Biomarkers of Isoflavone Intake:

Validity at High Intakes

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ABSTRACT

Isoflavones are biologically active plant chemicals (phytoestrogens) which are ordinarily present in human diets. There is considerable research interest in their potential to prevent or treat several chronic diseases. Biomarkers can demonstrate compliance during dietary interventions and validate associations between intake of isoflavones and health outcomes.

The objectives of this study were to validate 24-hour urine collections, timed spot urine samples and timed plasma samples as biomarkers of isoflavone intake up to 165mg/day.

Healthy volunteers (20 women and 11 men) consumed 55mg/d, 110mg/d or 165mg/d soy isoflavones or placebo for seven consecutive days in a randomised, double-blind, cross-over study. Timed blood samples, timed spot urine samples (taken in the afternoon, 5-7 hours after consuming the isoflavone supplement) and 24-hour urines were obtained at baseline and during each intervention. Isoflavone content of the samples was assayed by liquid chromatography and mass spectrometry. 24-hour urines were validated by percentage PABA recovery.

The relationship between daily isoflavone intake and 24-hour urinary isoflavone excretion was:
$y = 6.63132x^{0.7421} \equiv x = (y \div 6.63132)^{1.3475}$
where y = isoflavone excretion in μg/24h and x = isoflavone intake in μg/24h
$r^2 = 0.86$; $p < 0.001$; n = 109 samples from 31 volunteers

The relationship between daily isoflavone intake and plasma isoflavone concentration was:
$y = (3.3543x10^{-3})x^{0.4889} \equiv x = (y \div 3.3543x10^{-3})^{2.0454}$
where y = plasma isoflavone in μg/ml and x = isoflavone intake in μg/24h
$r^2 = 0.61$; $p < 0.001$; n = 100 samples from 30 volunteers

The relationship between daily isoflavone intake and spot urine isoflavone concentration was:
$y = (2.0324x10^{-3})x^{0.8009} \equiv x = (y \div 2.0324x10^{-3})^{1.2486}$
where y = isoflavone excretion in μg/ml and x = isoflavone intake in μg/24h
$r^2 = 0.69$; $p < 0.001$; n = 143 samples from 31 volunteers

It was concluded that 24-hour urine collections, timed plasma samples and timed spot urine samples are valid biomarkers of isoflavone intakes up to 165mg/day.
A curvilinear relationship was defined between a) isoflavone dose and bioavailability in plasma and b) isoflavone dose and 24-hour urinary excretion.

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INTRODUCTION

An overview of the field of phytoestrogen research will be presented before elaborating the chemical classification and dietary sources of isoflavones and the pharmacology and pharmacokinetics of these compounds. The rationale for this study will be presented in the context of current research into isoflavones in humans.

BACKGROUND

Plants contain a diverse array of chemicals which can have a substantial impact on human health, particularly if consumed over the course of a lifetime. Food phytochemicals in particular have attracted a great deal of interest in recent years in light of their potential to reduce the risk of chronic diseases, or improve the prognosis once the disease process has initiated. Indeed, it has been suggested that the major influence on the risk for Western diseases relates to an inadequate intake of plant products rather than to any other dietary factor (Griffiths *et al.*, 1996).

Phytoestrogens (PE) are among the most investigated food phytochemicals. As defined by the Commission on Toxicology (COT) Working Group, PE are plant substances and their human metabolites that induce biological responses and can mimic or modulate the actions of endogenous oestrogens, usually by binding to oestrogen receptors (COT, 2003b).

These responses include influences on the production, metabolism and cellular activity of sex steroids; influences on growth factors; on protein synthesis; on intracellular enzymes and signalling cascades; on free radical reactions and on the

proliferation, differentiation and metastasis of malignant cells (Adlercreutz & Mazur, 1997).

PE are present in a wide range of foods and medicinal plants and include isoflavones in soy foods and red clover; prenyl flavonoids in hops and beer; coumestans in alfalfa; lignans in linseeds, whole grains and berries (Cos *et al.*, 2003); resveratrol in nuts, grapes and red wine (Kris-Etherton *et al.*, 2002); glabridin (Tamir *et al.*, 2000) and licochalcone-A (Rafi *et al.*, 2000) from liquorice; ginsenosides in ginseng (Chan *et al.*, 2002) and others (Zava *et al.*, 1998). In plants, the PE glycosides genistin and daidzin are among the phytoalexins which protect the plant against pathogens, so their concentration in the plant depends upon the presence or absence of pathogenic attack, as well as on the season and variety of the plant (Dixon & Ferreira, 2002).

There has been increasing interest in PE since the mid-1940s, when fertility disorders consistent with an anti-oestrogenic effect were observed in ewes grazing on subterranean clover (*Trifolium subterraneum*) which is rich in isoflavones (Bennetts *et al.*, 1946). The ewes' intestinal microflora metabolised formononetin in the clover to equol, a potent PE.

After the chance discovery of equol in human urine (Axelson *et al.*, 1982), studies of isoflavones in humans began in the 1980s. Much of the early PE research focused on the relative oestrogenicity of these compounds – using assays such as growth promotion in oestrogen receptor-positive breast cancer cell lines or increases in uterine weights of mice to assess their stimulatory potential in oestrogen-responsive tissues.

PE are capable of producing both oestrogenic and anti-oestrogenic effects depending upon the tissue type, the proportion of α- to β-type oestrogen receptors and the concentration of their endogenous ligands (Ososki & Kennelly, 2003). PE induce a partial response upon binding to oestrogen receptors, so in an oestrogen-deprived state (for example, in the ovariectomised rodent or in the relatively low-oestrogen environment after menopause) PE increase the transcription of oestrogen-dependent genes. In an oestrogen-rich environment, these weak partial agonists effectively act as oestrogen antagonists by blocking endogenous oestrogens from receptors. For this reason, PE are classed as selective oestrogen receptor modulators or SERMs.

Interest in the effects of PE on human health grew rapidly after the observation that Asian populations with a high dietary intake of PE (and particularly of soya isoflavones) have a lower incidence of hormone-dependent cancers relative to Western populations whose habitual PE intake is low. The incidence of breast and prostate cancers in Asians increases within one or two generations after migration to a Western country, evidence that protection against these cancers is attributable to some aspect of Asian diet or lifestyle (Griffiths *et al.* 1996). Further epidemiological studies investigated Asian diets and the incidences of chronic diseases such as cancers and cardiovascular disease in these populations. Many of these studies highlighted soy as a potential protective factor (Adlercreutz, 2003; Dai *et al.*, 2002; Fournier *et al.*, 1998; Greenstein *et al.*, 1996; Hirayama, 1986; Hirose *et al.*, 1985; Nagata, 2000; Nomura *et al.*, 1978; Yuan *et al.*, 1995). Simultaneously, mechanistic studies were undertaken to elucidate a mechanism by which the Asian diet could protect against these Western diseases (Adlercreutz & Mazur, 1997; Anderson *et al.*, 1999; Messina *et al.*, 1994). Isoflavones in soy were a candidate for mediating such a protective effect.

CHEMISTRY OF PHYTOESTROGENS

Phytochemicals with oestrogenic properties fall into a number of chemical classes. The relationships between classes of PE commonly responsible for physiological effects in humans are shown in Table 1 but this is by no means an exhaustive list of phytochemicals with oestrogenic properties. Compounds shown in brackets are metabolites of PE.

Table 1: Classes of PE

Flavonoids			Non-Flavonoids
Isoflavones	***Prenyl flavonoids***	***Coumestans***	***Lignans***
Genistein 4',5,7-trihydroxyisoflavone	8-prenylnaringenin	Coumestrol	Lariciresinol
Daidzein 4',7-dihydroxyisoflavone	6-prenylnaringenin		Isolariciresinol
Glycitein 4',7-dihydroxy-6-methoxyisoflavone	Xanthohumol		Matairesinol
Biochanin A 5,7-dihydroxy-4'-methoxyisoflavone	Isoxanthohumol		Secoisolariciresinol
Formononetin 7-hydroxy-4'-methoxyisoflavone			[enterodiol 2,3-bis(3-hydroxybenzyl-butane-1,4-diol]
[equol 7-hydroxy-3-(4'-hydroxyphenyl)-chroman]			[enterolactone *trans*-2,3-bis(3-hydroxybenzyl)-butane-1,4-diol]

Of these compounds, those found most often and in the greatest concentration in human blood and urine are isoflavones and mammalian lignans. Enterodiol and enterolactone are the mammalian lignans, so named because they are formed by the action of microflora in the mammalian intestine upon the plant lignans (Adlercreutz &

Mazur 1997). Isoflavonoids are thought to have evolved about 120 million years ago via the flavone biosynthetic pathway (Adlercreutz, 1998).

Equol, an isoflavone derivative (or more correctly an isoflavan), is a product of intestinal bacterial enzymatic action on daidzein (Setchell *et al.*, 2002). In foods, isoflavones are usually present as glycosides (Coward *et al.*, 1993) or their methyl derivatives which may be conjugated to acetyl or malonyl groups at the sugar moiety. Genistein, daidzein and glycitein glucosides are named genistin, daidzin and glycytin.

Biochanin A and formononetin are the 4'-methyl derivatives of genistein and daidzein and constitute the aglycone moieties of ononin and sissotrin (COT 2003b). Glycitein is the 6-methoxy analogue of daidzein but is not converted to daidzein or equol to any appreciable extent *in vivo* because of the proximity of the 6-methoxyl group to the 7-hydroxyl group which hinders demethylation (Setchell *et al*. 2002). Structural formulae of isoflavones and their glucosides are illustrated in Appendix 1.

Conjugation to glucose in the plant or to glucuronide and sulphate in mammals increases the hydrophilicity of isoflavones. These glucuronide and sulphate conjugates can be hydrolysed by acid and/or heat as well as by enzymatic degradation. Fermentation of soy beans (as in the production of miso and tempeh) hydrolyses the glycosides (COT 2003b). Isoflavone aglycones are heat stable (Setchell, 1998), hydrophobic and acid stable (COT 2003b), the presence of phenolic groups in both ends of the molecule rendering exceptional stability upon these compounds (Adlercreutz & Mazur 1997).

All PE are structurally similar to oestradiol (Mazur & Adlercreutz, 2000; Miksicek, 1995) as they bear phenolic rings in similar positions and are close in molecular weight. In addition, the A ring of isoflavones can mimic the A ring of oestrogens and there is an almost identical distance (11Å) between the two aromatic hydroxyl groups of the isoflavone nucleus and the C_3 and C_{17} hydroxyl groups of oestradiol. The pattern of hydroxylation of isoflavones also appears to be a determinant of oestrogenicity, with hydroxyl groups in the 4', 5 and 7 positions (as in genistein) being optimal for oestrogen receptor binding (Miksicek 1995; Mazur and Adlercreutz 2000). The structural similarity of PE to oestradiol is illustrated in Appendix 2.

ISOFLAVONES IN THE DIET

These small, stable and physiologically active compounds occur commonly in the human diet. Dietary exposure to isoflavones comes principally from soy (raw soy beans are estimated to contain 142mg total isoflavones per 100g (Ritchie, 2003a) – and high isoflavone intakes in Asia are attributable mainly to soy foods (Arai *et al.*, 2000). In the West, the major source of isoflavones is 'hidden soy' in bakery products and processed meats, although consumption of soy milks and 'second generation' soy foods such as soy-based meat substitutes is becoming increasingly widespread (Ritchie *et al.*, 2005a). Most studies have found that the richest sources of isoflavones in the Western diet are soy products, bakery products and dried fruits (Ritchie *et al.* 2005a)(Clarke & Lloyd, 2004; Horn-Ross *et al.*, 2000). Isolated soya isoflavones as supplements are also widely available over the counter, widely used and currently being investigated for their clinical utility, safety and pharmacological properties.

Genistein and daidzein are the most prevalent dietary isoflavones, comprising 90% of the total intake of oestrogenic isoflavonoids (Pillow *et al.*, 1999). Although food processing can alter the concentrations of genistein and daidzein in foods and may influence the ratio of genistein to daidzein (Coward *et al.*, 1998; Slavin *et al.*, 1998), the presence of genistein, daidzein and glycitein in the ratio 1 : 0.8 : 0.1 in soybeans and their derivative foods is typical (Clarke & Lloyd, 2004). Biochanin A and formononetin are found mainly in clover and alfalfa sprouts (Franke *et al.*, 1998; Mazur *et al.*, 1996) and are therefore much less prevalent in the average diet than genistein and daidzein.

