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Bioavailability and Tissue Distribution of Dietary Phytoestrogens in Rats

Estatira Sepehr, Ph.D.

This thesis is submitted as a partial fulfillment of the Ph.D. program in Cellular Molecular Medicine.

University of Ottawa
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ABSTRACT

The fate of isoflavones in rats after oral and intravenous injection (IV) of soy was investigated.

A liquid chromatography-mass spectrometry method to determine isoflavones in rat plasma was developed with a short run time of 16 minutes.

Serum samples from a multigeneration reproductive study conducted to examine the effects of diets containing alcohol-washed soy protein supplemented with increasing amounts of Novasoy^{TM}, in male and female Sprague Dawley (SD) rats, were analyzed for isoflavones. Gender and age differences were observed and warranted additional investigations into the effects of gender, source of isoflavones and the role of glycosidation of isoflavones on the biovailability and pharmacokinetics of soy isoflavones.

The bioavailability of isoflavones from Novasoy^{TM}, a mixture of synthetic glucosides, and a mixture of synthetic aglyconees was examined in female and male (3-month-old) SD rats, and male Fisher 344 (20-month-old) rats. For the synthetic aglycone and synthetic glucoside sources, bioavailability was independent of gender. However, for Novasoy^{TM}, a gender difference was observed for daidzein, with higher plasma concentrations found in male rats. Bioavailability values for daidzein, genistein and glycitein were significantly ($p <$ 0.05) higher (up to sevenfold) from Novasoy^{TM} and the synthetic glucosides compared with the synthetic aglyconees. Therefore, the source of isoflavones had significant effects on isoflavone bioavailability.

Similar studies using aged (20-month-old) male rats, revealed significantly ($p < 0.05$) higher bioavailability values for glycitein from Novasoy^{TM} (27 ± 13%) and the synthetic glucosides (21 ± 10%) compared with the aglycone mixture (8 ± 3%). Significantly ($p < 0.05$) higher values for bioavailability of daidzein were observed in aged rats dosed with Novasoy^{TM} and the glucoside form compared with the aglycone mixture. Thus, the source of isoflavones had significant effects on daidzein and glycitein bioavailability in aged male rats.

These findings will be of importance in risk-benefit analyses of soy isoflavones by regulatory agencies.

DEDICATION

To my loving Family,

Bijan, Jasmine and Hameed Reza

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Ph.D. studies and I relied on this support many a time for continued encouragement. My sincerest thanks!

ABREVIATIONS:

ACN	acetonitrile
ANOVA	analysis of variance
AUC	area under the curve
Cl	plasma clearance
C_{max}	maximum concentration
DAD	diode-array detection (or diode-array detector)
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
Equol	7-hydroxy-3-(4'-hydroxyphenyl)-chroman
ESI	electrospray ionization
FA	formic acid
GC	gas chromatography
HPLC	high-performance liquid chromatography
IV	intravenous injection
PK	pharmacokinetics
LPE	liquid phase extraction
LOD	limit of detection
LOQ	limit of quantification
MS	mass spectrometry
MUF	methylumbelliferone
O-DMA	*O*-desmethylangolensin
PDA	photodiode array detector

RCF	relative centrifugal force
RP	reversed phase
RSD	relative standard deviation
SD	standard deviation
SIM	selected ion monitoring
S/N	signal-to-noise ratio

CHAPTER 1

Literature review

1.0 Phytoestrogens

Phytoestrogens are polyphenolic compounds that are constituents of many fruits, vegetables, legumes, and plant leaves (Bandele and Osheroff, 2007), have natural roles in plant pathogen defense and root nodulation (Rafii *et al.*, 2007).

Phytoestrogens comprise three major classes: isoflavones, lignans and coumestans (Setchell, 1998; Setchell and Cassidy, 1999; Thompson *et al.*, 2006; Humfrey, 1998). Isoflavones are not widely distributed in plants, occurring almost exclusively in soybeans and other leguminous plants (Coward *et al.*, 1993). Soybeans, soy protein products and soy based infant formula are the most significant sources of isoflavones in the human diet (Michihiro 2006). The precise content of isoflavones in soy foods varies with the type of soy food, soybean variety, geographical and environmental conditions, and soy food processing procedures (Wang and Murphy, 1994). Soybeans are composed of the cotyledons, the part of the seed that forms the first plant leaves; the hypocotyls, the part of the axis of the plant embryo below the cotyledon and the hull, the dry outer covering of the seed (Price and Fenwick, 1985). The cotyledon, which constitutes more than 90% of the soybean weight, accounts for 88% of the total isoflavones (Gugger, 2002). Although the hypocotyl (germ) represents only 2% of the soybean weight, it is five to six fold more concentrated in isoflavones than the cotyledon and it is enriched with glycitein and daidzein glucosides compared with genistein glucosides (Gugger, 2002).

1.1 Structure of Soy isoflavones

The structure of isoflavone compounds includes the flavone nucleus of two benzene rings (A and B) linked via a heterocyclic pyrane C ring (Fig. 1; Larkin *et al.,* 2008; Messina, 1999; Setchell, 1998).

The major isoflavones present in soy are predominantly in their glycoside forms (daidzin, genistin, and glycitin), with daidzin and genistin being the most abundant), which are conjugated to a malonyl or acetyl molecule to form 6'-*O*-malonylglucosides, 6'-*O*-acetylglucosides or ß-glucosides, respectively (Fig.1; Larkin *et al.,* 2008; Messina, 1999; Setchell, 1998). Before 1990, the composition of isoflavones in soyfoods was thought to be largely determined by whether the foods were fermented (Eldridge and Kwolek, 1983; Coward *et al.*, 1998). In fermented soy products such as miso or tempeh, the unconjugated aglyconees, daidzein (4', 7-dihydroxyisoflavone), genistein (4', 5, 7- trihydroxy-isoflavone) and glycitein (4', 7-dihydroxy-6-methoxyisoflavone), are the predominant forms (Fig. 2), because the fermentation process causes cleavage of the glucoside bond (Larkin *et al.,* 2008). However, non fermented soy foods [soymilk, tofu, soy flour, soy protein concentrate, and isolated soy protein (ISP)] contain their ß- D-glucoside conjugates (daidzin, genistin, and glycitin) with the sugar molecule attached at the 7 position of the A ring (Fig. 1; Larkin *et al.,* 2008; Messina, 1999; Setchell, 1998). Formononetin and biochanin A, the 4'-*O*-methoxylated isoflavone derivatives and precursors to daidzein and genistein, respectively (Fig. 2), occur in alfalfa and clover seeds and sprouts, and in chick peas (Wang and Murphy, 1994).

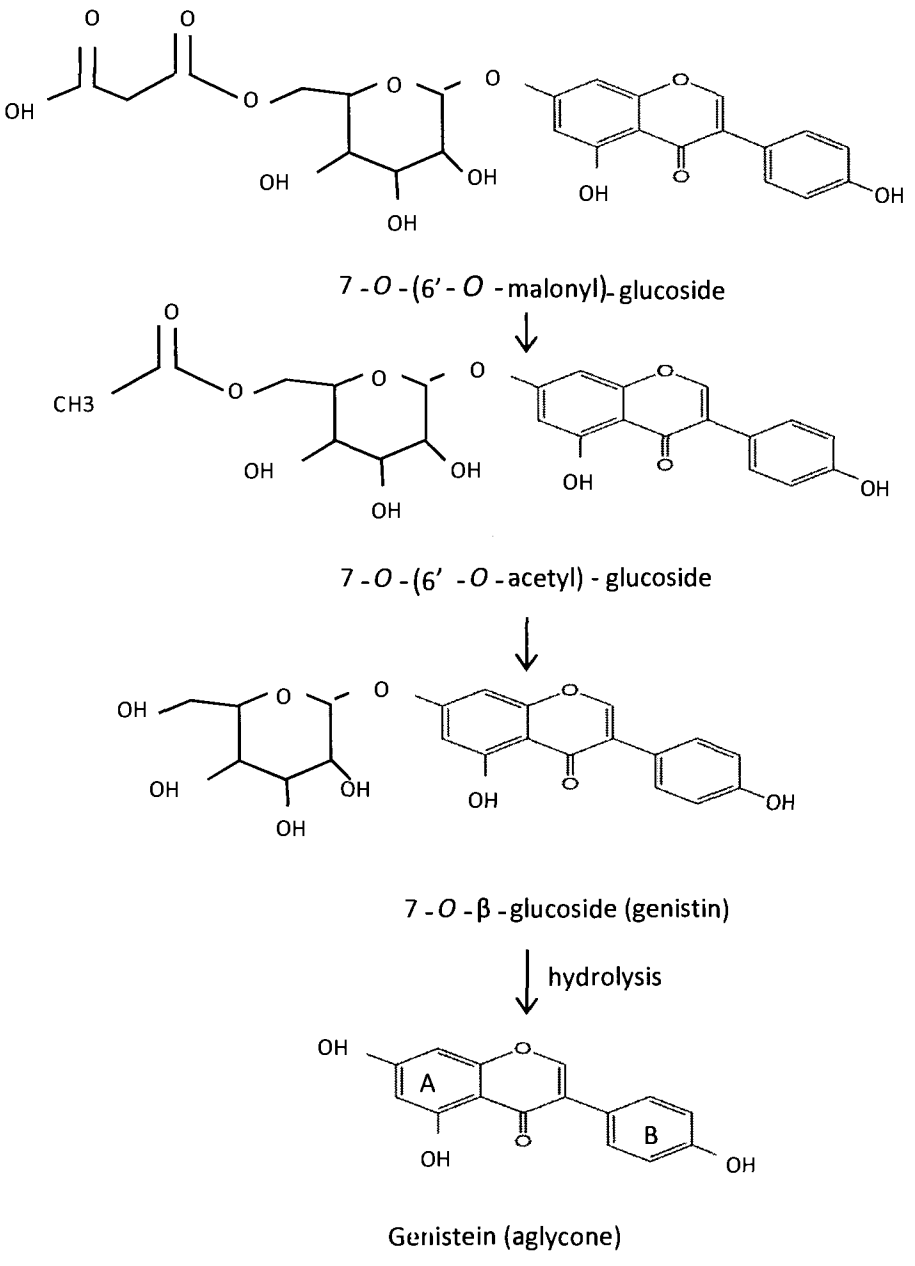


Figure 1: Glycosidic forms of isoflavone genistein in soy foods.

Analogous structures of daidzein and glycitein are also present in soybeans (Adapted from Larkin *et al.,* 2008; Messina, 1999; Setchell, 1998).

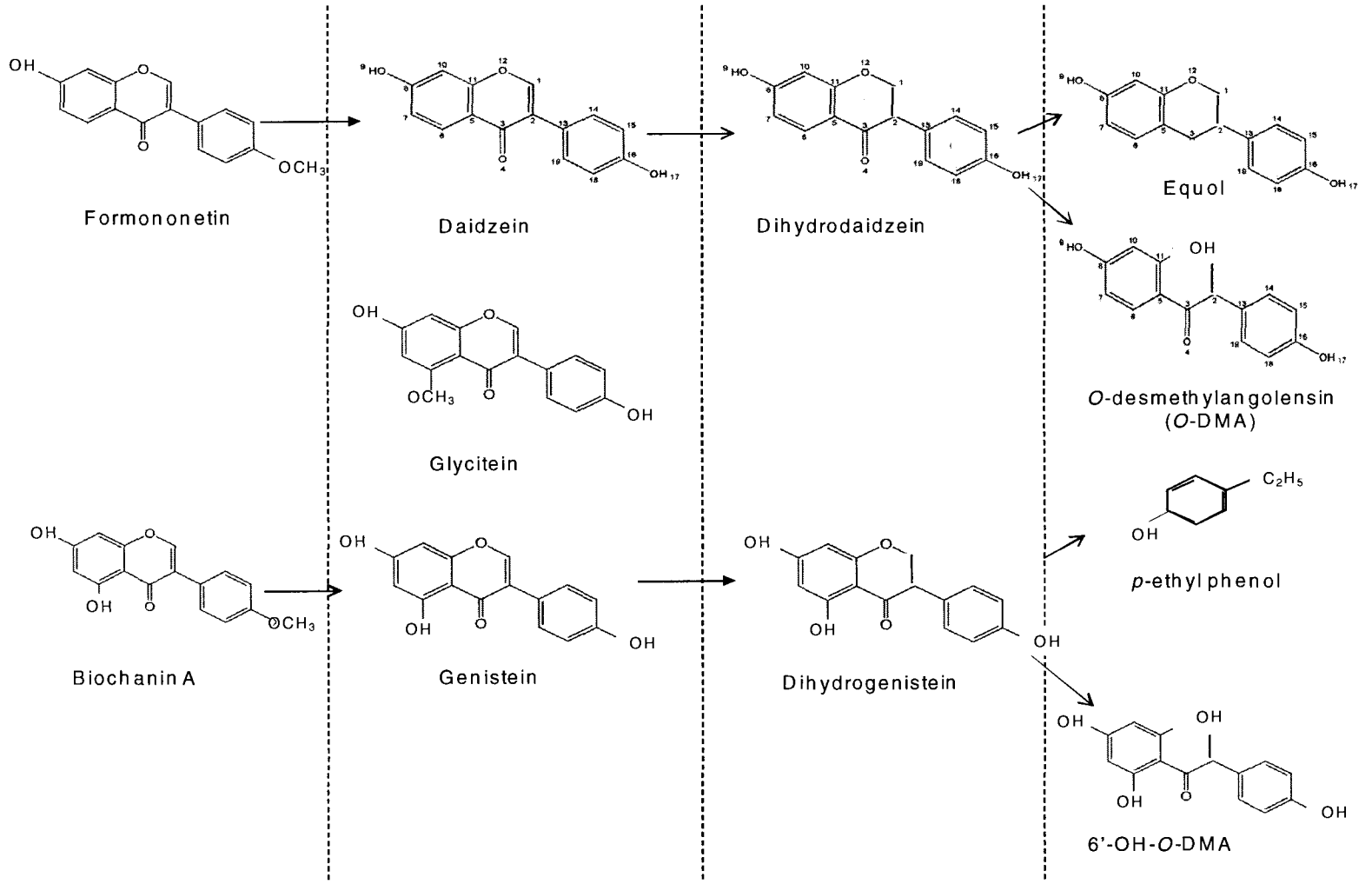


Figure 2: Structures of the major isoflavones and their metabolites.
Adapted from Larkin *et al.*, 2008.

1.2 Isoflavone synthesis.

Isoflavones are synthesized by a branch of the phenyl-propanoid pathway of secondary metabolism (Jung *et al.*, 2000; Jackson and Rupasinghe, 2002). Other branches of the phenyl-propanoid pathway produce lignin, and anthocyanin pigments (Fig. 3; Jung *et al.*, 2000; Jackson and Rupasinghe, 2002).

1.3 Soy Isoflavones; Dietary Intake

Soy foods have been a dietary component in some Asian countries for centuries (Adlercreutz *et al.*, 1995). Epidemiological evidence has demonstrated a low incidence of cardiovascular disease (Adlercreutz, 1990), hormone dependent cancers of the breast, prostate and colon (Yu *et al.*, 1991), menopausal symptoms and osteoporosis (Adlercreutz *et al.,* 1992; Clarkson, 2000) in Asian populations compared with countries where soy is not typically consumed (Chen and Bakhiet 2006, Setchell, 2006). However, migration to Western countries and adaptation to a Western diet increases the incidence of breast, prostate and colon cancers among migrant Asians to an occurrence similar to Western populations (Whittemore *et al.,* 1995). Thus diet, and in particular soy isoflavones may have a role in some protection against the development of these hormone dependent cancers.

Diets low in fat and high in fibre and complex carbohydrates from vegetables, fruits, grains and legumes have long been associated with a reduced risk of many cancers (Messina, 2002). In addition to such diets, it is well accepted that Asian populations consume more soy isoflavones than Western

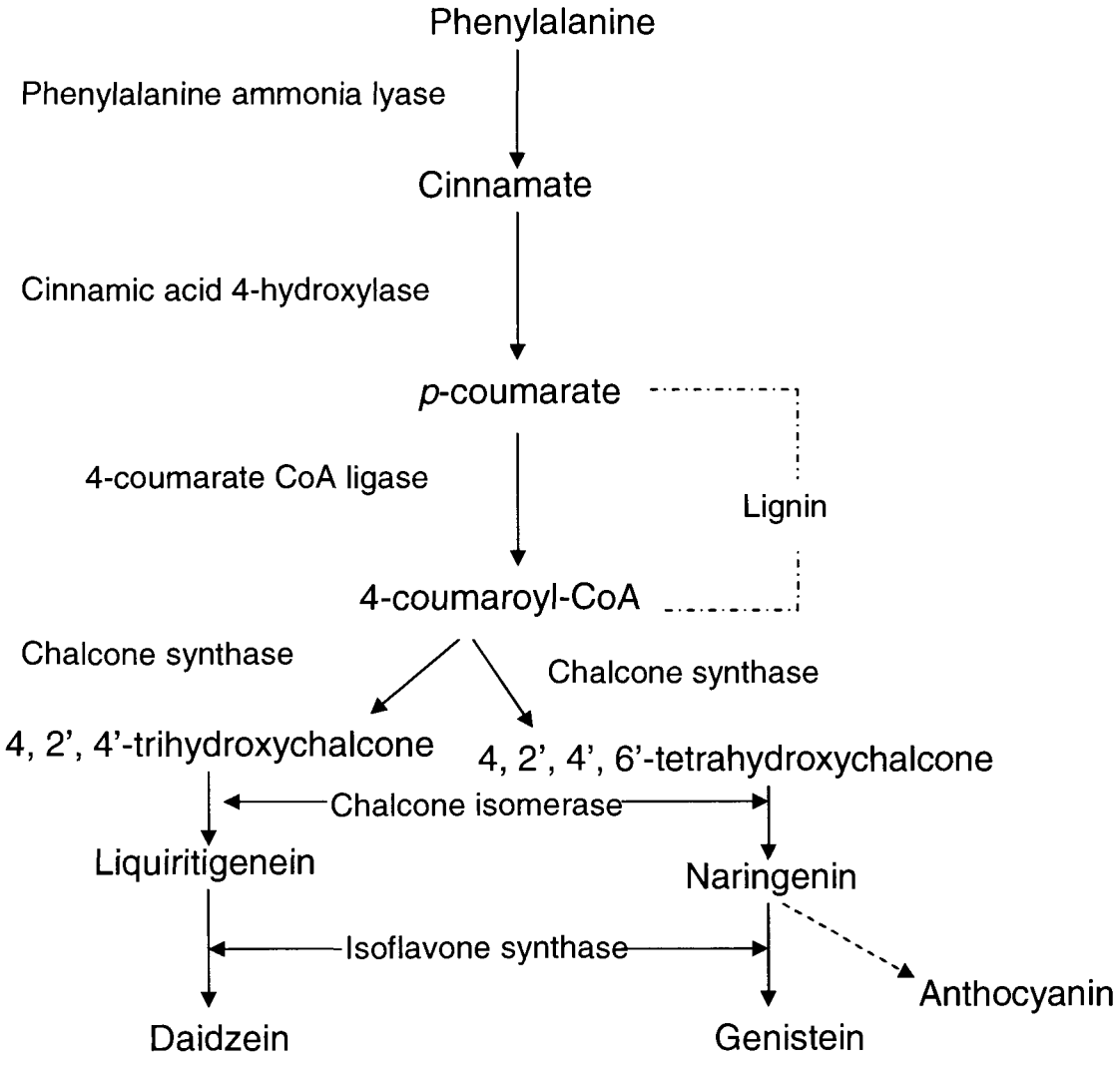


Figure 3: A diagram of the phenylpropanoid pathway showing intermediates and enzymes involved in isoflavone synthesis.

Branches for lignin and anthrocyanin synthesis are marked dotted lines represents multiple enzymatic steps (Adapted from Jung *et al.*, 2000; Jackson and Rupasinghe, 2002).

populations (Messina, 2002). The potential use of soybean protein products in the USA is expected to increase significantly following a FDA (US Food and Drug Administration, 1999) decision which will allow food manufacturers to make health claims for soy protein products; and the USDA's (United States Department of Agriculture) rule change allowing suitable alternate protein products (such as soybean products) to replace 100% of meat products in the National School Lunch Program, the Summer Food Service Program, and the Child and Adult Care Food Program. Prior to this USDA's rule change alternate protein products could replace no more than 30% of meat products. The consumption of soy products is estimated to be highest in Japanese populations, with levels of up to 200 mg/day isoflavones in the diet. Throughout Asia, the consumption of legumes is estimated to supply 25-45 mg total isoflavones in the diet each day, compared with Western countries with a consumption of less than 5 mg/day (Vanden Berghe *et al.*, 2006).

While intake of soy isoflavones varies among individuals (Wakai *et al.*, 1999), the absorption, metabolism, serum and urine levels of isoflavones are also known to vary among individuals (Rowland *et al.*, 2003; Adlercreutz *et al.*, 1993). The latter may be attributable to variations in intestinal microflora (Adlercreutz *et al.*, 1993) since, as will be discussed in the following sections, intestinal microflora is of crucial importance to the absorption and metabolism of isoflavones (Rowland *et al.*, 2003; Setchell *et al.*, 2002).

1.4 Isoflavone absorption

The natural isoflavones in soybeans and unfermented soyfoods occur as glucose conjugated forms (Kano *et al.,* 2006; Wang and Murphy, 1994). Once ingested, isoflavone glucosides are poorly absorbed in the small intestine because of their hydrophilicity and large molecular weight (Xu *et al.,* 1995). The conjugated isoflavones are hydrolyzed in the gut by both intestinal mucosal and bacterial ß-glucosidases releasing the aglyconees (Cassidy *et al.*, 2006). The significance of ß-glucosidases in the absorption of isoflavones was demonstrated by Setchell *et al.* (2002) where the ingested glucoside form of isoflavones did not appear in the blood circulation, indicating that isoflavones in their glucoside form were not being absorbed intact across the intestinal epithelium (Setchell *et al.*, 2002). This indicated that ingested glucoside isoflavones had to undergo hydrolysis of their malonyl, acetyl or glucoside moieties in order to enter the circulation (Setchell *et al.*, 2002). The differential ability of the aglycone, but not the glucoside form, to be absorbed, is related to the phenolic nature of the isoflavone structure becoming exposed after removal of the glucoside moiety. This is relevant because the phenolic structure of isoflavones allows for passive diffusion through the lipophilic intestinal wall (Setchell *et al.*, 2002). A review of the literature by Rowland *et al.* (2003) proposed that, although the main site for microfloral activity was the distal ileum and the colon, the rapid absorption of isoflavones after ingestion, suggests that hydrolysis occurs in the upper gastrointestinal tract.

Day *et al.,* 1998 reported the involvement of mammalian β-glucosidase in hydrolyzing isoflavone glucosidic form and the role of this enzyme in early step of isoflavone glucosidic uptake. Glycosidase activity can occur in the cells of the gastrointestinal mucosa or the enzyme can be secreted by the colon microflora (Prasain *et al.,* 2002). A number of mammalian β-glucosidases have been identified in the small intestine, including cytosolic β-glucosidase and the membrane-bound lactase phlorizin hydrolase (LPH) enzyme (Day *et al.,* 1998). LPH is present on the luminal side of the brush border in the small intestine and can deglycosylate genistin and daidzin within the gut lumen to release genistein and daidzein which can then diffuse into the epithelial cells (Day *et al.,* 2000). Therefore, the intestinal mucosa plays an important role in the deglycosylation of isoflavones and confirms that isoflavone absorption begins in the proximal small intestine and occurs along its length (Day *et al.,* 1998; Xu *et al.,* 1995; Setchell *et al.,* 2003). The cytosolic β-glucosidase enzyme has been identified in the small intestine, liver and kidney of mammals with the small intestine having a faster rate of hydrolysis compared with the liver (Day *et al.,* 1998; Day *et al.,* 2000). Studies with rats and humans have also provided evidence that the isoflavone aglyconees are absorbed more rapidly compared with glucosides. Piskula *et al.,* 1999, investigated the absorption of daidzein, genistein and their corresponding glucosidic forms in anesthetized rats over a period of 30 minutes after their oral administration. Genistein and daidzein (measured after enzymatic hydrolysis of glucuronides, sulfates, and glucosidase) were detectable in tail vein blood 3 minutes after the administration of aglyconees but after 10 minutes post

administration of the glucosidic forms. Consistent with this study, peak plasma genistein concentration occurred earlier and was substantially higher after an oral dose of genistein compared with a soy glucoside extract in rats (King *et al.,* 1996). In humans, peak concentrations of daidzein and genistein were reached earlier after consumption of aglyconees compared with glucosides (Setchell *et al.*, 2001; Izumi *et al.,* 2000). These researchers concluded that isoflavone aglyconees are more efficiently transported across the wall of the gastrointestinal tract than their glucoside form.

Additionally, another study by Qiu *et al.,* 2005 reported that daidzein aglycone has been detected in plasma as soon as 0.4h after oral administration of a daidzein solution and after 5h post oral administration of daidzein suspension in rats. An early increase in plasma concentration of daidzein aglycone form suggests that dosage forms have a great influence on the absorption of daidzein (Qiu *et al.,* 2005).

1.5 Isoflavone metabolism

After initial absorption through the intestinal epithelium, the isoflavones enter the submucosal capillaries that take them to the intestinal vein. The intestinal vein transports the isoflavones through the hepatoportal vein system to the liver (Fig. 4; Boroujerdi, 2001). In the liver, the metabolism of isoflavones occurs in two phases. In the first phase, cytochrome P450 (CYPs) enzymes oxidize isoflavones, and make them more susceptible to conjugation reactions (Boroujerdi, 2001; Vesell, 2000). Groups such as hydroxyl (OH), carboxyl (COOH) are introduced into the isoflavone molecule so that the metabolites can

undergo the second phase of their metabolism (Boroujerdi, 2001). During Phase II metabolism, the OH groups of the isoflavones provide sites for glucuronidation and sulfation by UDP-glucuronosyl-transferases and sulfotransferases in the liver and intestinal wall (Setchell *et al.,* 2001; Shelnutt *et al.*, 2002). There are two conjugation sites on genistein and daidzein, and each of these sites can be sulfated or glucuronidated (Fig. 5; Shelnutt *et al.*, 2002). The glucuronide and sulphate conjugates can be transported via the systemic circulation to tissues, from where they will be excreted by the kidney, or they can be secreted in bile and returned to the intestine (Shelnutt *et al.*, 2002; Xu *et al.*, 1995). After deconjugation by intestinal bacteria, isoflavone aglyconees can be reabsorbed then returned to the liver for reconjugation and either further enterohepatic circulation or renal excretion (King, 2002). The portion of the isoflavone aglyconees that was not absorbed via the small intestine can be further metabolised by intestinal microflora in the large intestine into other metabolites such as 7–hydroxyl–3-(4'-hydroxyphenyl)–chroman (equol) or O-desmethylangolensin (O-DMA) from daidzein, p-ethyl phenol (from genistein) and di-hydroglycitein (from glycitein) (Fig. 2, Cassidy *et al.*, 2006, Jackson and Rupasinghe, 2002; Setchell *et al.*, 1984; Setchell *et al.*, 2005).

The metabolism of isoflavones has been investigated in a wide range of animals including sheep (Lindsay and Kelly, 1970), cows (Lundh *et al.*, 1988), horses (Marrian and Haslewood, 1932), monkeys (Monfort *et al.*, 1984) and rodents (King *et al.*, 1996; Coldham *et al.*, 1999; Coldham and Sauer 2000). In rats, the portal vein contains predominantly glucuronide isoflavones suggesting

the primary site of glucuronidation is the intestinal wall (Barnes *et al.,* 1996; Coldham and Sauer, 2000).

In the human circulation, glucuronides are the predominant forms of isoflavones followed by the sulphated conjugates (Shelnutt *et al.*, 2003; Zhang *et al.,* 2003). Other conjugates also exist, including sulphoglucuronides, while aglyconees only represents a small portion of total isoflavones and there is a high percentage of binding of isoflavones to plasma proteins (Coldham and Sauer, 2000; Shelnutt *et al.*, 2003; Zhang *et al.,* 2003; Rowland *et al.*, 2003). Glucuronides of genistein and daidzein made up 48% and 33% of the total isoflavone concentration in plasma of men and women after consumption of a soy protein isolate drink, while the percentages of sulfates of genistein and daidzein in plasma were 8% and 26% respectively. Additionally, approximately 30% of the total genistein and daidzein was comprised of mixed conjugates (Shelnutt *et al.,* 2003).

1.6 Equol

Equol (7–hydroxyl–3-(4'-hydroxyphenyl)–chroman) is a key metabolite of daidzein (Arora *et al.,* 1998). The discovery of very high urinary concentrations of equol and the observation that isoflavones in urine and blood increased to levels far greater than endogenous estrogen levels after soy consumption, accelerated the research in this field (Setchell *et al.*, 2001, Setchell *et al,* 1984). Equol is exclusively produced by intestinal bacteria, but only about 35% of subjects excrete substantial amounts of equol following dietary soy consumption

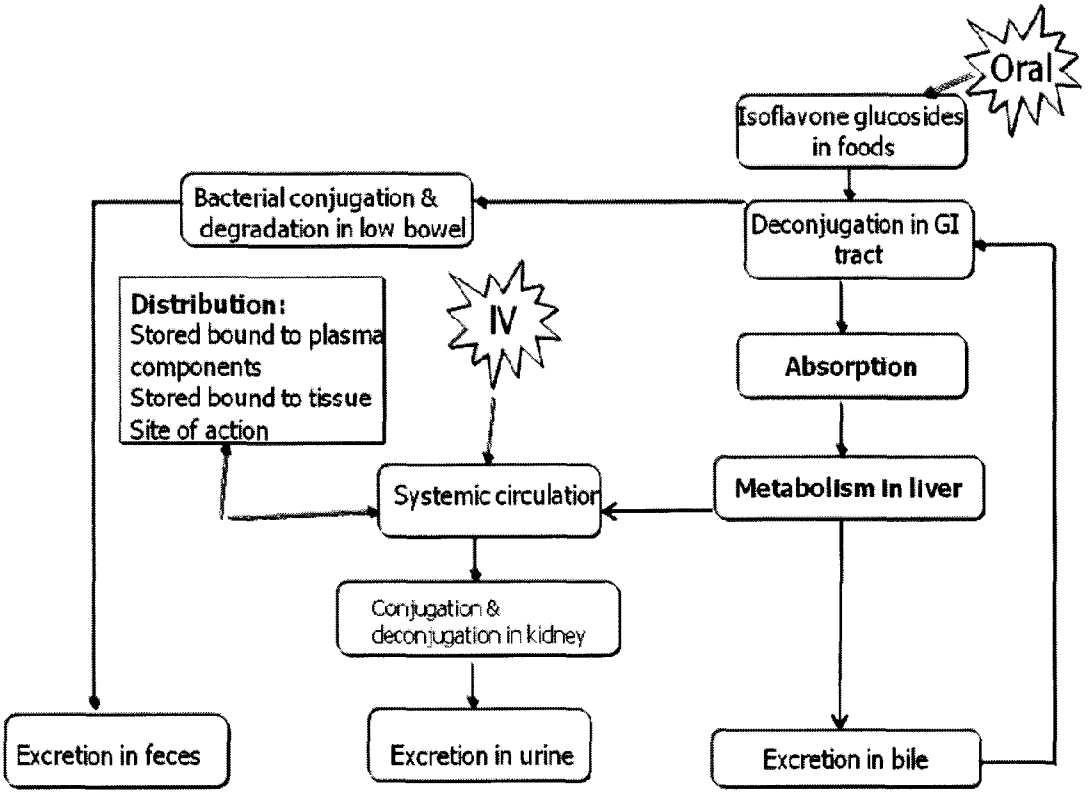


Figure 4: Metabolism of isoflavones in humans.

Adapted from Brody (2005).

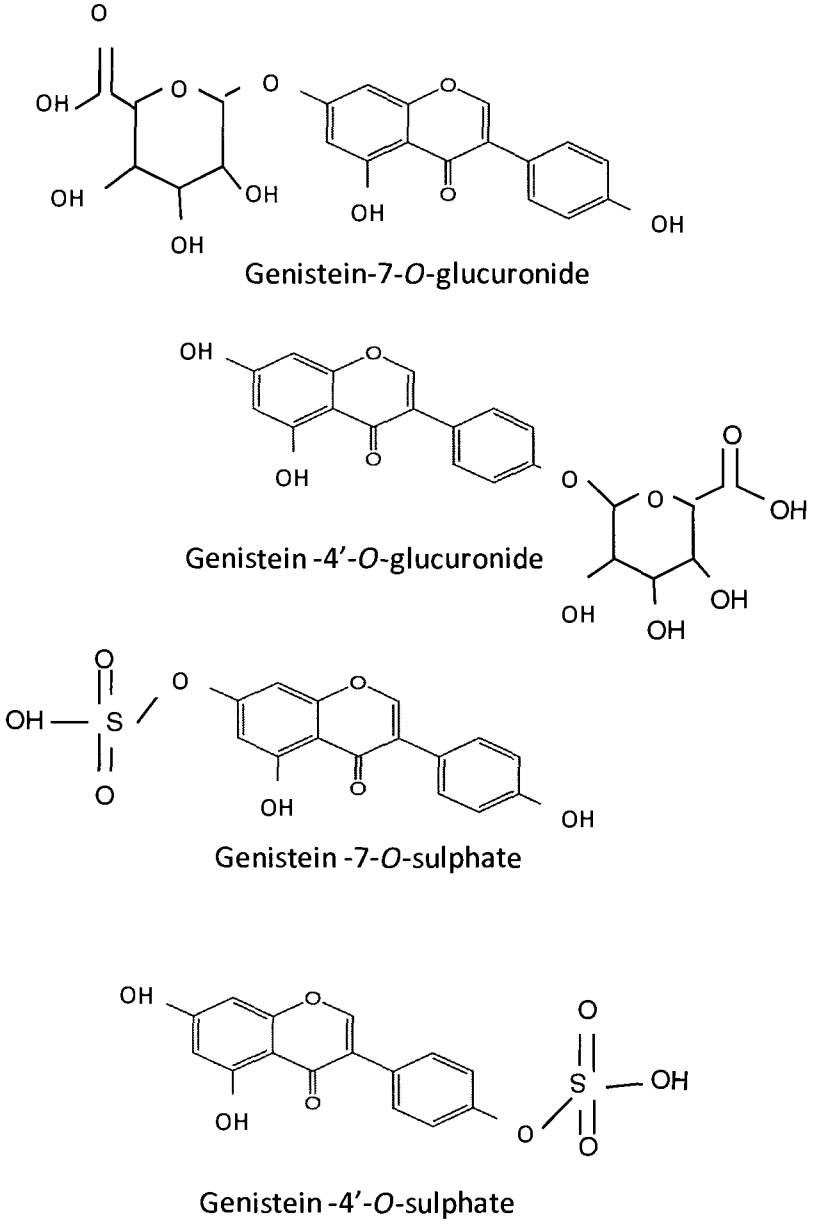


Figure 5: Glucuronide and sulphate conjugates of genistein.

Analogous structures of daidzein and glycitein are also present in the circulation. Adapted from Larkin *et al,* (2008).

(Xu *et al.*, 1998; Bowey *et al.,* 2003; Rowland *et al.*, 1999; Cassidy *et al.*, 1994; Cassidy *et al.*, 1995; Lampe *et al.*, 1998). The ability or inability of persons to produce equol seems to remain the same for at least several years (Setchell *et al*, 2002; Karr *et al.*, 1997).

Antibiotic treatment of human subjects decreases the excretion of equol, providing evidence supporting the role of bacterial flora in production of equol (Adlercreutz *et al.*, 1986). The composition of the intestinal flora and the presence or absence, of appropriate enzymes in the intestinal microflora plays a major role in equol production (de-Pascual-Teresa *et al.*, 2006; Manach *et al.*, 2004). Inoculation of germ free rats with human flora from equol producers confers on these rats, the capacity to produce equol, whereas colonization with flora from non-equol producers leaves the rats incapable of producing equol (Bowey *et al.*, 2003). The production of metabolites is very limited in infants (Setchell, 1998) due to lack of a fully developed intestinal microflora and lack of metabolic activity of enzymes in the intestine. The absence of equol in the urine of 4-month-old infants fed soy formula further highlights the essential role of intestinal bacteria in the formation of isoflavone metabolites (Setchell, 1998; Cruz *et al.*, 1994). The mouse, rat, and monkey, metabolize soy isoflavones differently from human adults, for example only about 25% of women possess the gut microorganisms to produce equol, whereas the gut flora of rats produce very large quantities of equol (Gu *et al.,* 2006; Setchell *et al.*, 2005, Setchell *et al.*, 2002). Because the metabolism of daidzein by colonic bacteria plays a key role in the production of equol, the differences in the metabolism of daizein by colonic

bacteria of experimental animals should be considered when results from experimental animals are extrapolated to humans (Raffi *et al.,* 2007). Gu *et al.,* 2006 suggested that female pigs had an overall metabolic profile closer to women than to either the female monkeys or rats, and pigs may be a better animal model for studying the health effects of isoflavones in non equol producers.

The appearance of equol in human plasma, after ingestion of daidzein, is time-dependent, and usually takes 6-8 hours after intake of bolus dose to appear which is consistent with its colonic origin (Setchell *et al.*, 2001; Setchell *et al.,* 2003).

Equol has the ability to be a hydrogen/electron donor via hydroxyl groups, and can scavenge free radicals (Arora *et al.*, 1998). In comparison with daidzein, equol has significantly enhanced antioxidant activity (Arora *et al.*, 1998). The chemical structure of equol is also unique in that it has a chiral centre at C-3 and therefore, can occur as two distinct enantiomeric forms; S-equol and R-equol (Setchell *et al.*, 2005). The binding of R- and S- equol to both α- and ß-estrogen receptors has been investigated (Setchell *et al.*, 2005). S-equol has high binding affinity for β-estrogen receptor, while R-equol binds less strongly to estrogen receptor α (Muthyala *et al.*, 2004). When the structure of equol (metabolite of daidzein) and structure of estradiol are overlaid, they can be virtually superimposed and the distance between the hydroxyl groups at each end of both molecules is nearly identical (Fig. 6, Setchell, 1998). However, the orientation of

hydroxyl group in equol is reversed, with the B-ring hydroxyl interacting with the 3-hydroxyl group of the aromatic A ring of estradiol.

It is well established, that the biological activities of isoflavones are dependent upon their respective chemical structures. The anti cancer activity of 4-hydroxy-equol has been studied and found to inhibit the growth of prostate cells in culture much more effectively than the parent compound daidzein (Sandler *et al.*, 2003). The question of whether one can enhance intestinal conversion of daidzein to equol, is one of great interest (Setchell *et al.*, 2002) as equol is approximately 100-fold more estrogenic than daidzein in binding to estogen receptors (Sathyamoorthy and Wang, 1997).

Human equol producers tend to consume less fat and more carbohydrates, as percentage of energy, than do non-equol producers (Lampe *et al.*, 1998; Rowland *et al.*, 2000). Lu *et al* (1996) reported an increase in equol production in urine after one month of isoflavone consumption in healthy women. A more comprehensive knowledge of the factors that may influence equol production is required as equol producers might gain more benefits from soy consumption than would non-equol producers (Setchell *et al.*, 2002). Earlier studies using an *in vitro* model of human colonic fermentation, found that in a high carbohydrate environment, colonic fermentation was stimulated and this increased the rate of conversion of daidzein to equol (Setchell, 1998; Setchell, 1999; Lampe *et al.*, 1998). In mice, equol production increased with the addition of fructo-oligosaccharides in the diet (Ohta *et al.*, 2002).

1.7 Isoflavone distribution

Following their absorption and metabolism, the ability of isoflavones to act biologically in target tissues, is dependent upon their distribution within the body. Ingested isoflavones have been quantified in plasma, bile, urine, feces and prostatic fluid as well as breast tissue and breast milk (Morton *et al.,* 1997; Maubach *et al.,* 2003; Franke *et al.*, 1998).

In Japanese men, plasma/serum levels of isoflavones range from 276-492 nM for genistein, 107-282 nM for daidzein and 5.5-99 nM for equol (Morton *et al.*, 2002; Adlercreutz *et al.*, 1993), while plasma daidzein in European men (United Kingdom) were at 35 nM (Morton *et al.*, 1997). The distribution of isoflavones in the body depends upon factors such as blood flow, plasma protein binding, and body composition, each of which may be altered with age (Dipiro *et al.*, 2002). The volume of distribution of water soluble therapeutic products will be decreased by age, whereas lipophilic therapeutic products will exhibit an increased volume of distribution with age (Dipiro *et al.*, 2002).

1.8 Isoflavone elimination

In clinical terms, isoflavone clearance is defined as the milliliters of blood cleared of the isoflavones per minute (Schoenwald, 2002). The kidney plays an important role in elimination of therapeutic products and their metabolites (Gibaldi, 1990). Within the liver, isoflavone elimination occurs either via biotransformation of parent chemical to one or more metabolites, or excretion of unchanged isoflavones into bile or via both mechanisms (Katzung, 2004).

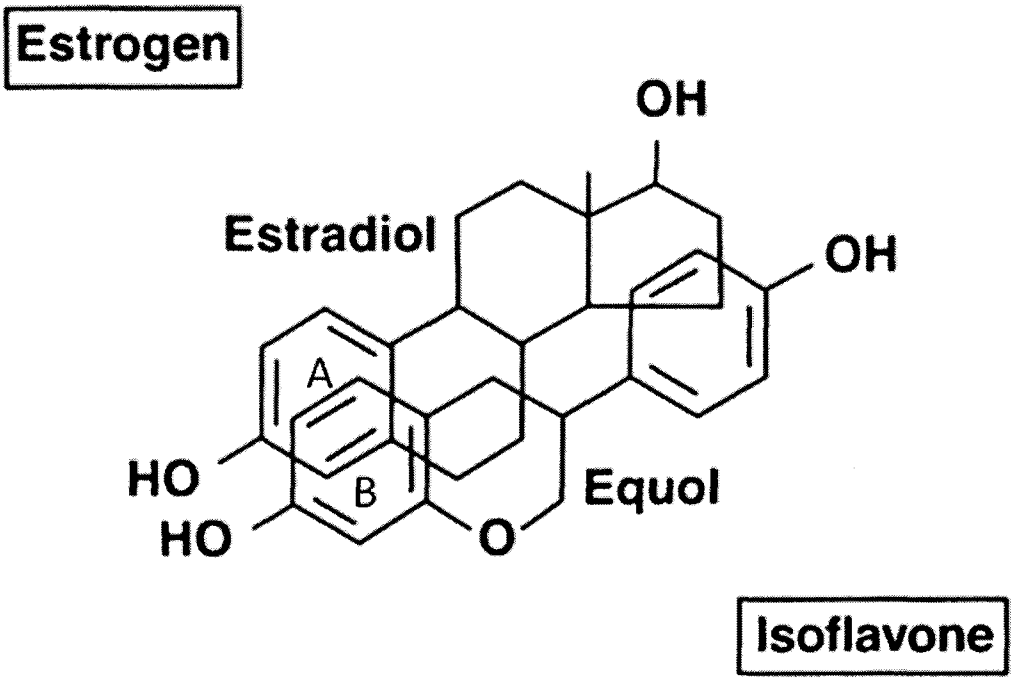


Figure 6: Structural similarity between estradiol and the isoflavone metabolite equol.

Adapted from Setchell, (1998)

1.9 Isoflavone bioavailability

The bioavailability of an ingested compound is defined as the amount that is absorbed and reaches the systemic circulation from where it can be distributed to tissues for their physiological effects (Rowland *et al.*, 2003; Wiseman, 1999).

Most of the previously published studies on the plasma pharmacokinetics of soy isoflavones (mainly daidzein and genistein), reported bioavailability by calculating the area-under-the concentration time curves (AUC) following oral administration of isoflavones in human or animal models (Cassidy *et al.*, 2006; Kano *et al.*, 2006; Izumi *et al.,* 2000; Setchell *et al.*, 2001; Zubik and Meydani 2003; Xu *et al.*, 2000).

Several reports have measured only the concentration of isoflavones excreted in urine (Xu *et al.*, 2000; Xu *et al.*, 1994; Tew *et al.*, 1996; Setchell *et al.*, 2003). This method can only be used if urinary excretion of unchanged chemical is the main mechanism of elimination of the chemical and urine samples have been collected in as short as possible intervals to allow for measuring the rate and amount of excretion (Schoenwald, 2002). The limitation of this method is the high degree of variability associated with the cumultative amount of chemical excreted in the urine, and the method is less reliable compared with the estimation of bioavailability from plasma concentration time profiles (Dipiro *et al.*, 2002; Schoenwald, 2002).

Isoflavones such as genistein are also considered to be pharmaceutical agents. Bioavailability in pharmacology and toxicology refers to the ratio of AUC after oral ingestion to the AUC after IV injection (NTP-CERHR-Soy Formula,

2006). Therefore, the bioavailability of isoflavones should be determined by comparing the AUC of the plasma concentration time curve after intravenous (IV) administration with the AUC after oral administration (NTP-CERHR-Soy Formula, 2006; Janning *et al.*, 2000; Coldham *et al.*, 2002).

$$\% \text{ Bioavailability} = [AUC_{oral} \times dose_{IV}] / [AUC_{IV} \times dose_{oral}] \times 100$$

The assumption which underlies this formula is that the isoflavone distribution and elimination is considered linear at the administered doses post IV injection. Supko and Malspeis, 1995, investigated the plasma pharmacokinetics of genistein in mice and suggested a linera relationship between AUC_{IV} and administered doses of genistein (9.2 - 52.4 mg/kg) post IV administration (Fig. 7).

1.10 Pharmacokinetic parameters

1.10.1 Area under the plasma drug concentration time curve (AUC)

The area under the plasma concentration and time curves (AUC: units of µg.h/mL, ng.h/mL, etc.) represents the body's total exposure to the chemical and is a function of the fraction of the chemical dose that enters the systemic circulation via the administered route and clearance (Fig. 8, Dipiro *et al.*, 2002). Therefore, a chemical given by the intravenous route will have a 100% bioavailability while drugs given by other routes usually have bioavailability of less than 100% (Shargel, 1999). When bioavailability is less than 100% for a compound administered orally, either the dosage form did not release all the

chemical contained in it or some of the chemical was eliminated or destroyed by stomach acid, or through other means, before it reached the systemic circulation. Also, in oral administration of a chemical, part of the dose will be metabolized by enzymes contained in the liver or gastrointestinal tract wall before it reaches the systemic circulation (Dipiro *et al.*, 2002).

Following the administration of a dose of chemical, its effect usually shows a characteristic temporal pattern. Onset of the effect is preceded by a lag period, after which, the concentration of a drug increases to a maximum and then declines. If a further dose is not administered, the effect will disappear as the drug is eliminated. This time course reflects changes in the drug's concentration as determined by the pharmacokinetics of its absorption, distribution, and elimination (Brunton *et al.*, 2006).

1.10.2 Estimation of C_{max}, t_{max}

Maximum observed concentration (C_{max} with units of µg/mL or ng/mL) and the time required to reach the maximum chemical concentration after chemical administration (t_{max} with units of hours, minutes). The C_{max} and t_{max} are obtained directly from the plasma concentration time profile (Dipiro *et al.*, 2002; Schoenwald, 2002 and Brunton *et al.*, 2006).

