskens, E. J., Kampman, E., Hollman, P. C., & Van 't Veer, P. (2007). Relation between plasma enterodiol and enterolactone and dietary intake of lignans in a Dutch endoscopy-based population. *J Nutr, 137*(5), 1266-1271.

Mishra, S. I., Dickerson, V., & Najm, W. (2003). Phytoestrogens and breast cancer prevention: what is the evidence? *Am J Obstet Gynecol, 188*(5 Suppl), S66-70.

Mitani, K., Fujioka, M., & Kataoka, H. (2005). Fully automated analysis of estrogens in environmental waters by in-tube solid-phase microextraction coupled with liquid chromatography-tandem mass spectrometry. *J Chromatogr A, 1081*(2), 218-224.

Mitchell, J. H., Cawood, E., Kinniburgh, D., Provan, A., Collins, A. R., & Irvine, D. S. (2001). Effect of a phytoestrogen food supplement on reproductive health in normal males. *Clin Sci (Lond), 100*(6), 613-618.

Miyanaga, N., Akaza, H., Takashima, N., Nagata, Y., Sonoda, T., Mori, M., . . . Fujioka, T. (2003). Higher consumption of green tea may enhance equol production. *Asian Pac J Cancer Prev, 4*(4), 297-301.

Moccia, R. D., Fox, G. A., & Britton, A. (1986). A quantitative assessment of thyroid histopathology of herring gulls (Larus argentatus) from the Great Lakes and a hypothesis on the causal role of environmental contaminants. *J Wildl Dis, 22*(1), 60-70.

Molamma, P. P., Conrad, O. P., & Suresh, V. (2005). Quantificatin of isoflavones in soymilk and tofu from South East Asia. *International Journal of Food Properties, 8*, 113-123.

Moon, D. G., Sung, D. J., Kim, Y. S., Cheon, J., & Kim, J. J. (2001). Bisphenol A inhibits penile erection via alteration of histology in the rabbit. *Int J Impot Res, 13*(5), 309-316.

Moorman, W. J., Cheever, K. L., Skaggs, S. R., Clark, J. C., Turner, T. W., Marlow, K. L., & Schrader, S. M. (2000). Male adolescent exposure to endocrine-disrupting pesticides: vinclozolin exposure in peripubertal rabbits. *Andrologia, 32*(4-5), 285-293.

Morales-Suarez-Varela, M. M., Toft, G. V., Jensen, M. S., Ramlau-Hansen, C., Kaerlev, L., Thulstrup, A. M., Bonde, J. P. (2011). Parental occupational exposure to endocrine disrupting chemicals and male genital malformations: a study in the Danish National Birth Cohort study. *Environ Health, 10*(1), 3.

Morton, M., Arisaka, O., Miyake, A., & Evans, B. (1999). Analysis of phyto-oestrogens by gas chromatography-mass spectrometry. *Environ Toxicol Pharmacol, 7*(3), 221-225.

Morton, M. S., Arisaka, O., Miyake, N., Morgan, L. D., & Evans, B. A. (2002). Phytoestrogen concentrations in serum from Japanese men and women over forty years of age. *J Nutr, 132*(10), 3168-3171.

Morton, M. S., Matos-Ferreira, A., Abranches-Monteiro, L., Correia, R., Blacklock, N., Chan, P. S., . . . Griffiths, K. (1997). Measurement and metabolism of isoflavonoids and lignans in the human male. *Cancer Lett, 114*(1-2), 145-151.

Mousavi, Y., & Adlercreutz, H. (1993). Genistein is an effective stimulator of sex hormone-binding globulin production in hepatocarcinoma human liver cancer cells and suppresses proliferation of these cells in culture. *Steroids, 58*(7), 301-304.

Mulligan, A. A., Welch, A. A., McTaggart, A. A., Bhaniani, A., & Bingham, S. A. (2007). Intakes and sources of soya foods and isoflavones in a UK population cohort study (EPIC-Norfolk). *Eur J Clin Nutr, 61*(2), 248-254.

Munguia-Lopez, E. M., Gerardo-Lugo, S., Peralta, E., Bolumen, S., & Soto-Valdez, H. (2005). Migration of bisphenol A (BPA) from can coatings into a fatty-food simulant and tuna fish. *Food Addit Contam, 22*(9), 892-898.

Munro, B. H. (2000). *Statistical Methods for Health Care Research* (4 ed.). New York: Lippincott Williams & Wilkins.

Mustafa, A. M., Malintan, N. T., Seelan, S., Zhan, Z., Mohamed, Z., Hassan, J., . . . Ito, N. (2007). Phytoestrogens levels determination in the cord blood from Malaysia rural and urban populations. *Toxicol Appl Pharmacol, 222*(1), 25-32.

Muthyala, R. S., Ju, Y. H., Sheng, S., Williams, L. D., Doerge, D. R., Katzenellenbogen, B. S., Katzenellenbogen, J. A. (2004). Equol, a natural

estrogenic metabolite from soy isoflavones: convenient preparation and resolution of R- and S-equols and their differing binding and biological activity through estrogen receptors alpha and beta. *Bioorg Med Chem, 12*(6), 1559-1567.

Nagata, C., Iwasa, S., Shiraki, M., Ueno, T., Uchiyama, S., Urata, K., . . . Shimizu, H. (2006). Associations among maternal soy intake, isoflavone levels in urine and blood samples, and maternal and umbilical hormone concentrations (Japan). *Cancer Causes Control, 17*(9), 1107-1113.

