Changes in the isoflavone profile of bread caused by the manufacturing process and the implications for human health

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By David Michael Scott

Supervised by Professor Ian Shaw

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Abstract

A recent increase in adverse effects on the reproductive capabilities of animals and humans has been observed. Reduced sperm count and increased testicular and breast cancer are of particular concern. The cause of these observations is likely the disruption of the endocrine system. One such endocrine disruptor is genistein, abundant in soy and a common addition to everyday foods such as bread and tofu. It has been shown that isoflavone levels alter throughout the bread making process, with the total amount decreasing and inter-conversion between different isoflavones as well as different glycoside and aglycone forms occurring. A possible explanation for this may be the metabolic activity of S*accharomyces cerevisiae* or bacteria in the case of sour dough, causing modification or the breakdown of these compounds. This may result in the health effects of baked bread being not simply a sum of the health effects of the breads ingredients. Yeast metabolism of these compounds has not been looked at before and could be a significant factor unaccounted for.

Isoflavone transformations throughout food preparation and processing can play a large role in the context of functional foods as the isoflavone aglycone is more bioactive and bioavailable than the naturally occurring sugar conjugate, which is responsible for the pharmacological activity and thus the changes to health that may occur. While release of the aglycones during food manufacturing processes increases the functionality of isoflavone containing fermented foods, the potential metabolic inter-conversion of isoflavones is likely a far more important factor, as different isoflavones have different levels of pharmacological activity, while the microflora in our digestive tract are also capable of releasing the aglycone. For this reason bacterial metabolic conversion from an isoflavone with higher estrogenicity (e.g. genistein; EQ = 2.6×10^{-4}) to lower estrogenicity (e.g. daidzein; EQ = 1.1×10^{-4}) during the food manufacturing process might reduce functionality. An example of this could be women eating soy bread as a post-menopausal pseudo-hormone replacement but not receiving as much benefit from it as initially thought.

My project involves determining what metabolism or interconversion of isoflavones occurs during the bread making process. This helps lead to a better understanding of the level of estrogenic effects caused by the addition of soy to bread.

List of Abbreviations

a.a – Amino acid

AF-1 – Activation function 1

AF-2 Activation function 2

BPA – Bisphenol A

cAMP - cyclic adenosine monophosphate

CBG – Cytosolic β-glucosidase

CHR – Chalcone reductase

CHS – Chalcone synthase

CoA – Coenzyme A

DBD – DNA binding domain

DDT – dichloro-diphenyl-trichloroethane

DNA – Deoxyribonucleic acid

EDCs – Endocrine disrupting chemicals

EGFR – Epidermal growth factor receptor

EQ – Equivalence factor

ER – Estrogen receptor

EREs – Estrogen response elements

ESR – Environmental sciences and research

ESR1 – Estrogen receptor 1

ESR2 – Estrogen receptor 2

FDA – Food and drug association

GDP – Guanosine diphosphate

GSH – Glutathione

GTP – Guanosine triphosphate

GTPases - Guanosine triphosphatases

HPLC – High performance liquid chromatography

HSP90 – Heat shock protein 90

ICAM – Intercellular adhesion molecule-1

IGF-1 – Insulin-like growth factor 1

IL-1β – Interlueukin-1β

LBD – Ligand binding domain

LBD – Ligand binding domain

LPH – Lactase-phloricin hydrolase

MCP-1 – Monocyte chemoattractant protein-1

NCoR – Nuclear co-repressor

MAPK – Mitogen-activated protein kinase

PCBs – Polychlorinated biphenyls

SHBG – Sex hormone-binding globulin

SOD – Super oxide dismutase

SMRT – Silencing mediator of retinoic acid and thyroid hormone receptors

TNF-α – Tumour necrosis factor-α

YPD – Yeast peptone dextrose media

3β-HSD – 3β-hydroxysteroid dehydrogenase

γ-GCS – γ-Glutamyl cysteine synthase

Chapter 1 – Introduction

1.1 Steroid hormones

1.1.1 Types of steroid hormones

Steroid hormones have a large range of functions including, but not limited to, the regulation of; inflammation, immune function, sodium balance, metabolism and development of sexual characteristics. There are two main types of steroid hormones, corticosteroids and sex steroids. A major corticosteroid is cortisone (Figure 1.1) which is released by the adrenal cortex in response to stress. Sex steroids are predominately synthesised in the gonads or placenta and can be further classified into androgens, progestogens and estrogens. A major androgen is testosterone (Figure 1.1) which plays a major role in the development of male reproductive tissue. A major progestogen is progesterone (Figure 1.1) which is involved in the menstrual cycle, pregnancy and the development of the embryo. A major estrogen is 17β-estradiol (Figure 1.1) which is the primary female sex hormone. 17β-estradiol is responsible for development and maintenance of the female reproductive tissues as well as having important effects in many secondary tissues. Steroids can regulate the functions of many tissues throughout the body in addition to their primary effects; for example, 17β-estradiol is also an important hormone for the regulation of brain, bone, kidney, heart, lung, prostate, intestinal and endothelial cells (Frye 2009).

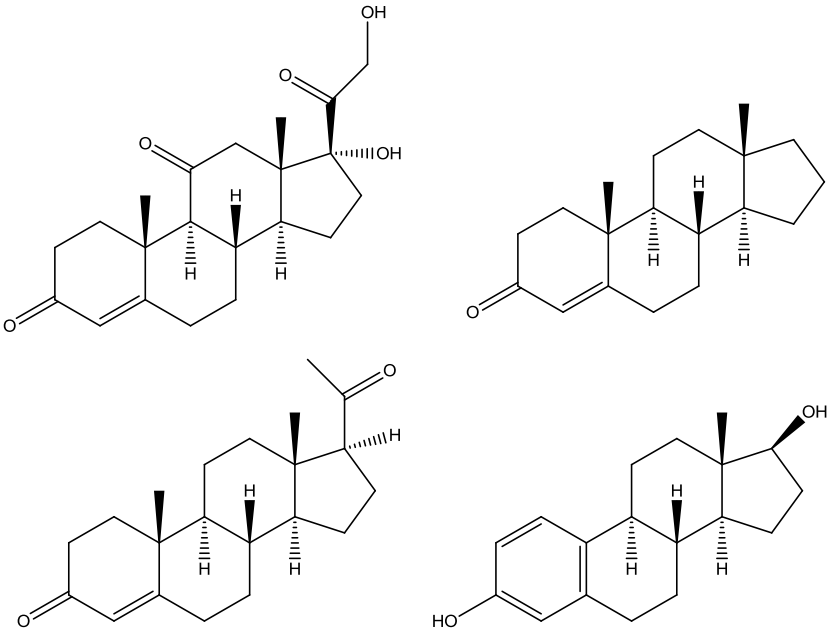

Figure 1.1 Structure of steroid hormones, Top Left: The corticosteroid, cortisone Top Right: The androgen, testosterone. Bottom Left: The progestogen, progesterone. Bottom Right: The estrogen, 17β-estradiol.

In the blood steroid hormones are generally bound to specific carrier proteins such as sex hormone-binding globulin, a glycoprotein that binds androgens and estrogens, or transcortin, an alpha-globulin that binds corticosteroids and progestogens. This protein binding is necessary as steroids have a low solubility in water, which constitutes 92 % of blood plasma. The natural steroid hormones are fat soluble lipids synthesized from cholesterol and can enter cells by diffusing through their phospholipid bilayer. Once inside the cell they bind to steroid hormone receptors such as in the case of 17β-estradiol binding to the estrogen receptor.

1.1.2 Estrogens

Estrogens are synthesized in all vertebrates as well as in certain insects (Ryan 1982; Mechoulam 2005). There are three major naturally occurring estrogens present in both males and females; estrone, 17β-estradiol and estriol with estetrol also being present in women during pregnancy (Figure 1.2). 17β-Estradiol is an aromatized C18 steroid with hydroxyl groups at 3-β and 17-β positions, resulting in a rigid hydrophobic structure with polar groups at both ends.

17β-Estradiol is both the most prevalent and biologically active estrogen during reproductive years. During menopause estrone is at the highest concentrations and during pregnancy estriol is the most abundant. Estriol is the weakest estrogen and 17β-estradiol the strongest, being about 80 times more potent than estriol. While estrogen levels are significantly lower in males compared to females, estrogens nevertheless also have important physiological roles in males, effecting; brain structure, pituitary hormone secretion, immune function and cardiovascular function (Lombardi 2001; Burger 2002).

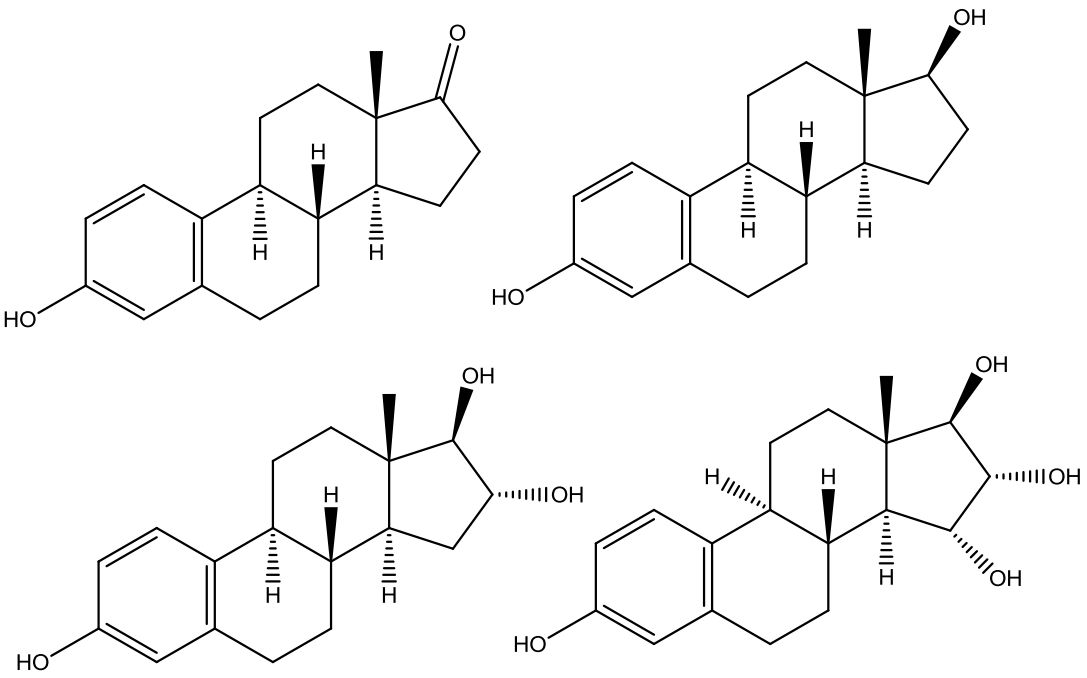

Figure 1.2 Structures of estrogens. Top left: estrone. Top right: 17β-estradiol. Bottom left: estriol. Bottom right: estetrol.

1.1.3 Estrogen receptors

Estrogen receptors (ERs) are members of the nuclear receptor superfamily, with two genes that code for two ER forms, ERα on chromosome 6q24-q27 (Gosden et al. 1986) and ERβ on chromosome 14q22-q24 in humans (Enmark et al. 1997). Gene organization is well conserved despite the distinct locations, estrogen receptor 1 (ESR1), the protein coding gene for ERα, and estrogen receptor 2 (ESR2), the protein coding gene for ERβ, both contain eight exons separated by seven long intronic sequences.

ERα consists of 595 amino acid residues and has a molecular weight of approximately 66 KDa, ERβ is 530 residues and has a molecular weight of approximately 60 KDa (Figure 1.3). They both have similar secondary and tertiary structures, consisting of 12 α-helixes arranged in an antiparallel layer with a sandwich topography.

Like all members of the nuclear receptor superfamily the ERs have 6 regions; A, B, C, D, E and F (Figure 1.3). Exon 1 encodes the A/B region in ERα and ERβ. Exon 2 and 3 encode part of the C region, exon 4 the remainder of the C, the D

and part of the E region. Exons 5 to 8 encode the rest of the E region with exon 8 also encoding the F region (Ascenzi et al. 2006).

The ERs have three key domains (Figure 1.3), activation function 1 (AF-1), the deoxyribonucleic acid (DNA) binding domain (DBD) and activation function 2 (AF-2). AF-1 is found at the A/B region and can transactivate gene transcription in the absence of a bound ligand, the activation is weak and more selective than the ligand bound activation. The DBD is at the C region and binds to specific DNA sequences found in the regulatory regions of estrogen-responsive genes called estrogen response elements (ERE) (Figure 1.4). AF-2 is at the E and F region and includes the ligand binding domain (LBD) and sites for co-activators and co-repressor proteins to bind. There is also a hinge domain at the D region which is variable in length and supports autonomous activation.

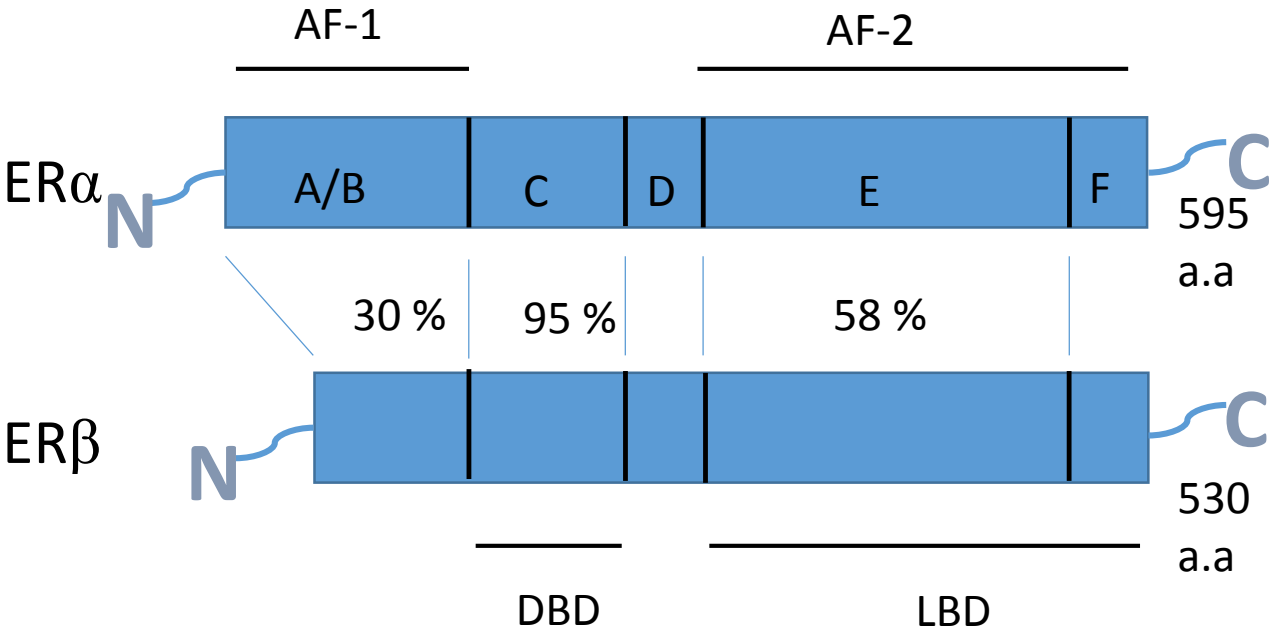

Figure 1.3 Domain organisation of human ERα and ERβ. Showing regions (A-F), domains (AF-1, DBD, AF-2 and LBD), N and C terminals, number of amino acid (a.a) residues and sequence homology.

The ERs have a high degree of homology, with the most conserved domain being the DBD (the C region), with 95 % homology between α and β subtypes (Figure 1.3). This high degree of homology suggests similar target promoter sites of both receptors. The A/B domain is located at the N terminus of the

protein and comprises the AF-1 domain which is responsible of independent transactivation. This is the least conserved domain with only 30 % homology and is functional only in the α subunit (Hall & McDonnel 1999). The D domain comprises the hinge region which contains the nuclear localization signal of the ERs as well as sites for post translational modifications (Sentis et al. 2005). This region has only 30 % homology between subunits and information on the structure/function relationship is very limited. The E and F regions comprises the AF-2 domain, which is a ligand dependent transactivation domain, as well as the LBD and the homo/hetero dimerization site. These regions have 58 % homology between subtypes resulting in potential differences in ligand binding between the two receptors (Figure 1.3). 17β-Estradiol binding to the LBD induces the ER to undergo a conformational change, resulting in the receptor dimerizing, binding to DNA (Figure 1.4) and stimulating gene expression.

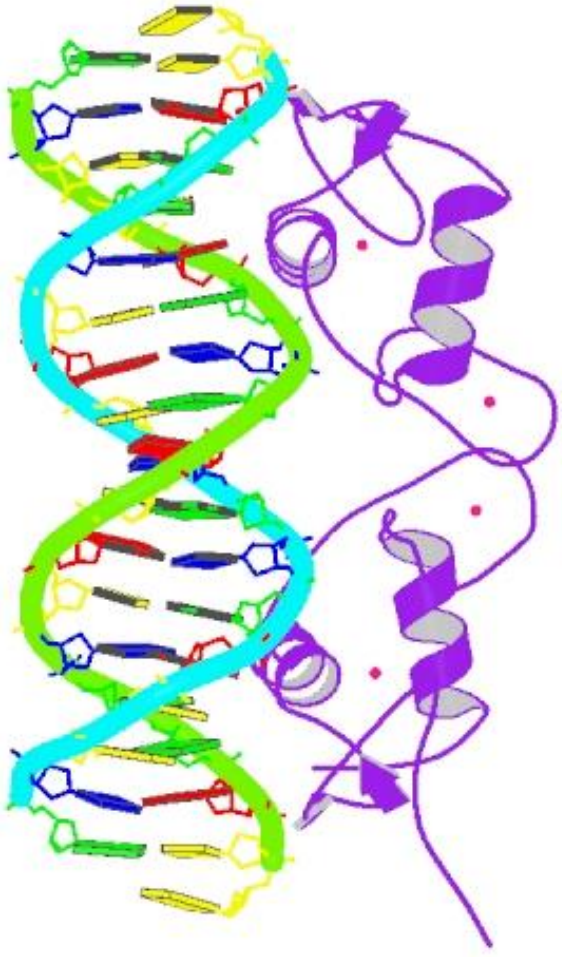

Figure 1.4 DBD domain dimer of Human ERα binding to an ERE. From PDB 1HCQ

The primary ligands for the ERs are 17β-estradiol and its two metabolites, estrone and estriol. The first estrogen receptor (ERα) was identified in 1968; however, it wasn't until 1996 that the gene for the ERβ was identified in rat prostate and ovary using degenerate ERα primers. This method involved using a mix of oligonucleotide sequences as primers for the ERβ, that are ERα based primers where some positions contained a number of possible bases (Jensen 2003; Kuiper et al. 1996).

The ERs have been historically regarded as residing in the cytoplasm when not ligand bound, but green fluorescent protein tagged ERα have been visualized predominately in the nucleus, with only a small amount in the cytoplasm (Htun et al. 1999).

The ERs distribution in human tissues vary as shown in Table 1.1, with both ERs being widely expressed in different tissue types but with some distinct differences. The ERα is the only ER found in the liver, adrenal gland and the vagina. The ERβ is the only ER found in the lung, bladder, epididymis, pituitary gland, gastrointestinal tract, colon, small intestine, prostate, testes, thymus and fallopian tube (Hess 2003; Babiker et al. 2002). This varying distribution causes the estrogenic effect of a certain compound on cells or tissues to depend on the receptor phenotype of these cells or tissues.

Table 1.1 Distribution of ERs in human tissues.

	ERα	ERβ		ERα	ERβ
Heart	✓	✓	Adrenal	✓	×
Lung	×	✓	Kidney	✓	✓
Vascular	✓	✓	Prostrate	×	✓
Bladder	×	✓	Testes	×	✓
Epididymis	×	✓	Brain	✓	✓
Pituitary	×	✓	Thymus	×	✓
Liver	✓	×	Breast	✓	✓
Muscle	×	×	Uterus	✓	✓
Fat	×	×	Endometrium	✓	✓
Gastrointestinal tract	×	✓	Vagina	✓	×
Colon	×	✓	Fallopian tube	×	✓
Small intestine	×	✓	Ovary	✓	✓
Bone	✓	✓			

1.1.4 ER activation pathway

Estrogens exert their effects on tissues by binding to the ERs, which can function as transcription factors when activated by a ligand. The biological action of ERs is complex with broad mechanisms, two main mechanisms have been described, genomic and non-genomic.

The genomic action of the ERs occurs in the nucleus of cells, where the receptor binds EREs and regulates transcription (Figure 1.5). In the absence of a ligand the ERs are associated with heat shock proteins. Heat shock protein 90 (HSP90) and heat shock protein 70 (HSP70) are chaperone proteins that inhibit many steroid receptors but stabilize the LBD making it accessible by a ligand. When the ERs have an estrogen bound they change conformation, displacing HSP90/HSP70 causing the ER to dimerize and become phosphorylated. Once dimerized they are able to recruit co-activators which can participate in the interaction with DNA. With 17β-estradiol bound the ERs induce chromatin remodelling and increase transcription of estrogen regulated genes (Berno et al. 2008).

Figure 1.5 Model representing the genomic mechanism of ERs. Ligand activation causes ER DNA interaction altering gene expression. From Heldring et al. 2006.

Since ERα and ERβ are often co-expressed, dimers that form may be ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers (Li et al. 2004).

Co-activators and co-repressors can bind in a shallow hydrophobic leucine containing binding site on the surface of the ER located between helices H3 and H5 in the AF-2 domain (Figure 1.6).

Binding of an ER antagonist in the LBD causes a conformational change resulting in helix 12 occupying the co-factor binding site blocking binding of co-activators and subsequent DNA interactions. When an agonist is present helix 12 is not in the co-factor binding site allowing co-activator recruitment and subsequent DNA interaction leading to induction of transcription (Leclercq 2011) (Figure 1.6). Co-activators include steroid receptor co-activator-1 and

steroid receptor co-activator-3 which function as histone acetyltransferases, acylating histones, which package DNA, causing downstream DNA to become more accessible for transcription (Onate et al. 1995).

Widely expressed co-repressors such as nuclear co-repressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) (Horlein et al. 1995) can also bind to the ERs at the DNA binding site, rather than the LBD, and block the ERs interaction with DNA (Varlakhanova et al. 2010).

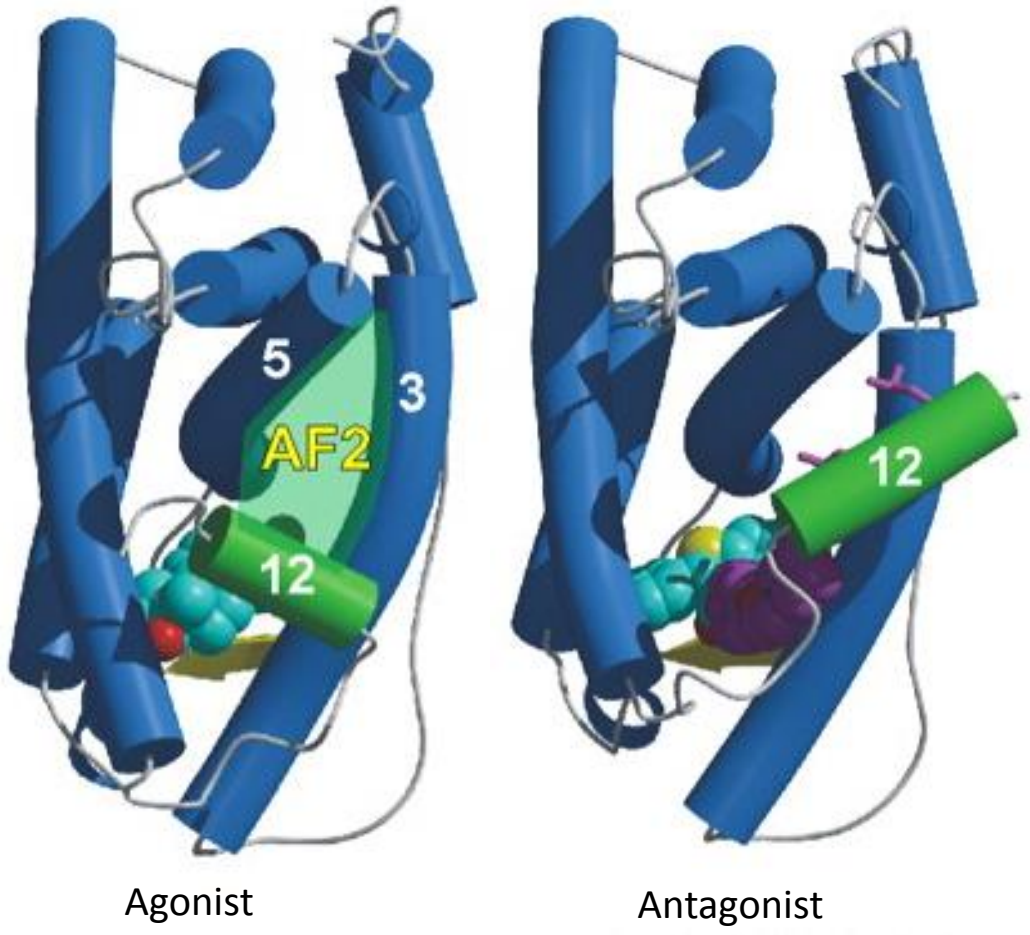

Figure 1.6 Schematic representation of ERs in agonistic (left) and antagonistic conformations. From Heldring et al. 2006. The agonist ER conformation allows co-factor binding with helix 12 blocking co-factor binding in the ER antagonist conformation.

Non-genomic ER activation was first noticed in 1967 when a 17β-estradiol dose increased cyclic adenosine monophosphate (cAMP) levels in the uterus of rats within 15 seconds (Szego & Davis 1967). This quick response is considered too fast for a genomic action. ERs can associate with the cell surface membrane and become rapidly activated by exposure of cells to estrogens causing signalling cascades leading to a change in cell function (Figure 1.7). The ERs on

the cell membrane attach to caveolin-1, a scaffolding protein that produces lipid rafts, forming complexes with guanine nucleotide-binding proteins (G proteins) and tyrosine kinases such as epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 (IGF-1). G proteins are guanosine triphosphatases (GTPases) that act as molecular switches inside cells. They are regulated by factors such as ERs that control their ability to bind and hydrolyse guanosine triphosphate (GTP) to guanosine diphosphate (GDP). When GTP is bound they are active and when GDP is bound they are inactive. When active G proteins cause a cascade of further intracellular signalling resulting in regulation of a variety of functions including; metabolic enzymes, ion channels, transporter proteins, transcription and secretion, leading to a regulation of system functions including; homeostasis, learning and memory and embryo development. Tyrosine kinases are enzymes that can transfer a phosphate from ATP to proteins, acting as an on/off switch. EGFR is essential for duct development of the mammary glands and IGF-1 plays an important role in growth and development (Lu et al. 2004; Kato et al. 1995).

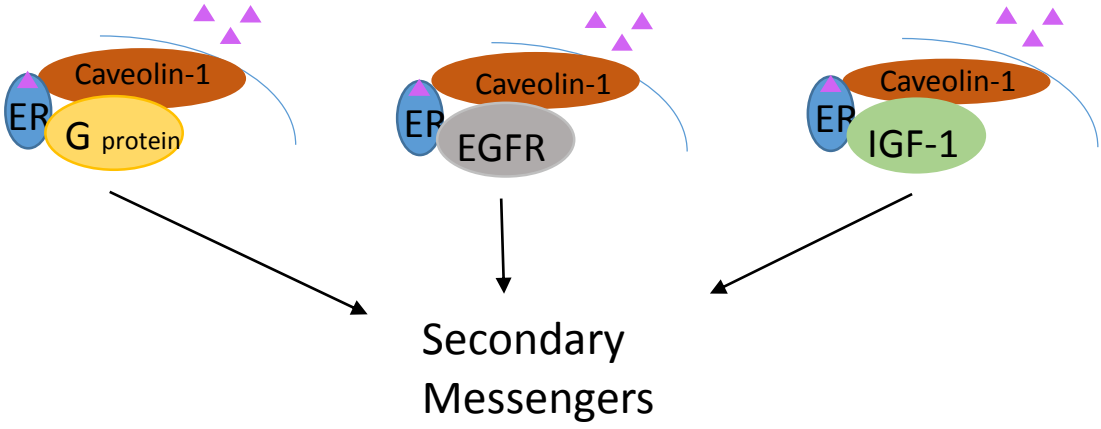

Figure 1.7 Model of non-genomic ER activation. Membrane associated ERs complex with G proteins, EGFR or IGF-1 to produce secondary messenger signalling cascades leading to rapid altered cellular function.

Ligands can have different binding affinity's for ERα and ERβ arising from structural differences in the LBD, for example estrone has a greater binding affinity for the ERα and estriol has a greater binding affinity for the ERβ with estradiol binding equally well to both (Zhu et al. 2006). ERs can also associate with different co-factors depending on binding affinities and relative abundance (Halachmi et al. 1994), with differences in ERα and ERβ co-factor binding (Suen et al. 1998). This preferential binding of ligands and co-factors to ERs can result in specific ligand signalling and differing biological effects by ligands and differing biological effects depending on the ratio of ERα/ERβ and

co-factors present in specific tissues. The results can be the same ligand acting asan agonist in tissues where co-activators are predominant and an antagonist in tissues where co-repressors are dominant. These factors effecting the overall biological impact of a specific ER ligand allow for a precise, targeted response to natural hormones.

An example of differing cell response to the same compound is the drug tamoxifen. Tamoxifen is an ER antagonist in breast cells, combating breast cancer and an ER agonist in bone cells and the endometrium, preventing osteoporosis and increasing the risk of uterine cancer (Shang & Brown 2002; Deroo & Korach 2006).

The multiple mechanisms and compounds involved for ER signalling provides many points for potential signal alteration by estrogens and estrogen mimicking compounds, with small structural changes between ligands potentially resulting in significantly different responses.

1.1.5 Estrogen biosynthesis

Follicle-stimulating hormone, a glycoprotein polypeptide hormone secreted by the anterior pituitary gland which regulates development, growth, puberty and reproductive process, stimulates the ovarian production of estrogens by the granulosa cells of the ovarian follicles and corpora lutea in females. The liver, adrenal glands, fat cells and breasts are also secondary producers of estrogens.

The ovaries contain roughly spherical shaped cell aggregations called ovarian follicles. Each ovarian follicle contains an immature oocyte (egg cell) which, when stimulated to grow releases a mature oocyte at ovulation. The oocyte is surrounded in granulosa cells which have a thin layer of extracellular matrix surrounding them known as the basal lamina. The basal lamina has theca interna and theca externa surrounding it. Once the oocyte has been released the corpus luteum develops from the ovarian follicle during the luteal phase of the menstrual cycle. The oocyte travels down the fallopian tube to the uterus but the corpus luteum remains in the ovary. The corpus luteum can have a diameter between 2-5 cm. The change from an ovarian follicle to the corpus luteum involves follicular theca cells luteinizing into small luteal cells and follicular granulosa cells luteinizing into large luteal cells (Figure 1.8).