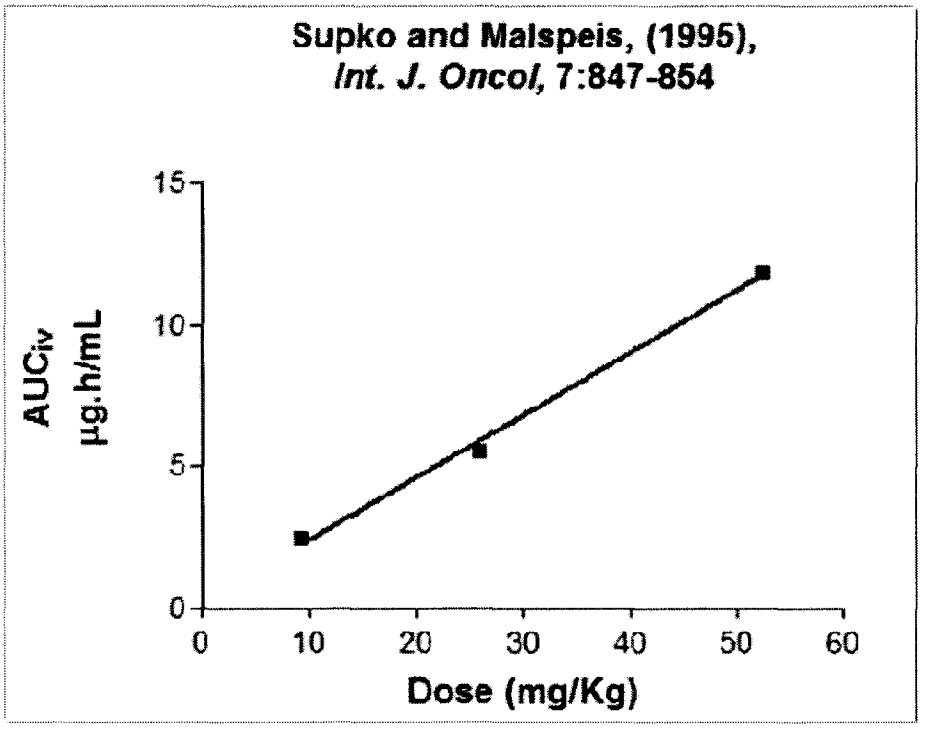


Figure 7: Linear relationship between AUCIV and the administered doses of genistein post IV injection.

Adapted from Supko and Malspeis, (1995).

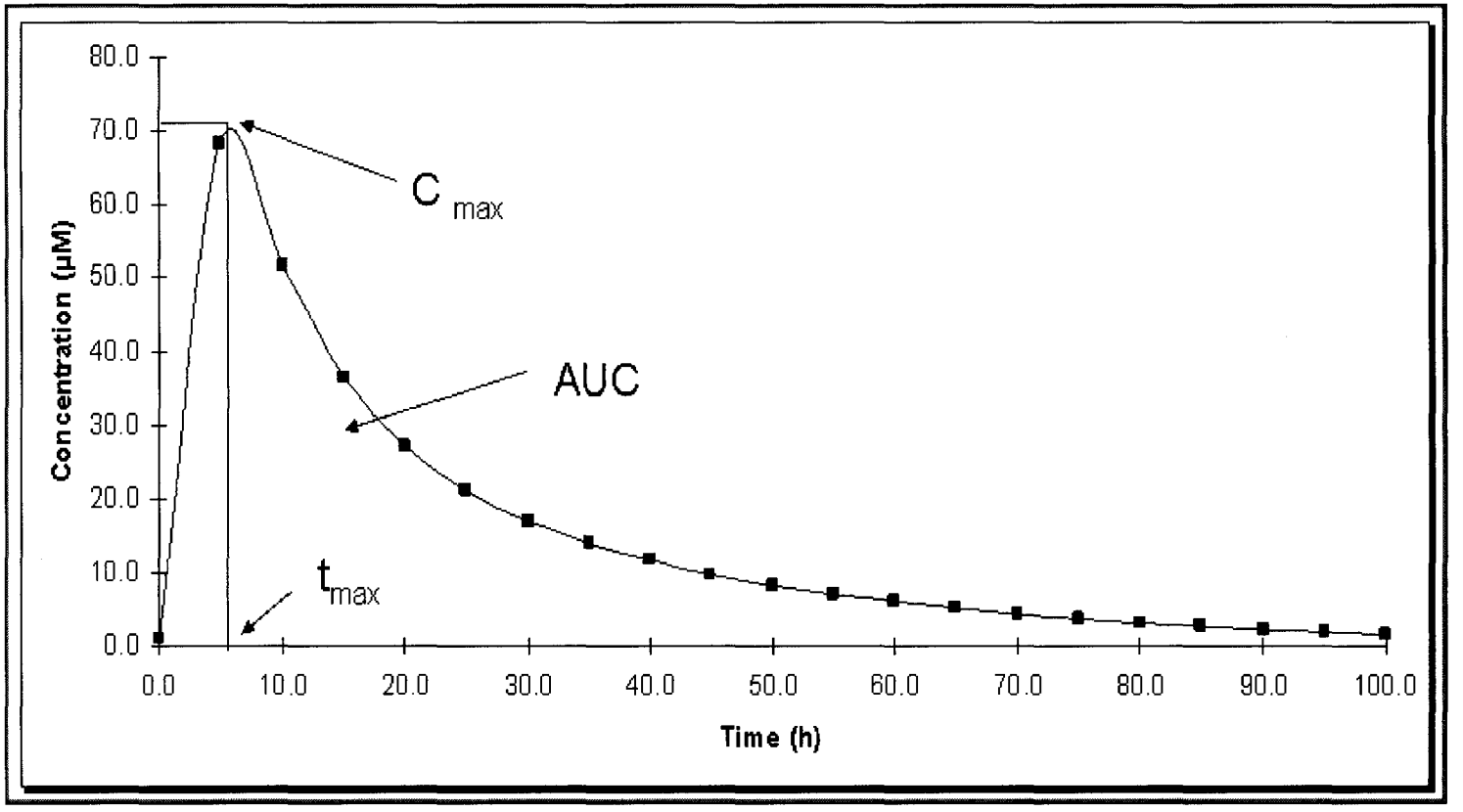


Figure 8: Area under the concentration time curve (AUC) after administration of an extravascular dose.

Bioavailability can also be calculated as absolute or relative bioavailability (Shargel, 1999).

1.10.3 Estimation of $t_{1/2}$, apparent Vd, Cl rate, and elimination rate constant (K)

Elimination half life is the time taken for the plasma concentration to fall by one-half after administration of single IV bolus dose (Gibaldi and Perrier, 1982).

$$t_{1/2} = 0.693/K$$

$$K = -2.303xS$$

S = Slope of elimination phase

Apparent volume of distribution formula based on $AUC_{(0-t)}$ trapezoid calculation and elimination rate after administration of single IV bolus dose:

$$Vd = FD/ AUC_{(0-t)} K \qquad \text{(Gibaldi and Perrier, 1982)}$$

F= Fraction of dose absorbed (For IV injection F=1)

D= Dose amount

K= Elimination rate constant

$AUC_{(0-t)}$ = AUC from the moment of injection to the time that it has reached zero or basal level. The $AUC_{(0-t)}$ is considered when AUC_{∞} is exaggerated due to high last concentration (Gibaldi and Perrier, 1982).

There is usually a considerable difference between the apparent volume of distribution (Vd) of a therapeutic products and the actual volume in which it distributes (Gibaldi, 1990).The apparent Vd is a proportionality constant relating the plasma concentration to the actual amount of therapeutic products in the body (Gibaldi, 1990). Depending on the degree of binding to plasma proteins and

tissues, the apparent Vd of therapeutic products may vary in man from 0.04 L/Kg to 20 L/Kg (Gibaldi, 1990). Two major plasma proteins to which therapeutic products can bind, are albumin and α-acid glycoprotein, and concentrations of these proteins may change with concurrent pathologies seen with increasing age (Dipiro *et al.*, 2002).

Based on the chemical structure of isoflavones, unconjugated genistein and daidzein are very lipophilic (hydrophobic), which favors retention and accumulation of these compounds in other tissues, such as the liver (Coldham and Sauer, 2000). Higher disposition of isoflavones in the liver would allow for the reduction of the systemic plasma concentration of genistein and daidzein.

Clearance rate can be calculated by:

$$Cl = FD/ AUC_{(0-t)} \qquad \text{(Gibaldi and Perrier, 1982)}$$

F= Fraction of dose absorbed (For IV injection F=1)

D= Dose amount

$AUC_{(0-t)}$ = AUC from the moment of injection to the time that it has reached zero or basal level. The $AUC_{(0-t)}$ is considered when AUC_{∞} is exaggerated due to high last concentration (Gibaldi and Perrier, 1982).

The total body clearance and the apparent Vd of therapeutic products are the two parameters that determine the therapeutic product elimination rate constant and half life:

$$K = Cl / Vd$$

$$Cl / Vd = 0.693 / t_{1/2} \qquad \text{(Hedaya, 2007)}$$

Elimination rate constant is inversly related to the half-life of the therapeutic product (Hedaya, 2007).

The production of urine and bile are both important excretory routes for isoflavones (Rowland *et al.*, 2003). Short term studies have shown that no more than 30% of an ingested dose of isoflavones is recovered from the urine and only 1- 2% is recovered from the feces of humans (Xu *et al.*, 1994; Rowland *et al.*, 2003). It has been suggested, that this low recovery is the result of extensive microbial degradation of isoflavones, producing phenols, such as p-ethylphenol (Coldham *et al.*, 1999; Setchell, 1998). The percentages of ingested isoflavones excreted in urine and feces are highly variable among individuals (Xu *et al.*, 1995). This variability has been correlated to the variability in intestinal bacterial strains among individuals (Xu *et al*, 1995, Rowland *et al.*, 2003).

1.11 Factors influencing bioavailability and pharmacokinetic profiles of isoflavones

Isoflavones present in soy foods include three aglyconees and nine glucosides. The most obvious structural differences are between the aglyconees, genistein, daidzein and glycitein and their respective glucosides. Therefore, there is a potential for differences in the bioavailability of these chemical forms, both in terms of the possible influence of structure on cellular transport, as well as on resistance to bacterial metabolism.

Other factors that influence the bioavailability and pharmacokinetic profiles of isoflavones are food matrix and dosage forms. Humans absorb isoflavones from a range of different soy-rich foods (Cassidy, 2006). Many studies have used a liquid matrix, like soy milk (Zhang *et al.*, 1999; Setchell *et al.*, 2001; Faughnan *et al.,* 2004), and determined that the time taken to reach peak plasma isoflavone concentrations was around 6 hour for daidzein and genistein. However, Cassidy *et al.,* (2006) reported that the peak serum concentrations of daidzein and genistein occurred about 2 hours later for solid food matrixes like tempeh and textured vegetable protein compared with soy milk. Qiu *et al.,* 2005 investigated the influence of dosage forms (liquid and suspension) on pharmacokinetics of daidzein in Wistar rats. Significant ($p < 0.05$) differences in absorption of daidzein after administration of daidzein solution (t_{max} = 0.4h) was observed compared with daidzein suspension (t_{max} = 5h). The reason for the significant differences was the poor solubility of daidzein. Physical properties of isoflavones (e.g. hydrophobicity and solubility) have a role in isoflavone bioavailability. As daidzein with plane chemical structure shows poor hydrophilicity and lipophilicity (Qiu *et al.,* 2005). However, by dissolving daidzein in weak alkaline solution and formation of the sodium salt at the hydroxyl groups increased its water solubility (Qiu *et al.,* 2005).

Direct interactions between nutrients and some component of food, such as binding to proteins and polysaccharides, can occur, and these interactions may affect absorption from the intestinal tract (Manach *et al.*, 2004). Dietary fibre, certain oligosaccharides, and resistant starch, which escape digestion in

the small intestine, could alter the total microfloral population of the bowel, and the proportion of the bacterial species and this in turn may influence the extent of hydrolysis both of the glycosidic form of the nutrient entering the bowel from the small intestine, and of the glucuronides entering with the bile, thus modulating the levels of the more efficiently absorbed aglycone form of the nutrients (King, 2002). Bioavailability could be altered by dietary components, such as fibre, which influence transit time and a shorter transit time may lead to a shorter exposure of the nutrients to the absorptive surface of the bowel and hence to reduce uptake (King, 2002).

The species composition of gut microflora and total activity of enzymes vary widely between individuals (Day *et al.,* 1998; Salminen *et al.,* 1998). Large differences among normal human subjects are caused by genetically controlled polymorphisms of drug-metabolizing enzymes, drug transporters, and drug receptors which would have an effect on bioavailability as well as safety and efficacy of many drugs (Vesell, 2000). At least part of this variability may be explained by dietary differences. Diet has been shown to alter cytochrome P450 expression and activity in both humans and animal models (Harris *et al.,* 2003; Bailey *et al.*, 1998). Rats fed diets containing soy protein isolates compared to casein had reduced levels of CYP1A1 protein in liver and CYP1A1, CYP1A2, and CYP1B1 proteins in mammary gland (Badger *et al.,* 2002).

The CYP3A enzymes are the major P450 forms expressed in human fetal and neonatal liver (Stevens *et al.,* 2003). Li and Shay (2004) demonstrated induction of the human CYP3A1 orthologue CYP3A4 in HepG2 hepatoma cells

transfected with a CYP3A4 reporter construct and induction in primary human hepatocytes *in vitro* after treatment with isoflavone extracts and purified isoflavones, including genistein and equol. However, these effects on CYP3A4 transcription were reported at isoflavone concentrations above 10 μ*M*, much higher than can be attained *in vivo* following dietary consumption of soy protein isolates. Ronis *et al.*, 2006 reported that soy protein isolate consumption also significantly up-regulates CYP3A1 mRNA expression in the rat jejunum. This suggests that soy consumption may also increase first pass as well as hepatic metabolism of CYP3A substrates (Ronis *et al.*, 2006).

Lactase phorizin hydrolase (LPH) which has a role in intestinal absorption of isoflavone aglyconees is also responsible of hydrolysis of lactose, and deficiency of this enzyme causes lactose intolerance (Day *et al.*, 1998). For those individuals who are deficient in LPH, isoflavone absorption in the small intestine may be reduced which would result in more isoflavones reaching the colon for microbial metabolism, including metabolism of daidzein to equol (Day *et al.*, 2000).

Another factor influencing bioavailability of isoflavones is circadian differences. All organisms are highly organized according to circadian rhythms (Baraldo, 2008). These temporal cycles influence different physiological functions and, consequently, can influence the pharmacokinetic phases of drugs. A drug's pharmacokinetics can be modified according to the time of drug administration (Baraldo, 2008).

Finally, in animal studies it is worth to consider the effect of coprophagy in bioavailability of isoflavones. Many animals, including rats, rabbits, dogs and monkeys eat excrement, which is called coprophagy (Gibson, 2001). This could alter the physiological conditions in the gastrointestinal tract, and thereby lead to an uncontrolled study situation. Furthermore, isoflavones or metabolites that have been excreted by faeces could be reabsorbed, which will obscure interpretation of bioavailability data. Coprophagy has thus to be prevented by using cages with bottom screens and by placing plastic collars around the animals neck, preventing the animal from reaching its anus (Gibson, 2001).

1.12 Health benefits of isoflavone consumption

For many years, nutritionists have noted the health benefits of soy based diets, citing the much lower incidence of breast, colon and prostate cancers in south-east Asians compared with Americans and Western Europeans (Barnes 1995; Green *et al.*, 2005). Other potential benefits of soy consumption include: the prevention of osteoporosis (Humfrey, 1998) as well as providing antioxidant effects, and antiestrogenic / estrogenic activities (Arora *et al.*, 1998; Barnes 1997; Fotsis *et al.*, 1995). Animal (Lin *et al.*, 2004; Moriyama *et al.,* 2004; Yamakoshi *et al.*, 2000; Kirk *et al.*, 1998) and human (Anderson *et al.*, 1999; Anthony *et al.,* 1998) studies have also shown that consumption of soy protein has beneficial impacts on the risk factors for cardiovascular disease including lowering liver or blood triglyceride, total and low density lipoprotein (LDL) cholesterol levels, increasing high density lipoprotein (HDL) cholesterol and the

ratio of HDL/LDL cholesterol in healthy men and women (Merz-Demlow *et al.,* 2000; Crouse *et al.,* 1999).

Isoflavones possess weak estrogenic activity due to their structural similarities to the human hormone, estradiol (Adlercreutz *et al.*, 1997). Based on the presence of the hydroxylated aromatic A-ring and second oxygen in the phenolic B-ring (Fig. 3), and molecular weight similar to those of the steroidal estrogens, the isoflavones are known as plant estrogens (Boersma *et al.*, 1999).

The estrogenic-activity of isoflavones is 10^5-fold less than estradiol in mammals (Messina *et al.*, 2001). Isoflavones can bind to estrogen receptors (ERs) where two known estrogen receptors are α and ß. Although the affinity of the ERα for isoflavones is weak (Verdeal *et al.*, 1980), the ERß has a strong affinity for genistein, comparable to that of the physiological estrogen agonist, estradiol (Kuiper *et al.*, 1997). In the absence of physiological estrogens and at very low concentration (nanomolar), genistein stimulates the growth of ER-positive human breast cancer cell lines (Wang and Kurzer, 1997). However, at micromolar concentrations, genistein is an inhibitor of estrogen stimulated cell growth (Peterson and Barnes, 1991). Therefore, isoflavones can act as either agonists or antagonists of estrogen receptors, or alter sex hormone production and metabolism (Bandele and Osheroff, 2007).

The estrogenic effects of isoflavones are believed to reduce the risk of osteoporosis (Mathey *et al.*, 2007) and alleviate menopausal symptoms (Welty *et al.*, 2007; Messina, 2002). Meta-analyses of randomized controlled trials suggest that isoflavone significantly reduces bone loss of the spine (Ma *et al.,* 2007).

Studies in postmenopausal women have shown similar results (Marini *et al.,* 2007; Lydeking *et al.,* 2004). However, no significant effects of isoflavones on bone mass density or biomarkers of bone metabolism have been reported in other studies in which soy protein was supplemented and isoflavone-poor soy protein was used as a control (Cheong *et al.,* 2007; Gallagher *et al.,* 2004). These data indicate that soy protein may interfere with the effects of isoflavones either by masking or antagonizing its effect. Currently, there is no existing health claim for bone health of soy intake. The UK Committee on Toxicity (2003) stated that long-term studies are needed before conclusions can be made about the effectiveness of isoflavones in improving bone health.

Two systematic reviews of the effects of soy isoflavones on menopausal symptoms have been published with conflicting results (Huntley and Ernst, 2004; Krebs *et al.,* 2004). Huntley and Ernst (2004) suggest some efficacy for the reduction of menopausal symptoms post soy isoflavone consumption while the review by Krebs *et al.,* (2004) does not support the hypothesis that isoflavones from a range of sources improves menopausal symptoms. The main outcome measure in the studies was the change in the number of hot flashes following intervention, which was either qualitatively assessed (using climacteric scale) or quantified using diaries.

Intake of soy supplements for treatment of menopausal symptoms in patients with early breast cancer did not show any significant effect on menopausal symptom scores or quality of life after 12 weeks compared with control group (MacGregor *et al.,* 2005). Of the 12 studies reviewed by the UK

Committee on Toxicity (2003), half reported that soy diets or isoflavone supplementation reduced the frequency of hot flashes, while the other half reported no effect on hot flashes. Therefore, there is no consistent evidence to support any beneficial effect of soy intake on menopausal symptoms.

The anti-estrogenic effects of isoflavones are thought to reduce the risk of breast cancer (Messina *et al.*, 2006). However, it is the non-hormonal properties that account for most of the interest in the potential chemo-preventive properties of isoflavones and have led to the speculation that isoflavones may reduce the risk of both hormone-dependent and independent cancers (Messina, 2002).

Isoflavones have various anti-carcinogenic properties (Bagchi and Preuss, 2005).The B-ring of genistein and daidzein is structurally similar to that of the amino acid tyrosine. Genistein is a potent inhibitor of specific protein tyrosine kinases (e.g. members of the Src family of kinases, Lck, Lyn, Fyn, and Blk) (Akiyama *et al.*, 1987; Constantinou, 2002) where it blocks the action of growth factors such as platelet-derived growth factor and basic fibroblast growth factor involved in the growth of the atherosclerotic lesion (Wilcox and Blumenthal, 1995). Genistein also inhibits the proliferation of many vascular cells including vascular epithelial cells (Raines and Ross, 1995), smooth muscle cells (Fugio *et al.*, 1993) and inhibits microsomal lipid peroxidation (Jha *et al.*, 1985) and angiogenesis (Fotsis *et al.*, 1993). In addition isoflavones have proapoptotic effects, decreasing the expression or function of several proteins that are involved in cell-cycle progression, inhibiting both the NF-*k*B and A*k*t signaling pathways (Bandele and Osheroff, 2007), inhibiting the activity of growth-

promoting steroid hormones by inhibiting the enzymes progesterone 5α-reductase and 17ß-hydroxysteroid dehydrogenase (Busby *et al.*, 2002), inhibiting protein-tyrosine-kinase-mediated signal transduction (Akiyama *et al.*, 1987), inhibiting DNA topoisomerase II (Knodo *et al.*, 1991; Markovits *et al.*, 1989) and have antioxidant activities (Arora *et al.*, 1998).

1.13 Potential risks of Isoflavone consumption

Besides all the reported beneficial roles of soy isoflavone consumption, reproductive dysfunction in animals [e.g. Sheep fertility problems in Western Australia (Bennetts *et al.*, 1946); reproductive abnormalities in New Zealand rabbits (Kendall *et al.*, 1950); and the inability of a captive cheetah to reproduce (Setchell *et al.*, 1987)] post ingestion of phytoestrogens, such as isoflavones have been reported. Excessive soy intake has been reported to be responsible for the development of goiter, including thyroid enlargement, in iodine-deficient rodents (Kimura *et al.,* 1976; Wilgus *et al.,* 1941). Additionally, soy components stimulate the development of thyroid hyperplasia in iodine deficient rats (Ikeda *et al.,* 2000; Son *et al.*, 2001).

In the United States and Canada, over 20-25% of newborns are fed soy-based formulas for various reasons (e.g. allergies to cow's milk). Blood isoflavone concentrations in those infants are 13,000-22,000 times higher than plasma estradiol levels in early life and are 6 to 11 fold higher on a body-weight basis than the dose that has hormonal effects in adults consuming soy foods (Fig. 9; Morton *et al*, 2002; Setchell *et al*, 1997). However, the impacts of

excessive intake of isoflavones on early development and reproductive function in humans remain unclear.

In 1950s and 1960s, 12 cases documented goitrogenic effect of soy flour infant formula that resulted in substituting soy flour with soy protein isolate and supplementing it with iodine (Hydovitz, 1960; Shepard *et al.,* 1960; Fomon, 1993). Additionally, infants with congenital hypothyroidism fed soy-based infant formula have an increased requirement for thyroxine by as much as 18-25% (Jabbar *et al.,* 1997). Isoflavones in soy-based infant formula may influence thyroid function in infants by acting as a mild goitrogen, reducing absorption of iodine from the gut, increasing loss of thyroxine via the entrohepatic circulation, and inhibiting the activity of thyroid peroxidase (UK Committee on Toxicity, 2003).

In Pre-menopausal women, and men, Hampl *et al.* (2008) found correlation between ingested isoflavones (from boiled natural soybeans at 2 g/kg/day) and changes in thyroid related proteins and hormones in healthy young men and women over 7 days, but these were modest and transitory changes without clinical-level impact on thyroid function. Dillingham *et al.* (2007) further notes that up to 61.7 mg/day isoflavones for 57 days caused no perturbations in blood levels of total T3, free T3, total T4, free T4, TSH, and thyroid binding globulin in healthy adult men.

For post-menopausal women, it appears that 2 mg/kg daily of soy isoflavones from a soy protein isolate is safe for up to 7 weeks on thyroid function since no significant alteration of thyroid levels were detected (Teas *et al.* 2007).

Persky *et al.* (2002) investigated the use of soy protein isolates containing 56 mg/day and 90 mg/day isoflavones and showed only marginal effects on thyroxine and TSH levels over 3 to 6 months. Bruce *et al.* (2003) conducted a study in which iodine-replete post-menopausal women on 90 mg/day isoflavones over 6 months showed no effect on TSH, T3, or T4 levels.

A significant dose-dependent risk (up to 2.4 fold) for development of vascular dementia and brain atrophy from consumption of tofu (two or more serving per week) reported in over 7000 men from Honolulu heart program study (White *et al.,* 2000). The author concluded that regular dietary exposure to soy isoflavones over many years during middle life may be associated with the appearance of brain aging in later life due to chronically sub-optimal neural plasticity. The specific means by which isoflavones might exert such influence could involve competition with endogenous estrogens for estrogen receptors in neurons and/or reduction in estrogen concentration in the brain by inhibition of the aromatization of androgens (White *et al.,* 2000). Isoflavones in tofu might exert their influence through interference with tyrosine kinase dependent mechanisms required for optimal hippocampal function, structure and plasticity (White *et al.,* 2000).

Although potential health benefits and adverse effects have been suggested for soy consumption, consistent and direct evidence to support these effects are lacking, inadequate and outdated in most of the cases. Future studies should pay more attention to identification of the bioactive components in soy and elucidation of the molecular mechanisms involved.

1.14 Direction of research

A vast amount of evidence suggests beneficial roles of soy consumption, particularly in relation to hormone dependent cancers. However, there are variations in the results of dietary interventions in terms of isoflavone bioavailability and clinical outcome measures. Background diet may have a role in isoflavone bioavailability and thus their physiological effects. Additionally, the role of gut microflora, liver and intestinal enzymes may be particularly important in the production of the isoflavone metabolites, particularly equol, which may confer more beneficial health effects than their precursor.

1.15 Thesis Hypothesis

Based on the limited and controvertial evidence, it is hypothesized that soy isoflavone bioavailability will be dependent on the dietary source in which the soy component is contained. Further, it is also hypothesized that gender and age may have a role in isoflavone bioavailability.

1.16 Overview of Thesis

This thesis has been divided into five chapters. Following the general introduction in chapter 1, about soy isoflavones,

> In chapter 2, the development of an accurate and reproducible method for the quantitative analysis of isoflavones and their metabolites in rat plasma using liquid chromatography-mass spectrometry combined with photodiode array detection is discussed.

> In chapter 3, the bioavailability of soy isoflavones in young adult rats, based on comparison of AUC time curves of short term oral and IV exposure to different sources of isoflavones, was investigated. Effects of gender and source of isoflavones were detected. Concentrations of isoflavones and equol in plasma samples were determined using the LC/MS method described in chapter 2.

> In Chapter 4, bioavailability of soy isoflavones in aged male rats was determined using the same methodology described in chapter 3.

>Chapter 5 is devoted to a general discussion of all the experimental results.

>Appendix 1: The developed HPLC/MS method was used for analyzing the serum concentration of isoflavones (genistein, daidzein, glycitein and equol) in male and female SD rats from a three-generation study.

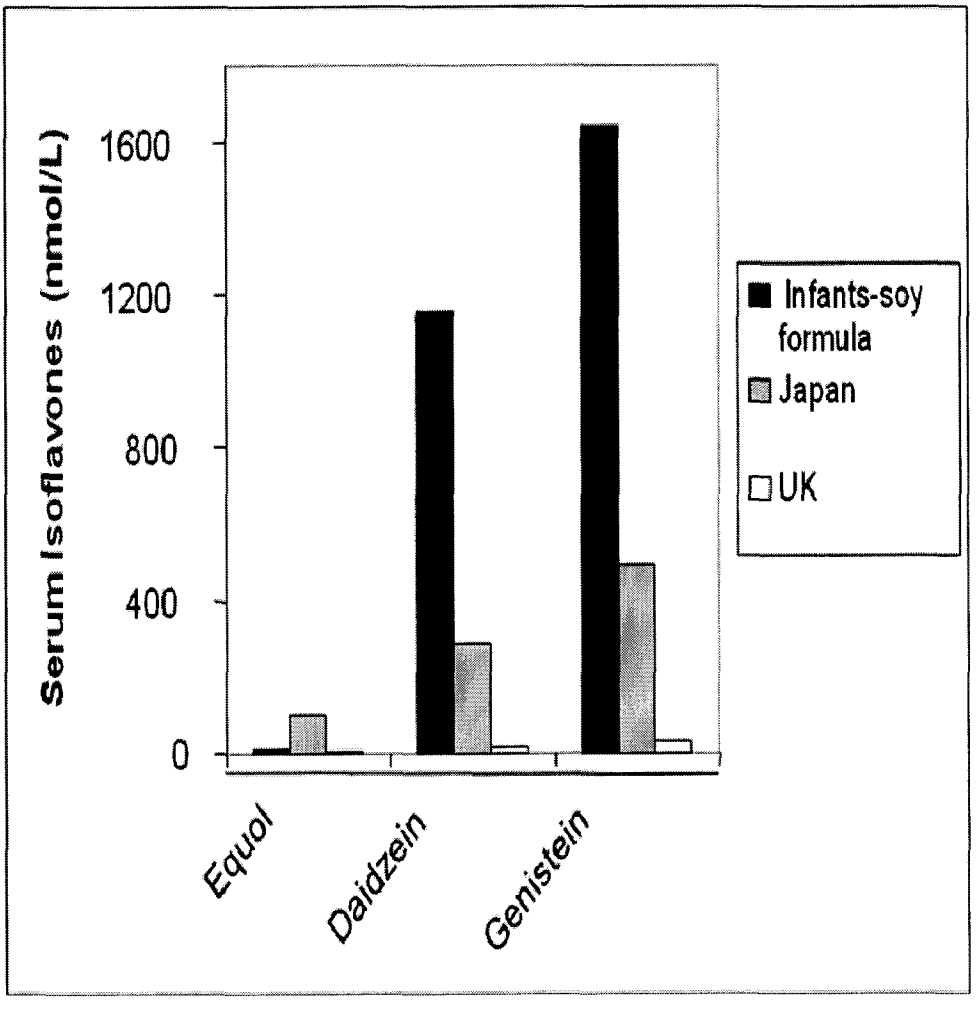


Figure 9: Phytoestrogen concentrations in the sera of men from Japan and UK and infants fed soy based formula.

Adapted from Morton *et al*, (2002); Setchell *et al*, (1997).

CHAPTER 2

2.0 An accurate and reproducible method for the quantitative analysis of isoflavones and their metabolites in rat plasma using liquid chromatography-mass spectrometry combined with photodiode array detection.

Estatira Sepehr, Patrick Robertson, G. Sarwar Gilani, Gerard Cooke, Benjamin Pui-Yan Lau., "An accurate and reproducible method for the quantitative analysis of isoflavones and their metabolites in rat plasma using liquid chromatography-mass spectrometry combined with photodiode array detection" *J. AOAC. Int* (2006); 89 (4) 1158-1167.

Study Context and Collaborations:

The initial focus of my research project was to develop an accurate HPLC/MS combined with photodiode array detection method for determining the concentration and composition of isoflavones and their metabolites in rat plasma samples. I was extensively involved in a literature review of the previously published methods of isoflavone analysis and provided a step by step method design. The development of the method was a collaborative effort of myself and Mr. Patrick Robertson.

Dr. Benjamin Pui-Yan Lau provided us with high-level technical support throughout the design and implementation of the method.

Ample amounts of rat plasma samples were used throughout method development, which were kindly provided by the Toxicology Research Division at Health Canada.

I presented the following two posters based on the developed method:

Estatira Sepehr, G. Sarwar Gilani, Patrick Robertson, Gerard Cooke, Benjamin P.-Y.Lau, Ivan H. A. Curran. Improved method for the extraction and analysis of isoflavones and their metabolites in rat blood using liquid chromatography combined with mass spectrometry and photodiode array detection. *Health Canada Science Forum, Ottawa, Ont.,* October, 2005.

Estatira Sepehr, G. Sarwar Gilani, Patrick Robertson, Gerard Cooke, Benjamin P.-Y.Lau, An accurate method for the quantitative analysis of isoflavones and their metabolites in rat blood using liquid chromatography combined with mass

spectrometry and photodiode array detection. *48th Annual Meeting Canadian Federation of Biological Societies, Guelph, Ont.*, June, 2005.

Additionally, I was involved in preparation of a manuscript which was published in *Journal of Nutrition.*

G. Sarwar Gilani, Estatira Sepehr, 2003, Protein digestibility and quality in products containing antinutritional factors are adversely affected by old age in rats, *J Nutr.* 133(1):220-5.

Abstract:

To study the safety and potential health benefits of soy isoflavones, a rapid and simple method based on liquid chromatography combined with mass spectrometry (LC/MS) and photodiode array detector (PDA) was developed for the determination of isoflavones in rat plasma. The analytes included daidzein, genistein, glycitein, equol, 4-ethyl phenol and Biochanin A over a concentration range of 1.0 – 4320.0 nM using 75 µL of rat plasma. Rat plasma samples were hydrolyzed by adding an enzyme mixture from *Helix pomatia* containing glucuronidase and sulfatase to convert isoflavone β-glycosides daidzin, genistin and glycitin to their active aglycone forms. A liquid-liquid extraction method using ethyl acetate as the extraction solvent was used to extract aglyconees and the internal standards (phenolphthalein β-D glucuronide, 4-methylumbelliferyl sulfate, and apigenin) from digested plasma samples. The extract was evaporated to dryness under a nitrogen stream, reconstituted with 0.1% formic acid in water-acetonitrile (85 +15), and injected into a Zorbax SB-CN reversed-phase column (4.6 X 75 mm, 3.5 µm particle size). The Micromass ZQ detector was operated in the positive ion selected-ion monitoring mode. The flow rate for the LC was 1.0 mL/ min, with a split where 25% of the effluent was introduced into the electrospray ionization probe of the MS instrument and 75% into the PDA. The chromatographic run time was 16.0 min, with delay of 10 min/injection. The interday precision and accuracy of the standard samples were <2.6% relative standard deviation (RSD) and <10% relative error, respectively. Recovery of the reported isoflavones with this method varied from 86 to100 %.

2.1 Introduction:

The current AOAC INTERNATIONAL *Official Methods of Analysis* (*Official Methods of analysis,* 2003) does not provide a method for the analysis of isoflavones and their metabolites in biological fluids. The only existing AOAC Method, **2001.10** (*Official Methods of analysis;* 2003), is for the determination of isoflavones in soy and selected foods containing soy by using extraction, saponification, and liquid chromatography (LC). Because of sample matrix differences, this method may not be applicable for biological fluids.

Development of a suitable analytical method for determining isoflavones and their metabolites in biological matrixes has been difficult due to the large number of phytoestrogens that exist and the range of chemical forms in which they can occur within various biological matrixes (Jackson and Gilani, 2002).

In 1985, gas chromatography/mass spectrometry (GC/MS) was the principle method for the quantitative analysis of isoflavones and other polyphenols in biological fluids (Wang *et al.,* 2002). These methods are relatively long, requiring purification, fractionation, hydrolysis, and derivatization, and they analyze only specific isoflavones of interest (Adlercreutz *et al.*, 1991, Adlercreutz *et al.*, 1993; Adlercreutz *et al.*, 1994; Adlercreutz *et al.,* 1995; Setchell *et al.*, 1984). During the last decade, there have been advancements in the field of MS with the development of new interfaces and ionization and detection techniques. High-performance LC/MS has become the method of choice for the analysis of isoflavones and their metabolites in food, rat plasma, and urine (Griffith and Collison, 2001; Holder *et al.*, 1999; Fang *et al.*, 2002). Most previous studies

have determined daidzein and/or genistein in plasma samples using a chromatographic separation of 30 min or longer (Holder *et al.*, 1999; Chang *et al.*, 2000). Compared with GC/MS, isoflavones can be analyzed using HPLC/MS without the need for derivatization and extraction from the biological matrixes, so the LC/MS procedure is much simpler. Furthermore, the sensitivity of LC/MS (1-500 fmol) is higher compared with GC/MS (50 fmol; Wang *et al.,* 2002).

To our knowledge, the method described in the present study is the first method for the determination of soy isoflavone aglyconees derived from dietary sources present in rat blood samples, as well as their metabolites. The method involves a single run of 16.0 min using LC/MS combined with a photodiode array detector (PDA). This state-of-the art LC/MS method will be a key determinant in generating reproducible and reliable data that will be used subsequently in the evaluation and interpretation of pharmacokinetic and bioavailability studies involving soy products, and it could be useful for the determination of isoflavones derived from other plant sources and fed to other animals.

2.2 Experimental

2.2.1Reagents

(**a**) *Hydrolytic enzyme.*-A mixture of sulfatase and glucuronidase from *Helix pomatia* type H-5 (S3009), containing 400-600 units/mg of glucuronidase activity and 15-40 units/mg of sulfatase activity (Sigma-Aldrich Co., St. Louis, MO).

(**b**) *HPLC- grade acetonitrile (ACN) and ethyl acetate.*- EMD Chemicals Inc. (Gibbstown, NJ)

(**c**) *Formic acid.*- Sigma-Aldrich Co.

(**d**) *Dimethyl sulfoxide (DMSO).*-99.9% HPLC grade (Sigma-Aldrich Co.).

(**e**) *Water.*-Deionized, NANO-pure [Diamond ultra-violet (UV) ultrapure water purification system; Barnstead International, Essex, UK].

(**f**) *ACN-water.*- 50+50 (v/v).

(**g**) *Mobile phase A.*-0.1% formic acid in water.

(**h**) *Mobile phase B.*- 0.1% formic acid in ACN.

(**i**) *0.1% Formic acid in water- CAN.- 85* +15 (v/v).

(**j**) *Sodium citrate buffer (25 mM, pH 5.0)*- Prepared by weighing an appropriate amount of sodium citrate (Sigma-Aldrich Co.) in water and adjusting the pH of the solution to 5.0 using acetic acid.

2.2.2 Standards

(**a**) *Daidzin, genistin, glycitin, daidzein, genistein, glycitein and equol (metabolite of daidzein).*- LC Labs. (Woburn, MA).

(**b**) *Biochanin A, 4-ethyl phenol (metabolite of genistein) and 3 internal standards; phenolphthalein β-D glucuronide, 4-methylumbelliferyl sulfate and apigenin.*- Sigma-Aldrich Co.

(**c**) *Stock standard solution of each standard (genistein, daidzein, glycitein, genistin, daidzin, glycitin, biochanin A, equol, 4 ethylphenol) and 3 internal standards (phenolphthalein β-D glucuronide, 4-methylumbelliferyl sulfate and Apigenin*).-Prepared by accurately weighing out each standard into 10 mL of DMSO. The concentration of the stock was calculated to be 8 mM.

Table 1: Stability test of the stock standards in DMSO at room temperature

% Degradation (Months)

Compound	1	3	4	7	11
Daidzein	0.0	0.0	0.0	4.2	4.2
Glycitein	0.0	0.0	0.0	4.2	3.4
Equol	0.0	5.0	8.2	18.3	29.6
Genistein	0.0	3.8	3.8	4.2	4.6
Biochanin A	[a]ND	[a]ND	14.9	18.9	20.7

[a]ND: not determined.

Stability of stock solutions was checked over a period of one year (Table 1). This solution was stable for 1 month at room temperature.

(**d**) *Working standards* 1 (8 µM).- Prepared by appropriately diluting the stock solution with CAN-water (50 + 50, v/v).

(**e**) *Working standards 2 (1.0- 4320.0 nM).*- Prepared from the working standards 1 by adding appropriate volumes of analyte and internal standards into 0.1% formic acid in water and acetonitrile mixture (85 + 15, v/v). Working standards 1 and 2 were prepared daily. Each working standard 2 was initially infused into the mass spectrometer in the positive selected-ion monitoring (SIM) mode (span= 0.05 Da, and interchannel delay time =0.1s) in order to determine the right cone voltage and maximum peak intensity for each $[M + H]^+$ ion. Mass spectrometer operating conditions were reported in table 2.

2.2.3 Enzyme

Enzyme sulfatase type H-5 from *H. pomatia*, containing 29 units/mg solid sulfatase activity was purchased from Sigma- Aldrich Co. A solution containing 23 units of sulfatase activity was prepared according to (Holder *et al.*, 1999) by dissolving an appropriate amount of enzyme in 1.0 mL of the sodium citrate buffer.

2.2.4 Apparatus:

(**a**) *Analytical evaporator (N–EVAP).*- Complete with heated water bath and nitrogen tank attached; capable of holding 12 vessels/run (Organomation Associates Inc., Berlin, MA).

(**b**) *Balance.*- Analytical, weighing to 0.00001g (Mettler, Toledo, Columbia, MD).

(**c**) *HPLC.*- The LC separations were performed by using Waters (Milford, MA) Alliance 2695 liquid chromatograph and a Zorbax SB-CN reversed-phase column (4.6 X 75 mm, 3.5 µm particle size; Agilent Technologies, Wilmington, DE) with gradient elution of (0.1% formic acid and 85 +15% water-acetonitrile) using the gradient steps shown in table 3. The flow rate was 1.0 mL/ min, with a split (using a Tee union splitter) where 25% of the effluent was introduced into the electrospray ionization (ESI) probe of the MS instrument and 75% into the PDA. Solvents were degassed by the online degasser of the Alliance 2695 system.

(**d**) *PDA.*- UV detection was monitored at 276.4 nm for the detection of 4-ethyl phenol in the same run using Waters 996 PDA.

(**e**) *MS system.*- A Waters Micromass ZQ single quadrupole mass spectrometer was operated in the positive ion SIM mode, (span= 0.05 Da, and interchannel delay time= 0.1s). For glycoside and aglycone analysis, 6 time functions were utilized: first time function (0.0 -8.0 min), second time function (8.0-10.8 min), and third time function (10.8 -12.5) monitored the $[M+H]^+$ ions for 4-methylumbelliferone (*m/z* 177.1, dwell time = 0.3 s) at cone voltage 35V, daidzin (*m/z* 417.3, dwell time = 0.3 s) and genistin (*m/z* 433.8, dwell time = 0.3 s) and glycitin (*m/z* 447.4, dwell time = 0.3 s) at cone voltage 25 V. In the second time function (8.0-10.8 min), daidzein (*m/z* 255.2, dwell time = 0.4 s), and glycitein (*m/z* 285.2, dwell time = 0.6 s) were monitored at a cone voltage of 45V. In the third time function (10.8 -12.5), the $[M+H]^+$ ions for equol (*m/z* 243.1, dwell time = 0.7s) and genistein (*m/z* 271.2, dwell time = 0.3 s) were monitored at cone voltages of 22V and 45V, respectively. In the fourth time function (12.5-14.0 min)

the $[M+H]^+$ ions for phenolphthalein (*m/z* 319.2, dwell time = 1.0 s) were monitored at a cone voltage of 30V. In the fifth time function (14.0-16.0 min), biochanin A (*m/z* 285.2, dwell time = 0.5 s) was monitored at a cone voltage of 45V and apigenin (*m/z* 271.2, dwell time = 0.5s) at a cone voltage of 50V. Mass spectrometer operating conditions are reported in Table 3. The entire system from sample injection to data acquisition was computer -controlled with Empower software (Waters).

2.2.5 Standard Curve

Three linear calibration curves (low, medium, and high) with a minimum of 5 concentrations/curve (total of 17 levels) were prepared daily by diluting the stock solutions with 0.1% formic acid and water-acetonitrile (85 +15). A constant concentration of internal standard (4-methylumbelliferone, 800.0 nM) was chosen for all 3 calibration curves to achieve the following range of the standard concentration: low (1.0 – 48.0 nM), medium (48.0 – 720.0 nM), and high (720.0 – 4320.0 nM) for all the standards except equol and 4 -ethyl phenol. Equol ranges were 30.0- 1920.0 nM and 1920.0 – 11526.0 nM for medium and high calibration curve, respectively (no low calibration curve was set for equol). 4-Ethyl phenol standard concentrations ranges were 1200.0 – 3600.0 nM and 3600.0 – 21620.0 nM for medium and high calibration curve, respectively (no low calibration curve was set for 4-Ethyl phenol). These standard mixtures (all 17 levels) were injected before or after analysis of the unknowns. Also, 2 levels of standards (low and high) were injected between every 2 unknown samples to ensure system stability

Table 2: Mass spectrometer operating conditions

MS	Waters ZQ 2000
Ion Mode	ESI^+
Capillary Cone Voltage, kV	3.2
Extractor, V	3.0
Source Temperature, °C	120
Desolvation Temperature, °C	350
Cone Gas Flow, L/h	50
Desolvation Gas Flow, L/h	600

Table 3: HPLC pump gradient elution of the isoflavones for each run

Step	Time, min	Flow, mL/min	%A, 0.1% FA[b] in H_2O	%B, 0.1% FA[b] in ACN
Initial	0	1	85	15
2	7.5	1	82	18
3	8.5	1	70	30
4	12.0	1	70	30
5	13.0	1	55	45
6	16.0	1	55	45
7	17.0	1	5	95
8	18.0	1	5	95
9	19.0	1	85	15

[a]All gradients are linear.
[b]FA= Formic acid.

during the whole run time. Only calibration curves from all analytes (r^2 >0.998) in the expected calibration range were used (data not shown). The calibration of the instrument was repeated when the calibration curves showed an r^2 lower than 0.998.

For each calibration curve, peak area ratios of the analyte to internal standard were plotted against the known analyte concentration. The resulting slope and y-intercept values for each analyte were used to determine analyte values in unknown specimens using the best -fit line equation

$$(y=mx+b)$$

All of the analytes were detected against 4-methylumbelliferone internal standard. Analytes below the limit of detection (LOD) were reported as nondetectable. Samples exceeding the highest standard of the high standard curve were diluted and reanalyzed in the linear range of the assay.

2.2.6 Rat Plasma Sample Collection

Blood samples (0.5 mL) from isoflavone-fed and control rats dosed with 1000 mg/kg of soy protein isolate plus NOVASOY™ (Archer Daniels Midland Company, Decatur, IL) and casein (90% purity, ICN Biomedicals, Costa Mesa, CA), respectively, were collected in microtainer tubes with lithium heparin and plasma separator (Becton Dickinson and Company, Franklin lakes, NJ). After blood collection, capped tubes were inverted several times. Tubes were centrifuged at 4^0C, 8000 rpm in an IEC Centra MP4R refrigerated centrifuge, Cat. No. 2438 (Needham Heights, MA) for 3 min and about 250 µL of the plasma

(supernatant) was transferred to 0.5 mL tubes and frozen at -80 ^{0}C until the day of the analysis.

2.2.7 Extraction and Cleanup

On the day of the analysis, plasma samples were thawed at room temperature and mixed on a Vortex mixer, and 75 µL aliquots were added to equal volumes of ACN and 10 µL of the internal standard solution (12.8 µM) in 1.5 mL Eppendorf tubes. Tubes were mixed on a Vortex mixer, sonicated for 10 min, and centrifuged at 14000 rpm for 6 min at 4^{0}C to precipitate proteins. An aliquot of the supernatant (100 µL) was combined with 1 mL sodium citrate buffer and incubated at 37^{0}C for 45 min.