Estimates of the mean (and range of) total dietary intakes of isoflavones in various populations are presented in Table 2. Where mean intakes were not reported, the range alone is given in brackets.

Table 2: Isoflavone intakes in various populations

Authors	**Population**	**Mean isoflavone aglycone intake (mg/d)**	**Methods used**	**Sample size**
Clarke and Lloyd 2004	UK	3	Direct food analysis extrapolated to average adult food consumption	20 diet samples
Verkasalo *et al.* 2001	UK	(0-34)	7-day food diaries	80
Clarke *et al.* 2002	UK (vegetarians)	10.5	Duplicate diet analysis	35
Guthrie *et al.* 2000	Australian	(0-340)	FFQ	354
Lampe *et al.* 1999	USA	(0.5-2)	5-day food diaries	103
Horn-Ross *et al.* 2000	USA	2.6	FFQ	447
De Kleijn *et al.* 2001	USA	0.2	FFQ	964
Nagata *et al.* 2000	Japanese	29	3-day food diaries	6000 families
Arai *et al.* 2000	Japanese women	46.5	3-day food diaries	106
Yamamoto *et al.* 2001	Japanese	50	FFQ	215
Yamamoto *et al.* 2001	Japanese	38	4x 7-day food diaries	215
Ritchie *et al.* 2005	Scottish omnivores	1.2	7-day weighed diaries	9
Ritchie *et al.* 2005	Scottish vegetarians	7.4	7-day weighed diaries	10
Ritchie *et al.* 2004	Scottish women	(0-53.1)	7-day food diaries	15
Ritchie *et al.* 2004	Scottish	3.7 (0.05-39)	24-hour food records	21

FFQ = food frequency questionnaire

Of note is the particularly wide range of intakes observed by Guthrie and colleagues in Australia (0-340mg/d), which might be due to the presence of a significant Indonesian minority in this population. Some authors put much higher estimates on Japanese isoflavone intake than are indicated above, for example, Cassidy *et al.* (1994) base their estimate of 150-200mg/d on their own measured concentrations of isoflavones in soy foods and on Japanese dietary patterns reported by other researchers. The distribution of isoflavones in Western population is positively skewed by high intakes in a small subset of the population who consume soy on a regular basis (Keinan-Boker *et al.*, 2004; Ritchie *et al.*, 2004; Verkasalo *et al.*, 2001). With increasing availability and popularity of isoflavone supplements, usually self-prescribed for menopausal and menstrual symptoms, very high intakes may be observed in this subset of the population.

Phytoestrogens and Lifestyle

Because PE are present in appreciable concentrations only in certain foods, PE intake varies greatly with dietary habit. Dietary habits are in turn associated with other social variables and lifestyle choices which therefore correlate with PE intake. Keinan-Boker *et al*. (2004) reported that in 15555 Dutch women aged between 49 and 70, those in the highest quartile of PE intake were significantly older at delivery of their first child, smoked significantly less, were significantly taller, had significantly lower BMI and significantly higher physical activity. They also had significantly higher daily intakes of energy, fibre, fruit, vegetables and soy produce. This would seem to point towards a "high-phytoestrogen lifestyle" in which many of the attributes of affluence and health-consciousness are clustered. This study also reported no significant relation between isoflavone or lignan intake and breast cancer incidence,

despite the apparently 'healthier' lifestyle of the high PE consumers. However, some lifestyle characteristics of affluent women, such as older age at delivery of their first child, are positively associated with breast cancer risk. In China, where affluence is associated with a more Westernised lifestyle, breast cancer is sometimes colloquially referred to as "the rich woman's disease" (Plant, 2000).

The traditional Asian diet which has been observed to protect against cancers and other Western diseases is also low in dairy produce (Plant 2000), fat and red meat and rich in vitamin D (Adlercreutz, 2003) and n-3 fatty acids. B-carotene has also been observed to confound associations of PE with cancer in some studies (Murkies *et al.*, 2000).

Adlercreutz has suggested that lignans may serve as a "biomarker of a healthy diet" (2003) due to their presence alongside high intakes of vegetables, fruits and fibre. Fibre and urinary and serum lignan concentration correlate significantly but not strongly (Grace *et al.*, 2004).

PHYSIOLOGICAL EFFECTS OF ISOFLAVONES

Isoflavones, being lipid soluble, stable in structure and of a low molecular weight, are able to cross the cell membrane by passive diffusion and act upon nuclear oestrogen receptors. Every organ system in the body expresses oestrogen receptors to a greater or lesser extent in its various tissue types, hence the oestrogen receptor-mediated activities of PE are extremely widespread.

Isoflavones may also interact with membrane receptors and exert effects that are expressed through second messengers in the cytoplasm. Non-oestrogen receptor-mediated actions include protein tyrosine kinase inhibition (Akiyama *et al.*, 1987); inhibition of cytochrome P450 enzymes (which are capable of metabolising pro-carcinogens to active carcinogens) (Badger *et al.*, 2002); inhibition of angiogenesis (Fotsis *et al.*, 1993); reduction in oxidative damage of both DNA (Djuric *et al.*, 2001; Mitchell & Collins, 1999) and lipoproteins (Vedavanam *et al.*, 1999); altering the expression or function of enzymes including topoisomerase (Bertrand *et al.*, 1993; McCabe & Orrenius, 1993; Yamashita *et al.*, 1990), glutathione peroxidase, catalase, superoxide dismutase, glutathione reductase and glutathione-S-transferase (Cai & Wei, 1996); inhibition of the transcriptional regulator NFκB (Davis *et al.*, 1999; Dijsselbloem *et al.*, 2004); modulation of cyclins and cyclin-dependent kinase inhibitors (Choi *et al.*, 2000; Sarkar & Li, 2002); modulating expression of pro-and anti-apoptotic genes such as Bax and Bcl-2 (Sarkar & Li 2002); and regulation of growth factors and growth factor receptors in both malignant and non-malignant cells (Boersma *et al.*, 2001). Thus, this group of molecules are considered to have immense potential to modify physiological and pathophysiological processes.

Among the most studied clinical effects of PE are in the prevention and management of cancer, cardiovascular disease and other hormone-related diseases, such as osteoporosis.

Phytoestrogens and Endocrine Physiology

Premenopausal women – sex hormone metabolism and the menstrual cycle

Increases in menstrual cycle length by three and a half days, accompanied by decreases in serum oestradiol and progesterone, have been observed after one month of soy supplementation (approximately 100mg/d genistein plus 100mg/d daidzein) (Lu *et al.*, 1996). A significant increase in the length of the follicular phase of the cycle was observed in six women supplemented with only 45mg/d isoflavones for one month (Cassidy *et al*. 1994). This increase correlated significantly with urinary equol excretion, indicating that this isoflavone metabolite may be largely responsible for the effects on the menstrual cycle. Increase in follicular phase length was accompanied by statistically significant increase in the concentration of circulating oestradiol in the follicular phase and suppression of the mid-cycle peaks of LH and FSH.
Circulating oestrogen concentrations are considerably lower in the follicular than in the luteal phase of the cycle, so lengthening of the follicular phase is associated with a reduced exposure to oestrogens over time. Reducing exposure to oestrogen may be significant for breast cancer prevention as well as for conditions of oestrogen excess such as menorrhagia, uterine fibroids and some forms of premenstrual syndrome. However, lengthening of the menstrual cycle may be considered an adverse effect in women with oligomenorrhoea or polycystic ovarian syndrome.

One study has assessed the impact of soy protein containing 68mg/d isoflavones on premenstrual symptoms after two months of supplementation. Significant improvements compared to placebo were seen in breast tenderness, headaches, cramps and swelling but differences in total symptoms were not significant (Bryant *et al.*, 2005).

Effects on sex hormone metabolism in postmenopausal women

In postmenopausal women, neither 56mg/d nor 90mg/d isoflavones administered as part of a soy protein isolate for three- or six-month periods had any effect on serum hormones (Persky *et al.*, 2002). Similarly, Wu *et al*. (2005) showed no effect of supplementing 50mg/d for 8 weeks and Foth & Nawroth (2003) reported no effect upon any serum hormones after supplementing 20mg/d for 24 weeks. Isoflavones extracted from red clover (26mg biochanin A, 16mg formononetin, 1mg genistein and 0.5mg daidzein) did not produce any alterations in circulating oestradiol, FSH (follicle stimulating hormone) or LH (luteinising hormone) in postmenopausal women after one year of supplementation (Atkinson *et al.*, 2004). A study of 144 postmenopausal women in Singapore found that soy intake (as assessed by food frequency questionnaire) was significantly associated with serum oestrone independently of any non-dietary factors, with those in the highest quartile of soy intake having 15% lower circulating oestrone than those in the lowest quartile (Wu *et al.*, 2002).

Phytoestrogens, sex hormones and breast cancer risk

In postmenopausal women, circulating oestrogens are low and oestrone dominates in the plasma. The ratio of breast tissue to plasma oestradiol concentration is 1:1 premenopausally but rises to 10:1 or 50:1 after menopause. Oestradiol concentrations in the breast are primarily regulated by aromatase, oestrogen sulphatase and 17β-hydroxysteroid dehydrogenase (17β-HSD). The entry of oestrone into breast cells is controlled by the de-conjugating enzyme oestrone sulphatase (Dai *et al.*, 2003). The activity of these enzymes may therefore be much more important than circulating oestrogen levels for determining the carcinogenic effects of oestrogens on the breast in postmenopausal women. Genistein, biochanin A, enterolactone, enterodiol and, to a lesser degree, equol, formononetin, daidzein and coumestrol inhibited 17β-HSD in genital skin fibroblasts (Evans *et al.*, 1995) . Genistein inhibits this enzyme in breast cancer cells in culture (Wong & Keung, 1997). Daidzein inhibits oestrone sulphatase *in vitro* (Adlercreutz *et al.*, 1993).

In one study which reported an inverse relationship between urinary isoflavones and breast cancer risk (Makela *et al.*, 1994) the association was significant only in women who had low levels of sulphated oestrone (indicating higher levels of oestrone sulphatase activity), high levels of oestradiol and low levels of sex hormone binding globulin (SHBG). These three factors would create a more oestrogenic environment and consequently a higher risk of breast cancer. It could therefore be speculated that PE might exert the observed anti-carcinogenic effect by competing with oestrogen at the receptor level, upregulation of SHBG synthesis, inhibition of oestrone sulphatase or all of these actions. Alternatively, it may be that there is no mechanistic relation between this triad of high-oestrogen states and PE chemoprevention, but simply that

the risk in this high-oestrogen group is sufficiently high for a protective effect of PE to reach statistical significance.

Lower circulating concentrations of SHBG are associated with increased breast cancer risk (Adlercreutz *et al.*, 1989; Lipworth *et al.*, 1996). SHBG binds oestradiol and has been found to antagonise its growth-promoting on MCF-7 human breast cancer cells in culture (Fortunati *et al.*, 1996). SHBG synthesis may be stimulated by PE including genistein, daidzein and equol (Adlercreutz *et al.*, 1987; Loukovaara *et al.*, 1995; Mousavi & Adlercreutz, 1993). Adlercreutz and Mazur (1997) reported significant increase in SHBG (from 55.7 to 71.2nmol/l; $p<0.01$) in postmenopausal women after three months' supplementation with soy and flaxseed but other dietary intervention studies have reported no effect of PE on SHBG concentrations in plasma (Cassidy *et al.*, 1994; Cassidy *et al.*, 1995; Nagata *et al.*, 1997; Phipps *et al.*, 1993). Higher plasma concentrations of SHBG are observed in vegetarians and subjects with high urinary PE excretion (Adlercreutz *et al.*, 1987; Setchell, 1998) but at present it is not possible to determine whether associations with SHBG are due to an effect of PE or some other aspect of the plant-based diet. For example, vegetarians generally have lower plasma insulin levels, which may increase plasma SHBG independently of dietary phytochemicals (Loukovaara *et al.*, 1995). Circulating concentrations of SHBG show great variation both between and within individuals so detecting an effect of small magnitude upon circulating SHBG is very difficult (Adlercreutz & Mazur 1997). In the above studies, basal SHBG was already relatively high, reducing the potential for increase by PE.