The ovarian cycle consists of the follicular phase, where the ovarian follicle is present, ovulation, where the oocyte is release into the fallopian tube, and the luteal phase, where the corpus luteum is present.

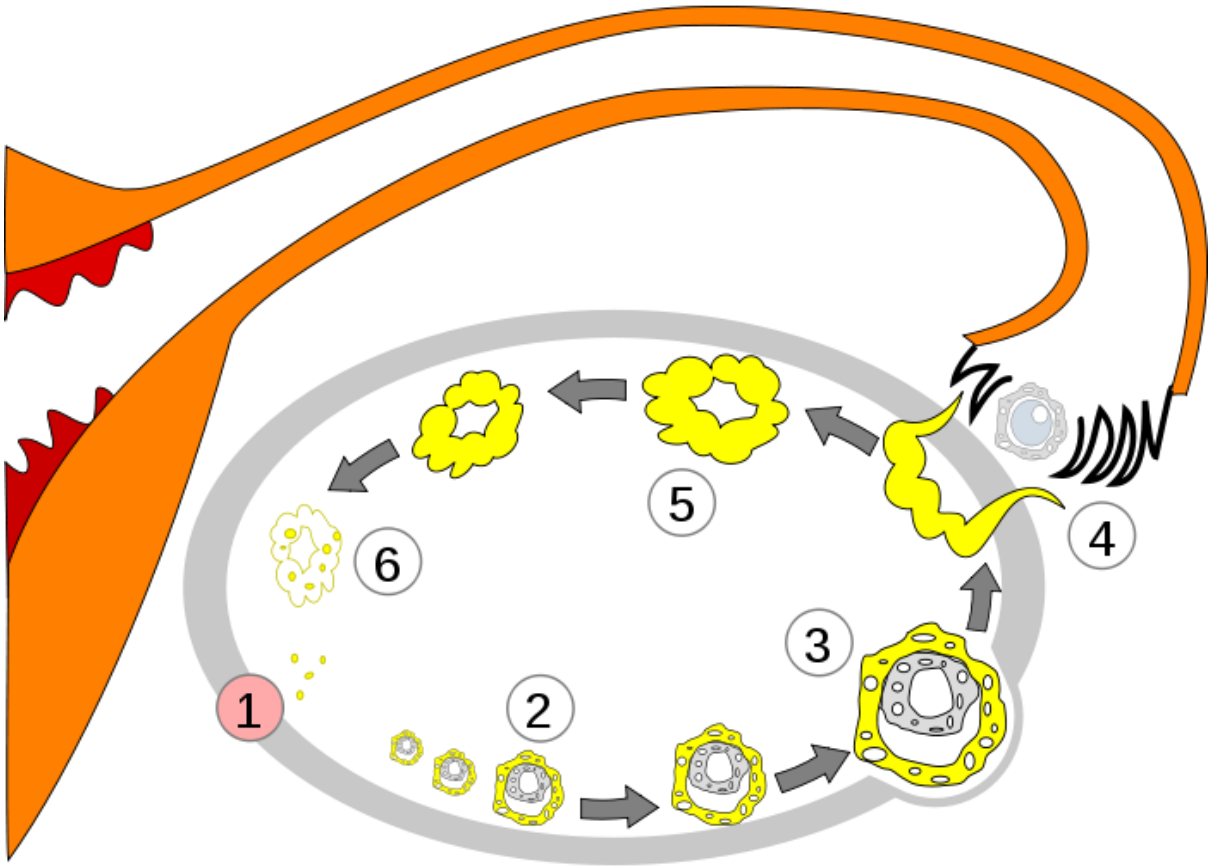

Figure 1.8 Changes in the ovary throughout the menstrual cycle. 1. Menstruation. 2. Maturing follicle. 3. Mature follicle. 4. Ovulation. 5. Corpus luteum. 6. Deterioration of corpus luteum. From wikimedia commons.

For the synthesis of estrogens in the ovaries the starting location is the theca interna cells and the starting compound cholesterol. Androstenedione is produced from cholesterol and is a weak androgenic compound serving as a precursor for more potent androgens (Figure 1.9). Androstenedione can cross the basal membrane into the surrounding granulomas cells where it is converted into estrone or estradiol via testosterone (Figure 1.9). Both granulosa and theca cells are essential for estrogen production in the ovaries. Granulosa cells lack 17α-hydroxylase and 17,20-lyase, used to convert

cholesterol to androstenedione and theca cells express these enzymes but not aromatase, used to convert androstenedione to estrone (Figure 1.9). Throughout the menstrual cycle estrogen levels vary with highest levels near the end, just before ovulation (Nelson & Bulun 2001).

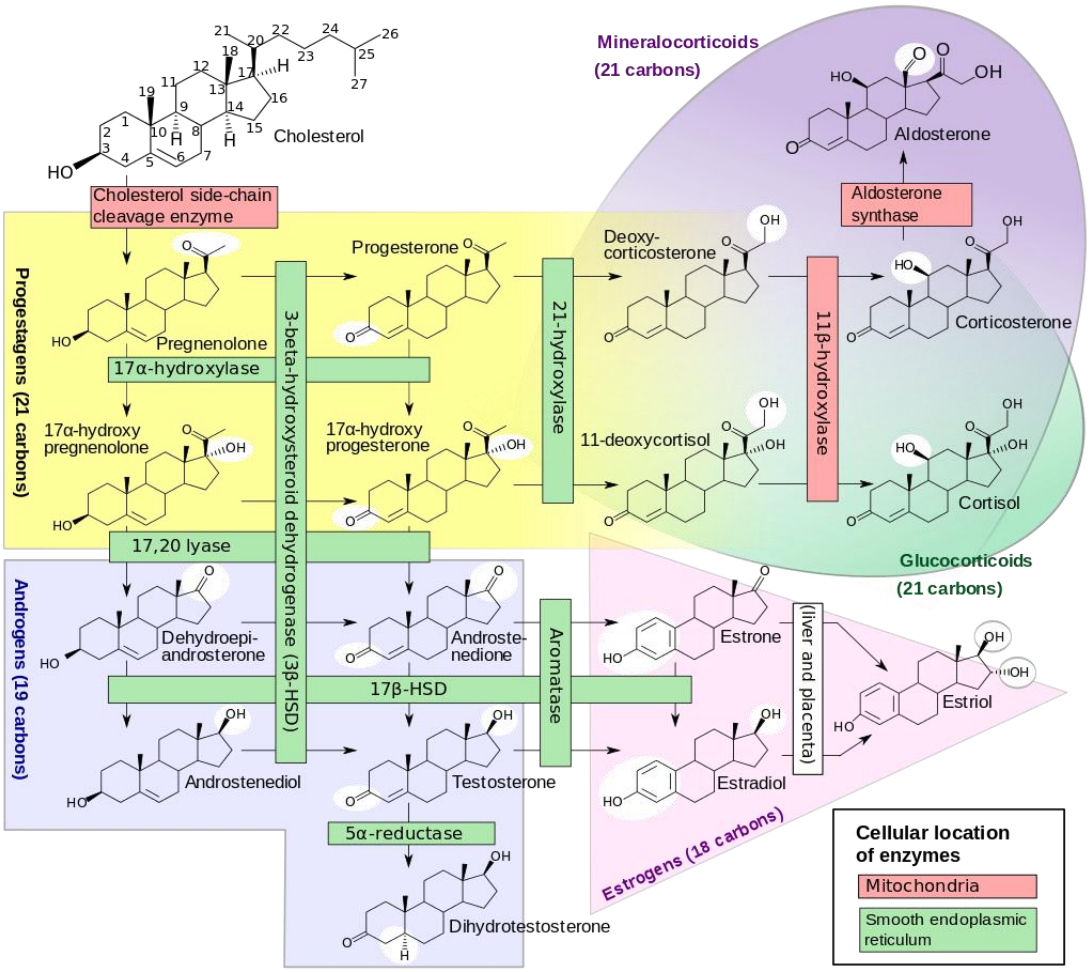

Figure 1.9 Biosynthesis of steroid hormones from cholesterol. From Wikimedia commons (Haggstrom & Richfield 2014).

1.2 Endocrine disruption

1.2.1 Types of endocrine disruption

Endocrine disruption is caused by chemicals that interfere with the endocrine system. Endocrine disrupting chemicals (EDCs) can interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body resulting in a deviation from normal homeostatic control or reproduction. (Crisp et al. 1998). These disruptions can cause obesity, diabetes, male and

female reproductive problems, male and female hormone-sensitive cancers, thyroid problems and neurodevelopmental problems, depending on which hormone is disrupted (Gore 2015).

EDCs act by many different mechanisms, including; nuclear receptors, non-nuclear receptors, non-steroid receptors, orphan receptors, enzymatic pathways involved in steroid biosynthesis and/or metabolism, as well as numerous other mechanism. An example of altered hormone biosynthesis is aromatase (Figure 1.9) inhibition by the fungicide fenarimol (Hirsch et al. 1987). An example of altered hormone secretion can occur as release of many protein hormones depends on activation of secondary messenger pathways such as cAMP, tyrosine kinase and Ca^{2+}. This causes an interference in the activation of these pathways, altering the serum levels of many hormones. Several metal cations have been shown to disrupt pituitary hormone release by interfering with the Ca^{2+} flux (Cooper et al. 1987).

EDCs are highly heterogeneous, representing a broad range of molecules such as; pesticides, fungicides, solvents, lubricants, pharmaceutical agents, plastics and plasticizers, fuels as well as many other chemicals that are widely present in the environment or are in widespread use.

There are critical development periods in humans where exposure to EDCs has a greater effect. In cases where disruption of programing a function occurs, such as reproductive health, an interference with early life organisation can be followed by a latent period with the dysfunction becoming obvious once the function becomes activated. This means fetal life is most vulnerable as there are rapid structural and functional events occurring (Diamanti-Kandarskis et al. 2009).

1.2.2 Xenoestrogens

Xenoestrogens are a sub class of EDCs that are exogenous compounds; xenos is Greek for strange or foreign, that mimic the effects of endogenous estrogens. These chemicals are cause for concern as they interfere with the normal endocrine process, which may lead to adverse health effects in the exposed individual and/or their offspring. As such they are classified as EDCs and have been the source of great interest in the last three decades. Synthetic and naturally occurring xenoestrogens are ubiquitous in the environment, and in most cases enter the body through ingestion, inhalation or cutaneously.

Sources of exposure to EDCs are diverse, changing and varying around the world as regulations banning chemicals were put into place decades ago in some countries but just recently in others. There are examples of toxic spills or contaminations of EDCs that show a direct relationship between a chemical and the manifestation of an endocrine or reproductive dysfunction in humans and wildlife.

BPA is a xenoestrogen found in many plastics, dental materials, covering receipt paper and lining many metal food and infant formula cans. Animals exposed to low levels of BPA can have elevated rates of diabetes, mammary and prostate cancers, decreased sperm count, reproductive problems, early puberty, obesity and neurological problems (vom Sal & Hughes, 2005).

DDT is another example of a xenoestrogen that has been used as a pesticide against lice, flies and mosquitoes and is now widely present in nature. It was designed to have a long half-life which has turned out to be detrimental to humans and wildlife and can be detected in virtually every tested animal or human. DDT interferes with reproductive development, inhibits development of female reproductive organs, decreased fertility in males and increases childhood obesity (Tiemann 2008; Hallegue et al. 2003; Verhulst et al. 2009). DDT has been found in remote glaciers of the Himalayas and Antarctic snow, which are far removed from sights of use or production. This is due to transportation by water and air currents as well as migratory animals. (Szlinder et al. 2008; Peterle 1969; Daly & Wania 2005).

Phthalates are used as plasticizers and can be found in soft toys, medical equipment, cosmetics, flooring and air fresheners. They have been linked with a rise in birth defects of the male reproductive system, such as hypospadias and cryptorchidism by altering testosterone levels (Barrett 2005).

To determining the actual biological net effect of phytoestrogens is a difficult task complicated by factors such as; the route of administration, bioavailability, metabolism, timing, level of exposure, endogenous estrogen concentration as well as various non-hormonal effects (Cassidy 1999).

The concentration of xenoestrogens is not the only factor in terms of estrogenic impact as xenoestrogens have varying levels of estrogenicity. To compare the estrogenic potency of xenoestrogens their estrogenicity is expressed relative to 17β-estradiol. This gives the equivalence factor (EQ), which is the concentration of a xenoestrogen required to give a specified result

in an estrogenicity assay divided by the concentration of 17β-estradiol required to give the same response.

Effects of weakly estrogenic compounds are additive (Payne et al. 2000), so even if intake of a particular xenoestrogen is low it is still important as it contributes to the cocktail of xenoestrogens to produce the overall exposure effect.

The detrimental health effects caused by xenoestrogens is estimated to be so vast that it has caused economic impact estimated to be twice the economic impact of the effects caused by mercury and lead contamination in the European Union. (Leonardo et al. 2015).

The most impactful xenoestrogens from the diet are BPA and the phytoestrogen genistein, with isoflavones accounting for 29-36 % of xenoestrogenicity (Thomson et al 2003).

1.3 Isoflavones

The most abundant natural xenoestrogens in a human context are the plant-produced phytoestrogens. Phytoestrogens are diphenolic non-steroidal estrogen-like substances found in all plants, being present at the highest concentration in legumes (Baber 2010).

The greatest estrogenic activity of the phytoestrogens is found in flavones, flavanals, flavanones, lignans, chalcones and isoflavones. The difference between flavones and isoflavones is the position of the phenyl group, for flavones it is located on carbon 2 and for isoflavones it is on carbon 3, (Figure 1.10). The most abundant group of phytoestrogens is the isoflavones, which can bind to the human estrogen receptors (ERs) to produce estrogenic or anti estrogenic effects.

Isoflavones have EQs of between 10^{-2} and 10^{-4} that of 17β-estradiol and can be present in blood at levels up to 10,000 times that of steroidal estrogens (Adlercreutz 1991; Mitisick 1994; Duncan 2003). Isoflavones at concentrations capable of exerting a physiologically relevant estrogenic effect are found in soybeans (*Glycine max*) and food derived from soybeans (Mazur 1997).

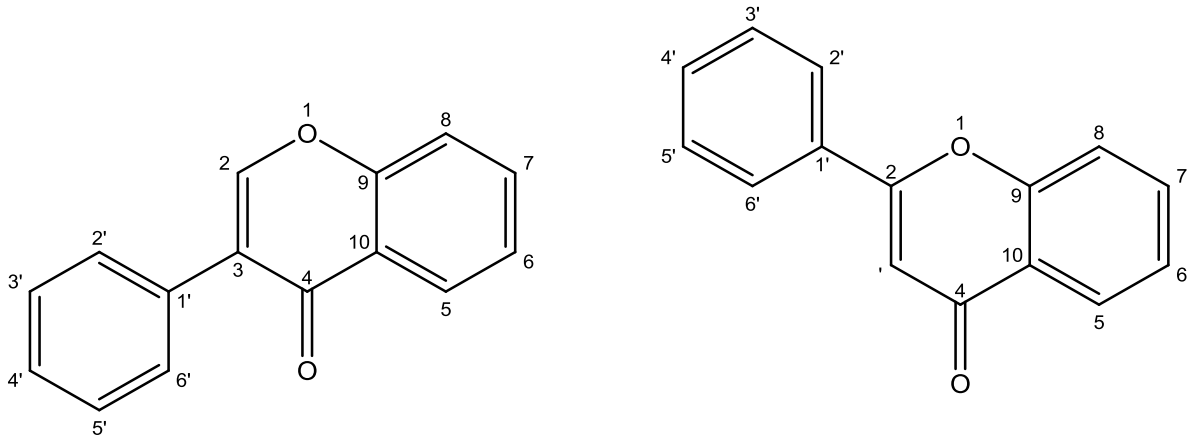

Figure 1.10 Backbone structures of isoflavones (left) and flavones (right). Isoflavones have a phenyl group on carbon 3 and flavones a phenyl group on carbon 2.

1.3.1 Soy isoflavones

Soy is the primary plant that produces isoflavones, containing 1-2 mg/g. There is only a small amount in other plants with chickpeas containing 1 % the isoflavones of soy (Dixon 2004).

The isoflavones present in soy include genistein (4'-5,7-trihydroxyisoflavone), genistin, 6"-0-malonyl genistin and 6"-0-acetyl genistin (Figure 1.11).

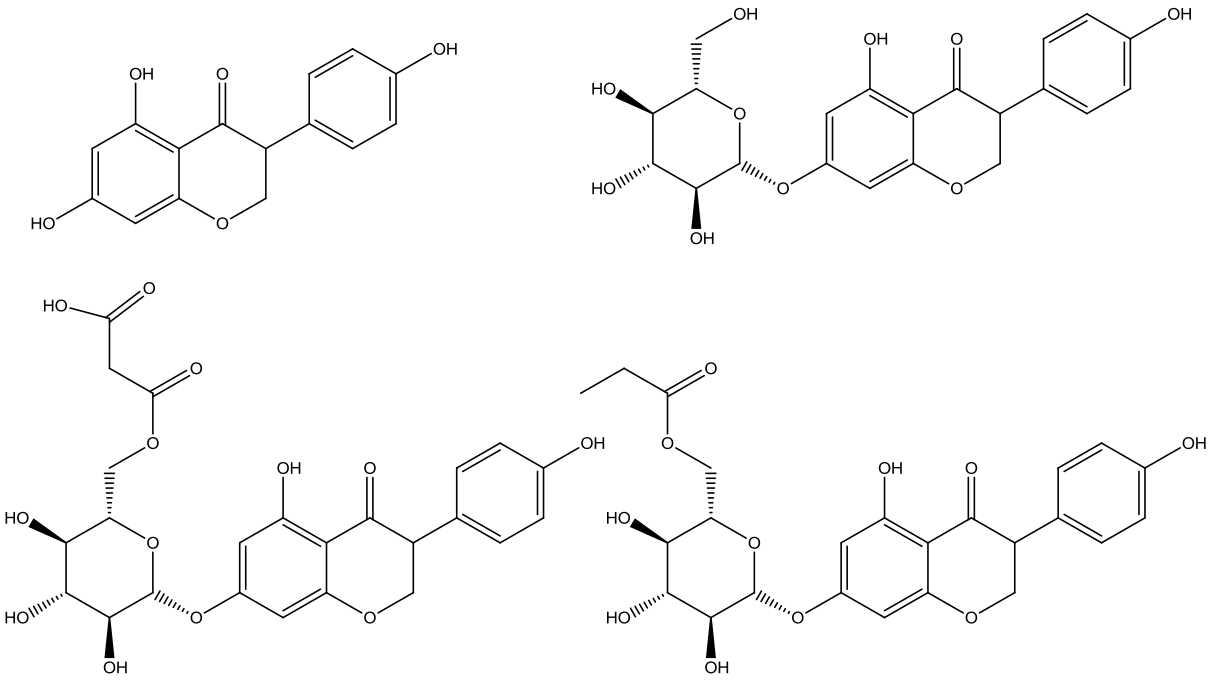

Figure 1.11 Genistein and its sugar conjugates, top left; genistein, top right; genistin, bottom left; , 6"-0-malonyl genistin, bottom right; 6"-0-acetyl genistin.

Daidzein is also present in soy, along with its sugar conjugated forms, daidzin, 6”-0-acetyl daidzin and 6”-0-malonyl daidzin (Figure 1.12).

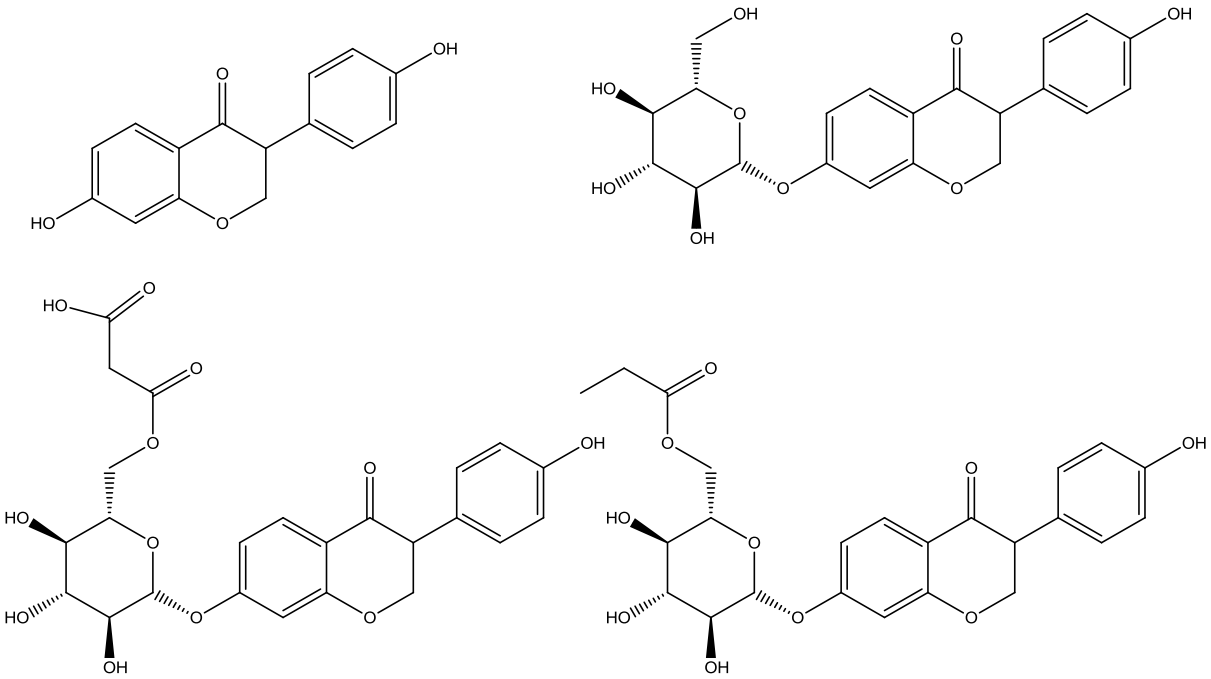

Figure 1.12 Daidzein and its sugar conjugates, top left; daidzein, top right; daidzin, bottom left; , 6”-0-malonyl daidzin, bottom right; 6”-0-acetyl daidzin.

Soy also contains glycitein along with its sugar conjugated froms, glycitin, 6”-0-acetyl glycitin and 6”-0-malonyl glycitin (Figure 1.13).

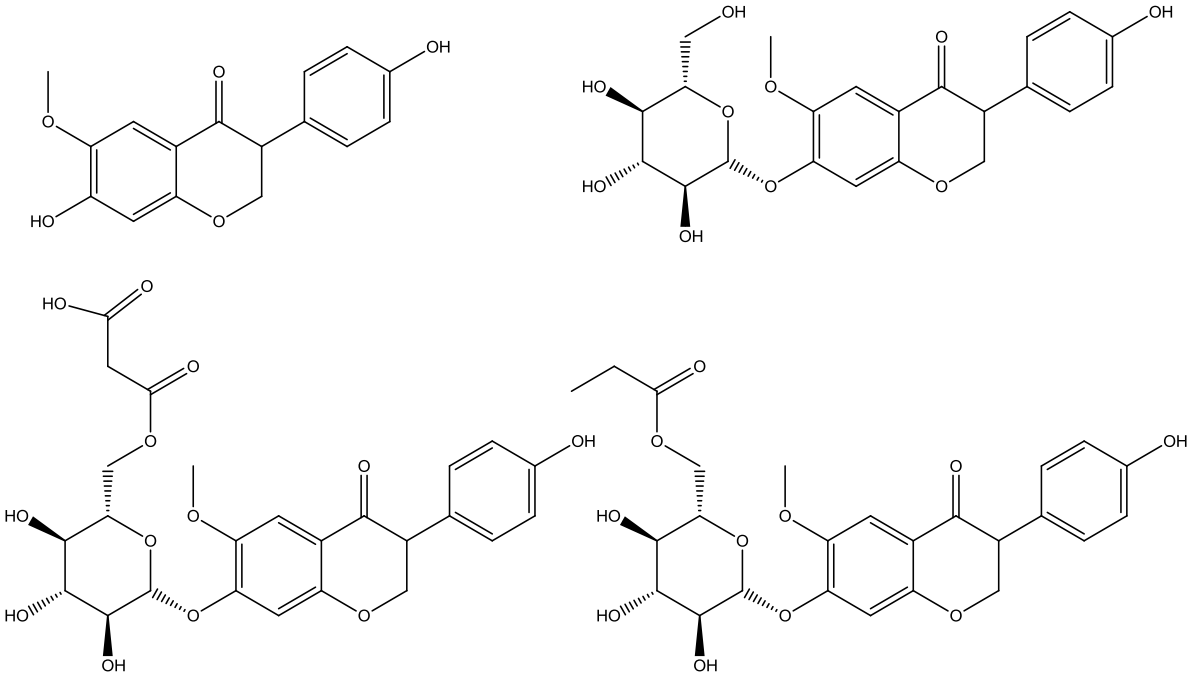

Figure 1.13 Glycitein and its sugar conjugates, top left; glycitein, top right; glycitin, bottom left; , 6”-0-malonyl glycitin, bottom right; 6”-0-acetyl glycitin.

This gives a total of 12 different isoflavone forms found in soy, 3 aglycones each with three glycoside forms.

Isoflavones distribution is not equal in all tissues of the soybean plant. The isoflavone content depends on factors such as the type of tissue, morphogenesis of the tissue and changes caused by biotic and abiotic stresses. Growing soy seedlings in the dark for example, causes reduced amounts of daidzein and genistein as well as their glycosides in the roots and significantly higher levels in cotyledons (Graham 1991).

The malonyl conjugated isoflavone forms are predominant in tissues of the mature soy plant, with acetyl conjugated forms being present in trace amounts or not at all. These acetyl glucosides are thought to arise from the decarboxylation of malonyl glucosides (Kim and Chung 2007).

The relative amount of the different soy isoflavones and the distribution among the various forms is effected by soybean variety and growing conditions (Coward et al 1998).

Isoflavones play an important role in maintaining the symbiotic relationship between plant and microbe in the leguminous plants. They are excreted by active transport through efflux pumps in plant roots, where they interact with proteins responsible for inducing the biosynthesis of bacterial nodulating signals. This causes the initiation of nodule formation, where the bacteria rhizobia grow and convert atmospheric nitrogen (N_2) to less inert forms (NH_4^+ or NO_2) in the process of nitrogen fixing (Mathesius 2009).

Isoflavones also play an important role in defence against microbial plant pathogens. They achieve this by being precursors to pterocarpans and isoflavans, which often lack C-5 hydroxylation. These are anti-microbial compounds which are either biosynthesised in response to a pathogen attack or pre-emptively, and are constantly present in plant cells to prevent infection (Shaw 2006).

1.3.2 Binding of isoflavones to the ERs

Genistein has two phenolic hydroxyls separated by 12.1 Å with similar spatial arrangements to 17β-estradiols having two hydroxyls in position 3 and 17β approximately 10.8 Å apart (Figure 1.14). This similar structure allows

isoflavones to mimic the steroidal estrogens, fitting into and activating the ERs (Pike 1999).

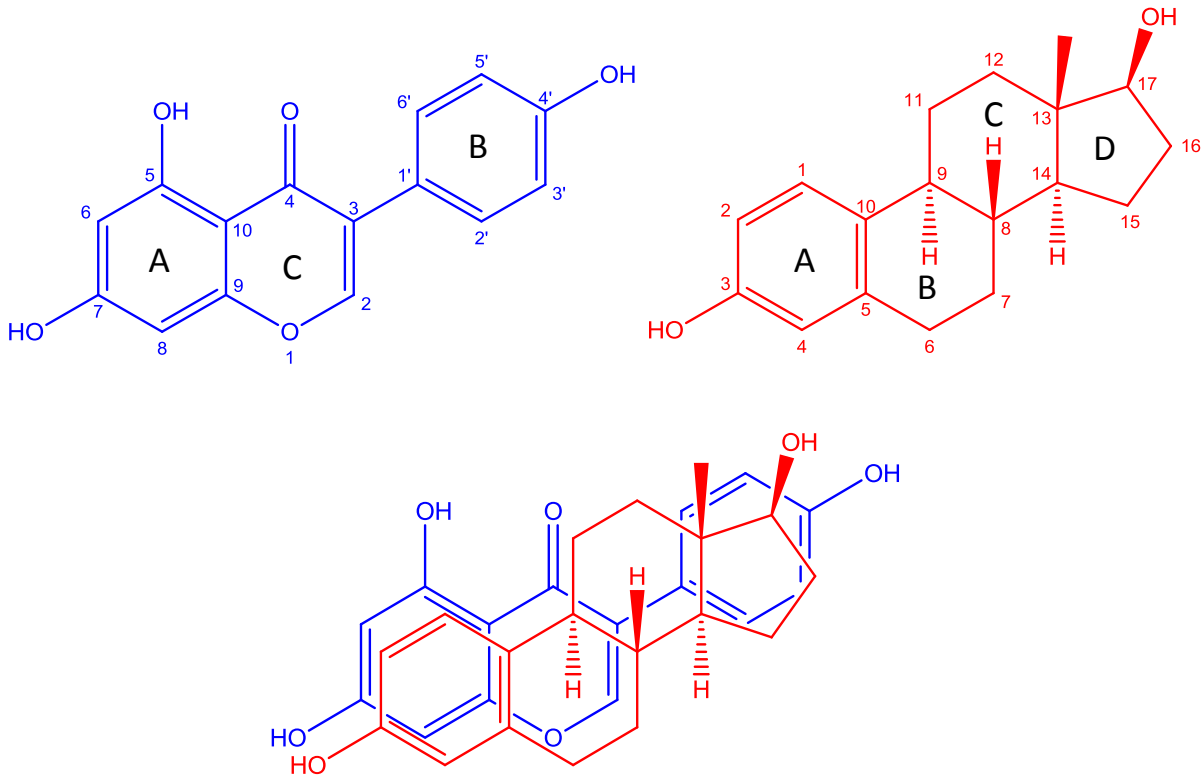

Figure 1.14 Structures of genistein (left) and 17β-estradiol (right). Superimposition of genistein and 17β-estradiol (bottom) allows visualization of similar characteristics with close alignment of peripheral hydroxyls and overlapping phenolic ring.

The ligand binding site of the ERs (Figure 1.15) binds estrogens by having polar amino acid residues that associate, directly or through co-ordinating a water molecule, with the peripheral hydroxyls. The core backbone of the estrogen molecules fit into a hydrophobic pocket created by hydrophobic amino acids.

Due to genistein's similar shape and chemical nature it is also able to interact with the ligand binding site of the ERs through association of its 4' and 7-phenolic hydroxyls (Figure 1.14) across the pocket of receptor usually occupied by estradiol and delimited by helices H3 and H6 as well as a β hairpin (Figure 1.16) (Manas 2004).