The time course of isoflavone hydrolysis by the mixture of enzymes was determined at 37^{0}C over the intervals of 0, 15, 30, 45, 60, 120, 180, and 240 min and 24 h. Maximum hydrolysis was monitored by the disappearance of the conjugated forms of the internal standards (phenolphthalein β-D glucuronide and 4-methylumbelliferyl sulfate) and the appearance of the deconjugated forms (phenolphthalein and 4- methylumbelliferone). Failure to detect unconjugated phenolphthalein and 4-methylumbelliferone in the expected amounts can identify incomplete enzyme hydrolysis with this method (data not shown).

After 45 min of incubation at 37^{0}C, isoflavone aglyconees were extracted into ethyl acetate solvent (3 x 2 mL). Addition of ethyl acetate to the hydrolyzate causes enzyme precipitation. After 15 min sonication, followed by 5 min centrifugation at 3000 relative centrifugal force (RCF) at 8^{0}C, the supernatant

was removed and evaporated to dryness under a nitrogen stream using the N–EVAP at 37^{0}C. Following the 3 extractions, the residue was reconstituted in 100 µL of HPLC mobile phase [0.1% formic acid in water- acetonitrile in (85 +15)]. Ten µL of each prepared sample was injected into a Zorbax SB-CN reversed-phase column. The isoflavone aglycone content of plasma was determined by omitting enzymatic hydrolysis.

2.2.8 Calculations

The concentrations (µM) of all isoflavones and their metabolites in the sample extract were determined from linear regression analysis of the appropriate standard curve. In case of using external calibration curves, the concentration of each isoflavone in test samples was calculated by using the following equation:

$$\textbf{Isoflavone (µM)} = C_s \times (V_m / V_i) \times (V_e / V_a) \times (1 / V_p)$$

Where C_s= concentration of isoflavone calculated from the standard curve (µM), V_m= mobile phase volume added (100µL), V_i= volume injected (10µL), V_e= volume of the analyte without the precipitation (160µL), V_a= volume of the aliquot (100µL), V_p= volume of the plasma used at the beginning of extraction (75µL).

2.2.9 Method Validation study

Accuracy.- To demonstrate the accuracy of the method, individual standards were subjected to complete extraction and analytical processes, and measured values compared to calculated values. The closeness of agreement between the calculated value and the measured value was determined for each

standard sample using 6 different concentrations of isoflavones added to a control rat plasma matrix (control rat plasma containing undetectable amounts of isoflavones). Plasma samples were injected 3 times on 3 different days. Results were reported as relative error. Accuracy of the standard samples was < 10% relative error (Table 4).

Precision.-The precision of the method was determined by preparing and assaying 4 replicates each of 6 different concentrations of isoflavones. Table 5 demonstrates single laboratory relative standard deviation (RSD) values between 0.1 and 2.6% for the 6 concentrations: 10.0-2410.0 nM for daidzein, genistein, glycitein and 30.0-6360.0 nM for equol.

Recovery of isoflavones from rat plasma.- To measure an analyte in a complex sample matrix (plasma), a spike recovery method was performed. Because other components of the matrix may interfere with the separation, detection, or accurate quantitation of the analyte, potential effects from matrix components were investigated. The analyte reference standard was added to a blank matrix (75 µL of plasma) at various levels. Plasma samples were run through the entire analytical procedure from sample preparation through final analysis. A minimum of 3 replicate measurements were performed at each level on different days and by 2 different technicians. Recoveries were determined by comparing the peak areas for the isoflavones in the extracts of spiked samples with the peak areas observed by injecting the standards directly into the LC/MS instrument (external standards). Also, an injection of the blank matrix was made to determine the matrix background. Recovery results are shown in Table 6.

Repeatability.- Homogeneous plasma samples prepared with isoflavones at 5 different concentrations and containing constant amounts of 4-methylumbelliferone sulfate and phenolphthalein β-D glucuronide as internal standards were analyzed in triplicate on 3 different days (Table 6). These samples were subjected to the entire analytical procedure from sample preparation through final analysis.

2.2.10 LOD and Limit of quantification (LOQ).-

The detection limits for isoflavones were estimated by making solutions with known amounts of isoflavones and a constant amount of internal standard (800nM), using the lowest concentration of the standards and defining the LOD as signal-to-noise ratio (S/N) of 3:1 and the LOQ as a S/N of 10:1. Table 7 shows the LODs and LOQs of isoflavones.

2.2.11 System Stability.-

The stability of the system was checked by injecting a known concentration of the standard 20 times in one day.

Table 4: Accuracy evaluation using isoflavone standard solutions fortified into control rat plasma

Isoflavone	Expected Concn, nM	Mean of Observed Concn[a], nM	Relative error, %	Accuracy, % added
Daidzein	2.0	2.0	0.6	100
	12.0	13.0	9.2	108
	24.0	23.0	1.4	96
	240.0	239.0	0.2	100
	480.0	464.0	3.1	97
	960.0	920.7	4.1	96
Genistein	2.0	2.0	1.7	100
	12.0	11.0	0.5	92
	24.0	22.0	4.2	92
	239.0	220.0	8.3	92
	479.0	434.0	9.5	91
	959.0	876.0	8.7	91
Glycitein	2.0	2.0	5.4	100
	12.0	12.0	2.3	100
	24.0	25.0	5.9	104
	240.0	249.0	3.8	104
	480.0	483.0	0.7	101
	960.0	971.0	1.2	101
Equol	30.0	29.0	4.7	97
	128.0	127.0	1.1	102
	640.0	631.0	1.5	105
	1921.0	1873.0	2.5	106
	3842.0	3871.0	0.7	101
	6403.0	6358.0	0.7	99

[a]The mean isoflavone concentrations (n=3) are shown with the respective relative error (%), and the accuracy compares all measurements with the concentrations added.

Table 5: Precision evaluation using isoflavone standard solutions as unknown samples

Isoflavone	Mean of Observed Concn[a], nM	Relative standard deviations from the mean, %
Daidzein[b]	10.0	1.7
	50.0	1.6
	680.0	2.6
	1450.0	0.6
	2410.0	0.2
Genistein	10.0	1.7
	50.0	0.1
	250.0	1.2
	700.0	0.9
	1450.0	0.6
	2410.0	0.8
Glycitein	10.0	1.7
	50.0	0.8
	230.0	1.2
	700.0	1.5
	1450.0	0.7
	2410.0	0.2
Equol	30.0	2.4
	130.0	0.7
	630.0	1.4
	1870.0	1.3
	3870.0	0.5
	6360.0	1.2

[a]n=4 for all injections.
[b]Five levels of concentrations were used for daidzein.

Table 6: Recovery and repeatability of isoflavones from plasma after enzyme hydrolysis as determined by LC/MS and calculated against external calibration curve analyzed on 3 different days.

	Concentration	Recovery (%)			
	nM	Day 1	Day 2	Day 3	mean ± S.D
Daidzein	2.0	96.5	93.0	93.6	94.4 ± 1.9
	10.0	108.7	105.2	108.2	107.3 ± 1.8
	20.0	95.1	87.8	91.8	91.5 ± 3.6
	240.0	91.6	93.4	104.4	96.4 ± 6.9
	480.0	92.5	90.6	90.4	91.1 ±1.1
	960.0	88.7	85.3	99.0	91.0 ± 7.1
Genistein	2.0	104.5	100.3	100.9	101.9 ± 2.2
	10.0	94.0	97.7	100.4	97.3 ± 3.2
	20.0	99.4	92.7	88.1	93.4 ±5.6
	240.0	80.5	83.6	94.2	86.1 ± 7.1
	480.0	87.6	85.0	89.0	87.2± 2.0
	960.0	85.1	90.3	91.8	89.1 ± 3.5
Glycitein	10.0	99.1	101.8	103.3	101.4 ±2.1
	20.0	99.9	101.7	101.3	100.9 ± 0.9
	240.0	96.4	98.7	105.7	100.2 ± 4.8
	480.0	97.0	101.6	93.7	97.4 ±3.9
	960.0	96.6	101	98.3	98.6 ± 2.2
Equol	30.0	100.7	110.3	95.8	102.2 ± 7.3
	60.0	101.4	98.9	95.8	98.7 ± 2.8
	600.0	85.7	92.4	102.6	93.5 ± 8.5
	1200.0	90.0	94.6	94.2	92.9 ± 2.5
	2400.0	99.9	97.7	96.8	98.1± 1.6
4MUF[a]	800.0	101.5	100.0	99.9	100.4± 0.7
Phenolphthalein	800.0	90.9	95.6	89.5	92.0± 2.6
Apigenin	800.0	82.2	89.2	86.2	86.0± 2.8

[a]4-Methylumbelliferone

2.3 Results and Discussion:

In this study, a rapid, accurate method based on HPLC/MS combined with a PDA has been developed that permits the sensitive measurement of isoflavones and their metabolites in rat plasma samples. As far as we are aware, this is the first report of a method to determine the plasma concentration of aglycone isoflavones and metabolites of daidzein and genistein in one fast run of 16.0 min with well separated peaks using a Zorbax SB-CN reversed-phase column. This method builds on a previously published technique (10) and results in improved selectivity, sensitivity, and precision, as well as shorter run time for the detection of a full spectrum of isoflavones in rat plasma. Figures 1-3 present representative HPLC/MS chromatograms of isoflavones. Figure 2 shows a representative chromatogram for the standard isoflavone glucosides (daidzin, glycitin, genistin), aglyconees (daidzein, genistein, glycitein), Biochanin A, equol and 3 internal standards (Apigenin, phenolphthalein β-D glucoronide, and 4-methylumbelliferyl sulfate). The retention times were for daidzin, 2.9 min; glycitin, 3.3 min; genistin, 5.2 min; 4-methylumbelliferone, 5.3 min; daidzein, 9.7 min; glycitein, 10.0 min; equol, 11.3 min; genistein, 11.7 min; apigenin, 12.6 min; phenolphthalein, 13.0 min; and Biochanin A, 14.9 min. Genistin and 4-methylumbelliferone coeluted with very close retention times of 5.2 and 5.3 min, respectively, however, further analysis with MS showed no mass interference between those 2 compounds. Isoflavone concentrations in rat plasma samples were analyzed with and without enzyme hydrolysis (Figure 1 and 3, respectively).

Quantitative measurements of isoflavones and their metabolites using chromatographic methods required appropriate internal standards to correct for unknown losses during the procedure used. These standards range from ^{2}H – and ^{13}C- labeled stable isotope forms of the isoflavones of interest, or compounds with similar chemical structure and properties that are not naturally present in the sample to be studied (Wang *et al.,* 2002). For LC-based techniques, apigenin, 4-methylumbelliferyl-sulfate, and phenolphthalein β-D-glucuronide have been used to correct for extraction and incomplete hydrolysis (Griffith and Collison, 2001; Barnes *et al.,* 1999; Coward *et al.,* 1996). The use of isotopically labeled compounds (^{2}H or ^{13}C) as internal standards was mostly reported in GC/MS methodologies; because of their extensive set of workup steps, these methods have the greatest need for stable internal standard (Wang *et al.*, 2002).

A choice of apigenin as an internal standard was first introduced by Barnes *et al.* (1999) for LC/MS analysis of isoflavones; however, apigenin did not resolve from genistein using an isocratic HPLC separation. Apigenin was later separated from genistein by Griffith and Collison (2001) with a long run of 55.0 min. In the gradient LC/MS method reported here, genistein and apigenin are well resolved with retention times of 11.7 and 12.6 min, respectively (Figure 2). Furthermore, apigenin is commercially available, and it has a chemical structure very similar to the isoflavone aglyconees (analog of genistein, 5, 7, 4'-trihydroxyflavone) and is more hydrophobic than genistein. This chemical

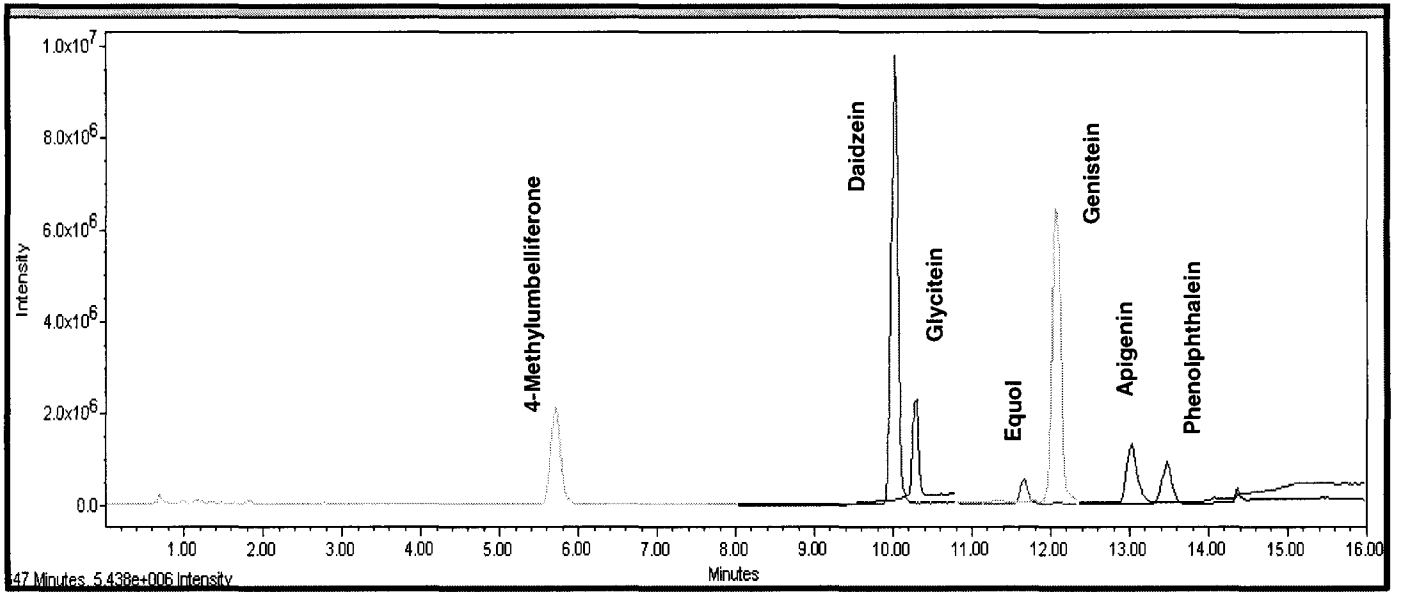


Figure 1: A representative HPLC/MS chromatogram of isoflavones in a rat plasma sample after enzyme hydrolysis.

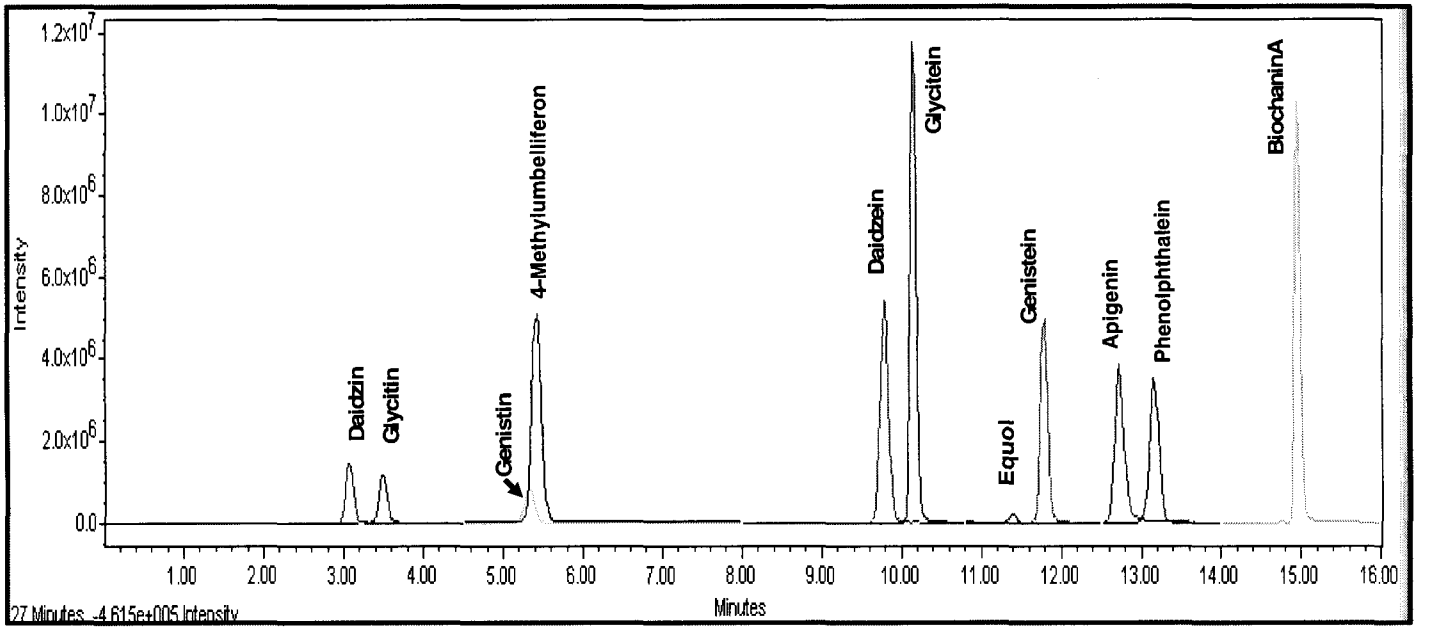


Figure 2: A representative HPLC/MS chromatogram of isoflavone standards.

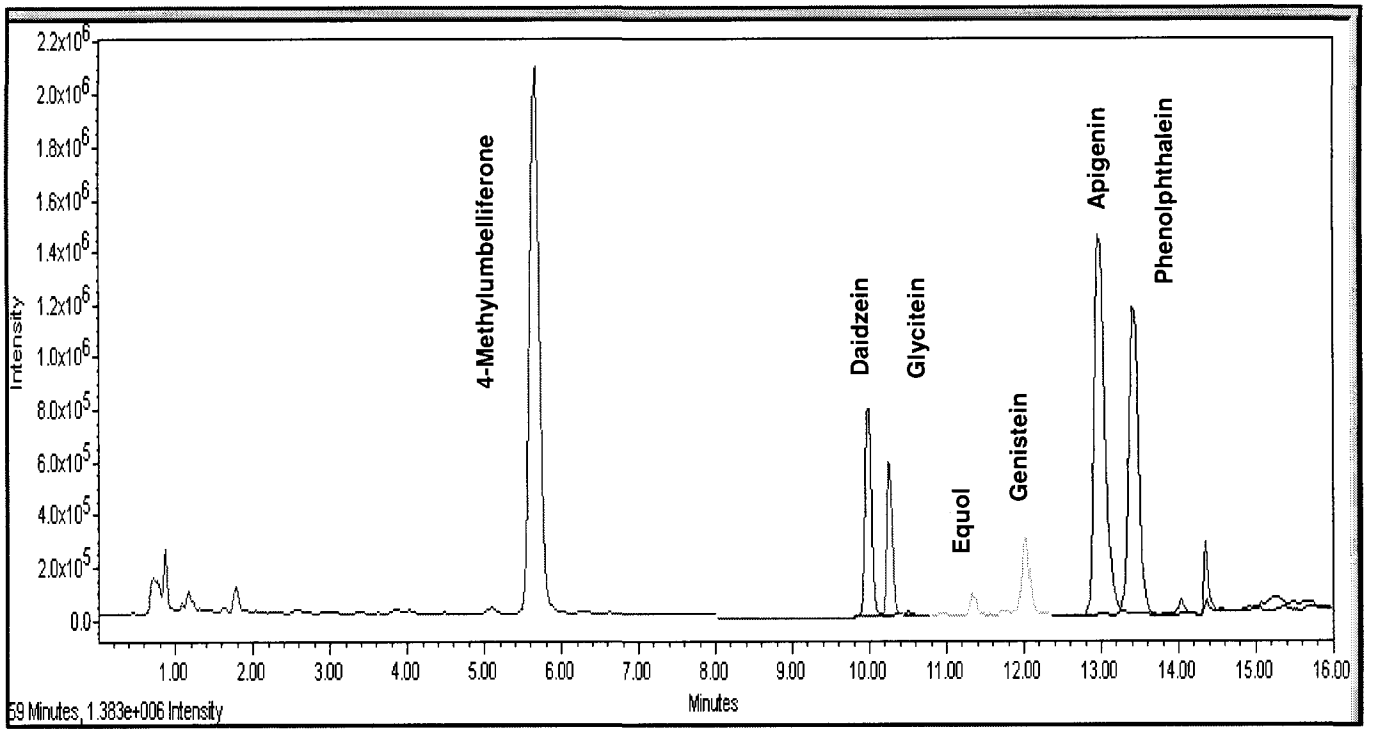


Figure 3: A representative HPLC/MS chromatogram of isoflavones in a rat plasma sample without enzyme hydrolysis.

property of apigenin is useful for detection of any hydrophobic loss of the isoflavones during the extraction and analysis (Griffith and Collison, 2001).

Two other internal standards were added at the beginning of the extraction (4-methylumbelliferyl-sulfate, and phenolphthalein β-D-glucuronide) to check for the efficiency of enzymatic hydrolysis in each sample. Detection of unconjugated phenolphthalein and 4-methylumbelliferone and the disappearance of phenolphthalein β-D-glucuronide and 4-methylumbelliferyl-sulfate was an indication that the enzyme hydrolysis was efficient. Recovery [mean (%) ± standard deviation (SD)] of isoflavones from plasma after enzyme hydrolysis and liquid-phase extraction, as determined by LC/MS and calculated against external calibration curve analyzed on 3 different days, are reported in Table 6. Average recovery of 6 different concentrations of daidzein, genistein, glycitein, and equol were 94.9 ± 7.3, 90.6 ± 5.4, 102.5 ± 9.2, and 94.1 ± 6.5, respectively. It was demonstrated that this extraction procedure was 86-100 % efficient in recovery of isoflavone aglyconees, which was higher in comparison to previously reported data (Holder *et al.*, 1999). However, for the Biochanin A, a low recovery (75 - 85%) was obtained at high concentrations due to the chemical nature of this compound. Bioachanin A is the most hydrophobic compound of interest, and low recovery is due to the hydrophobic loss of this compound at high concentration (results not shown).

LODs obtained from authentic standards ranged between 0.2 and 20.0 nM and LOQs ranged between 0.5 and 60.0 nM, allowing for sensitive quantitation of isoflavones (Table 7).

Soy isoflavones circulate in blood in several molecular forms, including glucuronide and sulfate conjugates (95%), freely circulating aglyconees, and protein-bound aglyconees (5%; Barnes *et al.*, 1999; Coward *et al.*, 1993). There are 2 conjugation sites on genistein, daidzein and glycitein, and each of these sites can be sulfated or glucuronidated. Thus, there are monoglucuronides, monosulfates, diglucuronides, disulfates and mixed conjugates with one site glucuronidated and one site sulfated (Shelnutt *et al.*, 2002). In order to obtain the total isoflavone concentration in rat plasma samples, there is a need to use enzymatic digestion of the isoflavone conjugates with subsequent detection of aglyconees. Accurate quantification of the isoflavone concentration depends on the complete hydrolysis of their conjugated forms. Therefore, a time-course experiment was conducted from 0 to 24 h (0, 15, 30, 45, 60, 120, 180, and 240 min, and 24 h) incubation time. Maximum hydrolysis was monitored by the disappearance of the conjugated forms of the internal standards [phenolphthalein β-D glucuronide (*m/z* 495.5) and 4-methylumbelliferyl sulfate (*m/z* 257.0)] and the appearance of the deconjugated forms [phenolphthalein (*m/z* 319.2) and 4-methylumbelliferone (*m/z* 177.1)], monitored by LC/MS in positive ion mode. The time course of isoflavone hydrolysis by commercial hydrolytic enzymes was previously determined to be maximal at 30 min (Holder *et al.*, 1999). In this study, maximum hydrolysis was obtained after 45 min incubation at 37^0C. Incomplete incubation time would result in incomplete hydrolysis of conjugated isoflavones and a low result. Failure to detect unconjugated phenolphthalein and 4-methylumbelliferone in the expected amounts identified insufficient enzyme

hydrolysis by this method. Deconjugation of 4-methylumbelliferyl-sulfate to 4-methylumbelliferone was very rapid, while for phenolphthalein β-D-glucuronide, quantitative formation phenolphthalein occurred after 45 min. In this study, enzyme solution was prepared daily, however according to Twaddle *et al.* (2002), enzymatic activity is stable to at least 5 freeze-thaw cycles, and stable in the frozen state for at least 2 weeks.

The resulting unconjugated isoflavones were then extracted by liquid - liquid extraction using ethyl acetate. Liquid-liquid extraction has the advantage over solid- phase extraction (SPE) in that most of the electrolytes in the plasma sample (Na^+, K^+, and phosphate) are left behind in the aqueous phase (Barnes *et al.*, 1999). Because more-hydrophilic compounds prefer the polar aqueous phase, whereas more-hydrophobic compounds will be found mainly in the organic solvent (Snyder, 1997), polar solvents have been recommended for efficient extractions of isoflavones (Coward *et al.,* 1993; Setchell *et al.,* 1987). In our laboratory, the best extraction efficiency was found using 3 extractions each with 2 mL ethyl acetate. A fourth extraction with 2 mL solvent improved the recovery of the isoflavones by less than 2% and was, therefore, not utilized. The presented method for extraction of isoflavones and their metabolites in rat plasma is shown in Figure 4.

Table 7: Limits of detection and quantitation for isoflavones analyzed with the proposed LC/MS method.

Analyte	Limit of detection, nM	Limit of quantitation, nM
Daidzein	0.3	0.8
Genistein	0.5	1.5
Glycitein	0.2	0.5
Daidzin	0.4	1.2
Genistin	1.5	5.0
Glycitin	1.5	5.0
4-methylumelliferone	1.0	3.0
Biochanin A	0.3	1.0
Equol	20.0	60.0

In this study, in addition to liquid-phase extraction, SPE with Strata-X [Phenomenex (Torrance, CA), 30 mg, 1 mL, 33 µm particle size, 85 A pore size, and 800 m^2/g surface area] was performed for standard isoflavone aglyconees (daidzein, genistein, glycitein); equol; Biochanin A; phenolphthalein; and 4-methylumbelliferon. Potential interferences were removed by the addition of a wash solution [1 mL, ACN-methanol (50 +50)]. Recovery of the standards was above 90%, except for Biochanin A and phenolphthalein (75.1 and 75.3%, respectively).

Twaddle *et al.* (2002), demonstrated and validated a reversed-phase SPE method with Isolute ENV+, 25 mg columns (Jones Chromatography, Lakewood, CO) for serum samples containing genistein, daidzein and equol in the 96-well format for subsequent LC/ESI-MS/MS or LC/ESI-MS analysis.

A Zorbax SB-CN column in a combination with an ACN-formic acid (0.1% in water) elution system exhibited the best selectivity, recovery, and peak shape, and the shortest retention time for all the analytes of interest compared with other HPLC columns (Phenomenex, Luna C18, 150 x 2.1 mm, 3 µm and Phenomenex ultracarb, 150 x 2.0 mm, 5 µm) with different mobile phases and different acidity levels. The choice of the more hydrophilic column (silica-based packing with cyanopropyl stationary phase; Zorbax SB-CN) compared with C18, allows better peak separation for equol from genistein, phenolphthalein, and 4-methylumbelliferone and from the rest of the compounds. The chromatographic run time with this column was reduced from 55.0 min with the C18 column to 16.0 min.

4-Ethyl phenol (a metabolite of genistein with the lowest molecular weight of all compounds of interest, Mw= 122.1) was detected by running standard and the internal standard (4-Methylumbelliferone) through the PDA detector. Because of the chemical structure of this compound and the lack of extra electron pair donation from nitrogen groups for protonation in the MS detector, we were able to detect it in the same run with the PDA detector by Tee-splitting the flow of the effluent, 75% to PDA and 25% to MS detectors. Monitoring was carried out at 276.4 nm to achieve sensitive detection at or very near the absorption maximum of 4-ethyl phenol, which had a retention time of 7.6 min (Figure 5). In plasma, no detectable level of 4-ethyl phenol was obtained.

In conclusion, the HPLC/MS combined with a PDA detection method described in this study allowed the measurement of isoflavones, including aglyconees as well as the metabolites of daidzein and genistein, in a short run time of 16.0 min. It also required fewer steps for sample preparation, extraction, and less technician time compared with the previously published methods. Recently, further advances in LC, termed ultra- performance (UPLC), offers significant theoretical advantages in resolution, speed, and sensitivity for analytical determinations, particularly when coupled with mass spectrometers capable of higher-speed acquisitions compared with LC/MS (Churchwell *et al.,* 2005).

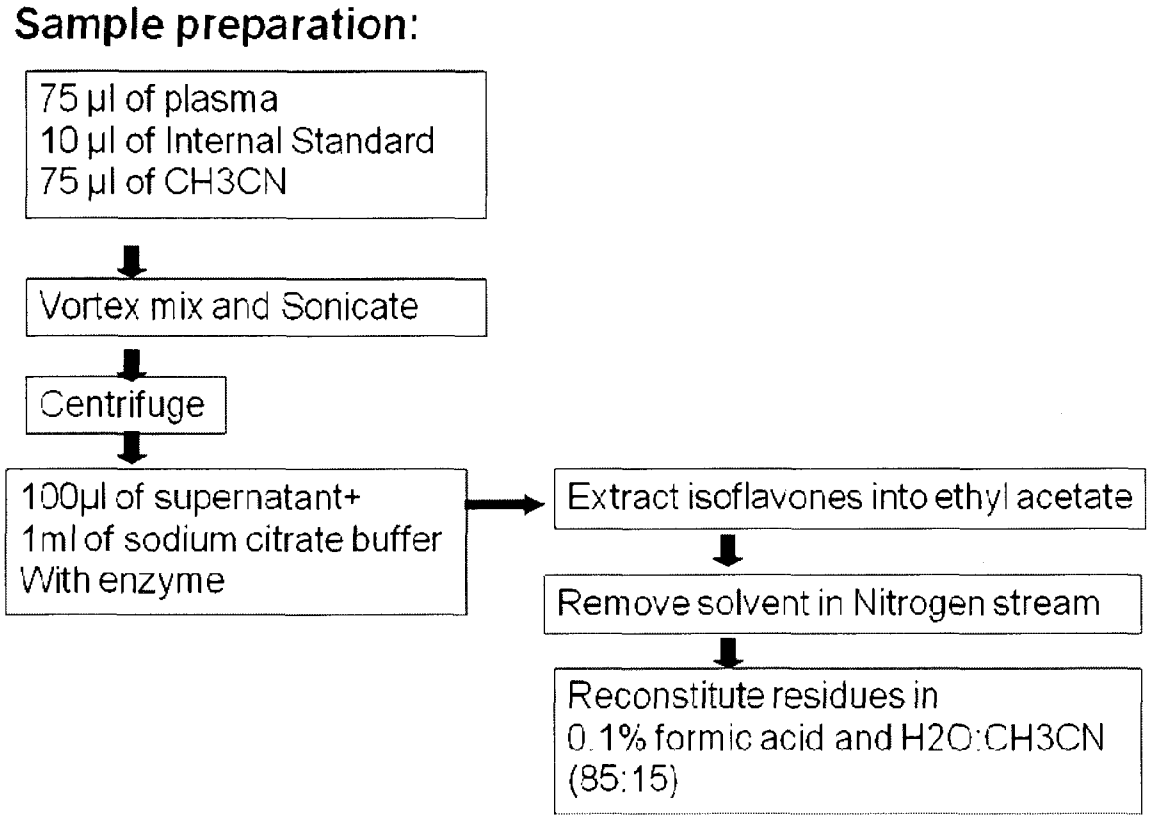


Figure 4: Sample preparation and extraction for isoflavone conjugates in rat plasma

2.4 Acknowledgments

We thank Robert Peace and Nick Hidiroglou, Health Canada, Health Products and Food Branch, Nutrition Research Division, Ottawa, Ontario, Canada; Daniel R. Doerge, National Centre for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR; and Pierluigi Delmonte, U.S. Food and Drug Administration, College Park, MD, for critical reviewing the manuscript. The high-level technical support of Dr. Daniel R. Doerge is gratefully acknowledged. The tested Zorbax SB-CN column (4.6 X 75 mm, 3.5 µm particle size) was a kind gift of Agilent Technologies.

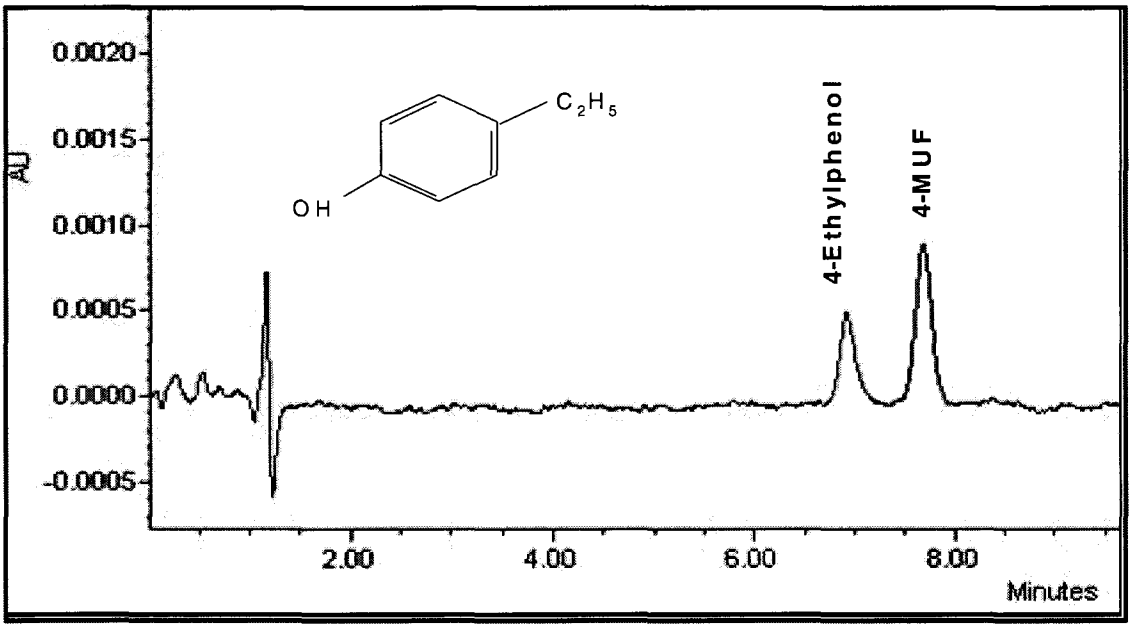


Figure 5: Structure and chromatogram detected by UV of 4-Ethyl phenol

Note to Chapter 2:

Standards:

(i) Daidzin, genistin, glycitin, daidzein, genistein, glycitein and equol (metabolite of daidzein) were purchased from LC Labs. (Woburn, MA, USA). The purity of each standard used was verified using LC/MS method (Sepehr *et al.*, 2006). LC/MS spectra agreed with the structure of each isoflavone and matched the spectra obtained from a purchase standard of each isoflavone, indicating a purity of above 99% for each lot.

(ii) Biochanin A, 4-ethyl phenol (metabolite of genistein) and apigenin- Sigma-Aldrich Co. The purities of these three compounds were above 97%, 99%, and 95% respectively. For apigenin, LC/MS spectra indicated one major peak and minor impurities in some of the lots. Probe/MS testing in different lots of apiginin indicated one major component with minor components, suggesting little impurities. Therefore apiginin did not use as an internal standard in analyzing plasma samples.

National Centre for Complementary and Alternative Medicine (NCCAM) guidlines were followed in assuring quality of isoflavone-sourced materials.

To ensure stability, the bulk chemical was stored at -80 ^{0}C, protected from light in the original shipping containers. Purity was periodically measured during the study; no degradation of the bulk chemical was detected.

Sample centrifugation: Speed of 14000 rpm, for 6 min at 4^{0}C with relative centrifugal forces of 16,750 g in an IEC Centra MP4R refrigerated centrifuge, Cat. No. 2438 (Needham Heights, MA) was used to precipitate proteins.

Enzyme hydrolysis:

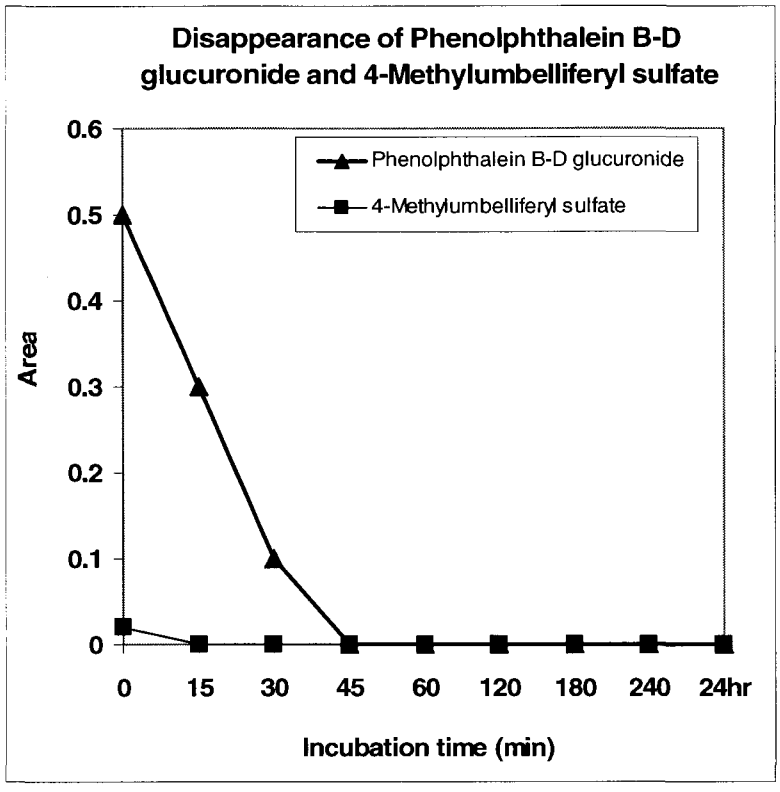
There are two conjugated sites on genistein, daidzein, and glycitein and each of these sites can be sulfated or glucuronidated (Shenutt *et al.,* 2002). Without liquid chromatography-double mass spectrometry (LC-MS-MS), the lack of commercially available standards for counjugates has led our laboratory to use enzymatic digestion of isoflavone conjugates with subsequent detection of the aglyconees. Therefore, a time-course experiment was conducted from 0 to 24 h (0, 15, 30, 45, 60, 120, 180, and 240 min and 24 h) to determine the maximum incubation time required to reach maximum hydrolysis.

On the day of the analysis, plasma samples of rats on casein based diet were thawed at room temperature and mixed on a Vortex mixer, and 75 µL aliquots were added to equal volumes of ACN and 10 µL of the internal standard solution (12.8 µM) of phenolphthalein β-D glucuronide and 4-methylumbelliferyl sulfate in 1.5 mL Eppendorf tubes. Tubes were mixed on a Vortex mixer, sonicated for 10 min, and centrifuged at 14000 rpm (16,750 x g) for 6 min at 4^0C to precipitate proteins. An aliquot of the supernatant (100 µL) was combined with 1 mL sodium citrate buffer and incubated at 37^0C for 15, 30, 45, 60, 120, 180, and 240 min and 24 h. Maximum hydrolysis was monitored by the disappearance of the conjugated forms of the internal standards (phenolphthalein β-D glucuronide and 4-methylumbelliferyl sulfate) and the appearance of the deconjugated forms (phenolphthalein and 4- methylumbelliferone). The disappearance of phenolphthalein β-D glucuronide completed at 45 minutes and

4-methylumbelliferyl sulfate completed at 15 minutes. Addittionally, at 45 minutes formation of phenolphthalein was completed and increased incubation may have less than 2% effect in phenolphthalein formation. Failure to detect unconjugated phenolphthalein and 4-methylumbelliferone in the expected amounts, if it occurred, would indicate insufficient enzyme hydrolysis.

System Stability

The stability of the system was checked by injecting a known concentration of standard twenty times in one day before and after of each batch of about sixteen samples running every day.



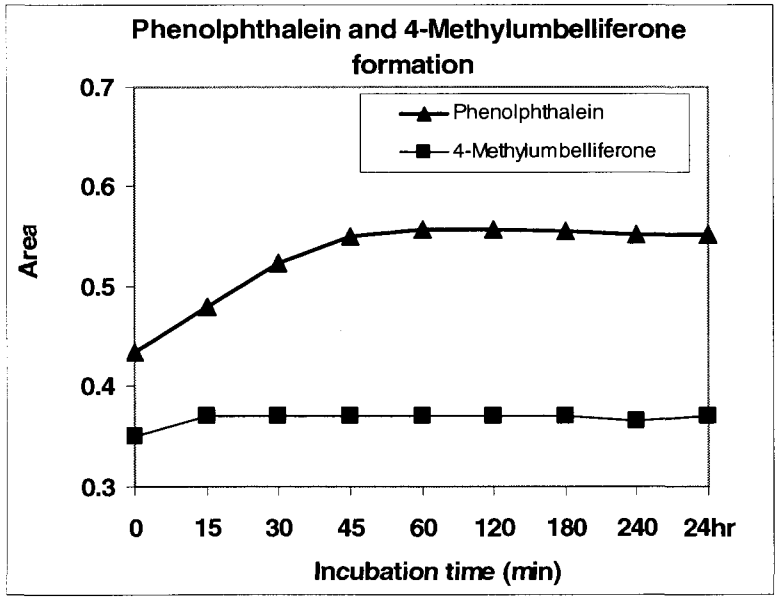


Figure 6: Time course of enzyme hydrolysis of two internal standards (phenolphthalein β-D glucuronide and 4-methylumbelliferyl sulfate) at 37°C over the interval of 0-24h.

CHAPTER 3

3.0 Bioavailability of soy isoflavones in rats. Part I: Application of accurate methodology for studying the effects of gender and source of isoflavones

Estatira Sepehr, Gerard M Cooke, Patrick Robertson, G. Sarwar Gilani "Bioavailability of soy isoflavones in rats. Part I: Application of accurate methodology for studying the effects of gender and source of isoflavones", *Mol. Nutr. Food Res.* 2007, 51, 799-812

Study Context and Collaborations:

The development of a HPLC/MS method for analysing rat plasma sample concentration of soy isoflavones (Chapter 2), and gender differences observed in plasma isoflavone concentration from the multigeneration rat study (Appendix.1, Tables 2-5) warranted an additional investigation of the pharmacokinetics and bioavailability of soy isoflavones. Additionally, development of an accurate methodology for measurement of bioavailability based on oral and IV administration of purified synthetic isoflavones (daidzein, genistein, glycitein and their respective β-glucosides) and Novasoy^{TM}, a commercial dietary isoflavone supplement, was investigated in detail.

I was responsible for animal study design, implementation and co-ordination of the study. I was extensively involved in all aspects of animal project such as organizing meetings, designing control and experimental diets, random assigning of rats to metabolic cages. Saphenous vein blood collections were done by the highly trained technicians of the Animal Resources Division at Health Canada. I was involved in collection of rat blood, urine and fecal samples at all different time points. Mr. Robertson was partially involved in collection of the rat blood, urine and fecal samples. However, he was involved in mantainance of LC/MS instruments throughout the project.

I analysed blood samples obtained from rats at different time points, pharmacokinetic analysis and statistical analysis of the results.

I presented the following four posters based on the results obtained from this Chapter:

Estatira Sepehr, G. Sarwar Gilani, Patrick Robertson, Gerard Cooke, Benjamin P.-Y.Lau. , Jocelyn Fournier. Bioavailability of soy isoflavones as affected by gender, age and food matrix in rats, *6th international symposium of soy, Chicago, IL., U.S.A,* October 30- November 2, 2005.

Estatira Sepehr, Gerard Cooke, Jocelyn Fournier, Patrick Robertson, G. Sarwar Gilani, Effects of sex, age and source of soy isoflavones on their bioavailability in a rat model, *Health Canada Research Forum, Ottawa, Ont.*, October, 2003.

Estatira Sepehr, Gerard Cooke, Patrick Robertson, G. Sarwar Gilani, Bioavailability of Dietary soy isoflavones in a rat model. *5th international symposium of soy, Orlando, Fl., U.S.A,* September, 2003.

Estatira Sepehr, Gerard Cooke, Patrick Robertson, G. Sarwar Gilani, Effect of the gender on the metabolism of soy isoflavones in rats. *46th Annual Meeting Canadian Federation of Biological Societies, Ottawa, Ont,* June, 2003.

Additionally, I was involved in preparation of a manuscript which was published in *Journal of AOAC Int.*

Gilani G.S, Cockell K.A, **Sepehr E**, 2005, Effects of antinutritional factors on protein digestibility and amino acid availability in foods. *J AOAC Int.*, 88(3):967-87.

Abstract:

There are limited and controversial reports about the effects of gender and source of isoflavones on their bioavailaibility. Moreover, several previous studies have not used appropriate methodology to determine the bioavailability of soy isoflavones, which requires comparing the area under the plasma concentration-time curve after both oral and intravenous (IV) administration. Therefore, the present study was conducted to determine the bioavailability of isoflavones from different sources following both oral and IV administration in male and female rats. Three sources of isoflavones; Novasoy^{TM} (a commercial supplement), a mixture of synthetic aglyconees (daidzein, genistein and glycitein) and a mixture of synthetic glucosides (daidzin, genistin and glycitin) were tested. Following administration, blood samples were collected at several time points (0, 10, 30 min and 1, 2, 8, 24, 48 h post oral gavage and 0, 10, 30, 45 min and 1, 2, 3, 4, 8 h post-IV dosing) and plasma isoflavones were measured by LC/MS. Bioavailability values for daidzein, genistein and glycitein were significantly (P<0.05) higher (up to sevenfold) in Novasoy^{TM} and the glucoside forms of isoflavones compared with those of the aglycone forms. Moreover, significant (P<0.05) gender differences in the bioavailability of 7–hydroxyl–3-(4'-hydroxyphenyl)–chroman (a metabolite of daidzein), glycitein and daidzein were observed for Novasoy^{TM}, with higher values in male rats. In summary, the source of isoflavones and the sex of rats had significant effects on isoflavone bioavailability.

Abbreviations:

AUC, area under the concentration-time curve; C_{max}, maximum observed peak plasma; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; Equol, 7–hydroxyl–3-(4'-hydroxyphenyl)–chroman; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; IV, intravenous injection; MS, mass spectrometry; O-DMA, O-desmethylangolensin; Cl_{or}, plasma clearance; SD, standard deviation; $t_{1/2}$, elimination half-life; Vd_{or}, volume of distribution.

3.1. Introduction:

Phytoestrogens have gained increasing interest in recent years because of their potential health benefits in cases of certain cancers, cardiovascular diseases, and alleviation of symptoms of menopause and bone loss in postmenopausal women [Setchell, 1998; Setchell and Cassidy, 1999; Thompson *et al.*, 2006]. These naturally occurring plant compounds with estrogenic or anti-estrogenic activities comprise three major classes: isoflavones, lignans and coumestans [Setchell, 1998; Setchell and Cassidy, 1999; Thompson *et al.*, 2006]. Soybeans, soy protein products and soy- based infant formula are the most significant sources of isoflavones [Michihiro, 2006]. Moreover, a number of isoflavone-rich supplements have become available for over the counter sale.