Nagata, C., Takatsuka, N., Inaba, S., Kawakami, N., & Shimizu, H. (1998). Effect of soymilk consumption on serum estrogen concentrations in premenopausal Japanese women. *J Natl Cancer Inst, 90*(23), 1830-1835.

Nagata, C., Takatsuka, N., Kurisu, Y., & Shimizu, H. (1998). Decreased serum total cholesterol concentration is associated with high intake of soy products in Japanese men and women. *J Nutr, 128*(2), 209-213.

Nagata, C., Takatsuka, N., Shimizu, H., Hayashi, H., Akamatsu, T., & Murase, K. (2001). Effect of soymilk consumption on serum estrogen and androgen concentrations in Japanese men. *Cancer Epidemiol Biomarkers Prev, 10*(3), 179-184.

Nahar, M. S., Liao, C., Kannan, K., Harris, C., & Dolinoy, D. C. (2015). In utero bisphenol A concentrationmetabolism and global DNA methylation across matched placenta, kidney, and liver in the human fetus. *Chemosphere, 124*, 54-60.

Nelson, R. E., Grebe, S. K., DJ, O. K., & Singh, R. J. (2004). Liquid chromatography-tandem mass spectrometry assay for simultaneous measurement of estradiol and estrone in human plasma. *Clin Chem, 50*(2), 373-384.

Newbold, R. R. (2010). Impact of environmental endocrine disrupting chemicals on the development of obesity. *Hormones (Athens), 9*(3), 206-217.

Newton, K. M., Reed, S. D., Uchiyama, S., Qu, C., Ueno, T., Iwashita, S., . . . Lampe, J. W. (2015). A cross-sectional study of equol producer status and self-reported vasomotor symptoms. *Menopause, 22*(5), 489-495.

Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., . . . Gustafsson, J. A. (2001). Mechanisms of estrogen action. *Physiol Rev, 81*(4), 1535-1565.

Noorafshan, A., Asadi-Golshan, R., Monjezi, S., & Karbalay-Doust, S. (2014). Sodium metabisulphite, a preservative agent, decreases the heart capillary volume and length, and curcumin, the main component of Curcuma longa, cannot protect it. *Folia Biol (Praha), 60*(6), 275-280.

Nunez, A. A., Kannan, K., Giesy, J. P., Fang, J., & Clemens, L. G. (2001). Effects of bisphenol A on energy balance and accumulation in brown adipose tissue in rats. *Chemosphere, 42*(8), 917-922.

Oka, K., Hirano, T., & Noguchi, M. (1988). Changes in the concentration of testosterone in serum during the menstrual cycle, as determined by liquid chromatography. *Clin Chem, 34*(3), 557-560.

Olsen, L., Lampa, E., Birkholz, D. A., Lind, L., & Lind, P. M. (2012). Circulating levels of bisphenol A (BPA) and phthalates in an elderly population in Sweden, based on the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS). *Ecotoxicol Environ Saf, 75*(1), 242-248.

Ortiz-Zarragoitia, M., & Cajaraville, M. P. (2006). Biomarkers of exposure and reproduction-related effects in mussels exposed to endocrine disruptors. *Arch Environ Contam Toxicol, 50*(3), 361-369.

Orwoll, E., Lambert, L. C., Marshall, L. M., Phipps, K., Blank, J., Barrett-Connor, E., . . . Cummings, S. (2006). Testosterone and estradiol among older men. *J Clin Endocrinol Metab, 91*(4), 1336-1344.

Paris, F., Balaguer, P., Terouanne, B., Servant, N., Lacoste, C., Cravedi, J. P., . . . Sultan, C. (2002). Phenylphenols, biphenols, bisphenol-A and 4-tert-octylphenol exhibit alpha and beta estrogen activities and antiandrogen activity in reporter cell lines. *Mol Cell Endocrinol, 193*(1-2), 43-49.

Park, H. Y., Kim, S. H., Kim, G. B., Sim, J. Y., Lim, S. S., Kim, M. J., . . . Kwon, Y. S. (2010). A new isoflavone glycoside from the stem bark of Sophora japonica. *Arch Pharm Res, 33*(8), 1165-1168.

Park, S. K., Sakoda, L. C., Kang, D., Chokkalingam, A. P., Lee, E., Shin, H. R., . . . Hsing, A. W. (2006). Rising prostate cancer rates in South Korea. *Prostate, 66*(12), 1285-1291.

Patisaul, H. B., & Jefferson, W. (2010). The pros and cons of phytoestrogens. *Front Neuroendocrinol, 31*(4), 400-419.

Payne, J., Rajapakse, N., Wilkins, M., & Kortenkamp, A. (2000). Prediction and assessment of the effects of mixtures of four xenoestrogens. *Environ Health Perspect, 108*(10), 983-987.

Peeters, P. H., Slimani, N., van der Schouw, Y. T., Grace, P. B., Navarro, C., Tjonneland, A., Bingham, S. A. (2007). Variations in plasma phytoestrogen concentrations in European adults. *J Nutr, 137*(5), 1294-1300.

Perabo, F. G., Von Low, E. C., Ellinger, J., von Rucker, A., Muller, S. C., & Bastian, P. J. (2008). Soy isoflavone genistein in prevention and treatment of prostate cancer. *Prostate Cancer Prostatic Dis, 11*(1), 6-12.

Peterson, G., & Barnes, S. (1993). Genistein and biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate, 22*(4), 335-345.