The 4' hydroxyl of genistein, corresponding to the A ring phenolic hydroxyl of estradiol (Figure 1.14) is involved in hydrogen bonds with Glu-305 and Arg-346 of ERβ (Glue-353 and Arg-394 in ERα) as well as a buried molecule of water (Figure 1.16). The 7-phenolic hydroxyl of genistein associates through a hydrogen interaction with His-475 of ERβ (His-524 of ERα) as occupies the

same region that the D ring 17β-hydroxyl of 17β-estradiol would. The core scaffold fills the remainder of the primarily hydrophobic pocket (Figure 1.16).

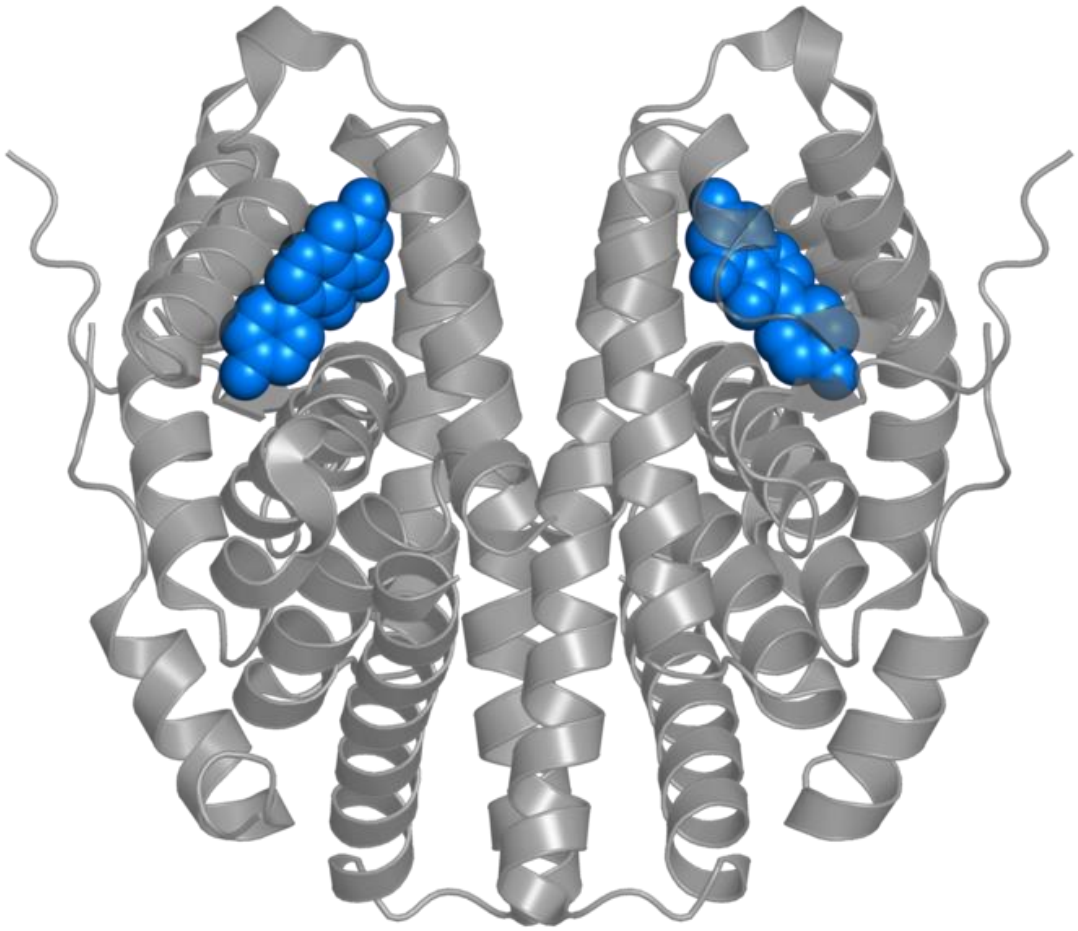

Figure 1.15 Genistein in binding clefts of a human ERβ dimer. From PDB 1QKM.

Isoflavones have a greater binding affinity for the ERβ than the ERα with genistein being almost 40-fold more selective for the ERβ. Despite this the site for ligand-binding is almost identical (figure 1.16)(Pike 1999).

The genistein B-ring (Figure 1.14) is very close to the ERα Leu-384, ERβ Met-336 residue, with the B-ring centroid approximately 4-4.5 Å from the ERβ Met-336 C atom, compared to 6.2 Å from the ERα Leu-384 Cδ1 atom. The C on position 3 of the isoflavone ring is approximately 4.2-4.6 Å from the ER-β Met-336 Sδ atom versus 4.8 Å from the ER-α Leu-384 Cδ2 atom (Figure 1.16). The volume difference between the binding pocket of ERα and ERβ causes steric effects contributing to the preferential binding to ERβ.

The repulsion between genistein's hydroxyl group on carbon 5 (Figure 1.14) with the methionine sulphur group on ERα Met-421 is another factor causing more preferential ERβ genistein binding as there is no methionine in a similar place for the ERβ (Figure 1.16).

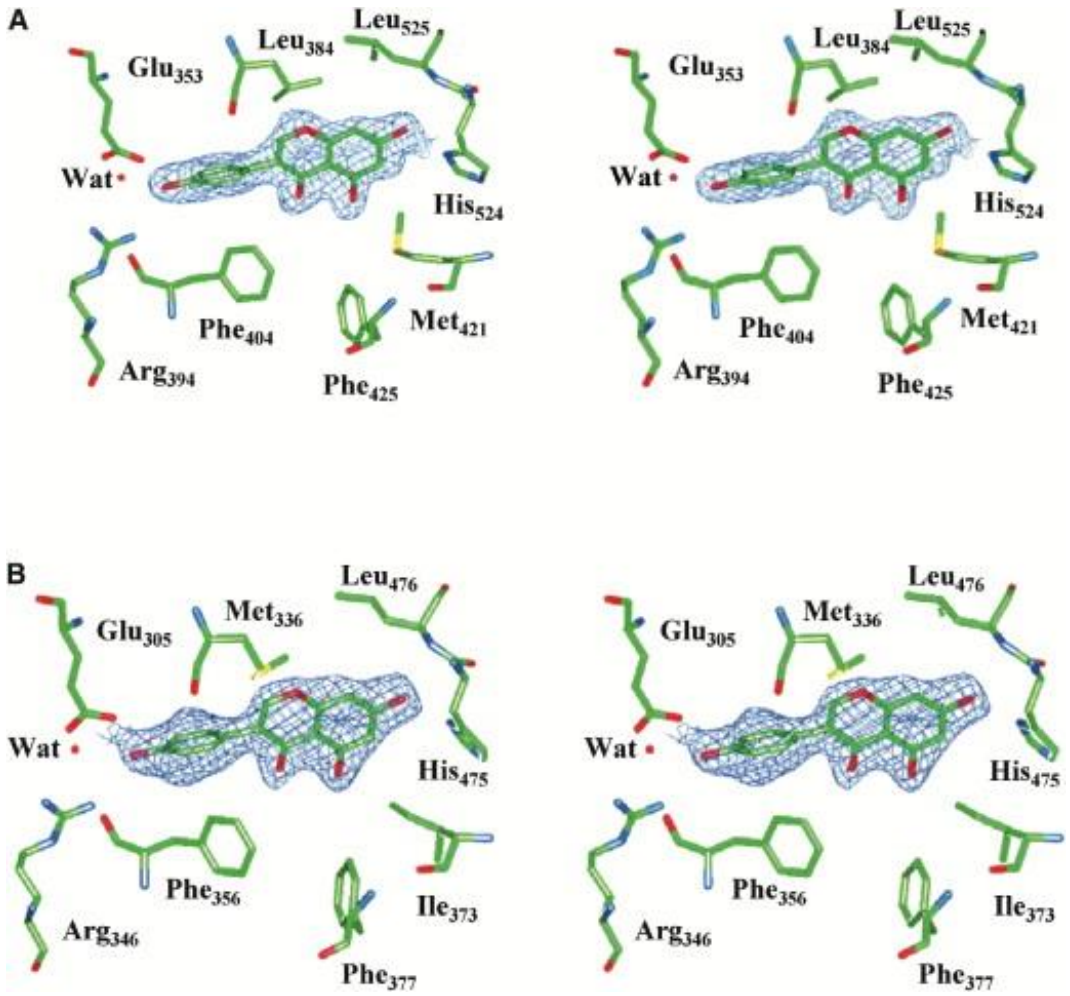

Figure 1.16 Sterioimage showing electron density for genistein when complexed with (A) ERα and (B) ERβ. From Manas 2004.

1.3.3 Biosynthesis of isoflavones

Isoflavones are biosynthetically derived from the phenypropenoid pathway. The crucial step is conversion of flavanones into their respective isoflavononones by 2-hydroxyisoflavanone synthase followed by dehydration to form the isoflavones. The dehydration step is done by the enzyme 2-hydroxyisoflavone dehydroxase (Figure 1.19) (He 2011).

Chromismate, found in the shikimate pathway responsible for the biosynthesis of the aromatic amino acids, is transformed into prephenate by a chromismate muatase. Prephenate is converted to phenylpyruvate, by prephenate dehydratase, which is converted into L-phenylalanine by a transaminase (Figure 1.17).

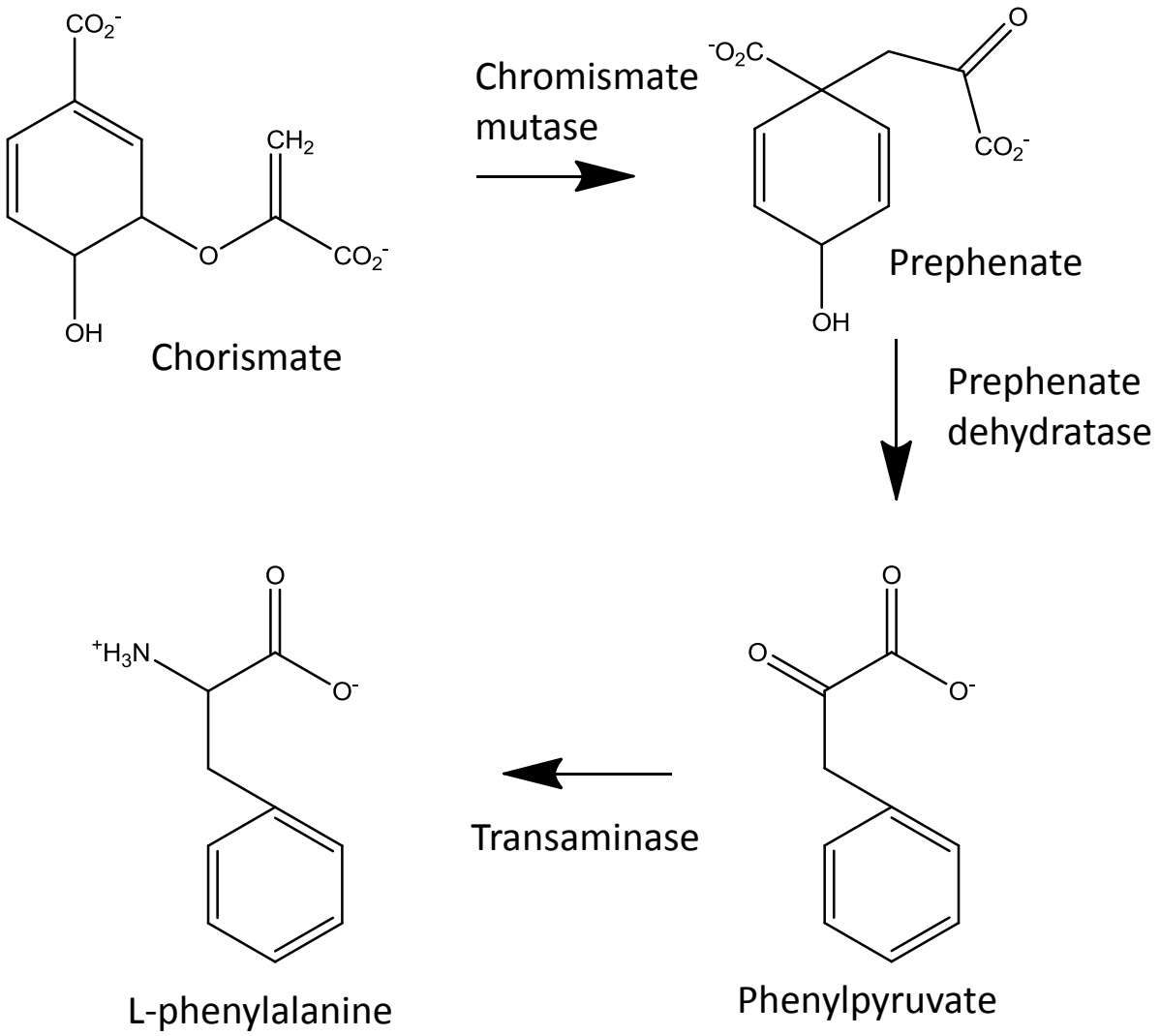

Figure 1.17 Biosyntetic pathway from chorismate to L-phenyalanine.

Trans-cinnamate is produced from L-phenylalanine, catalysed by the enzyme phenylalanine ammonia lyase. Trans-cinnamate is transformed into p-coumaric acid by a cinnamate 4-hydroxylase which is in turn converted to p-coumaroyl-S-coenzyme A (CoA) by the action of 4-coumarate:CoA ligase (Figure 1.18).

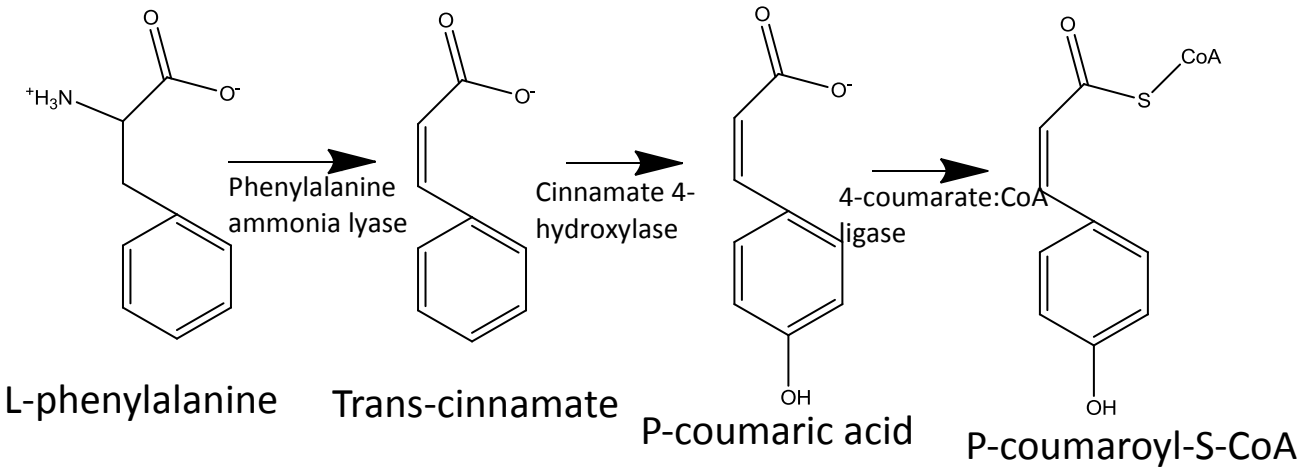

Figure 1.18 Biosynthetic pathway from L-phenylalanine to P-coumaroyl-S-CoA

P-coumaroyl-S-CoA is the start of isoflavone biosynthesis with two competing branches, one leading to genistein production and the other daidzein. It reacts with 3 malonyl S-CoA units in the preseince of chalcone synthase (CHS) and chalcone reductase (CHR) or CHS alone. This produces 4,2',4'-trihydroxychalcone (isoliquirtigenin) and 4,2',4'6'-trihydroxychalcone (naringenin chalcone) respectively (Figure 1.19).

Cytochrome P_{450} mono-oxygenase and chalcone isomerase respectively steriospecifically catalyse the cyclization of 4,2',4'-trihydroxychalcone into 7,4'-dihydroxyflavanone (liquritigenin) and 4,2',4'6'-trihydroxychalcone into 5,7,4'-diydroxflavanone (naringenin) (Figure 1.19).

Daidzein and genistein are produced by the enzyme 2-hydroxyisoflavone synthase, a cytochrome P_{450} enzyme (CYP93). is responsible for the formation of a 2-3 double bond and the regioselective migration of the B phenolic ring from the 2 to position 3 double bond (Figure 1.19) (Crombie 1992).

Once the isoflavone aglycone has been produced in the soy plant it is converted to a 7-0-β-glucoside by a glucosyltransferase and then to a 6-0-malonyl form by a malony transferase. This malonyl conjugated isoflavone is stored in cell vacuoles until required for cell defence or in the nitrogen fixation process.

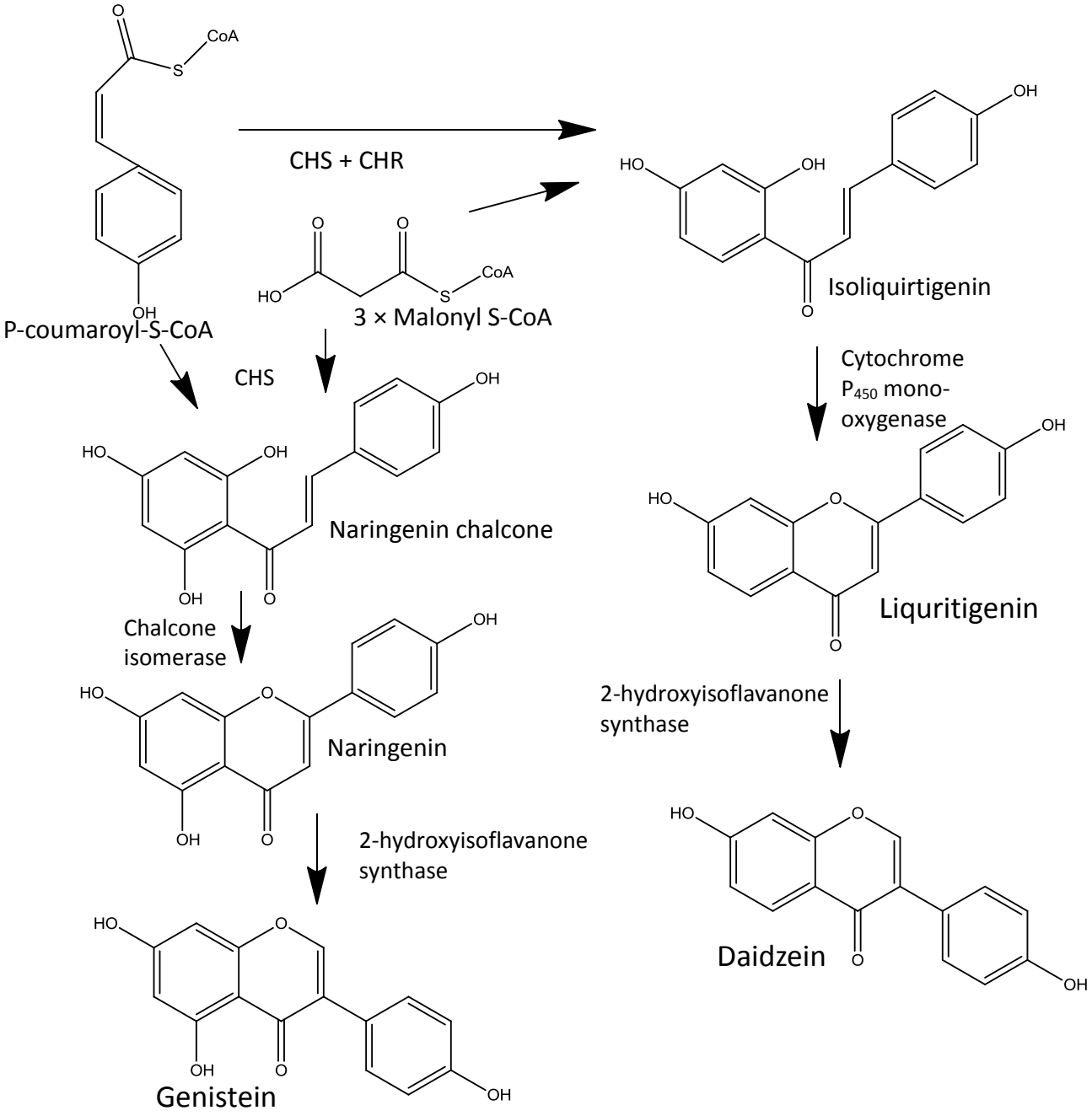

Figure 1.19 Biosynetic pathway from P-coumaroyl-S-CoA to genistein and daidzein

1.4 Soy in bread

1.4.1 History of soy

Soybeans have a long history as a domesticated plant tracing back to the 11th century BC in China. From there it is believed missionaries brought the plant to Korea and Japan in the 3rd and 4th centuries. Soybeans further spread around

the world reaching Europe in the early 1700s and USA by 1765 (Hymowitz 1990).

Between 1961 and 2009 soy production expanded nearly tenfold, with 270 million tonnes being produced in 2012, with that expected rise to 514 million tonnes by 2050 as shown in Figure 1.20 (WWF 2016).

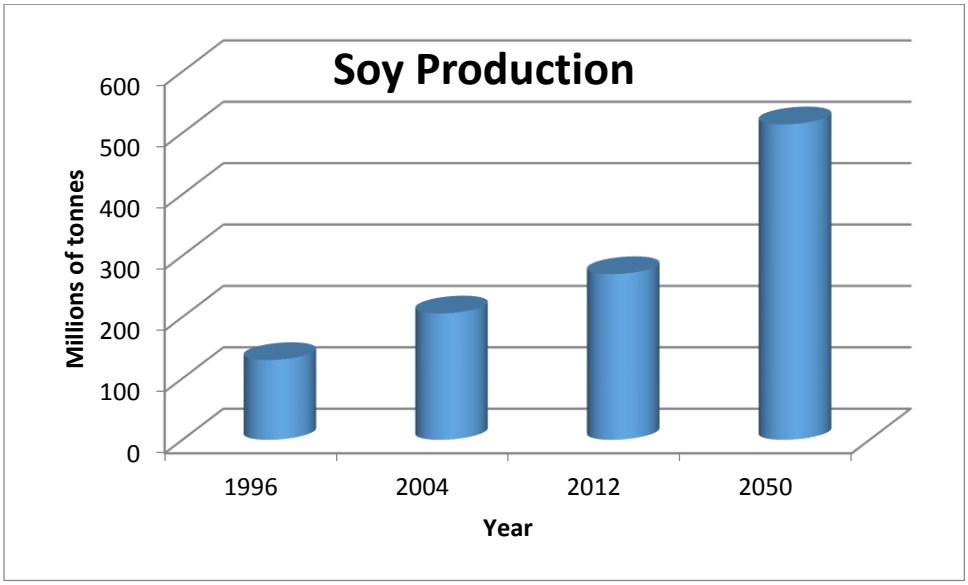

Figure 1.20 Soy production from 1996 to 2012 and predicted growth in 2050 from (WWF 2016)

Asian and Central American countries have a higher soy consumption than the west. East Asian diets contain an estimated 17-47 mg/day of isoflavones (Glodin 1986; Vergne 2009).

Adding soy to bread was first considered in the 1950's with the idea of adding more high quality protein to common and inexpensive foods to improve diets, especially in developing countries. In recent years, a push towards a greater consumption of functional foods, defined by the International Food Information Council as foods that provide health benefits beyond their nutritional value, has led to increased soy products and consumption (food insight 2016).

Increase in soy products was further spurred on in 1999 when the Food and Drug Administration (FDA) approved a health claim for soy protein and its role in reducing cholesterol and heart disease (FDA 2016).

1.4.2 Food processing effects on isoflavones

The isoflavone content of foods varies greatly and is affected by many factors, such as raw material variety, growth and environmental conditions, methods of processing and storage as well as analysis (Caldwell et al 2005; Lee et al 2003; Rostango et al 2009).

In non-fermented soy foods, the glucoside forms of the isoflavone are the most abundant, >95% (Wang and Murphy 1994). In fermented soy foods the opposite is true with the aglycones being the most abundant (Wang and Murphy 1994; Murphy et al 1997). In general fermentation causes the removal of the glucosidic group releasing the aglycone, likely due to the activity of endogenous β-glucosidase present on yeast cell surfaces (Kuo et al 2006).

Isoflavones are relatively stable at temperatures up to 260 °C, they are not destroyed by heat but interconverting between different forms depending on the specific conditions. Various other interconversions' of isoflavones are possible during the processing of food products, Figure 1.21 shows some known interconversions (Chein et al 2005).

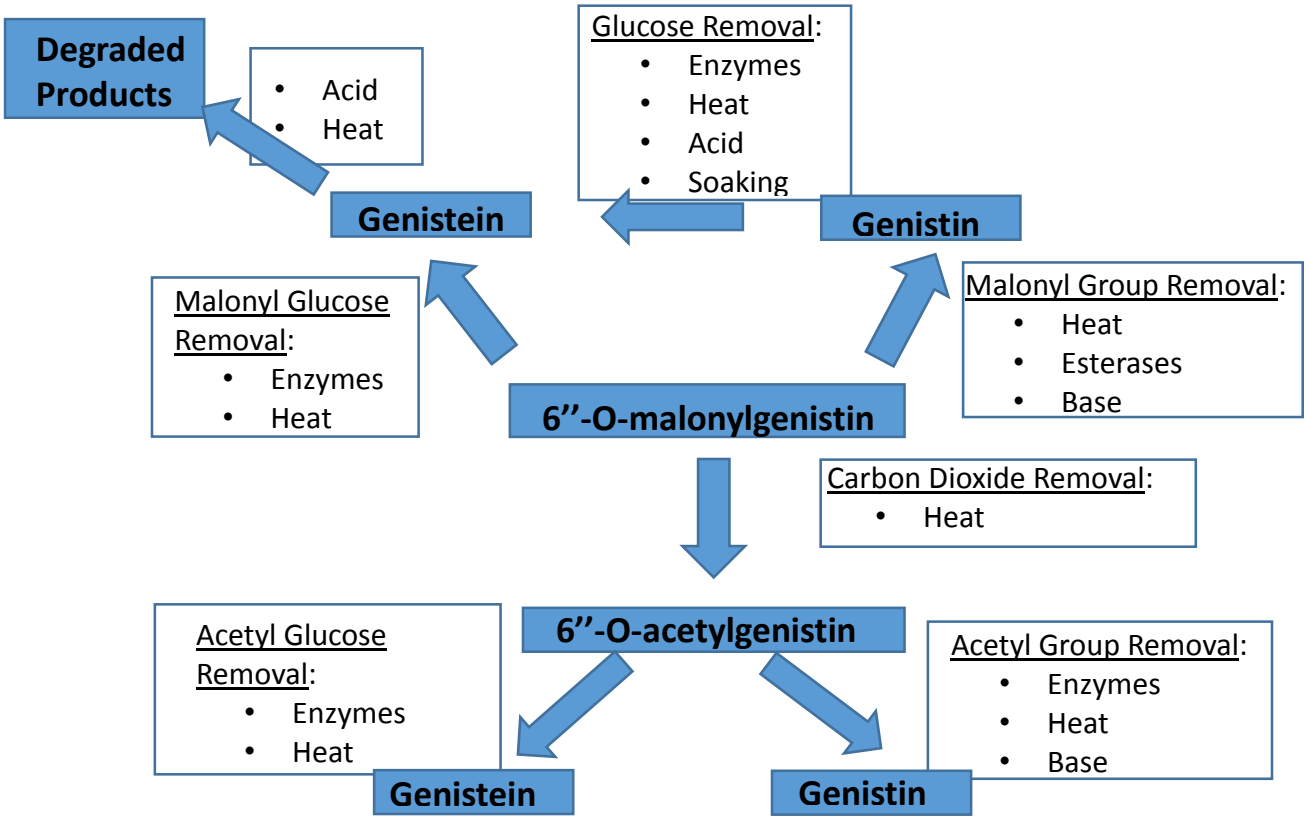

Figure 1.21 Possible interconversions and degredation of genistein and conjugates during food processing.

The use of live organisms, such as yeast in the case of fermentation, is often used in food preparation. Another common example is the use of *Aspergillus niger* in sofu (fermented tofu) production. These processes are termed bioconversion or biotransformation and are used to develop flavours and increase functional components such as increased isoflavone aglycones and active peptides causing greater potential health benefits (Hong 2004; Mejia 2006). If the culture process is long enough for bacterial metabolism additional oxidative metabolism may occur, likely involving introduction of hydroxyl groups into the 6- and 8- positions on the A ring. 8-hydroxygenistein and 6-hydroxydaidzein (Figure 1.22) have been detected in tempeh, a traditional Indonesian food produced from fermented soybeans. These compounds likely arise from the metabolic activity of *Microbacterium aborescens, Brevibacterium epidermidis or Micrococcus luteus,* all bacterial strains from tempeh (Esaki 1999).

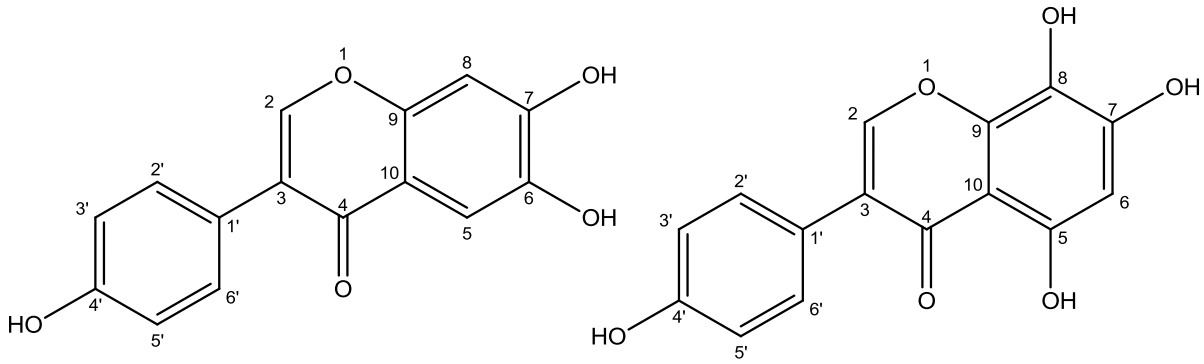

Figure 1.22 Structures of daidzein and genistein bacterial metabolites found in tempeh, left; 6-hydroxydaidzein, right; 8-hydroxygenistein.

1.5 Metabolism and bioavailability of isoflavones

When dietary isoflavones enter the human digestive tract, a large proportion of genistein and related compounds are transported to the colon. During their passage through the small and large intestine they are readily hydrolysed by intestinal microflora to simpler chemical compounds and eliminated in the faeces. A large proportion of genistein and genistin, 40 – 58 % of the total isoflavone dose is eliminated via the luminal effluent (Andlauver 2000).