The major isoflavones present in soy are predominantly in their glycoside forms (daidzin, genistin, and glycitin), with daidzin and genistin being the most abundant [Setchell, 1998; Boersma *et al.,* 2001; Wang and Murphy, 1994]. However, in fermented soy products such as miso or tempeh, the unconjugated aglyconees (daidzein, genistein, glycitein) are the predominant forms, because the fermentation process causes cleavage of the glucoside bond [Wang and Murphy, 1994].

Isoflavones, when ingested predominantly as the ß-glucoside form, are hydrolyzed in the gut by both intestinal mucosal and bacterial ß-glucosidases releasing the aglyconees. Aglyconees are in turn either absorbed intact or further metabolised by intestinal microflora in the large intestine into other metabolites such as 7–hydroxyl–3-(4'-hydroxyphenyl)–chroman (equol) or *O*-

desmethylangolensin (*O*-DMA) (from daidzein), p-ethyl phenol (from genistein) and di-hydroglycitein (from glycitein) [Gilani and Anderson, 2002; Cassidy *et al.*, 2006].

Controversial evidence exists regarding the bioavailability of the aglycone and glucoside forms of isoflavones in animal and human studies [Izumi *et al.*, 2000; Setchell *et al.*, 2001; Zubik and Meydani, 2003]. Some studies have reported that aglyconees were absorbed more efficiently than glucosides [Izumi *et al.*, 2000], while other data suggest that the resulting bioavailability of daidzein and genistein was greater when soy isoflavones were ingested as glucosides rather than aglyconees [Setchell *et al.*, 2001]. However, in one study, there was no difference in bioavailability following consumption of aglycone or glucoside tablets [Zubik and Meydani, 2003].

There is also limited information regarding gender influences on the bioavailability of isoflavones in humans or animal models. Cassidy *et al.* 2006 reported significant effects of gender and food matrix on the area under the concentration-time curve (AUC) of isoflavones in humans, and Chang *et al.* 2000 reported significant gender differences in the elimination half-life ($t_{1/2}$) and AUC in adult rats.

In most of these earlier studies, the determination of bioavailability was based on the oral administration of isoflavones, which is an acceptable method for the determination of bioavailability in food sources. Some isoflavones such as genistein, are also considered to be pharmaceutical agents. Therefore, the bioavailability of these compounds (as drugs) should be determined by

comparing the AUC of the plasma-concentration time curve after intravenous (IV) administration with the AUC after oral administration [Janning *et al.*, 2000; Coldham *et al.*, 2002; Moon *et al.*, 2006].

The objectives of the present study were to develop accurate methodology for measurement of bioavailability based on oral and IV administration of purified synthetic isoflavones (daidzein, genistein, glycitein and their respective β-glucosides) and Novasoy™, a commercial dietary isoflavone supplement, and to investigate the effects of the three isoflavone sources and of gender on the bioavailability and pharmacokinetics of soy isoflavones in rats.

3.2. Materials and methods:

3.2.1 Chemicals

The following chemical with the indicated specification were used.

(i) Sodium citrate buffer (25 mM, pH 5.0).

(ii) Hydrolytic enzyme. - *Helix pomatia* type H-5 (S3009), containing 29 units/mg solid sulfatase activity was purchased from Sigma- Aldrich Co (St. Louis, MO, USA). A solution containing 23 units of sulfatase activity was prepared by dissolving an appropriate amount of enzyme in 1.0 mL of the sodium citrate buffer [Sepehr *et al.*, 2006].

(iii) Dimethyl sulfoxide (DMSO). - 99.9% HPLC grade (Sigma-Aldrich Co.).

(iv) Water. - Deionized, NANO-pure (Diamond UV ultra-pure water purification system; Barnstead International, Essex, UK).

3.2.2 Apparatus

(i) HPLC. - The LC separations were performed by using a Waters (Milford, MA, USA) Alliance 2695 liquid chromatograph equipped with a Zorbax SB-CN reversed-phase column (4.6 X 75 mm, 3.5 µm particle size; Agilent Technologies, Wilmington, DE, USA).

(ii) MS system. A Waters Micromass ZQ single quadrupole mass spectrometer was operated in the positive ion SIM mode. The entire system from sample injection to data acquisition was computer-controlled with Empower software (Waters).

3.2.3 Experimental Diets:

(i) NOVASOY™ (concentrate #152-400) soy isoflavone (Archer Daniels Midland Company, Decatur, IL, USA). One gram of Novasoy™ contains 248 mg isoflavones (the remainder was moisture, carbohydrate, protein, fat and ash). The total content of genistein, daidzein and glycitein in the Novasoy™ was determined by Waters HPLC linear gradient with UV detection monitored at 254 nm [Wang and Murphy, 1994].

To prepare the 20 mg/kg oral dose of Novasoy™, 80.645 mg of Novasoy™ powder was used according to the following calculation:

(1mg x 20 mg/Kg) / 0.248 mg = 80.645 mg /Kg rat body weight.

(ii) Daidzin, genistin, glycitin, daidzein, genistein, glycitein. - LC Labs. (Woburn, MA, USA).

The ratio of genistein, daidzein and glycitein in Novasoy™ was 1.0: 0.5: 0.2 respectively. Synthetic glucosides and respective aglyconees were prepared with the same ratio of 1.0: 0.5: 0.2 for genistein, daidzein, and glycitein respectively. Thus the aglycone diet (20 mg/kg body weight) composed of 11.76 mg genistein, 5.88 mg daidzein and 2.36 mg glycitein.

The glucoside dose (20 mg/kg body weight) was prepared taking into account the differences in molecular weight between the aglycone and glucoside form. Therefore the glucoside dose was composed of: 19.09 mg genistin, 9.62 mg daidzin and 3.70 mg glycitin. The molecular weights for isoflavone glucosides and aglyconees are reported in Table 1.

(iii) Casein protein (90% purity, ICN Biomedicals, Costa Mesa, CA, USA). The composition of the casein based isoflavone-free diet is reported in Table 2.

3.2.4 Preparation of diets:

Oral gavage:

A mixture of synthetic glucosides (genistin, daidzin, glycitin); or a mixture of synthetic aglyconees (genistein, daidzein, glycitein) or Novasoy™ were suspended in ultra-pure water and the administered volume was adjusted to 2.5 mL to provide a dose of 20 mg /kg body weight of rats. Solutions were sonicated for one hour prior to the oral administration.

Intravenous (tail vein) injection

A mixture of synthetic glucosides (genistin, daidzin, glycitin in the same proportion as for oral gavage); or a mixture of synthetic aglyconees (genistein,

Table 1: Molecular weight for isoflavone glucosides and aglyconees

Isoflavone glucoside	MW a	MWg	MWa/MWg
Genistin	270.23	438.37	0.616
Daidzin	254.23	416.36	0.611
Glycitin	284.27	446.21	0.637

Table 2: Composition of the casein based isoflavone-free diet

Ingredient	g/kg
Vitamin free casein[a)]	222.20
Corn starch	477.30
Sucrose	100.00
Fibre (Sulfa-Floc)	50.00
Soybean oil	100.00
AIN-93-G Mineral Mix[b)]	35.00
AIN-93-V Vitamin Mix[b)]	10.00
Choline Bitartrate	2.50
DL-methionine	3.00
Tert-Butylhydroquinone	0.01
Actual isoflavone content[c)] ,mg/kg diet	0.00

a) Casein from ICN Biomedicals contains 90% crude protein.
b) AIN-93-G Mineral Mix [Reeves *et al.*, 1993] and AIN-93-V Vitamin Mix [Lampe *et al.*, 1998] were from ICN Biomedicals.
c) The actual content of isoflavones was determined by Waters HPLC linear gradient with UVdetection monitored at 254 nm [Wang and Murphy, 1994].

daidzein, glycitein, also in the same proportion as for oral gavage) was suspended in 50% v/v aqueous DMSO and the volume adjusted to provide 10 mg/kg body weight of rats such that the intended doses were delivered in a volume of 1.0 µL/g rat body weight. This amount of DMSO has been shown to be well tolerated by mice when administered by rapid IV or extra vascular injection [Supko and Malspeis, 1995].

3.2.5 Animals, isoflavones administration and samples collection:

Forty female and male 90-day-old (250 - 400 g) Sprague Dawley rats (Charles River, St- Constant, QC, Canada), were individually caged in rooms where the temperature was maintained at 23 ± 2°C and lights were on a 12-h light/dark cycle. All aspects of the experimental protocol were reviewed and approved by the Health Canada, Ottawa, Animal Care Committee.

During an adjustment period of 10 days, rats were fed an isoflavone-free casein based control diet formulated according to the American Institute of Nutrition (AIN-93G) recommendations [Reeves *et al.*, 1993]. After the adjustment period, rats of each sex were randomly assigned to groups of four animals; those rats destined for oral administration were gavaged with a single oral dose of one of the three sources of isoflavones (Novasoy^{TM}, a mixture of synthetic aglyconees or a mixture of synthetic glucosides). Rats assigned for IV injection, were injected (tail vein) with one of the two sources of isoflavones (a mixture of synthetic aglyconees or a mixture of synthetic glucosides) by using a 26-gauge needle (Becton Dickinson, Rutherford, NJ, USA) without anesthesia. Post-oral or

IV administration, rats were assigned to metabolic cages with free access to isoflavone-free casein based diet and tap water for the total period of the study.

3.2.6 Rat Plasma Sample Collection:

Blood samples (0.4 mL) were collected from the saphenous vein in lithium heparin microtainers according to the method of Hem *et al.*, 1998 at 0, 10, 30 min, and 1, 2, 8, 24, 48h (post oral) for aglycone and Novasoy™ source and at 0, 2, 8, 24, 48h (post oral) for glucoside source.

To investigate the possible presence of the multiple peaks observed in the previous studies reported by Janning *et al.* 2000 and Supko and Malspeis, 1995 before 2 h, three time points (10, 30, 60 min) were added to the original (0, 2, 8, 24, 48 h) blood collection for the two groups of 90-day-old male and female Sprague-Dawley rats as the minor amendment to the original protocol. A total of 16 (8 male and 8 female) rats were gavaged with Novasoy™ and synthetic aglycone at 20 mg/Kg dose.

Blood samples were also collected at 0, 10, 30, 45 min, 1, 2, 3, 4, 8 h (post IV) for aglycone and glucoside groups. Accumulative multiple sampling was maintained at about 15% of the total rat blood circulatory volume [Diehl *et al.*, 2001].

Collected blood was centrifuged at 4◦C, 8000 rpm in an IEC Centra MP4R refrigerated centrifuge (Needham Heights, MA, USA) for 3 minutes and 250-µL aliquots were stored frozen at -80 ◦C until the day of analysis.

3.2.7 Plasma isoflavone analysis:

Plasma concentrations of isoflavones were determined by LC/MS as previously described [Sepehr *et al.*, 2006]. Briefly, following enzymatic hydrolysis of isoflavone conjugates with mixed glucuronidase/sulfatase enzyme, the resultant aglyconees were extracted with ethyl acetate, centrifuged and the supernatant was diluted with mobile phase (0.1% formic acid in 85:15 water: ACN) and injected into a Zorbax SB-CN reversed-phase column (4.6 X 75 mm, 3.5 µm particle size). The chromatographic run time was 16.0 min, with a delay of 10 min/injection.

3.2.8 Determination of plasma isoflavones pharmacokinetics:

Pharmacokinetic analysis of plasma isoflavone profiles for each rat was conducted using non-compartmental analysis software (PK Solutions^{TM} version 2.0.2 package, Summit Research Services, Ashland, Ohio, USA). The pharmacokinetic parameters were calculated using the residuals method of analysis, assuming first order disposition kinetics. The depletion kinetics was modeled for the elimination phase after oral dosing. The pharmacokinetic parameters determined were: the terminal elimination half-life, $t_{½}$ (the time taken for the plasma concentration to decrease by half); C_{max} (the maximum observed peak plasma isoflavone concentration); t_{max} (time point at C_{max}); $AUC_{(0\text{-}t)}$, the area under the concentration-time curve (reflecting the exposure of plasma to isoflavone from time zero to time t when the plasma concentration of isoflavones returned to baseline); $AUC_{(0\text{-}t)}$ was estimated using the linear trapezoidal rule and

calculated using data to the last quantifiable time point. All pharmacokinetic analyses of the rats dosed with glucoside, aglycone and Novasoy™ were calculated using 5 time points (0, 2, 8, 24, 48 h). However, only minor differences in bioavailability parameters were observed when all 8 time points were used for the analysis.

The absolute oral bioavailability was calculated from the percentage ratio of the AUCs derived from plasma isoflavone concentrations after oral and IV dosage of different sources of isoflavones to male and female rats.

$$\% \text{ Bioavailability} = [(AUC_{oral}) \times Dose_{iv} / (AUC_{iv}) \times Dose_{oral}] \times 100$$

Novasoy™ source was not injected IV, however bioavailability parameters were calculated by comparing AUC_{oral} post Novasoy™ administration by AUC_{iv} post glucoside IV injection.

3.2.9 Statistical analyses:

Statistical analyses were carried out using Sigma stat version 3.1 (2004 Systat Software Inc, Richmond CA, USA). All data were expressed as mean ± SD, and were analyzed using two-way ANOVA involving two main effects (source of isoflavones, and gender). The interaction between these two main effects (source of isoflavones x gender) was also analyzed. When warranted, post hoc analysis was performed using Holm-Sidak test. Differences were considered significant at $p < 0.05$.

3.3. Results:

3.3.1 Phase1 (oral gavage):

The profiles of plasma isoflavones (daidzein, genistein, glycitein and equol) following oral administration of the synthetic glucoside source, synthetic aglycone source and Novasoy™ to male and female 90-day-old SD rats are presented in Figs. 1-3.

Plasma concentration-time curves from rats dosed with the isoflavone glucosides revealed a significant effect of time (daidzein, $p < 0.001$; glycitein, $p < 0.001$; equol, $p = 0.018$; and genistein, $p < 0.001$) and gender x time interactions (genistein, $p = 0.025$) but no significant effect of gender (Fig. 1). Rats dosed with the aglycone form showed a significant effect of time (daidzein, glycitein, genistein, $p < 0.001$); gender (daidzein, $p = 0.020$); and gender x time interactions (daidzein, $p = 0.001$; genistein, $p = 0.036$) on the plasma concentration-time curves (Fig. 2). Rats dosed with Novasoy™ showed a significant effect of time (daidzein, glycitein, equol and genistein, $p < 0.001$); gender (daidzein, $p = 0.001$, and genistein, $p = 0.023$) and gender x time interactions (daidzein, $p < 0.001$; equol, $p = 0.037$; and geniestin, $p < 0.001$) on the plasma concentration-time curves (Fig. 3).

Pharmacokinetic data analysis from the plasma curves of the synthetic isoflavone aglycone, glucosides and Novasoy™ were examined post single–bolus oral exposure to 90-day-old SD rats (Table 3-6).

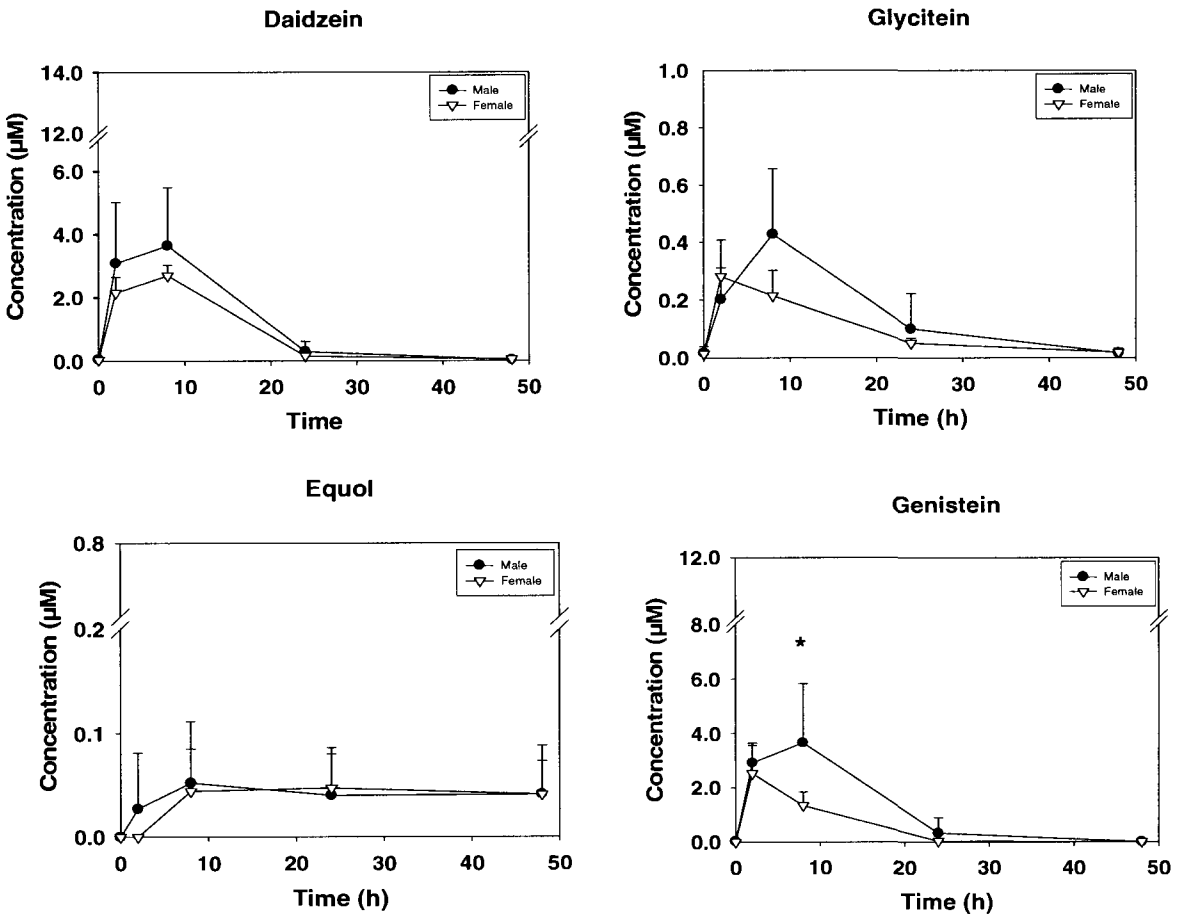


Figure 1: Plasma profiles of isoflavones in male and female (3 months) SD rats post oral gavage of a mixture of synthetic isoflavone glucosides at 20 mg/kg rat body weight.

Results are expressed as mean ± SD on a sample size of 4 rats / group. An effect of gender at specified time points is denoted by an asterisk (*), $p < 0.05$.

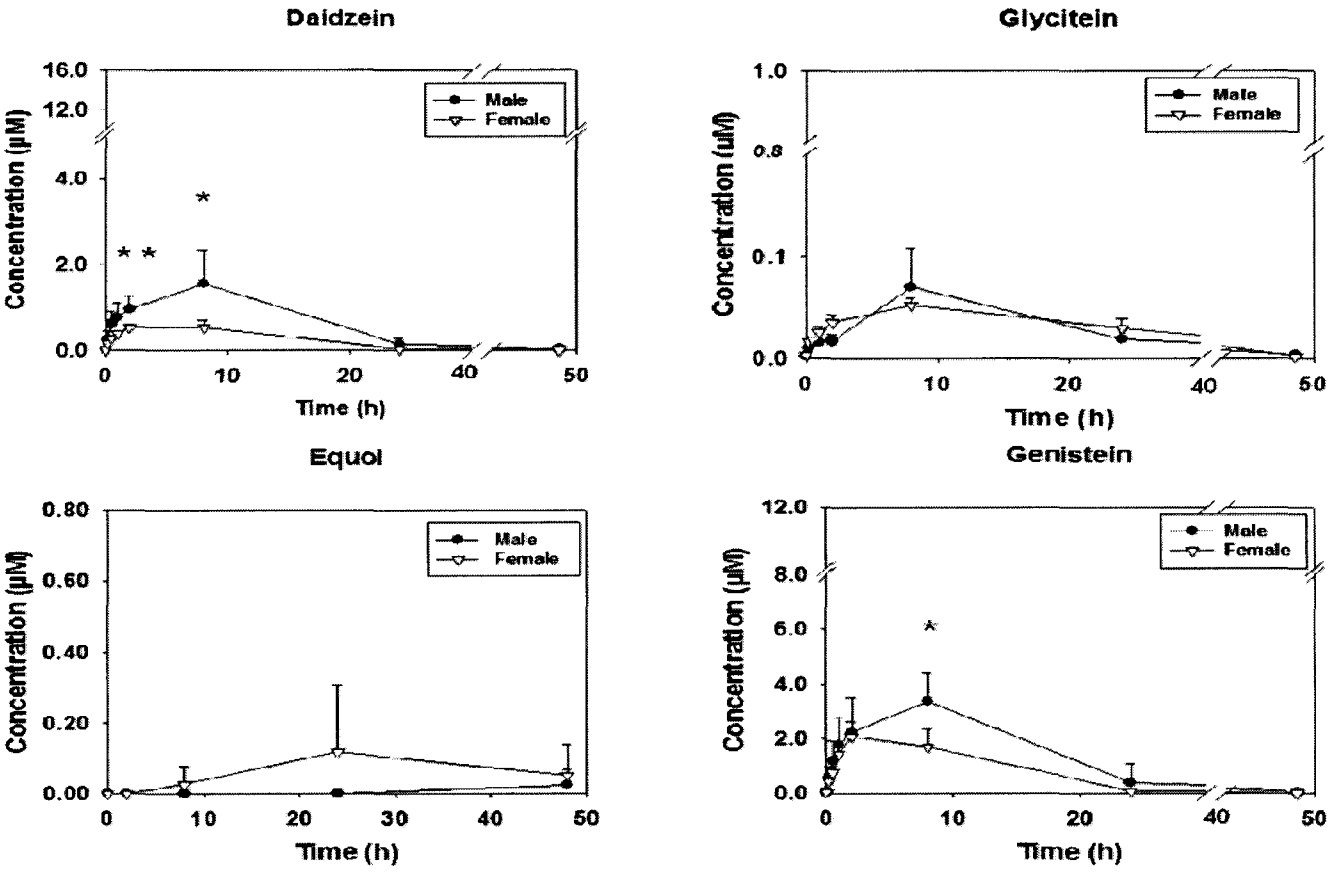


Figure 2: Plasma profiles of isoflavones in male and female (3 months) SD rats post oral gavage of a mixture of synthetic isoflavone aglyconees at 20 mg/kg rat body weight.

Results are expressed as mean ± SD on a sample size of 4 rats / group. An effect of gender at specified time points is denoted by an asterisk (*), $p < 0.05$.

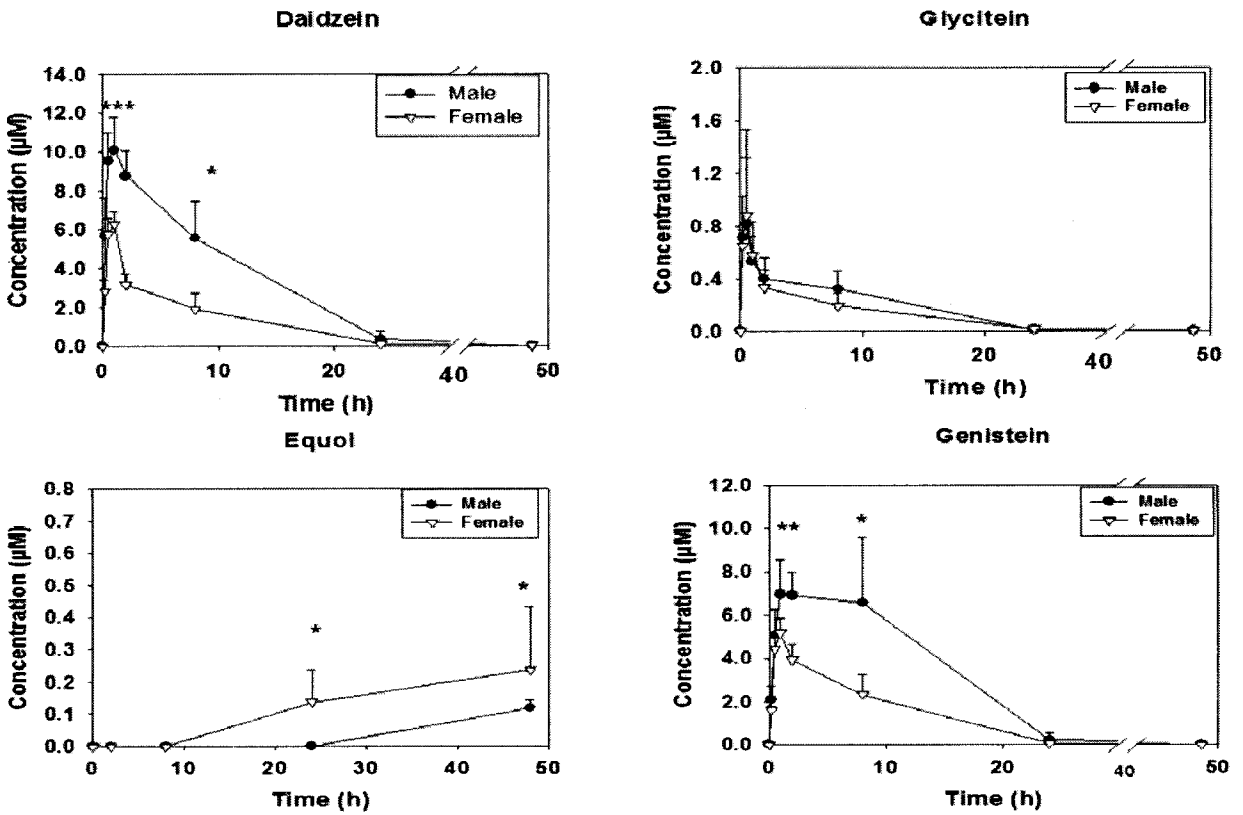


Figure 3: Plasma profiles of isoflavones in male and female (3 months) SD rats post oral gavage of a mixture of isoflavone Novasoy™ at 20 mg/kg rat body weight.

Results are expressed as mean ± SD on a sample size of 4 rats / group. An effect of gender at specified time points is denoted by an asterisk (*), $p < 0.05$.

The maximum observed peak plasma (C_{max}) daidzein concentration was significantly higher ($p < 0.05$) in glucoside (~ 3-fold) and Novasoy™ (~ 7-fold) dosed rats compared with aglycone-dosed rats. In addition, gender differences in daidzein C_{max} were observed in rats dosed with glucoside ($p = 0.002$) and Novasoy™ ($p = 0.000$) (Table 3). Genistein C_{max} in rats dosed with Novasoy™ was significantly higher (~ 2 fold; $p < 0.05$) than in rats dosed with glucoside and aglycone, and both daidzein and genistein concentrations were ~60% higher in male rats compared with female rats (Table 3). Glycitein concentration reached their maximum levels in rats dosed with Novasoy™ significantly earlier, compared with glucoside ($p < 0.048$) and aglycone ($p = 0.000$) dosed rats (Table3).

A significant effect of gender ($p = 0.004$) on $t_{½}$ for glycitein was observed within the glucoside treatment with longer $t_{1/2}$ (~ 2.4- fold) in female rats (Table 6).

The AUC_{or} values post oral administration of daidzein and glycitein were found to be significantly greater ($p < 0.05$) for the glucoside-dosed rats than the aglycone- dosed rats. Following Novasoy™ treatment, the AUC_{or} for daidzein, genistein and glycitein were significantly higher ($p < 0.05$) compared with both the aglycone and glucoside treated rats. In addition, a significant effect of gender ($p < 0.05$) was obtained on AUC_{or} for daidzein and genistein where greater values (~ 2-fold), were obtained for male rats (Tables 4-6).

Volume of distribution (Vd_{or}) was significantly larger ($p < 0.05$) for daidzein, genistein and glycitein in rats dosed with aglyconees compared with rats dosed with glucosides or Novasoy™. Glycitein and daidzein exhibited larger

Vd_{or} values (> 10-fold and 0.5-fold, respectively) compared with genistein in rats dosed with the aglyconees (Tables 4-6).

Plasma clearance (Cl_{or}) rates for daidzein, genistein and glycitein were significantly faster ($p < 0.05$) in rats dosed with aglyconees compared with rats dosed with glucosides or Novasoy™. Also, a significant effect of gender on the Cl_{or} rates for both daidzein ($p = 0.000$) and genistein ($p = 0.012$) were obtained in rats dosed with aglyconees. Cl_{or} rates of daidzein and genistein were faster (> 1.5-fold and > 0.5-fold, respectively) in females than males dosed with aglycone source (Tables 4 - 6).

3.3.2 Phase 2 (IV injection):

Following IV administration of two sources of isoflavones, the plasma profile of isoflavones demonstrated a rapid increase in plasma concentration of isoflavones followed by an elimination phase (Figs 4, 5). The profiles of plasma isoflavones in 90-day-old SD rats post-IV injection of mixtures of isoflavone glucosides or aglyconees reached C_{max} for daidzein, genistein and glycitein within 2 h post-administration of the doses (Figs. 4, 5).

Plasma concentration-time curves for rats dosed with the mixture of glucosides revealed a significant effect of time [daidzein, $p < 0.001$; glycitein, $p < 0.001$; equol, $p = 0.013$; and genistein, $p < 0.001$); a significant effect of gender (daidzein, $p = 0.038$; genistein, $p = 0.041$); and gender x time interactions (daidzein, $p < 0.001$; glycitein, $p = 0.005$; equol, $p = 0.013$; and genistein, $p < 0.001$) (Fig. 4).

Table 3: Pharmacokinetic parameters of plasma isoflavones in male and female (3 months) SD rats following gavage of a single dose (20 mg/kg) of three different sources of soy isoflavones.

Sources of Isoflavones	Daidzein		Genistein		Glycitein	
	C_{max} (μmol/L)	t_{max} h	C_{max} (μmol/L)	t_{max} h	C_{max} (μmol/L)	t_{max} h
Aglycone						
Male	1.63±0.69	6.50±3.00	3.53±1.03	6.50±3.00	0.05±0.06	8.00±0.00
Female	0.60±0.08	5.00±3.46	2.38±0.31	5.00±3.50	0.05±0.06	8.00±0.00
Glucoside						
Male	4.28±1.13	5.00±3.46	3.93±1.87	5.00±3.46	0.45±0.24	8.00±0.00
Female	2.20±0.41	3.50±3.00	2.58±0.93	3.50±3.00	0.30±0.14	3.50±3.00
Novasoy™						
Male	8.78±1.31	2.00±0.00	7.68±1.67	4.75±3.77	0.45±0.13	3.50±3.00
Female	3.15±0.54	2.00±0.00	3.93±0.71	2.00±0.00	0.28±0.15	3.50±3.00
Statistics (ANOVA)				*P*-value		
Source effect	< 0.001	0.035	<0.001	0.315	<0.001	0.002
Gender effect	<0.001	0.367	<0.001	0.143	0.080	0.100
Source x gender	<0.001	0.809	0.085	0.895	0.433	0.075
Holm-sidak						
Novasoy™ vs aglycone	0.000	0.011	0.000		0.000	0.000
Novasoy™ vs glucoside	0.000	0.106	0.000		0.000	0.048
Glucoside vs aglycone	0.000	0.272	0.627		0.000	0.025
Gender within aglycone	0.091	0.433	0.197		1.000	1.000
Gender within glucoside	0.002	0.433	0.133		0.155	0.008
Gender within Novasoy™	0.000	1.000	0.000		1.000	1.000

a) Values are mean ± SD, n=4 rats in each gender were tested in each dietary treatment group.

Table 4: Pharmacokinetic parameters of plasma daidzein in male and female (3 months) SD rats after oral and IV exposure to three different sources of soy isoflavones

	$t_{½ or}$ h	AUC_{or} (µmol.h)/L)	Vd_{or}/Kg L/Kg bw	Cl_{or}/Kg L/h/Kg bw	AUC_{IV} (µmol.h)/L)	%Bioavailability
Daidzein						
Aglycone						
Male	9.34±1.48	8.46±3.15	14.80±7.29	1.08±0.45	18.85±0.42	23.00±4.48
Female	8.29±2.25	3.68±0.38	14.13±3.39	2.77±0.58	7.48±1.38	25.07±3.57
Glucoside						
Male	7.74±2.39	23.26±10.17	3.33±1.94	0.28±0.11	17.03±4.15	56.93±8.04
Female	7.21±0.49	13.89±1.77	5.15±0.60	0.50±0.06	10.45±1.60	54.49±7.76
Novasoy™						
Male	8.26±2.66	40.28±11.11	1.65±0.52	0.14±0.03	-----	97.66±2.63
Female	6.39±2.39	18.13±3.23	3.70±1.64	0.40±0.08	-----	88.46±13.09
Statistics (ANOVA)						
Source effect	0.314	<0.001	<0.001	<0.001	0.979	<0.001
Gender effect	0.194	<0.001	0.460	<0.001	<0.001	0.157
Source x gender	0.811	0.002	0.688	<0.001	0.046	0.027
Holm-sidak						
Novasoy™ vs aglycone		0.000	0.000	0.000	-----	0.000
Novasoy™ vs glucoside		0.000	0.378	0.456	-----	0.000
Glucoside vs aglycone		0.001	0.000	0.000	0.147	0.000
Gender within aglycone		0.309	0.786	0.000	0.000	0.858
Gender within glucoside		0.036	0.465	0.326	0.046	0.599
Gender within Novasoy™		0.000	0.413	0.257	------	0.004

a) Values are mean ± SD, n=4 rats in each gender were tested in each dietary treatment group.

Table 5: Pharmacokinetic parameters of plasma genistein in male and female (3 months) SD rats after oral and IV exposure to three different sources of soy isoflavones

	$t_{½\ or}$ h	AUC_{or} (µmol.h)/L)	Vd_{or}/Kg L/Kg bw	Cl_{or}/Kg L/h/Kg bw	AUC_{IV} (µmol.h)/L)	%Bioavailability
Genistein						
Aglycone						
Male	7.02±1.77	19.05±6.69	9.43±4.69	0.92±0.43	34.55±13.29	29.14±12.35
Female	8.29±2.25	13.41±1.16	10.30±5.30	1.60±0.38	17.60± 3.62	38.93±5.78
Glucoside						
Male	3.59±2.65	30.38±21.08	2.58±1.08	0.64±0.41	31.33±16.23	57.78±17.40
Female	4.65±2.11	14.10±4.90	7.28±2.89	1.16±0.41	10.40±2.91	62.34±8.07
Novasoy™						
Male	5.75±2.14	49.54±10.35	2.20±0.76	0.27±0.07	-----	62.34±8.07
Female	5.79±2.09	20.38±2.08	5.93±2.90	0.67±0.17	-----	90.46±5.00
Statistics (ANOVA)						
Source effect	0.236	<0.001	0.006	<0.001	0.351	0.013
Gender effect	0.568	<0.001	0.038	0.001	0.004	0.094
Source x gender	0.229	0.005	0.515	0.736	0.718	0.522
Holm-sidak						
Novasoy™ vs aglycone		0.000	0.003	0.000	-----	0.006
Novasoy™ vs glucoside		0.000	0.617	0.022	-----	0.136
Glucoside vs aglycone		0.534	0.009	0.048	0.351	0.143
Gender within aglycone		0.250	0.719	0.012	0.046	0.643
Gender within glucoside		0.090	0.065	0.048	0.017	0.541
Gender within Novasoy™		0.000	0.137	0.110	-----	0.065

a) Values are mean ± SD, n=4 rats in each gender were tested in each dietary treatment group.

Table 6: Pharmacokinetic parameters of plasma glycitein in male and female (3 months) SD rats after oral and IV exposure to three different sources of soy isoflavones

	$t_{½\ or}$ h	AUC_{or} (µmol.h)/L)	Vd_{or}/Kg L/Kg bw	Cl_{or}/Kg L/h/Kg bw	AUC_{IV} (µmol.h)/L	%Bioavailability
Glycitein						
Aglycone						
Male	9.21±2.84	0.28±0.13	108.03±71.68	7.68±3.35	2.85±0.66	4.85±2.10
Female	8.72±2.32	0.29±0.01	76.05±23.28	6.00±0.85	2.28±0.46	6.59±1.11
Glucoside						
Male	5.59±0.25	1.54±0.36	7.43±4.59	0.90±0.53	1.60±0.17	57.81±15.75
Female	12.78±5.97	2.28±0.76	19.55±10.35	1.03±0.13	2.35±0.97	38.32±4.82
Novasoy™						
Male	6.03±2.26	2.59±0.96	8.83±3.37	1.07±0.45	-----	68.14±19.92
Female	7.82±1.53	1.76±0.67	7.98±2.59	1.40±0.37	-----	40.18±16.52
Statistics (ANOVA)						
Source effect	0.291	< 0.001	<0.001	<0.001	0.132	<0.001
Gender effect	0.037	0.165	0.594	0.524	0.969	0.013
Source x gender	0.060	0.432	0.368	0.364	0.089	0.108
Holm-sidak						
Novasoy™ vs aglycone	0.200	0.000	0.000	0.000		0.000
Novasoy™ vs glucoside	0.157	0.489	0.950	0.729		0.382
Glucoside vs aglycone	0.886	0.000	0.000	0.000		0.000
Gender within aglycone	0.825	0.974	0.164	0.135		0.858
Gender within glucoside	0.004	0.488	0.589	0.905		0.058
Gender within Novasoy™	0.420	0.084	0.970	0.755		0.009

a) Values are mean ± SD, n=4 rats in each gender were tested in each dietary treatment group.

Rats dosed with a mixture of isoflavone aglyconees showed a significant effect of time [daidzein, glycitein, genistein, $p < 0.001$); a significant effect of gender (daidzein, $p = 0.002$); and significant gender x time interactions (daidzein, $p < 0.001$; genistein, $p = 0.009$) on the plasma concentration time curves (Fig. 5).

The AUC_{IV} values post-IV administration for daidzein, genistein and glycitein were not significantly different for the glucoside and aglycone dosed rats but significant effects of gender ($p < 0.05$) within both glucoside- and aglycone-dosed rats were found for daidzein ($p < 0.05$) and genistein ($p < 0.05$) where higher values (~one to threefold) were obtained for males (Tables 4-6).

3.3.3 Formation of equol:

Plasma profiles (Figs. 1 - 5) of equol exhibited a time lag in its appearance after a single-bolus of the isoflavones, and it took > 8 h before equol appeared in substantial amounts after either oral or IV administration of the doses. Equol production also differed with gender, with significantly ($p = 0.035$) higher concentrations produced by female rats post oral administration of 20 mg/kg of three different mixtures of soy isoflavones. However, no significant effects of source or gender x source interactions were obtained (Fig. 6)

The AUC for plasma equol in male and female rats after IV injection of 10 mg/kg of the two different mixtures of soy isoflavones are shown in Fig. 7. The plasma concentration-time curve revealed no significant effects of gender ($p = 0.356$), time ($p = 0.448$) or gender x time interactions ($p = 0.448$) for equol post aglycone injection. However, significant effects of time ($p = 0.013$) and gender x

time interactions (p = 0.013) were observed for equol, post glucoside injection (Fig. 7).

3.4. Discussion:

The importance of isoflavones in the prevention of a range of chronic diseases, and data from human and animal studies attest to the biological activity of these compounds and their potential role in human health [Setchell and Cassidy, 1999; Gilani and Anderson, 2002; de-Pascual-Teresa *et al.*, 2006]. If soy isoflavones are to be effective in preventing diseases, they must be bioavailable, and thus understanding the factors which may alter their bioavailability, such as gender and food sources, need to be elucidated. Most published studies on the pharmacokinetics of soy isoflavones have focused on systemic studies of the absorption, metabolism and excretion of isoflavones (mainly daidzein and genistein) using the areas under the concentration-time curves following oral administration of isoflavones in humans or animal models [Cassidy *et al.*, 2006; Izumi *et al.*, 2000; Setchell *et al.*, 2001; Zubik and Meydani, 2003; Xu *et al.*, 2000]. Several reports have measured only the concentration of isoflavones excreted in urine, which may only be deemed appropriate if the compound is 100% bioavailable and recovered completely in the urine. As is evident from many studies, this is not the case for soy isoflavones; [Setchell *et al.*, 2003; Xu *et al.*, 1994; Tew *et al.*, 1996].

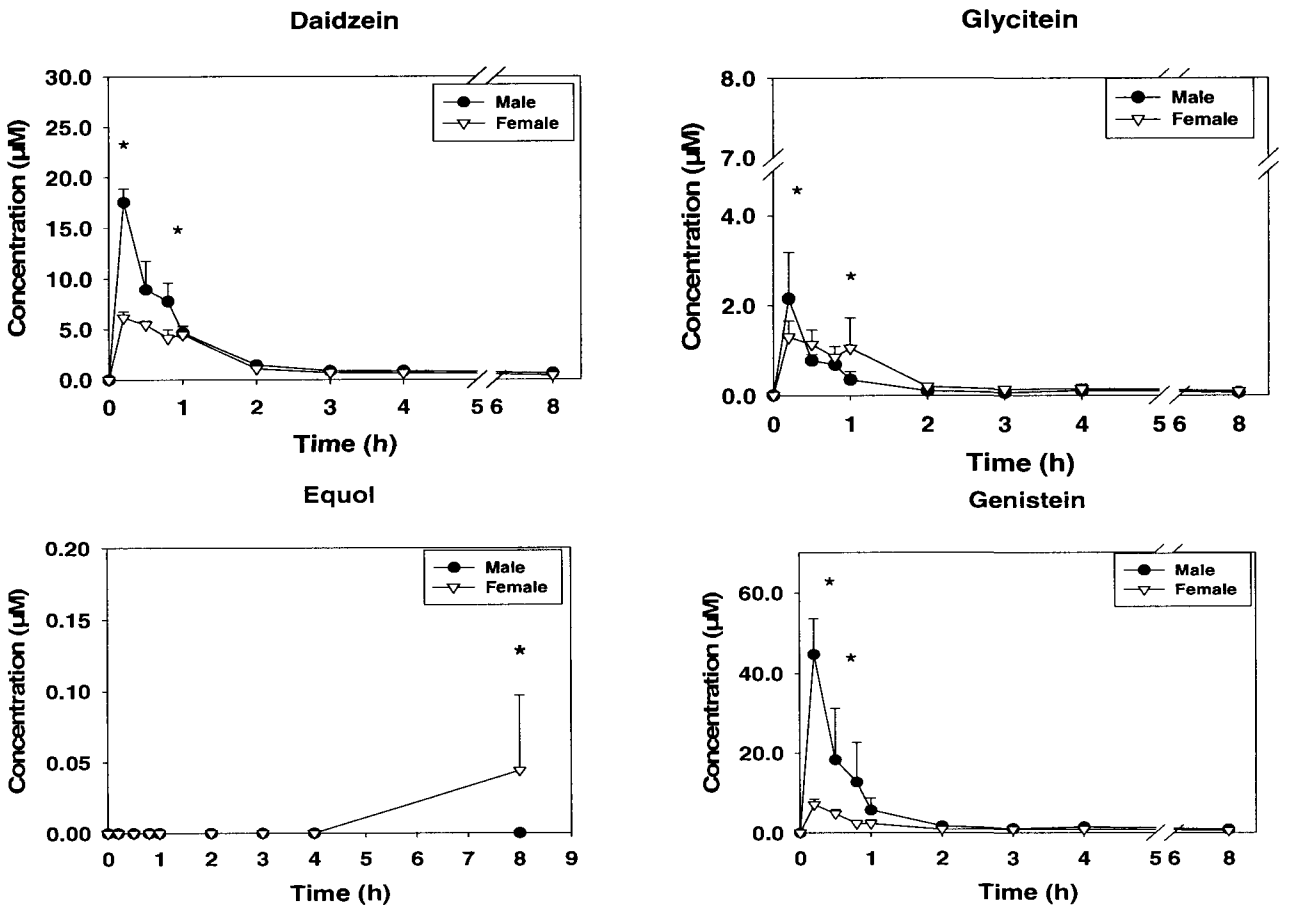


Figure 4: Plasma profiles of isoflavones in male and female (3 months) SD rats post IV injection of a mixture of synthetic isoflavone glucosides at 10 mg/kg rat body weight.

Results are expressed as mean ± SD on a sample size of 4 rats / group. An effect of gender at specified time points is denoted by an asterisk (*), $P < 0.05$.

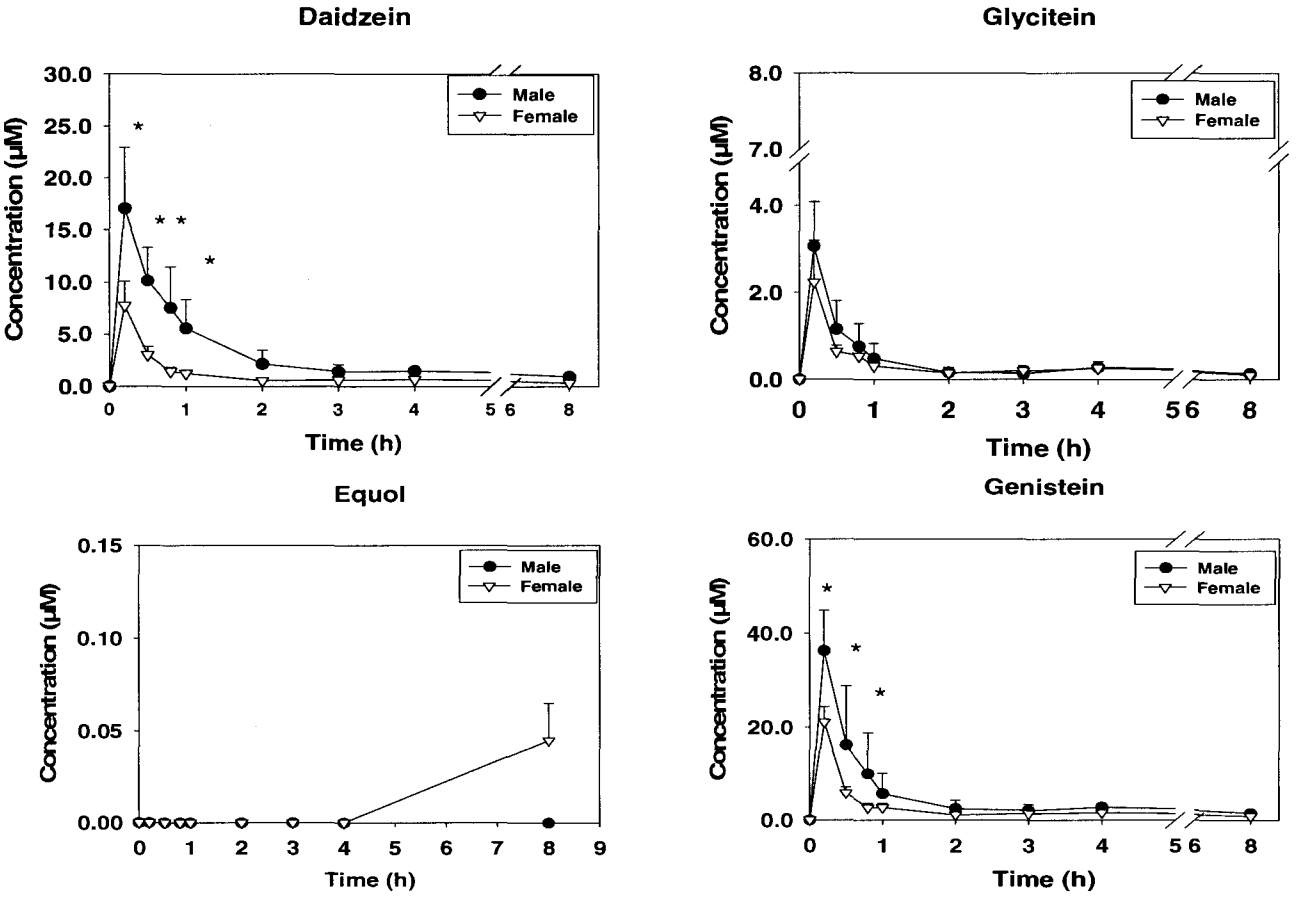


Figure 5: Plasma profiles of isoflavones in male and female (3 months) SD rats post IV injection of a mixture of synthetic isoflavone aglycone 10 mg/kg rat body weight.