Posnack, N. G., Idrees, R., Ding, H., Jaimes, R., 3rd, Stybayeva, G., Karabekian, Z., . . . Sarvazyan, N. (2015). Exposure to phthalates affects calcium handling and

intercellular connectivity of human stem cell-derived cardiomyocytes. *PLoS One, 10*(3), e0121927.

Prins, G. S., Tang, W. Y., Belmonte, J., & Ho, S. M. (2008). Perinatal exposure to oestradiol and bisphenol A alters the prostate epigenome and increases susceptibility to carcinogenesis. *Basic Clin Pharmacol Toxicol, 102*(2), 134-138.

Pusparini, Yenny, & Hidayat, A. (2015). Effect of soy isoflavone supplementation on endothelial dysfunction and oxidative stress in equol-producing postmenopausal women. *Endocr Metab Immune Disord Drug Targets, 15*(1), 71-79.

Reddy, S., Iden, C. R., & Brownawell, B. J. (2005). Analysis of steroid conjugates in sewage influent and effluent by liquid chromatography-tandem mass spectrometry. *Anal Chem, 77*(21), 7032-7038.

Reijnders, P. J. (1986). Reproductive failure in common seals feeding on fish from polluted coastal waters. *Nature, 324*(6096), 456-457.

Rodriguez-Mozaz, S., de Alda, M. J., & Barcelo, D. (2004). Monitoring of estrogens, pesticides and bisphenol A in natural waters and drinking water treatment plants by solid-phase extraction-liquid chromatography-mass spectrometry. *J Chromatogr A, 1045*(1-2), 85-92.

Rudel, R. A., Brody, J. G., Spengler, J. D., Vallarino, J., Geno, P. W., Sun, G., & Yau, A. (2001). Identification of selected hormonally active agents and animal mammary carcinogens in commercial and residential air and dust samples. *J Air Waste Manag Assoc, 51*(4), 499-513.

Rudel, R. A., Camann, D. E., Spengler, J. D., Korn, L. R., & Brody, J. G. (2003). Phthalates, alkylphenols, pesticides, polybrominated diphenyl ethers, and other endocrine-disrupting compounds in indoor air and dust. *Environ Sci Technol, 37*(20), 4543-4553.

Rudel, R. A., Gray, J. M., Engel, C. L., Rawsthorne, T. W., Dodson, R. E., Ackerman, J. M., . . . Brody, J. G. (2011). Food packaging and bisphenol A and bis(2-ethyhexyl) phthalate exposure: findings from a dietary intervention. *Environ Health Perspect, 119*(7), 914-920.

Rui, X., Xu, X., Wu, H., Li, W., Chen, X., Jiang, M., & Dong, M. (2014). A survey of equol contents in Chinese stinky tofu with emphasis on the effects of cooking methods. *Int J Food Sci Nutr, 65*(6), 667-672.

Sajiki, J. (2001). Determination of bisphenol A in blood using high-performance liquid chromatography-electrochemical detection with solid-phase extraction. *J Chromatogr B Biomed Sci Appl, 755*(1-2), 9-15.

Sajiki, J., Miyamoto, F., Fukata, H., Mori, C., Yonekubo, J., & Hayakawa, K. (2007). Bisphenol A (BPA) and its source in foods in Japanese markets. *Food Addit Contam, 24*(1), 103-112.

Santell, R. C., Chang, Y. C., Nair, M. G., & Helferich, W. G. (1997). Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. *J Nutr, 127*(2), 263-269.

Santhi, V. A., Hairin, T., & Mustafa, A. M. (2011). Simultaneous determination of organochlorine pesticides and bisphenol A in edible marine biota by GC-MS. *Chemosphere*.

Santhi, V. A., Sakai, N., Ahmad, E. D., & Mustafa, A. M. (2012). Occurrence of bisphenol A in surface water, drinking water and plasma from Malaysia with exposure assessment from consumption of drinking water. *Sci Total Environ, 427-428*, 332-338.

Schecter, A., Lorber, M., Guo, Y., Wu, Q., Yun, S. H., Kannan, K., Birnbaum, L. S. (2013). Phthalate concentrations and dietary exposure from food purchased in New York State. *Environ Health Perspect, 121*(4), 473-494.

Schiffer, C., Muller, A., Egeberg, D. L., Alvarez, L., Brenker, C., Rehfeld, A., Strunker, T. (2014). Direct action of endocrine disrupting chemicals on human sperm. *EMBO Rep, 15*(7), 758-765.

Schiliro, T., Gorrasi, I., Longo, A., Coluccia, S., & Gilli, G. (2011). Endocrine disrupting activity in fruits and vegetables evaluated with the E-screen assay in relation to pesticide residues. *J Steroid Biochem Mol Biol, 127*(1-2), 139-146.

Schiliro, T., Porfido, A., Spina, F., Varese, G. C., & Gilli, G. (2012). Oestrogenic activity of a textile industrial wastewater treatment plant effluent evaluated by the E-screen test and MELN gene-reporter luciferase assay. *Sci Total Environ, 432*, 389-395.

Schmitt, E., Dekant, W., & Stopper, H. (2001). Assaying the estrogenicity of phytoestrogens in cells of different estrogen sensitive tissues. *Toxicol In Vitro, 15*(4-5), 433-439.

Schonfelder, G., Wittfoht, W., Hopp, H., Talsness, C. E., Paul, M., & Chahoud, I. (2002). Parent bisphenol A accumulation in the human maternal-fetal-placental unit. *Environ Health Perspect, 110*(11), A703-707.

Schug, T. T., Janesick, A., Blumberg, B., & Heindel, J. J. (2011). Endocrine disrupting chemicals and disease susceptibility. *J Steroid Biochem Mol Biol, 127*(3-5), 204-215.