Enterohepatic circulation of genistein occurs, which is the cycle of excretion from the liver to the bile followed by entry back into the small intestine where reabsorption can occur. This is typically observed as fluctuations in the blood concentration over time, an initial drop is observed followed by an increase,

where reabsorption is occurring. Enterohepatic circulation is important because it extends the elimination process and increases the area under the concentration-time curve. The accumulative amount reabsorbed in mice was approximately 6 % of an administered dose (Supko & Malspeis 1995).

Genistin is poorly absorbed and becomes deglycosylated by intestinal β-glucosidase and enzymes from gut microflora. Two broad specificity enzymes responsible for isoflavone deglycosylation in the small intestine have been identified, lactase-phloricin hydrolase (LPH) and cytosolic β-glucosidase (CBG) (Nemeth 2003). LPH is located on the apical membrane in human small intestine epithelial cells and promotes absorption by localising β-glucosides to membrane and increasing their lipophilicity. CBG hydrolysis β-glucosides that have already been transported into epithelial cells. This transportation is done via sodium dependent glucose transporter 1, present in the intestinal brush border. Ecoli HGH21 and HGH6 are microflora present in intestinal wall that are capable of genistin hydrolysis (Hur 2000).

In the small and large intestine as well as the liver, genistein is conjugated with glucuronic acid and/or sulfate groups at the 4’ and 7 hydroxyl groups (Figure 1.23). This is termed phase 2 metabolism which produces conjugates with increased polarity and molecular weight, reducing bioactivity and increasing excretion in water based fluids such as urine. Sulfotransferase is responsible for addition of the sulfate groups and uridine-5’-diphospho-glucuronosyltransferase is responsible for addition of the glucuronic acid moiety to produce glucuronides. Seven different genistein conjugates are possible. Glucuronides make up to 78% of an initial isoflavone dose, the aglycone 5% and sulfates 25% (Shelnutt 2002).

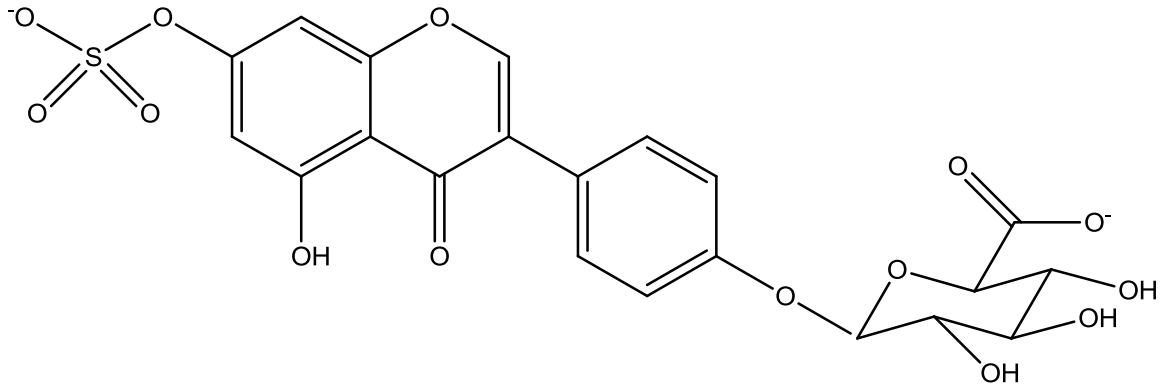

Figure 1.23 Genistein-7-sulfate 4’-β-D-glucuronide, a human genistein metabolite made from the addition of a sulfate group by sulfotransferase on the 7’ hydroxyl and glucuronic acid on the 4’ hydroxyl by uridine-5’-diphospho-glucuronosyltransferase.

Oxidative metabolism by cytochrome P_{450} enzymes in the liver produces hydroxylated genistein at any position on the A or B ring, often at multiple locations. This produces compounds that are less biologically active and more easily excreted than their parent compound (Kulling 2001).

The majority of genistein compounds pass through intestinal lumen without being absorbed, some are biotransformed by gut microflora. Many intestinal microflora can metabolize aglycones to potentially physiologically active or inactive substances. Examples of this include *Ecbacterium ramulus*, which accounts for 0.16% of total intestinal bacteria, degrading genistein to 6'-hydroxy-O-desmethylangolensin (Schuefer 2002). Clostrdium like bacteria taken from human faeces can convert genistein into dihydrogenistein (Tamura 2007). The two above strains have been shown to be present in healthy humans feed soy (Joannou 1995). These are just some known examples of gut microflora metabolism, many other bacteria also possesse the ability to metabolise genistein and isoflavones.

The impact of gut microflora is difficult to measure as gut microflora is not consistent in individuals or throughout the digestive tract. There is inter-individual variation in gut microflora, with established flora remaining consistent even with dietary changes. Dietary changes can induce significant changes in microflora metabolic activity with differences in diet effecting isoflavone metabolism (Lampe et al. 1998). Changes in microbe populations occur throughout the digestive tract as transitions of structural and functional characteristics result in ecological changes in gut microflora, resulting in favourable colonization by particular microbes. These microflora changes potentially have a large impact on the potential form and bioavailability of isoflavones with gut microflora metabolizing potential as great as that of the liver (Orrhage & Nord 2000). Further identification of involved microbes and factors influencing their activity is required for a more complete picture of isoflavone metabolism and bioavailability.

The form and concentration of bioactive compounds in plasma following consumption is what determines pharmacological activity. For ingested isoflavones only approximately 1 % reach the plasma (Arai et al. 2000; Shaw & McCully 2002).

1.6 Health Effects

1.6.1 Estrogenic activity of isoflavones

Genisteins estrogen mimicking ability allows it to fit the ERs and function as an agonist or antagonist depending on the level of hormones present, target tissue and species under investigation. Isoflavones are capable of producing an estrogenic effect in either the absence or presence of endogenous estrogens (Adlercreutz 1997).

There is concern that phytoestrogens, alone and in conjunction with the additive effects of other xenoestrogens, have detrimental human health effects. These include reduced sperm count and quality (Carlsen et al. 1992; Swan et al. 2000), cryptorchidism, hypospadias and increased occurrence of male breast and testicular cancer (Shaw 2001; Safe 2000).

In addition to genisteins negative health effects, through its estrogenic activity, genistein is also associated with health benefits. This benefits arise in part from genistein's anti-estrogenic effects, by competing for binding to ERs. This is done by suppressing the more harmful effects of estrogens, such as estrogen sensitive cancer cell proliferation. An example of this has been shown in the MCF-7 cell line, these are breast cancer cells known to be estrogen receptor positive. In these cells genistein is able to produce 50% inhibition by competing with 17β-estradiol binding to the ERs (Wang 1996).

Genistein may also reduce estrogenic activity by its ability to induce the production of sex hormone-binding globulin (SHBG), which transports estrogens, but also binds them in a state where they are not bioavailable. This leads to faster clearance of sex hormones, and a reduced risk of hormone sensitive breast and prostate cancer (Mousavi 1993; Messina 2009; Smith 2008).

Genistein may also inhibit enzymes involved in the biosynthesis and metabolism of steroid hormones. An example of this is genistein inhibiting 3β-hydroxysteroid dehydrogenase (3β-HSD), as well as down regulating its transcription. 3β-HSD catalysis the biosynthesis of progesterone from pregnenolone (Figure 1.9), with inhibition and down regulation leading to a decrease in cellular progesterone (Head 1998; Tiemann 2006). Progesterone is an important metabolic intermediate in the production of sex hormones and corticosteroids (Figure 1.9). Progesterone also plays a role in the regulation of the menstrual cycle.

A diet rich in soy protein and thus genistein has an impact on the hormonal status and regulation of the menstrual cycle of premenopausal women (Cassidy 1994). A daily intake of 60 g of soy protein, which contains 45 mg of isoflaovones, for 1 month significantly increased the follicular phase length by 2.5 days and/or delay menstruation. The follicular phase is typically day 1-14 where estrogen levels are low (Figure 1.24) and the follicle matures in the ovary (Figure 1.8), Increased length to the follicular phase increases the proportion of time estrogens are at a lower concentration and consequently could have a protective effect against breast cancers, which undergo mitosis four times faster in the luteal phase. The luteal phase is typically day 14-28, when estrogen levels are much higher (Cassidy 1994) (Figure 1.24).

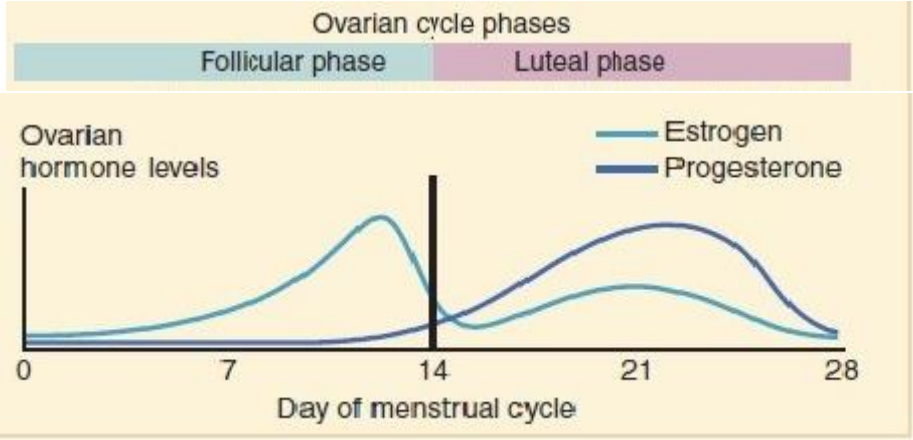

Figure 1.24 Ovarian hormone levels throughout the menstrual cycle. From wikimedia commons (Anatomy & Physiology 2013)

The biological effects of genistein are effected by its metabolism. As well as the phase 2 metabolites, conjugation to glucuronic acid or sulphuric acid occurs in liver or intestinal epithelium, intracellular metabolism to produce novel metabolites also effects its cellular actions. Genistein glucronides and sulfates isolated from human urine have also demonstrated interactions with the ERs, showing that metabolism by hepatic enzymes may affect estrogenic activity (Kinjo 2004).

As well as genistein's estrogenic activity it exhibits a wide range of biological effects that contribute to its potential health benefits, such as anticancer, antioxidant and anti-inflammatory activity.

1.6.2 Anticancer activity of isoflavones

Genistein was first looked at as an anticancer agent when it was shown there was a link between genistein consumption and a decreased risk of mortality from several cancers, particularly prostate and breast. This was noticed from populations with a higher dietary isoflavone, typically Asian, linked with lower cancer rates (Pavese 2010). While this is not necessarily a cause/effect link it generated interest in studying genistein as an anticancer agent

Epidemiological studies suggest a relationship between isoflavone ingestion and a protective effect against breast cancer in premenopausal women and women with high soy intake in adolescence. (Peeters 2003).

Genistein is a potent inhibitor of breast cancer cell growth, whereas the glycosylated form, genistin, is not. The effects of genistein on breast cancer cell proliferation where studied in ER negative (MDA-468) and positive (MCF-7 and MCF-7-D-40) cell lines. This showed that the presence of the ER was not required for genistein to inhibit cancer cell growth as it can also inhibited the ER negative MDA-468 cell line (Peterson 2003). This effect of genistein is due to its or its metabolites' ability to block the cell cycle progression through direct effects on intracellular signalling (Nguyen 2006). This has also been shown *in vitro* to cause a cell cycle block in the G_2-M phase transition (Santell 2000). Genistein also causes increased expression of the cyclin-dependent kinase inhibitor $p21^{WAFI}$ in breast cancer cells leading to cell cycle arrest (Chinni 2003).

Genistein also inhibits proliferation and induces apoptosis in prostate cancer. In rats it has been shown to have a dose-dependent relationship between inhibition of N-methylnitrosourea-induced prostate invasive adenocarcinomas and levels of genistein feed over their lifetimes (Wang 2002).

Genistein can also induce apoptosis (Sergeev 2004), inhibit tyrosine kinase (Mitropoulou 2002), modulate mitogen-activated protein kinase (MAPK) signalling (Li 2006), alter phosphatidylinositol 3-kinase cascade (Lee 2001) and inhibit DNA topoisomerases (McCabe 1993).

The level of anticancer effect and the dietary level of genistein required to produce them is difficult to determine. It is also important to weigh up the risk vs benefit by keeping in mind the detrimental phytoestrogenic effects.

1.6.3 Antioxidant activity of isoflavones

Antioxidants are popular dietary supplements and are under investigated for the prevention of diseases such as cancer and coronary heart disease. This is because oxidative stress is either a cause or a consequence of these diseases. Genistein has relatively strong antioxidant activities as it can scavenges free radicals (Kim 2010), chelate metals (Dowling 2010), inhibit the production of oxidizing species such as H_2O_2 (Sethy 2005) and enhance the activity of endogenous antioxidant enzymatic systems such as catalase (Cai 1996).

Physiologically achievable concentrations of 5 nM genistein increased intracellular reduced glutathione (GSH) levels by 10% in human endothelial cells. GSH is an important antioxidant in cells that prevents damage to cellular components by reactive oxygen species. The mechanism behind increased GSH may include the effect of isoflavones on enzymes, such as γ-glutamyl cysteine synthetase (γ-GCS) which catalysis the first step of GSH biosynthesis, (L-glutamate + L-cysteine + ATP ⇌ γ-glutamyl cysteine + ADP + P_i) (Guo 2002) (Figure 1.25).

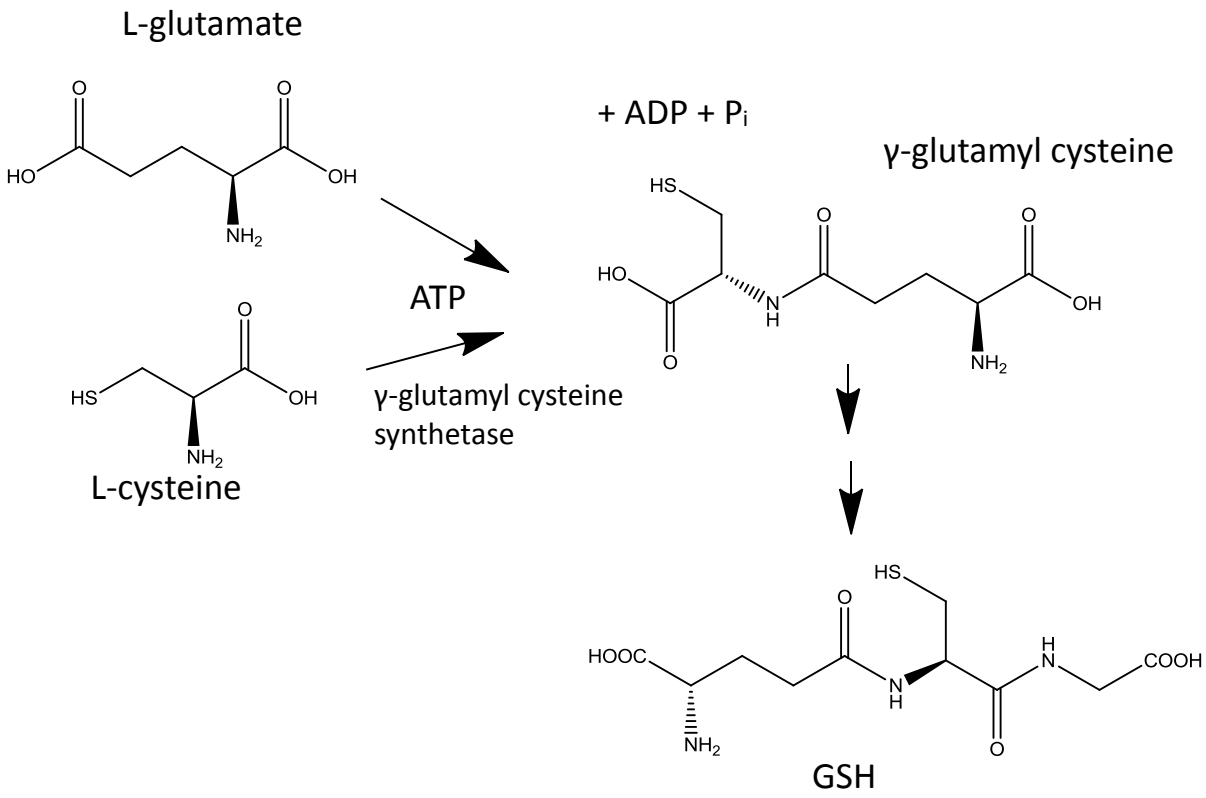

Figure 1.25 The first step of GSH (bottom right) biosynthesis, γ-glutamyl cysteine (top right) is made from L-glutamate (top left) and L-cysteine (Middle left) by the enzyme γ-glutamyl cysteine synthetase using ATP as an energy source.

A 30-day administration of genistein in mice increased the activity of other antioxidant enzymes such as catalase and superoxide dismutase (SOD). Catalase converts hydrogen peroxide to water and oxygen, and SOD catalysis the dismutation of superoxide ($O^{\cdot-}_2$) to molecular oxygen (O_2) or hydrogen peroxide (Cai 1996).

1.6.4 Anti-Inflammatory activity of isoflavones

Anti-inflammatory diets have been linked to reduced heart disease risk, reduced blood triglycerides, reduced blood pressure and reduced tendon and arthritic pain (Estruch et al. 2006).

Genistein has been shown to have an effect on the production of inflammatory mediators were it was shown it can inhibit cytokine-induced up-regulation of pro-inflammatory mediators such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM)-1 as well as cytokine-induced transmigration of blood leukocytes in a dose-dependent manner in human brain microvascular endothelial cells (Lee 2008). TNF-α and IL-1β are cytokine cell signalling proteins involved in systemic inflammation, produced mainly by activated macrophages. ICAM-1 is a vascular cell adhesion protein which binds leucocytes causing inflammation. Inhibition of these pro-inflammatory mediators results in reduced inflammation.

Population studies show a correlation of high isoflavone consumption to a lower risk of inflammatory bowel disease (Loftus 2004). Orally administered genistein showed beneficial anti-inflammatory effects by inhibiting molecular and biochemical inflammatory markers in the colon of rats with acid induced chronic colitis, this is likely the mechanism by which it lowers the risk of inflammatory bowel disease (Seibel 2009).

1.7 Bread making process

1.7.1 Yeast based bread

The process of bread making typically consists of mixing the ingredients to form the dough, proofing and baking. At Coupland's bakery yeast (*saccharomyces cerevisiae*) is used to raise the bread, with the dough containing yeast equal to 2 % the weight of flour. The proofing stage at

Coupland's lasts for 48 min and is done at 42 ^{0}C and 80 % humidity. This is where the yeast cause the dough to rise by releasing carbon dioxide in the process of fermentation. Enzymes in the flour and yeast create sugars, used by the yeast to produce carbon dioxide and alcohol. The grain enzyme diastase converts starch, a polysaccharide consisting of a large number of glucose molecules connected via a glyosidic bond, to maltose, a disaccharide made from two units of glucose. Maltose is then converted into glucose by the yeast enzyme maltase. Invertase converts added sucrose to glucose and fructose. One glucose molecule is converted into two ethanol molecules and two carbon dioxide molecules over the process of fermentation.

Baking at Coupland's is done at 257 ^{0}C for 21 min with the core temperature of the bread reaching 97-98 ^{0}C. Loafs weigh 550 g after baking with a loss of 11.25 % in weigh.

When the yeast cells die they release high quantities of a protease, which chop up proteins and result in a softer bread.

1.7.2 Sour dough bread

Adding a sour dough starter is another way of producing risen bread and creating flavor. A sour dough starter contains yeast and naturally occurring lactobacilli, which produce lactic and acetic acids, creating a sour flavor and better inherent keeping qualities.

At Memphis Belle the sour dough starter was originally started 12 years ago by yeast and bacteria in the air at the time. As the sour dough starter remains open to the air its yeast and bacteria make up can be constantly changing based on the conditions it is exposed to.

The dough contains approximately 40 % sour dough starter with the proofing process at Memphis Belle taking between 5-10 h depending on the temperature, with an ideal temperature of 21 ^{0}C.

1.8 Hypothesis

The bread making process alters soy-derived isoflavones changing the possible health effects of the products.

1.9 Aims

- To investigate yeast's and sourdough culture's effect on isoflavones in a bread making context.
- To investigate heat's effects on isoflavones in a bread making context.
- To track isoflavones throughout the bread making process.
- To determine importance of breads contribution as a functional food

Chapter 2 – Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were of analytical reagent grade except for high performance liquid chromatography (HPLC) grade methanol (Thermo Fisher Scientific, Auckland, New Zealand) and acetonitrile (Scharlau, Barcelona, Spain). Genistein, genistin and daidzein, >99 %, were purchased from LC Laboratories, Woburn, Massachusetts, United States.

2.1.2 Biological Products

The yeast used was a strain of *Saccharomyces cerevisiae,* Pinnacle fresh compressed yeast from Coupland's Bakeries, Christchurch, New Zealand. The sour dough culture used was supplied by Memphis Belle Bakery, Christchurch, New Zealand. Yeast peptone dextrose media (YPD) was purchased from Sigma-Aldrich, Auckland, New Zealand.

2.1.3 Equipment

- High performance liquid chromatography (Shimadzu Corporation, Kyoto, Japan)
    - SCL-10A system controller
    - LC-10A liquid chromatography
    - DGC-14AL degasser
    - SPD-M10A diode array detector
    - SIL-10A Auto injector
    - Analysed in Lab Solutions version 6.11SP2 licenced to ESR
- Champion Multicook oven
- Super wiz duo electronic food processor (Breville, Melbourne, Australia)
- Thermistor Ecoscan thermometer probe (Thermo Fisher Scientific, Auckland, New Zealand)
    - Calibrated to +/- 0.0 ^{0}C at 60 and 120 ^{0}C
- Rotary evaporator, technite AG, 461 water bath (Buchi Labortechnik, Flawil, Switzerland)
- Orion 230A pH meter (Thermo Fisher Scientific, Auckland, New Zealand)
- Magnetic mixer (IKA, Selangor, Malaysia)
- Vortex minishaker MS1 (IKA, Selangor, Malaysia)

- Centrifuge, Heraeus Megafuge (Thermo Fisher Scientific, Auckland, New Zealand)
- Electronic scales, classic AB204-S (Mettler Toledo, Columbus, Ohio, United States)
    - Calibrated every 6 months

2.2 High performance liquid chromatography

2.2.1 HPLC system

The HPLC system used was based on that used for daidzein and genistein analysis from *Mangifera* fruit (Khoo & Ismail 2008). Samples were analysed for isoflavones (genistein, genistin and daidzein) by reverse phase HPLC using a gradient elution profile with UV absorbance detection at 260 nm using a photodiode array detector. An injection volume of 50 µL was used except where otherwise stated. The column, a 220 µm by 4.6 µm C18, was equilibrated using the starting solvent at a flow rate of 1.5 mL/min as well as repeating the first sample analysis.

2.2.2 HPLC mobile phase

The mobile phase was prepared from;
Buffer; 0.2 mol/L ammonium acetate (aq), pH adjusted to 4.6 using hydrochloric acid.
Solvent; 1:5 acetonitrile to methanol

Mobile phase A consisted of 90 % buffer and 10 % solvent. Mobile phase B consisted of 20 % buffer and 10 % solvent. The mobile phase, once prepared, was filtered through a 0.45 µm polyethersulfone filter. A flow rate of 1.5 mL/min was used along with the following gradient programs:

100 % method: 100 % solvent A at 0 min going to 100 % solvent B at 20 min in a linear fashion.

90 % method: 100 % solvent A at 0 min going to 90 % solvent B at 20 min in a linear fashion

80 % method: 100 % solvent A at 0 min going to 80 % solvent B at 20 min in a linear fashion.

70 % method: 100 % solvent A at 0 min going to 70 % solvent B at 20 min in a linear fashion.

70 % extended method: 100 % solvent A at 0 min going to 87.7 % solvent B at 25 min in a linear fashion.

The column is prepared for the next run by going from 100 % B to 0 % B at 30 to 31 min and holding at 0 % B till 40 minutes.

2.2.3 Calibration graphs

Genistein, genistin and daidzein standards where prepared by weighing the selected compound transferring to a volumetric flask and filling to the appropriate volume with methanol. A plastic lid was added and the solution was mixed by inverting until properly dissolved. An approximate concentration of 500 mg/L was targeted (e.g. 5 mg compound in 10 mL methanol). HPLC ready samples were then prepared by pipetting methanol and isoflavone standard into HPLC flasks at volumes to give 1 mL of desired concentrations (e.g 100 µL of 500 mg/L genistein standard and 900 µL of methanol to give 1 mL of desired 50 mg/L genistein sample).

Standards were prepared for isoflavones at 1, 2.5, 5, 10, 20 and 40 mg/L which were analysed by HPLC at the beginning of every batch. A methanol blank was run after the standards and a standard at 10 mg/L was run every 6 runs to check for variance within the batch. UV absorbance was measured at 260 nm and a calibration graph prepared to be used as reference for determining isoflavone concentrations in samples. The coefficient of determination (R^2) and the coefficient of variation (CV) was calculated for all calibration graphs.

2.2.4 Limit of detection and quantification

The limit of detection (LOD) was measured as 2-3 times the signal to noise ratio and the limit of quantification (LOQ) is measured as 5-6 times the signal to noise ratio. This was measured using a genistein standard at a low concentration (0.5 mg/L) and comparing the relative height of the standards

peak to the variance in the baseline, the signal to noise ratio, to determine concentration.

2.2.5 Spiked samples

To identify the presence of a particular isoflavones, the UV absorbance spectrum of the peak in question was visually compared to that of the isoflavone standard. Additionally, spiked samples were prepared by adding an isoflavone standard to the sample being analysed. If the peaks co-chromatographed (i.e. there was no peak shoulder) and the peak was larger the sample was identified as the isoflavone standard. Recoveries were measured in this manner in duplicate at both high and low spike concentrations for all starting material, i.e. yeasty media, sour dough culture, soy and linseed and daily white dough, proofed dough and bread. 2 g samples of starting material were spiked with isoflavones standards to give concentrations of 50 mg/L or 5 mg/L.

2.2.6 Isoflavone extraction from bread

Bread samples containing soy were analysed using this method. Two bread samples were tested, Coupland's daily white and Coupland's soy and linseed. Samples of each are blended in a food processor and weighed into 50 ml centrifuge tubes to give approximately 2 g.

A calibration graph is run at the beginning of each series of runs. 8ml of MeOH and 2 ml of H_20 were added such that 10 ml of 80% MeOH was added. Samples were then vortexed for 30 s each at approximately 2000 rpm, causing any clumped doughy samples to disperse. Following vortexing samples where centrifuged for 15 min at 3500 rpm. HPLC vials then had 1 ml of supernatant pippeted in and were sealed with 10 mm HPLC auto sampler screw caps to be analysed by HPLC.

2.2.7 Cleaning column

The C18 column used for isoflavone analysis was washed when required (when peaks appeared in methanol blanks) using multiple methanol runs. If a more thorough wash was required the column was washed by running water for 30

min (45 mL), methanol for 30 min (45 mL), dichloromethanol for 13.3 min (20 mL), methanol for 30 min and H_20 for 30 min.

2.3 Non-soy bread

A non-soy bread was made at Coupland's bakery using the same recipe as Coupland's daily white bread except the soy flour was left out. Spiked samples of this was analysed by HPLC to determine recoveries for the daily white bread without interference.

2.4 Yeast incubation

Experimental work was undertaken at the food safety labs in environmental science and research (ESR) Christchurch division. This is a PC2 laboratory and accordingly PC2 lab protocols where followed (AS-NZS 2243-3 2010).

For the yeast incubations, isoflavone standards were made up using the method described in "Calibration graphs" but using DMSO instead of methanol. A 20 g/L yeast sample was prepared by weighing yeast in conical flasks 150 mL and adding yeast peptone dextrose media (YPD) from a measuring cylinder 100 mL which had been placed in a 37 ^{0}C kaiapoi coldstore an h earlier. This mixture was then swirled to disperse any yeast clumps. A 2 mL sample (to be used as a blank) is pippeted out into a centrifuge tube containing 10 mL of 80 % methanol and 20 % water which is vortexed at a speed of 2000 rpm for 30 seconds. The isoflavone standard in DMSO is then pippeted in to a conical flask at a volume to give the intended final concentration when made up with YPD. (e.g. 2 g yeast into 101 mL YPD, 2mL pippeted out to be used as a blank, then 1.15 mL of 260 mg/L genistein added). Cotton wool is then placed in the neck of the conical flask and it is incubated at 37 ^{0}C incubator. 2 mL samples are taken at 15 min, 30 min 60 min, 180 min and 1320 min. The samples were vortexed for 30 s and placed in the freezer were they are stored until the isoflavones are extracted and analysed using the method described in "Isoflavone extraction from bread" starting at the vortexing stage.

A conical flask (150 mL) containing just water and isoflavone and one containing YPD and isoflavone, both without yeast, were incubated in the same manner as negative controls. Samples for these are taken at the same stages as

the ones with both YPD and yeast, before isoflavone addition, after and at 15 min, 30 min, 60 min and 180 min.

This method was carried out in duplicate for genistein, genistin and daidzein.

2.5 Sour dough incubation

For the sour dough incubations genistein standards where made up in DMSO instead of methanol otherwise following the method described in "Calibration Graph". A sour dough culture obtained from Memphis Belle bakery was feed every two days with one teaspoon of sugar and two tablespoons of organic white flour along with enough water to produce a culture of the required consistency, liquid but very thick, approximately 50-120 mL. When the culture became too large all but half a cup was discarded. The culture was kept at room temperature covered but with holes in the lid to allow air flow.

Incubation flasks where prepared by weighting 30 g into a conical flask along with 30 mL of water to allow samples to be pippeted. This was placed into a 37 ^{0}C incubator to equilibrate to the correct temperature. A blank sample of 2 mL was pippeted into 10 mL 80 % methanol before isoflavone standards were added of genistein, genistin and daidzein, with one standard per flak, repeated in duplicate. Another 2 mL sample was taken and the isoflavone spiked culture was then placed back into the incubator. Samples in 80 % methanol were vortexed and placed straight into the freezer. Additional samples were taken at 15 min, 30 min, 60 min, 180 min, 270 min and 1380 min. The isoflavone spiked culture had a cotton wool bung placed in the neck of the conical flask and was stirred before each sample was taken.

2.6 Yeast-based bread fermentation samples

Samples of Coupland's daily white and Soy and linseed bread were taken from Coupland's bakery after mixing, after proofing and after baking. Two samples for each stage were taken at the same time with the whole loaf or dough being placed into airtight bags and taken straight to the lab. Samples were weighed and added to 80 % methanol within an hour of being taken from the bakery.

The isoflavones were extracted using the “Isolavone extraction from bread” procedure and they were analysed on HPLC by the “70 % extended method”.