Results are expressed as mean ± SD on a sample size of 4 rats / group. An effect of gender at specified time points is denoted by an asterisk (*), $P < 0.05$

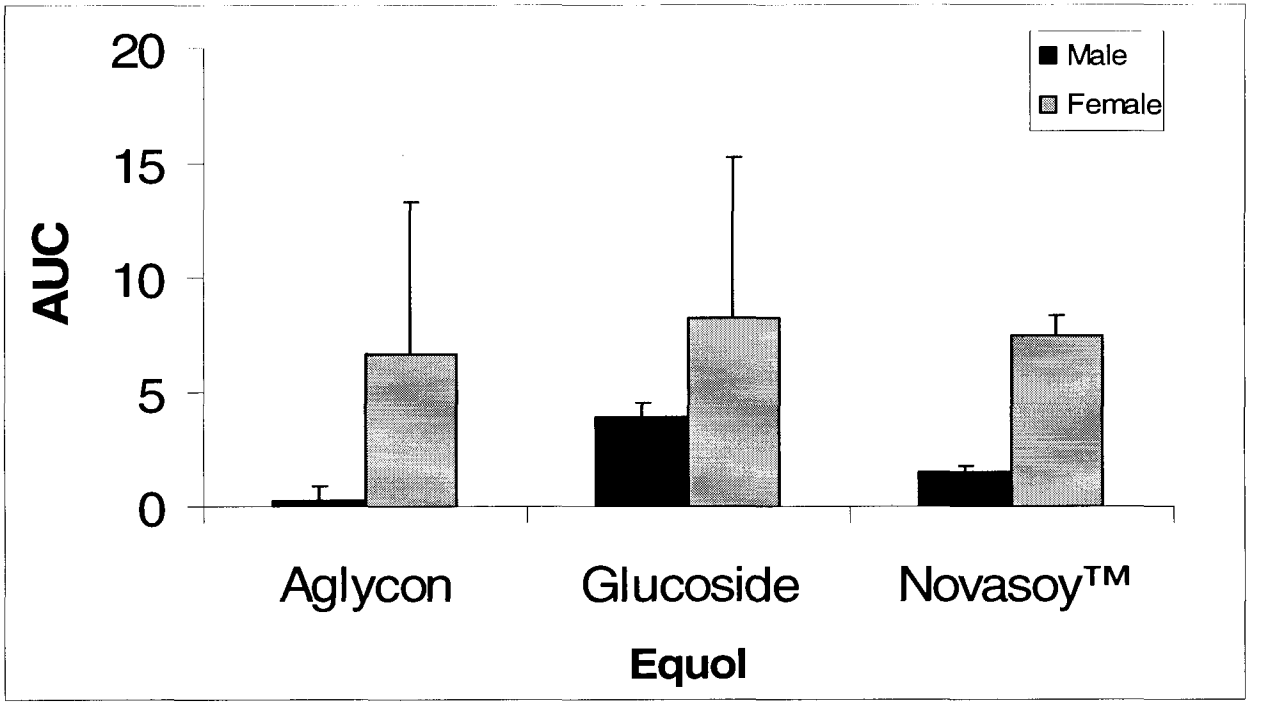


Figure 6: Area under the curve of plasma equol in male and female (3 months) SD rats after consumption of single oral dose (20 mg/kg) of three different mixtures of soy isoflavones.

Results are expressed as mean ± SD on a sample size of 4 rats / group.

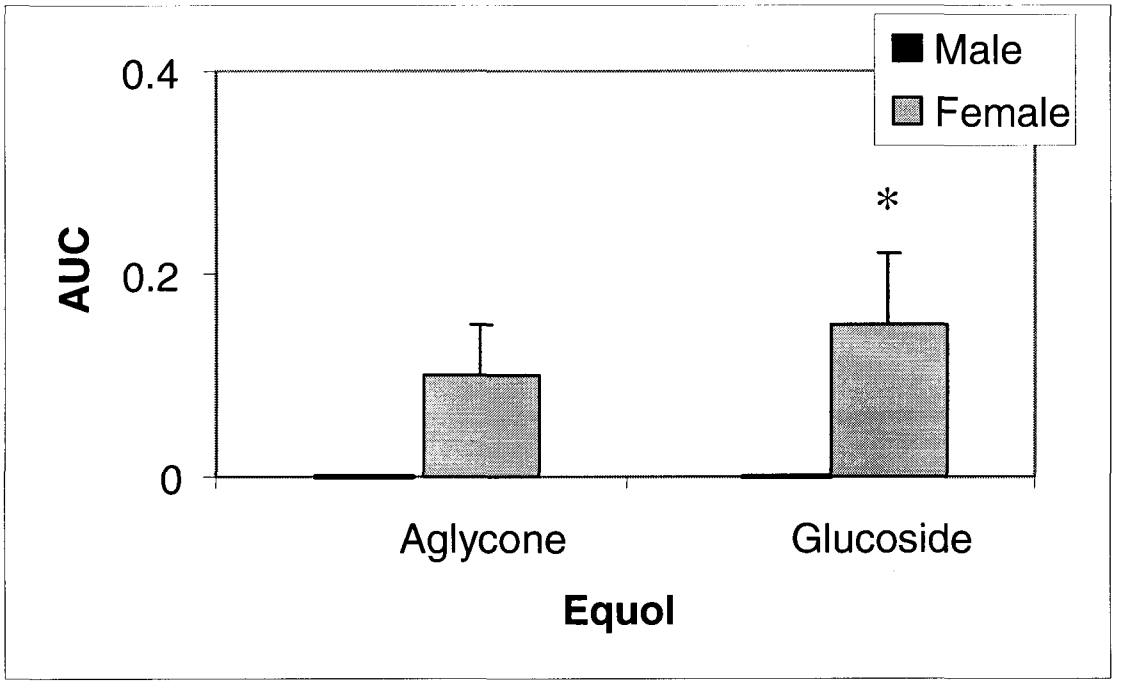


Figure 7: Area under the curve of plasma equol in 3-month old male and female SD rats after IV injection of (10 mg/kg) of two different mixtures of soy isoflavones.

Results are expressed as mean ± SD on a sample size of 4 rats / group. An effect of gender at specified time points is denoted by an asterisk (*), $P < 0.05$.

Therefore, conflicting results are present in the literature regarding the bioavailability of the aglycone and glucoside forms of isoflavones, mainly daidzein and genistein [Izumi *et al.*, 2000; Setchell *et al.*, 2001; Zubik and Meydani, 2003]. Accurate measurements of bioavailability would ideally compare the AUC after both oral and IV administration of the pure compounds [Janning *et al.*, 2000, Supko and Malspeis, 1995; T'ien-Li *et al.*, 1977]. The accuracy of this determination is dependent upon obtaining multiple blood samples during the elimination phase and sampling times should ideally be extended to at least five half-lives beyond the time that steady-state levels are reached in the blood [de-Pascual- Teresa *et al.*, 2006; Setchell *et al.*, 2003]. Only a few studies of isoflavone bioavailability have taken the above points into consideration [Setchell *et al.,* 2001; King and Bursill, 1998; Watanabe *et al.*, 1998; Setchell *et al.*, 1997], and several studies have only used two time points to compute the pharmacokinetics [Izumi *et al.*, 2000; Xu *et al.*, 2000]. These inconsistencies in study design may account for discrepancies in the available literature.

The present study is the first part of isoflavone bioavailability study in our laboratory that measures bioavailability based on AUC of both oral and IV administration of two sources of isoflavones; a mixture of synthetic aglyconees (daidzein, genistein and glycitein) and a mixture of synthetic glucosides (daidzin, genistin and glycitin). Glycitein was included in our study because it may also contribute to soy's health effects. Knowing the bioavailability of glycitein is important because many commercially available supplements contain high levels

of glycitein and limited information exists about its biological properties [Setchell *et al.*, 2001].

Our data clearly show that the bioavailability of daidzein and glycitein in rats dosed with these glucosides is significantly ($p < 0.05$) higher compared with the bioavailability of daidzein and glycitein (> twofold) in aglycone-dosed rats. The bioavailability of isoflavones was also significantly ($p < 0.05$) higher in rats dosed with Novasoy^{TM} than in rats dosed with pure sources of isoflavone glucosides and aglyconees. The glucoside moiety in isoflavones glucosides and in Novasoy^{TM}, in conjunction with other compounds in Novasoy^{TM} (phytic acid, saponins, oligosaccharides, proteases inhibitors and phytosterols), may be protecting against biodegradation of the isoflavone structure [Setchell *et al.*, 2001]. Our results are in agreement with the previously published report [Setchell *et al.*, 2001]. This increase of absorption from Novasoy^{TM} compared with pure chemicals may be an important consideration for regulators.

In our study, the lower bioavailability in rats dosed with aglycone sources is most likely explained by the reduced absorption of isoflavones with increasing level of intake (20 mg/kg body weight of rats). Earlier studies have demonstrated a linear dose–response relationship for isoflavone absorption at low doses [Setchell *et al.*, 2001]; however, at higher doses (0.4 - 1.8 mg/kg body weight) a curvilinear relationship was evident [Setchell *et al.*, 2003]. Usually, when a compound exhibits non-linear pharmacokinetics the AUC_{or} increases in a manner that is disproportionate to the applied dose. When the AUC_{or} is lower than would

be expected from a linear relationship, this is indicative of either increased elimination, or reduced absorption [Setchell *et al.*, 2001; Setchell *et al.*, 2003].

Cl_{or} rates for daidzein, genistein and glycitein in rats dosed with Naovasoy™ and glucosides, were significantly ($p < 0.05$) slower compared with the aglycone dosed rats, consistent with the high bioavailability of these isoflavones in Novasoy™- and glucoside-dosed rats compared with the aglycone-dosed rats. The pharmacokinetic data from the analysis of the plasma curves showed that Cl_{or} of daidzein (~ 1-fold) and genistein (>1.5-fold) were significantly higher in females than males.

In this study, the Vd_{or} was significantly ($p < 0.05$) larger for daidzein, genistein and glycitein in rats dosed with aglyconees compared with rats dosed with glucosides or Novasoy™, indicating extensive tissue distribution of isoflavone aglyconees. Furthermore, within the aglycone-dosed rats, genistein was more bioavailable (~1 – 6-fold) than daidzein and glycitein. The Vd for both glycitein (~108 L/Kg bw) and daidzein (~14 L/Kg bw) exhibited much higher values than genistein (~9 L/kg bw), indicating extensive tissue distribution of glycitein and daidzein in rats dosed with aglycone source. The lower Vd_{or} of genistein compared with daidzein and glycitein in the aglycone-dosed rats explains why the AUC_{or} for genistein in plasma is exceeded by daidzein and glycitein. When isoflavones reach the circulation, it is possible that they are bound extensively to proteins such as albumin (the most abundant protein in plasma), lipoproteins, sex hormone-binding globulin and α_1-acid glycoprotein. It has been reported that at 0.5 h, and 5 h post oral dosing of genistein, in male

rats 80.6 ± 2.0 and 77.3 ± 4.7 % and in female rats 97.7 ± 6.2 and 78.1 ± 4.7 % were bound to plasma protein, respectively [Coldham *et al.*, 2002]. This will limit the distribution of isoflavones into tissues, resulting in a lower Vd, which may be the case for rats dosed with Novasoy™ and the glucosides. It is also possible that in aglycone-dosed rats, isoflavones bind preferentially to tissues at the expense of plasma and therefore, the plasma concentration of isoflavones would be significantly lower compared with the glucoside and Novasoy™-dosed rats. This may be the reason for the very large Vd_{or} values, which were even larger than the actual volumes of the rats themselves (> 1L/kg).

We observed significant ($p < 0.05$) gender differences in the bioavailability of daidzein and glycitein for the isoflavone Novasoy™ ($p < 0.05$). Gender related differences in bioavailability might be due to differences in absorption of isoflavones and differences in the biotransformation, excretion and enterohepatic circulation [Coldham *et al.*, 2002]. Previously published studies have reported peak plasma concentrations at time points within 2 h of the oral administration of isoflavones [Janning *et al.*, 2000; Supko and Malspeis, 1995; T'ien-Li and Hsiu-Yuan, 1977]. They also reported fluctuations in the plasma concentration at time points within the first 2 h of the oral administration of the doses and attributed them to entro-hepatic circulation. In other studies [Setchell *et al.,* 2001; King and Bursill,1998; Watanabe *et al.*, 1998], peak concentrations of daidzein or genistein were achieved within 2-8 h post oral administration of daidzein and / or genistein and a sharp decrease was observed 8 h after the administration. In order to investigate the possible presence of the multiple peaks observed in the previous

studies reported by Janning *et al.* (2000) and Supko and Malspeis, (1995) before 2 h, three time points (10, 30, 60 min) were added to the original (0, 2, 8, 24, 48 h) blood collection for the two groups of 90-day-old male and female Sprague-Dawley rats as the minor amendment to the original protocol. A total of 16 (8 male and 8 female) rats were gavaged with Novasoy^{TM} and synthetic aglycone at 20 mg/Kg dose. In our study, we could not detect an early peak before C_{max} that would represent entero-hepatic circulation. In spite of the addition of extra time points, however, the study was designed to obtain bioavailability data. Future studies may address entro-hepatic circulation by increasing the number of rats in each group and bleeding each rat only at three or four time points [Coldham *et al.*, 2002; Coldham and Sauer, 2000].

IV injection of isoflavones bypasses first-pass metabolism [Coldham *et al.,* 2002]. The metabolic transformation on first-pass absorption is important in both human and rats, because it is the unconjugated form of isoflavone that is available for receptor occupancy. IV administration of isoflavones will enable a more substantial role for renal excretion since first pass metabolism and enterohepatic circulation of isoflavones will be reduced [Coldham *et al.,* 2002].

By contrast, oral administration of isoflavones leads to significant phase II metabolism and isoflavone glucuronides become the major circulating forms in plasma [Setchell *et al.*, 2002]. This conjugation happens during transport across the enterocytes and in the liver [Coldham and Sauer, 2000]. An additional factor to consider is the extent of protein binding which differs considerably for daidzein, genistein and equol. The intestine provides a key barrier to limiting the biological

activity of isoflavones administered orally. Not only do the enterocytes conjugate isoflavones on first pass, but there are also specific intracellular efflux pumps, such as the multidrug resistance protein (MRP2) that shunt isoflavones back into the intestinal lumen. Therefore, events with the lumen and across the enterocytes serve to account for the limited absorption of isoflavones when given orally [Setchell *et al.*, 2002].

In this study, daidzein C_{max} was significantly higher ($P < 0.05$) in glucoside and Novasoy^{TM}-dosed rats (> 2 and > 6 times, respectively) compared with aglycone-dosed rats (Table 3). Genistein C_{max} in rats dosed with Novasoy^{TM} was significantly higher (~ 2-fold; $p < 0.05$) compared with glucoside- and aglycone-dosed rats, and both daidzein and genistein concentrations were 60% higher in male rats compared with female rats (Table 3). Based on the chemical structure of isoflavones, unconjugated genistein and daidzein are very lipophilic, which favors retention and accumulation of those compounds in other tissues such as liver [Coldham and Sauer, 2000]. Higher disposition of isoflavones in the liver would allow the reduction of the systemic plasma concentration of genistein and daidzein as reflected by lower plasma pharmacokinetic values for C_{max}. Gender-related differences in tissue concentrations of isoflavones have already been reported in rat liver due to a number of factors, including the presence of binding proteins, biotransformation, and clearance at the site [Coldham and Sauer, 2000]. C_{max} of ingested isoflavone aglycone and glucoside were attained within 5.0 - 8.0 h and 3.5 – 8.0 h, respectively. This is in agreement with other human and rodent studies [Cassidy *et al.*, 2006; Setchell *et al.*, 2001; King and Bursill,

1998; Watanabe *et al.,* 1998; Messina in Gilani and Anderson (Eds), 2002]. Values of $T_{1/2}$ for aglycone and glucoside dosed rats in the present study, are similar to $t_{½}$ for genistein in mice [Supko and Malspeis, 1995], and for genistein and daidzein in humans [Cassidy *et al.*, 2006; Setchell *et al.*, 2001; King and Bursill, 1998; Watanabe *et al.*, 1998]. Moreover, significant ($p < 0.05$) gender differences on $t_{½}$ for glycitein were observed within the glucoside treatment where the $t_{1/2}$ in female rats was longer compared with males. These values are similar to the previously reported $t_{1/2}$ for the pure compounds [Setchell *et al.*, 2001; Setchell *et al.,* 2003] and are similar to values reported by others allowing for some limitations in the methods used [Cassidy *et al.,* 2006; King and Bursill, 1998; Watanabe *et al.,* 1998; Messina in Gilani and Anderson (Eds), 2002]. It is clear that the t_{max} and $t_{½}$ values for the pure compounds are longer than for Novasoy™, which is also consistent with previously reported data [King and Bursill, 1998; Watanabe *et al.,* 1998; Messina in Gilani and Anderson (Eds), 2002].

In the present study, the late appearance (> 8h) of equol in plasma samples after both oral and IV administration is consistent with its production being in the colon region of the large intestine [Cassidy *et al.,* 2006; Setchell *et al.*, 2001]. Rats dosed with glucoside and Novasoy™ produced more equol compared with aglycone dosed rats (Fig. 6). Earlier studies using an *in vitro* model of human colonic fermentation, found that in a high carbohydrate environment, colonic fermentation was stimulated and this increased the rate of conversion of daidzein to equol [setchell, 1998; Setchell and Cassidy, 1999;

Lampe *et al.,* 1998]. Equol production also differed with gender, with significantly higher concentrations produced by female rats post oral and IV administration of the glucoside source, possibly due to the enhanced ability of female rats to convert more daidzein to equol compared with male rats. It has been reported that variation in equol production is due to the absence of certain bacterial species in the intestine [Setchell *et al.,* 2005; Fujioka *et al.*, 2004]. For example, only about 25% of women possess the gut microorganisms to produce equol, whereas the gut flora of rats produces very large quantities of equol [Cooke, 2006]. The appearance of equol in blood following IV administration of isoflavones indicates the transfer of daidzein into the intestine since the conversion to equol is thought to be mediated only by the gut microflora. It is possible that once absorbed by the liver, some daidzein will be excreted in the bile which would allow for the transfer of daidzein into the intestine. Recently, transfer to the bile has been indicated for genistein metabolites [Prasain *et al.*, 2006].

Establishing the validity of a model species for investigating the bioavailability of isoflavones is important in order to extrapolate their effects in humans. The pharmacokinetic parameters (C_{max}, t_{max}, and $t_{1/2}$) for genistein, daidzein and glycitein in the present study were very similar to those reported for humans [Cassidy *et al.,* 2006; Setchell *et al.*, 2001; King and Bursill, 1998; Watanabe *et al.,* 1998; Messina in Gilani and Anderson (Eds), 2002] and support the use of rats as a model species for human isoflavone pharmacokinetics.

3.5. Acknowledgements:

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Note to Chapter 3:

Animal care:

The animal care and handling procedures were conducted according to the guidelines of the Canadian Council on Animal Care, and the experimental protocol was approved by the Health Canada, Ottawa, Animal Care Committee prior to the initiation of the study. Sprague-Dawley rats (Charles River, St-Constant, PQ, Canada) were individually caged in stainless steel cages with hardwood chip bedding (maple or ash) and kept in an environmentally controlled room with an illumination intensity of 150-300 lux (fluorescent bulbs on automated timer) and 12 hours illumination, humidity 40-60%, temperature 23 ± 2 °C and free access to isoflavone-free casein based control diet formulated according to the American Institute of Nutrition (AIN-93G) recommendations (Reeves *et al.,* 1993) and fresh water available *ad libitum*.

Post-oral or IV administration of isoflavone sources, rats were assigned to metabolic cages with bottom screens to prevent coprophagy during bioavailability study.

Isoflavone Sources:

Three lots of NOVASOY^{TM} concentrate #152-400 (600g of Lot # 9809151, 2400g of Lot # 0011101, and 2000g of Lot # 0104181; Archer Daniels Midland Company, Decatur, IL, USA) were mixed together and used for all studies conducted. The actual content of isoflavones (genistein, daidzein and glycitein) in the Novasoy^{TM} source was determined by Waters HPLC linear gradient with UV

detection after acid hydrolysis and liquid –liquid extraction of samples according to the method of Wang and Murphy, (1994) in Dr. Gilani's laboratory by Mr. Robertson.

The preparation of Novasoy^{TM} contained a total isoflavone concentration of 24.1% (genistein 12.5%, daidzein 8.6%, and glycitein 3.0%) the remainder being moisture, carbohydrate, protein, fat and ash.

Standards:

(i) Daidzin, genistin, glycitin, daidzein, genistein, glycitein and equol (metabolite of daidzein) were purchased from LC Labs. (Woburn, MA, USA). The purity of each standard used was verified using LC/MS method (Sepehr *et al.*, 2006). LC/MS spectra agreed with the structure of each isoflavone and matched the spectra obtained from a purchase standard of each isoflavone, indicating a purity of above 99% for each lot.

(ii) Biochanin A, 4-ethyl phenol (metabolite of genistein) and apigenin- Sigma-Aldrich Co. The purities of these three compounds were above 97%, 99%, and 95% respectively. For apigenin, LC/MS spectra indicated one major peak and minor impurities in some of the lots. Probe/MS testing in different lots of apiginin indicated one major component with minor components, suggesting little impurities. Therefore apiginin did not use as an internal standard in analyzing plasma samples.

National Centre for Complementary and Alternative Medicine (NCCAM) guidelines were followed in assuring quality of isoflavone-sourced materials.

To ensure stability, the bulk chemical was stored at -80 ^{0}C, protected from light in the original shipping containers. Purity was periodically measured during the study; no degradation of the bulk chemical was detected.

Sample centrifugation:

Samples were centrifuged for 6 min at 4^{0}C at 16,750 x g in an IEC Centra MP4R refrigerated centrifuge, Cat. No. 2438 (Needham Heights, MA) in order to precipitate proteins.

Determination of plasma isoflavones pharmacokinetics:

Pharmacokinetic analysis of plasma isoflavone profiles for each rat was conducted using non-compartmental analysis software (PK Solutions^{TM} version 2.0.2 package, Summit Research Services, Ashland, Ohio, USA). The pharmacokinetic parameters were calculated using the residuals method of analysis (curve stripping), assuming first order disposition kinetics. Figures 8 and 9 are representative examples of the curve stripping method for pharmacokinetic analysis of daidzein aglycone administered IV and oral in female (3 months) SD rats.

Clarification of determination of bioavailability values for rats administered with the aglycone source of isoflavones (Tables 4-6 of Chapter 3).

In chapter 3, bioavailability data for rats dosed with synthetic aglycone source of isoflavones were calculated from AUCs of oral and IV exposure of individual animals by random assignation of the AUC_{or} to AUC_{IV} from four rats in

the same diet group. However, since IV and oral exposure studies were not both performed in each rat, it was considered appropriate to determine the bioavailability in each group by comparing the average of AUCs of IV exposure of four rats in one group, with AUC_{or} of each rat in the same diet group (Table 7).

No significant differences were observed for bioavailability results in Chapter 3 compared with new method.

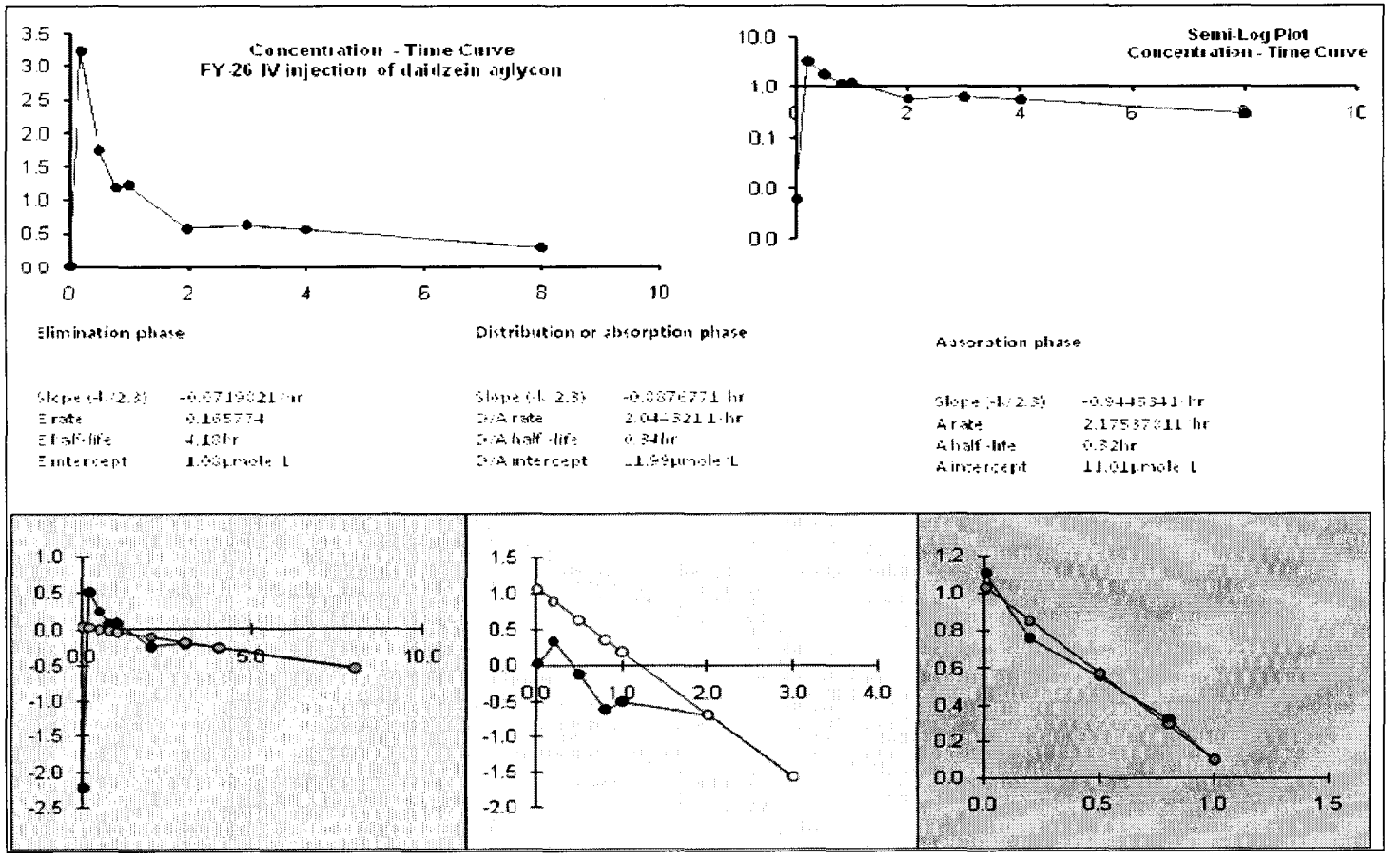


Figure 8: Pharmacokinetic parameters were calculated using the residual method of analysis (curve stripping) post IV injection of isoflavones.

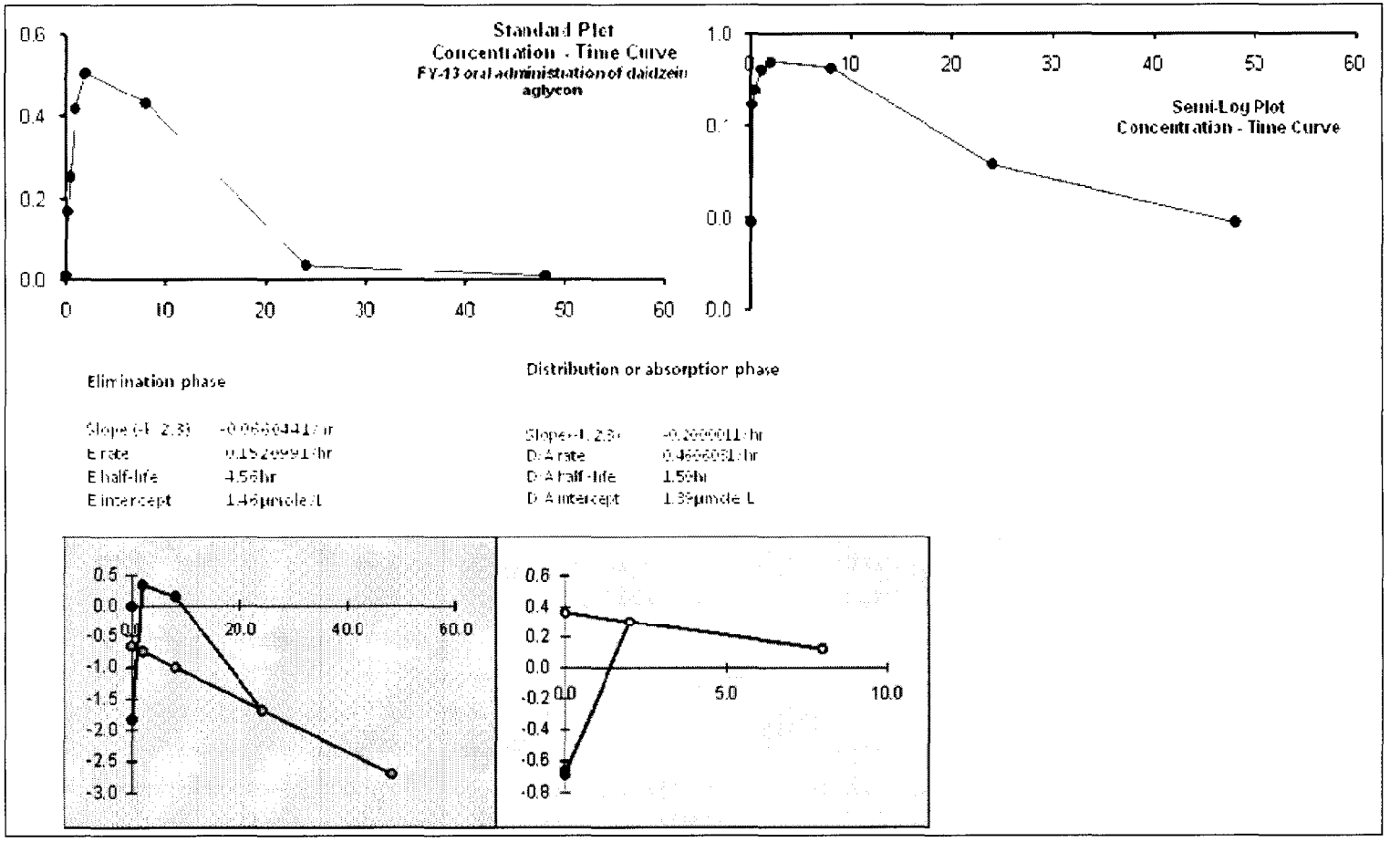


Figure 9: Pharmacokinetic parameters were calculated usinf the residual method of analysis (curve stripping) post oral administration of isoflavones.

Table 7: Bioavailability of plasma daidzein in female (3 months) SD rats after oral and IV exposure to aglycone source of isoflavone daidzein.

Aglycone Daidzein	AUC_{or} µmol.h/L	AUC_{IV} µmol.h/L	% Bioavailability[a]	% Bioavailability[b]
Female	3.66	7.30	25.07	24.48
	4.21	8.70	24.20	28.16
	3.52	8.30	21.20	23.55
	3.34	5.60	29.82	22.34
Average	3.68	7.48	25.07	24.63
Standard deviation	0.38	1.38	3.57	2.51

[a]Bioavailability based on the random assigning of the AUC_{or} to AUC_{IV} from four rats in the same diet group.
[b]Bioavailability based on comparing the average of AUC_{IV} with each AUC_{or}

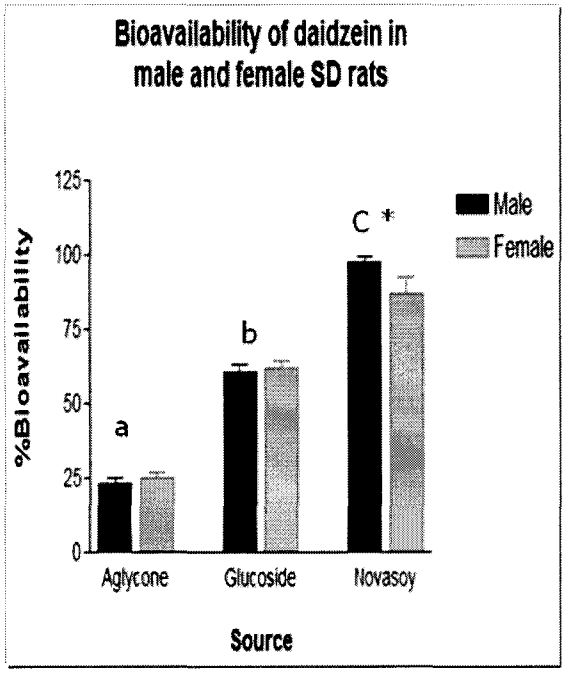
An additional suggestion with respect to the bioavailability calculation concerned the groups of rats that were administered the synthetic source of glucoside form and Novasoy™ source of isoflavones. In Chapter 3, the bioavailabilities of the synthetic glucoside form and Novasoy™ form were calculated based on AUC_{or} for the synthetic glucoside and the AUC_{or} for Novasoy™ and these were then compared with AUC_{IV} of the synthetic glucosidic form. This was based on the consideration that isoflavones are presented in food mainly as glucosides. Although there is evidence to suggest that particular members of a related class of flavonoids are absorbed in their naturally occurring glucosidic forms (Hollman and Katan, 1997), this does not appear to be the case for the isoflavones. After soy intake, isoflavones are absorbed as aglyconees, which are more readily absorbed than the parent glucosides due to their higher hydrophobicity and lower molecular weight (Xu et al., 1995; Liu and Hu, 2002). This is supported by the evidence from Setchell *et al.,* (2002), which indicates that isoflavone glucosides are not absorbed intact across the enterocytes of healthy adults and shows that uptake requires hydrolysis of the isoflavone glucoside to their aglycone form. Therefore, bioavailability of Novasoy™ and synthetic glucoside form of isoflavones (daidzein, genistein and glycitein) after oral and IV exposure in male and female SD rats were recalculated based on the consideration that aglyconees arising from Novasoy™ and glucosidic form are the absorbable form of isoflavones. Results were compared with the ones already been reported in chapter 3 (Figs 10-12).

The significant gender effect that was observed in the bioavailability of daidzein in rats dosed with Novasoy™ prior to re-evaluation based on AUC_{IV} of glucoside dosed rats was not changed after the re-evaluation based on AUC_{IV} of aglycone dosed rats (Fig. 10).

The significant source effects observed in the bioavailability of daidzein, genistein, and glycitein in rats dosed with Novasoy™ and synthetic glucosides compared with synthetic aglycone dosed rats, did not reveal any additional gender or source effects in the bioavailability of isoflavones following re-evaluation based on AUC_{IV} of aglycone dosed rats (Figs 10-12).

The significant gender effect that was observed in the bioavailability of glycitein in rats dosed with Novasoy™ prior to re-evaluation based on AUC_{IV} of glucoside dosed rats was not observed after the re-evaluation based on AUC_{IV} of aglycone dosed rats (Fig. 12).

Bioavailability of daidzein reported in Chapter 3:



Bioavailability of daidzein based on AUC_{IV} aglycon

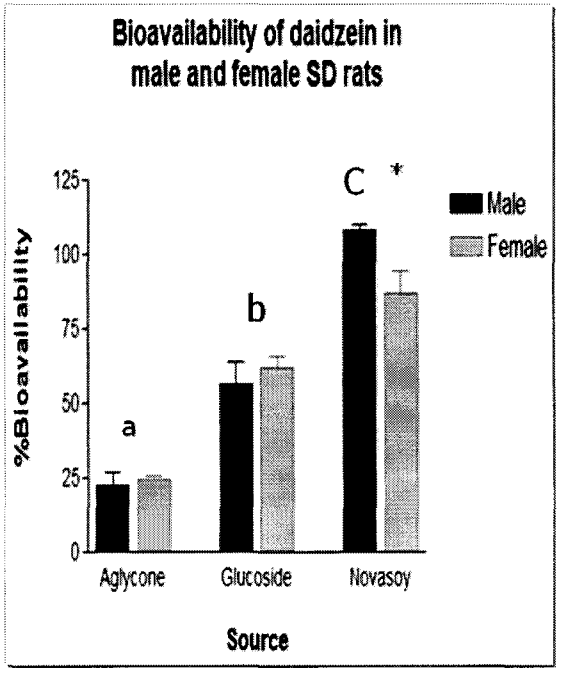
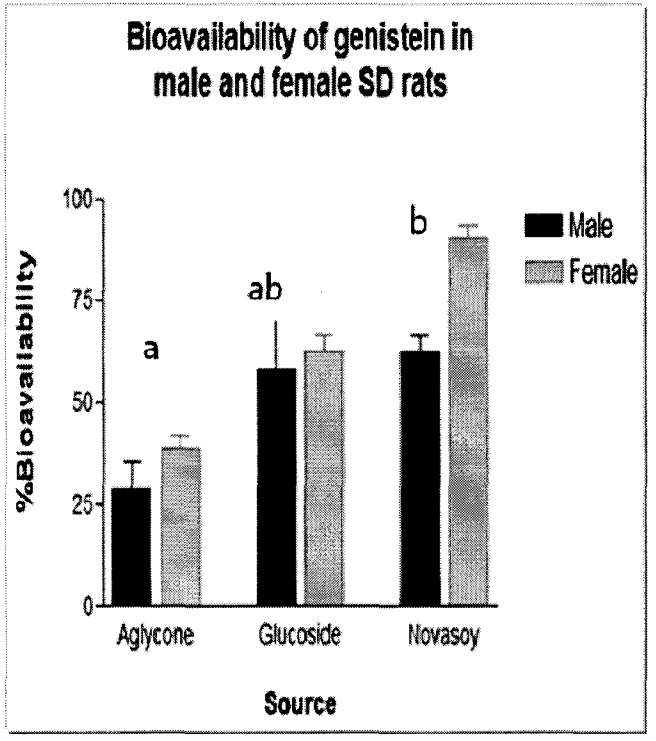


Figure 10: Bioavailability of daidzein calculated based on the AUCor of all sources

*Significant gender effecr;
[a, b, c] Significant source effect

Bioavailability of genistein reported in Chapter 3:



Bioavailability of genistein based on AUC_{IV} aglycon

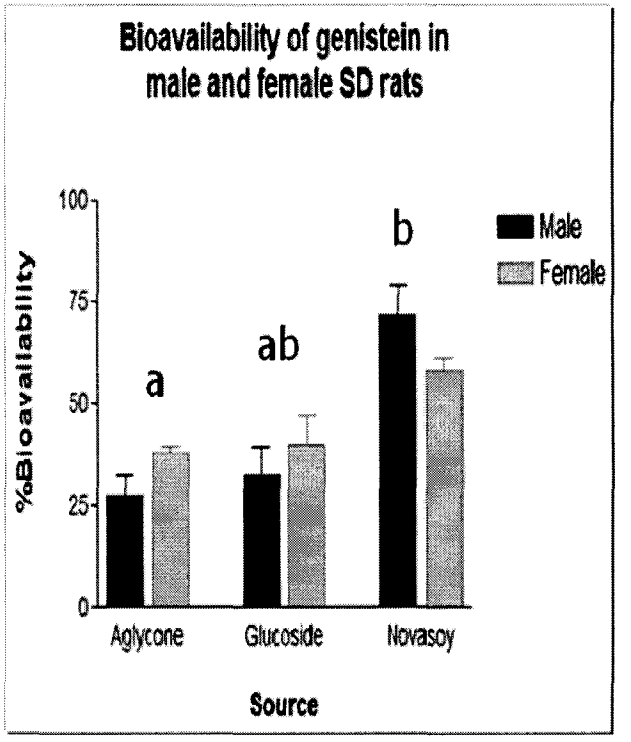
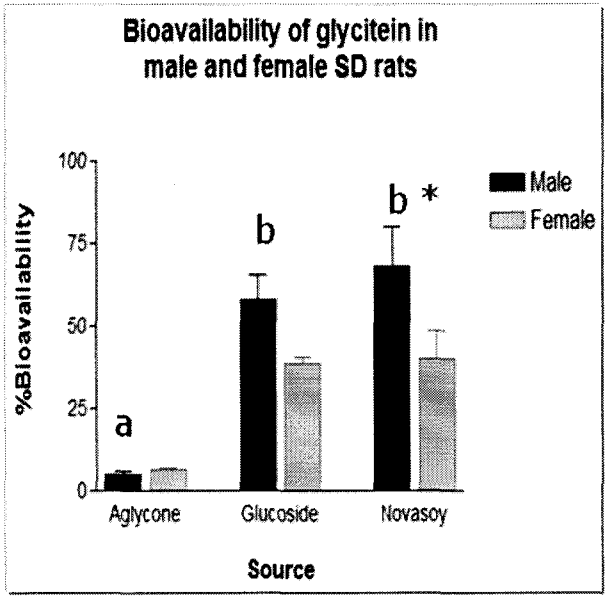


Figure 11: Bioavailability of genistein calculated based on the AUCor of all sources comparing

*Significant gender effecr
[a, b, c] Significant source effect

Bioavailability of glycitein reported in Chapter 3:



Bioavailability of glycitein based on AUC_{IV} aglycon.

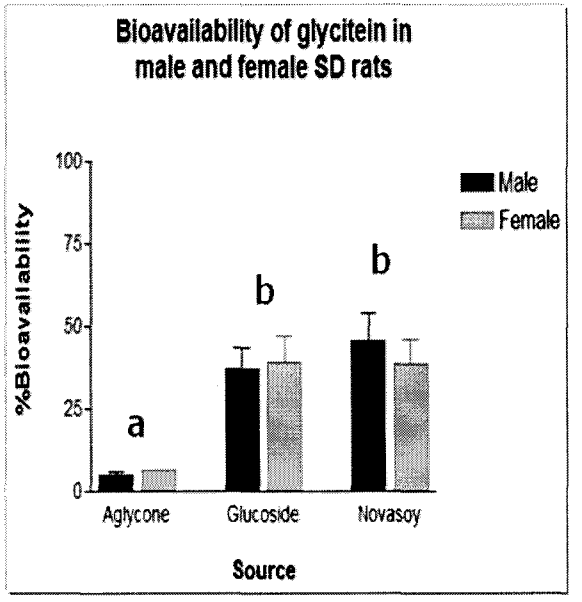


Figure 12: Bioavailability of glycitein calculated based on the AUCor of all sources comparing with

*Significant gender effecr
[a, b, c] Significant source effect

Pharmacokinetic parameters:

In Chapter 3, the pharmacokinetic parameters (e.g. the apparent Vd, Cl rate, and $t_{1/2}$) were calculated using the residuals method of analysis (curve stripping), post oral administration of isoflavone sources. The errors that would occur in the calculation of the above parameters from the oral administration relate to the declining concentrations during the elimination phase which would include elimination as well as an unknown absorption rate. Besides the issue of overlap of elimination, distribution and absorption phases, more data points were needed to perform the analysis in a statistically appropriate manner (Fig. 9).

The apparent Vd is a proportionality constant relating the plasma concentration to the total amount of the therapeutic product in the body (Gibaldi, 1990). In practice, it is impossible to measure the amount of therapeutic product in the body but, immediately after IV administration (at time zero), the amount of drug is equal to the dose (Hedaya, 2007).

Therefore, a more accurate, way of calculating apparent Vd, Cl rate, and and $t_{1/2}$ for isoflavones is based on the IV administration of the synthetic sources of isoflavones. Novasoy^{TM} source was not injected IV, however bioavailability parameters were calculated by comparing AUC_{oral} post Novasoy^{TM} administration by AUC_{iv} post aglycone IV injection. While IV dosing will provide an accurate pharmacokinetic results, when IV injection is impossible (in case of Novasoy^{TM}), the PK values obtained from oral dosing would be inaccurate due to the issue of overlapping phases (elimination, distribution and absorption), and not enough data point collected during elimination phase of the oral administration.

Elimination rate constants (K) for different sources of isoflavones were calculated based on the consideration that K = Cl / Vd. Elimination rate constant reflects the the fraction of isoflavones removed from the blood per unit of time. Tables 9-11 are the representative of pharmacokinetic parameters of plasma daidzein, genistein and glycitein in male and female SD rats based on IV exposure to synthetic sources of isoflavones and comparison of the IV results with the pharmacokinetic results reported in chapter 3.

Table 9: Pharmacokinetic parameters of plasma daidzein in male and female SD rats

	$t_{1/2\ IV}$ h	C_{maxIV} (µmol.h)/L	Vd_{IV}/Kg L/Kgbw	Cl_{IV}/Kg L/h/Kgbw	$tmax_{IV}$ h	K h^{-1}
Daidzein Aglycone						
Male	4.93 ± 1.12	34.28 ± 32.47	3.70 ± 1.00	0.38 ± 0.15*	0.15 ± 0.10	0.12 ± 0.06
Female	3.87 ± 0.67	17.43 ± 8.10	8.48 ± 3.33	1.18 ± 0.28	0.10 ± 0.12	0.15 ± 0.06
Glucoside						
Male	4.30 ± 0.85	37.13 ± 39.24	5.70 ± 4.72	0.30 ± 0.03	0.05 ± 0.10	0.09 ± 0.07
Female	4.57 ± 0.47	110.93 ± 171.06	4.68 ± 2.42	0.47 ± 0.23	0.30 ± 0.48	0.12 ± 0.06

Chapter 3:	$t_{1/2or}$ h	C_{maxor} (µmol.h)/L	Vd_{or}/Kg L/Kgbw	Cl_{or}/Kg L/h/Kgbw	$tmax_{or}$ h
Daidzein Aglycone		a	a	a	a
Male	9.34 ± 1.48	1.63 ± 0.69	14.80 ± 7.29	1.08 ± 0.45*	6.50 ± 3.00
Female	8.29 ± 2.25	0.60 ± 0.08	14.13 ± 3.39	2.77 ± 0.58	5.00 ± 3.46
Glucoside		b	b	b	b
Male	7.74 ± 2.39	4.28 ± 1.13*	3.33 ± 1.94	0.28 ± 0.11	5.00 ± 3.46
Female	7.21 ± 0.49	2.20 ± 0.41	5.15 ± 0.60	0.50 ± 0.06	3.50 ± 3.00

[a, b] Significant source effect; *Significant gender effect; K: elimination rate constant

The significant gender effect that was observed in Cl rate of daidzein post oral administration of synthetic aglycone source reported in chapter 3 is still evident post IV administration of daidzein from a synthetic aglycone source. However, the significant source effect reported in chapter 3 for Vd_{or} and Cl_{or} is no longer evident in Vd_{IV} and Cl_{IV}. No significant effect of gender (p = 0.26), source (p = 0.40), and gender x source interactions (p = 0.87) revealed for daidzein elimination rate constant.