Seow, A., Shi, C. Y., Franke, A. A., Hankin, J. H., Lee, H. P., & Yu, M. C. (1998). Isoflavonoid levels in spot urine are associated with frequency of dietary soy intake in a population-based sample of middle-aged and older Chinese in Singapore. *Cancer Epidemiol Biomarkers Prev, 7*(2), 135-140.

Setchell, K. D. (1998). Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am J Clin Nutr, 68*(6 Suppl), 1333S-1346S.

Setchell, K. D., Borriello, S. P., Hulme, P., Kirk, D. N., & Axelson, M. (1984). Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. *Am J Clin Nutr, 40*(3), 569-578.

Setchell, K. D., Brown, N. M., Desai, P., Zimmer-Nechemias, L., Wolfe, B. E., Brashear, W. T., . . . Heubi, J. E. (2001). Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J Nutr, 131*(4 Suppl), 1362S-1375S.

Setchell, K. D., Brown, N. M., & Lydeking-Olsen, E. (2002). The clinical importance of the metabolite equol-a clue to the effectiveness of soy and its isoflavones. *J Nutr, 132*(12), 3577-3584.

Setchell, K. D., Brown, N. M., Zimmer-Nechemias, L., Brashear, W. T., Wolfe, B. E., Kirschner, A. S., & Heubi, J. E. (2002). Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am J Clin Nutr, 76*(2), 447-453.

Setchell, K. D., & Cassidy, A. (1999). Dietary isoflavones: biological effects and relevance to human health. *J Nutr, 129*(3), 758S-767S.

Setchell, K. D., & Clerici, C. (2010). Equol: history, chemistry, and formation. *J Nutr, 140*(7), 1355S-1362S.

Setchell, K. D., & Cole, S. J. (2006). Method of defining equol-producer status and its frequency among vegetarians. *J Nutr, 136*(8), 2188-2193.

Setchell, K. D., & Lydeking-Olsen, E. (2003). Dietary phytoestrogens and their effect on bone: evidence from in vitro and in vivo, human observational, and dietary intervention studies. *Am J Clin Nutr, 78*(3 Suppl), 593S-609S.

Setchell, K. D., Zimmer-Nechemias, L., Cai, J., & Heubi, J. E. (1997). Exposure of infants to phyto-oestrogens from soy-based infant formula. *Lancet, 350*(9070), 23-27.

Settimi, L., Masina, A., Andrion, A., & Axelson, O. (2003). Prostate cancer and exposure to pesticides in agricultural settings. *Int J Cancer, 104*(4), 458-461.

Shao, B., Han, H., Li, D., Ma, Y., Tu, X., & Wu, Y. (2007). Analysis of alkylphenol and bisphenol A in meat by accelerated solvent extraction and liquid chromatography with tandem mass spectrometry. *Food Chemistry, 105*, 1236-1241.

Shao, B., Han, H., Tu, X., & Huang, L. (2007). Analysis of alkylphenol and bisphenol A in eggs and milk by matrix solid phase dispersion extraction and liquid chromatography with tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci, 850*(1-2), 412-416.

Shertzer, H. G., Puga, A., Chang, C., Smith, P., Nebert, D. W., Setchell, K. D., & Dalton, T. P. (1999). Inhibition of CYP1A1 enzyme activity in mouse hepatoma cell culture by soybean isoflavones. *Chem Biol Interact, 123*(1), 31-49.

Shimizu, H., Ross, R. K., Bernstein, L., Yatani, R., Henderson, B. E., & Mack, T. M. (1991). Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. *Br J Cancer, 63*(6), 963-966.

Shiue, I. (2015). Urinary heavy metals, phthalates and polyaromatic hydrocarbons independent of health events are associated with adult depression: USA NHANES, 2011-2012. *Environ Sci Pollut Res Int*.

Silva, E., Rajapakse, N., & Kortenkamp, A. (2002). Something from "nothing"--eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ Sci Technol, 36*(8), 1751-1756.

Sirtori, C. R., Arnoldi, A., & Johnson, S. K. (2005). Phytoestrogens: end of a tale? *Ann Med, 37*(6), 423-438.

Sohoni, P., & Sumpter, J. P. (1998). Several environmental oestrogens are also anti-androgens. *J Endocrinol, 158*(3), 327-339.

Sole, M., Morcillo, Y., & Porte, C. (1998). Imposex in the commercial snail Bolinus brandaris in the northwestern Mediterranean. *Environ Pollut, 99*(2), 241-246.

Sonnenschein, C., & Soto, A. M. (1998). An updated review of environmental estrogen and androgen mimics and antagonists. *J Steroid Biochem Mol Biol, 65*(1-6), 143-150.

Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., & Serrano, F. O. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect, 103 Suppl 7*, 113-122.

Srinivas, T., Prasad, T. A., Raffi, G. M., & Reddy, D. C. (1991). Effect of atrazine on some aspects of lipid metabolism in fresh water fish. *Biochem Int, 23*(3), 603-609.

Starcevic, B., DiStefano, E., Wang, C., & Catlin, D. H. (2003). Liquid chromatography-tandem mass spectrometry assay for human serum testosterone and trideuterated testosterone. *J Chromatogr B Analyt Technol Biomed Life Sci, 792*(2), 197-204.

Stattin, P., Bylund, A., Biessy, C., Kaaks, R., Hallmans, G., & Adlercreutz, H. (2004). Prospective study of plasma enterolactone and prostate cancer risk (Sweden). *Cancer Causes Control, 15*(10), 1095-1102.