If observational but not intervention studies report demonstrable effects of PE on SHBG concentrations, this may be because a high-PE diet must be consumed over a

long time period or early in life in order to produce an effect. Alternatively, it may indicate that co-existent lifestyle factors and not the PE concentration of the diet are responsible for maintaining high SHBG concentrations.

SHBG is of relevance not only for breast cancer risk reduction, but also for other conditions of oestrogen dominance such as polycystic ovarian syndrome, uterine fibroids and endometriosis, in which an increase in circulating SHBG would theoretically be beneficial.

The relative ratios of different oestrogen metabolites are of relevance for the risk of female cancers because while 2α-hydroxyoestrone (2OHE) has relatively little biological activity, 16α-hydroxyoestrone (16OHE) promotes hormone-dependent cancer growth (Brooks *et al.*, 2004). Xu and colleagues (2000) found that supplementation with 65mg/d isoflavones increased the urinary ratio of 2OHE relative to 16OHE in postmenopausal women. They also reported a decrease in urinary 4-hydroxyoestrone with both 65mg/d and 123mg/d isoflavone supplementation and an increase in the ratio of 2OHE : 4OHE at 7.1mg/d, 65mg/d and 123mg/d. Brooks *et al.* (2004) supplemented soy flour providing 41.9mg/d isoflavones to 46 postmenopausal women and found no significant relationship with the ratio of 2OHE to 16OHE. However, these studies are not directly comparable because a lower isoflavone dose was administered by Brooks *et al.*

Menopausal symptoms

Soy isoflavone and other PE supplements are commonly self-prescribed by women for the treatment of hot flushes and other menopausal symptoms (Quella *et al.*, 2000), although the findings of clinical trials assessing their efficacy have been, at best, mixed (Albertazzi *et al.*, 1998; Murkies *et al.*, 2000). A systematic review by Krebs *et al*. (2004) concluded that PE are ineffective for the treatment of hot flushes but other authors consider that PE do have a beneficial effect, albeit one that is significant only in women who have more than five flushes per day (Messina & Hughes, 2003). PE, either as supplements or included in the diet, are also frequently used by women for the treatment of menopausal symptoms after breast cancer or during anti-oestrogen treatment for breast cancer, when conventional hormone replacement therapy is contraindicated. Hot flushes in breast cancer survivors are a significant clinical problem and are often more severe when medically induced than as a consequence of natural menopause (Quella *et al*. 2000). During a double-blind crossover study of 149 breast cancer survivors suffering hot flushes severe enough to require intervention, 150mg of soy isoflavones in three divided doses for four weeks produced no effect discernible from placebo in terms of patient preference, frequency or severity of hot flushes (Quella *et al*. 2000).

There is some evidence of a beneficial impact of PE on cognition in postmenopausal women (File *et al.*, 2002), which will be discussed later.

Despite some negative findings, soy and PE supplements continue to be popular among menopausal women and anecdotal evidence of efficacy is widespread. It is conceivable that if introduction of PE into the diet brings about a general dietary change, some other aspect of this dietary change is responsible for the alleviation of symptoms and perceived efficacy of PE.

Hormonal effects of PE in men

In fifteen 18-35 year old men, two-month administration of a soy extract containing 40mg/d isoflavones induced no changes in oestradiol, testosterone, FSH or LH, testicular volume or sperm count, volume, motility or morphology (Mitchell *et al.*, 2001). An inverse correlation between soy product intake and oestrogen levels in men was observed by Nagata *et al*. (2000) although the same group found that dietary supplementation with 400ml soy milk daily for 8 weeks did not result in any alteration of total oestrogen concentrations in men, despite a reduction in serum oestrone.

Fertility and development

Concerns over the safety of PE have centred around their potential impact on human fertility and infant development. However, reliable clinical data in this area are extremely difficult to obtain. A review in 2002 of the health consequences of early soy consumption concluded that no long-term adverse effects of early exposure are evident up to young adulthood but that little is known about any effects (adverse or beneficial) on health in later life (Badger *et al.*, 2002). The body of evidence regarding PE effects on fertility and development is based almost entirely on rodent (and, to a lesser extent, primate) studies. These suggest that genistein produces a limited degree of oestrogenic effect such as advancement of puberty, mammary gland differentiation, irregular cycling and abnormal histology of the reproductive tract. Exposure in the perinatal, neonatal and prepubertal stages produce the most marked effects in animals. However, extrapolation to humans is extremely difficult because many of the animal studies have used subcutaneous administration of PE, bypassing intestinal and hepatic metabolism, or have fed soy-based diets without recording their PE content. Significant species differences also exist, both in the hormonal control of

sexual development (COT, 2003c) and in the metabolism of PE, making the data difficult to interpret.

Phytoestrogens and the thyroid

Isoflavones are structurally similar to the thyroid hormones, tri-iodothyronine (T3) and thyroxine (T4) and have shown some goitrogenic effects (although without producing hypothyroidism) in infants fed soy flour-based infant formula in the 1960s. Subsequently, iodine was added to formulae and no such effects have been reported since (COT, 2003d).

Isoflavones could theoretically increase concentrations of thyroid binding globulin (TBG), thereby lowering free thyroxine and consequently increasing thyroid stimulating hormone (TSH). In the healthy state, compensation would occur but if thyroid function were compromised, increases in TBG might reduce free thyroxine sufficiently to be clinically significant.

In postmenopausal women, no difference in TBG concentration was observed between women supplementing with 118mg/d isoflavones in soy protein isolate and those on placebo (Teede *et al.*, 2004). Persky *et al*. (2002) saw increases in TSH in postmenopausal women taking 90mg/d isoflavones compared to a placebo group. However, these increases were small and unlikely to be clinically significant. A study using the same dose of isoflavones did not find any differences in TSH, T4 or T3 between the isoflavone and placebo groups (Bruce *et al.*, 2003). A Japanese study (Ishizuki *et al.*, 1991) reported hypothyroid symptoms and increases in TSH in subjects eating 30g soybeans per day in addition to their usual diets. However, isoflavone content of the soybean supplement and of the background diet was not reported. Concentrations of inorganic iodide were lower during the soybean

administration and the effect upon serum TSH was most pronounced in the older group of subjects (mean age 61 years). Although TSH increased significantly, it remained within normal parameters and serum T3 and T4 remained unchanged. Overall, the data suggest that any effect of isoflavones on the thyroid is likely to be clinically important only when thyroid function is compromised, for example by iodine deficiency or advanced age.

Effects of Phytoestrogens in other physiological systems

Phytoestrogens and the cardiovascular system

Mortality due to cardiovascular disease (CVD) is lower in Eastern than in Western populations and this is thought to be at least partly due to dietary differences. There are a number of mechanisms by which soy foods might reduce CVD risk but no strong evidence that any of these is due to its isoflavone content.

The hypocholesterolaemic effect of soy has been established in a meta-analysis of 38 controlled clinical trials (Anderson *et al.*, 1995). Biomarkers of *in vivo* lipid peroxidation are also reduced and the resistance of LDL to oxidation is increased after soy consumption (Wiseman *et al.*, 2000).

However, studies using purified isoflavones have produced inconsistent results (COT, 2003f). Similarly, soy diets have been shown to reduce blood pressure in human studies (Teede *et al.*, 2001; Vigna *et al.*, 2000; Washburn *et al.*, 1999) although two studies have shown no effect of isolated isoflavones (Han *et al.*, 2002; Simons *et al.*, 2000) and another demonstrated an improvement in systemic arterial compliance without concomitant effect on arterial pressure (Nestel *et al.*, 1997).

Influences on vascular reactivity may be isoflavone-mediated, because both dietary soy protein and intravenous genistein produce an oestrogen-like vasodilatory effect in rhesus monkeys (Honore *et al.*, 1997). In cultured human umbilical vein endothelial cells the production of prostacyclin (a potent vasodilator) was significantly increased in a dose- and time-dependent manner by genistein and daidzein (Hermenegildo *et al.*, 2005).

There is sufficient evidence to recommend inclusion of soy in the diet to reduce the risk of CVD but as yet no basis for the recommendation of isoflavone supplements for this purpose. If there is an effect of isoflavones in reducing CVD mortality or biomarkers of CVD risk, then large, long-term intervention studies may be needed to detect it. Such studies should assess plasma and urinary isoflavone concentrations in relation to disease biomarkers and if isoflavone supplements are administered long-term, markers of compliance will undoubtedly be required.

Phytoestrogens and bone

Studies in rodents have shown that PE consistently prevent bone loss after ovariectomy (COT, 2003e). A major determinant of osteoporosis risk is the peak bone mass attained in early adulthood, which may be affected by pre-pubertal consumption of PE as occurs in countries where they form part of the traditional diet. Rates of osteoporosis are lower in these countries (COT 2003e).

In the middle-aged, at-risk population, it seems that a relatively low threshold dose of PE consumed over several years produces measurable effects on bone density (Anderson *et al.*, 1999). A two-year study by Lydeking-Olsen *et al*. (2004) reported

that bone mineral content and bone mineral density in the lumbar spine were maintained in a group taking two glasses of soy milk per day (containing 76mg/d isoflavones) but decreased in the group on isoflavone-poor placebo.
The effects of isoflavones on bone are thought to be mediated through the oestrogen receptors of osteoblasts (Anderson *et al.* 1999).

Phytoestrogens and the central nervous system

The biphenolic structure of isoflavones allows them to pass through the blood-brain barrier and act on the CNS, where animal studies have revealed effects on various brain structures such as the sexually dimorphic nucleus of the preoptic area and the anteroventral periventricular nucleus (Lephart *et al.*, 2005).

Isoflavones have demonstrable effects on cognition in humans and rodents, which appear to show gender variations (Hill & Dye, 2003). File *et al.* (2001) randomised 15 male and 12 female students to soy supplements containing either 0.5 or 100mg/d isoflavones for ten weeks before administering a battery of tests assessing four areas of cognition. They observed significant enhancement of long-term episodic memory (delayed verbal recall of pictures) and short-term non-verbal episodic memory in the high isoflavone group. Mental flexibility as measured by rule shifting and reversal improved slightly in the high isoflavone group while it deteriorated slightly in the low isoflavone group. In terms of verbal fluency (word generation) and planning ability, the women in the high isoflavone group showed improvements while the men showed impaired performance. In a later study, the same group supplemented isolated isoflavones (60mg/d) to 33 post-menopausal women (not taking HRT) for 12 weeks. After this time they observed a greater improvement in episodic memory and frontal

lobe functioning (sustained attention and speed of mental flexibility and planning ability) in the supplemented group compared to the placebo group (File *et al.* 2002). Whether these effects of isoflavones are oestrogen-receptor mediated is not known.

Phytoestrogens, immunity and inflammation

Oestrogens and xenoestrogens are well known to influence the immune system, therefore PE were hypothesised to exert immunomodulatory influences. However, experimental results to date have been contradictory.

Studies in rodents have produced reductions in thymus weight, lymphopenia and compromised humoral immunity after injected (2-200mg/kg bw/d) or dietary (1000-1500mg/kg diet) genistein (Yellayi *et al.*, 2002). In a study by Zhang *et al*. (1997), daidzein administered to mice by oral gavage produced increases in thymus weight, phagocytic activity, circulating T-cell concentrations and splenocyte-mediated cell lysis at 20 and 40mg/kg bw/d, although 10mg/kg bw/d showed no effect. The doses administered in these rodent studies were extremely large and, neglecting species differences in metabolism, would be equivalent to 140-14,000mg/d injected genistein; dietary genistein equivalent to a diet consisting entirely of raw soy beans (which contain approximately 142mg isoflavones per 100g); or 700, 1400 or 2800mg/d oral daidzein for a 70kg adult.

Cordle *et al.* (2002) and Ostrom *et al.* (2002) compared soy formula-fed infants with breast fed infants and showed no decrement in immune function based on antibody responses to vaccines, immune cell sub-populations and morbidity.

Anti-inflammatory effects have been observed in animal models of inflammatory bowel disease (0.1mg/kg bw/d) and collagen-induced arthritis (Verdrengh *et al.*, 2003).

Richard *et al*. (2005) observed diminished secretion of the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α) and interleukin-8 in human peripheral blood mononuclear and polymorphonuclear leukocytes *in vitro*, after stimulation with lipopolysaccharide and interferon gamma. TNF is a key mediator of inflammatory reponses and may play a pivotal role both in bone resorption and in the development of cancer (Huang *et al.*, 2005).