Table 10: Pharmacokinetic parameters of plasma genistein in male and female SD rats

	$t_{1/2\ IV}$ h	C_{maxIV} (µmol.h)/L	Vd_{IV}/Kg L/Kgbw	Cl_{IV}/Kg L/h/Kgbw	$tmax_{IV}$ h	K h^{-1}
Genistein Aglycone						
Male	5.33 ± 3.87	41.75 ± 15.00	3.25 ± 0.53	0.54 ± 0.23	0.15 ± 0.10	0.18 ± 0.09
Female	5.33 ± 3.39	22.08 ± 11.22	8.08 ± 4.33	0.78 ± 0.13	0.10 ± 0.12	0.13 ± 0.10
Glucoside						
Male	4.93 ± 2.02	45.55 ± 25.42	4.98 ± 5.63	0.48 ± 0.28	0.15 ± 0.10	0.17 ± 0.13
Female	5.50 ± 2.01	9.58 ± 4.48	10.18± 7.04	0.70 ± 0.34	0.10 ± 0.12	0.10 ± 0.07

Chapter 3:	$t_{1/2or}$ h	C_{maxor} (µmol.h)/L	Vd_{or}/Kg L/Kgbw	Cl_{or}/Kg L/h/Kgbw	$tmax_{or}$ h
Genistein Aglycone			a	a	a
Male	7.02 ± 1.77	3.53 ± 1.03	9.43 ± 4.69	0.92 ± 0.43*	6.50 ± 3.00
Female	8.29 ± 2.25	2.38 ± 0.31	10.30 ± 5.30	1.60 ± 0.38	5.00 ± 3.50
Glucoside			b	b	
Male	3.59 ± 2.65	3.93 ± 1.87	2.58 ± 1.08	0.64 ± 0.41*	5.00 ± 3.46
Female	4.65 ± 2.11	2.58 ± 0.93	7.28 ± 2.89	1.16 ± 0.41	3.50 ± 3.00

[a, b] Significant source effect; *Significant gender effect; K: elimination rate constant

The significant gender effect that was observed in Cl rate of genistein post oral administration of synthetic aglycone and glucoside sources reported in chapter 3 are no longer evident post IV administration of genistein from a synthetic aglycone and glucoside sources. Similarly, the significant source effects reported in chapter 3 for Cl_{or} and Vd_{or} are no longer evident for in Vd_{IV} and Cl_{IV}. No significant effect of gender ($p = 0.31$), source ($p = 0.71$), and gender x source interactions ($p = 0.89$) revealed for genistein elimination rate constant.

Table 11: Pharmacokinetic parameters of plasma glycitein in male and female SD rats

	$t_{1/2\ IV}$ h	C_{maxIV} (µmol.h)/L	Vd_{IV}/Kg L/Kgbw	Cl_{IV}/Kg L/h/Kgbw	$tmax_{IV}$ h	K h^{-1}
Glycitein Aglycone						
Male	4.93 ± 3.56	3.63 ± 1.52	6.93 ± 2.39	1.14 ± 0.32	0.05 ± 0.10	0.19 ± 0.11
Female	4.40 ± 4.25	3.60± 2.90	11.65 ± 9.46	1.18 ± 0.42	0.10 ± 0.12	0.21 ± 0.18
Glucoside						
Male	5.95 ± 3.14	6.17 ± 6.45	12.68 ± 8.15	1.45 ± 0.64	0.05 ± 0.10	0.15 ± 0.11
Female	6.08 ± 2.46	1.70 ± 0.67	8.65 ± 5.73	0.93 ± 0.31	0.43 ± 0.43	0.12 ± 0.04

Chapter 3:	$t_{1/2or}$ h	C_{maxor} (µmol.h)/L	Vd_{or}/Kg L/Kgbw	Cl_{or}/Kg L/h/Kgbw	$tmax_{or}$ h
Glycitein Aglycone		a	a	a	a
Male	9.21 ± 2.84	0.05 ± 0.06	108.03 ± 71.68	7.68 ± 3.35	8.00 ± 0.00
Female	8.72 ± 2.32	0.05 ± 0.06	76.05 ± 23.28	6.00 ± 0.85	8.00 ± 0.00
Glucoside		b	b	b	b
Male	5.59 ± 0.25*	0.45 ± 0.24	7.43 ± 4.59	0.90 ± 0.53	8.00 ± 0.00*
Female	12.78 ± 5.97	0.30 ± 0.14	19.55 ± 10.35	1.03 ± 0.13	3.50 ± 3.00

[a, b] Significant source effect; *Significant gender effect; K: elimination rate constant

The significant gender effect that was observed in t1/2 of glycitein post oral administration of synthetic glucoside source reported in chapter 3 is no longer present post IV administration of glycitein from a synthetic glucoside source. Similarly, the significant source effects that reported in chapter 3 for Cl_{or} and Vd_{or} are no longer presented in Vd_{IV} and Cl_{IV}. No significant effect of gender (p = 0.90), source (p = 0.34), and gender x source interactions (p = 0.74) revealed for glycitein elimination rate constant.

Conclusion:

Recalculation of bioavailability of Novasoy^{TM} and synthetic glucoside forms of isoflavones (daidzein, genistein and glycitein) after oral and IV exposure in male and female SD rats based on the consideration that aglyconees arising from Novasoy^{TM} and glucosidic form are the absorbable form of isoflavones did not affect bioavailability significances. The significant gender effect that was observed in the bioavailability of glycitein in rats dosed with Novasoy^{TM} source in Chapter 3 is not observed after the re-evaluation based on AUC_{IV} of aglycone dosed rats (Fig. 12).

The reported significant source effect in apparent Vd and Cl rate for daidzein post oral administration of the synthetic aglycone source, for genistein post oral administration of synthetic aglycone and glucoside sources, and for glycitein post oral administration of synthetic glucoside source in chapter 3 is no longer evident post IV administration of synthetic aglycone and glucoside sources.

The significant gender effect that was observed in Cl rate of genistein post oral administration of synthetic aglycone and glucoside sources reported in chapter 3 is no longer evident post IV administration of genistein from a synthetic aglycone and glucoside sources. Similarly, the significant gender effect on $t_{1/2}$ of glycitein post oral administration of synthetic glucoside source reported in chapter 3 is no longer evident post IV administration of glycitein from a synthetic glucoside source.

Recalculation of pharmacokinetic parameters such as apparent Vd, Cl rate and $t_{1/2}$ post IV administration of synthetic glucoside and synthetic aglycone sources revealed more accurate results as the confounding effect of re-absorption of isoflavones during the elimination phase for oral administration of isoflavone sources has disappeared.

CHAPTER 4

4.0 Effect of glycosidation of isoflavones on their bioavailability and pharmacokinetics in aged male rats.

Estatira Sepehr, Gerard M. Cooke, Patrick Robertson, G. Sarwar Gilani, "Effect of glycosidation of isoflavones on their bioavailability and pharmacokinetics in aged male rats, Journal of *Mol. Nutr. Food Res.* (2009), 53, S16-S26.

Study Context and Collaborations:

The development of a HPLC/MS method for analysing rat plasma sample concentration of soy isoflavones (Chapter 2), and age differences observed in plasma isoflavone concentrations from the multigeneration rat study (Appendix.1, Tables 4-7) warranted an additional investigation of the effect of glycosidation of isoflavones on the bioavailability and pharmacokinetics in aged male rats. Additionally, the bioavailability of isoflavones was determined using an accurate methodology reported in Chapter 3 [based on oral and IV administration of purified synthetic isoflavones (daidzein, genistein, glycitein) and their respective β-glucosides and Novasoy^{TM}, a commercial dietary isoflavone supplement].

I was responsible for animal study design, implementation and co-ordination of the study. I was extensively involved in all aspects of animal project such as organizing meetings, designing control and experimental diets, random assigning of rats to metabolic cages. Saphenous vein blood collections were done by the highly trained technicians of the Animal Resources Division at Health Canada. I was involved in collection of rat blood, urine and fecal samples at all different time points. Mr. Robertson was partially involved in collection of the rat blood, urine and fecal samples. However, he was involved in mantainance of LC/MS instruments throughout the project.

I analysed blood samples obtained from rats at different time points, pharmacokinetic analysis and statistical analysis of the results.

I presented the following two posters based on the results obtained from this Chapter:

Estatira Sepehr, G. Sarwar Gilani, Patrick Robertson, Gerard Cooke, Benjamin P.-Y.Lau. , Jocelyn Fournier, Bioavailability of soy isoflavones as affected by gender, age and food matrix in rats. *6th international symposium of soy, Chicago, IL., U.S.A,* October 30-November 2, 2005.

Estatira Sepehr, Gerard Cooke, Jocelyn Fournier, Patrick Robertson, G. Sarwar Gilani, Effects of sex, age and source of soy isoflavones on their bioavailability in a rat model, *Health Canada Research Forum, Ottawa, Ont.*, October, 2003.

Effect of glycosidation of isoflavones on their bioavailability and pharmacokinetics in aged male rats.

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This work was presented in part at the 5th international symposium of soy, September, 2003, Orlando, Fl., U.S.A. and at the 6th international symposium of soy, October 30-November 2, 2005 in Chicago, IL. USA.

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Abstract:

There are limited reports on the bioavailability and pharmacokinetics of isoflavones in elderly humans and aged animals. The present study was conducted to assess the effect of glycosidation of isoflavones on their bioavailability and pharmacokinetics in aged (20-month-old) male Fischer-344 (F-344) rats. The F-344 rat, developed by the National Institute on Aging, is an inbred rat model that is commonly used for aging studies and resembles many features of aging humans. Three sources of isoflavones; Novasoy™ (a commercial supplement), a mixture of synthetic aglyconees (daidzein, genistein and glycitein) and a mixture of synthetic glucosides (daidzin, genistin and glycitin) were tested. Following administration, blood samples were collected at different times (0- 48 h post-oral gavage and 0-8 h post-IV dosing). Plasma isoflavones and 7-hydroxy-3-(4'-hydroxyphenyl)-chroman (a metabolite of daidzein) were measured by LC/MS. The extent of absorption was determined by comparing the area under the curve (AUC) of the plasma-concentration time curve after intravenous (IV) administration with that following oral administration. The extent of bioavailability was then be calculated as:

$$\% \text{ Bioavailability} = \frac{AUC_{or}}{AUC_{IV}} \times \frac{Dose_{IV}}{Dose_{or}} \times 100$$

Bioavailabilities for genistein were significantly (p = 0.013) higher for the aglycone (35 ± 9%) compared with the glucoside forms (11 ± 3%). In contrast, the bioavailabilities for glycitein were significantly (p = 0.011) higher in Novasoy™ (27 ± 13%) and the glucoside form (21 ± 10%) compared with the

aglycone (8 ± 3%). No significant differences in the bioavailability of daidzein were observed in aged rats dosed with aglycone, glucoside or Novasoy™. However, aged rats were able to produce equol as early as 8 h post dosing. In summary, the source of isoflavones had significant effects on genistein and glycitein bioavailability in aged male rats.

Keywords: Bioavailability/ Isoflavones/ LC-MS/Plasma Pharmacokinetics

4.1 Introduction:

Phytoestrogens are a diverse group of polyphenolic plant compounds with hormonal activity and structural similarity to estrogen (Bandele and Osheroff, 2007). Three main classes of phytoestrogens are isoflavones, lignans, and coumestans (Bandele and Osheroff, 2007; Adlercreutz and Mazur, 1997). Isoflavones exist primarily in soybeans, in most soy foods and soy infant formula as a complex mixture of conjugated and unconjugated isoflavones. The β-glucosides, genistin, daidzin, glycitin and the 6'-*O*-malonyl glucosides and 6'-*O*-acetyl glucoside of genistein, daidzein and glycitein (Setchell *et al.*, 2002; Cassidy *et al.*, 2006). The absorption, distribution, metabolism, and excretion of isoflavones from glucoside and aglycone forms have been investigated in animals and humans (King *et al.*, 1996; Piskula *et al.*, 1999; Piskula, 2000; Xu *et al.*, 1994; Franke and Custer, 1996; Izumi *et al.*, 2000; Zubik and Meydani, 2003; de-Pascual-Teresa *et al.,* 2006). After ingestion, the glucoside forms of isoflavones are hydrolyzed to the aglycone form in the jejunum (Zubik and Meydani, 2003). The released aglycone forms of isoflavones are either absorbed into the enterocytes where extensive glucuronidation occurs or further metabolized by intestinal microflora into several products, including equol (7–hydroxyl–3-(4'-hydroxyphenyl)–chroman) a metabolite of daidzein, *p*-ethyl phenol, a metabolite of genistein and di-hydroglycitein, a metabolite of glycitein before absorption (Zubik and Meydani, 2003).

It is believed that dietary intake of soy isoflavones provides a number of health benefits (Bandele and Osheroff, 2007). Epidemiological studies suggest that Southeast Asians, who consume a soy-based diet, experience lower incidences of prostate cancer (Yu *et al.,* 1991), cardiovascular disease (Adlercreutz, 1990), osteoporosis (Adlercreutz *et al.*, 1992), age related disease, and inflammation than do their Eastern and Western populations (Bandele and Osheroff, 2007, Morton *et al.*, 1997; Messina *et al.*, 2006). Within a few generations migrants to the West experience an increase in the incidence of cancer, approaching the rates seen in Western populations. Epidemiological studies, therefore, support the role of dietary and/or life style factors against the development of cancers and cardiovascular diseases (Adlercreutz, 1990).

The potential use of soybean protein products in the USA increased significantly following the Food and Drug Administration (FDA) decision (Setchell *et al.,* 2001) that allowed food manufacturers to make cardiovascular health claims for soy protein products; and the United States Department of Agriculture's (USDA) rule change allowing suitable alternative protein products (such as soybean products) to replace 100% of meat products in the National School Lunch Program, the Summer Food Service Program, and the Child and Adult Care Food Program (Setchell *et al.,* 2001; Messina, 2002). In North America, isoflavone supplements are available for sale in pharmacies and health food stores with wide ranging health claims but there is little regulation regarding their manufacture or efficacy which has raised concerns about potentially dangerous effects from self-administered mega doses of these compounds

(Gilani and Betz, 2006). Soy isoflavones, as isolated pure compounds, are now marketed as concentrated powders, and tablets may soon become available for sale over-the-counter (Gilani and Betz, 200).

In 2004, it was reported that 465 million people (7.2% of the world's population) were over the age of 65 (United Nations Demographic Year book, 2004). About 52% of the world total elderly were in Asia, 25% in Europe, 8.6% in North America and 6.4% in Africa (United Nations Demographic Year book, 2004).

Aging has been defined as the progressive loss of function accompanied by decreasing fertility and increasing mortality and disability (McLean and Le Couteur, 2004). Age has a significant influence on food metabolism which is particularly important for the elderly (Mutschler and Derendorf, 1995). With advancing age, liver blood flow, gastric, and pancreatic secretions decrease. Alterations in biliary secretion, increases in intestinal mucosa permeability, immunological changes, and impairment of intestinal motility may all alter the rate of food metabolism (Del Piano *et al.*, 2004, Crooks *et al.,* 1976). Alterations in the pharmacokinetics of drugs and nutrients are now recognized as one of the consequences of increased longevity (Setchell *et al.*, 1997).

A Health Canada study on protein digestibility in young (5 wk old) and old (20 month old) Fischer-344 (F-344) rats indicated that old rats were more susceptible to nutritional insults, such as the presence of protein-associated bioactive components, compared with young rats (Gilani and Sepehr, 2003). Rats are commonly used as animal models of human diseases and biological

studies because they are easily available, relevant, and appropriate (Gilani and Sepehr, 2003; Wang *et al.*, 2001). The motivation for using the rat model for study of age–related pharmacokinetics and bioavailability in men in this project is, in part, because the F-344 rat developed by the National Institute on Aging, is an inbred rat model that is commonly used for aging studies. It has a longer lifespan, smaller body size, lower spontaneous tumor rate than non-inbred rats, conveniently available and resembles many features of aging humans (Pacher *et al.*, 2004).

The effect of gender on isoflavone bioavailability has been previously reported (Cassidy *et al.*, 2006, Sepehr *et al.,* 2007; Chang *et al.,* 2000). To date there are limited data on the effect of aging on isoflavone bioavailability (Cassidy *et al.*,2006, Faughnan *et al.*, 2004; Setchell *et al.*, 2003). Therefore, the objective of the present study was to investigate the effects of the three isoflavone sources [purified synthetic isoflavones (daidzein, genistein, glycitein and their respective β-glucosides) and Novasoy^{TM} (a commercial dietary isoflavone supplement)] on the bioavailability and pharmacokinetics of soy isoflavones in aged male rats.

4.2. Materials and methods:

4.2.1 Chemicals

The following chemicals with the indicated specifications were used.

(i) Sodium citrate buffer (25 mM, pH 5.0).

(ii) Hydrolytic enzyme. – A mixture of sulfatase and glucuronidase from *Helix pomatia* type H-5 (S3009), containing 400-600 units/mg of glucuronidase activity

and 15-40 units/mg of sulfatase activity was purchased from Sigma- Aldrich (St. Louis, MO, USA). A solution containing 23 units of sulfatase activity was prepared by dissolving an appropriate amount of enzyme in 1.0 mL of the sodium citrate buffer (Sepehr *et al.*, 2006).

(iii) DMSO- 99.9% HPLC grade (Sigma-Aldrich)

(iv) Water - Deionized, NANO-pure (Diamond UV ultra-pure water purification system; Barnstead International, Essex, UK).

All other chemicals including high performance liquid chromatography (HPLC) grade acetonitrile (ACN) and ethyl acetate were purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA).

4.2.2 Apparatus

(i) *HPLC* - The LC separations were performed using a Waters (Milford, MA, USA) Alliance 2695 liquid chromatograph equipped with a Zorbax SB-CN reversed-phase column (4.6 mm X 75 mm, 3.5 µm particle size; Agilent Technologies, Wilmington, DE, USA).

(ii) MS system. A Waters Micromass ZQ single quadrupole mass spectrometer was operated in the positive ion SIM mode. The entire system from sample injection to data acquisition was computer-controlled with Empower software (Waters).

4.2.3 Experimental Diets:

(i) NOVASOY^{TM} (concentrate #152-400) soy isoflavone (Archer Daniels Midland Company, Decatur, IL, USA). One gram of Novasoy^{TM} contains 248 mg

isoflavones as determined based on the method of Wang and Murphy, 1994. About 98% of isoflavones in Novasoy™ are present in their natural glycosidic form and the remainder are protein, sugar, fat, ash and moisture (Table 1). The total content of genistin, daidzin and glycitin in the Novasoy™ was determined by Waters HPLC linear gradient with UV detection monitored at 254 nm (Wang and Murphy, 1994).

To prepare the 20mg/kg oral dose of Novasoy™-derived isoflavones 80.645 mg of Novasoy™ powder was used according to the following calculation: (20 mg isoflavones/kg) / 0.248 mg isoflavones/mg Novasoy™ = 80.645 mg Novasoy™ /kg rat body weight.

(ii) Daidzin, genistin, glycitin, daidzein, genistein, glycitein- LC Laboratories. (Woburn, MA, USA). The ratio of genistin, daidzin and glycitin in Novasoy™ was 1.0: 0.5: 0.2 respectively. Synthetic glucosides and the respective aglyconees were prepared with the same ratio of 1.0: 0.5: 0.2 for genistein, daidzein, and glycitein respectively. Thus, the aglycone diet (20 mg/kg body weight) composed of 11.76 mg genistein, 5.88 mg daidzein and 2.36 mg glycitein.

The glucoside dose (20 mg/ kg body weight) was prepared taking into account the differences in molecular weight between the aglycone and glucoside form. Therefore, the glucoside dose was composed of 19.09 mg genistin, 9.62 mg daidzin and 3.70 mg glycitin. The molecular weights for isoflavone glucosides

Table 1: Composition of the Novasoy™ diet.

Ingredient	%
Total isoflavones	40
Other Natural Soy Phytocomponents	42
Protein	9
Sugar	1
Fat	1
Ash	3
Moisture	4

*Adapted from the technical data sheet for NOVASOY™ (concentrate #152-400) soy isoflavone (Archer Daniels Midland Company).

and aglyconees are reported in Table 2. Thus, both the aglycone and glucoside doses were equimolar with respect to the isoflavone moieties.

(iii) Casein protein (90% purity, ICN Biomedicals, Costa Mesa, CA, USA). The composition of the casein-based isoflavone-free diet is reported in Table 3.

4.2.4 Preparation of diets:

Oral gavage:

A mixture of synthetic glucosides (genistin, daidzin, glycitin); or a mixture of synthetic aglyconees (genistein, daidzein, glycitein) or Novasoy^{TM} were suspended in ultra-pure water and the administered volume was adjusted to 2.5 mL to provide a dose of 20 mg /kg body weight of rats. Solutions were sonicated for 1 h prior to the oral administration. The isoflavones oral dose of 20 mg/kg body weight of rats is representative of a high dietary level experience by infants fed on soy-based formulae (Irvine *et al.*, 1998; Zung *et al.,* 2001).

Intravenous (tail vein) injection:

A mixture of synthetic glucosides (genistin, daidzin, glycitin in the same proportion as for oral gavage); or a mixture of synthetic aglyconees (genistein, daidzein, glycitein, also in the same proportion as for oral gavage) was suspended in 50% v/v aqueous DMSO and the volume adjusted to provide 10 mg/kg body weight of rats such that the intended doses were delivered in a volume of 1.0 μL/g rat body weight.

Table 2: Molecular weights for isoflavone glucosides (MW_g) and aglyconees (MW_a).

Isoflavone Glucoside	Isoflavone aglycone	MW_g	MW_a	MW_a / MW_g
Genistin	Genistein	438.37	270.23	0.616
Daidzin	Daidzein	416.36	254.23	0.611
Glycitin	Glycitein	446.21	284.27	0.637

Table 3: Composition of the casein based isoflavone-free diet (g/kg diet).

Ingredient	Casein
Vitamin free casein[a)]	222.2
Sucrose	100.0
Cornstarch	477.3
Fibre (Sulka-Floc)	50.0
Soyabean oil	100.0
Mineral mix[b)]	35.0
Vitamin mix[b)]	10.0
Choline bitartrate	2.5
DL-Methionine	3.0
tert-Butylhydroquinone	0.014
Total isoflavones (mg/kg diet)[c)]	0.0
Genistein (mg/kg diet)[c)]	ND
Daidzein (mg/kg diet)[c)]	ND
Glycitein (mg/kg diet)[c)]	ND

ND, not detectable

a) Casein from ICN Biomedicals contains 90% crude protein.

b) AIN-93G Mineral mix and AIN-93G Vitamin mix were from ICN Biomedicals.

c) The actual content of isoflavones (genistein, daidzein and glycitein) was determined by Waters HPLC linear gradient with UV detection monitored at 254 nm by Mr Robertson.

Novasoy™ source was not injected IV, however bioavailability parameters were estimated by comparing AUC_{oral} post-Novasoy™ administration by AUC_{iv} post-glucoside IV injection.

4.2.5 Animals, isoflavones administration and samples collection:

Twenty male retired breeder 20 month old (400 g) F-344 rats (National Institute on Aging; Bethesda, MD, USA), were individually caged in rooms where the temperature was maintained at 23 ± 2°C and lights were on a 12 h light/dark cycle. Animal handling and care followed the guidelines of the Canadian Council for Animal Care and all aspects of the experimental protocol were reviewed and approved by the Health Canada, Ottawa Animal Care Committee.

During an adjustment period of 10 days, rats were fed an isoflavone-free casein-based control diet formulated according to the American Institute of Nutrition (AIN-93M) recommendations (Reeves *et al.*, 1993). After the adjustment period, rats were randomly assigned to five groups of four; those rats destined for oral administration were gavaged with a single oral dose of one of the three sources of isoflavones (Novasoy™, a mixture of synthetic aglyconees or a mixture of synthetic glucosides). Rats assigned for IV injection, were injected (tail vein) with one of the two sources of isoflavones (a mixture of synthetic aglyconees or a mixture of synthetic glucosides) by using a 26-gauge needle (Becton Dickinson, Rutherford, NJ, USA) without anesthesia. Post-oral or IV administration, rats were assigned to metabolic cages with free access to isoflavone-free casein- based diet and tap water for the total period of the study.

4.2.6 Rat Plasma Sample Collection:

Individual blood samples of approximately 400µL were collected from the saphenous vein of the same rat in lithium heparin microtainers at 0, 2, 8, 24, 48 h (post-oral) for aglycone, glucoside and Novasoy™ sources and at 0, 10, 30, 45 min, 1, 2, 3, 4, 8h (post-IV) for aglycone and glucoside groups as described previously (Sepehr *et al.,* 2007). Plasma was separated by centrifugation for 3 min at 4°C, 8000 rpm in an IEC Centra MP4R refrigerated centrifuge (Needham Heights, MA, USA) and stored at -80°C until the day of analysis.

4.2.7 Plasma isoflavone analysis:

Plasma concentrations of isoflavones were determined by LC/MS as previously described (Sepehr *et al.*, 2006). Briefly, following enzymatic hydrolysis of isoflavone conjugates with mixed glucuronidase/sulfatase enzyme, the resultant aglyconees were extracted with ethyl acetate, centrifuged and the supernatant was diluted with mobile phase (0.1% formic acid in 85:15 water: ACN) and injected onto a Zorbax SB-CN reversed-phase column (4.6 mm X 75 mm, 3.5 µm particle size). The chromatographic run time was 16.0 min, with a delay of 10 min/injection.

4.2.8 Pharmacokinetic analysis:

Pharmacokinetic parameters were first derived using non-compartmental methods (PK Solutions™ version 2.0.2 package, Summit Research Services, Ashland, Ohio, USA). The pharmacokinetic parameters were calculated using the

residuals method of analysis, assuming first order disposition kinetics. The depletion kinetics were modeled for the elimination phase after oral dosing. The pharmacokinetic parameters determined were: the terminal half-life, $t_{½}$ (the time taken for the maximum plasma concentration to decrease by half); C_{max} (the maximum observed peak plasma isoflavone concentration); t_{max} (time point at C_{max}); $AUC_{(0-t)}$, the area under the concentration-time curve (reflecting the exposure of plasma to isoflavone from time zero to time t when the plasma concentration of isoflavones returned to baseline); $AUC_{(0-t)}$ was estimated using the linear trapezoidal rule and calculated using data to the last quantifiable time point.

The absolute oral bioavailability was calculated from the percentage ratio of the AUCs derived from plasma isoflavone concentrations after oral and IV dosage of different sources of isoflavones to male rats.

$$\% \text{ Bioavailability} = \frac{AUC_{or}}{AUC_{IV}} \times \frac{Dose_{IV}}{Dose_{or}} \times 100$$

4.2.9 Statistical analyses:

Statistical analyses were done using SigmaStat version 3.1 (2004 Systat Software Inc, Richmond CA, USA). All pharmacokinetic data were expressed as mean ± SD, and were analyzed using one-way ANOVA for its source effect of isoflavones. Statistically significant differences between sources were identified using the Holm-Sidak test. A probability value of $p < 0.05$ was considered statistically significant.

4.3 Results:

4.3.1 Phase1 (oral gavage):

The profiles of plasma isoflavones (daidzein, genistein, glycitein and equol) following oral administration of the synthetic glucoside source, synthetic aglycone source and Novasoy^{TM} (20 mg/kg body weight) to aged (20 month old) male F-344 rats are presented in Fig. 1.

The results of the non-compartmental pharmacokinetic analysis from the plasma curves of the synthetic isoflavone aglycone, glucosides and Novasoy^{TM} post single–bolus oral exposure to aged male rats are summarized in Tables 4 and 5.

The maximum observed peak plasma (C_{max}) daidzein and glycitein concentrations were significantly higher (up to four-fold; $p < 0.05$) in Novasoy^{TM} dosed rats compared with aglycone-dosed rats (Table 4). Additionally, the C_{max} daidzein and genistein concentrations were significantly higher ($p < 0.05$) in Novasoy^{TM} (up to two-fold) dosed rats compared with glucosides-dosed rats (Table 4).

The AUC_{or} values post-oral administration of daidzein and glycitein were found to be significantly greater (up to three-fold) for the Novasoy^{TM}-dosed rats than the aglycone-dosed rats (Table 5). Following glucoside treatment, the AUC_{or} values for daidzein and glycitein were significantly higher (up to two-fold) compared with the aglycone treated rats (Table 5).

Volume of distribution (Vd_{or}) was significantly larger ($p < 0.05$) for glycitein in rats dosed with aglyconees compared with rats dosed with glucosides (up to

six-fold) and Novasoy™ (up to seven-fold). Additionally, Vd_{or} was significantly larger ($p < 0.05$) for daidzein in rats dosed with aglyconees compared with rats dosed with Novasoy™ (up to three-fold) (Table 5).

4.3.2 Phase 2 (IV injection):

Following IV administration of two sources of isoflavones (10 mg/kg body weight), the plasma profile of isoflavones demonstrated a rapid increase in plasma concentration of isoflavones followed by an elimination phase (Fig. 2). The profiles of plasma isoflavones in old male (20 month old) F-344 rats post-IV injection of mixtures of isoflavone glucosides or aglyconees reached C_{max} for daidzein, genistein and glycitein within the first 10 minutes post-administration of the doses (Fig. 2).

4.3.3 Bioavailability of isoflavones:

Bioavailability of daidzein did not significantly differ when administered as the aglycone, glucoside or Novasoy™. The bioavailability value for genistein was significantly ($p < 0.05$) higher (up to three-fold) in the aglycone form compared with those in the glucoside forms of isoflavones (Table 5). Bioavailability values for glycitein were significantly ($p < 0.05$) higher (up to four-fold) in Novasoy™ and the glucoside forms of isoflavones (up to three-fold) compared with those in aglycone forms (Table 5).

Table 4: Pharmacokinetic parameters of plasma isoflavones in aged (20 month old) male F-344 rats following gavage of a single dose (20 mg/kg/body weight) of three different sources (mixtures) of soy isoflavones.

Sources of Isoflavones	Daidzein		Genistein		Glycitein	
	C_{max} [a)] (µmol/L)	t_{max} h	C_{max} [a)] (µmol/L)	t_{max} h	C_{max} [a)] (µmol/L)	t_{max} h
Aglycone	1.63 ± 0.35^{A}	6.50 ± 3.00	3.23 ± 0.52^{AB}	3.50 ± 3.00	0.10 ± 0.00^{A}	8.00 ± 0.00
Glucoside	2.80 ± 1.05^{A}	6.50 ± 3.00	2.08 ± 0.82^{B}	6.50 ± 3.00	0.35 ± 0.13^{ab}	8.00 ± 0.00
Novasoy™	5.15 ± 1.68^{B}	6.50 ± 3.00	3.95 ± 1.13^{A}	8.00 ± 0.00	0.40 ± 0.08^{b}	6.50 ± 3.00

Means in a column with different superscripts differ significantly ($p < 0.05$).
a) Values are means ± SD, n = 4.

Table 5: Pharmacokinetic parameters of plasma daidzein, genistein and glycitein in aged (20 month old) male F-344 rats after oral or IV exposure to three different sources (mixtures) of soy isoflavones[a)].

	$t_{½\ or}$ h	AUC_{or} [a)] (µmol.h)/L)	Vd_{or}/Kg [a)] L/Kg bw	Cl_{or}/Kg [a)] L/h/Kg bw	AUC_{iv} (µmol.h)/L)	%Bioavailability
Daidzein						
Aglycone	6.23±2.94	10.66±1.72^{B}	7.00±2.50^{B}	0.82±0.17^{B}	16.30±4.61	34±6
Glucoside	6.88±1.58	15.39±7.73^{C}	4.28±2.40^{AB}	0.41±0.16^{A}	32.35±18.46	26±5
Novasoy™	5.75±1.11	32.37±11.79^{A}	1.68±0.57^{A}	0.22±0.10^{A}	-------	45±18
Genistein						
Aglycone	5.92±2.98	20.01±1.74	8.75±5.06	1.00±0.12^{B}	30.68±11.58	35±9^{A}
Glucoside	6.25±0.57	12.11±6.65	9.03±3.12	1.00±0.31^{B}	43.30±27.13	11±3^{B}
Novasoy™	5.63±4.43	21.31±7.67	4.60±1.21	0.37±0.20^{A}	-----	21±7^{AB}
Glycitein						
Aglycone	7.34±5.45	0.65±0.13^{B}	67.65±8.87^{B}	1.39±1.59	4.15±0.85	8±3^{B}
Glucoside	9.28±1.69	1.77±0.66^{A}	11.00±3.52^{A}	0.83±0.25	3.45±0.24	21±10^{A}
Novasoy™	8.15±0.62	2.23±0.79^{A}	9.23±5.14^{A}	0.77±0.39	-------	27±13^{A}

Means in a column for individual isoflavones with different superscripts differ significantly ($p < 0.05$).
a) Values are means ± SD, n = 4.

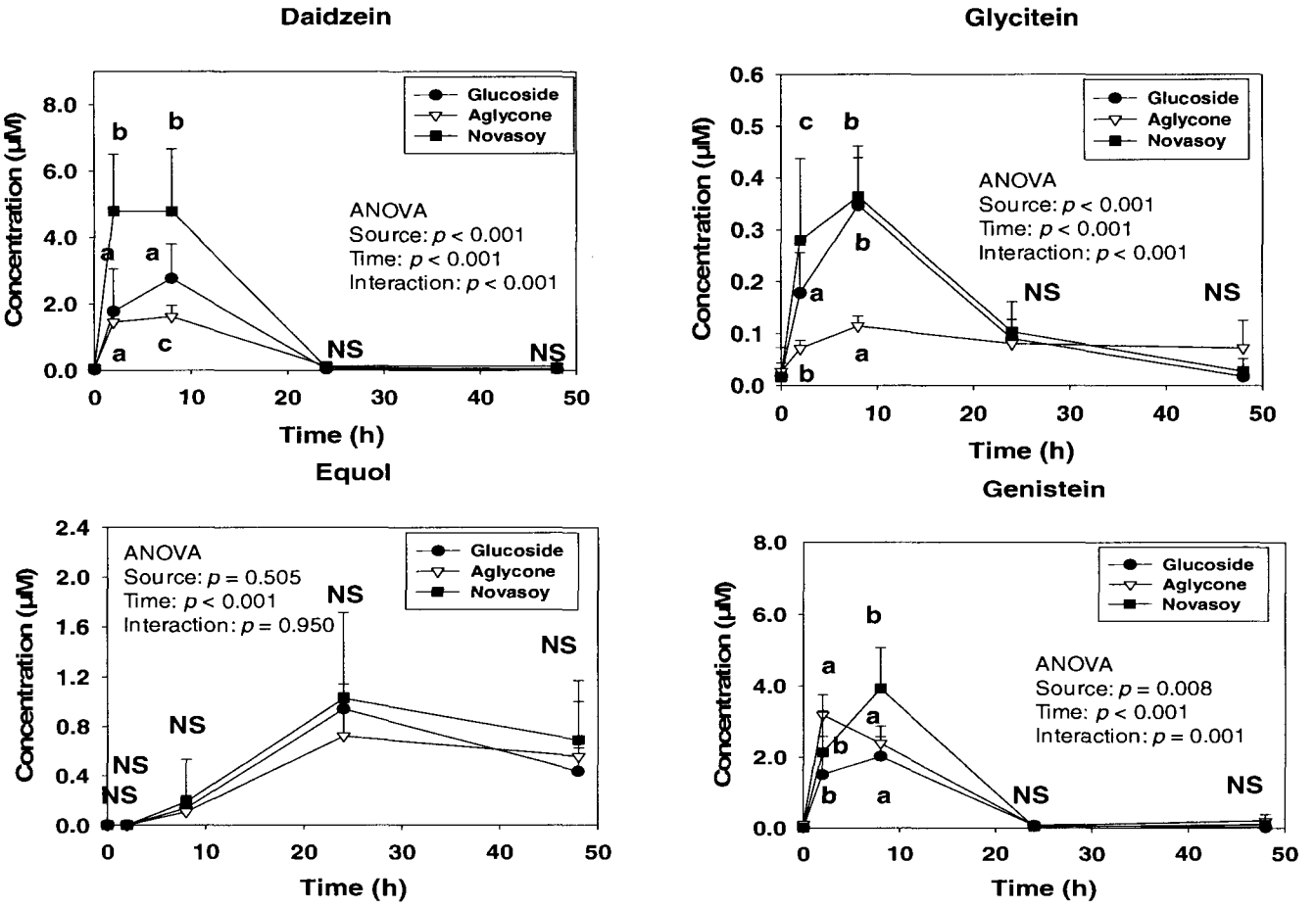

Figure 1: Plasma profiles of isoflavones in aged (20 month old) male F-344 rats following oral gavage of a mixture of synthetic isoflavone glucosides, aglyconees or NovasoyTM at 20 mg/kg body weight.

Results are expressed as means ± SD on a sample size of four rats / group. Significant effects of source at specific time points are indicated by different superscripts ($p < 0.05$). NS indicates no significant differences.

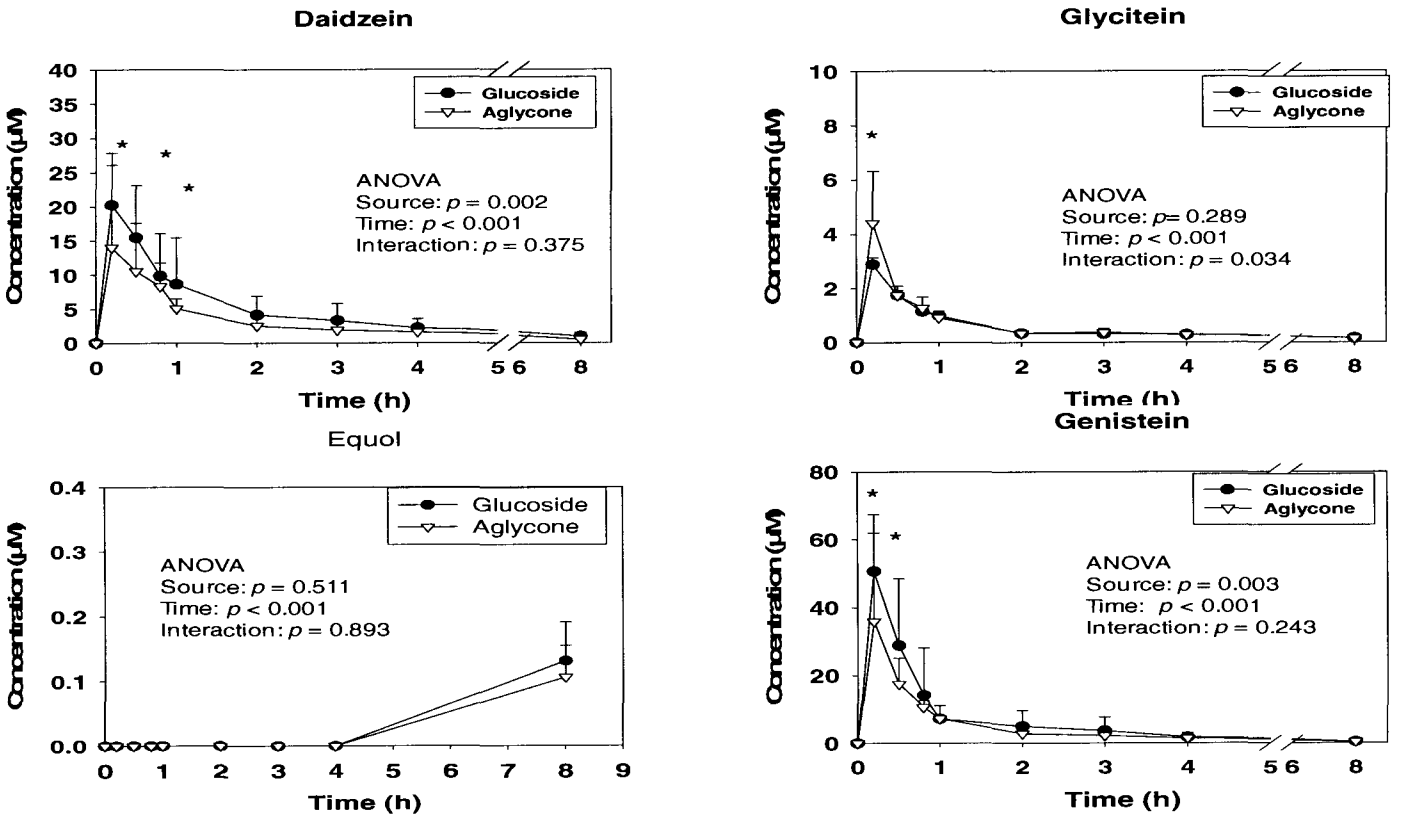

Figure 2: Plasma profiles of isoflavones in aged (20 month old) male F-344 rats following IV injection of a mixture of synthetic isoflavone glucosides or aglyconees at 10 mg/kg body weight.

Results are expressed as means ± SD on a sample size of four rats / group. Significant effects of source at specified time points are denoted by an asterisk (*), $p < 0.05$.

4.3.4 Formation of equol:

Plasma profiles of equol exhibited a time lag in its appearance after a single-bolus of the isoflavones, and it took ≥ 8 h before equol appeared in substantial amounts after either oral or IV administration of the doses (Figs. 1 and 2).

4.4 Discussion:

The bioavailability and pharmacokinetics of the isoflavones from glucoside and aglycone forms have been extensively investigated in animals and humans (Cassidy *et al.,* 2006; Piskula *et al.,* 1999; Izumi *et al.*, 2000; de-Pascual-Teresa *et al.*, 2006; Setchell *et al.*, 2001; Sepehr *et al.,* 2007; Coldham *et al.,* 2002; Coldham *et al.,* 2000; Janning *et al.,* 2000; Moon *et al.,* 2006; Busby *et al.*, 2002; Bloedon *et al.*, 2002; Kano *et al.*, 2006). However, relatively few studies have characterized the bioavailability and pharmacokinetics of these compounds in elderly humans and aged animals (Cassidy *et al.,* 2006, Faughnan *et al.*, 2004; Setchell *et al.,* 2003; Joshi *et al.,* 2007).

The present study is the second part of an isoflavone bioavailability study in our laboratory that determined bioavailability based on the AUC of both oral and IV administration of different sources of isoflavones, in aged (20 month old) rats. In our earlier study, the effects of the different isoflavone sources and of gender on bioavailability and pharmacokinetics of soy isoflavones in rats were investigated in detail (Sepehr *et al.*, 2007).

The dietary dose selected for use in this study was relevant to infants consuming soy formulas. A recent National Centre for Toxicological

Research/National Toxicology program study of life time exposure to dietary genistein demonstrates this connection (Chang *et al.*, 2000). In that study, rats consuming 5 μg/g and soy-free control diets had serum levels of total genistein (0.01-0.06 μM), similar to those in humans consuming a typical Western diet containing little or no soy; rats consuming a 100 μg/g genistein diet produced serum levels (0.6-0.9 μM) similar to those measured in adults consuming typical Asian diets (Adlercretz *et al.,* 1994); and rats consuming a 500 μg/g genistein diet had serum total genistein levels (6.0-7.9 μM) similar to infants consuming soy formulas (Setchell *et al.*, 1997).

In the context of bioavailability, the main difference between the IV and oral doses is the fact that in oral administration, chemicals are absorbed by the digestive system and enter the liver via the hepatic portal system before reaching the rest of the body (Aghazadeh-Habashi *et al.*, 2006). The liver metabolizes the chemicals sometimes to such an extent that only a small amount of active chemical emerges from the liver to reach the rest of the circulatory system (Aghazadeh-Habashi *et al.*, 2006). Alternative routes of administration such as IV, avoid the first-pass effect and chemicals can be absorbed directly into the systemic circulation and therefore their bioavailability is 100% (Aghazadeh-Habashi *et al.*, 2006).

Our data clearly show that the bioavailability of genistein was significantly ($p < 0.05$) higher (up to three-fold) in aglycone treated rats compared with glucoside forms of isoflavones (Table 5). It is presumed that in aged rats, genistein in the glucoside form is poorly absorbed from the gut compared with

the aglycone, which might be due to the higher hydrophilicity and greater molecular weight of isoflavone glucosides (Izumi *et al.,* 2000; Brown, 1988). Factors such as intestinal bacterial fermentation, intestinal transit time, and age are expected to have an influence on the metabolism and bioavailability of isoflavones (Piskula *et al.*, 1999; Zubik and Meydani, 2003; Hendrich, 2002; Lee *et al.*, 2007). Additionally, the observed short t_{max} (3.5 h) for genistein in plasma in rats treated with the aglycone mixture is attributed to the absorption of the aglycone form from the stomach, as was found in the study by Piskula *et al.*, (1999).

Glycitein comprises less than 10% of the total isoflavone amount in soybeans and soybean foods, which may be the reason that its absorption and metabolism has not been well-studied (Song *et al.*, 2002; Simons *et al.*, 2005). However, the bioavailability of glycitein is important as many commercially available supplements contain high levels of glycitein and limited information exists about its biological properties (Setchell *et al.*, 2001). Our data show that bioavailabilities for glycitein were significantly ($p < 0.05$) higher (up to four-fold) from Novasoy™ and the glucoside mixture (up to three-fold) compared with those from the aglycone mixture (Table 3).

The relatively low bioavailability of glycitein in aglycone dosed rats might be due to extensive first pass metabolism (i.e. enterohepatic recycling) (Jia *et al.,* 2004). Oral administration of glycitein in rats dosed with the aglycone mixture resulted in a significantly more rapid clearance rate (1.39 L/h/kg body weight) than from Novasoy™ (0.77 L/h/kg body weight) and the glucosides mixture (0.83

L/h/kg body weight) and furthermore, a larger volume of distribution (~67.65 L/kg body weight) compared with the glucosides (11 L/kg body weight) and Novasoy™ (9.23 L/Kg body weight) sources. This suggests that the aglycone form of glycitein in aged rats is more likely to enter, and perhaps be sequestered by tissues, faster than the glucoside and Novasoy™ forms of glycitein. The rapid clearance rate is likely due to rapid metabolism of glycitein. The major pathways of glycitein metabolism by human intestinal microflora are reduction to dihydroglycitein (7, 4'dihydroxy-6methoxy-isoflavanone) followed by demethylation to produce dihydro-6, 7, 4'-trihydroxyisoflavone (6, 7, 4'-trihydroxyisoflavanone) or, alternatively, C ring cleavage of dihydroglycitein to produce 5'-methoxy-*O*-desmethylangolensin (Simons *et al.*, 2005). Additionally, the chemical structure of glycitein is similar to daidzein with the exception of the methoxy group at the six-position, and in fact, Setchell *et al.,* (2001) and Simons *et al.*, (2005) have reported that humans fed glycitein showed minute concentrations of daidzein in plasma due to direct demethoxylation of glycitein at the six-position. Therefore, minor pathways include direct demethoxylation of glycitein to daidzein (7, 4'-dihydroxyisoflavone) and reduction of dihydroglycitein to 6-*O*-Me-equol (7, 4'-dihydroxy-6-methoxy-isoflavan) (Simons *et al.*, 2005). Since demethoxylation of glycitein to daidzein may not be a major pathway of glycitein metabolism in humans and rats, the exact identification of glycitein metabolites warrants further investigation.