Stelmach, I., Majak, P., Jerzynska, J., Podlecka, D., Stelmach, W., Polanska, K., . . . Hanke, W. (2015). The effect of prenatal exposure to phthalates on food allergy and early eczema in inner-city children. *Allergy Asthma Proc, 36*(4), 72-78.

Strom, A., Hartman, J., Foster, J. S., Kietz, S., Wimalasena, J., & Gustafsson, J. A. (2004). Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc Natl Acad Sci U S A, 101*(6), 1566-1571.

Sungur, S., Koroglu, M., & Ozkan, A. (2013). Determination of bisphenol a migrating from canned food and beverages in markets. *Food Chem, 142*, 87-91.

Swaby, R. F., Sharma, C. G., & Jordan, V. C. (2007). SERMs for the treatment and prevention of breast cancer. *Rev Endocr Metab Disord, 8*(3), 229-239.

Szafran-Urbaniak, B. (2008). Application of validated method for determination of selected polychlorinated biphenyls in human adipose tissue samples. *Environ Toxicol Pharmacol, 25*(2), 131-135.

Szczepanska, N., Namiesnik, J., & Kudlak, B. (2016). Assessment of toxic and endocrine potential of substances migrating from selected toys and baby products. *Environ Sci Pollut Res Int*.

Szymczak, J., Milewicz, A., Thijssen, J. H., Blankenstein, M. A., & Daroszewski, J. (1998). Concentration of sex steroids in adipose tissue after menopause. *Steroids, 63*(5-6), 319-321.

Tai, S. S., & Welch, M. J. (2005). Development and evaluation of a reference measurement procedure for the determination of estradiol-17beta in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry. *Anal Chem, 77*(19), 6359-6363.

Taioli, E., Im, A., Xu, X., Veenstra, T. D., Ahrendt, G., & Garte, S. (2010). Comparison of estrogens and estrogen metabolites in human breast tissue and urine. *Reprod Biol Endocrinol, 8*, 93.

Takeuchi, T., & Tsutsumi, O. (2002). Serum bisphenol a concentrations showed gender differences, possibly linked to androgen levels. *Biochem Biophys Res Commun, 291*(1), 76-78.

Tan, B. L., & Ali Mohd, M. (2003). Analysis of selected pesticides and alkylphenols in human cord blood by gas chromatograph-mass spectrometer. *Talanta, 61*(3), 385-391.

Tan, B. L., & Mustafa, A. M. (2003). Leaching of bisphenol A from new and old babies' bottles, and new babies' feeding teats. *Asia Pac J Public Health, 15*(2), 118-123.

Tan, B. L., & Mustafa, A. M. (2004). The monitoring of pesticides and alkylphenols in selected rivers in the State of Selangor, Malaysia. *Asia Pac J Public Health, 16*(1), 54-63.

Tanaka, M., Fujimoto, K., Chihara, Y., Torimoto, K., Yoneda, T., Tanaka, N., . . . Hirao, Y. (2009). Isoflavone supplements stimulated the production of serum equol and decreased the serum dihydrotestosterone levels in healthy male volunteers. *Prostate Cancer Prostatic Dis, 12*(3), 247-252.

Tang, L., Lee, A. H., Xu, F., Zhang, T., Lei, J., & Binns, C. W. (2015). Soya and isoflavone intakes associated with reduced risk of oesophageal cancer in north-west China. *Public Health Nutr, 18*(1), 130-134.

Taylor, C. K., Levy, R. M., Elliott, J. C., & Burnett, B. P. (2009). The effect of genistein aglycone on cancer and cancer risk: a review of in vitro, preclinical, and clinical studies. *Nutr Rev, 67*(7), 398-415.

Taylor, J. I., Grace, P. B., & Bingham, S. A. (2005). Optimization of conditions for the enzymatic hydrolysis of phytoestrogen conjugates in urine and plasma. *Anal Biochem, 341*(2), 220-229.

Tenover, J. S., & Bremner, W. J., (1991). Circadian rhythm of serum immunoreactive inhibin in young and elderly men. *J Gerontol, 46*, M181-184.

Tharp, A. P., Maffini, M. V., Hunt, P. A., VandeVoort, C. A., Sonnenschein, C., & Soto, A. M. (2012). Bisphenol A alters the development of the rhesus monkey mammary gland. *Proc Natl Acad Sci U S A, 109*(21), 8190-8195.

Thomas, B. F., Zeisel, S. H., Busby, M. G., Hill, J. M., Mitchell, R. A., Scheffler, N. M., Jeffcoat, A. R. (2001). Quantitative analysis of the principle soy isoflavones genistein, daidzein and glycitein, and their primary conjugated metabolites in human plasma and urine using reversed-phase high-performance liquid chromatography with ultraviolet detection. *J Chromatogr B Biomed Sci Appl, 760*(2), 191-205.

Thomas, H. V., Key, T. J., Allen, D. S., Moore, J. W., Dowsett, M., Fentiman, I. S., & Wang, D. Y. (1997). A prospective study of endogenous serum hormone concentrations and breast cancer risk in premenopausal women on the island of Guernsey. *Br J Cancer, 75*(7), 1075-1079.

Todaka, E., & Mori, C. (2002). Necessity to establish new risk assessment and risk communication for human fetal exposure to multiple endocrine disruptors in Japan. *Congenit Anom (Kyoto), 42*(2), 87-93.