2.7 Thermal degradation

For wet heat, 1 mL of 80 mg/L genistein in methanol was pippeted into a 100 mL shot bottle and 10 mL of water added. This was done twice, the lids screwed on and one shot bottle was placed in the fridge as a control and one into an oven measured to be 98 ^{0}C by a temperature probe. After 21 min in the oven or fridge 1 mL samples where pippeted into HPLC tubes and these prepared for HPLC.

For dry heat, 1 mL of 80 mg/L genistein in methanol was pippeted into a round bottom flask, the methanol was evaporated off using a rotary evaporator for approximately 20 min with the water bath at 45 ^{0}C. This was done twice with 1 placed in the fridge as a control and 1 into an oven at 257 ^{0}C as measured by a temperature probe. After 21 min in the oven or fridge samples were analysed by HPLC using the “70 % extended method”.

Chapter 3 – Results

3.1 HPLC method

3.1.1 Genistein calibration graph

The first run of a genistein standard on the 100% HPLC method gave an peak area of 8547126 at a retention time of 16.551 min for a 20 µL injection of a 100 mg/L genistein standard in MeOH.

A 20 µL injection of a 200 mg/L genistein standard gave an absorbance of 16956355, with a retention time of 16.619 min. Duplicates of the 100 and 200 mg/L injections gave peak areas of 8671993 and 17007023 with retention times of 16.669 min and 16.656 min. Methanol blanks ran after the standard injection showed minimal absorbance and no peak near 16.6 min.

A calibration graph for genistein using standards at 100, 50, 25, 10, 5, 2 and 1 mg/L all with similar retention times of around 16.6 min resulted in the calibration graph shown in Figure 3.1 below.

Average Absorbance ÷ Concentration = 285716.8
S.D. = 8163
CV = 2.86 %

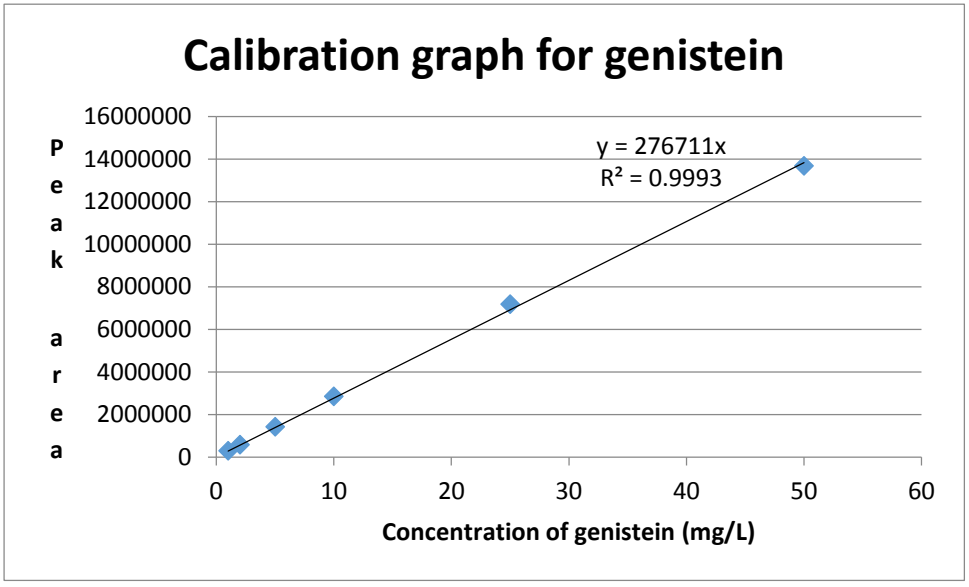

Figure 3.1 Calibration graph for genistein prepared using genistein standards ranging from 1-100 mg/L analysed on "100 % method"

3.1.2 HPLC bread analysis

Two types of bread were analysed using the set up HPLC method,

A) Coupland's Soy and Linseed
B) Coupland's Daily White

The two bread samples were analysed using the 100 % method, in duplicate, along with a single genistein spiked sample. The genistein spike was made with a standard of 110 mg/L made up using 1.1 mg of genistein in 10 mL methanol. Spiked samples of each type of bread had 0.909 µL of this standard added, then the isoflavone extraction method was applied to the samples.

A genistein standard of 50 mg/L gave a peak area of 14556791, an peak area/concentration of 291135.8 and a retention time of 16.503 min. Table 3.1 gives values for the weight of bread samples, the absorbance of a peak with a corresponding retention time to that of the genistein standard and the calculated genistein concentrations assuming the peak is genistein. The sample concentration has been worked out from the concentration present in the HPLC tube by correcting for the dilution that the isoflavone extraction method contains, as 20 mL of 80 % methanol is added (Concentration in HPLC tube x (20/Sample weight)

Table 3.1 Samples of Coupland's daily white (A) and soy and linseed (B) along with genistein samples at 4 mg/L, showing weight of samples, peak area, genistein concentration in HPLC tube and in bread.

	Weight (g)	Peak area	Genistein (mg/L)	Genistein in Bread (mg/L)
A1	1.98	90597	0.311	3.143
A2	2.11	89097	0.306	3.091
A3 – genistein spiked (4 mg/L)	1.8	1306463	4.487	45.327
B1	1.8	40383	0.138	1.401
B2	2.1	19296	0.066	0.669
B3 - genistein spiked (4 mg/L)	1.9	1286028	4.417	44.618

A chromatogram of a genistein standard, a bread sample and a spiked bread sample is shown in Figure 3.2.

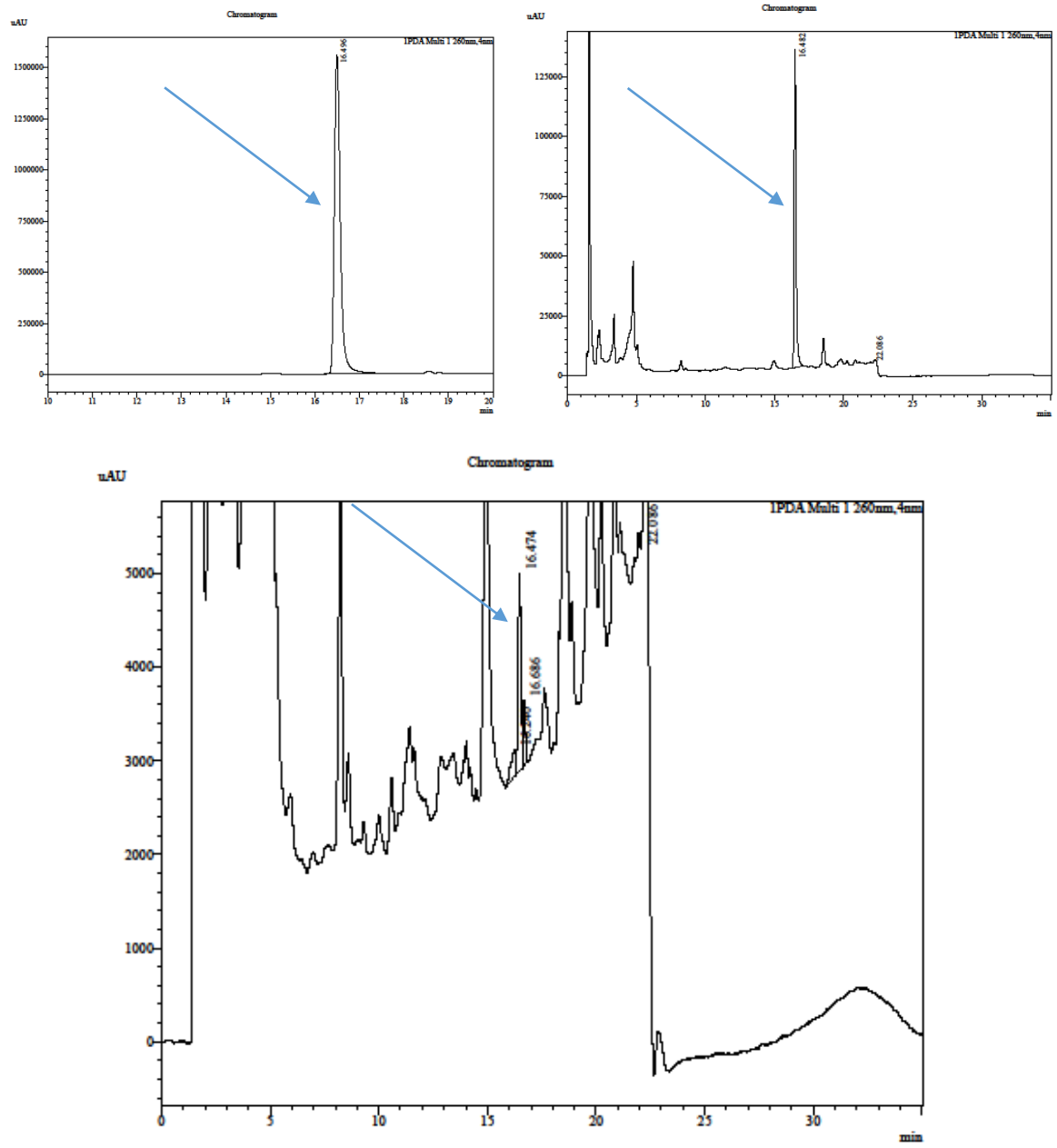

Figure 3.2 Top Left: Chromatogram of a 50 mg/L genistein standard. Top Right: Chromatogram of a bread sample spiked with 50 mg/L genistein. Bottom: Chromatogram of bread sample. Analysis was done using the 100 % HPLC method and the blue arrows show the peak corresponding to genistein.

3.1.3 Spiked bread recoveries

A calibration graph was prepared for genistein using standards at 0.5, 1, 2.5, 5, 10, 20 and 50 mg/L. This was then used to determine the concentration of genistein present in Coupland's soy and linseed bread and of spiked samples of this bread. Duplicates of the bread, along with 1, 5 and 50 mg/L spiked samples were run on the 100 % HPLC method and recoveries calculated.

Table 3.2 Soy and linseed samples showing weight, peak area, genistein concentration in the HPLC vial, bread sample and the recovery %.

Sample	Sample Weight	Peak area	Genistein in HPLC vial (mg/L)	Genistein in Bread sample (mg/kg)	Average	Recovery %
blank	2.06	89575	0.31	3.04	3.03	
blank	1.98	85930	0.30	3.03		
1 mg/kg	2.11	105539	0.37	3.49		45.92
1 mg/kg	2.04	99706	0.35	3.41		37.94
5 mg/kg	1.91	210273	0.73	7.69		93.06
5 mg/kg	2.11	199664	0.70	6.61		71.47
50 mg/kg	1.88	1341159	4.68	49.81		93.55
50 mg/kg	1.94	1519811	5.31	54.69		103.32

Recoveries were also determined from a non-soy daily white Coupland's bread. The bread along with 5 and 50 mg/L spiked samples were analysed. Chromatograms of this non-soy daily white and spiked non-soy daily white are shown in Figure 3.3 with recoveries calculated and shown in Table 3.3.

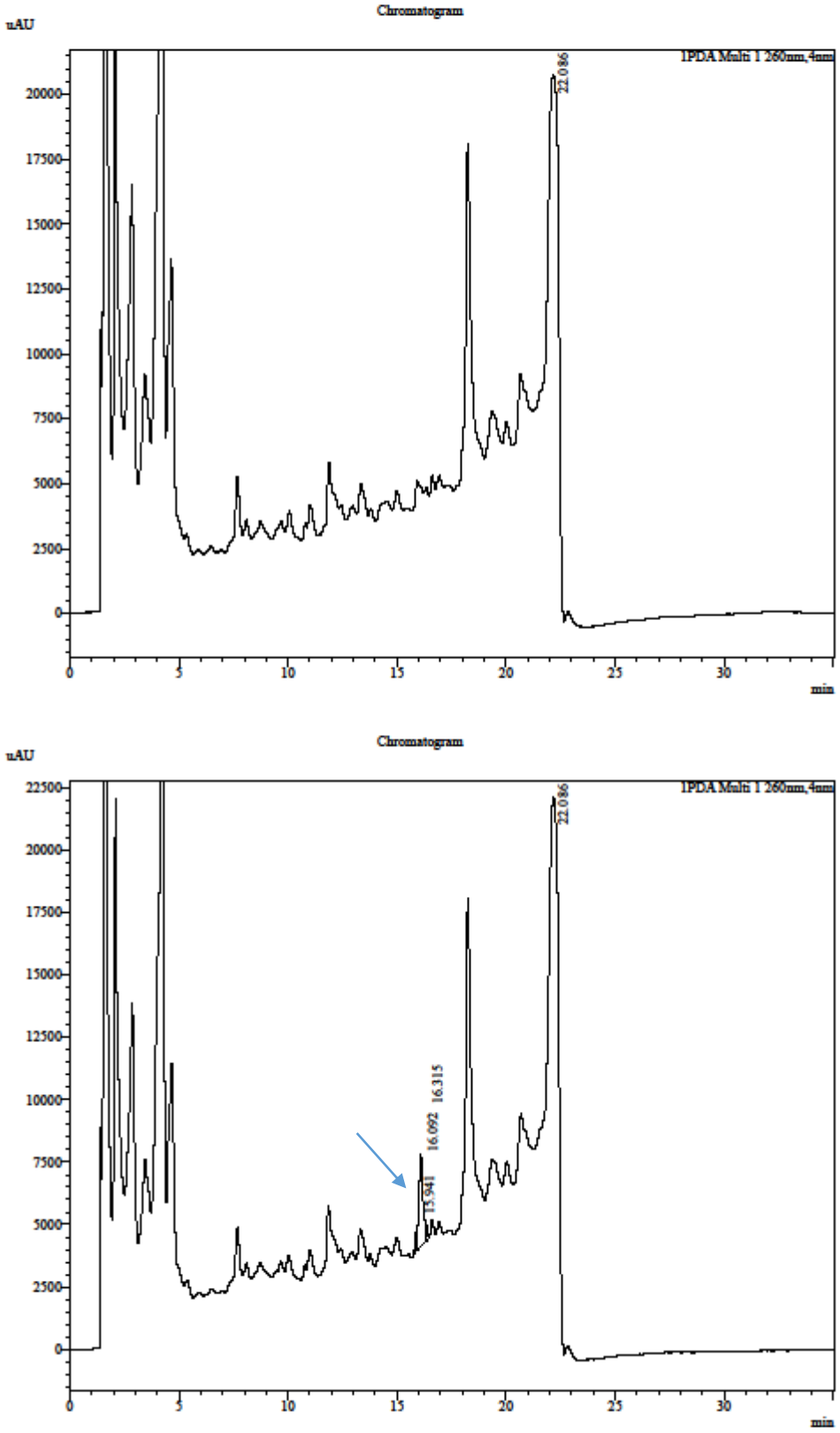

Figure 3.3 Top: Chromatogram of non-soy daily white bread. Bottom: Chromatogram of genistein spiked non-soy daily white, analysis was done using the 100 % HPLC method and the blue arrow indicates the peak arising from genistein absorbance

Table 3.3 Non-soy daily white bread samples showing weight, peak area, genistein concentration in the HPLC vial, bread sample and the recovery %.

Sample	Sample weight (g)	Peak area	Genistein in HPLC vial (mg/L)	Genistein in bread (mg/kg)	Recovery %
blank	2.07	0	0	0	
blank	2.12	0	0	0	
blank	2.05	0	0	0	
5 mg/kg	1.99	57362	0.655	6.582	131
5 mg/kg	2.01	45898	0.524	5.214	104
5 mg/kg	1.97	62609	0.715	7.257	145
50 mg/kg	2.13	476596	5.442	51.094	102
50 mg/kg	2	550988	6.291	62.909	125
50 mg/kg	2.03	506984	5.788	57.029	114

3.1.4 Yeast analysis

A yeast and YPD mixture was made using 2.04 g of yeast and 20 mL of YPD media. A sample of this was prepared for HPLC analysis along with a sample containing 800 µL of yeasty media and 200 µL of 110 mg/L genistein standard. This was run along with a 110 mg/L genistein standard.

The yeasty YPD mixture contained no peaks comparable to genistein's retention time and a spiked sample gave a recovery of 106.6 %. Chromatograms for yeasty YPD and spiked yeasty YPD are shown in Figure 3.4

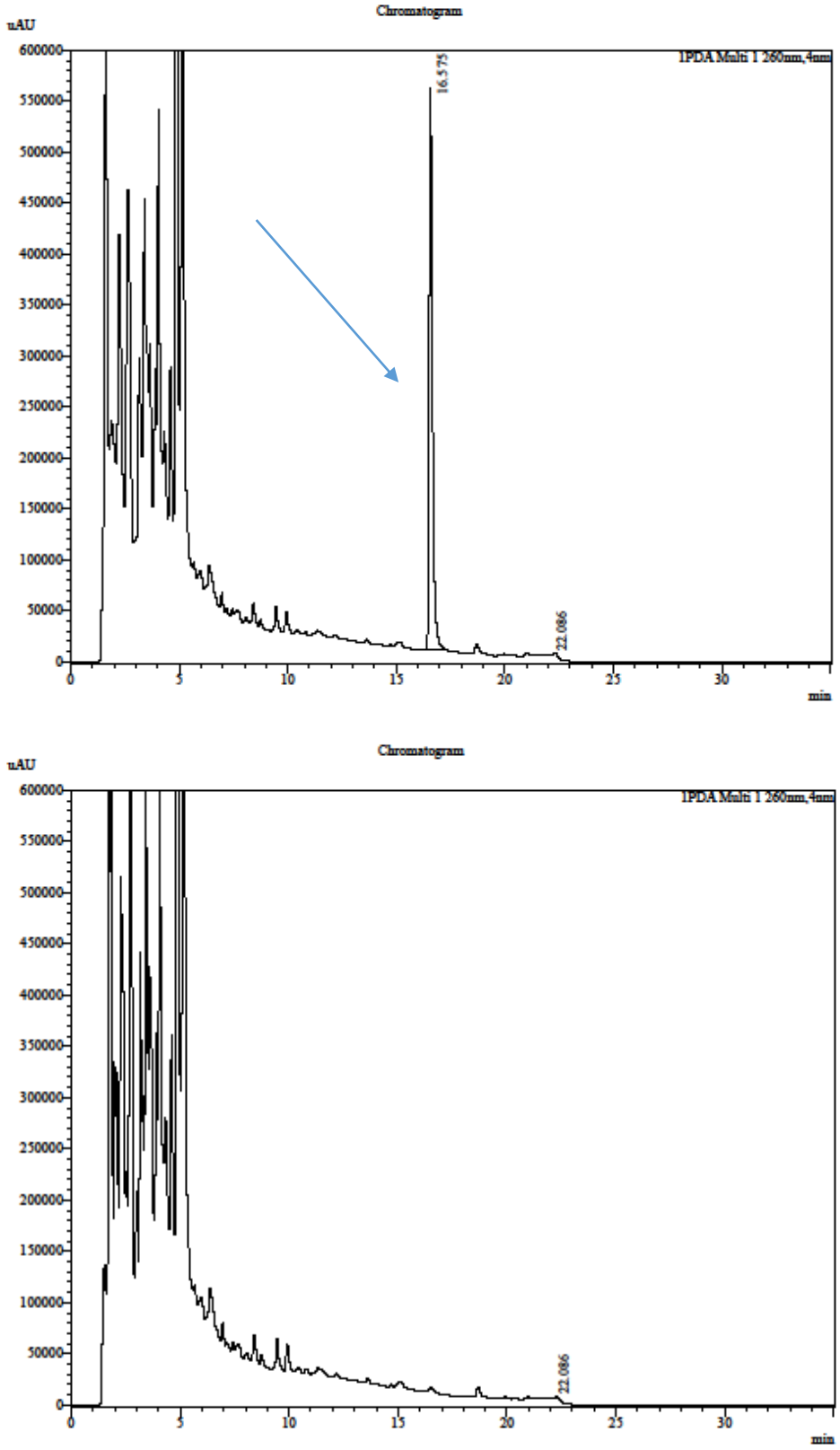

Figure 3.4 Top: Chromatogram of spiked yeasty YPD mixture. Bottom: Chromatogram of yeasty YPD mixture. Analysis was done using the 100 % HPLC method and the blue arrow indicates the peak arising from genistein absorbance

Yeast recoveries were also done using 1 and 10 mg/L spiked yeasty media samples. The results of these are shown in Table 3.4 below.

Table 3.4 Genistein spiked yeasty media showing peak area, concentration and recoveries

Sample	Peak area	Genistein (mg/L)	Average (mg/L)	Recovery (%)
1 mg/L	291495	1.01	0.979	97.90
1 mg/L	269868	0.942		
10 mg/L	2651852	9.257	9.477	94.80
10 mg/L	2778195	9.698		

3.1.5 Limit of detection and quantification

A LOD was determined from a genistein standard, shown in Figure 3.5, with an average signal to noise ratio with a height of 1000 µAU. This gives a LOD with a height of 2000-3000. A 0.5 mg/L genistein standard gave a peak with a height of 14000. This was used as reference to determine the concentration for the LOD and LOQ. The LOD is 0.07 – 0.1 mg/L and the LOQ 0.17-0.21 mg/L.

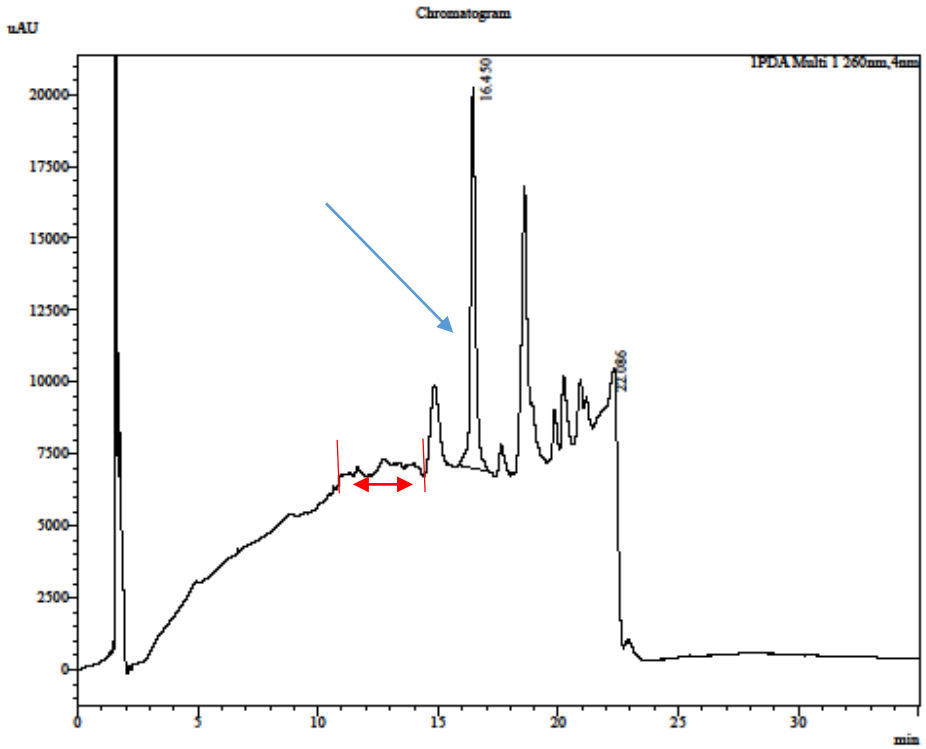

Figure 3.5 Chromatogram of 0.5 mg/l genistein standard used to determine limit of detection and quantification. Analysis was done using the 100 % HPLC method with the blue arrow indicating the peak arising from genistein absorbance, with the area used to calculate the noise indicated in red.

3.2 Yeast incubations

A genistein yeast incubation was performed using 0.53 g and 0.50 g of yeast and 1.61 mL of 630 mg/L genistein in DMSO, this was made up to 25 mL total by adding YPD media. This underwent the yeast incubation procedure and 2 mL samples were taken at T -1 min, T 0 min, T 15 min, T 30 min, T 60 min and T 180 min and added into 8 mL of 80 % methanol which underwent the isoflavone extraction procedure before they were analysed by HPLC using the 100 % method. The results of this are shown in Table 3.5. T -1 min samples where spiked with yeast at 50 mg/L (0.192 mL of 260 mg/L genistein in DMSO made to 1 mL with T-1 sample) and 5 mg/L (0.0384 ml), and recoveries are shown in Table 3.6.

Table 3.5. Genistein concentration over time for two, A and B, genistein incubations in yeast. Showing peak area, genistein concentration in HPLC vial and genistein concentration in incubation.

A			
Time (min)	Peak area	Genistein in HPLC vial (mg/L)	Genistein in incubation (mg/L)
0	1753282	5.3	26.4
15	1744922	5.2	26.2
30	1758829	5.3	26.4
60	1765058	5.3	26.5
180	1715901	5.2	25.8
B			
0	1769811	5.3	26.6
15	1772387	5.3	26.6
30	1836180	5.5	27.6
60	1981967	6.0	29.8
180	1772419	5.3	26.6

Table 3.6. Recoveries for genistein spiked yeasty YPD media, also showing peak area, genistein concentration in HPLC vial and genistein concentration in incubation.

Genistein pike (mg/L)		Peak area	Genistein in HPLC vial (mg/L)	Genistein in yeasty YPD (mg/L)	Recovery %
5		323241	1.0	4.9	98
5		295061	0.9	4.4	88
25		1384403	4.2	20.8	83.2
25		1284385	3.9	19.3	77.2

A genistein yeast incubation was then done again, this time with two negative controls, a YPD + genistein and a water + genistein, both without yeast. A spike was also done at T -1 min and T 0 min of 40 mg/L. Our conical flask contained 1.01 g of yeast, 2.15 mL of 930 mg/L genistein in DMSO and 47.85 mL of YPD media. The results are shown in Table 3.7 with the genistein concentration over time graphed in Figure 3.6

Table 3.7 Results of genistein yeast incubations including spikes. The two negative controls also gave recoveries near 100 % for T -1 min and T 0 min spikes along with a constant genistein concentration of around 40 mg/L over the 20 h period. Showing peak area, genistein concentration in HPLC vial and genistein concentration in incubation. Spikes were performed with 40 mg/L genistein.

Time (min)	Peak area	Genistein in HPLC vial (mg/L)	Genistein in yeasty YPD (mg/L)
-1	45371	0.19	0.97
0	1872039	8.02	40.11
15	1929999	8.27	41.36
30	1876984	8.04	40.22
60	1810573	7.76	38.80
180	1891644	8.11	40.53
1200	1937004	8.30	41.51
-1 Spiked	1946406	8.34	41.71
0 Spiked	4376281	18.75	93.77

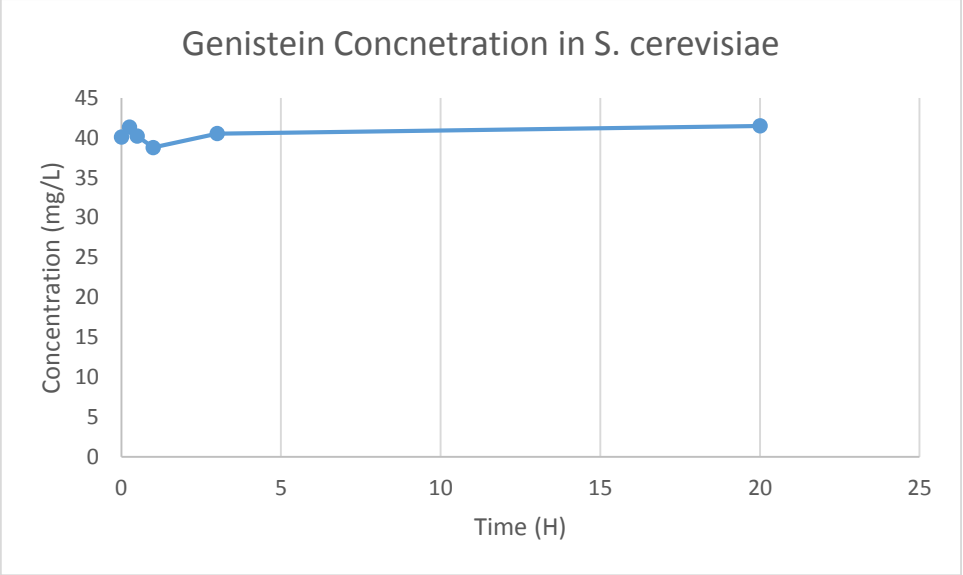

Figure 3.6 Genistein concentration over the yeast incubation.

A standard of genistin was run on the HPLC using the same method as for genistein. Genistin eluted at a time of 10.725 min. This however was a similar time to a peak in the yeasty YPD media at 10.686 min, as shown in Figure 3.7.

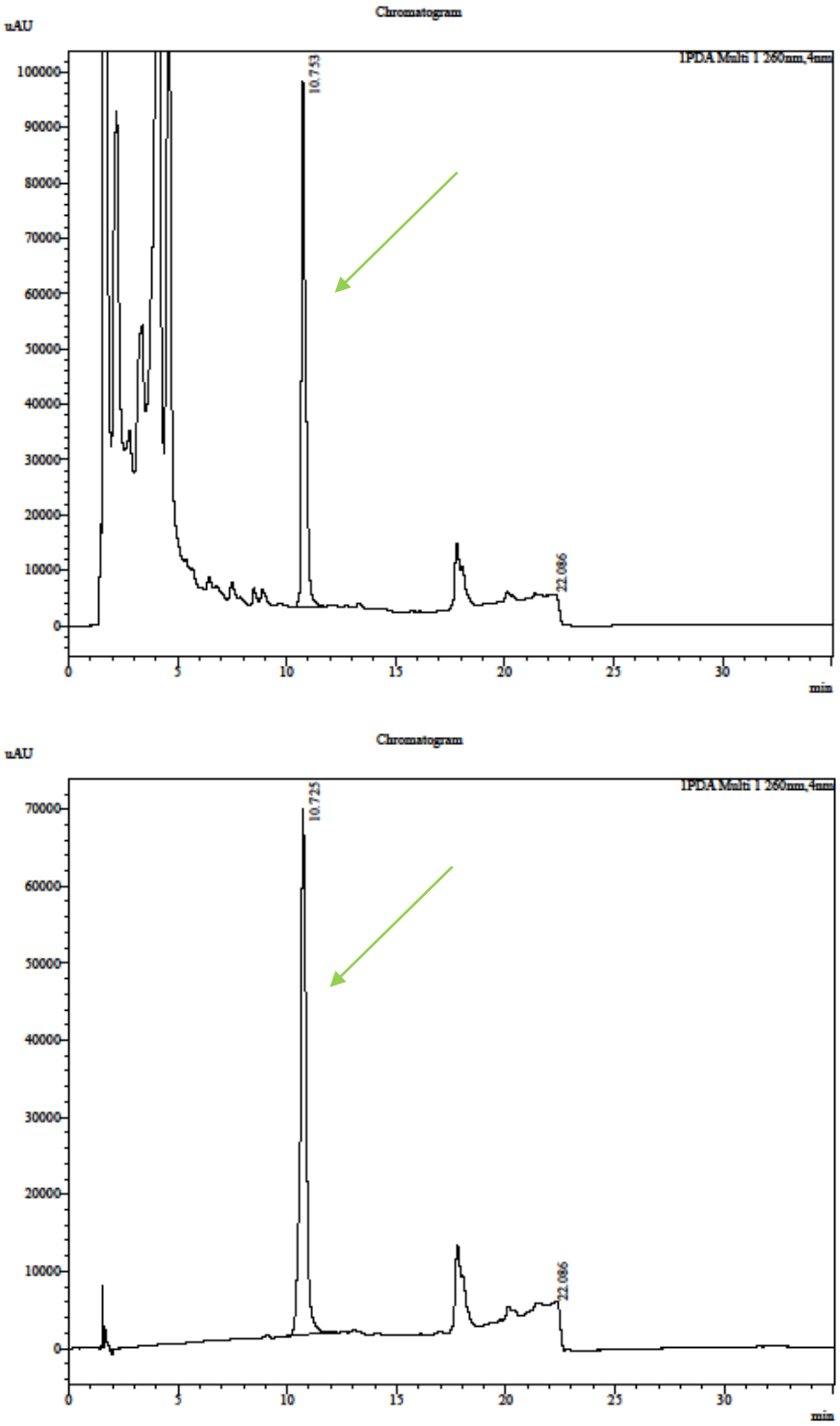

Figure 3.7 Top: Chromatogram of a genistin spiked yeasty YPD mixture. Bottom: Chromatogram of a genistin standard. The green arrows indicates peaks arising from genistin absorbance.