TNF inhibiting properties were confirmed in a human study by Huang *et al*. (2005), who found 67% decreased serum TNF-α, 57% decreased serum interleukin-1α and 14% decreased blood monocytes after 10 weeks consuming 36 ounces soy milk per day, compared with pre-supplementation levels. This equates to 1.02 litres soy milk, likely to contain approximately 8320mg total isoflavones. Jenkins *et al.* (2002) found no effect of 10mg/d or 73mg/d isoflavones in 50g soy protein upon C-reactive protein, serum amyloid A or TNF-α in 41 hypercholesterolaemic men and postmenopausal women, although there was a trend towards increase in interleukin-6 after the 73mg/d phase. This negative finding may support the hypothesis that large doses of isoflavones are required to produce a measurable impact on circulating TNF-α., but further intervention studies would be required to verify it.

Phytoestrogens and the digestive system

Some research suggests that a PE-rich diet may modulate the luminal environment of the GIT. Wiseman *et al.* (2004) noted an increase in faecal bacterial β-glucosidase activity after 10 weeks' consumption of a high-soy diet, indicating a modulation of the intestinal microfloral population which might have physiological effects for the host. Isoflavones may also have a role in chemoprevention of cancers of the colon and rectum (Lechner *et al.*, 2005).

Phytoestrogens and renal function

In animal models and in human subjects with chronic kidney disease, soy protein has been shown to limit or reduce proteinuria and preserve renal function (Velasquez & Bhathena, 2001). It is not clear whether these effects can be attributed to the isoflavone content, although their effects on cell proliferation, the extracellular matrix, inflammation and oxidative stress may be highly relevant to renal disease.

Summary

With so many diverse pharmacological properties in all physiological systems, the impact of PE - and especially of isoflavones such as genistein and daidzein – on human health has been the topic of considerable debate and research. However, some data lack generalisability from experimental model to humans or are weakened by methodological shortcomings (Yang *et al.* (1996) give examples of common methodological pitfalls). There is a need for rigorous investigation of the biological and dietary correlates of isoflavone exposure in humans, in relation to physiologically and clinically relevant outcome measures.

ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION

These highly biologically active molecules are ordinarily present in human diets and human bodies. Isoflavones and lignans have been found in human fluids including urine, plasma (Franke *et al.*, 2002), prostatic fluid (Finlay *et al.*, 1991; Morton *et al.*, 1997), saliva (Finlay et al. 1991), semen (Dehennin *et al.*, 1982), amniotic fluid (Foster *et al.*, 2002) and breast milk (Franke *et al.*, 1998). High concentrations of isoflavones and lignans are found in the blood and urine of vegetarians (Adlercreutz *et al.*, 1986; Adlercreutz *et al.*, 1982) and Asian populations (Adlercreutz *et al.*, 1991; Adlercreutz *et al.*, 1993; Morton *et al.*, 2002).

Transformation and uptake of phytoestrogens

Isoflavones exist primarily as glycosides in plants and are hydrolysed in the jejunum by the action of bacterial β-glucosidases (Setchell, 2000). The total absorption of isoflavones appears to be similar regardless of whether glycosides or aglycones are ingested, though aglycones are absorbed faster (discussed later).

Absorption of intact glycosides has not been observed (Hendrich, 2002; Setchell, 2000), hence the bioavailability of isoflavones is dependent on the ability of the gut microflora to hydrolyse their glycosides. Intestinal microflora also largely determine the metabolic fate of isoflavones. In a simulator of the human intestinal microbial ecosystem, inoculation with a faecal sample from an equol-producing individual was followed by equol production in the simulator (Decroos *et al.*, 2005). Inter-individual

variation in metabolic pathways may also be due to differences in gut transit time (Hendrich 2002) or polymorphisms in liver enzymes (Kulling *et al.*, 2000). Individuals may be categorised as 'equol producers' (plasma equol > 83nmol/L or 20μg/L; urinary equol > 1000nmol/L or 242μg/L) or 'non-equol producers' (plasma equol < 40nmol/L or 10μg/L; urinary equol < 1000nmol/L or 242μg/L) (Setchell *et al.*, 2002). These cut-off values have been derived empirically because there is a large demarcation between individuals who produce equol and those who do not.

Rats, due to their large caecum and abundant microflora, produce very large quantities of equol which is therefore the predominant isoflavone derivative in rat plasma (Brown & Setchell, 2001). Germ-free animals do not excrete equol (Axelson & Setchell, 1981) and equol is not present in the urine of infants whose microflora have not yet developed (Setchell *et al.*, 1998), illustrating the importance of intestinal bacteria in the production of this metabolite.

Most studies show that only about a third of Caucasians produce equol in response to ingestion of daidzein (Setchell *et al.* 1998). However, studies in Japanese women have observed 50-54% prevalence of equol production (Arai *et al.*, 2000; Uchiyama *et al.*, 2001). Equol is excreted in urine predominantly on days 2 and 3 after ingestion, with only traces of equol excreted during the first 24 hours. Serum concentrations of 13C equol peak 24h after administration of 13C daidzein (Setchell *et al.*, 2003a).

Isoflavone aglycones are rapidly conjugated at the hydroxyl groups by UDP-glucuronyl transferases and sulphotransferases (Hendrich 2002) before undergoing enterohepatic cycling (Setchell, 2000). It is generally assumed that glucuronide and sulphate conjugates are biologically inactive (Hendrich 2002) but displacement of oestradiol from receptors in murine uterine cytosol and enhancement of natural killer

cell-mediated cytotoxicity have been demonstrated by daidzein and genistein glucuronides (Zhang *et al.*, 1999).

Glucoronide conjugates predominate over sulphates; the proportions of free aglycones in plasma are small and the proportion of free aglycone in urine is smaller still (Hendrich 2002).

PE may interact with oxidants such as HOCl, HOBr and $ONOO^-$ produced by inflammatory cells to form brominated, chlorinated or nitrated derivatives. Boersma *et al*. (2001) discovered a novel chlorinated metabolite of biochanin A formed by human polymorphonuclear cells (PMN) upon stimulation of a respiratory burst. The biochemical properties of such compounds are completely unknown but the presence of a chlorine group, particularly in the 3'-position on the B-ring, may augment the antioxidant properties of isoflavones. The capacity of human neutrophils to chlorinate and nitrate genistein was later confirmed by D'Alessandro *et al*. (2003). Reactions occurring in PMN are relevant to atherosclerotic plaques and tumours because PMN aggregate in these lesions (Boersma *et al.* 2001). A low level of nitration of the compound was constitutive while the extent of chlorination was markedly increased by stimulation of the neutrophils.

Phytoestrogens in the blood

Watanabe and colleagues (1998) reported that plasma concentrations of genistein and daidzein increased within two hours after ingestion of baked soybean powder and peaked after 6 hours. Other studies have reported peak plasma isoflavone concentrations at 5.6 hours (Bloedon *et al.*, 2002) genistein peaks at 5.5 hours and daidzein peaks at 7.4 hours (Setchell 2003). These data are not contradictory - since genistein is the dominant isoflavone in plasma, peak genistein concentrations rather than daidzein concentrations, will determine the t_{max} of total isoflavones.

Studies using isolated isoflavones have reported shorter times to reach peak plasma concentration, for example, 4 hours (Izumi *et al.*, 2000) and 3-5 hours (Busby *et al.*, 2002).

In a recent study (Kano *et al.*, 2006) orally administered isoflavone aglycones in soy milk produced peak concentrations in plasma after only one hour, compared with 6 hours for isoflavone glucosides. However, in one study of 16 female subjects in which ^{13}C-labelled genistein and daidzein were administered orally, peak plasma genistein concentrations were observed at 5.5 hours and peak plasma daidzein concentrations at 7.4 hours, resembling more the pattern of conjugated or food-state isoflavones. The serum half-lives of genistein and daidzein in this study were 7.77 hours and 7.75 hours, respectively. It is clear that the absorption kinetics of isoflavones vary according to the formulation used but for isoflavone glycosides peak plasma concentrations between 5 and 7 hours after ingestion appear reproducible.

Setchell *et al.* (2003a; 2003b) have reported evidence suggesting that isoflavone bioavailability may not increase linearly with increasing dose. Specifically, they report that percentage urinary recovery of daidzein at 0.4mg/kg was 34.5% and at

0.8mg/kg was 25.6% (Setchell *et al.*, 2003a). It must be stated that no conclusions regarding the non-linearity of relationship between dose and percentage recovery can be reached from only two dose groups, but the same authors also reported a non-linear relationship when plasma isoflavone bioavailability (as measured by AUC_{inf}) was plotted against three different doses of isoflavones from soy nuts (Setchell *et al.*, 2003b). Again, three points cannot prove a relationship but the data certainly warrant further investigation.

Genistein concentrations in plasma are consistently higher than daidzein concentrations (Adlercreutz *et al.*, 1993; Arai *et al.*, 2000; Djuric *et al.*, 2001; Franke *et al.*, 2002; Izumi *et al.*, 2000; Morton *et al.*, 2002; Morton *et al.*, 1994; Sanders, 2002). In urine, concentrations of daidzein are higher than those of genistein. Higher daidzein than genistein concentrations in urine correspond to more rapid clearance by the kidney and a larger volume of distribution for daidzein (Setchell *et al.* 2003a).

Isoflavones bind to plasma proteins with a lower affinity than do endogenous oestrogens, so they are more able to enter the cells. Nagel *et al.* (1998) demonstrated that isoflavones have a greater binding affinity to oestrogen receptors (relative to oestradiol) when applied to cell cultures in 100% serum compared to their application in a serum-free medium. This has profound implications for the interpretation of many *in vitro* studies, most of which use serum-free or 10% serum media for carcinogenetic studies in breast and prostate. In these media, the effective free fraction of isoflavones is reduced so their biological activity may be underestimated.

The half-life of plasma disappearance of isolated isoflavones is 7.9 hours after a single 50mg oral dose (Setchell, 1998). For isoflavones from baked soybean powder, the plasma half life was 5.79h for daidzein and 8.36h for genistein (Watanabe *et al.* 1998). Plasma appearance and disappearance is similar in pre- and post-menopausal women (Setchell 2000) and no clear age-related differences are reported (Setchell 1998).

Progressive accumulation of isoflavones in plasma during a four-week period of supplementation was reported by Izumi *et al.* (2000) but could not be supported by subsequent investigations (Ritchie *et al.*, 2004a; Ritchie *et al.*, 2004b; Setchell *et al.*, 2003a) so may have been an artefact of one study.

Plasma concentrations of isoflavones from populations consuming different habitual diets are presented in Table 3.

Plasma isoflavone concentrations are markedly higher in Japanese people than in Europeans and higher in vegetarians than in omnivores.

Table 3: Plasma concentrations of isoflavones from populations consuming different habitual diets

Authors	Subjects	Analytical Method	Genistein nmol/L	Daidzein nmol/L	Equol nmol/L	Enterolactone nmol/L
Morton *et al.* 2002	Japanese men	GC-MS	**492***	**282***	**99.1***	**32.6***
Morton *et al.* 2002	British men	GC-MS	**33.2***	**17.9***	**0.57***	**24.4***
Morton *et al.* 2002	Japanese women	GC-MS	**501.9***	**246.8***	**57.6***	**22.7***
Morton *et al.* 2002	British women	GC-MS	**27.7**	**12.5**	**2.2**	**18.7**
Adlercreutz *et al*. 1993	Japanese men	GC-MS	**276**	**107**	**ND**	**ND**
Arai *et al.* 2000	Japanese women	TR-FIA	**206.1****	**72.5****	**ND**	**ND**
Adlercreutz *et al.* 1993	Finnish women (omnivore)	GC-MS	**9.25**	**6.3**	**ND**	**ND**
Adlercreutz *et al.* 1993	Finnish women (vegetarian)	GC-MS	**50**	**59.2**	**ND**	**ND**
Heald *et al.* 2006	Scottish men	GC-MS	**33.79****	**18.00****	**0.67**$^{\$**}$	**ND**

ND = no data presented; * serum concentrations; ** median plasma concentrations; $ median of equol producers only

By comparison with circulating concentrations of oestradiol, serum concentrations of isoflavones are very high. Thus, although PE have 100 or 1000 times lesser binding affinity for oestrogen receptors, their presence in 10-10,000 times the concentration of oestradiol means that they are capable of exerting physiological influence via oestrogen receptor action. Appendix 3 illustrates this disparity between serum concentrations of PE and oestradiol.