Trenholm, R. A., Vanderford, B. J., Holady, J. C., Rexing, D. J., & Snyder, S. A. (2006). Broad range analysis of endocrine disruptors and pharmaceuticals using gas chromatography and liquid chromatography tandem mass spectrometry. *Chemosphere, 65*(11), 1990-1998.

Tsuchida, K., Mizushima, S., Toba, M., & Soda, K. (1999). Dietary soybeans intake and bone mineral density among 995 middle-aged women in Yokohama. *J Epidemiol, 9*(1), 14-19.

Tsuda, H., Naito, A., Kim, C. K., Fukamachi, K., Nomoto, H., & Moore, M. A. (2003). Carcinogenesis and its modification by environmental endocrine disruptors: in vivo experimental and epidemiological findings. *Jpn J Clin Oncol, 33*(6), 259-270.

Tuohy, P. G. (2003). Soy infant formula and phytoestrogens. *J Paediatr Child Health, 39*(6), 401-405.

Tyagi, V., Garg, N., Mustafa, M. D., Banerjee, B. D., & Guleria, K. (2015). Organochlorine pesticide levels in maternal blood and placental tissue with reference to preterm birth: a recent trend in North Indian population. *Environ Monit Assess, 187*(7), 4369.

Tzatzarakis, M. N., Vakonaki, E., Kavvalakis, M. P., Barmpas, M., Kokkinakis, E. N., Xenos, K., & Tsatsakis, A. M. (2015). Biomonitoring of bisphenol A in hair of Greek population. *Chemosphere, 118*, 336-341.

Uehar, M., Arai, Y., Watanabe, S., & Adlercreutz, H. (2000). Comparison of plasma and urinary phytoestrogens in Japanese and Finnish women by time-resolved fluoroimmunoassay. *Biofactors, 12*(1-4), 217-225.

Usui, T. (2006). Pharmaceutical prospects of phytoestrogens. *Endocr J, 53*(1), 7-20.

Utian, W. H., Jones, M., & Setchell, K. D. (2015). S-equol: a potential nonhormonal agent for menopause-related symptom relief. *J Womens Health (Larchmt), 24*(3), 200-208.

Vafeiadou, K., Hall, W. L., & Williams, C. M. (2006). Does genotype and equol-production status affect response to isoflavones? Data from a pan-European study on the effects of isoflavones on cardiovascular risk markers in post-menopausal women. *Proc Nutr Soc, 65*(1), 106-115.

Valentin-Blasini, L., Blount, B. C., Caudill, S. P., & Needham, L. L. (2003). Urinary and serum concentrations of seven phytoestrogens in a human reference population subset. *J Expo Anal Environ Epidemiol, 13*(4), 276-282.

Valsta, L. M., Kilkkinen, A., Mazur, W., Nurmi, T., Lampi, A. M., Ovaskainen, M. L., . . . Pietinen, P. (2003). Phyto-oestrogen database of foods and average intake in Finland. *Br J Nutr, 89 Suppl 1*, S31-38.

van den Beld, A. W., de Jong, F. H., Grobbee, D. E., Pols, H. A., & Lamberts, S. W. (2000). Measures of bioavailable serum testosterone and estradiol and their relationships with muscle strength, bone density, and body composition in elderly men. *J Clin Endocrinol Metab, 85*(9), 3276-3282.

Van Eeckhaut, A., Lanckmans, K., Sarre, S., Smolders, I., & Michotte, Y. (2009). Validation of bioanalytical LC-MS/MS assays: evaluation of matrix effects. *J Chromatogr B Analyt Technol Biomed Life Sci, 877*(23), 2198-2207.

van Erp-Baart, M. A., Brants, H. A., Kiely, M., Mulligan, A., Turrini, A., Sermoneta, C.,Valsta, L. M. (2003). Isoflavone intake in four different European countries: the VENUS approach. *Br J Nutr, 89 Suppl 1*, S25-30.

Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs, D. R., Jr., Lee, D. H., Myers, J. P. (2012). Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses. *Endocr Rev, 33*(3).

Vandenberg, L. N., Maffini, M. V., Schaeberle, C. M., Ucci, A. A., Sonnenschein, C., Rubin, B. S., & Soto, A. M. (2008). Perinatal exposure to the xenoestrogen bisphenol-A induces mammary intraductal hyperplasias in adult CD-1 mice. *Reprod Toxicol, 26*(3-4), 210-219.

Vandenberg, L. N., Gerona, R. R., Kannan, K., Taylor, J. A., van Breemen, R. B., Dickenson, C. A., …. Woodruff, T. J. (2014). A round robin approach to the

analysis of bispehnol a (BPA) in human blood samples. *Environmental Health, 13*(25).

Vanderford, B. J., Pearson, R. A., Rexing, D. J., & Snyder, S. A. (2003). Analysis of endocrine disruptors, pharmaceuticals, and personal care products in water using liquid chromatography/tandem mass spectrometry. *Anal Chem, 75*(22), 6265-6274.

Vanharanta, M., Voutilainen, S., Lakka, T. A., van der Lee, M., Adlercreutz, H., & Salonen, J. T. (1999). Risk of acute coronary events according to serum concentrations of enterolactone: a prospective population-based case-control study. *Lancet, 354*(9196), 2112-2115.

Vedrine, N., Mathey, J., Morand, C., Brandolini, M., Davicco, M. J., Guy, L., . . . Manach, C. (2006). One-month exposure to soy isoflavones did not induce the ability to produce equol in postmenopausal women. *Eur J Clin Nutr, 60*(9), 1039-1045.