Genistin spiked yeasty media samples were performed at 2.5, 5 and 50 mg/L and recoveries calculated as shown in Table 3.9 shown below. As the recoveries were not adequately close to 100 %, likely due to the yeasty YPD

media containing peaks at the same elution time as genistin, the HPLC was altered in an attempt to obtain better recoveries at low concentration. The interfering peaks are shown in the top left of Figure 3.9 with retention times of 11.205 min and 11.929 min. Samples of genistin spiked yeasty YPD, yeasty YPD and a genistin standard were run on the HPLC under different gradient systems, using the 100 %, 90 %, 80 % and 70 % HPLC methods. Figure 3.8 shows genistins retention time getting longer as the solvent gradient decreases, with retention times going from 10.847 min on the 100 % method, 11.556 min on the 90 % method, 12.252 min on the 80 % method and 13.143 min on the 70 % method.

Table 3.8 Recoveries for genistin spiked yeasty media, two genistin standards (10 mg/L) were used to determine genistin concentration in samples. Showing genistin concentration in HPLC vial, genistin concentration in spiked sample, along with values obtained for these by correcting for the peak from the yeast media.

Genistin standards (mg/L)	Peak area	Peak area / Genistin concentration		
10	1220079	122007.9		mean
10	1217760	121776		121892
Genistin spikes (mg/L)	Peak area	Genistin in HPLC vial (mg/L)	Genistin in sample (mg/L)	Recovery %
2.5	127618	1.047	5.235	209.395
2.5	119498	0.980	4.902	196.072
5	184131	1.511	7.553	151.061
5	178010	1.460	7.302	146.039
50	1376446	11.292	56.462	112.923
50	1348417	11.062	55.312	110.624
blank peak in YPD + yeast at same time = 45233				
	Corrected peak area	Genistin in HPLC vial (mg/L)	Genistin in sample (mg/L)	Recovery %
2.5	82385	0.676	3.379	135.177
2.5	74265	0.609	3.046	121.854
5	138898	1.140	5.698	113.952
5	132777	1.089	5.447	108.930
50	1331213	10.921	54.606	109.212
50	1303184	10.691	53.457	106.913

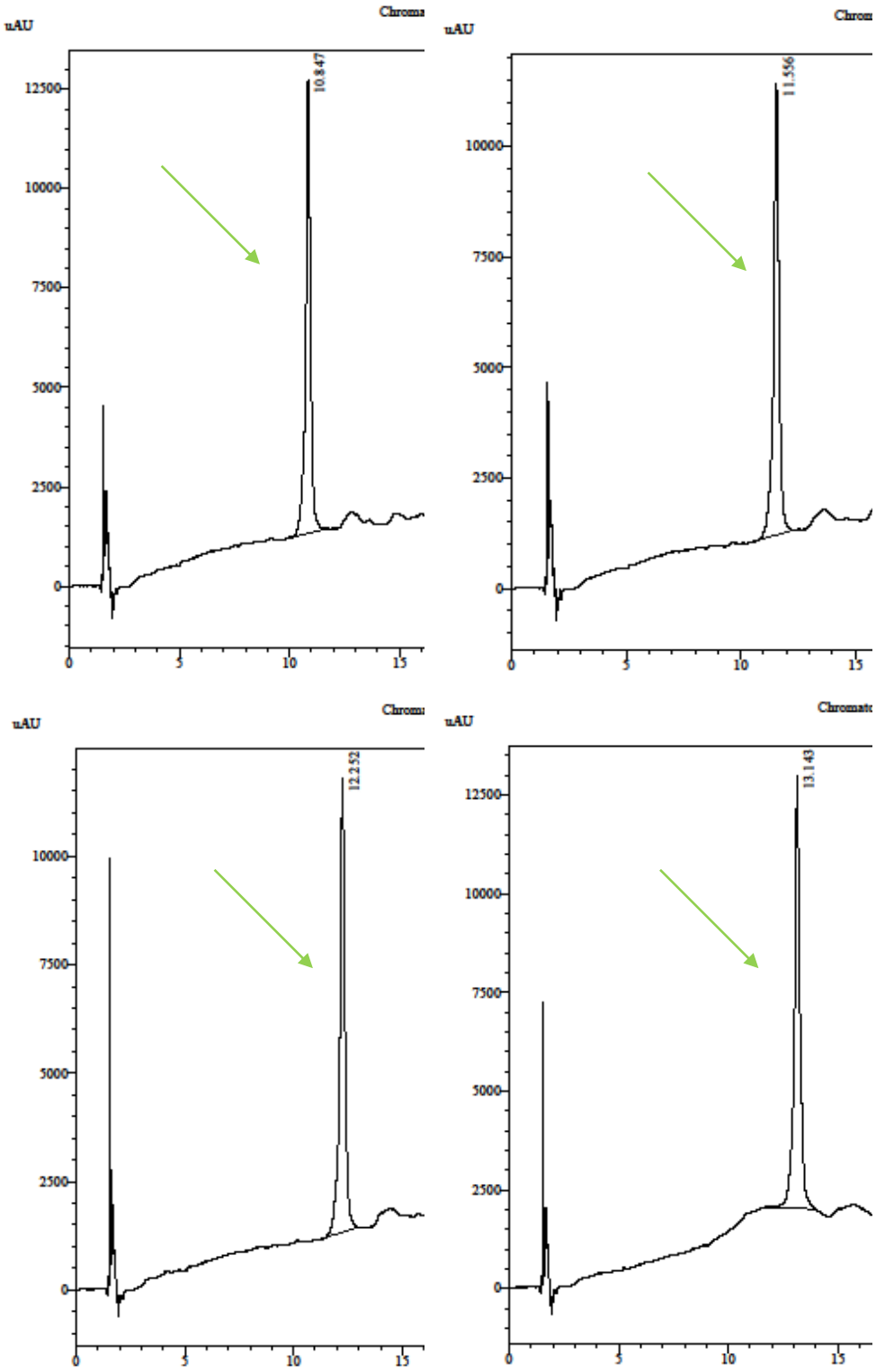

Figure 3.8 Top Left: Chromatogram for genistin standard using 100 % method. Top Right: Chromatogram for genistin standard using 90 % method. Bottom Left: Chromatogram for genistin standard using 80 % method. Bottom right: Chromatogram for genistin standard using 70 % method. The green arrows indicates peaks arising from genistin absorbance.

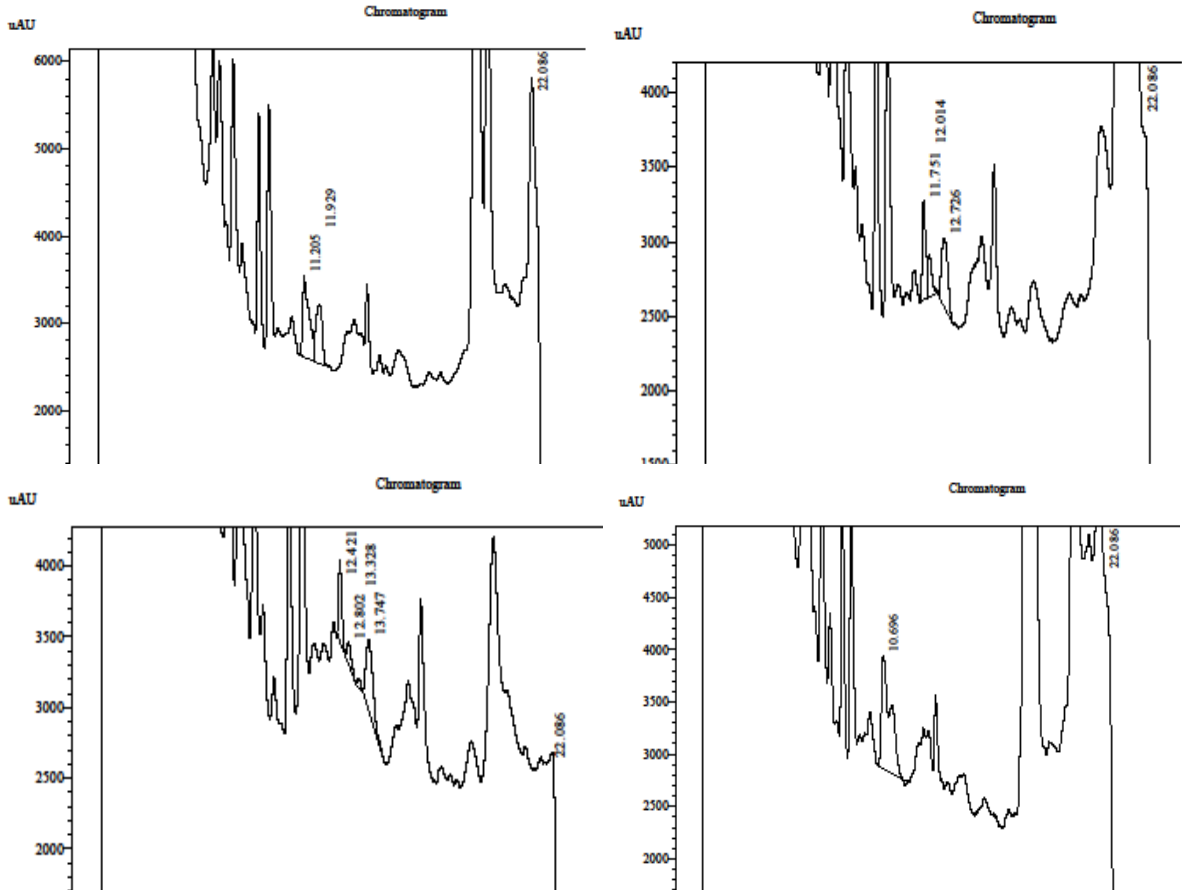

Figure 3.9 Top Left: Chromatogram for yeasty media using 100 % method. Top Right: Chromatogram for yeasty media using 90 % method. Bottom Left: Chromatogram for yeasty media using 80 % method. Bottom Right: Chromatogram for yeasty media using 70 % method.

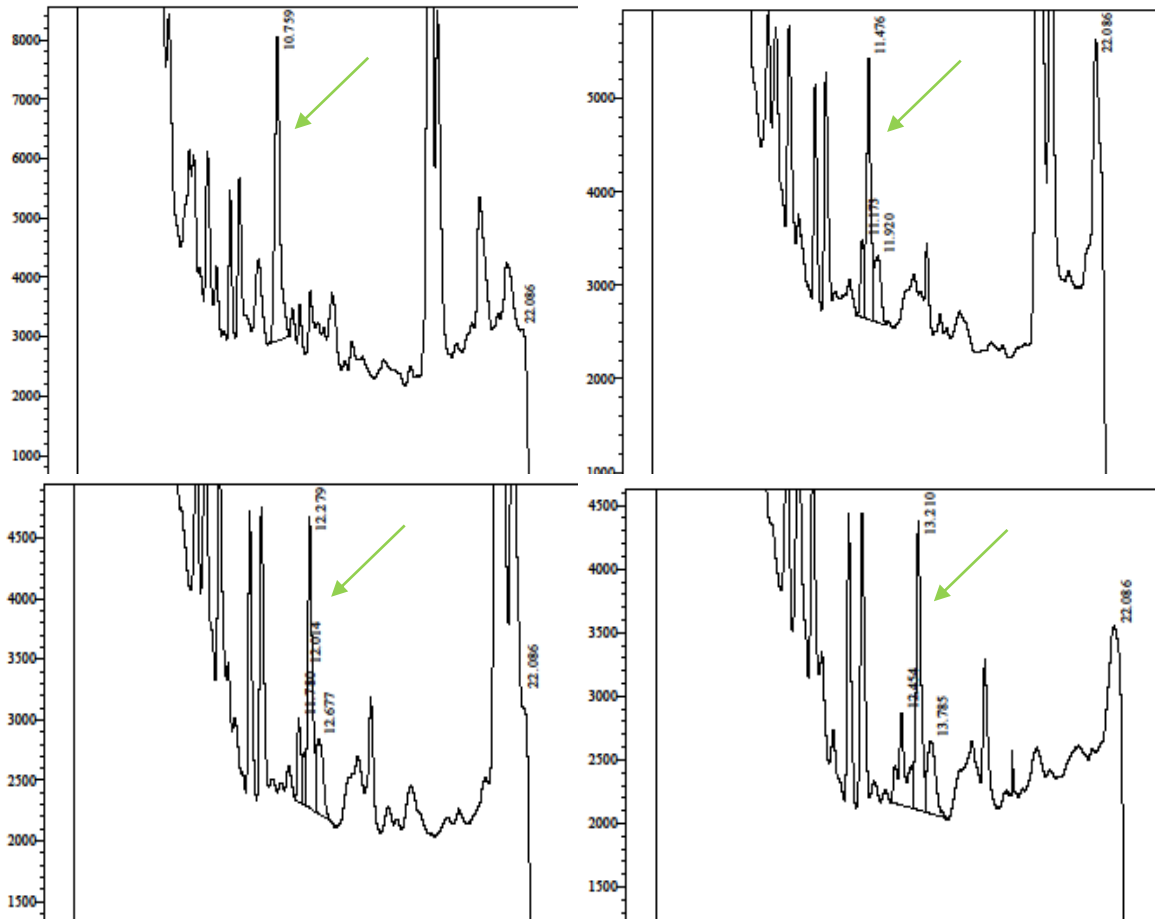

Figure 3.10 Top Left: Chromatogram for genistin spiked yeasty YPD media using 100 % method. Top Right: Chromatogram for genistin spiked yeasty YPD media using 90 % method. Bottom Left: Chromatogram for genistin spiked yeasty YPD media using80 % method. Bottom Right: Chromatogram for genistin spiked yeasty YPD media using 70% method. The green arrows indicates peaks arising from genistin absorbance.

Table 3.9 Genistin recoveries in yeasty YPD media using changing gradient systems.

100 % method	Peak area	Genistin in HPLC vial (mg/L)	Genistin in sample (mg/L)	Recovery %
1 mg/l	66913	0.380	1.900	189.95
90 % method				
1mg/l	39197	0.223	1.113	111.27
80 % method				
1 mg/l	34401	0.195	0.977	97.65
70 % method				
1 mg/l	35370	0.201	1.004	100.40
1 mg/l standard	176133			

Figure 3.9 shows the two interfering peaks from the yeasty YPD media also changing to have longer retention times as well as a larger gap between them as the solvent gradient slows down. The genistin spiked yeasty YPD media chromatograms are shown in Figure 3.10 for the four different HPLC methods. You can see that as the gradient change slows down the interfering peaks begin to separate on either side of the genistin peak. The recoveries for these are shown in Table 3.10, with the 0-70 % solvent B method having a recovery of 100.4 % and for this reason that was the chosen method to progress with. Duplicate genistin spikes in yeasty YPD media were then performed at both high and low concentration (40 and 5 mg/L) giving recoveries close to 100 % as shown in Table 3.11.

Table 3.10 Showing spikes at high and low concentrations for genistin in yeasty YPD media and calculated recoveries.

Standards (mg/L)	Peak area	Peak area / concentration		
Genistin 40	6511303	162782.575		
Genistin spikes	Peak area	Genistin in HPLC vial (mg/L)	Genistin in sample (mg/L)	Recovery %
40	1318566	8.100	40.501	101.25
40	1274697	7.831	39.153	97.88
5	156947	0.964	4.821	96.41
5	144030	0.885	4.424	88.48

A calibration graph was prepared for both genistin (Figure 3.11) and genistein using the 70 % HPLC method. This was followed by a yeast incubation according to the yeast incubation method described earlier. The flasks contained 1.1 g yeast, 2.27 mL of 880 mg/l genistin in DMSO and 49.73 mL of YPD, 49.73 mL of water and 2.27 mL of genistin standard for the water negative control and 49.73 mL of YPD and 2.27 mL of genistin standard for the media negative control.

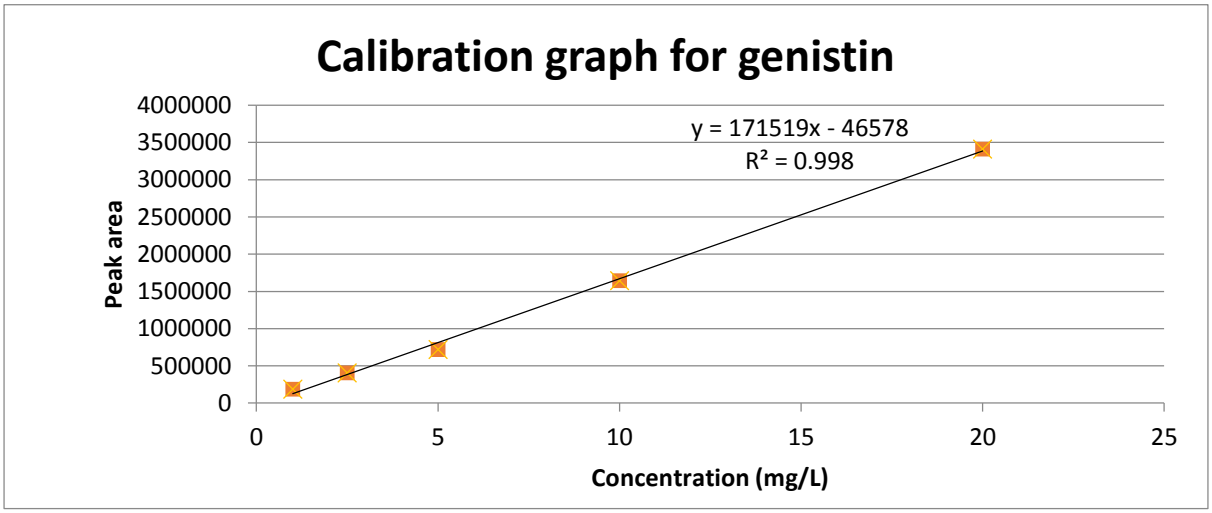

Figure 3.11 Calibration graph for genistin using 70 % method used to calculate genistin concentration in yeast incubations.

Table 3.11 Genistin and Genistein concentration throughout yeast incubation.

Yeast + YPD + Genistin					
Time (min)	Peak area, Genistin	Genistin (mg/L)	Recovery %	Peak area Genistein	Genistein (mg/L)
Spiked -1	1218755	36.57	91.44		
Spiked 0	2486908	74.63	93.29		
-1	0	0.00	0.00		
0	1194298	35.84	89.60	0	0
15	1138937	34.18	85.45	16686	0.279
30	1213100	36.40	91.01	43954	0.737
60	1063986	31.93	79.82	115984	1.945
180	796563	23.90	59.76	353490	5.928
1320	0	0.00	0.00	1130537	18.960

The genistin and genistein concentrations over time for the genistin incubation in yeast are showing in Figure 3.12. The data for the control of YPD + genistin (no yeast) is shown in Table 3.12.

Table 3.12 Genistin concentration throughout incubation for genistin spiked YPD media containing no yeast, used as a negative control. Spikes were performed with 40 mg/L genistin.

YPD + Genistin			
Time (min)	Peak area, Genistin	Genistin (mg/L)	Recovery %
Spiked -1	1201886	36.07	90.17
Spiked 0	2484356	74.55	93.19
-1	0	0.00	0.00
0	1276977	38.32	95.80
15	1254130	37.64	94.09
30	1261859	37.87	94.67
60	1241001	37.24	93.10
180	1237834	37.15	92.87
1320	1625287	48.77	121.94

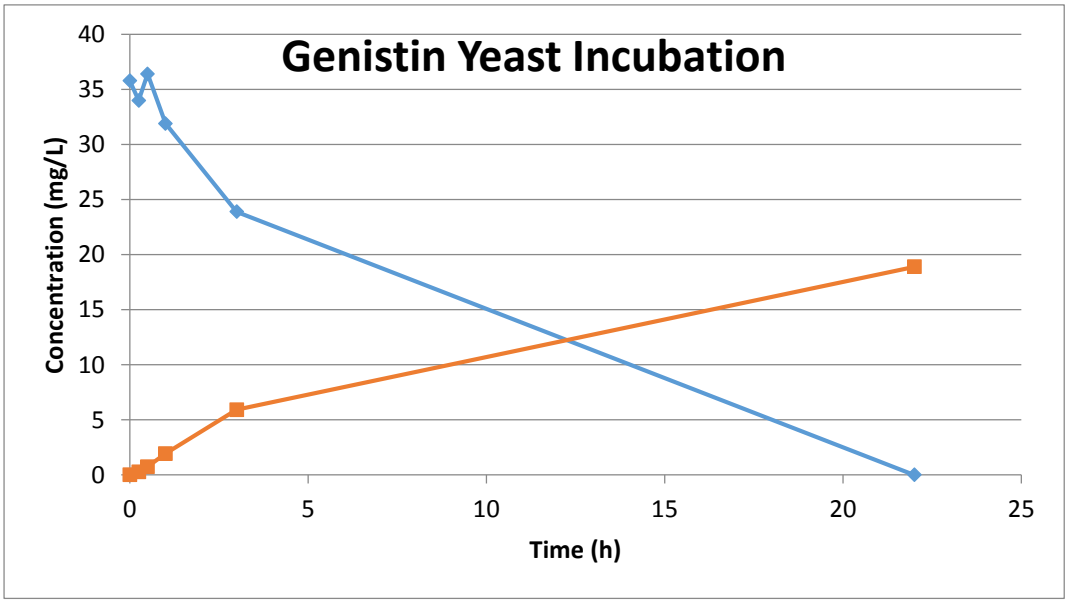

Figure 3.12 Change in genistin and genistein concentration over time, throughout genistin incubation in yeasty YPD media.

3.3 Sour dough recoveries

The Initial results for sour dough spikes with genistin, daidzein and genistein gave poor results as there was a lot of interference from the HPLC system. A methanol blank is shown in Figure 3.13, the column was then washed with 90 % CH3CN, 10 % H_20 overnight and another methanol blank was analysied (Figure3.13).

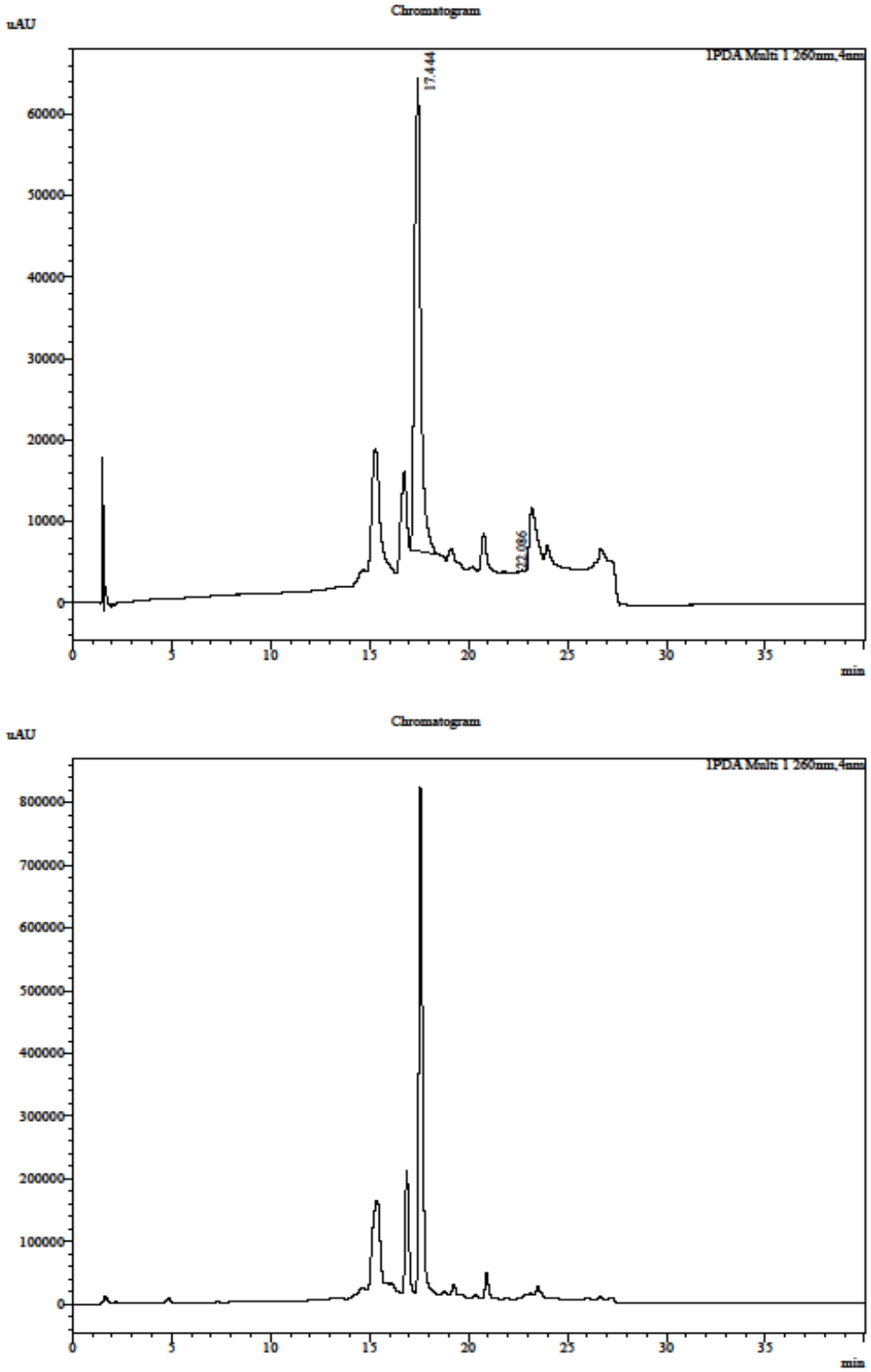

Figure 3.13 Top: Chromatogram of methanol blank before washing column. Bottom: Chromatogram of methanol blank after washing column.

Genistein and daidzein spike sour dough samples were run again to determine recovery. These results are shown in Table 3.13 below.

Table 3.13 Recoveries for genistein and daidzein spiked sour dough cultures, using single standards as reference.

Standards (10 mg/L)	Peak area	Peak area / concentration	
Daidzein	2285004	228500.4	
Genistein	2258576	225857.6	
Spikes (mg/L)	Peak area	concentration	Recovery %
Daidzein 10	2497393	10.93	109.29
Genistein 10	2455635	10.87	108.72

3.4 Sour dough isoflavone incubations

A calibration graph was then made for genistein, daidzein and genistin and a sour dough incubation with these three isoflavones was performed. T-1 and T0 samples were spiked. Spiked samples all gave recoveries close to 100 % and the concentration of genistein and daidzein in sour dough cultures remained relatively unchanged as shown in Table 3.14, throughout the incubation. Genistin was converted to genistein, over time in the sour dough culture similar to the genistin yeast incubation as shown in Figure 3.15.

Table 3.14 Daidzein concentration throughout sour dough incubation.

Time (min)	Peak area	Daidzein in HPLC vial (mg/L)	Daidzein in sample (mg/L)	% of initial
0	1388749	8.32	41.58	103.96
15	1084930	6.50	32.49	81.22
30	1373657	8.23	41.13	102.83
60	1425908	8.54	42.70	106.74
180	1422360	8.52	42.59	106.48
270	1237653	7.41	37.06	92.65
1320	1529995	9.16	45.81	114.53

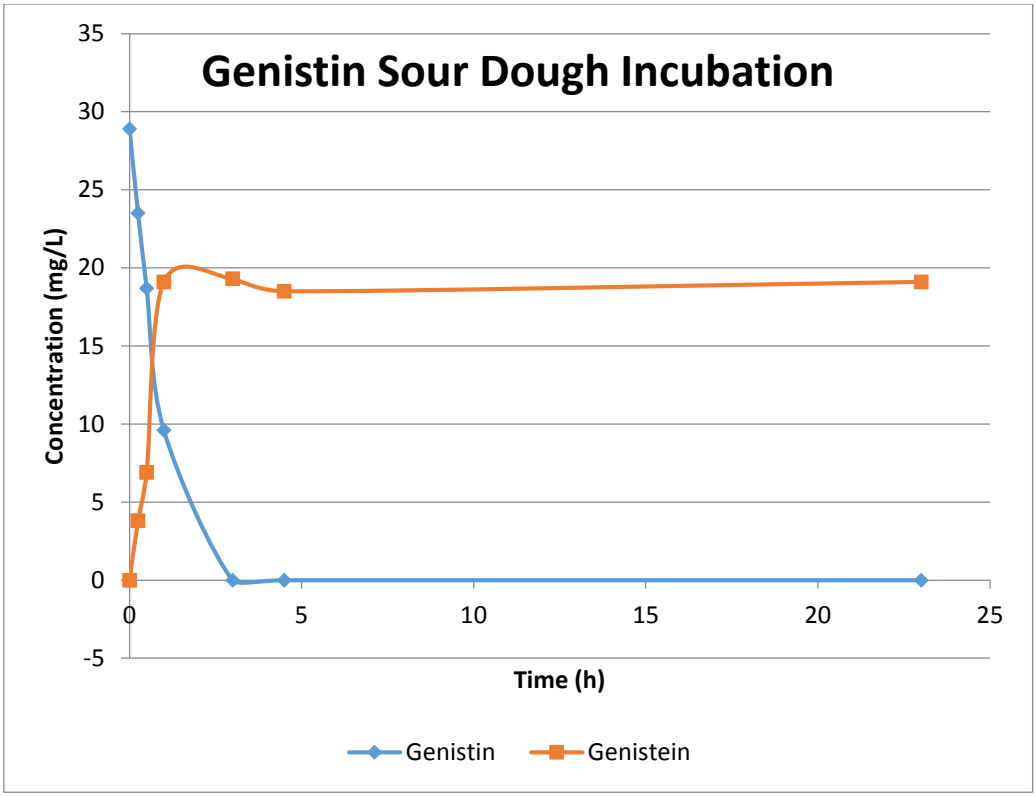

Figure 3.14 Graph of genistin decreasing and genistein increasing for genistin sour dough incubation

3.5 Daily white bread isoflavone changes

Samples for bread improver, flour, dough after mixing, dough after proofing and baked bread was taken of Coupland's daily white bread from the Coupland's factory. These samples were taken in triplicate and analysed in duplicate using the isoflavone extraction method and analysed on HPLC. A calibration graph was made for genistein, daidzein and genistin and ran on the same run. Results for the sample weights, peak area and concentration of genistein, daidzein and genistin are shown in Table 3.15 below and the measured isoflavone concentrations throughout the bread making process shown in Figure 3.15. Recoveries for the dough, proofed dough, baked bread and improver were all done in duplicate at high and low concentration and all obtained results close to 100 % recovery, as shown in Table 3.16.