Isoflavone excretion

Isoflavones are excreted in urine and minimally in faeces (Setchell *et al.* 2003a). Isoflavone metabolites identified in human urine include methylequol, daidzein, dihydrodaidzein, O-desmetholangolensin (O-DMA), genistein, 3'7-dihydroxyisoflavan and formononetin. In faeces, daidzein, genistein, equol and O-DMA have been measured (Adlercreutz & Mazur 1997). Other metabolites of genistein and daidzein remain to be identified, as evidenced by stable isotope-labelled studies (Setchell *et al*. 2003a). The percentage of the ingested dose excreted in urine varies between 9-13% of genistein and 30-38% of daidzein (Setchell *et al.* 2003a; Arai *et al.* 2000; Ritchie *et al.* 2004) and may decrease with increasing dose (Setchell *et al.* 2003b). The elimination half-life may be influenced by the food matrix, with isoflavones being more rapidly excreted when ingested in a liquid matrix than a solid matrix (Setchell 2000). Studies report elimination half-lives for genistein and daidzein (respectively) of 10.1 and 8.0 hours (Setchell *et al.* 2003b), 5.7 and 4.7 hours (King & Bursill, 1998) and 6.0 and 3.8 hours (Shelnutt *et al.*, 2000)

Table 4 shows 24-hour urinary excretion of PE in different populations. Urinary isoflavone excretion is highest in the Japanese and higher in vegetarians than in omnivores.

Table 4: Urinary phytoestrogen concentrations in different populations

Author	Subjects	Analytical Method	Descriptor	Genistein µmol/day	Daidzein µmol/day	Equol µmol/day	O-DMA µmol/day	Enterolactone µmol/day	Enterodiol µmol/day
Arai *et al*. 2000	106 Japanese women	HPLC-UV	Median	10.79	20.54	15.74	1.64	ND	ND
Adlercreutz *et al.* 1995	10 Japanese women	GC-MS	Mean	ND	2.5	0.5	ND	ND	ND
Adlercreutz *et al.* 1995	10 recent Oriental immigrants to Hawaii	GC-MS	Mean	ND	0.2	0.08	ND	ND	ND
Adlercreutz *et al.* 1982	10 omnivorous American women	GC-MS	Mean	ND	ND	0.14	ND	2.3	0.32
Adlercreutz *et al.* 1982	10 vegetarian American women	GC-MS	Mean	ND	ND	0.14	ND	3.18	0.21
Ingram *et al*. 1997	144 Australian women	GC-MS	Median	ND	0.91	0.11	ND	3.1	0.32
Adlercreutz *et al*. 1995	10 Finnish omnivores	GC-MS	Mean	ND	0.2	0.1	ND	ND	ND
Adlercreutz *et al.* 1995	10 Finnish vegetarians	GC-MS	Mean	ND	0.41	0.09	ND	ND	ND
Wiseman *et al.* 2004	76 British adults	LC-MS	Mean	1.0	2.5	0.5	0.4	ND	ND
Ritchie *et al.* 2004	14 Scottish adults	LC-MS	Mean	2.7	6.9	ND	ND	ND	ND

ND = no data presented

Isoflavones in tissues

The concentrations of isoflavones in tissues have yet to be adequately studied but the large volume of distribution identified in pharmacokinetic studies (Setchell *et al.* 2003a; Setchell 2000) indicates a wide tissue distribution. Tissue concentrations of PE will be a key area for future research because activities seen *in vitro* are concentration dependent and have, at best, been compared with the concentrations achievable in plasma rather than tissues.

Unpublished observations by Petrakis, Coward, Kirk and Barnes (1996) cited in Peterson *et al*. (1998) suggest that free genistein is either readily and selectively absorbed by breast tissues or deconjugated in the breast, because nipple aspirate fluid from women consuming soy contained large quantities of unconjugated genistein, moderate amounts of genistein-7-sulphate and little genistein β-glucuronide while their blood contained mostly genistein β-glucuronide.

Preliminary work by Maubach *et al*. (2003) showed a 10-fold predominance of equol in the breast tissue of one human subject relative to the concentrations of this metabolite in serum and urine after isoflavone ingestion.

In rats, the ratios of free to conjugated genistein vary from 1:9 in the testis and 1:4 in the female thyroid to 100% free genistein in the uterus and the male brain (Chang & Doerge, 2000). In rats with implanted accessory sex gland tumours, very high doses of genistein (50mg/kg bw administered subcutaneously) concentrated threefold less in the tumours than in the blood (Schleicher *et al.*, 1999).

Given that isoflavone aglycones are lipophilic, it is reasonable to question whether they may enter and accumulate in adipose cells. This has never been examined in humans but might have implications for the effects of isoflavones on organs such as the breast which have a large fat component and may act as a 'sump' for a variety of lipophilic chemicals which may then be locally available to exert effects on the breast epithelial cells. It is also unknown whether expression of intracellular oestrogen receptors concentrates PE intracellularly. Expression of oestrogen receptor β might be particularly important, given its higher affinity for PE.

Genistein and biochanin A have shown significantly different degrees of metabolic transformation between normal and malignant breast cells in culture which may explain the differential growth inhibition seen in these cells in response to these compounds. MCF-7 malignant breast cells form the 7-*O*-sulphate ester of genistein which is rapidly excreted from the cells. Normal breast epithelial cells in culture appear to maintain higher intracellular concentrations than malignant MCF-7 cells after application of the same initial isoflavone dose (Peterson *et al.*, 1996), which seems to support the findings of Schleicher *et al.* (1999).

STUDYING PHYTOESTROGENS IN HUMANS

Epidemiological studies have been limited by difficulties in quantifying PE intake. The use of dietary records to assess PE intake is always an approximation since the concentration of PE in plants varies according to the season, variety and presence or absence of plant pathogens. It is also difficult to estimate the percentage of soy in processed foods which have not previously been analysed for PE. Development and validation of databases of the PE content of foods is time-consuming and expensive. Biomarkers are therefore an attractive alternative to quantify the PE intake of individuals.

Some intervention studies (Maskarinec *et al.*, 2002; Maskarinec *et al.*, 2003) have measured urinary PE as a marker of compliance but have simply demonstrated a significant difference in mean urinary PE between the intervention and placebo phases. These two studies used a soy food intervention (average 12 servings of soy foods per week) and a supplement of 100mg/d isolated isoflavones, respectively. Without a means of translating this level of prescribed intake to expected excretion, individual compliance and the relationship between compliance and outcome (which is especially important in long-term studies and those which require a major dietary change) cannot be assessed.

The validity of 24-hour urine samples as biomarkers of isoflavone intake has been assessed for intakes up to 53mg/d total isoflavones while the validity of spot urine and plasma samples has been assessed for intakes up to 77mg/d.

Plasma and 24-hour urine samples have been validated as biomarkers against duplicate diet analysis ($r = 0.92$ and $r = 0.97$ respectively, $p<0.001$) (Ritchie *et al.* 2004). The mean total isoflavone intake in this study was 11mg/d.

Ritchie *et al.* (2004b) validated 24hour urine collections as a biomarker of intake in supplemented and unsupplemented diets ranging from 0-53mg/d. Significant correlation was found between total isoflavone intake and 24-hour urinary isoflavone excretion ($r = 0.89$; $p<0.001$).

The same group validated timed spot urine and plasma samples as biomarkers of intake using cereal bar supplements providing 28mg/d total isoflavones. Subjects consumed their usual diets and dietary isoflavone intake which was estimated from food diaries brought the range of intake to 0.05-77.3mg/d. The correlation of intake with timed spot urine sample isoflavone concentration was $r = 0.86$, and of intake with timed plasma sample concentrations was $r = 0.88$ ($p<0.001$ in both cases) (Ritchie *et al.* 2004a).

Huang *et al.* (2000) found no correlation ($r^2 = -0.05$, $p = 0.85$) between dietary and urinary daidzein in 18 Caucasian women with a mean isoflavone intake of 2.2mg/d.

Mascarinec *et al.* (1998) found a significant correlation ($r^2 = 0.62$, $p = 0.0001$) between dietary and urinary isoflavones in a multi-ethnic sample of Hawaiian women with isoflavone intakes between 5mg/d and 38mg/d.

Grace *et al.* (2004) investigated the relationships between dietary, urinary and serum PE including isoflavones. This study involved large numbers of women, used sensitive analytical methodology and assessed the relationship between biomarkers and the range of intakes commonly observed in the UK. However, dietary isoflavones were assessed using dietary records and a database of published values of the isoflavone concentration of foods. This is significantly less accurate than duplicate

diet analysis or the use of a food composition database validated for the purpose by duplicate diet analysis. This study did find significant, though weak, correlations between creatinine-adjusted spot urinary isoflavones and isoflavone intake ($r^2 = 0.27$; $p<0.001$) and between serum isoflavones and isoflavone intake ($r^2 = 0.31$; $p< 0.001$) for intakes up to 1.2mg/d.

Verkasalo *et al*. (2001) selected 80 participants on the basis of FFQ-assessed soy intake to obtain a range of intakes before comparing isoflavone intake from 7-day food diaries with plasma genistein and daidzein. Spearman correlation coefficients for diary-based estimates of intake were between 0.66 and 0.8, while for the FFQ-based estimates they were between 0.24 and 0.74. Isoflavone intakes in this study ranged from almost zero in the lowest soy intake group to 49mg/d (assessed by FFQ) or 33.7mg/d (assessed by 7d diary) in the highest soy group.

Takashima *et al*. (2004) compared genistein and daidzein content of hospital diets with serum isoflavones in 22 prostate cancer patients and 66 controls. Overall, dietary genistein correlated with serum genistein ($r = 0.27$) and dietary daidzein correlated with serum daidzein ($r = 0.33$) but correlations were stronger in non-equol producers ($r = 0.52$ for genistein; $r = 0.67$ for daidzein).

Using supplements of soy protein providing up to 36mg/d total isoflavones, Karr *et al*. (1997) found a highly linear relationship between soy intake and isoflavone concentration in 3 successive 24-hour urine collections but did not report correlation coefficients. After adjustment for individual subject effect, they found that soy dose accounted for 85% of the variability in daidzein excretion and 84% of the variability in genistein excretion.

A study published by Arai *et al.* (2000) reported correlations (r^2) between log plasma genistein and log urinary genistein of 0.499; between log plasma daidzein and log

urinary daidzein of 0.499; between log genistein intake and log urinary genistein of 0.346; between log daidzein intake and log urinary daidzein of 0.365; between log genistein intake and log plasma genistein of 0.429; and between log daidzein intake and log plasma daidzein of 0.335. This study used the authors' previously published tables of the isoflavone content of Japanese foods and dietary records to estimate isoflavone intake, which may explain why correlations with biomarkers were not strong. Plasma samples were also taken after an overnight fast, further weakening the association with isoflavone intake since the half-life of plasma disappearance of isoflavones is between 6 and 8 hours (Watanabe *et al.* 1998; Setchell 1998) and the last consumption of isoflavone-containing foods would most likely have been at the evening meal, perhaps 12 hours before blood sampling.

Atkinson *et al*. (2002) assessed the correlation between 24hour urine collections and intake of soy foods assessed by food frequency questionnaire (FFQ) and dietary records. However, as this study was carried out in an American population and other authors have found the greatest contribution to isoflavone intake in Western populations comes not from soy foods but from soy additives in other foods such as bread and processed meats (Ritchie *et al.* 2005a; Clarke & Lloyd 2004; Horn-Ross *et al.* 2000), it is likely that their estimates of intake were insufficiently accurate to validate a biomarker. A similar study conducted in a Singapore Chinese population (Seow *et al.*, 1998) found no significant correlation between the frequency of soy food consumption and isoflavone concentration in spot urine samples.

Other studies (Bhakta *et al.*, 2005; Frankenfeld *et al.*, 2003; Frankenfeld *et al.*, 2002; Heald *et al.*, 2006; Yamamoto *et al.*, 2001) have assessed the correlation between biomarkers and soy or isoflavone intake as assessed by FFQ but this is generally to

validate the FFQ rather than the biomarker. FFQ is an insufficiently accurate means of assessing dietary intake to be employed in validating biomarkers. Duplicate diet analysis or supplementation that is simple for participants to comply with are more appropriate methods to measure this variable.