Vihma, V., Wang, F., Savolainen-Peltonen, H., Turpeinen, U., Hamalainen, E., Leidenius, M., Tikkanen, M. J. (2016). Quantitative determination of estrone by liquid chromatography-tandem mass spectrometry in subcutaneous adipose tissue from the breast in postmenopausal women. *J Steroid Biochem Mol Biol, 155*(Pt A), 120-125.

Wagner, M., & Oehlmann, J. (2009). Endocrine disruptors in bottled mineral water: total estrogenic burden and migration from plastic bottles. *Environ Sci Pollut Res Int, 16*(3), 278-286.

Wagner, M., & Oehlmann, J. (2011). Endocrine disruptors in bottled mineral water: estrogenic activity in the E-Screen. *J Steroid Biochem Mol Biol, 127*(1-2), 128-135.

Wang, L., Xue, J., & Kannan, K. (2015). Widespread occurrence and accumulation of bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ether (BFDGE) and their derivatives in human blood and adipose fat. *Environ Sci Technol, 49*, 3150-3157.

Wang, H. J., & Murphy, P. A. (1994). Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. *J Agric Food Chem, 42*, 1674-1677.

Wang, S., Cyronak, M., & Yang, E. (2007). Does a stable isotopically labeled internal standard always correct analyte response? A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma. *J Pharm Biomed Anal, 43*(2), 701-707.

Waring, R. H., Ayers, S., Gescher, A. J., Glatt, H. R., Meinl, W., Jarratt, P., . . . Harris, R. M. (2008). Phytoestrogens and xenoestrogens: the contribution of diet and environment to endocrine disruption. *J Steroid Biochem Mol Biol, 108*(3-5), 213-220.

Watanabe, S., Yamaguchi, M., Sobue, T., Takahashi, T., Miura, T., Arai, Y., . . . Adlercreutz, H. (1998). Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako). *J Nutr, 128*(10), 1710-1715.

Watson, C. S., Bulayeva, N. N., Wozniak, A. L., & Finnerty, C. C. (2005). Signaling from the membrane via membrane estrogen receptor-alpha: estrogens, xenoestrogens, and phytoestrogens. *Steroids, 70*(5-7), 364-371.

Welshons, W. V., Murphy, C. S., Koch, R., Calaf, G., & Jordan, V. C. (1987). Stimulation of breast cancer cells in vitro by the environmental estrogen enterolactone and the phytoestrogen equol. *Breast Cancer Res Treat, 10*(2), 169-175.

Westgard, J. O., Carey, R. N., & Wold, S. (1974). Criteria for judging precision and accuracy in method development and evaluation. *Clin Chem, 20*(7), 825-833.

Wetherill, Y. B., Akingbemi, B. T., Kanno, J., McLachlan, J. A., Nadal, A., Sonnenschein, C., Belcher, S. M. (2007). In vitro molecular mechanisms of bisphenol A action. *Reprod Toxicol, 24*(2), 178-198.

White, S. S., & Birnbaum, L. S. (2009). An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev, 27*(4), 197-211.

Wietrzyk, J., Grynkiewicz, G., & Opolski, A. (2005). Phytoestrogens in cancer prevention and therapy--mechanisms of their biological activity. *Anticancer Res, 25*(3c), 2357-2366.

Wolff, M. S. (2006). Endocrine disruptors: challenges for environmental research in the 21st century. *Ann N Y Acad Sci, 1076*, 228-238.

Wolff, M. S., Teitelbaum, S. L., Windham, G., Pinney, S. M., Britton, J. A., Chelimo, C., Calafat, A. M. (2007). Pilot study of urinary biomarkers of phytoestrogens, phthalates, and phenols in girls. *Environ Health Perspect, 115*(1), 116-121.

Wood, C. E., Register, T. C., Franke, A. A., Anthony, M. S., & Cline, J. M. (2006). Dietary soy isoflavones inhibit estrogen effects in the postmenopausal breast. *Cancer Res, 66*(2), 1241-1249.

Wu, Q., Wang, M., & Simon, J. E. (2004). Analytical methods to determine phytoestrogenic compounds. *J Chromatogr B Analyt Technol Biomed Life Sci, 812*(1-2), 325-355.

Wuttke, W., Jarry, H., & Seidlova-Wuttke, D. (2010). Definition, classification and mechanism of action of endocrine disrupting chemicals. *Hormones (Athens), 9*(1), 9-15.

Xiao, X., & McCalley, D. (2000). Quantitative analysis of estrogens in human urine using gas chromatography/negative chemical ionisation mass spectrometry. *Rapid Commun Mass Spectrom, 14*(21), 1991-2001.

Xu, L., & Spink, D. C. (2008). Analysis of steroidal estrogens as pyridine-3-sulfonyl derivatives by liquid chromatography electrospray tandem mass spectrometry. *Anal Biochem, 375*(1), 105-114.

Xu, X., Duncan, A. M., Merz, B. E., & Kurzer, M. S. (1998). Effects of soy isoflavones on estrogen and phytoestrogen metabolism in premenopausal women. *Cancer Epidemiol Biomarkers Prev, 7*(12), 1101-1108.

Xu, X., Harris, K. S., Wang, H. J., Murphy, P. A., & Hendrich, S. (1995). Bioavailability of soybean isoflavones depends upon gut microflora in women. *J Nutr, 125*(9), 2307-2315.

Xu, X., Roman, J. M., Issaq, H. J., Keefer, L. K., Veenstra, T. D., & Ziegler, R. G. (2007). Quantitative measurement of endogenous estrogens and estrogen metabolites in human serum by liquid chromatography-tandem mass spectrometry. *Anal Chem, 79*(20), 7813-7821.