Table 3.15 Sample weights, peak area and concentration in HPLC for dough, proofed dough and baked daily white bread.

Sample	Peak area, Genistein	Peak area, Genistin	Peak area, Daidzein	Genistein in HPLC vial (mg/L)	Genistin in HPLC vial (mg/L)	Daidzein in HPLC vial (mg/L)	Weights (g)
D 1a	21534	16807	5388	0.100	0.100815	0.037031	4.1
D 1b	23154	21446	12491	0.108	0.128641	0.085848	4.9
D 2a	22564	11873	9313	0.105	0.071219	0.064006	4.15
D 2b	20116	18885	15674	0.094	0.113279	0.107724	4.85
D 3a	25200	14865	11463	0.117	0.089166	0.078783	3.91
D 3b	21508	19771	14108	0.100	0.118594	0.096961	4.51
P 1a	49751	38519	16287	0.232	0.231052	0.111937	3.94
P 1b	28718	107963	15828	0.134	0.647603	0.108782	4
P 2a	20623	111332	?	0.096	0.667812	?	3.6
P 2b	24374	125068	16931	0.114	0.750205	0.116363	4.17
P3a	25923	98121	14914	0.121	0.588567	0.102501	3.77
P3b	pressure overload						4.8
B 1a	16683	21371	20986	0.078	0.128191	0.144232	4.21
B 1b	14454	24464	15761	0.067	0.146744	0.108322	3.85
B 2a	14654	21567	44648	0.068	0.129367	0.306856	4.03
B 2b	13651	13963	16258	0.064	0.083755	0.111738	4.23
B 3a	14635	32675	12299	0.068	0.195997	0.084528	4.26
B 3b	15411	42343	16421	0.072	0.253989	0.112858	4.03

Table 3.16 Sample isoflavone concentration for dough, proofed dough and baked daily white bread.

sample	Genistein (mg/kg)	Genistin (mg/kg)	Daidzein (mg/kg)
I 1	24.02216	157.3935899	17.621373
I 2	22.8642	143.9030582	17.614359
F 1	0.420002	0.831030628	0
F2	0.447758	0.755573622	0
D 1a	0.489232	0.492	0.181
D 1b	0.440154	0.525	0.350
D 2a	0.506457	0.343	0.308
D 2b	0.386344	0.467	0.444
D 3a	0.600341	0.456	0.403
D 3b	0.44422	0.526	0.430
P 1a	1.176196	1.173	0.568
P 1b	0.668757	3.238	0.544
P 2a	0.53361	3.710	?
P 2b	0.544459	3.598	0.558
P3a	0.640499	3.122	0.544
P3b	?	?	?
B 1a	0.369119	0.609	0.685
B 1b	0.349705	0.762	0.563
B 2a	0.338708	0.642	1.523
B 2b	0.300607	0.396	0.528
B 3a	0.320005	0.920	0.397
B 3b	0.356205	1.260	0.560

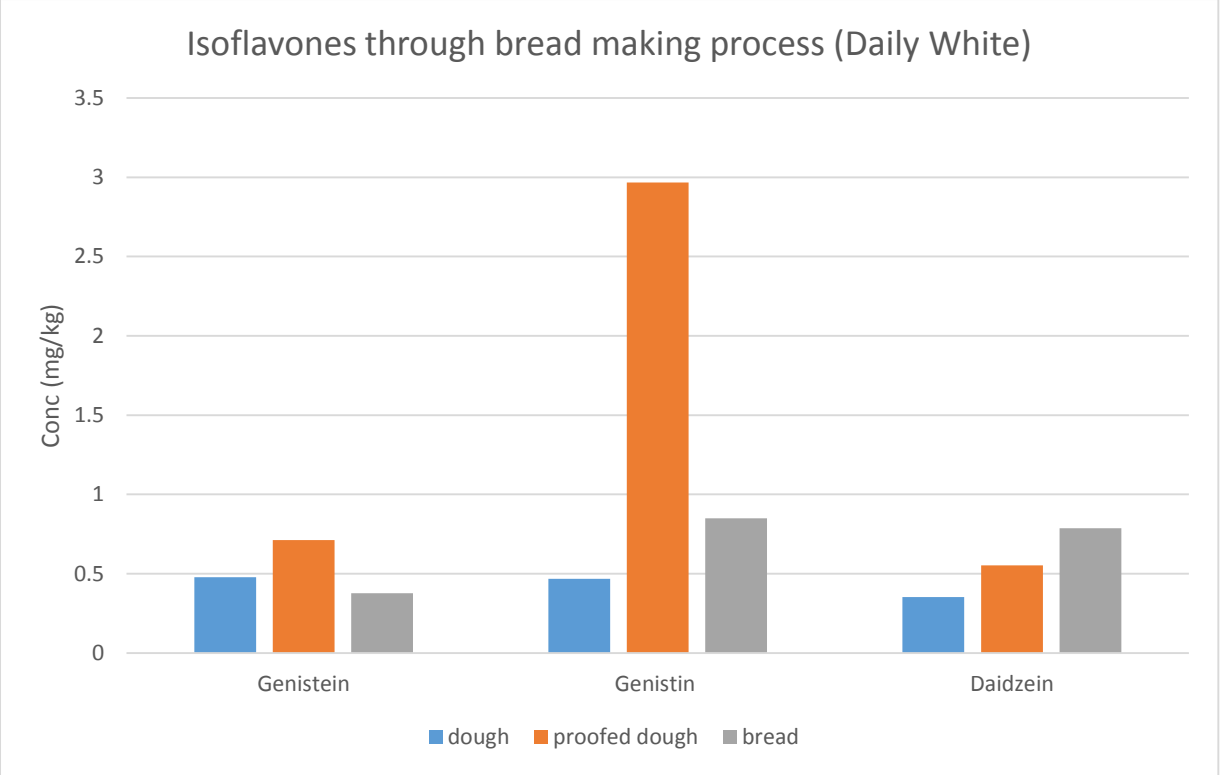

Figure 3.15 Graph showing the concertation of genistein, genistin and daidzein in dough, proofed dough and baked bread.

The Improver added to the bread was also analysed by HPLC and the results for isoflavone concentration are shown in Table 3.17.

Table 3.17 Isoflavone analysis of soy Improver added to bread, showing peak area and Isoflavone concentrations in HPLC vial and in improver.

Sample	Peak area, Genistein	Peak area, Genistin	Peak area, Daidzein	Genistein in HPLC vial (mg/L)	Genistin in HPLC vial (mg/L)	Daidzein in HPLC vial (mg/L)	Weights (g)
I 1	1052199	5352828	523043	4.900520093	32.10829	3.59476	4.08
I 2	1077572	5265876	562560	5.018692507	31.58672	3.866352	4.39

sample	Genistein in improver (mg/kg)	Genistin in improver (mg/kg)	Daidzein in improver (mg/kg)
I 1	24.02216	157.3935899	17.621373
I 2	22.8642	143.9030582	17.614359

Recoveries were measured at high (10 mg/L) and low (2.5 mg/L) concentration in improver, dough, dough after proofing, and baked bread. The results for these are shown in Table 3.18.

Table 3.18 Recoveries for spiked samples at high and low concentrations (2.5 and 10 mg/L) in Improver, dough, proofed dough as well as baked bread.

Sample	Peak area, Genistein	Peak area, Genistin	Peak area, Daidzein	Recovery % Genistein	Recovery % Genistin	Recovery % Daidzein
I 2.5a	1308221	5844757	702533	119.23	295.07767	123.35955
I 10	2120360	6859679	1469516	121.41	239.0058706	155.83276
I 10a	1771936	6541302	1123504	83.802	178.2229442	103.17093
D 2.5	608341	470131	416143	113.33	112.8009612	114.40239
D 2.5a	601028	414603	430783	107.96	94.73408285	114.54865
D 10	didn't add	1709140	2215311	?	101.387905	151.17624
D 10a	2320579	1883143	1780406	106.90	112.0663997	121.57559
P 2.5a	617367	464311	443783	105.74	102.1624757	117.52346
p 10	2181966	1580882	1926438	100.48	87.32524472	131.23624
P 10a	2354777	1783188	1842500	108.53	99.46032582	125.46737
B 2.5	?	446767	430933	?	102.06	112.69
B 2.5a	?	429648	431087	?	99.73	114.04
B 10	13497719	476428	5498282	628.00	27.74	376.76
B 10a	2765038	1810334	2136847	128.14	107.75	145.74

3.6 Mass spectrometry

To determine the identity of peaks in the soy improver they were manually collected for MS analysis following HPLC chromatography. 4.06 g underwent the isoflavone extraction method and two samples were prepared for HPLC. The first run was to be used as reference for peak collection the following run. 11 peaks were collected from the HPLC waste flow tube, collection started as soon as peaks started to appear on the UV absorbance detector and collection stopped just after the peaks had finished. A chromatogram used as reference for peak collection is shown in Figure 3.16 with the 11 collected peaks labelled.

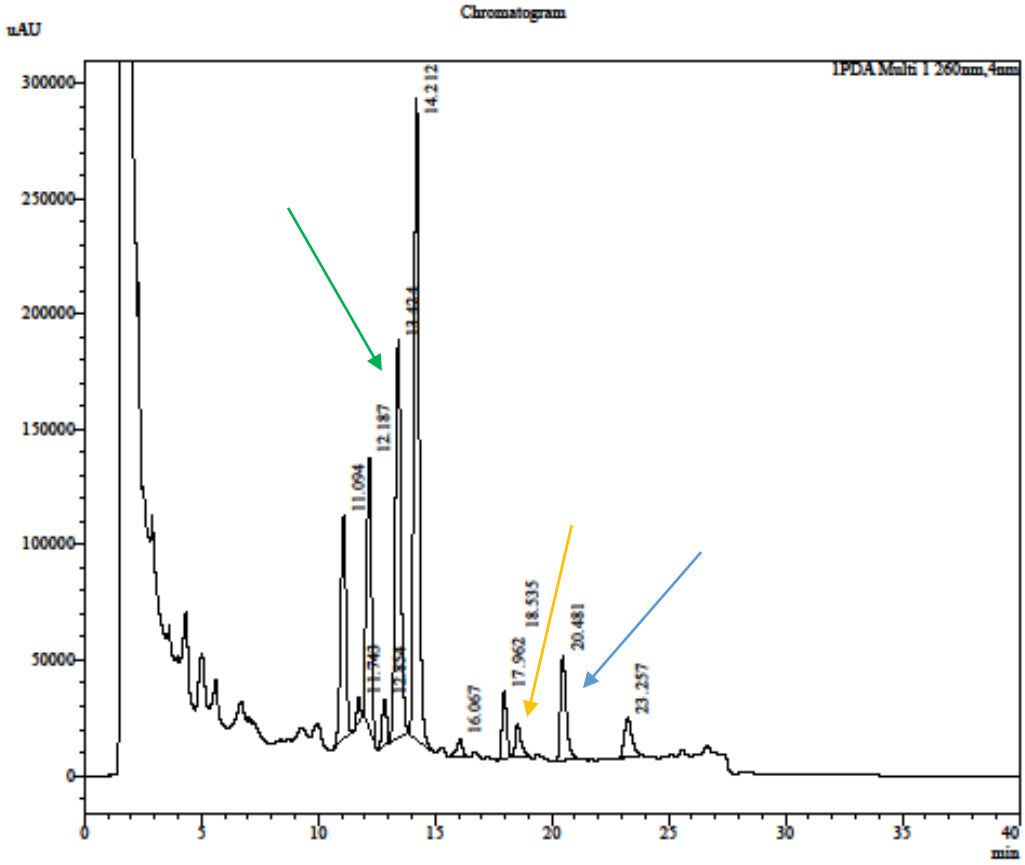

Figure 3.16 HPLC chromatogram of peaks collected from soy improver for MS analysis. Analysis was done using the 70 % HPLC method, with the green arrow indicating the peak arising from genistin absorbance, the yellow daidzein and the blue genistein.

These peaks where then analysed by a technician on a quadrupole time of flight MS. The results fail to clearly identify any isoflavones in all 11 collected peaks as there was a lot of background ions being read. The MS of Peak 8, showed a ion with a m/z of that of daidzein + sodium (276) (Figure 3.17) and most analysed peaks looked like peak 6 (Figure 3.17) with no ions having a mass to charge ration of the molecular weight of an isoflavone plus a proton (1) or Na^+ (22.99).

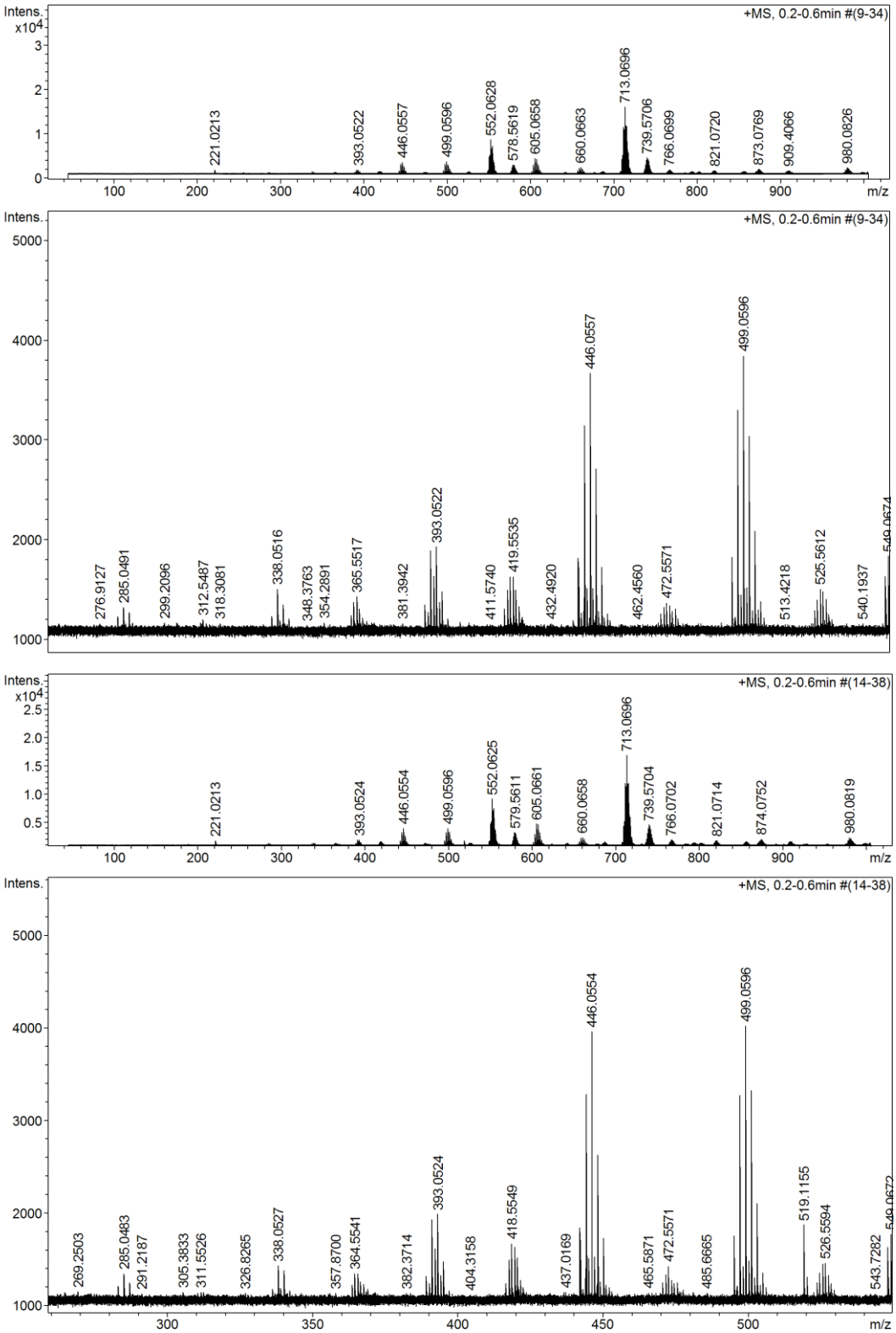

Figure 3.17 Top: MS of peak 8 showing an ion for daidzein + Na^+ with an m/z of 276. Bottom: Ms of peak 6 showing a typical MS for the 11 collected peaks and no ions with an m/z for soy isoflavones.

3.7 Thermal degradation studies

Following the method for genistein thermal degradation studies the wet heat control gave a similar concentration to the wet heat standard. The peak area is shown in Table 3.16 and the HPLC chromatograms in Figure 3.18. The dry heat sample gave an absorbance for genistein that was 10.1 % of the standard. The peak areas are also shown in Table 3.19 and the HPLC chromatograms in Figure 3.19. For the genistein dry heat sample a new peak appeared at 15.228 min with a peak area of 53665.

Table 3.19 Absorbance's for wet and dry heat degradation of genistein

Genistein					
Wet heat	Peak area		Dry heat		Peak area
peak area control	802121		peak area control		959530
peak area sample	842070		peak area sample		97446
Genistein remaining	104.98%		Genistein remaining		10.1556 %

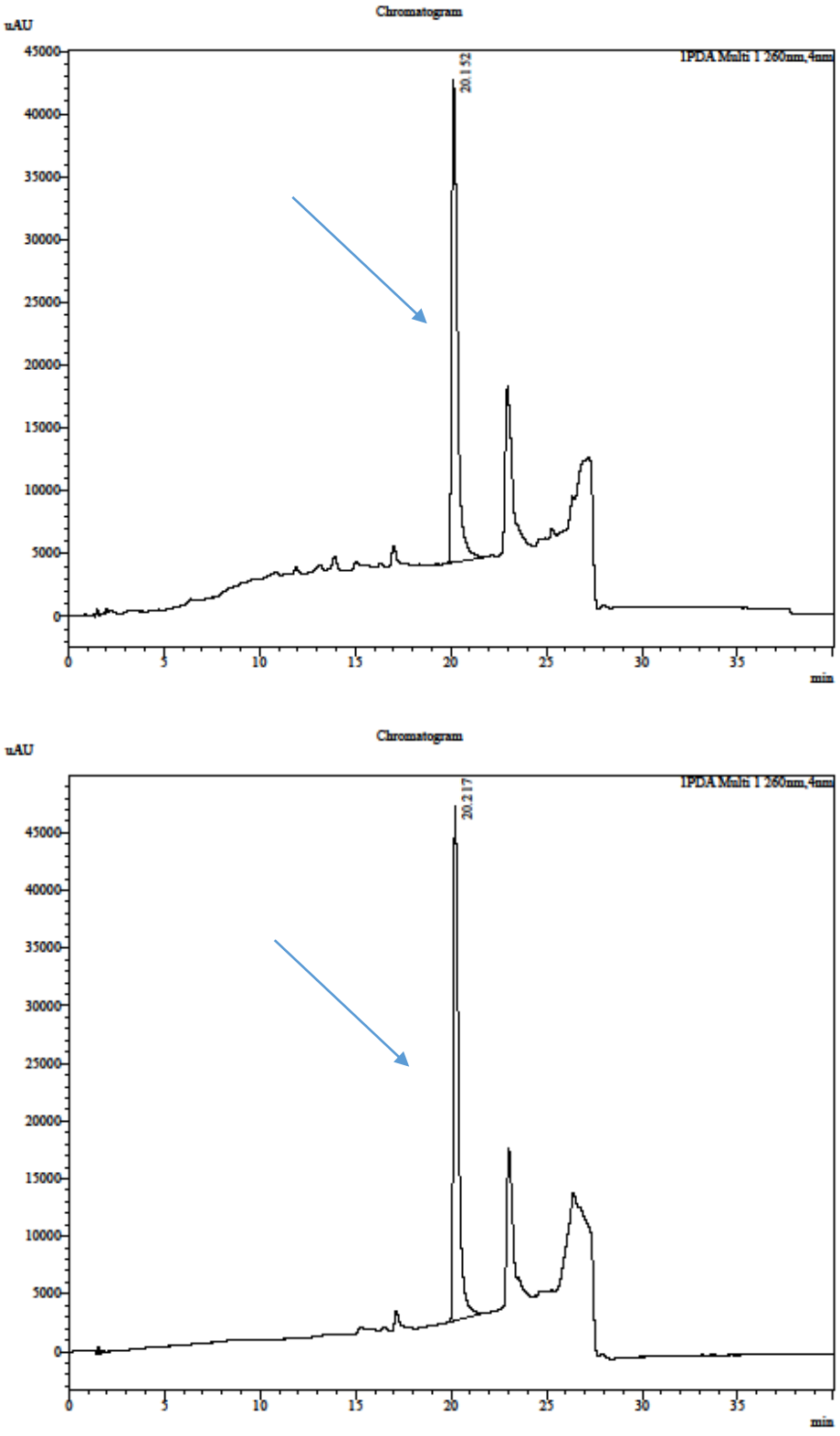

Figure 3.18 Top: HPLC chromatogram of genistein wet heat control. Bottom: HPLC chromatogram of genistein wet heat sample. The blue arrow indicates the peak arising from genistein absorbance. Analysis was done using the 70 % HPLC method.

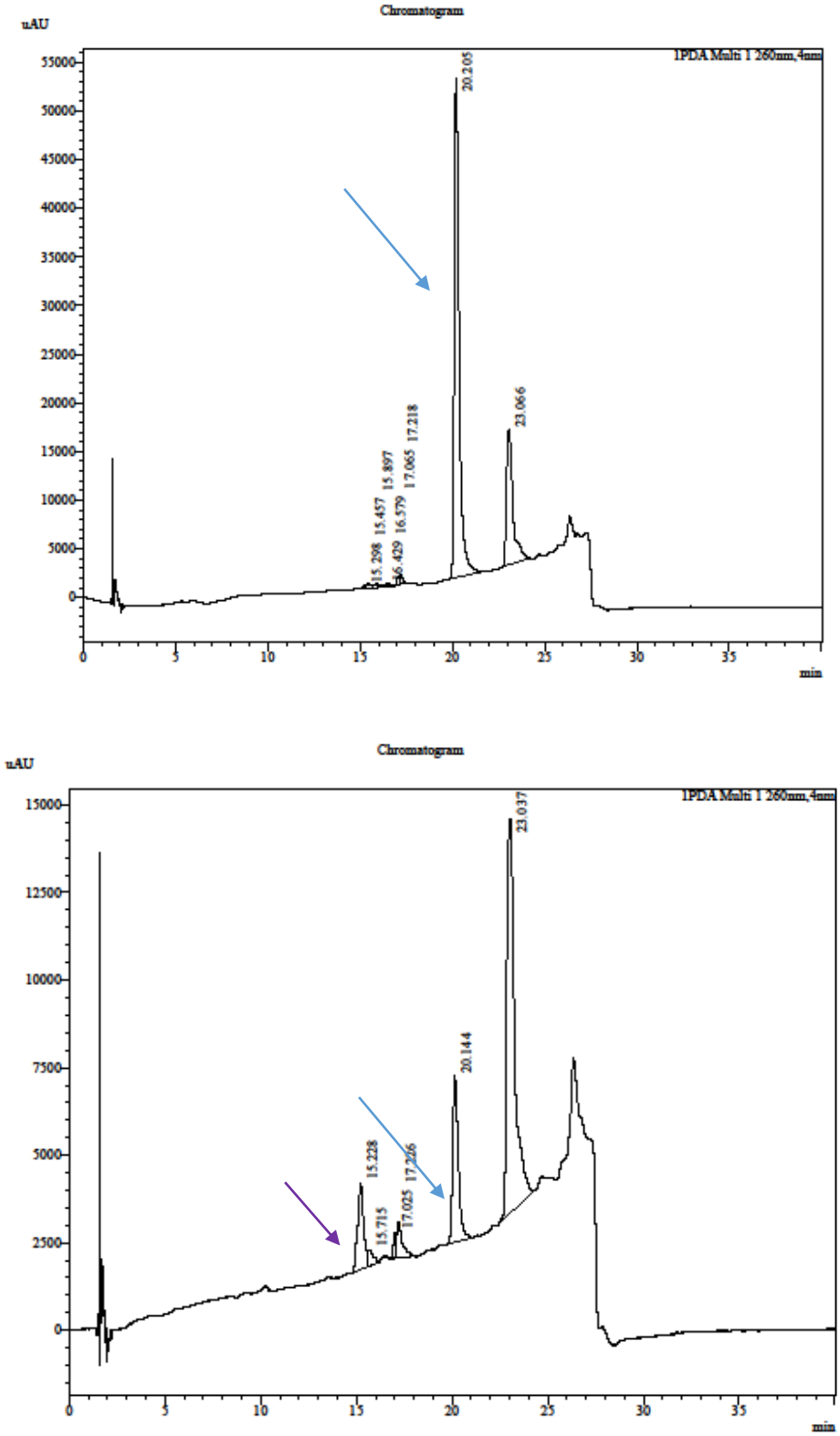

Figure 3.19 Top: HPLC chromatogram of genistein dry heat standard. Bottom: HPLC chromatogram of genistein dry heat sample. The blue arrows indicate peaks arising from genistein absorbance, and they purple arrow indicating the new peak formed from genistein heat degradation. . Analysis was done using the 70 % HPLC method.

3.8 Soy and linseed bread isoflavone changes

Dough, proofed dough and baked bread samples gathered in triplicate from soy and linseed bread from Coupland's factory underwent isoflavone extraction method and was then analysed by HPLC. Values for weights of samples, peak area, concentration in extract and concentration in sample are given for the dough (Table 3.20), the proofed dough (Table 3.21) and the baked bread (Table 3.22). With a graph of the analysed isoflavone concentrations being shown for the analysed samples in Figure 3.20.

Table 3.20 Showing values weights, peak area and concentration for genistein, genistin and daidzein for dough from soy and linseed bread made at Coupland's.

	Dough	Peak area		
Sample	Weight (g)	Genistin	Daidzein	Genistein
D1a	4.52	117816	60398	160286
D1b	4.5	124379	63604	156920
D2a	4.55	121155	59246	147614
D2b	4.1	106433	67654	166839
D3a	4.38	128586	60943	154281
D3b	4.42	148537	65448	159139

Concentration in HPLC Vial (mg/L)		
Genistin	Daidzein	Genistein
0.778	0.453	0.764
0.821	0.477	0.748
0.800	0.444	0.704
0.702	0.508	0.795
0.849	0.457	0.736
0.980	0.491	0.759

Concentration in sample (mg/kg)				Average		
Genistin	Daidzein	Genistein		Genistin	Daidzein	Genistein
3.440	2.005	3.381		3.723	2.144	3.412
3.648	2.121	3.325				
3.515	1.954	3.093				
3.426	2.476	3.880				
3.875	2.088	3.359				
4.436	2.222	3.433				

Table 3.21 Showing values weights, peak area and concentration for genistein, genistin and daidzein for proofed dough from soy and linseed bread made at Coupland's.

	Proofed Dough			
		Peak area		
Sample	Weight (g)	Genistin	Daidzein	Genistein
P1a	4.07	116940	67825	196793
P1b	3.9	98487	64606	182132
P2a	3.86	127305	67518	187542
P2b	4.26	114396	68476	202063
P3a	4.36	335787	117292	318900
P3b	4.09	277973	118869	325440

Concentration in HPLC vial (mg/L)		
Genistin	Daidzein	Genistein
0.772	0.509	0.938
0.650	0.485	0.868
0.840	0.507	0.894
0.755	0.514	0.963
2.216	0.880	1.520
1.834	0.892	1.552

Concentration in sample (mg/kg)				Average		
Genistin	Daidzein	Genistein		Genistin	Daidzein	Genistein
3.792	2.500	4.610		3.756	2.506	4.555
3.333	2.486	4.453				
4.353	2.625	4.633				
3.544	2.412	4.523				
10.165	4.036	6.974				
8.971	4.361	7.587				

Table 3.22 Showing values weights, peak area and concentration for genistein, genistin and daidzein for baked bread from soy and linseed bread made at Coupland's.