In general, a stronger correlation between intake and biomarkers has been observed when the range of isoflavone intakes is greater. This is to be expected, and indicates that a study aiming to validate a biomarker should assess the relationship between intake and excretion over a wide range of intakes to ensure the best-fitting model for relating the two variables.

Many studies have examined the effect of supplementary intakes far in excess of those for which biomarkers have been validated (for example, 90mg/d (Bruce *et al.*, 2003); 114mg/d (Teede *et al.*, 2004); 132mg/d (Xu *et al.*, 2000); 83mg/d (Adams *et al.*, 2004); 90mg/d (Anderson *et al.*, 2002); 96mg/d (Gallagher *et al.*, 2004); 5mg/kg bw (Hutchins *et al.*, 2005); 100mg/d (Maskarinec *et al.*, 2002); 100mg/d (Mori *et al.*, 2004)). It is not yet known whether or not these higher intakes result in proportionally greater availability of isoflavones to the target tissues.

Summary

Isoflavones are ordinarily present in human biological fluids - their concentration dependent on dietary intake - and exhibit a wide range of biological activity. The pharmacological properties of these compounds, investigated in *in vitro* and animal studies, coupled with the associations between isoflavone intake and health variables in human populations, warrants further exploration into clinical and dietetic applications. From the presently available data it is not possible to conclude whether a) a diet high in isoflavones, or b) supplements of isoflavones can be recommended for the treatment or prevention of disease. In order to draw such conclusions, research in humans is required.

Such research must include biological measures of isoflavone exposure because estimation of dietary isoflavones in free-living studies is extremely difficult. Intervention studies should include biological markers of compliance, particularly if the intervention requires a major dietary change or adherence to the protocol for a prolonged period of time.

Before conclusions can be drawn regarding the efficacy or safety of large doses of isoflavones and before the results of *in vitro* studies using high concentrations of isoflavones can be extrapolated to humans, it is important to determine the oral bioavailability of these compounds at higher doses.

Aims

The primary aim of this study was to validate a biomarker suitable for application in observational studies and intervention studies as a reliable measure of exposure to dietary isoflavones.

A secondary aim was to determine whether plasma isoflavone concentrations or urinary isoflavone concentrations during isoflavone supplementation up to 165mg/d will reach a maximum beyond which they will not increase with increasing supplement dose. This will be referred to as a plasma concentration plateau or a urinary concentration plateau.

METHODS

In order to investigate the relationship between isoflavone intake and isoflavone concentration in 24-hour urine collections, timed plasma samples and timed spot urine samples, a cross-over study was carried out in which each participant received three different doses of isoflavones between 55 and 165mg total isoflavones per day. 24-hour urine collections, timed plasma samples and timed spot urine samples were taken at baseline and during each supplement phase for isoflavone analysis by liquid chromatography and mass spectrometry (LC-MS). Correlations between the biomarkers and between each biomarker and intake were assessed.

Study Design

Healthy volunteers were recruited by poster advertisement to a randomised, double-blind, placebo-controlled, cross-over study. Each participant took oral supplements of soy-derived isoflavones providing 48mg genistin, 5mg glycitein and 41mg daidzin. 48mg genistin is equivalent to 29.996mg genistein and 41mg daidzin is equivalent to 25.017mg daidzein. Thus the total genistein plus daidzein content of one tablet was 55mg. Full details of the isoflavone tablets and placebo tablet used are given in Appendix 4.

A diagram of the study protocol is shown in Figure 1.

Ethical approval was granted by Fife NHS Research Ethics Committee and by the Bute Medical School Ethics Committee.

Figure 1: Study Protocol

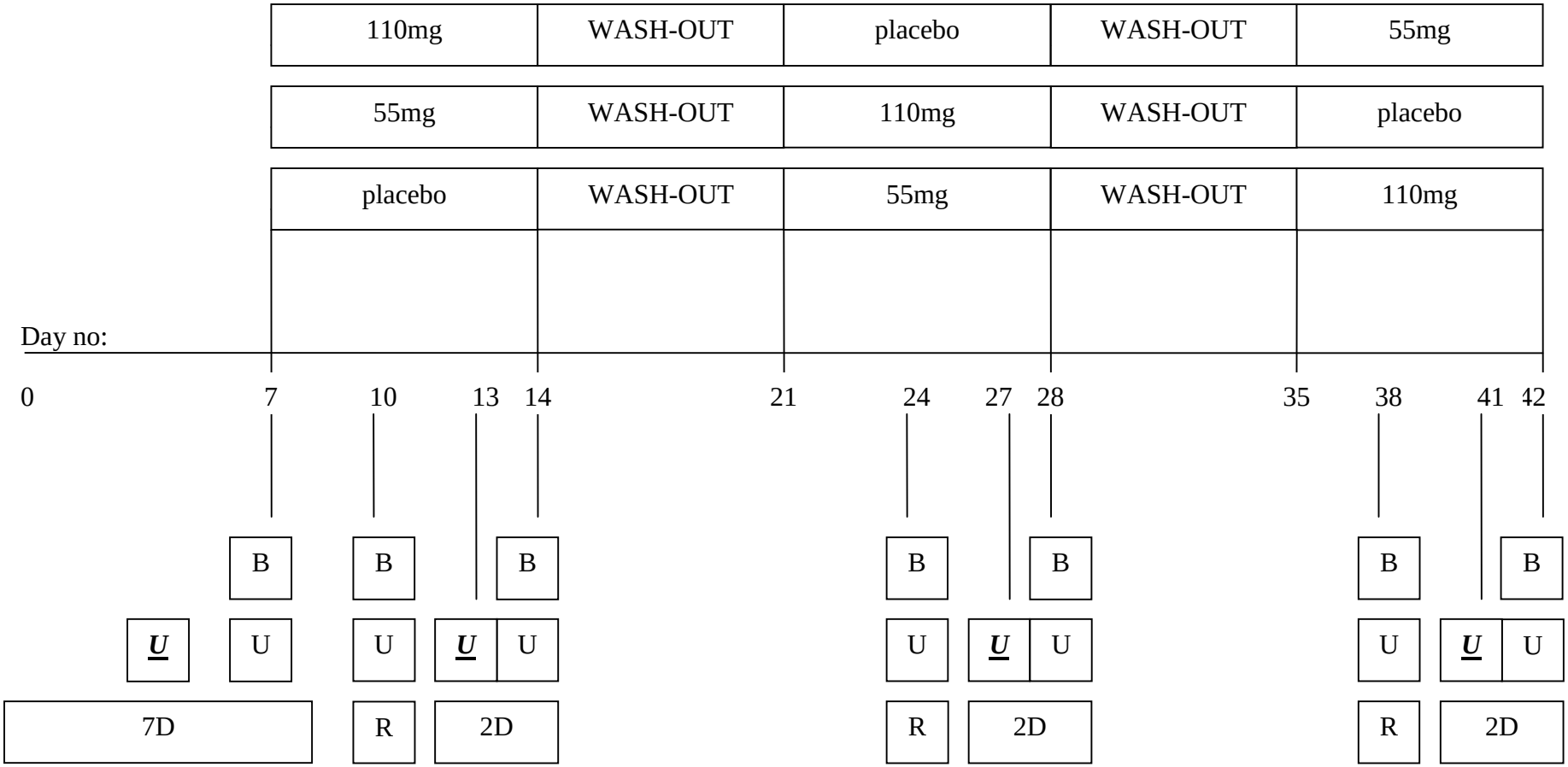
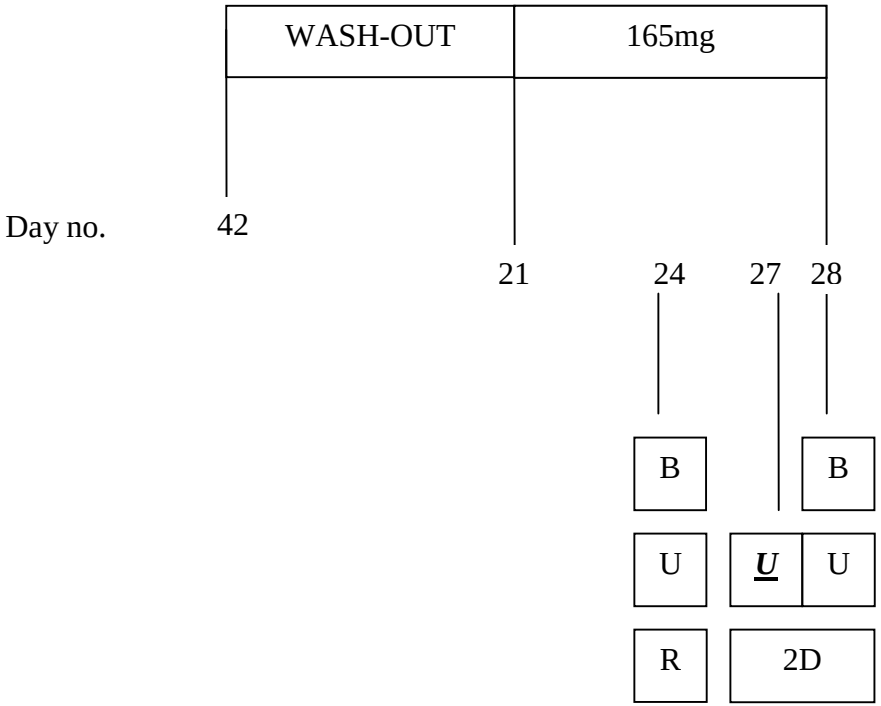


Figure 1 continued:
Optional Open Phase:



B = timed blood sample
U = timed urine sample
U = 24-hour urine sample
R = 24-hour dietary recall
7D = 7-day food diary
2D = 2-day food diary

Each volunteer was screened prior to recruitment to ensure that they met the inclusion and exclusion criteria, which were as follows:

Inclusion Criteria	**Exclusion Criteria**
In good general health	Antibiotic use within 6 weeks prior to study commencement
Aged between 18 and 65 years	Concurrent pregnancy
	Concurrent participation in another research trial
	Any systemic illness affecting nutritional status

Suitable volunteers were provided with an information sheet and invited to attend an initial visit before giving written consent to participate. All volunteers were weighed at the beginning and end of the study and their height measured. This was to ensure that normal dietary intake was maintained during the study and for the purposes of calculating basal metabolic rate (BMR) and body mass index (BMI).

Each volunteer completed three intervention phases, each one week long, in which they received either 55mg isoflavones, 110mg isoflavones or placebo. The order of the intervention phases was randomised and the phases were separated by wash-out phases of one week duration. Participants who wished to continue with the study entered an optional open phase after a further week of wash-out at the end of the double blind phase. In this phase participants received 165mg of isoflavones per day and attended study visits as before. Dietary intake of isoflavones was assessed for the 24-hours preceding each sample and estimated dietary intake added to supplementary intake for each participant.

Timed blood samples, timed spot urine samples, 24-hour urine samples and 7-day dietary records were obtained prior to supplementation to establish background dietary intake and baseline levels of PE in blood and urine.

Timed blood samples and timed spot urine samples were obtained at two study visits during each intervention phase. A 24-hour dietary recall was obtained for the period up to the first timed samples. Participants provided a 24-hour urine collection between the two timed samples and a two-day dietary record over the period in which they were collecting urine and providing the second timed blood and urine samples of each phase. Spot urine samples were taken within half an hour of blood sampling. Blood samples were drawn into heparinised vacutainers and kept upright at 4^{o}C overnight to settle before separation of plasma by aspiration using a disposable pipette. Blood and urine samples were stored at -20^{o}C until they were required for analysis. Holder *et al*. (1999) have analysed isoflavone the content of plasma by LC-MS before and after two freeze-thaw cycles and demonstrated a lack of effect on plasma isoflavone concentrations of freezing and thawing.

The recruitment poster; pre-enrolment telephone interview schedule; participant information sheet; consent form; letter to general practitioners; dietary recording instructions and 24-hour urine collection instructions are given in Appendices 5 to 11.