There was no difference in the bioavailability of daidzein from aglycone or glucoside forms in aged rats. Additionally, there was no difference in t_{max} and

C_{max} values for daidzein between the aglycone and glucoside forms. Our finding agrees with some reports indicating that the bioavailability of daidzein is not influenced by the presence of free or conjugated forms in the diet of humans (Zubik and Meydani, 2003; Richelle *et al.*, 2002).

We observed age differences in the bioavailability of daidzein (57 vs 26%), genistein (58 vs 11%) and glycitein (58 vs 21%) for the isoflavone glucosides and daidzein (98 vs 45%), genistein (62 vs 21%) and glycitein (68 vs 27%) for the Novasoy^{TM} sources, where lower bioavailability values (~ 40%) were observed in aged (20 month old) male rats compared with young adult (3 month old) male Sprague Dawley rats reported earlier (Sepehr *et al.*, 2007). The age differences are likely due to the changes in physiology associated with advancing age, particularly decreases in total body water and lean body mass and, most importantly, a decline in renal function as well as differences in absorption of isoflavones and differences in the biotransformation, excretion and enterohepatic circulation (Sepehr *et al.,* 2006; Coldham *et al.,* 2002; Marchbanks *et al.*, 1990). However, such a comparison between different strains of rats has drawbacks that future research may address.

When isoflavones are ingested, the conjugated isoflavones are hydrolyzed in the gut by both intestinal mucosal and bacterial ß-glucosidases releasing the aglyconees (Cassidy *et al.,* 2006). Aglyconees are in turn either absorbed intact or further metabolised by intestinal microflora in the large intestine into other metabolites such as 7–hydroxyl–3-(4'-hydroxyphenyl)–chroman (equol) or *O*-desmethylangolensin (*O*-DMA) from daidzein, *p*-ethyl phenol (from genistein) and

di-hydroglycitein (from glycitein) (Cassidy *et al.,* 2006). The next metabolic step in the metabolism of aglyconees involves the re-conjugation of the aglycone with glucuronide or sulphate within the intestinal absorbing cells and in the liver (Shenutt *et al.,* 2002; Rowland *et al.,* 2000). As a result, isoflavones are found primarily as conjugates in serum, with only a small portion present as free aglyconees (Rowland *et al.,* 2000). There are two conjugation sites on genistein and daidzein, and each of these sites can be sulphated and/or glucuronidated. Thus, there are monoglucuronides, monosulphates, diglucuronides, disulphates, and mixed conjugates of aglyconees with one site glucuronidated and one site sulphated (Shenutt *et al.*, 2002). Structural determination based on LC/MS and ^{1}H NMR showed that both 7-and 4' glucoronides of genistein were present in rat and human blood (Doerge *et al.*, 2002). Circulating concentrations of genistein aglycone, measured after oral administration, are quite low in adult Sprague-Dawley rats (about 2% of total genistein; Holder *et al.*, 1999) and in humans (about 1%; Setchell *et al.*, 2001). This is in agreement with our previous report (Sepehr *et al.*, 2006) where LC/MS chromatograms of isoflavones in rat plasma samples, following oral administration of isoflavones without enzyme hydrolysis, revealed very low concentrations of free isoflavone aglyconees and also that the isoflavone glucoside concentrations were below the LOD of the reported method. An article by Janning *et al.*, (2000), reported daidzein conjugates (glucuronides/sulfates) are also the main circulating metabolites in female Da/Han rats post oral and IV administration of daidzein.

Further metabolism of isoflavones and their metabolites occurs through their incorporation into bile acids followed by enterohepatic circulation including reabsorption into the bloodstream (Rowland *et al.,* 2000). Bile excreted into the intestine is met by bacterial ß-glucuronidase and sulphatase-enzymes which deconjugate the isoflavones and isoflavone metabolites and allow them to be reabsorbed into the circulation (Rowland *et al.*, 2000).

In the present study, the late appearance (~8h) of equol in plasma samples after both oral and IV administration is consistent with its production being in the large intestine (Cassidy *et al.,* 2006; Setchell *et al.,* 2001). It is probable that enterohepatic circulation is the probable mechanism by which IV injected daidzein may reach the colon, where it can be converted to equol. Interestingly, we found aged (20 month old) male rats are capable of producing equol at 8 hr post IV administration of aglycone (0.106 ± 0.04µM) and glucoside (0.131 ± 0.06 µM) sources of isoflavones compared with undetectable equol production in young adult (3 month old) male rats (Sepehr *et al.*, 2007). Additionally, a higher concentration of equol was produced in aged male rats following oral administration of aglycone (0.72 ± 0.4 µM), glucoside (0.94 ± 0.1 µM) and Novasoy^{TM} (1.02 ± 0.6 µM) than in young adult male rats [aglycone and Novasoy^{TM} (0.0 ± 0.0µM) and glucoside (0.04 ± 0.02 µM)] (Sepehr *et al.*, 2007). This might be due to the enhanced ability of aged (20 month old) male rats to convert more daidzein to equol compared with young adult male rats. It has been reported that variation in equol production is due to the absence or presence of certain bacterial species in the intestine (Izumi *et al.,* 2000; Liu and Hu, 2002;

T'ien-Li and Hsiu-Yuan, 1977; Watanabe *et al.,* 1998) as evidenced from the finding that infants fed soy formula up to the age of 4 months (Setchell *et al.,* 1997; Setchell, 1998) and germ-free rats fed soy-containing diets do not produce equol (Axelaon and Setchell, 1981). Rats and mice are equol producers (Setchell *et al.*, 2005). In contrast, humans are unique among animals as only 20-35% of the adult population produce equol after ingesting soy foods or pure isoflavones (Setchell *et al.,* 2005). It is unknown why the colonization of equol –producing bacteria varies between individuals. Diet has been suggested as a contributing factor for equol production, especially when it contains prebiotics and/or probiotics (Vatanparast and Chilibeck, 2006). A few bacterial groups make up 50-70% of the dominant bacteria in the human intestinal tract, comprising mainly the *Clostridium coccoides-Eubacterium rectale* cluster, the genus *Bacteroides*, the *Clostridium leptum* subgroup (including the genus *Faecalibacterium*), and the genus *Bifidobacterium* (Clavel *et al.*, 2005). Three strains of bacteria which are able to convert daidzein to equol *in vitro* are *Streptococcus intermedius*, *Ruminococcus productus*, and *Bacteroides ovatus* (Ueno and Uchiyama, 2001). Although several studies have investigated bacterial populations in the adult large bowel (Gorbach *et al.*, 1967; Franks *et al.,* 1998; Finegold *et al.,* 1983; Benno *et al.,* 1991), relatively little information is available concerning the effect of age on gut microflora. With advancing age, species such as bifidobacteria are thought to decline in numbers, whereas clostridia and enterobacterial populations increase (Gorbach et al., 1967; Hopkins and Macfarlane, 2002). Determination of

gut microflora types prior to and after soy isoflavones intake would be useful in identifying those bacteria that are involved in equol production.

In conclusion, our data clearly show that the source of isoflavones had significant effects on isoflavone genistein and glycitein bioavailability and pharmacokinetics in aged male (20 month old) rats. These findings will provide valuable information to policy and regulatory agencies in assessing safety and efficacy of isoflavones with regards to elderly humans.

4.5. Acknowledgements:

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The high-level statistical advice of Mr. S. Hayward is gratefully acknowledged. Many thanks are extended to all members of the Animal Resources Division of Health Canada, for their technical assistance with the animal portion of this study.

Note to Chapter 4:

Animal care:

The animal care and handling procedures were conducted according to the guidelines of the Canadian Council on Animal Care, and the experimental protocol was approved by the Health Canada, Ottawa, Animal Care Committee prior to the initiation of the study. Sprague-Dawley rats (Charles River, St-Constant, PQ, Canada) were individually caged in stainless steel cages with hardwood chip bedding (maple or ash) and kept in an environmentally controlled room with an illumination intensity of 150-300 lux (fluorescent bulbs on automated timer) and 12 hours illumination, humidity 40-60%, temperature 23 ± 2 °C and free access to isoflavone-free casein based control diet formulated according to the American Institute of Nutrition (AIN-93G) recommendations (Reeves *et al.,* 1993) and fresh water available *ad libitum.*

Post-oral or IV administration of isoflavone sources, rats were assigned to metabolic cages with bottom screens to prevent coprophagy during bioavailability study.

Isoflavone Sources:

Three lots of NOVASOY^{TM} concentrate #152-400 (600g of Lot # 9809151, 2400g of Lot # 0011101, and 2000g of Lot # 0104181; Archer Daniels Midland Company, Decatur, IL, USA) were mixed together and used for all studies conducted. The actual content of isoflavones (genistein, daidzein and glycitein) in the Novasoy^{TM} source was determined by Waters HPLC linear gradient with UV

detection after acid hydrolysis and liquid –liquid extraction of samples according to the method of Wang and Murphy, (1994) in Dr. Gilani's laboratory by Mr. Robertson.

The preparation of Novasoy^{TM} contained a total isoflavone concentration of 24.1% (genistein 12.5%, daidzein 8.6%, and glycitein 3.0%) the remainder being moisture, carbohydrate, protein, fat and ash.

Standards:

(i) Daidzin, genistin, glycitin, daidzein, genistein, glycitein and equol (metabolite of daidzein) were purchased from LC Labs. (Woburn, MA, USA). The purity of each standard used was verified using LC/MS method (Sepehr *et al.*, 2006). LC/MS spectra agreed with the structure of each isoflavone and matched the spectra obtained from a purchase standard of each isoflavone, indicating a purity of above 99% for each lot.

(ii) Biochanin A, 4-ethyl phenol (metabolite of genistein) and apigenin- Sigma-Aldrich Co. The purities of these three compounds were above 97%, 99%, and 95% respectively. For apigenin, LC/MS spectra indicated one major peak and minor impurities in some of the lots. Probe/MS testing in different lots of apiginin indicated one major component with minor components, suggesting little impurities. Therefore apiginin did not use as an internal standard in analyzing plasma samples.

National Centre for Complementary and Alternative Medicine (NCCAM) guidelines were followed in assuring quality of isoflavone-sourced materials.

To ensure stability, the bulk chemical was stored at -80 ^{0}C, protected from light in the original shipping containers. Purity was periodically measured during the study; no degradation of the bulk chemical was detected.

Sample centrifugation:

Samples were centrifuged for 6 min at 4^{0}C at 16,750 x g in an IEC Centra MP4R refrigerated centrifuge, Cat. No. 2438 (Needham Heights, MA) in order to precipitate proteins.

Determination of plasma isoflavones pharmacokinetics:

Pharmacokinetic analysis of plasma isoflavone profiles for each rat was conducted using non-compartmental analysis software (PK Solutions^{TM} version 2.0.2 package, Summit Research Services, Ashland, Ohio, USA). The pharmacokinetic parameters were calculated using the residuals method of analysis (curve stripping), assuming first order disposition kinetics as shown in note to chapter 3 (Fig. 8).

Clarification of determination of bioavailability values for rats administered with aglyconece source of isoflavones (Table 5 of Chapter 4).

In chapter 4, bioavailability data for rats dosed with synthetic aglycone source of isoflavones were calculated from AUCs of oral and IV exposure of individual animals by random assigning of the AUC_{or} to AUC_{IV} from four rats in the same diet group. However, since IV and oral exposure studies were not both performed in each rat, it was considered appropriate to determine the

bioavailability in each group by comparing the average of AUCs of IV exposure of four rats in one group, with AUC_{or} of each rat in the same diet group (Table 6).

The same method was also used for all rats administered with synthetic aglycone sources of isoflavone.

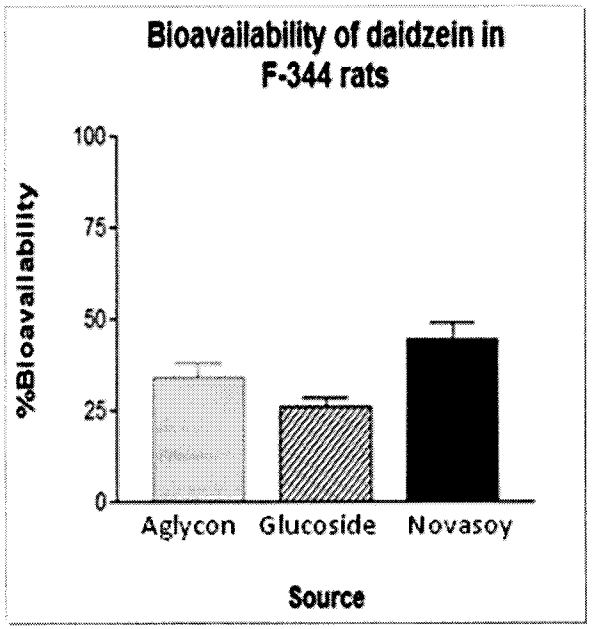
Table 6: Bioavailability of plasma glycitein in aged (20 months) male F344 rats after oral and IV exposure to aglycone source of isoflavone glycitein

Aglycone Glycitein	AUC_{or} µmol.h/L	AUC_{IV} µmol.h/L	% Bioavailability[a]	% Bioavailability[b]
Male old	0.79	3.20	12.34	9.52
	0.63	3.80	8.29	7.59
	0.69	5.20	6.63	8.31
	0.49	4.40	5.57	5.90
Average	0.65	4.15	8.21	7.83
Standard deviation	0.13	0.85	2.98	1.51

[a]Bioavailability based on the random assigning of the AUC_{or} to AUC_{IV} from four rats in the same diet group.
[b]Bioavailability based on comparing the average of AUC_{IV} with each AUC_{or}

An additional suggestion with respect to the bioavailability calculation concerned the groups of rats that were administered the synthetic source of glucoside form and Novasoy™ source of isoflavones. In Chapter 4, the bioavailabilities of the synthetic glucoside form and Novasoy™ form were calculated based on AUC_{or} for the synthetic glucoside and the AUC_{or} for Novasoy™ and these were then compared with AUC_{IV} of the synthetic glucosidic form. This was based on the consideration that isoflavones are presented in food mainly as glucosides. Although there is evidence to suggest that particular members of a related class of flavonoids are absorbed in their naturally occurring glucosidic forms (Hollman and Katan, 1997), this does not appear to be the case for the isoflavones. After soy intake, isoflavones are absorbed as aglyconees, which are more readily absorbed than the parent glucosides due to their higher hydrophobicity and lower molecular weight (Xu et al., 1995; Liu and Hu, 2002). This is supported by the evidence from Setchell *et al.,* (2002), which indicates that isoflavone glucosides are not absorbed intact across the enterocytes of healthy adults and shows that uptake requires hydrolysis of the isoflavone glucoside to their aglycone form. Therefore, bioavailability of Novasoy™ and synthetic glucoside form of isoflavones (daidzein, genistein and glycitein) after oral and IV exposure in aged male F344 rats were recalculated based on the consideration that aglyconees arising from Novasoy™ and glucosidic form are the absorbable form of isoflavones. Results were compared with the ones already reported in chapter 4 (Figs 3-5).

Bioavailability of daidzein reported in Chapter 4:



Bioavailability of daidzein based on AUC_{IV} aglycone

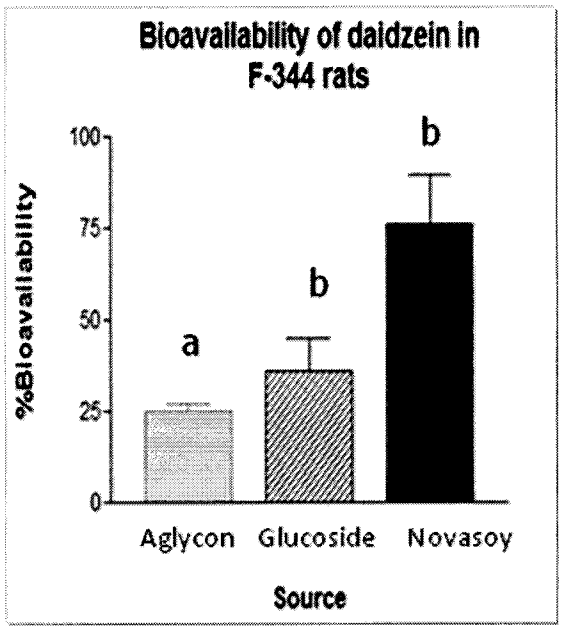
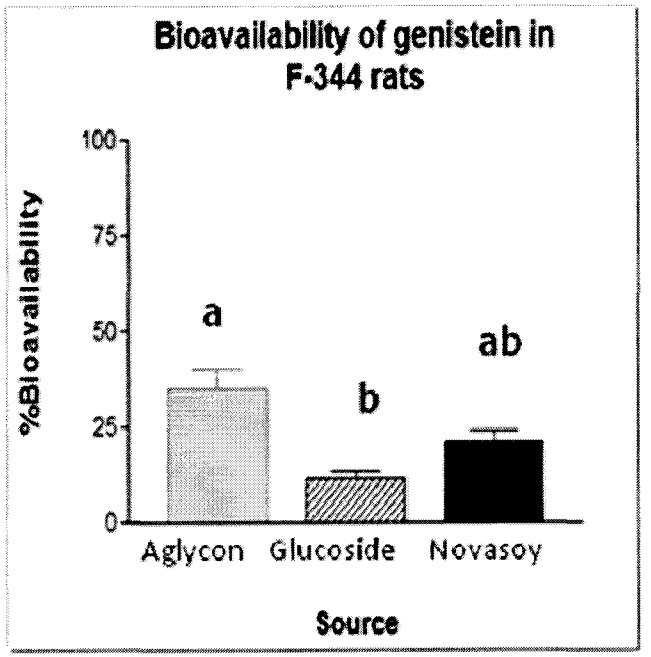


Figure 3: Bioavailability of daidzein calculated based on the AUC_{or} of all sources comparing with AUC_{IV} of synthetic aglycone source.

[a,b] Significant source effect

Bioavailability of genistein reported in Chapter 4:



Bioavailability of genistein based on AUC_{IV} aglycon

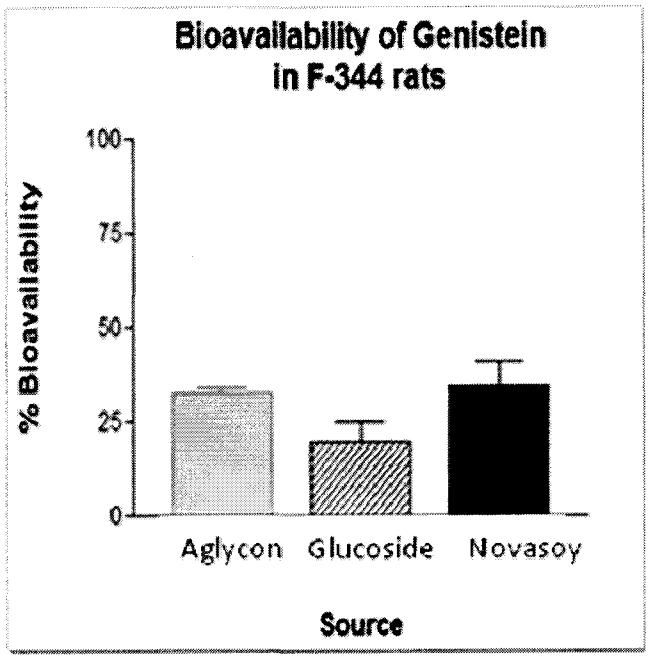
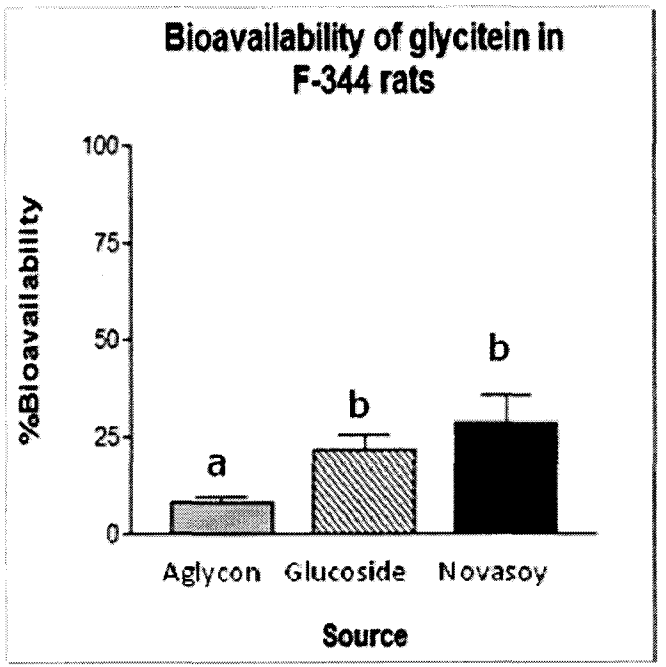


Figure 4: Bioavailability of genistein calculated based on the AUC_{or} of all sources comparing with AUC_{IV} of synthetic aglycone source.

[a,b] Significant source effect

Bioavailability of glycitein reported in Chapter 4:



Bioavailability of glycitein based on AUC_{IV} aglycone

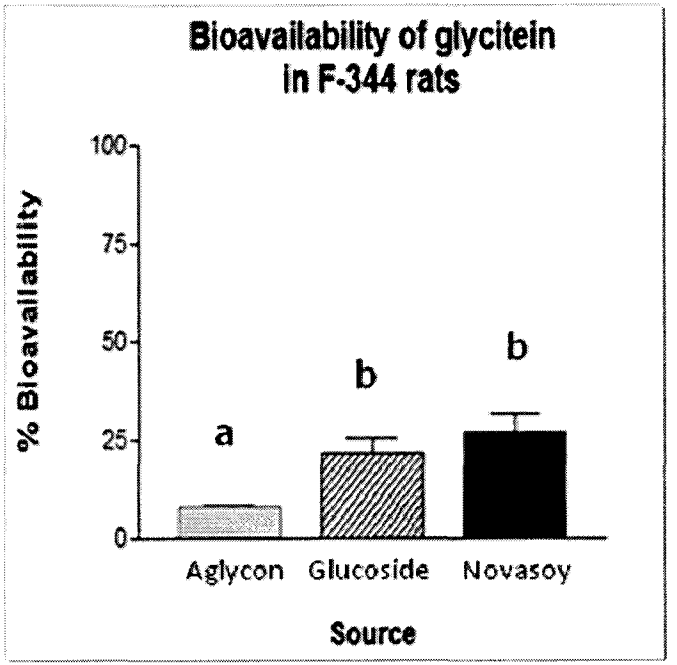


Figure 5: Bioavailability of glycitein calculated based on the AUC_{or} of all sources comparing with AUC_{IV} of synthetic aglycone source.

[a,b] Significant source effect

Pharmacokinetic parameters:

In Chapter 4, the pharmacokinetic parameters (e.g. the apparent Vd, Cl rate, and $t_{1/2}$) were calculated using the residuals method of analysis (curve stripping), post oral administration of isoflavone sources. The errors that would occur in the calculation of the above parameters from the oral administration relate to the declining concentrations during the elimination phase which would include elimination as well as an unknown absorption rate. Besides the issue of overlap of elimination, distribution and absorption phases, more data points were needed to perform the analysis in a statistically appropriate manner.

The apparent Vd is a proportionality constant relating the plasma concentration to the total amount of therapeutic products in the body (Gibaldi, 1990). In practice, it is impossible to measure the amount of therapeutic product in the body but, immediately after IV administration (at time zero), the amount of drug is equal to the dose (Hedaya, 2007).

Therefore, a more accurate, way of calculating apparent Vd, Cl rate, and and $t_{1/2}$ for isoflavones is based on the IV administration of the synthetic sources of isoflavones. Novasoy^{TM} source was not injected IV, however bioavailability parameters were calculated by comparing AUC_{oral} post Novasoy^{TM} administration by AUC_{iv} post aglycone IV injection. While IV dosing will provide an accurate pharmacokinetic results, when IV injection is impossible (in case of Novasoy^{TM}), the PK values obtained from oral dosing would be inaccurate due to the issue of overlapping phases (elimination, distribution and absorption), and not enough data point collected during elimination phase of the oral administration.

Elimination rate constants (K) for different sources of isoflavones were calculated based on the consideration that K = Cl / Vd. Elimination rate constant reflects the the fraction of isoflavones removed from the blood per unit of time. Table 8 is the representative of pharmacokinetic parameters of plasma daidzein, genistein and glycitein in aged male F344 rats based on IV exposure to synthetic sources of isoflavones and comparison of the IV results with the pharmacokinetic results reported in chapter 4.

Table 8: Pharmacokinetic parameters of plasma daidzein, genistein and glycitein in aged male F344 rats after IV exposure to two different sources (mixtures) of soy isoflavones

	$t_{1/2\ IV}$ h	C_{maxIV} (µmol.h)/L	tmaxIV h	Vd_{IV}/Kg L/Kgbw	Cl_{IV}/Kg L/h/Kgbw	K h^{-1}
Daidzein						
Aglycone	2.53 ± 1.52	28.25 ± 20.40	0.05 ± 0.10	2.00 ± 0.88	0.58 ± 0.33[b]	0.29 ± 0.13
Glucoside	2.43 ± 1.23	23.33 ± 8.51	0.05 ± 0.10	1.30 ± 1.00	0.21 ± 0.13[a]	0.26 ± 0.20
Genistein						
Aglycone	2.60 ± 2.34	41.80 ± 24.33	0.05 ± 0.10	2.63 ± 2.20	0.72 ± 0.26	0.40 ± 0.21
Glucoside	2.53 ± 1.53	49.67 ± 13.16	0.10 ± 0.10	1.50 ± 1.28	0.38 ± 0.20	0.35 ± 0.17
Glycitein						
Aglycone	3.93 ± 2.25	4.58 ± 1.72	0.15 ± 0.10	5.35 ± 1.78	0.78 ± 0.34	0.17 ± 0.12
Glucoside	3.90 ± 3.39	3.28 ± 0.68	0.10 ± 0.10	5.63 ± 3.05	0.51 ± 0.19	0.17 ± 0.20

	$t_{1/2\ or}$ h	C_{maxor} (µmol.h)/L	$tmax_{or}$ h	Vd_{or}/Kg L/Kgbw	Cl_{or}/Kg L/h/Kgbw
Daidzein					
Aglycone	6.23 ± 2.94	1.63 ± 0.35	6.50 ± 3.00	7.00 ± 2.50	0.82 ± 0.17[b]
Glucoside	6.88 ± 1.58	2.80 ± 1.05	6.50 ± 3.00	4.28 ± 2.40	0.41 ± 0.16[a]
Genistein					
Aglycone	5.92 ± 2.98	3.23 ± 0.52	3.50 ± 3.00	8.75 ± 5.06	1.00 ± 0.12
Glucoside	6.25 ± 0.57	2.08 ± 0.82	6.50 ± 3.00	9.03 ± 3.12	1.00 ± 0.31
Glycitein					
Aglycone	7.34 ± 5.45	0.10 ± 0.00	8.00 ± 0.00	67.65 ± 8.87[b]	1.39 ± 1.59
Glucoside	9.28 ± 1.69	0.35 ± 0.13	8.00 ± 0.00	11.00 ± 3.52[a]	0.83 ± 0.25

[a, b] Significant source effect; K: Elimination rate constatnt. No significant effect of source revealed for daidzein ($p = 0.80$), genistein ($p = 0.72$), and glycitein ($p = 0.98$) elimination rate constant.

Conclusion:

Recalculation of bioavailability of Novasoy™ and synthetic glucoside form of isoflavone glycitein after oral and IV exposure in aged male F344 rats based on the consideration that aglyconees arising from Novasoy™ and glucosidic form are the absorbable form of isoflavones did not affect glycitein bioavailability significances.

The higher value for the bioavailability of daidzein from Novasoy™ and glucoside form compared with the aglycone form, which was not statistically significant, became significant after re-evaluation using the comparison with the AUC_{IV} aglycone.

For genistein, the significantly higher bioavailability value from the aglycone form compared with the glucoside was no longer statistically significantly different.

Recalculation of Cl rate and $t_{1/2}$ post IV administration of synthetic glucoside and synthetic aglycone sources did not affect their significances. The reported significant source effect in apparent Vd for glycitein post oral administration of synthetic aglycone source in chapter 4 is no longer present post IV administration of synthetic aglycone and glucoside sources. The difference observed after recalculation of apparent Vd can be due to the elimination of the confounding effect of re-absorption during the elimination phase for oral administration of isoflavone sources.

CHAPTER 5

5.1 General Discussion

An analytical method, based on LC/MS which allows the determination of isoflavones in small volumes of rat plasma following exposure to different dietary sources, was developed, validated, and used to determine their absolute bioavailability and pharmacokinetic characteristics in the plasma following oral and IV administration.

In chapter 3, the bioavailability of isoflavones from Novasoy™ (a supplement source) and from a mixture of synthetic glucosides and a mixture of synthetic aglycone forms was investigated in female and male (3-month-old) SD rats. The results of this study show that for synthetic aglycone and synthetic glucoside sources of isoflavones the bioavailability is independent of the gender. However, when Novasoy™ source was administered to SD rats, a gender difference was observed in the bioavailability of daidzein, with higher plasma concentration values in male rats. Bioavailability values for daidzein, genistein and glycitein were significantly higher (up to sevenfold) in Novasoy™ and the synthetic glucoside forms of isoflavones compared with those of the synthetic aglycone forms. Therefore, the source of isoflavone had significant effects on isoflavone bioavailability.

In chapter 4, the effect of glycosidation of isoflavones on bioavailability and pharmacokinetics in aged (20-month-old) male F344 rats revealed significantly higher bioavailability values for glycitein in Novasoy™ and the glucoside form compared with the aglycone mixture. Significantly higher values for bioavailability of daidzein were observed in aged rats dosed with Novasoy™

and the glucoside form compared with the aglycone mixture. Thus, the source of isoflavones had significant effects on daidzein and glycitein bioavailability in aged male rats.

In order to quantify isoflavones in biological samples, they must first be extracted from these complex matrices (Delmonte *et al.*, 2006; Adlercreutz *et al.,* 1991). Development of a suitable analytical method for determining isoflavones and their metabolites in biological matrices has been difficult due to the large numbers of phytoestrogens that exist and the range of chemical forms in which they can occur in various biological matrices (Jackson and Gilani, 2002). The majority of the methods reported in the literature have been optimized for the analysis of specific isoflavones of interest and were long, requiring purification, fractionation, hydrolysis, and derivatization (Adlercreutz *et al.*, 1991, Adlercreutz *et al.*, 1993; Adlercreutz *et al.*, 1994; Adlercreutz *et al.,* 1995; Setchell *et al.*, 1984; Official Methods of Analysis, 2005; Delmonte *et al.*, 2006; Griffith and Collison, 2001; Prasain *et al.*, 2003; Wang *et al.,* 2002; Merken and Beecher, 2000). Reliable measurement of plasma, urinary and food isoflavone levels are essential for interpretation of isoflavone pharmacokinetics and their bioavailability.

This led our laboratory to develop a method for the quantitative analysis of isoflavones and their metabolites in rat plasma samples (Sepehr *et al.*, 2006). A liquid chromatographic system comprising of Waters 2695 separation module and Waters 996 PDA detector connected to Waters Micromass ZQ single quadrupole mass spectrometer with Empower chromatography software for

processing the data generated were used to develop for the quantitative analysis of isoflavones and their metabolites in rat plasma samples (Sepehr *et al.*, 2006). High recovery (86-100%) of the reported isoflavones with this method is a desirable outcome of sample preparation, and is therefore an important characteristic of the complete extraction procedure. Additionally, this method required a shorter run time (16 minutes) compared with previously published methods using LC/MS (Holder *et al.*, 1999; Chang *et al.*, 2000; Griffith and Collison, 2001) and fewer steps for sample preparation, extraction, and less technical time compared with the previously published methods using gas chromatography/mass spectrometry which required purification, fractionation, hydrolysis, and derivatization (Wang *et al.,* 2002; Heinonen *et al.,* 1999; Heinonen *et al.,* 2001; Fotsis *et al.,* 1982).

A lack of standards for the isoflavone conjugates and the difficulty in measuring a diverse range of isoflavones that circulate in blood, including glucuronide and sulfate conjugates; freely circulating aglyconees; and protein-bound aglyconees (Barnes *et al.,* 1999; Coward *et al.,* 1993) means that most investigators have reported the combined total of aglycone and glucoside forms of isoflavones after enzymatic or acid hydrolysis (Vergne *et al.,* 2008; Halm *et al.,* 2007; Kano *et al.*, 2006).

The developed LC/MS method (Sepehr *et al.*, 2006) was used to examine the serum levels of soy isoflavones in male and female SD rats from a multigeneration-rat study of soy isoflavones conducted at the Toxicology Research Division at Health Canada. Participation in the multi-generational study

will contribute to an assessment of the long term effects at every stage of life of diets including differing amounts of isoflavones. Consumption of soy isoflavones has been associated with a variety of beneficial effects in animals and humans, but concerns have also been raised regarding potential adverse effects of isoflavones, particularly with regard to reproductive toxicity and the induction or potentiation of carcinogenesis, due to its weak estrogenic activity. Because of these concerns, Novasoy^{TM} as a supplement source was selected to be examined using a protocol designed to evaluate the effects of multigenerational and long-term exposures to doses of estrogenic agents that may produce different toxic effects in exposed SD rats. Serum concentrations of isoflavones in different stages of the study are reported here, and results from the multigenerational reproductive toxicology will be reported separately by Toxicology Research Division at Health Canada. These results are critical to the development of Health Canada guidelines.

The F0 (Parental; 120, 240, 360 days); F1 [Off-spring; postnatal day (PND) 28, 70, 120, 240, and 360 days], F2 and F3 (PND 28 and 70 days) rats were continuously exposed to graded levels of the soy isoflavone extract Novasoy^{TM} in the diet (Appendix 1, Table 1) such that the isoflavone intake resembled human intake levels ranging from low to no consumption (North American diet; 0 to 1 mg isoflavones/kg bodyweight/ day (mg/kg/d)), to modest vegetarian and Asian consumption levels; 1 to 3 mg/kg/d, to high infant consumption levels; 10 mg/kg/d, as some assessments of soy intake in humans

have revealed that intake may lead to a reduction in some cancer risks such as breast cancer in adulthood (Cooke, 2006).

Female rats in both F0 (day 360), and F2 (day 28) generations treated with isoflavones at the 1046.6 mg isoflavones/kg diet did exhibit significantly lower overall body weights in comparison to casein control diet and lower isoflavone treatment groups (Table 9). This finding is in agreement with the National Toxicology Program (NTP) technical report 539, 2008 on the multigenerational reproductive toxicology study of genistein in SD rats. Female rats given genistein in a high dose of 500 ppm (mg/kg diet) as part of their diet had lower body weights, accelerated sexual maturation, and altered estrus cyclicity compared to unexposed rats.

Serum concentrations of isoflavones in male and female Sprague Dawley rats fed to the soy isoflavone extract Novasoy^{TM} (SPI+ 74.5 and SPI+235.8) from F0 (parental) and F1 generations exhibited a gender difference in serum concentration with higher concentrations of total isoflavones obtained in female rats compared with male rats (Appendix 1, Tables 2-5).

In the F1 generation, 28 day old male and female rats treated with isoflavones at 1046.6 mg/kg diet level (Table 4-5) exhibited higher levels of total isoflavones in comparison with 70,120, 240, 360 day old rats. However, through generations F2 and F3 the levels of total isoflavones decreased in 28 day old rats compared with F1 (Tables 4-7).

The gender and age differences (Appendix 1, Tables 4-7) observed in plasma isoflavone concentrations from the multi-generation rat study warranted an investigation of the phamacokinetics and bioavailability of soy isoflavones.

Most of the previously published studies on the plasma pharmacokinetics of soy isoflavones have considered soy isoflavones as a food source and focused on systemic studies of the absorption, metabolism and excretion of isoflavones (mainly daidzein and genistein) by calculating the areas under the concentration time curves following oral administration of isoflavones in human or animal models (Cassidy *et al.*, 2006; Kano *et al.*, 2006; Izumi *et al,* 2000 ; Setchell *et al.*, 2001 ; Zubik and Meydani 2003; Xu *et al.*, 2000; Xu *et al.,* 1994; Zhang *et al.,* 1999b; Doerge *et al.,* 2000). Different factors have been suggested to influence the absorption of isoflavones based on oral administration. In addition to inter-individual (Larkin *et al.,* 2008; Bowey *et al.,* 2003; Rowland *et al.*, 2000), species variation (Gu *et al.,* 2006), different study designs, isoflavone sources, and food matrices between and within studies; other factors such as gut motility and transit time, gut pH, biliary secretion and gut microflora (enterohepatic circulation) can have a major influence on isoflavone absorption.
In this project in order to minimize the above effects in bioavailability study, the bioavailability of isoflavones was determined by comparing the AUC of the plasma concentration-time curve after IV administration with the AUC after oral administration.

Additionally, studies examining the bioavailability of isoflavones ingested as glucosides versus aglyconees have reported conflicting findings (Izumi *et al.*,

2000; Setchell *et al.,* 2001; Tsunoda *et al.,* 2002; Richelle *et al.,* 2002; Zubik and Meydani, 2003; Tsangalis *et al.,* 2005). Izumi *et al.*, (2000) reported a greater bioavailability of aglyconees, whereas Setchell *et al.*, (2001) reported a more efficient use of glucoside. Other studies showed that absorption between aglyconees and glucosides did not differ significantly (Tsunoda *et al.,* 2002; Richelle *et al.,* 2002; Zubik and Meydani, 2003; Tsangalis *et al.,* 2005).

In Chapter 3, the profiles of plasma isoflavones following oral administration of the synthetic glucoside source, synthetic aglycone source and Novasoy^{TM} to male and female 90-day-old SD rats (Figs. 1-3), post IV administration (Figs. 4, 5), the total $AUC_{or,}$ C_{max}, and t_{max} parameters in female SD rats were consistent with a slower rate of absorption than male SD rats. These differences could be attributed to gender differences, or differences in source or chemical form. They could occur because of differences in bioavailability or systemic kinetics (distribution, elimination). Therefore, the purpose of the pharmacokinetic analysis in this project was to explain the differences seen in the plasma concentration curves after oral and IV administration of the isoflavones. The findings of this thesis will be relevant to the understanding of some of the factors (e.g. gender, source and the role of glycosidation of isoflavones) that have an effect on the systemic kinetics (distribution, elimination) and bioavailability of isoflavones in rats.

In chapter 3, the results of the bioavailability study show that when Novasoy^{TM} as a supplement source was administered to SD rats a gender difference was observed in the bioavailability of daidzein, with lower plasma

concentration values in female rats and decreased bioavailability compared with male rats. Lower plasma concentration levels of these isoflavones in female rats suggested that a greater proportion of isoflavones may be distributed to the tissues, perhaps as a result of higher tissue density of estrogen receptors. Maubach *et al.*, (2003) suggested that isoflavones could occupy estrogen receptor sites or accumulate in lipophilic tissues such as breast tissue, in which the authors quantified isoflavone levels. Other studies have reported gender differences in isoflavone bioavailability (Lu and Anderson, 1998; Zhang *et al.,* 1999b; Cassidy *et al.*, 2006, Chang *et al.*, 2000). Further Coldham and Sauer (2000) reported gender differences in rats following administration of radioactive genistein, with female rats having higher levels in the liver. These authors suggested that there was greater isoflavone retention in the liver of females than males and also reported higher levels of genistein in reproductive tissues than other peripheral organs, supporting the idea that distribution may be related to estrogen receptor distribution and density. Additionally, because of their estrogenic potential, isoflavones were proposed to have a role in improving human health by acting in several organs and to reduce cardiovascular risk, osteoporosis (Mathey *et al.*, 2007) and alleviate menopausal symptoms (Welty *et al.*, 2007). Finally, lower plasma concentration values in female rats compared with male rats can be explained by higher clearance rate of isoflavones in female rats which may be due to faster metabolism (e.g conjugation) and excretion (Busby *et al.,* 2002).

The results of this study (Chapter 3) also revealed significant source effects in the bioavailability of daidzein, genistein and glycitein in rats dosed with Novasoy™ and synthetic glucosides compared with synthetic aglycone dosed rats. The low bioavailability of synthetic aglycone source is most likely explained by a reduction in the absorption of aglycone source as it appears from AUCs (Fig. 2, Chapter 3) or induction of metabolizing enzymes. In previous research using Caco-2 cells to model absorption and metabolism of genistein, Oitate *et al.,* (2001) found that genistein permeated Coca-2 monolayer cell membranes in a dose and temperature-dependent manner and that the permeation of the monolayer could be saturated. Other flavones such as rutin, and quercetin, can competitively inhibit transcellular transport of genistein (Oitate *et al.,* 2001). This indicates that absorption of genistein and other phytoestrogens occurs in the gastrointestinal tract by a mechanism other than simple diffusion such as active transportation. Chen *et al.,* (2006) concluded that the main reason for the low bioavailability of genistein is not only its poor absorption but also its significant first-pass metabolism (glucuronidation and sulfatation). Glucuronidation is the primary metabolic route for isoflavones in humans (Doerge *et al.*, 2000) and rat (Coldham and Sauer, 2000; Zhou *et al.,* 2008). Low levels of free isoflavones have been found in plasma of humans, but sulfo-conjugates and glucuronides are found in greater concentrations, the most important circulating glucuronide conjugate being 7-*O*-glucuronide (Doerge *et al.*, 2000). These findings were confirmed by Shelnutt *et al.,* (2002) who reported that glucuronides represent 48% of the total isoflavone concentration in human plasma, wheras sulfates and

sulfoglucuronides correspond to approximately 8% and 30%, respectively. In rats, the major genistein metabolite is genistein-7-*O*-β-D-glucuronide; other metabolites include genistein-4'-*O*-sulfate and genistein-4'-O-sulfate-7-*O*-β-D-glucuronide; but they are formed in smaller amounts (Prasain *et al.,* 2006; Zhou *et al.,* 2008). It has been reported that when rats were orally administered genistein, the glucuronidation occurred in the intestine, and not in the liver (Sfakianos *et al.,* 1997). Some of the glucuronidated metabolite is absorbed into blood; the majority is excreted into the intestinal lumen by the intestinal epithelium (Chen *et al.,* 2003). Because large quantities of the glucuronidated metabolites of genistein enter the intestinal lumen via bile and intestinal epithelial excretion, glucuronidated genistein can be deconjugated by glucuronidase in the intestine to release genistein (Chen *et al.,* 2005; Jia *et al.,* 2004). The released genistein can be absorbed, metabolized, and excreted for a second time, through enteric recycling and enterohepatic circulation (Chen *et al.,* 2005; Jia *et al.,* 2004).

In this project, it is considered that aglyconees arising from glucosidic form of isoflavones are the absorbable form of isoflavones, which are more readily absorbed than the parent glucosides due to their higher hydrophobicity and lower molecular weight. Additionally glucosides of isoflavones have not been identified in plasma. Therefore, a more accurate way of calculating bioavailability is based on using AUC_{IV} aglycone rather than AUC_{IV} glucosides.

Very high bioavailability (97%-120%) of daidzein from Novasoy™ source compared with synthetic aglycone and synthetic glucoside form in our study (Chapter 3) may be due to the synergistic effect of other compounds (e.g. lipids, phytic acid, saponins, oligosaccharides, proteases inhibitors and phytosetrols) present in the Novasoy™ formulation which may have a protective role against biodegradation of daidzein in the Novasoy™ source (Setchell *et al.,* 2001). A recent investigation by Qui *et al.,* (2008) reported the influence of the dosage form on the pharmacokinetics of daidzein and its metabolite daidzein -7-*O*-glucuronide in rats. Daidzein (free plus conjugated form) as a compound with poor hydrophilicity and lipophilicity due to the typical plane structure showed higher bioavailability from a solution as opposed to a suspension (47% bioavailability in solution vs 12.2% bioavailability in suspension)

In Chapter 3, gender differences were also detected in equol production in female rats. Female rats were capable of producing more equol compared with male rats post oral and IV exposure to different synthetic mixtures and Novasoy™ source. There is considerable interindividual variation in the production of certain metabolites, particularly equol and *O*-DMA (Bowey *et al.,* 2003). For example, only about 30-50% of individuals from various populations excrete equol after consuming soy products (Lampe *et al.,* 1998; Lampe *et al.*, 2001; Slavin *et al.,* 1998; Rufer *et al.*, 2006), the factors related to the metabolism of daidzein to equol remain unknown. Even when adult humans were administered pure daidzein, which removes the influence of food matrix, a high percentage of humans do not convert daidzein to equol (Rowland *et al.,* 2000;

Rufer *et al.,* 2006). The clear distinction between individuals in their capacity or inability to produce equol (Rowland, 1999) may be an inherent characteristic related to genetics or habitual diet which is not modifiable by dietary interventions (Karr *et al.*, 1997; Lampe *et al.*, 2001). Previous studies examining the bacterial composition of human fecal samples from individuals known to produce equol, have identified a number of bacteria as playing a role in the metabolism of daidzein to equol *including Enterococci, Lactobacilli, Bifidobacteria, Streptococci, Ruminococci* and *Bacteroids* (Tsangalis *et al.*, 2002; Decroos *et al.,* 2005). Based on antibiotic effects on equol production in human fecal samples, Atkinson *et al.,* (2004) suggested that the conversion of daidzein to dihydrodaidzein and of dihydrodaidzein to equol might be carried out by different bacteria and that the bacteria involved may differ between individuals. Bowey *et al.,* (2003), reported the presence of dihydro derivatives of genistein and daidzein in germ free rats indicating that these derivatives are mainly a consequence of mammalian processes, presumably cytochrome P450-mediated reactions, and not due to gut bacterial metabolism. It was apparent, however, that the amount of the dihydroderivatives and their ratio to the parent compounds were higher in human flora associated rats, suggesting that the microflora may play a role in their formation, possibly by modulating hepatic enzyme activity. In our study (Chapter 3) the low concentration of equol detected in female rats post IV administration of synthetic glucoside and aglycone sources might be partly due to the cytochrome P450-mediated reaction and partly by the action of the intestinal microflora.

The role of the gut microflora in this conversion may allow for potential modification of this activity through dietary modification including prebiotic treatment. In particular, the type and amount of dietary carbohydrate available *in vivo* to intestinal microflora may have an important role for equol production capacity (Lampe *et al.*, 2001; Rowland, 1999). The role of gut bacteria in metabolism is complicated by the differences in gut flora between animals and men. In order to overcome these problems, human flora associated rats have been developed, where germ free rats are colonized with a complete human fecal microflora. Such rats retain the bacteriological and metabolic characteristics of the human flora (Bowey *et al.*, 2003).

Our study in aged (20-month-old) male rats, the significantly higher bioavailability of daidzein from Novasoy™ source compared with the aglycone form, may be due to the synergistic effect of other compounds (e.g. lipids, phytic acid, saponins, oligosaccharides, proteases inhibitors and phytostrols) presented in the Novasoy™ formulation which may have a protective role against biodegradation of isoflavone daidzein in Novasoy™ source (Setchell *et al.*, 2001).