Xu, X., Wang, H. J., Murphy, P. A., Cook, L., & Hendrich, S. (1994). Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J Nutr, 124*(6), 825-832.

Yamashita, K., Okuyama, M., Watanabe, Y., Honma, S., Kobayashi, S., & Numazawa, M. (2007). Highly sensitive determination of estrone and estradiol in human serum by liquid chromatography-electrospray ionization tandem mass spectrometry. *Steroids, 72*(11-12), 819-827.

Yang, L., Lan, C., Liu, H., Dong, J., & Luan, T. (2006). Full automation of solid-phase microextraction/on-fiber derivatization for simultaneous determination of endocrine-disrupting chemicals and steroid hormones by gas chromatography-mass spectrometry. *Anal Bioanal Chem, 386*(2), 391-397.

Ye, X., Kuklenyik, Z., Needham, L. L., & Calafat, A. M. (2005). Quantification of urinary conjugates of bisphenol A, 2,5-dichlorophenol, and 2-hydroxy-4-methoxybenzophenone in humans by online solid phase extraction-high performance liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem, 383*(4), 638-644.

Youlden, D. R., Cramb, S. M., Yip, C. H., & Baade, P. D. (2014). Incidence and mortality of female breast cancer in the Asia-Pacific region. *Cancer Biol Med, 11*(2), 101-115.

Yue, W., Santner, S. J., Masamura, S., Wang, J. P., Demers, L. M., Hamilton, C., & Santen, R. J. (1998). Determinants of tissue estradiol levels and biologic responsiveness in breast tumors. *Breast Cancer Res Treat, 49 Suppl 1*, S1-7; discussion S33-37.

Zamora-Ros, R., Jimenez, C., Cleries, R., Agudo, A., Sanchez, M. J., Sanchez-Cantalejo, E., Gonzalez, C. A. (2013). Dietary flavonoid and lignan intake and mortality in a Spanish cohort. *Epidemiology, 24*(5), 726-733.

Zava, D. T., Blen, M., & Duwe, G. (1997). Estrogenic activity of natural and synthetic estrogens in human breast cancer cells in culture. *Environ Health Perspect, 105 Suppl 3*, 637-645.

Zhang, Q., Aft, R. L., & Gross, M. L. (2008). Estrogen carcinogenesis: specific identification of estrogen-modified nucleobase in breast tissue from women. *Chem Res Toxicol, 21*(8), 1509-1513.

Zhang, T., Sun, H., & Kannan, K. (2013). Blood and urinary bisphenol A concentrations in children, adults and pregnant women from China: partitioning between blood and urine and maternal and fetal cord blood. *Environ Sci Technol, 47*, 4686-4694.

Zhang, Z., Aft, R. L., & Gross, M. L. (2011). Urinary bisphenol A concentrations and their implications for human exposure in several Asian countries. *Environ Sci Technol, 45*, 7044-7050.

Zhao, J. H., Sun, S. J., Arao, Y., Oguma, E., Yamada, K., Horiguchi, H., & Kayama, F. (2006). Identification of equol producers in a Japanese population by high-performance liquid chromatography with coulometric array for determining serum isoflavones. *Phytomedicine, 13*(5), 304-309.

Zhao, M., Baker, S. D., Yan, X., Zhao, Y., Wright, W. W., Zirkin, B. R., & Jarow, J. P. (2004). Simultaneous determination of steroid composition of human testicular fluid using liquid chromatography tandem mass spectrometry. *Steroids, 69*(11-12), 721-726.

Zheng, B., Liu, R., Liu, Y., Jin, F., & An, L. (2014). Phenolic endocrine-disrupting chemicals and intersex in wild crucian carp from Hun River, China. *Chemosphere, 120*, 743-749.

Zheng, W., Hou, Y., & Yao, W. (2014). Lactulose increases equol production and improves liver antioxidant status in barrows treated with Daidzein. *PLoS One, 9*(3), e93163.

Zhou, Q., Miao, M., Ran, M., Ding, L., Bai, L., Wu, T., Li, D. K. (2013). Serum bisphenol-A concentration and sex hormone levels in men. *Fertil Steril, 100*(2), 478-482.

Zierau, O., Kolba, S., Olff, S., Vollmer, G., & Diel, P. (2006). Analysis of the promoter-specific estrogenic potency of the phytoestrogens genistein, daidzein and coumestrol. *Planta Med, 72*(2), 184-186.

Zimmer, J., Lange, B., Frick, J. S., Sauer, H., Zimmermann, K., Schwiertz, A., Enck, P. (2012). A vegan or vegetarian diet substantially alters the human colonic faecal microbiota. *Eur J Clin Nutr, 66*(1), 53-60.

Zimmerman-Downs, J. M., Shuman, D., Stull, S. C., & Ratzlaff, R. E. (2010). Bisphenol A blood and saliva levels prior to and after dental sealant placement in adults. *J Dent Hyg, 84*(3), 145-150.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. 3rd International Public Health Conference & 20th National Public Health Colloquium, Kuching Sarawak, August 2013. Oral presentation: Distribution of selected endocrine disrupting chemicals in male vegetarian and non-vegetarians.
2. International Conference on Medical Science Technology, Bangi Putrajaya Hotel, November 2016. Poster presentation: Correlation studies of oestrogenic EDCs in male vegetarians.
3. Plasma isoflavones in Malaysian men according to vegetarianism and by age. Asia Pac J of Clin Nutr 2016; 25(1): 89-96.