Dietary analysis

Dietary records were analysed using the software program Microdiet®, into which were imported values for the PE content of foods from the database validated and published by Ritchie (2003a). Portion sizes were converted to average weights using a Ministry of Agriculture, Fisheries and Food (MAFF) booklet (Nelson *et al.*, 1997). Microdiet provided a measure of estimated PE intake and energy intake (kcal). Accuracy of food recording was checked by comparing EI (energy intake) and BMR (basal metabolic rate). Accuracy was assumed if the EI: BMR was greater than 1.2, indicating that reported energy intake was sufficient to maintain health. BMR was calculated using the Schofield equations published by the Department of Health (1991).

24-hour urine collections

Participants were provided with two 2 litre containers containing 1g/l boric acid (as a preservative), a plastic measuring jug, three 80mg PABA tablets, and a urine collection record sheet. PABA recovery was used to measure completeness of collections. Participants were instructed to discard the first urine that they passed on the day of the collection but to record the time at which they did this. They were instructed to collect every drop of urine passed until the same time the next morning. PABA tablets were to be taken at approximately breakfast, lunch and dinner times, although the last tablet had to be taken by 8pm. (Bingham & Cummings, 1982).

The total volume of urine passed was recorded. The entire collection was then thoroughly shaken to ensure complete mixing and aliquoted into three labelled universal containers and stored at -20^{o}C. The remaining urine was discarded.

Validation of urine collections using para-aminobenzoic acid

240mg/day para-aminobenzoic acid (PABA) was administered orally to subjects while they were collecting 24-hour urine samples and its recovery in urine was measured by spectrophotometry using the method of Bingham and Cummings (1982) to assess the completeness of urine collections.

Sample preparation and methods

Standard solutions of PABA (Fluka) were prepared by dissolving 240mg PABA in one litre of double-distilled water in a volumetric flask with the addition of six sodium hydroxide pellets. Standard solutions of 180mg/l, 120mg/l and 60mg/l PABA were prepared by dilution from the original stock solution and subsequently stored in glass Duran bottles at 4^{o}C.

All urine samples were prepared in triplicate. 100µl aliquots were pipetted into glass stoppered boiling tubes and 5ml 2M NaOH added to each tube. 100µl aliquots of each standard solution were also prepared in triplicate with the addition of 5ml NaOH and were kept at room temperature for two hours while the urine samples were heated in a water bath at 100^{o}C. Heatproof silicone grease was used to lubricate the ground-glass stoppers of the sample tubes to prevent sticking after heating, and stoppers were removed while tubes were warm. After cooling, the following reagents were added to each tube, in sequence, vortexing after the addition of each reagent: 3ml 5M HCl; 0.5ml 0.2% (w/v) sodium nitrite; 0.5ml 1% (w/v) ammonium sulphamate; 0.5ml 0.2% (w/v) naphthylenediamine dihydrochloride. Samples were then allowed to stand for 45 minutes to allow colour development. Absorbance of each sample at 540nm was measured (in triplicate) using a Camspec double beam spectrophotometer.

A standard curve was plotted from the average absorbance of each of the PABA standard samples. The equation of the line of the standard graph was used to calculate the PABA concentration in each urine sample from the average absorbance of the three tubes. PABA recovery in mg/litre was multiplied by 24-hour urine volume, giving recovery in mg/24h which was then expressed as a percentage of the original 240mg ingested. Urine collections with PABA recovery greater than 85% were deemed sufficiently complete for further analysis.

Analytical Methodology

Background

Separation and analysis of phytoestrogenic compounds followed the discovery of oestrogenic activity in food and plant extracts. With the introduction of increasingly sensitive technologies phytoestrogen analysis has advanced considerably. However, the isolation and quantification of phytoestrogens is considered particularly difficult even with today's technology, due to the similarity of their structures and chemical properties and the structural and chemical similarity of PE to other compounds in the complex biological matrices in which they occur. (COT, 2003b)

The predominant analytical methods in current use are: gas chromatography with mass spectrometric detection (GC-MS); liquid chromatography with mass spectrometric detection (LC-MS); high-performance liquid chromatography with ultraviolet detection (HPLC-UV) and fluorescent- or radio-immunoassays (FIA or RIA).

GC-MS is sufficiently sensitive to measure concentrations of PE of less than parts per million (COT 2003b & refs. therein), the exact limits of sensitivity depending on the laboratory. However, PE require transformation to trimethylsilyl derivatives to attain sufficient volatility for gas chromatographic analysis. This adds considerably to the time and expense of sample preparation.

LC-MS allows equal sensitivity of detection but without the necessity for derivatisation prior to analysis. Thus, LC-MS has the potential to be used for non-destructive analysis of PE glycosides and glucuronide/sulphate conjugates but this is

not yet commonplace due to the expense of obtaining suitable internal standards (COT 2003b).

The great advantage of GC-MS and LC-MS technologies is that they enable the use of isotopically labelled internal standards, chemically identical to the PE of interest but distinguishable by MS. This allows definitive identification of the compound of interest in the chromatogram and permits quantification of PE relative to a known amount of the isotopically labelled internal standard, thus correcting for losses in sample preparation. ^{13}C labelled compounds are the standards of choice, being considerably more stable during analysis than the alternative deuterium (^{2}H) labels. (COT 2003b)

HPLC-UV allows rapid, simultaneous purification and quantification of complex mixtures but with a higher and more variable limit of detection than mass spectrometric techniques. UV detection does not permit the use of isotopically labelled internal standards, so quantification is based on comparisons with calibration curves derived from reference standards of the analytes. This does not correct for losses during sample preparation and may also lead to inaccurate measurements of the analyte due to co-elution in the sample with substances not present in the reference standard (COT 2003b).

Immunoassays employ antibodies raised against PE aglycones and quantification is achieved by using radiolabelled or fluorescently labelled PE in competitive assays. This is cheaper, faster and less time-consuming than GC-MS or LC-MS but is less specific. The antibodies raised against daidzein and genistein may cross-react with

other PE or with other similar compounds such as steroids in biological samples. (COT 2003b)

LC-MS was chosen for this study because of its high sensitivity and specificity, the relative ease of sample preparation by comparison with GC-MS and the availability of internal standards.

Prior to quantification, PE must first be extracted from the matrix (blood or urine samples in this study). Because this process is not 100% efficient, the internal standard is added to the sample prior to extraction to enable determination of the relative concentration of PE analyte to a known concentration of internal standard.

In urine and blood, PE are present predominantly as conjugates, bound to glucuronide or sulphate residues (Hendrich, 2002). The proportion of free PE is very low and very difficult to detect with the required degree of accuracy, so enzymatic hydrolysis is used to cleave PE from their conjugating groups and enable quantification of the 'total PE' content of the sample. When investigating relationships between intake and blood or urine concentrations of PE it is necessary to use total PE measurements because it is not known whether the proportion of free to bound PE alters with increasing dose. Measurement of PE glucuronides and sulphates is now possible with LCMS technology but requires labelled PE glucuronide/sulphate internal standards which are not yet readily available. As this study was intended to develop a biomarker that would be widely applicable in clinical and epidemiological studies, it was decided to measure total PE concentration, employing mass-labelled daidzein and

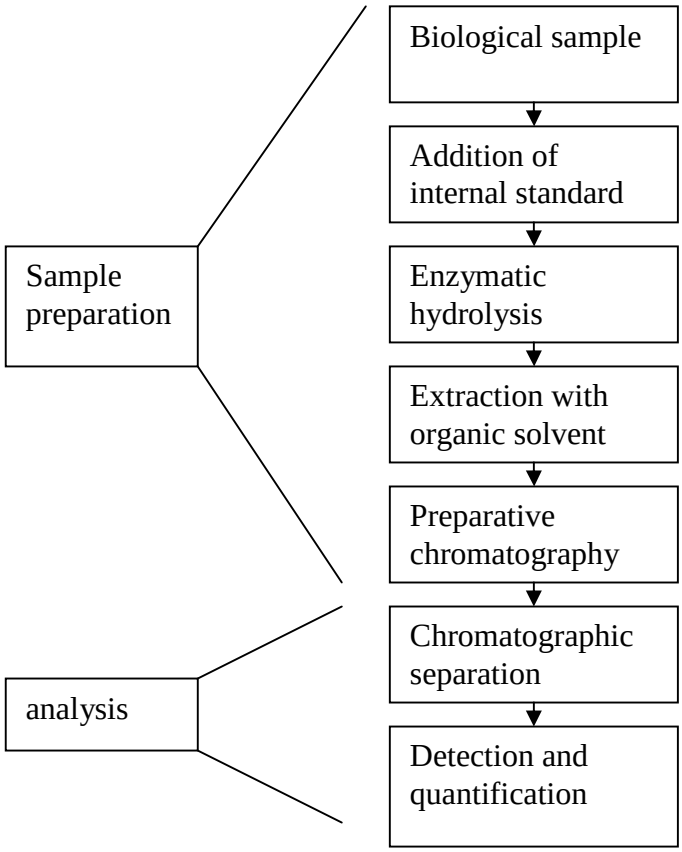
genistein in the unconjugated forms as internal standards so that the method can be more easily replicated in other laboratories.

After hydrolysis and extraction, PE are separated from chemically similar constituents of the biological matrix (such as steroids in the case of urine and blood samples) in a process referred to as sample 'clean-up' or preparative chromatography. The narrower spectrum chemical extract which results from this process is then separated and its components quantified. Sample clean-up eliminates the majority of contaminants which might foul the analytical column, impairing its capacity to separate PE with consistent retention times and discrete peaks, and eliminates some contaminants with the same mass as a compound of interest which would create an artefactual peak in the mass chromatogram.

Some laboratories have employed ready-made solid-phase extraction cartridges to simplify sample clean-up but in this study, less-expensive glass columns packed with LH-20 lipophilic sephadex were used.

The sequence of PE extraction and analysis is depicted in Figure 2.

Figure 2: Sequence of PE extraction and analysis



Sample preparation

Chemicals and reagents:

Methanol, HPLC grade

Chloroform, HPLC grade

Heptane, HPLC grade

Ethyl acetate, Analar grade

Sephadex LH-20 bead size 25-100μ

B-glucuronidase with sulphatase from *Helix pomatia* type H-3AF; 127,000 units/ml (one unit will liberate 1μg phenolpthalien from phenolpthalien glucuronide per hour at 37^{o}C and pH 5.0)

Sodium acetate buffer 0.1M acidified to pH 5.0 using glacial acetic acid.

All reagents were purchased from Sigma unless otherwise stated.

Analytes:

Daidzein (7,4'-dihydroxyisoflavone), genistein (5,7,4'-trihydroxyisoflavone), equol (7,4'dihydroxyisoflavane) and triply ^{13}C-labelled internal standards of genistein and daidzein were a gift from Dr M. Morton, Cardiff University, Wales.

A working dilution of PE analytes was prepared in methanol in a volumetric flask to give a concentration of 250ng/ml. This was used as an external standard in preparation of the calibration curves.

Similarly, a methanolic solution of ^{13}C PE was prepared to a concentration of 250ng/50μL. 50μL of this solution was added to each sample and to each calibration standard. Methanolic stock solutions were stored in a refrigerator at 4^{o}C.

All glassware was silanised prior to use according to the method of Pumford *et al.* (2002). Briefly, glassware was soaked in a solution of 3% dichlorodimethylsilane in toluene for five minutes then rinsed once with toluene, twice with methanol and air dried in a fume cupboard.

Hydrolysis and Extraction

Urine and plasma samples were prepared according to the method of Pumford *et al.* (2002) and Morton *et al.* (2002) but omitting the derivatisation step.

Plasma and urine samples were defrosted fully and shaken before aliquotting. Aliquot volume taken was adjusted according to supplementation phase and sample matrix to ensure that the final concentration of PE in the prepared sample would fall within the range which produces a reliable, linear response from the analytical method. For all blood samples, aliquots of 500μL were used. For baseline and placebo urine samples, 250μL aliquots, for urine samples during 55mg/d supplementation 50μL aliquots, for urine samples during 110mg/d supplementation 40μL aliquots and for urine samples during 165mg/d supplementation 25μL aliquots were taken. If, after LC-MS analysis, the concentration of PE was found to be outside the calibrated linear range then sample preparation was repeated with a smaller volume of urine or blood.

Aliquots of plasma or urine were pipetted into labelled, ground-glass stoppered boiling tubes (Quickfit NS19/26) and the weight of the aliquot recorded. β-glucuronidase/sulphatase was mixed with 5ml sodium acetate buffer in a test tube and divided among the samples allowing 1000units phenolpthalien-liberating activity per sample. A further 3ml sodium acetate buffer was added to each sample.