Significantly higher bioavailability value of daidzein (up to 1.5-fold) from glucoside source compared with the aglycone form is due to the significantly ($p < 0.05$) slower Cl rate of daidzein from glucoside source compared with aglycone source. The volume of distribution value determined in this study for daidzein in the aglycone dosed rats (2.0 L/Kg bw) is higher compared with the Vd glucoside source (1.3 L/Kg bw). This may be associated with greater retention of the daidzein in the body which may be associated with sequestration of daidzein in

tissues or fat, especially in organs with a high density of estrogen receptors (Maubach *et al.*, 2003). Calculation of K and $t_{1/2}$ revealed no significant differences between aglycone and glucoside source for daidzein.

The bioavailability values for glycitein were significantly higher (up to 4-fold) from Novasoy™ and the glucoside mixture (up to 3-fold) compared with those from the aglycone mixture. In aglycone dosed rats, very low quantities of glycitein were detected in plasma which is due to faster clearance rate of glycitein in aglycone source (0.7 L/h/Kg bw) compared with glucoside source (0.5 L/h/Kg bw). However, the differences were not statistically significant ($p > 0.05$). It is worth mentioning that our analysis is based on using four rats in each source group and probably not sufficiently powered to see all significant differences. This problem can be resolved by increasing number of rats in each source group.

Aged male rats were capable of producing more equol than young adult male rats which is, presumably due to the enhanced ability of aged male rats to convert more daidzein to equol. It has been reported that the absence or presence of certain bacterial species in the intestine are responsible for equol production (Izumi *et al.*, 2000; Liu and Hu, 2002; T'ien-Li and Hsiu-Yuan, 1977). Therefore, it would be beneficial to further investigate the potential for probiotic and prebiotic intake to increase isoflavone bioavailability as this may provide some insight into favorable gastrointestinal characteristics for enhanced isoflavone bioavailability and metabolism, especially to equol. Determination of gut microflora types prior to and after probiotic or prebiotic intake in relation to isoflavone bioavailability would be useful in identifying those bacteria that are

involved in this process and also to identify particular microflora profiles that are responsive for metabolism of isoflavones to their metabolites especially in elderly humans.

5.2 Conclusions

The substantial evidence supporting the potential for beneficial effects of soy consumption has led to much wider use of traditional soy in Western countries and to the development of new isoflavone and soy-enriched foods and supplements.

This thesis has focused on the importance of understanding the factors influencing bioavailability and pharmacokinetics of soy isoflavones in a rat model. The effects of different sources, gender and age have been shown to have a role in isoflavone bioavailability. Furthermore, the effects of the different sources of isoflavones on the production of isoflavone metabolites, such as equol, were investigated.

While animal studies can provide explanations about bioavailability and pharmacokinetics of isoflavones *in vivo,* they do not provide sufficient evidence on their own to support effectiveness of isoflavones in humans without clinical trials. There are important limitations of using animal models to predict the effects of isoflavones in humans (Cooke, 2006; Messina *et al.*, 2006; Duffy *et al.,* 2007; Raffi *et al.,* 2007). For instance, while the gut flora of rats able to metabolize large quantities of daidzein to equol, only 30-50% of humans contain the gut flora necessary to metabolize daidzein to equol (Duffy *et al.*, 2007, Gu *et al.,* 2006;

Atkinson *et al.,* 2005; Bowey *et al.,* 2003). Additionally, the equol produced in humans is the S-enantiomer and binds preferentially to the β-estrogen receptor. Whether this is the case in rodents is not known yet (Setchell *et al.,* 2005; Cooke, 2006). In infants, the production of metabolites is very limited due to lack of a fully developed intestinal microflora and the inactivity of metabolic enzymes (Setchell, 1998).

Another major difference between species is in the cytochrome P450s (CYP) and other therapeutic product metabolizing enzymes. Differences in CYP enzymes between males and females are prominent in rodents and much less evident in humans (Anderson, 2004). Diet has been shown to alter cytochrome P450 expression and activity in both humans and animal models (Ronis *et al.,* 2006). The CYP3A family is the major group of P450 enzymes expressed in human liver and it appears that they are affected by soy consumption (Ronis *et al.,* 1999). The CYP3A enzymes are important in the metabolism of endogenous compounds, such as bile acids, estrogens, and most therapeutic products (Xie *et al.,* 2001; Ronis *et al.,* 1999) and are the major P450 forms expressed in human fetal and neonatal liver (Stevens *et al.,* 2003). It has been reported that rats fed soy protein isolate have elevated CYP3A. For children fed soy formula, it would be extremely important, because therapeutic product efficacy in these infants may be reduced.

All these issues suggest caution is warranted when extrapolating available animal data to humans.

5.3 Future work:

This study was conducted based on the assumption that isoflavone aglycone is the active form of isoflavones in rats. The bioavailability of active compounds must be known before investigating the potential physiological effects. As a consequence it is important to understand the mechanism through which all these compounds are absorbed, and to increase knowledge on factors influencing bioavailability before extrapolating rat data to humans. In addition, the role of gut microflora may be particularly important in the production of the equol, which may confer more health benefits than daidzein. Much more research needs to be carried out in this area with larger sample size to confirm the relationships between daidzein-metabolizing phenotypes and disease risk.

Future studies are needed to consider the conjugation pattern of isoflavones in their bioavailability. Analyzing total isoflavones present in plasma without enzyme hydrolysis and determination of the concentrations of their active forms are important factors in estimating bioavailability. There are reports suggesting that the conjugated forms of isoflavones may either have biological activity themselves or may act as a source of tissue aglyconees (Bursztyka *et al.,* 2008; Ronis *et al.*, 2006; Shelnutt *et al.*, 2002). For example, daidzein 7, 4'-di-*O*-sulfate competitively inhibits sterol sulfatase in hamster liver microsomes, whereas daidzein does not (Wong *et al.,* 1997). Similarly, sulfate conjugates of endogenous steroids are thought to possess biological activity and to be an important source of free cellular steroids after sulfatase hydrolysis (Shelnutt et al., 2002; Pasqualini *et al.,* 1990). Genistein glucuronides may also be active *in*

vivo because they have been shown to have weak estrogenic activity and can activate human natural killer cells *in vitro* (Zhang *et al.,* 1999a). The biological importance of conjugates may be partly due to inactivation and excretion of dietary isoflavones or by serving as an immediate source of aglyconees within target tissues (Bursztyka *et al.,* 2008; Shelnutt *et al.,* 2002). Therefore, to assess the potential risks and benefits of soy isoflavones in rats, it is essential to have data on the plasma pharmacokinetics of major isoflavone conjugates.

In order to complement existing bioavailability studies in rats, future studies would concentrate on the excretion and tissue distribution of isoflavones from the synthetic sources of aglyconees and glucosides as well as from the Novasoy^{TM} supplement source. The gender differences observed in the bioavailability of isoflavones from Novasoy^{TM} (Chapter 3), with lower plasma concentrations and higher volumes of distribution for daidzein and glycitein in females compared with males, warrants further investigation. The issue of tissue distribution of isoflavones after ingestion, particularly in relation to estrogen levels and estrogen receptor distribution may be of significance in comparing the biological effects of isoflavones in young adult and older female rats. This may be considerable value in interpreting the different effects of isoflavones seen in young and postmenopausal women and may assist in understanding the mechanisms of action of dietary soy isoflavones.

CHAPTER 6

6.1Literature cited

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APPENDIX 1

The developed HPLC/MS method (Sepehr *et al.*, 2006) was used for analyzing the serum concentration of isoflavones (genistein, daidzein, glycitein and equol) in male and female Sprague Dawley rats from a multigenerational study of soy isoflavones. The F0 (Parental; 120, 240, 360 days); F1 [Off-spring; postnatal day (PND) 28, 70, 120, 240, and 360 days], F2 and F3 (PND 28 and 70 days) were continuously exposed to graded levels of the soy isoflavone extract Novasoy^{TM} in the diet. Few studies to date have examined the effects of various concentrations of isoflavones over multiple generations of animals. This study design was based on two considerations: Firstly, constant exposure to isoflavones from the parental generation through to the first, second and third generations includes exposure during the early windows of development. Secondly, the design simulated humans consuming soy in the diet for several generations such as occurs in Asian populations. The diets used were relevant to human consumption of isoflavones, ranging from low to no consumption [North American diet; 0 to 1 mg isoflavones/ kg bodyweight/ day (mg/kg/d)], to modest vegetarian and Asian consumption levels (1 to 3 mg/kg/d), to high infant consumption levels (10 mg/kg/d) (Cassidy *et al.*, 1994; Irvine *et al.*, 1998; Wakai *et al.*, 1999; Zung *et al.*, 2001) and a diet level which represented approximately 5 times the infant and 10 times the adult consumption level, and was representative of a consumer of soy isoflavone supplements (30 mg/kg/d). Therefore, this study is useful for

determination of safe ranges of soy consumption and potential effects of soy consumption.

The animal portion of the study was completed by the time I began my doctoral studies. However, I was extensively involved in analyzing blood samples from the experimental rats (Total of 1740 rats). This study is part of the larger study at Toxicology Research Division at Health Canada, the results of which will be published elsewhere (Curran, I.H.A, Cooke G.M., Gilani G.S., and Sepehr E. in preparation).

Materials and methods

Chemicals

(**a**) *Sodium citrate buffer (25 mM, pH 5.0).*

(**b**) *Hydrolytic enzyme.* - *Helix pomatia* type H-5 (S3009), containing 29 units/mg solid sulfatase activity was purchased from Sigma- Aldrich Co (St. Louis, MO, USA). A solution containing 23 units of sulfatase activity was prepared by dissolving an appropriate amount of enzyme in 1.0 mL of the sodium citrate buffer (Sepehr *et al.*, 2006).

(**c**) *Dimethyl sulfoxide (DMSO).* - 99.9% HPLC grade (Sigma-Aldrich Co.).

(**d**) *Water.* -Deionized, NANO-pure [Diamond ultra-violet (UV) ultra pure water purification system; Barnstead International, Essex, UK].

Apparatus

(**a**) *HPLC.* - The LC separations were performed by using a Waters (Milford, MA, USA) Alliance 2695 liquid chromatograph equipped with a Zorbax SB-CN

reversed-phase column (4.6 X 75 mm, 3.5 µm particle size; Agilent Technologies, Wilmington, DE, USA).

(**b**) *MS system.* A Waters Micromass ZQ single quadrupole mass spectrometer was operated in the positive ion SIM mode. The entire system from sample injection to data acquisition was computer-controlled with Empower software (Waters).

Experimental Diets

Three lots of NOVASOY™ concentrate #152-400 (600g of Lot # 9809151, 2400g of Lot # 0011101, and 2000g of Lot # 0104181; Archer Daniels Midland Company, Decatur, IL, USA) were added to the diets by Dr. I. Curran and Dr. S. Gilani. The actual content of isoflavones (genistein, daidzein and glycitein) in the Novasoy™ was determined by Waters HPLC linear gradient with UV detection after acid hydrolysis and liquid –liquid extraction of samples according to the method of Wang and Murphy, (1994) in Dr Gilani's laboratory.

Commercial preparation of Novasoy™ contains a total isoflavone concentration of 24.1% (genistein 12.5%, daidzein 8.6%, and glycitein 3.0%) the remainder being moisture, carbohydrate, protein, fat and ash.

(**b**) Alcohol washed soy protein isolate contains 90% crude protein as determined by manufacturer (Pro Fam 930; Archer Daniels Midland Company, Decatur, IL, USA).

(**c**) Casein protein contains 90% crude protein as determined by manufacturer (ICN Biomedicals, Costa Mesa, CA).

Preparation of the diets

All diets were formulated according to the specifications of American Institute of Nutrition (AIN-93G; Reeves *et al.*, 1993). Diet 1, a casein-based, AIN-93G semi-purified basal diet containing AIN-93G Vitamin Mix and AIN-93 Mineral Mix (TestDiet®, Purina Mills, Richmond, IN, USA). A soya-free, casein protein (AIN93G), diet was used to control for effects of non-isoflavone soya components; Diet 2, casein was replaced by alcohol-washed soy protein isolate (PRO-FAM 930; Aliments UFL Corp., Boucherville, Quebec, Canada), AIN-93G Vitamin Mix and AIN-93 Mineral Mix; Diets 3-6, consisted of the soya-based complete basal diet 2 supplemented with increasing concentrations of isoflavones (36.1, 74.5, 235.8 and 1046.6 mg/kg diet) from Novasoy™.

Dietary analysis

The total content of genistein, daidzein and glycitein in the prepared diets was determined by Waters HPLC linear gradient with UV detection monitored at 254 nm after acid hydrolysis and liquid –liquid extraction of samples which had been ground to a homogenous powder. The procedure followed the published method Wang and Murphy, (1994) for analysis of dietary isoflavonoids. The composition of experimental diets is shown in Table 1. All diets were provided to the animals ad libitum in pelleted form.

Animals

The animal care and handling procedures (Fig. 1) were conducted according to the guidelines of the Canadian Council on Animal Care, and the experimental protocol was approved by the Health Canada Ottawa Animal Care

Committee prior to the initiation of the study. Pubertal Sprague-Dawley rats (Charles River, St-Constant, PQ, Canada) were pair housed in stainless steel cages with hardwood chip bedding (maple or ash) and kept in an environmentally controlled room with a 12 hours light/dark cycle (fluroscent bulbs on automated timer), humidity 40-60%, temperature 23 ± 2 °C and free access to food and fresh water. Parental generation (P) rats were acclimatized until 50 days of age. During an adjustment period, rats were fed an isoflavone free casein based control diet. At 50 days of age rats were assigned to experimental diets. After 70 days exposure to the experimental diets, rats were mated on postnatal day (PND) 120 and the resulting (F1) progeny were weaned at 21 days of age and provided with the same diet as their parents. Each 100 Kg batch of the prepared diets was analyzed for isoflavone content. Weekly food consumption (data not shown) and body weights were recorded at regular intervals throughout the study (Tables 8, 9).

Parental rats (10; from each diet and each gender) were sacrificed on PND 120, 240, and 360 days. F1 rats were sacrificed at PND 28, 70, 120, 240 and 360, and F2, F3 generations sacrificed at PND 28 and 70 days by exsanguination through cardiac puncture under general anesthesia with isoflurane. Blood samples were collected immediately and serum was separated using serum separator tubes (Becton Dickinson and Company, Franklin Lakes, NJ), centrifuged (1000g for 15 minutes), and stored at –80 °C until assayed.

Isoflavone analysis in rat serum

Analysis of blood samples from male and female rats from F0 (120, 240, 360 days), F1 (28, 70, 120, 240, 360 days), F2 (28 and 70 days) and F3 generation (28 and 70 days) were done using the LC-MS method as described in Sepehr *et al.*, 2006. Briefly, following enzymatic hydrolysis of isoflavone conjugates with mixed glucuronidase/sulfatase enzyme, the resultant aglyconees were extracted with ethyl acetate, centrifuged and the supernatant was diluted with mobile phase (0.1% formic acid in 85:15 water: acetonitrile) and injected into a Zorbax SB-CN reversed-phase column (4.6 X 75 mm, 3.5 µm particle size). The chromatographic run time was 16.0 min, with a delay of 10 min/injection (Sepehr *et al.*, 2006).

Results

Female rats in both F0 (day 360), and F2 (day 28) generations treated with isoflavones at the 1046.6 mg isoflavones/kg diet did exhibit significantly lower overall body weights in comparison to casein control diet and lower isoflavone treatment groups (Table 9). Significantly increased body weight was observed in male rats in F2 (day 28 and 120 day) generations treated with isoflavones at 235.8 compared with casein control treated rats. Additionally, significant body weight increase was observed in male rats in F2 (28 days) generation treated with 1046.6 mg isoflavones/kg diet. Food consumption (data not shown) was similar for all dose groups for the length of the study, indicating palatability of food was not a factor in weight change.

Serum isoflavone concentrations in male and female rats are presented in

Tables (2 -7). Rats (male and female) treated with isoflavones at 1046.6 mg/kg diet level exhibited higher levels of serum equol, daidzein, genistein and glycitein in comparison to both casein and alcohol washed soy protein control diets and lower isoflavone treatment groups (Tables 2 -7). However, with increasing doses of intake from 0 to 1046.6 mg/kg diet (representing approximately 5 times the infant and 10 times the adult consumption level) serum concentration of isoflavones showed a linear relationship (Tables 2 -7).

In F1 generation, 28 day old male and female rats treated with isoflavones at 1046.6 mg/kg diet level (Table 4-5) exhibited higher levels of total isoflavones in comparison with 70,120, 240, 360 day old rats. However, through generations F2 and F3 the levels of total isoflavones decreased in 28 day old rats compared with F1 (Tables 4-7).

The total level of isoflavones in male rats (70 day old) treated with isoflavones at 1046.6 mg/kg diet level (Table 4) increased through the generations from F1-F3 (Tables 4, 6, 7).

In F0 (Parental) and F1 generations male and female rats exhibited a decrease in serum total isoflavones concentrations from 120 days to 360 days exposure to soy isoflavone extract Novasoy^{TM} (SPI+74.5 and SPI+235.8) in the parental generation and from 28 days to 360 days in F1 generation. There is also a gender difference in serum concentration of isoflavones in F0 and F1 generation rats exposed to soy isoflavone extract Novasoy^{TM} (SPI+74.5 and SPI+235.8) with higher concentrations of total isoflavones obtained in female rats compared with male rats (Tables 1-5).

Because of the numerous numbers of rats, blood samples from the same rats (n=10) in each diet groups were pooled. To verify that the analysis of the serum pool would be representative of the data derived from individual samples, we compared the concentrations of the serum pool with the calculated mean isoflavones concentrations from the individual serum samples for two different time points (Fig. 2). As expected, when plotted on the same graph, the time course for the calculated mean was accurately super-imposable on that from the serum pool, providing confidence that the data derived from the pool would be a good approximation of those derived from individual samples. Each sample extracted twice on two different days to check instrument variability and the final result was the average of both extractions (Fig. 2).

Table 1: Composition of experimental diets (g/kg diet)

Ingredient	Casein	SPI+ 31.7§	SPI+ 36.1§	SPI + 74.5§	SPI + 235.8§	SPI + 1046.6§
Casein*	222·2	–	–	–	–	–
Soya protein†	–	222·2	222·2	222·2	222·2	222·2
Sucrose	100·0	100·0	100·0	100·0	100·0	100·0
Maizestarch	375·3	375·3	374·5	374·5	374·5	374·5
Dextronized Maizestarch	132·0	132·0	132·0	132·0	132·0	132·0
Soyabean oil	70·0	70·0	70·0	70·0	70·0	70·0
Cellulose	50·0	50·0	50·0	50·0	50·0	50·0
Mineral mix‡	35·0	35·0	35·0	35·0	35·0	35·0
Vitamin mix‡	10·0	10·0	10·0	10·0	10·0	10·0
Choline bitartrate	2·5	2·5	2·5	2·5	2·5	2·5
l-Cystine	3·0	–	–	–	–	–
l-Methionine	–	3·0	3·0	3·0	3·0	3·0
tert-Butylhydroquinone	0·014	0·014	0·014	0·014	0·014	0·014
Novasoy†	–	–	0.127	0.263	0.833	3.697
Genistein (mg/kg diet)§	ND	18·6	21.0	39.3	124.4	544.8
Daidzein (mg/kg diet)§	ND	10·5	12.3	27.6	90.9	412.3
Glycitein (mg/kg diet)§	ND	2·6	2.8	7.6	20.5	89.5

Diets were prepared by Dr. I. Curran and Dr. S. Gilani from three different Lot # of Novasoy™ (A 600 g of Lot # 9809151 was mixed with 2400 g of Lot # 0011101 and 2000 g of Lot # 0104181). §The actual content of isoflavones (genistein, daidzein and glycitein) in the prepared diets was determined by Waters HPLC linear gradient with UV detection after acid hydrolysis and liquid –liquid extraction of samples (Wang and Murphy, 1994) by Mr. P. Robertson. Each 100 Kg batch of the prepared diets was analyzed for isoflavone content.

SPI, soya protein isolate; ND, not detectable

*Casein from ICN Biomedicals contains 90 % crude protein.

†Alcohol-washed SPI contains 90 % crude protein, and Novasoy concentrate contains 30 % total isoflavones.

‡AIN-93G Mineral mix and AIN-93G Vitamin mix were from ICN Biomedicals.

Table 2: Parental (F0) serum results from male SD rats

F0 Generation serum results
Males

120 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.004	0.049	0.053	0.158	0.799	2.772
Glycitein	0.001	0.006	0.007	0.011	0.054	0.272
Equol	0.051[T]	0.143	0.198	0.309	0.977	3.538
Genistein	0.016	0.055	0.043	0.109	0.640	1.998
Total	0.072	0.254	0.302	0.587	2.470	8.580

240 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.002	0.036	0.045	0.094	0.321	1.486
Glycitein	ND	0.003	0.004	0.006	0.035	0.146
Equol	ND	0.185	0.224	0.266	0.780	2.813
Genistein	0.001	0.048	0.051	0.119	0.290	1.340
Total	0.003	0.272	0.324	0.485	1.426	5.785

360 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.003	0.014	0.022	0.046	0.223	1.502
Glycitein	0.001	0.020	0.002	0.006	0.020	0.197
Equol	ND	ND	ND	0.192	0.604	3.525
Genistein	0.003	0.015	0.029	0.052	0.233	1.667
Total	0.007	0.049	0.053	0.297	1.079	6.892

[a)]Pooled serum sample of 10-12 rats were hydrolyzed by enzyme *Helix Pomatia* H-5.
Each hydrolyzed sample was analyzed twice on two different days by LC/MS method (Sepehr *et al.*, 2006). No statistical analysis was performed as pooled serum samples were analyzed.
ND, not detectable <LOD; [T]trace for concentrations above LOD and below LOQ

Table 3: Parental (F0) serum results from female SD rats

F0 Generation serum results
Female

120 Days	Mean[a)]					
Compound (µM)	**Casein**	**SPI+ 31.7**	**SPI+ 36.1**	**SPI + 74.5**	**SPI + 235.8**	**SPI + 1046.6**
Daidzein	0.002	0.026	0.035	0.080	0.294	0.975
Glycitein	0.001	ND	ND	0.016	0.052	0.134
Equol	ND	0.259	0.320	0.425	1.617	3.703
Genistein	ND	0.031	0.039	0.082	0.234	0.783
Total	0.003	0.316	0.395	0.603	2.197	5.596

240 Days	Mean[a)]					
Compound (µM)	**Casein**	**SPI+ 31.7**	**SPI+ 36.1**	**SPI + 74.5**	**SPI + 235.8**	**SPI + 1046.6**
Daidzein	0.002	0.021	0.023	0.045	0.175	0.682
Glycitein	ND	0.002	0.002	0.009	0.026	0.109
Equol	0.046[T]	0.299	0.327	0.505	1.579	6.486
Genistein	0.001	0.026	0.033	0.048	0.164	0.828
Total	0.049	0.348	0.386	0.606	1.943	8.105

360 Days	Mean[a)]					
Compound (µM)	**Casein**	**SPI+ 31.7**	**SPI+ 36.1**	**SPI + 74.5**	**SPI + 235.8**	**SPI + 1046.6**
Daidzein	0.004	0.011	0.015	0.048	0.231	1.290
Glycitein	0.002	0.003	0.002	0.005	0.036	0.171
Equol	ND	ND	ND	0.383	1.414	4.858
Genistein	0.002	0.011	0.015	0.053	0.253	1.459
Total	0.009	0.025	0.032	0.490	1.935	7.777

[a)]Pooled serum sample of 10-12 rats were hydrolyzed by enzyme *Helix Pomatia* H-5.

Each hydrolyzed sample was analyzed twice on two different days by LC/MS method (Sepehr *et al.*, 2006).No statistical analysis was performed as pooled serum samples were analyzed.

ND, not detectable <LOD; [T]trace for concentrations above LOD and below LOQ

Table 4: First generation (F1) serum results from male SD rats

F1 Generation serum results
Males

28 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.005	0.169	0.193	0.525	1.893	5.818
Glycitein	0.003	0.026	0.024	0.097	0.271	1.157
Equol	ND	0.282	0.290	0.605	1.772	10.943
Genistein	0.006	0.234	0.237	0.537	1.781	7.343
Total	0.014	0.710	0.744	1.763	5.717	25.261

70 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.004	0.052	0.059	0.204	0.812	1.943
Glycitein	ND	0.001	0.001	0.003	0.043	0.131
Equol	ND	0.108	0.138	0.267	0.911	3.110
Genistein	ND	0.064	0.068	0.139	0.697	1.276
Total	0.004	0.224	0.265	0.612	2.462	6.459

120 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.008	0.043	0.067	0.143	0.527	2.383
Glycitein	ND	ND	0.006	0.003	0.039	0.195
Equol	ND	0.113	0.179	0.333	0.677	3.107
Genistein	0.001	0.051	0.078	0.140	0.468	2.298
Total	0.009	0.207	0.329	0.619	1.710	7.982

240 Days	Mean[a)]					
Compound (μM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.007	0.018	0.030	0.101	0.305	1.746
Glycitein	ND	0.001	ND	0.004	0.020	0.156
Equol	ND	0.065	0.069	0.235	0.599	2.600
Genistein	0.001	0.018	0.034	0.111	0.318	2.249
Total	0.008	0.102	0.133	0.450	1.242	6.750

360 Days	Mean[a)]					
Compound (μM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.001	0.010	0.016	0.047	0.240	0.996
Glycitein	ND	ND	ND	0.003	0.020	0.103
Equol	ND	0.048[T]	0.094	0.193	0.763	2.593
Genistein	ND	0.015	0.016	0.058	0.325	1.197
Total	0.001	0.073	0.126	0.301	1.348	4.889

[a)]Pooled serum sample of 10-12 rats were hydrolyzed by enzyme *Helix Pomatia* H-5.
Each hydrolyzed sample was analyzed twice on two different days by LC/MS method (Sepehr *et al.*, 2006).No statistical analysis was performed as pooled serum samples were analyzed.
ND, not detectable <LOD; [T]trace for concentrations above LOD and below LOQ

Table 5: First generation (F1) serum results from female SD rats

F1 Generation serum results
Females

28 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.004	0.159	0.214	0.461	1.629	4.698
Glycitein	0.001	0.021	0.030	0.071	0.284	0.721
Equol	ND	0.185	0.200	0.378	1.983	8.284
Genistein	0.005	0.195	0.247	0.496	1.755	5.361
Total	0.010	0.560	0.690	1.405	5.651	19.063

70 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.003	0.027	0.041	0.079	0.329	1.034
Glycitein	0.002	0.004	0.005	0.014	0.049	0.147
Equol	ND	0.130	0.233	0.500	1.402	3.161
Genistein	0.007	0.035	0.048	0.083	0.305	1.131
Total	0.012	0.196	0.327	0.676	2.084	5.472

120 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.002	0.033	0.034	0.072	0.221	1.069
Glycitein	ND	0.003	0.002	0.010	0.045	0.161
Equol	ND	0.258	0.289	0.494	1.502	4.350
Genistein	0.005	0.037	0.037	0.098	0.225	1.343
Total	0.007	0.331	0.362	0.674	1.993	6.923

240 Days	Mean[a)]					
Compound (μM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.002	0.014	0.022	0.047	0.198	0.963
Glycitein	ND	0.001	0.002	0.006	0.039	0.165
Equol	ND	0.152	0.157	0.428	1.019	3.245
Genistein	ND	0.015	0.026	0.055	0.209	1.345
Total	0.002	0.182	0.206	0.535	1.464	5.717

360 Days	Mean[a)]					
Compound (μM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.002	0.013	0.011	0.046	0.147	3.226
Glycitein	ND	0.001	0.001	0.005	0.035	0.087
Equol	ND	0.072	0.090	0.293	0.932	5.149
Genistein	ND	0.015	0.012	0.044	0.187	0.525
Total	0.002	0.101	0.114	0.387	1.300	8.986

[a)]Pooled serum sample of 10-12 rats were hydrolyzed by enzyme *Helix Pomatia* H-5.
Each hydrolyzed sample was analyzed twice on two different days by LC/MS method (Sepehr *et al.*, 2006).No statistical analysis was performed as pooled serum samples were analyzed.
ND, not detectable <LOD; [T]trace for concentrations above LOD and below LOQ

Table 6: Second generation (F2) serum results from male and female SD rats

F2 Generation serum results

Males

28 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	ND	0.197	0.173	0.293	1.230	5.712
Glycitein	ND	0.016	0.017	0.028	0.130	0.639
Equol	ND	0.222	0.261	0.372	1.694	8.512
Genistein	0.001	0.257	0.236	0.367	1.357	7.560
Total	0.001	0.692	0.687	1.060	4.411	22.423

70 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.003	0.027	0.029	0.103	0.679	3.879
Glycitein	ND	0.002	0.002	0.006	0.045	0.291
Equol	ND	0.098	0.132	0.235	0.621	3.589
Genistein	0.003	0.025	0.030	0.080	0.533	3.092
Total	0.006	0.152	0.193	0.424	1.878	10.851

Females

28 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.004	0.161	0.159	0.339	1.339	3.842
Glycitein	0.001	0.017	0.019	0.047	0.185	0.467
Equol	ND	0.206	0.284	0.552	1.534	7.448
Genistein	0.005	0.234	0.232	0.433	1.700	4.938
Total	0.010	0.618	0.694	1.371	4.758	16.695

70 Days	Mean[a)]					
Compound (μM)	**Casein**	**SPI+ 31.7**	**SPI+ 36.1**	**SPI + 74.5**	**SPI + 235.8**	**SPI + 1046.6**
Daidzein	0.001	0.013	0.015	0.066	0.330	0.599
Glycitein	ND	0.002	0.003	0.010	0.042	0.069
Equol	ND	0.114	0.116	0.500	1.556	4.228
Genistein	0.001	0.010	0.012	0.076	0.320	0.522
Total	0.002	0.139	0.146	0.652	2.248	5.418

[a)]Pooled serum sample of 10-12 rats were hydrolyzed by enzyme *Helix Pomatia* H-5.
Each hydrolyzed sample was analyzed twice on two different days by LC/MS method (Sepehr *et al.*, 2006).No statistical analysis was performed as pooled serum samples were analyzed.
ND, not detectable <LOD; [T]trace for concentrations above LOD and below LOQ

Table 7: Third generation (F3) serum results from male and female SD rats

F3 Generation serum results

Males

28 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.002	0.057	0.073	0.296	1.419	4.940
Glycitein	0.001	0.008	0.011	0.034	0.147	0.676
Equol	ND	0.049[T]	0.058[T]	0.224	0.910	4.632
Genistein	0.007	0.073	0.094	0.394	2.083	8.201
Total	0.010	0.187	0.236	0.948	4.559	18.449

70 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.024	0.041	0.049	0.119	0.559	4.391
Glycitein	ND	0.002	0.004	0.013	0.043	0.368
Equol	ND	0.070	0.102	0.185	0.651	2.506
Genistein	0.009	0.037	0.042	0.115	0.569	4.411
Total	0.033	0.150	0.197	0.432	1.822	11.676

Females

28 Days	Mean[a)]					
Compound (µM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	ND	0.056	0.062	0.207	0.884	4.158
Glycitein	0.001	0.008	0.010	0.027	0.112	0.633
Equol	ND	0.064	ND	0.139	0.771	4.660
Genistein	ND	0.049	0.091	0.343	1.284	6.786
Total	0.001	0.177	0.163	0.716	3.051	16.236

70 Days	Mean[a)]					
Compound (μM)	Casein	SPI+ 31.7	SPI+ 36.1	SPI + 74.5	SPI + 235.8	SPI + 1046.6
Daidzein	0.003	0.044	0.029	0.100	0.303	1.120
Glycitein	ND	0.003	0.005	0.018	0.047	0.146
Equol	ND	0.067	0.128	0.318	0.803	3.148
Genistein	ND	0.011	0.025	0.106	0.331	1.143
Total	0.003	0.125	0.187	0.542	1.484	5.557

[a)]Pooled serum sample of 10-12 rats were hydrolyzed by enzyme *Helix Pomatia* H-5.
Each hydrolyzed sample was analyzed twice on two different days by LC/MS method (Sepehr *et al.*, 2006).No statistical analysis was performed as pooled serum samples were analyzed.
ND, not detectable <LOD; [T]trace for concentrations above LOD and below LOQ

Table 8: Body weight of male SD rats from multi-generation study

Males

Body Weight (g)

Generation	Diet (Dose, mg/kg)	Males				
		Day 28	Day 70	Day 120	Day 240	Day 360
		*Mean	Mean	Mean	Mean	Mean
0	casein			601.4 ± 48.4	747.0 ± 70.3	957.7 ± 126.6
	SPI + 31.7			592.5 ± 61.8	804.4 ± 81.8	948.9 ± 133.2
	SPI + 36.1			609.1 ± 45.1	831.8 ± 113.2	909.6 ± 87.9
	SPI + 74.5			600.1 ± 32.6	791.8 ± 82.3	934.5 ± 112.4
	SPI + 235.8			590.9 ± 83.9	790.2 ± 112.9	944.5 ± 140.0
	SPI + 1046.6			575.3 ± 26.0	737.4 ± 67.3	933.9 ± 151.0
1	casein	99.1 ± 14.7	444.7 ± 52.6	623.9 ± 76.1	845.6 ± 112.3	981.2 ± 151.8
	SPI + 31.7	106.4 ± 16.4	453.23 ± 47.0	621.1 ± 79.7	859.0 ± 139.3	835.4 ± 140.1
	SPI + 36.1	105.7 ± 9.5	465.7 ± 26.3	631.4 ± 56.9	850.2 ± 90.4	924.9 ± 126.3
	SPI + 74.5	99.8 ± 9.2	436.4 ± 33.5	600.1 ± 45.1	869.7 ± 84.0	966.5 ± 146.2
	SPI + 235.8	110.0 ± 8.6	432.4 ± 34.7	680.5 ± 90.0	835.4 ± 141.9	931.1 ± 129.6
	SPI + 1046.6	106.3 ± 14.2	441.9 ± 30.7	578.3 ± 81.8	795.9 ± 81.5	897.6 ± 142.8
2	casein	88.9 ± 12.9	442.2 ± 30.6	535.8 ± 47.6	799.8 ± 72.7	
	SPI + 31.7	95.6 ± 12.7	448.4 ± 55.3	583.6 ± 57.1	782.9 ± 92.3	
	SPI + 36.1	99.8 ± 14.6	445.6 ± 37.5	582.4 ± 56.9	865.8 ± 87.6	
	SPI + 74.5	99.6 ± 7	456.6 ± 39.1	583.6 ± 44.1	813.7 ± 20.8	
	SPI + 235.8	101.3 ± 12.1[1]	457.1 ± 37.8	602.6 ± 69.8[1]	818.9 ± 30.3	
	SPI + 1046.6	100.7 ± 12[1]	438.5 ± 40.6	556.7 ± 62	769.4 ± 99.6	

* mean ± standard deviation of minimum 10 animals/group
[1]Dunnett's significant result ($p<0.05$) with Diet 1 control

Table 9: Body weight of female SD rats from multi-generation study

Females

Body Weight (g)

Generation	Diet (Dose, mg/kg)	Females				
		Day 28	Day 70	Day 120	Day 240	Day 360
		*Mean	Mean	Mean	Mean	Mean
0	casein			342.2 ± 28.6	387.4 ± 48.7	538.1 ± 90.0
	SPI + 31.7			329.0 ± 46.5	443.1 ± 46.9	532.0 ± 78.5
	SPI + 36.1			389.6 ± 73.1	410.7 ± 67.5	553.1 ± 124.9
	SPI + 74.5			331.2 ± 39.5	396.3 ± 50.3	509.7 ± 72.7
	SPI + 235.8			349.2 ± 30.1	428.8 ± 64.2	510.0 ± 93.5
	SPI + 1046.6			303.0 ± 31.5	379.0 ± 50.8	436.6 ± 84.7[1]
1	casein	94.8 ± 7.4	280.2 ± 20.9	338.4 ± 38.2	477.2 ± 122.4	592.2 ± 164.6
	SPI + 31.7	91.7 ± 12.1	301.3 ± 36.2	348.9 ± 44.2	471.1 ± 105.2	601.1 ± 114.7
	SPI + 36.1	91.6 ± 17.2	273.1 ± 29.3	348.4 ± 51.1	461.4 ± 56.4	530.6 ± 102.8
	SPI + 74.5	97.1 ± 8.8	273.1 ± 28.7	347.7 ± 39.8	452.4 ± 74.8	560.6 ± 165.9
	SPI + 235.8	94.2 ± 15.8	279.3 ± 39.6	347.0 ± 50.0	517.0 ± 116.1	698.6 ± 209.3
	SPI + 1046.6	94.8 ± 15.4	253.9 ± 31.7	323.9 ± 49.6	429.2 ± 84.0	444.4 ± 72.1
2	casein	80.2 ± 11.8	253 ± 35.3	315.9 ± 37.5	390.5 ± 63.6	
	SPI + 31.7	85.7 ± 13.6	267.6 ± 15.7	322.4 ± 25	429.3 ± 67.2	
	SPI + 36.1	87.8 ± 6.7	276.2 ± 32.7	336.3 ± 29	467.5 ± 83.3	
	SPI + 74.5	88.6 ± 10.4	288.9 ± 24.9[1]	340.9 ± 31.4	467.8 ± 93.4	
	SPI + 235.8	99.2 ± 10.7[1]	275.5 ± 29.7	330.9 ± 28	420.1 ± 60	
	SPI + 1046.6	94.2 ± 7[1]	241.2 ± 14.4	309.4 ± 24.9	358.2 ± 28.5	

* mean ± standard deviation of minimum 10 animals/group

[1]Dunnett's significant result ($p<0.05$) with Diet 1 Control

Three Generation reproductive study:

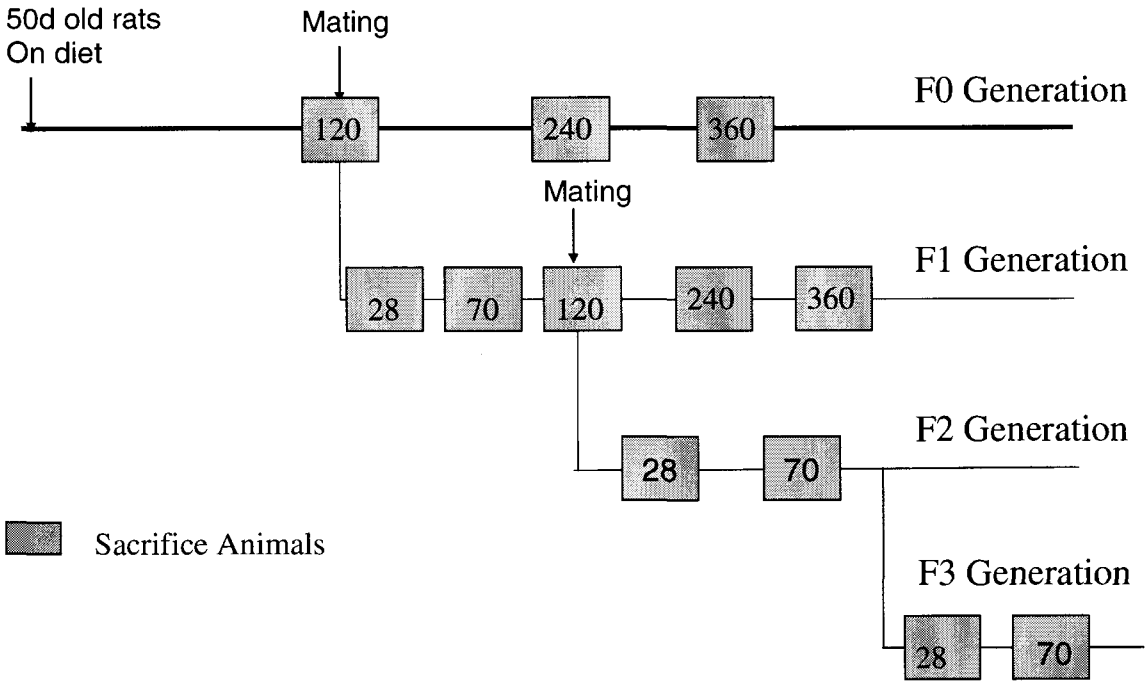

Figure 1: Three-generation breeding study

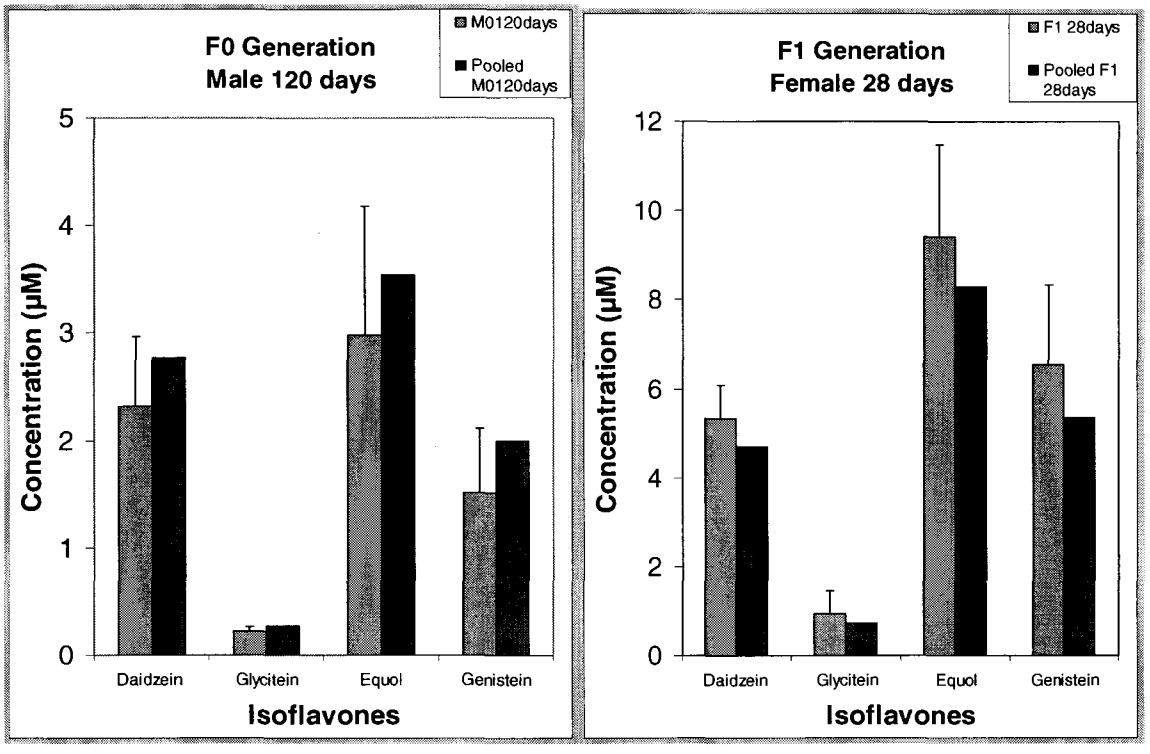

Figure 2: Serum concentrations of isoflavones from 6 individual subjects compared with those of plasma pooled from all 12 subjects.

Values represent determinations by LC/MS of aglyconees generated after hydrolysis with enzyme *Helix pomatia.*

LIST OF PUBLICATIONS

Estatira Sepehr, Gerard M. Cooke, Patrick Robertson, G. Sarwar Gilani, "Effect of glycosidation of isoflavones on their bioavailability and pharmacokinetics in aged male rats, ahead of publication in Journal of *Mol. Nutr. Food Res.* 2008.

Estatira Sepehr, Gerard M Cooke, Patrick Robertson, G. Sarwar Gilani "Bioavailability of soy isoflavones in rats. Part I: Application of accurate methodology for studying the effects of gender and source of isoflavones", *Mol. Nutr. Food Res.* 2007, 51, 799-812.

Estatira Sepehr, Patrick Robertson, G. Sarwar Gilani, Gerard Cooke, Benjamin Pui-Yan Lau., "An accurate and reproducible method for the quantitative analysis of isoflavones and their metabolites in rat plasma using liquid chromatography-mass spectrometry combined with photodiode array detection" *J. AOAC. Int* 2006, 89 (4) 1158-1167.

Gilani GS, Cockell KA, Sepehr E, Effects of antinutritional factors on protein digestibility and amino acid availability in foods. *J AOAC Int.* 2005, 88(3):967-87.

Gilani GS, Sepehr E, Protein digestibility and quality in products containing antinutritional factors are adversely affected by old age in rats. *J. Nutr*, 2003, 133(1):220-5.

Sepehr E, Peace RW, Storey KB, Jee P, Lampi BJ, Brooks SP, Folate derived from cecal bacterial fermentation does not increase liver folate stores in 28-d folate-depleted male Sprague-Dawley rats. *J. Nutr,* 2003, 133(5):1347-54.

Manuscripts in preparation

Estatira Sepehr, Gerard M. Cooke, G. Sarwar Gilani, Accurate methodology for studying bioavailability of isoflavones, for the Journal of *Current Pharmaceutical Analysis.*

Estatira Sepehr, Jagroop Dahiya, Gerard M. Cooke, G. Sarwar Gilani, Urinary and fecal isoflavone kinetics: the effect of gender, age and source of isoflavones, for the Journal of *Mol. Nutr. Food Res.*

CONFERENCE POSTER PRESENTATIONS

Estatira Sepehr, G. Sarwar Gilani, Patrick Robertson, Gerard Cooke, Benjamin P.-Y.Lau. , Jocelyn Fournier (2005) Bioavailability of soy isoflavones as affected by gender, age and food matrix in rats, *6th International Symposium on the Role of Soy in Preventing and Treating Chronic Disease,* Chicago, IL., U.S.A.

Estatira Sepehr, G. Sarwar Gilani, Patrick Robertson, Gerard Cooke, Benjamin P.-Y.Lau, Ivan H. A. Curran (2005) Improved method for the extraction and analysis of isoflavones and their metabolites in rat blood using liquid chromatography combined with mass spectrometry and photodiode array detection, *Health Canada Science Forum, Ottawa, Ont.,* Canada.

Estatira Sepehr, G. Sarwar Gilani, Patrick Robertson, Gerard Cooke, Benjamin P.-Y.Lau, (2005) An accurate method for the quantitative analysis of isoflavones and their metabolites in rat blood using liquid chromatography combined with mass spectrometry and photodiode array detection, *48th Annual Meeting Canadian Federation of Biological Societies, Guelph, Ont.*, Canada.

Estatira Sepehr, Gerard Cooke, Jocelyn Fournier, Patrick Robertson, G. Sarwar Gilani, (2003) Effects of sex, age and source of soy isoflavones on their bioavailability in a rat model, *Health Canada Research Forum, Ottawa, Ont.*, Canada.

Estatira Sepehr, Gerard Cooke, Patrick Robertson, G. Sarwar Gilani, (2003) Bioavailability of Dietary soy isoflavones in a rat model, *5th International Symposium on the Role of Soy in Preventing and Treating Chronic Disease,* Orlando, Fl., U.S.A.

Estatira Sepehr, Gerard Cooke, Patrick Robertson, G. Sarwar Gilani, (2003) Effect of the gender on the metabolism of soy isoflavones in rats, *46th Annual Meeting Canadian Federation of Biological Societies,* Ottawa, Ont., Canada.

Estatira Sepehr, G. Sarwar Gilani, Gerard Cooke, Eric Lok, Rekha Mehta, (2002) Safety assessment of dietary isoflavones as predicted by growth, blood isoflavones, and estrus cycle in rats, *Health Canada Research Forum,* Ottawa, Ont., Canada.