	Baked Bread			
		Peak area		
Sample	Weight (g)	Genistin	Daidzein	Genistein
B1a	4.09	273260	44597	127521
B1b	4.44	263729	47636	168772
B2a	4.27	217176	44400	163800
B2b	3.82	192245	38317	146167
B3a	4.35	236027	46163	151302
B3b	3.86	184852	46605	147983

Concentration in HPLC vial (mg/L)		
Genistin	Daidzein	Genistein
1.803	0.335	0.608
1.740	0.357	0.805
1.433	0.333	0.781
1.269	0.287	0.697
1.558	0.346	0.721
1.220	0.350	0.706

Concentration in sample (mg/kg)				Average		
Genistin	Daidzein	Genistein		Genistin	Daidzein	Genistein
8.818	1.636	2.973		7.249	1.619	3.479
7.840	1.610	3.624				
6.713	1.560	3.658				
6.642	1.505	3.648				
7.162	1.592	3.316				
6.321	1.812	3.655				

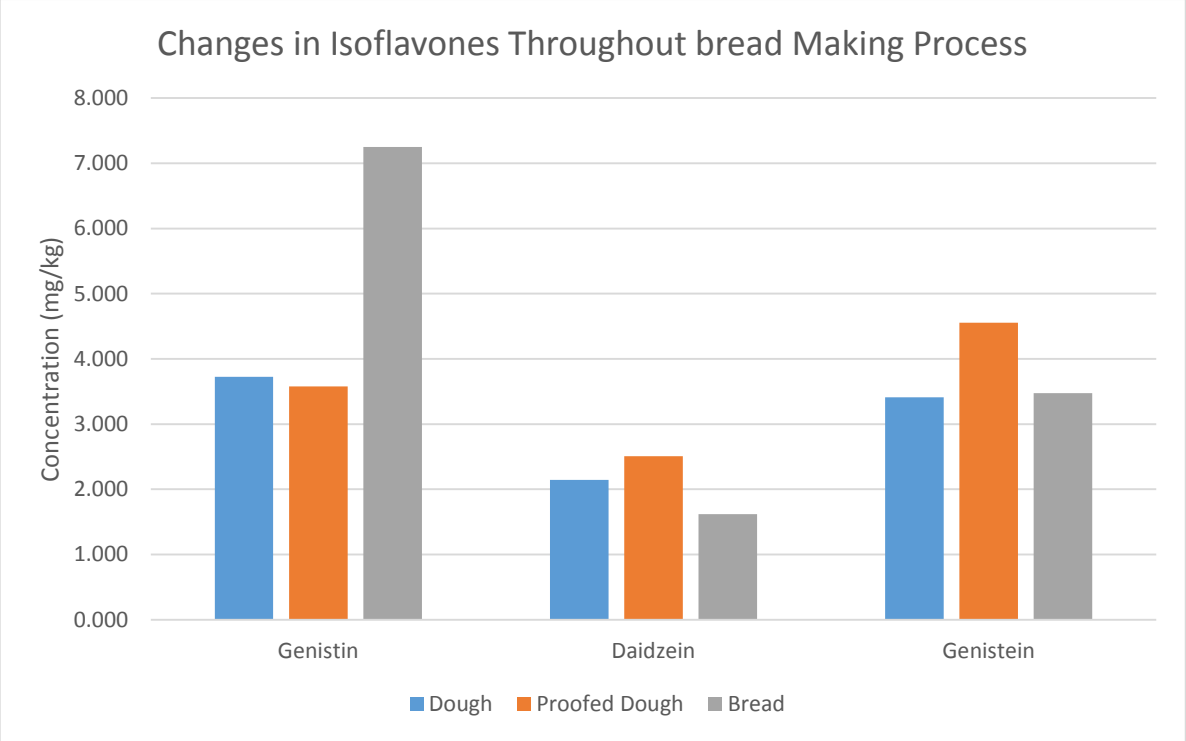

Figure 3.20 Genistin daidzein and genistein concentrations at various stages of the bread making process, dough, proofed dough and baked bread, for soy and linseed bread made at Coupland's bakery.

Discussion

4.1 Method suitability

4.1.1 Initial method trials

Initially genistein had a retention time of 16.551 min, this appeared to be a suitable retention time as it's short enough for multiple HPLC analysis runs to be performed without a large time burden being applied and also likely long enough that adequate peak separation can occur to give adequate levels of specificity and accuracy. As the concentration of standard was increased the absorbance increased in a like manner. When the concentration doubled, from 100 – 200 mg/L the peak area increased, from 8547126 to 16956355, an increase of 198.3 %, roughly double. This is ideal as it shows that standards are being prepared with suitable accuracy and that the reading at an absorbance of 260 nm is suitable to allow the concentration to be calculated from the absorbance with use of a calibration graph. The fact that methanol blanks had few and small peaks shows with none at a retention time around 16.5 min shows that there is no residual genistein being left on the column, injector or in the system to show up in future samples, and that the column and solvents are at a suitable grade for accurate qualitative and quantitative results to be obtained.

For production of the calibration graph a range from 1-100 mg/L was selected to cover a broad range of possible sample concentrations. A standard deviation of 8163 along with a CV of 2.86 % and a R^2 of 0.9993 all show that the calibration graph is indeed linear and accurate over this range.

4.1.2 Bread suitability

The two types of bread selected for analysis were the soy and linseed and daily white. The soy and linseed was selected as it has a high amount of soy four added, 6 %, and the daily white selected as it is the most consumed and produced bread by Coupland's also with the addition of soy, all be it at smaller concentrations. Coupland's breads were selected for analysis as they allowed access to their factory and to samples throughout the bread making process. The isoflavone extraction method from bread was based on one used previously in the same laboratory to analyse isoflavones in faeces of cheaters that had been feed soy. The use of this method for bread samples was confirmed as suitable as a peak in bread had the same retention time as the genistein standard. Spiked samples had a single peak at 16.5 min showing co-

elution of genistein standard and the corresponding peak from bread samples. A peak with a shoulder appearing would indicate that these two peaks were not the same compound. The same electrochemical properties (the same retention time and co-elution) as well absorbance properties (UV spectra) give a strong indication that the peak appearing in bread at 16.5 min is from genistein.

For soy and linseed samples the results obtained for genisteins concentration is, 3.09 and 3.14 mg/L, varying by 0.052 mg/L, a difference of 1.68 %. By running the samples in duplicates and obtaining two very similar results it shows the reliability of the method (weighing, pipetting, extracting…) and the accuracy of the results. For the daily white bread the two values obtained for the concentration of genistien are 1.40 and 0.67 mg/L. This is a variance of 0.73 mg/L and a difference of 108.95 %, which is much too large and shows why duplicate samples themselves are not ideal as when they do not match you cannot tell which one is closer to the real value. To avoid this problem in the future three different samples were run in duplicate.

Soy and linseed samples gave an average recovery of 105.54 % and daily white an average recovery of 108.96 %. For the daily white the recovery is not as close to 100 % likely due to one of the samples having a calculated genistein concentration that was inaccurate. The lower concentration of 0.67 mg/L is likely the incorrect one as the calculated recovery is too large. A larger genistein concentration in the sample would mean we were subtracting a greater proportion from the spiked samples, resulting in a lower recovery, closer to 100 %. Both recoveries are still relatively close to 100 % showing the suitability of the extraction method its ability to obtaining a large amount of genistein from the samples, and thus what we are measuring from the extracted samples is close to that which is in the actual sample. Recoveries must be tested for forms of analysed samples to show the suitability of the method to extract and detect the specific isoflavones. It is possible that interfering peaks, isoflavone binding or inadequate extraction could lead to a determined concentration that was not representative of the sample.

When determining the recoveries for the soy and linseed samples two soy and linseed un-spiked samples were also run. They produced results of 3.04 and 3.03 mg/L, very close to the initial results obtained of 3.09 and 3.14, showing reproducibility among different runs and using different calibration graphs,

along with the similarity of isoflavone concentration in different loafs from different batches of the same bread.

Spiked samples were done in duplicate at 1, 5 and 50 mg/L to determine recoveries at both high and low concentrations. This is standard practice when determining recoveries and checks that the extraction method works for samples with a range of isoflavone concentrations. At 1 mg/L recoveries of 45.92 % and 37.94 % were obtained, at 5 mg/L recoveries of 93.06 % and 71.47 % were obtained and at 50 mg/L recoveries of 93.55 % and 103.32 % were obtained. The spikes at low concentration give much lower recoveries, likely due to the fact that there is already genistein in the sample and to calculate the recoveries the values for genistein concentration obtained from non-spiked runs are being subtracted. There is more room for errors when spikes are at concentrations lower than that which is in the sample as it is difficult to accurately minus the isoflavone contribution form the bread, which has less of an effect when spikes are at high concentrations.

To accurately determine recoveries for the daily white bread, a non-soy daily white bread was specially made by Coupland's bakery. This was not done for the soy and linseed bread as it is not feasible to make a soy bread containing no soy, but is possible to make a white bread, normally containing small amounts of soy without the addition of soy. The ability to make this non-soy daily white means we are able to obtain the recoveries more accurately as the initial genistein concentration is not present and therefore does not need to be accounted for as all genistein found will be from what was added to spike the sample. Chromatograms of the non-soy daily white (Figure 3.3) showed no peak at 16.5 min. To determine recoveries genistein spiked samples were done at 5 and 50 mg/L in triplicate and produced recoveries of 127.02 % and 114.01 % respectively, confirming the suitability of this method of analysis for daily white bread. Additionally the fact that this bread contained no peak at 16.5 min is more evidence that the peak in the regular soy containing daily white is indeed genistein, as the only difference between the two bread samples is the soy addition.

4.1.3 Yeast suitability

To determine the recoveries for accurate determination of isoflavone concentrations in the yeast incubations an YPD and yeast mixture was analysed

by HPLC, along with a spiked sample and a standard. The yeasty YPD media contained no peaks at 16.5 min, the retention time of genistein. This is good as it this mixture does not contain any genistein and means there is nothing to interfere with the genistein peak when it is added. A spiked mixture shows an additional peak from the blank at 16.5 min, the same time as the peak of the genistein standard, as shown in Figure 3.4, and gives a recovery of 106.6 %. This is a value close to 100 % and therefore shows that genistein is not binding to anything in the media that would cause the concentration to not be determinable by HPLC analysis.

Yeast recoveries were done using duplicates at the low and high concentrations of 1 mg/L and 10 mg/L. The low spikes gave close recoveries with an average of 98.0 % and the high spike also had similar results with an average of 94.8 %. This confirms that this extraction and analysis procedure is suitable for genistein analysis from the yeast incubations.

For genistin the observed retention time of 10.72 min is expected as the additional glucose group increases the polarity of the molecule with respect to genistein, because of the increased polarity it associates with the hydrophobic C18 column less than genistein and elutes in a shorter time. The appearance of this peak also shows that analysis of genistin is still possible by measuring absorbance at 260 nm, as to be expected as genistein absorbs at this wavelength and genistin contains genistein.

Having a peak from the yeasty YPD media run at the same time as genistin, as shown in Figure 3.9, is not suitable as this media contains no genistin and therefore this peak is being made by something else with the same retention time. This will cause calculation of genistin levels higher than are actually present. This can be seen when recoveries are calculated from spiked samples. 2.5 mg/L spiked samples gave recoveries of 209.4 % and 196.1 %, 5 mg/L spiked samples gave recoveries of 151.1 % and 146.1 % and 50 mg/L spiked samples gave recovery's of 112.9 % and 110.6 %. These results are expected as at lower concentrations the portion of the peak contributed by the yeasty YPD media contributes a larger proportion than when a higher concentration of genistin is present.

One way of getting a more accurate genistin concentration is to run the yeasty YPD media blank samples, calculate the average size of the peak at 10.7 minutes and minus this from the peak when genistin is present to get the absorbance contributed by just the genistin. Using this method recoveries of

135.1 % and 121.9 % for 2.5 mg/L spikes, 114. 0% and 108.9 % for 5 mg/L and 109.2 % and 106.9 % for 50 mg/L. These values are much closer to the target 100 % but are still too high for the lower concentration spikes, suggesting that the absorbance contributed by the media has not been fully taken account of.

Another way of getting around this problem is to change the HPLC method as to cause the genistin and whatever else is there eluting at the same time from the yeasty YPD media to elute at different times. This could be done by using a different column, different solvents or changing the gradient of solvent change. The chosen option was to change the gradient, lowering the rate of solvent change causing increased retention times and greater separation of peaks. Several different slower rates were trailed with the best being the 70 % method. This was chosen as it caused the best separation of genistin from other unwanted media caused interference. The interference turned out to be caused by at least 2 different compounds, you can see the peaks slowly separating as the gradient is slowed down. Using the 70 % method one of these peaks has a shorter retention time than genistin and the other a longer retention time.

Calculating the recoveries at these different gradient was less accurate as the difference between one peak and another had to be defined arbitrarily using the split peak function. This arises as at least three compounds are eluting with similar retention times. You can see the recoveries getting closer to 100 % as you slow down the system going from 189.0 % at the initial rate, 111.3 % at 90 % gradient, 97.7 % at 80 % gradient and 100.4 % at the selected 70 % gradient.

The retention times also change in a predicted manner, getting longer as the gradient change is slowed down, going from 10.8 minutes at the initial gradient, 11.476 min at 90 %, and 12.3 minutes at the 80 % gradient and 13.2 min at the 70 % gradient. This change is because the gradient change of the solvents initially went from 100 % of the more polar solvent A (containing more H_2O and NH_4OAc) to 100 % of the more hydrophilic solvent B (containing more MeOH and CH_3CN), but this change was slowed down, and therefore the genistin took longer to elute as it bound tighter to the column when the solvent was more polar.

Recoveries for genistin in yeasty YPD media where then performed at high and low concentration, 40 mg/L and 5 mg/L, using the 70 % gradient system. Recoveries were close to 100 % and the method was deemed suitable for genistin analysis in yeast incubations.

Genistein recoveries were tested in the new method. A genistein spike and standard confirm that it is still suitable for analysis of this isoflavone. Genisteins new retention time was 20.49 min, and to accommodate for this the method was increased in length to 25 min, increasing at the same gradient, 70 % B at 20 min and 87.5 % at 25 min, before a change back to 100 % solvent A to re-equilibrate for the next run.

4.1.4 Sour dough suitability

Sour dough recoveries were tested for daidzein, genistin and genistein at high and low spikes (40 mg/L and 5 mg/L) into 50 % sourdough and 50 % water (to make the mixture pippetable). This was then added to 10 mL of 80 % methanol and the isoflavone extraction procedure was carried out before HPLC analysis was performed. Spiked sourdough samples all gave recoveries closet to 100 % so this method for analysis was determined to be of a suitable standard for analysis of sour dough culture.

4.1.5 LOD, LOQ

The calculated LOD was 0.07 – 0.1 mg/L and the LOQ 0.17-0.21 mg/L. The LOD represents the lowest noticeable genistein concentration, but quantification would not be accurate at this range. The LOQ represents the lowest accurately determinable genistein concentration. As these values are much lower than the values found in bread samples the detection limits of this method are suitable. Additionally it means that if interconversion of isoflavones is happening, in yeast or sour dough incubations, it would have to be at a rate that concentrations of the appearing isoflavone could not be higher than 0.07 – 0.1 mg/L.

4.1.6 Alternative method approaches

The approach used here of separating compounds based on their polarity and determining concentration by level of UV absorbance works well when compounds can be identified by use of a standard. However when a standard is unable to be obtained, accurate determination of concentration (lack of a calibration graph) and compound identification (co-elution and a comparable UV spectra) cannot be determined. For the research performed here standards

for genistein, daidzein and genistin were able to be purchased from LC laboratories at a reasonable price. Standards for the 9 other isoflavone forms present in soy, and likely Coupland's bread and improver, could not be obtained due to availability or price issues (e.g 6''-O-malonylgenistin is $4750 (US) for 25 mg from Toronto Research Chemicals Inc, Toronto, Canada).

Another approach typical of metabolism studies involves tracking an isotopically label standard. This could not be performed as isotopically labelled genistein (genistein-2',6'-d2) cost $9900 (US) and daidzein (daidzein-d6) cost $11250 (US) from Santa Cruz Biotechnology, Dallas, Texas, U.S.A.

Using an alternative detection system capable of adequate identification of compounds without standards following HPLC analysis would be another more robust way to analyse soy isoflavones. HPLC-MS would be able to identify the isoflavones based on their mass to charge alleviating the reliance on standards required in HPLC-UV. HPLC-MS was however not available.

MS identification of peaks by manual collection following HPLC-UV analysis is another feasible approach for quantification of unknown isoflavones. This approach did not work using NH_4OAc, MeOH and CH_3CN as the mobile phases for HPLC separation. A HPLC-UV method could be prepared with alternative mobile phases suitable for MS identification.

As malonyl isoflavones are at high concentrations in soy, a standard could be extracted as performed previously in the literature (Griffith & Collison 2001). This can be a lengthy process of extraction, qualification and quantification and the research done here is directed towards what happens to the isoflavones in the bread making process rather than the process of acquiring the isoflavones.

Another approach, that in hindsight would have been more suitable, is to copy an HPLC identification method from the journal of AOAC international (association of analytical communities). AOAC has released a report with information on an HPLC method that has been independently verified by different labs around the world. Identification of all 12 soy isoflavone forms can be done by replicating the method and using relative retention times along with analysing soy flour and heated soy flour as this converts the malonyl isoflavones to the acetyl form (Collison 2008).

4.2 Yeast isoflavone incubations

4.2.1 Genistein yeast incubations

A genistein yeast incubation was performed in an attempt to model what might happen to the genistein in the proofing stage of the bread making process. The yeast was incubated at 37 ^{0}C as this was the closest temperature of incubator available to model the temperature that bread is proofed at Coupland's, 41 ^{0}C. 0.5 g of yeast was added to 25 mL of YPD as this makes a 2 % yeast concentration, the same as that of Coupland's bread. The yeast added was compressed yeast obtained from Coupland's bakery, the same as which Coupland's add to their bread. Samples were taken before genistein addition (to use as a blank for recoveries), straight after genistein addition, at 15 and 30 minutes as well as 1 and 3 h. This time frame was selected as multiple samples are taken over the length of dough proofing done at Coupland's (41 minutes) and a much longer one, 3 h, to extend results and cause an abundance of any potential metabolites to appear for easier analysis.

The YPD media was preheated to 37 ^{0}C to cause the reactions to happen at a constant rate rather than change with temperature as the solution warms up in the incubator. When the 3 h sample was taken a good amount of yeast material was on the bottom of the conical flask, showing that the yeast are capable of metabolising and reproducing under these conditions. Additionally the solution itself was still cloudy indicating that some of the yeast was still in solution and potentially capable of interacting with the genistein in solution rather than all having settled on the bottom of the flask.

For yeast incubations standards were made up and added in DMSO rather than methanol, this is because the methanol added would harm the yeast as opposed to the DMSO, which would not cause any harm to the yeast at this concentration. Putting the incubation samples straight into 80% methanol causes the yeast to die and any metabolism they are potentially doing to cease, allowing analysis of samples selected stages throughout the incubation.

This incubation was done in duplicate and genistein concentrations remained relatively constant throughout the entire incubation, varying by no more than +/- 0.5 accept for one outlier 3 mg/L larger than the rest, 29.6 mg/L compared to 26.6 mg/L. This no change in genistein concentration was not the expected result, especially after 3 h when there is a large yeast concentration and plenty of time for them to metabolise genistein. HPLC chromatograms were analysed

and while some initial peaks increased in size, likely from compounds produced by the yeast (more evidence of their activity), no new peaks appeared.

The genistein yeast incubation was done again, introducing two negative controls, YPD + genistein and water + genistein. Along with this being done two new spiked samples were introduced, a spike at T-1 and at T0. These were done to make sure that adding the samples to the 80 % methanol did indeed cause the yeast to die and any metabolism to cease as well as to test if storing the samples overnight caused any change, as the samples were put into the freezer overnight and analysed the next day due to time constraints. The T0 sample was spiked at the time it was taken and the T-1 spiked the next morning before the extraction procedure was done.

Genistein and Daidzein concentrations remained constant again throughout the 3 h period in all 3 scenarios, isoflavone + yeasty YPD, YPD + isoflavone and water + isoflavone. There was very little variance throughout and the spiked samples all gave good recoveries. The lack of any new compounds detected in HPLC chromatograms and constant value of genistein and daidzein concentrations over time shows that yeast fail to metabolise isoflavone aglycones in any manor over this time period.

This model system gives strong evidence that the metabolic activity of *saccharomyces cerevisiae* is not responsible for a decrease in isoflavone aglycone concentration throughout the bread making process and that no unknown metabolites with unknown estrogenic and biological activity are produced.

Fungi, including yeast, have a cell wall in addition to a cell membrane. The cell wall is required to prevent cell lysis due to the high internal turgor pressure (Kollar, et al. 1997). The cell wall consists of proteins and polysaccharides, and could help to explain why yeast do not metabolise them compounds. Both estrogens and isoflavones can enter cells through passive diffusion through the plasma membrane. The cell wall of fungi may play a role in binding isoflavones and blocking them from entering the cell cytoplasm. Bacteria also have cell walls and some have been shown to metabolize isoflavones, but bacterial cell walls have a different structure to that of fungi. Another possible reason for the lack of metabolism is the absence or lack of specificity of key metabolising enzymes in yeast, yeasts do however possess cytochrome P_{450}'s (Kappeli 1986).

4.2.2 Genistin yeast incubations

A genistin and genistein calibration graph is prepared as it is expected that the β-glucosidases on the surface of yeasts cells can hydrolyse the genistin releasing glucose and genistein (Kaplan, 1966). The gradient of the genistin calibration graph is 171519, with a R^2 of 0.999 showing that genistins absorbance increases in a linear manner as the concentration increases. All spiked samples gave recoveries close to 100 % showing, just like for the genistein incubation that storage in a freezer overnight is suitable and the methanol causes the yeast and any β-glucosidases to stop functioning.

Figure 3.12 shows genistin decreasing and genistein increasing. This represents a cleaving of the conjugated glucose group and release of the aglycone. This is expected as previously mentioned due to the β-glucosidases present. From this graph you can see that the initial rate, up to the 3 h mark, is faster than the rate from the 3 h mark until the 22 h mark. This is likely because total interconversion occurs much sooner than the 22 h mark but it is only at this point that we sample and realise that. A sample was taken the next day after 22 h of incubation this time, as it was previously possible that yeast did metabolise genistein but at a rate much slower than that which would have any significance in the bread making process or to be visible as a new peak or a decreased genistein peak after 3 h of incubation. Just like the previous genistein incubation, once it is formed here no further metabolism occurs and the concentration does not decrease at all even at the 22 h mark.

As the isoflavone glycosides do not fit into the ligand binding site of the estrogen receptor the biological effects of isoflavones are predominately from the aglycones interaction with the ERs (Morito et al. 2001). This suggests that increased aglycone production from yeasts hydrolysing activity during the proofing stage of bread making, produces a more functional bread in the terms of biological activity, through positive estrogenic effects. These effects have the largest impact in tissues expressing ERβ, due to isoflavone aglycones greater affinity for it relative to the ERα, with soy containing yeast risen bread being a good source of phytoestrogens for the alleviation of postmenopausal symptoms such as prevention of osteoporosis.

Because the isoflavones β-glycoside conjugates are more hydrophilic they can be in solution which can lead to faster absorption and earlier peak serum concentrations as stomach emptying occurs later after ingestion of solid foods compared with liquid food matrices (Cassidy et al. 2006). As bread is consumed

as a solid this is likely to not play any effect and the aglycone will be absorbed faster than β-glycosides. The mean time to attain peak plasma concentrations for the aglycones genistein and daidzein is 5.2 and 6.6 h respectively, whereas with the corresponding β-glycosides the time until peak plasma concentrations is delayed to 9.3 and 9.0 h respectively. This corresponds to the time needed for hydrolytic cleavage of the glycoside moiety for absorption to occur (Setchell et al. 2001). Additionally isoflavones are absorbed in greater amounts in their aglycone form than when present as β-glycosides (Izumi et al. 2000). This all gives evidence that ingestion of isoflavones already in the aglycone form, as potentially in the case of the bread, increases the biological effect.

4.3 Sour dough isoflavone incubations

A sour dough isoflavone incubation was done to model what happens to the isoflavones from soy during the proofing stage of the sour dough bread making process. It was hypothesised that like the genistin incubation in yeast the glucose would be released and the aglycone formed, as yeast are also present in this incubation. For the aglycone, genistein, it was thought that further metabolism would occur as the sour dough mixture also contains bacteria, and many bacteria have been shown to metabolise genistein.

A sourdough incubation was performed with genistein, daidzein and genistin much the same way as the yeast ones were done, with much the same results. The sour dough mixture failed to metabolise genistein or daidzein at any level, even after 22 h. The sour dough culture bubbled throughout the incubation from release carbon dioxide showing the metabolic activity of present organisms.

For the genistin incubation the glucose was cleaved and the aglycone produced, similar to the yeast incubation. In this incubation the rate of conversion was much faster, as shown in Figure 3.14, with complete aglycone conversion happening before the 3 h mark. There are three possible reasons for the faster conversion rate, the bacteria present also contain β-glucosidase activity, the yeast strain present contains more β-glucosidase activity or the yeast is present at higher concentrations.

4.4 Mass spectrometry analysis

The soy improver added to the daily white bread likely contains many of the 12 soy isoflavone forms. HPLC analysis of this (Figure 3.17) had genistein, genistin and daidzein present as well as additional large, distinct peaks with retention times that would be expected for other isoflavones. It was thought that by collecting these peaks and analysing by MS other isoflavone forms could be identified and relative concentration based on relative peak areas of peaks with the same retention time in bread samples could be determined.

As previously mentioned 12 isoflavone forms are possible, the aglycones genistein, daidzein and glycitin can all be present in 3 different glycosylated forms. All of these forms contain the aromatic ring and would likely absorb at the 260 nm range.

HPLC analysis under the same conditions using a MS detector rather than the UV one was not viable as the column was potentially to dirty for a much more sensitive method like HPLC-MS, and the flow rate would have to be reduced to a maximum of 1 mL/min compared to the current 1.5 mL/min. HPLC-MS was ruled out for these reasons and manual peak collection was performed after a HPLC-UV run. The volume in the tube between the detector and coming out as waste was estimated to be such that collection of peaks should be done virtually as soon as they appeared on the UV detector. Collection was done into HPLC vials suitable for use on the MS, and about 1 mL was collected for large peaks and 0.5 mL for the smaller ones. By running the sample in duplicate, collecting from the second sample knowing where peaks would appear using the first sample as reference it was possible to collect all 11 peaks in Figure 3.16 with enough time in-between that no two samples should contain any remnants from the previous peak.

Peak 8, which we know to be from daidzein had an ion with a m/z of 276. This is equivalent to the molecular mas of daidzein + that of the positively charged ion sodium, giving it the charge required to fly. However this is at such a small relative amount that it is barely recognizable compared to the many other ions appearing in this run. Only one peak was shown using the UV detector at 260 nm so it is possible that other material with a similar retention time but not absorbing UV light at 260 nm was present and not picked up by the UV detector but was picked up by the MS detector. This however is unlikely as all 11 peaks collected and ran on the MS contained basically the same ions. This suggests that it is the solvent, as it is likely the only thing that would be able to

be in all 11 samples. MS results of other collected peaks showed no ions with m/z values that would be expected for any of the other possible isoflavones, +/- a proton or a sodium ion of their molecular mass. This includes peak 10, the peak collected for what we know is the genistein peak. The MS results showed no ions at a molecular mass of genistein + proton or Genistein + Na^+. This shows us that either the peak collection was done at the wrong time, though it would be hard to miss almost all the samples completely, or that the interference by a large reading from other unwanted ions caused the reading for the wanted isoflavone compounds to be at such a small relative amount they were missed.

4.5 Heat Isoflavone degradation

The effect of heat on genistein was investigated to model what might occur during the baking stage of the bread baking process.

During baking the centre of the bread reaches 98 ^oC and is still moist. The wet heat genistein experiments were done to model what might happen to genistein at the centre of the loaf, in wet conditions. That is why the temperature of 98 ^oC was chosen and the length of 21 minutes corresponds to the time taken to bake the loaf. Genistein remained stable at these conditions so it would be assumed the genistein inside the loaf would not degrade from heat over the course of baking bread.

The outside of the loaf would likely reach 257 ^oC, or close to it, as that is the temperature of the oven the bread is baked in. The dry heat genistein experiments were carried out at 257 ^oC for this reason and also over 21 minutes, the time in the oven. These experiments were performed in dry conditions as they are simulating the bread crust, which contains much less moisture than the interior of the bread. For these experiments the genistein concentration decreased by approximately 90 %. This suggests that it is likely the genistein present in the crust of the bread would be degraded by heat over the baking process.

The compound contributing to the peak that appeared following the degradation of genistein could not be identified. It also could not be identified in chromatograms of baked bread, likely due to its relatively small peak area and the presence of multiple interfering peaks with the same retention time.

4.6 Isoflavone levels throughout bread making process

4.6.1 Daily white bread

From Figure 3.15 you can see that the genistein levels increase after proofing, this is potentially from genistin being hydrolysed to genistein by enzymes from the yeast, as shown to happen in our earlier yeast incubations. Genistein then decreases to nothing after the baking process.

The genistin levels increase by nearly 6 fold after proofing, from 0.5 mg/L to 3 mg/L. This is an unexpected result as the genistein also increases, from our earlier yeast experiments a predicted result would be that genistin decreased during the proofing stage as the glucose can be cleaved by the yeast. The genistin then decreases by approximately 300 % from the proofed dough to baked bread, resulting in levels approximately double that of what was present in the initial dough.

Daidzein concentrations increase after proofing and again after baking, resulting in a concentration in the baked bread of approximately double that of the initial dough. An increase after proofing can be expected in a similar manner to genistein, as the daidzin glucose can likely be cleaved and daidzein formed. The reason for daidzein increasing again after baking is unknown but could be due to the yeast enzymes remaining active and continuing to convert the glucosides to aglycones until the bread all reaches a certain temperature.

4.6.2 Soy and linseed bread

Genistin concentration did not change significantly after proofing, staying at approximately 3.8 mg/g. This is unexpected as from the results of genistin yeast incubations it would be expected to decrease dramatically as it is possible for the glucose to be cleaved and genistein formed. However in the daily white bread a significant increase of genistin occurred and again it is unusual for the soy and linseed dough to act in such a different manner as the daily white dough. After baking the genistin concentration nearly doubles from that in the proofed dough. This again is different from what occurred in the daily white bread, which decreased dramatically.

The daidzein concentration increased by a small amount after proofing and decreased by a small amount after baking in the soy and linseed bread. A

decrease after baking is the opposite of what occurred in the daily white bread.

Genistein concentrations increased after proofing and decreased to the initial dough concentration after baking. This is the same as what occurred in the daily white bread and is what the results from the model systems would expect to occur. An increase from hydrolysis of conjugates and a decrease from heat degradation. The fact that the genistin concentration does decrease does not necessarily mean that the increase in genistein concentration does not arise from hydrolysis of conjugates as it is possible that the malonyl or acetyl conjugates are being hydrolysed. Another explanation for the lack of change of genistin is that the malonyl or acetyl conjugates are being converted to genistin and genistin to genistein.

4.7 Conclusion

The use of the model systems would suggest that during the bread making process the aglycone is released in both yeast risen bread and sour dough, with this occurring at a faster rate in the sour dough breads. And that baking the bread would degrade genistein in the crust but not the centre of the loaf.

The lack of production of phytoestrogenic or bioactive compounds from isoflavone aglycones by the metabolic activity of yeast or sour dough cultures is an important finding. It is one less factor that needs to be taken into consideration, with the use of the model systems helping to determine a clearer picture of the health effects of a “simple” everyday food item, bread.

Analysis at the different stages of bread making did not necessarily sync up with what was expected to occur from knowledge obtained from the model system experiments. The reasons for this may be that the model systems were not an accurate representation of the bread making process or that analysis of the three forms of soy isoflavones used was not suitable enough to obtain a clear view of what is occurring.

4.8 Future work

Tracking genistein, daidzein and genistin throughout the sour dough bread making process would be of value as it could likely produce results differing from the yeast risen bread.

The development of a method for the identification and quantification of all possible soy isoflavone forms would be able to provide a more complete picture on what was happening throughout the bread making process. This would make it viable to do yeast, sour dough or heat experiments on soy flour, giving a complete picture of what might happen to all the soy isoflavones in the bread making process.

Using an alternate solvent system or transferring to a mass spectrometry detection system would likely allow determination of the new peak arising after heat degradation of genistein following the dry heat treatment.

Ideally knowing all possible interconversions, metabolism or breakdown products that occurs in all soy or phytoestrogenic containing fermented or processed foods would allow for more precise quantification of risk vs benefit.

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