50µL of the internal standard cocktail was added to each sample using a 50µL glass syringe. Internal standard solution was allowed to equilibrate to room temperature for at least one hour before use. Samples were then vortexed and incubated in a water bath at 37^{o}C for 16 hours. After hydrolysis, samples were cooled and PE extracted by adding 4ml ethyl acetate to each tube. Samples were shaken for 1 minute and placed in a salt-ice bath to freeze the aqueous layer before decanting the organic layer into labelled test tubes. A further 4ml ethyl acetate was added to each sample and the extraction repeated, adding the second aliquot of ethyl acetate to the first. The test tubes were then evaporated to dryness in a Techne dri-block at 60^{o}C under a gentle stream of nitrogen.

Preparative Chromatography

Columns were prepared by cutting the tips from glass Pasteur pipettes to widen the opening and rounding the sharp edges in a Bunsen flame. Columns were then silanised following the procedure above, marked at 3.5cm from the neck and plugged with silanised glass wool. Supported in a rack and draining into waste-collecting tubes, the columns were packed with LH-20 lipophilic Sephadex to the 3.5cm mark. 350mg Sephadex per sample was required and the total quantity of Sephadex was soaked for approximately 15 minutes in of 50ml solvent mixture (10 parts chloroform, 10 parts heptane, 1 part methanol) to form a gel.

The dry extract from each sample was dissolved in 200µL of the same solvent mixture with vortexing, and transferred to the top of each correspondingly numbered column. A further 200µL solvent mixture was added and the washings again transferred to the top of the column. Samples were then washed onto the column with 3x 1ml plus 1x 0.6ml aliquots of solvent mixture, running each addition down the side of the glass to

avoid disturbing the surface of the Sephadex. The solvent mixture was discarded and PE eluted from the columns into clean, numbered, silanised test tubes with 4x 1ml aliquots of methanol. Methanol was evaporated from the samples under nitrogen at 60^{o}C and the samples re-dissolved in 80µL 50% v/v methanol/water before being transferred to silanised inserts in autosampler vials for LC-MS.

Preparation of Calibration Curve

Calibration standards were prepared using the methanolic stock solutions of labelled and unlabelled phytoestrogen standards as described previously. A different volume of unlabelled analyte solution was added to each vial with a constant volume of internal standard solution (volumes and the corresponding weights and concentrations of standard solutions are given in Table 5). The methanolic solutions in the vials were blown to dryness under nitrogen at 60^{o}C and the solid residue re-dissolved in 80µL of 50% v/v methanol/water and transferred to silanised inserts in the same manner as the prepared samples. A set of calibration standards was analysed with each batch of samples.

Table 5: Calibration Standards

Volume of internal standard (µL)	Mass of internal standard (ng)	Concentration of internal standard (ng/ml)	Volume of unlabelled analyte (µL)	Mass of unlabelled analyte (ng)	Concentration of unlabelled analyte (ng/ml)
50	250	3125	0	0	0
50	250	3125	200	50	625
50	250	3125	400	100	1250
50	250	3125	800	200	2500
50	250	3125	1000	250	3125
50	250	3125	1200	300	3750
50	250	3125	2000	500	6250
50	250	3125	4000	1000	12500
50	250	3125	8000	2000	25000

Liquid Chromatography

5µL injections of each sample were taken by an autosampler (Waters 2795 Separations Module) and separated on a reversed-phase, polar end-capped C18 column (Phenomenex Synergi HydroRP) preceded by two polar end-capped C18 guard cartridges (Phenomenex). The autosampler, LC and MS were controlled by MassLynx™ software (MassLynx Version 4.0 © Micromass Ltd 2002).

A flow rate of 0.22ml/min was used and the mobile phases were:

A. Water with 30ml 40mM ammonium acetate

B. 90% methanol, 10% water with 30ml 40mM ammonium acetate

Run time for each sample was 26 minutes with a gradient of 100% A to 100% B over 10 minutes followed by isocratic elution of analytes at 100% B for 10 minutes. Mobile phase was returned to 100% A over 0.5 min and the column re-equilibrated at 100% A for 5.5 min before injecting the next sample. The retention time of daidzein was 11.50-12.12 minutes and of genistein 12.10-13.02 minutes. Retention time was consistent within each batch of samples but varied slightly from day to day.

Before sample analysis the column was equilibrated using 100% A at 0.22ml/min for 30min to remove the storage solvent and establish operating conditions. Each run was started with a blank (injection of 5µL methanol) followed by reference standards to confirm correct operating conditions. A set of calibration standards (prepared as described above) were analysed with each batch of samples to verify the relationship between the absolute concentration of analyte and the response of unlabelled analyte

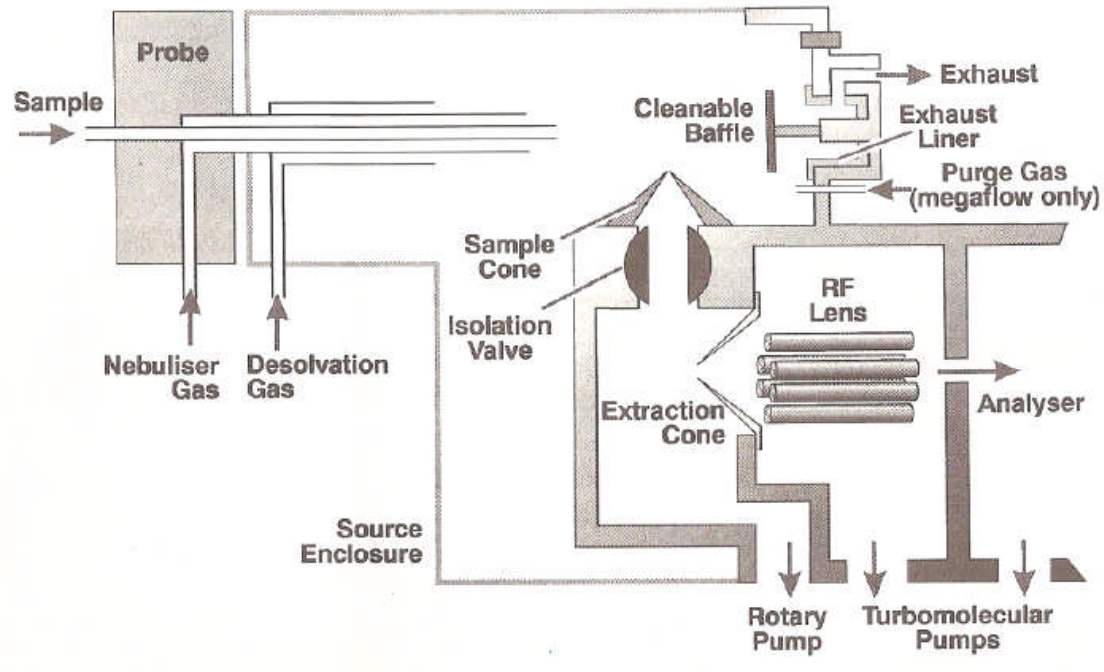
relative to internal standard. Calibration standards were interspersed throughout the sample list to confirm consistent performance throughout the run. Every third or fourth injection was a blank, used to ensure that peaks measured in samples were not artefacts or contaminants and also to remove strongly retained compounds which were not entirely eluted during the 10min isocratic gradient, preventing fouling of the column. After each batch of samples the column and guard cartridges were flushed with 5 column volumes of 100% water to remove buffer, then the column was flushed with 98% methanol/water (without buffer) at 0.2ml/min for 40-60 minutes to remove strongly retained contaminants. Guard cartridges were visually inspected after each run, replaced if soiled and if apparently clean, flushed with 98% methanol/water at 0.2ml/min for 10 minutes. Both column and guard cartridges were then washed with 5 column volumes 65% acetonitrile/ 35% water (storage solvent) and capped to prevent dessication during storage.

Mass Spectrometry

A Micromass LCT orthogonal acceleration time-of-flight mass spectrometer was employed for detection, identification and quantification of compounds.
The column effluent was introduced into the electrospray ionisation (ESI) interface where nitrogen gas was used to desolvate ions at 300^{o}C. In ESI, an aerosol of charged droplets is produced by applying a strong electrical charge to the eluent as it emerges from the nebuliser. As the solvent evaporates, these charged droplets reduce in size until they are sufficiently charge-dense to eject sample ions from the surface of the droplet (the Rayleigh limit). At this point a "Coulombic explosion" occurs and the droplet is ripped apart. This produces smaller droplets that can repeat the process as

well as naked charged analyte molecules. Charged analyte molecules (they are not, strictly speaking, ions) are then drawn through the sample cone aperture into the ion block before being extracted into the analyser. ESI was employed because it permits the analysis of intact molecules at high accuracy. It is a 'soft' ionisation method since the sample is ionised by the removal of a proton, with very little extra energy remaining to cause fragmentation of the sample ions, and is well-suited to the analysis of polar molecules ranging from less than 100 Da to more than 1,000,000 Da in molecular mass (Ashcroft, 2006). Figure 3 is a schematic of the ESI interface.

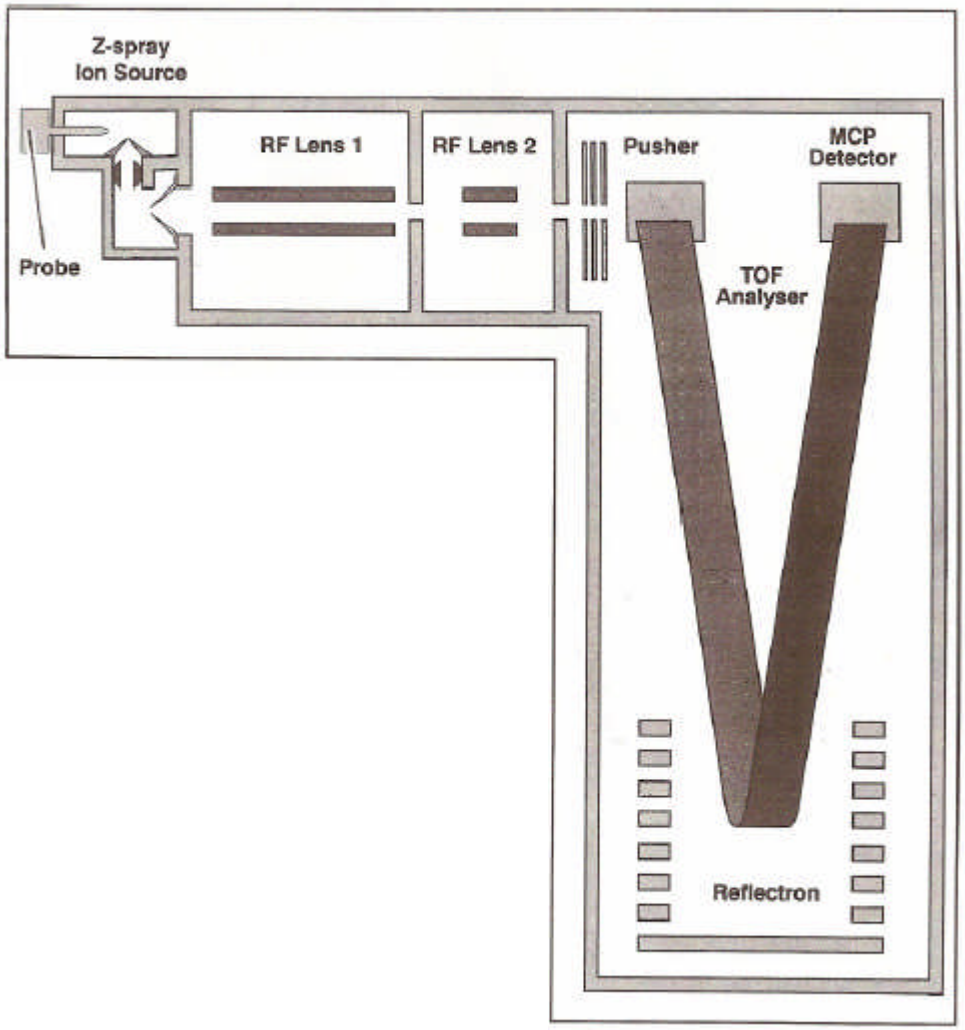
Figure 3: Electrospray ionisation interface



Ions generated in the Z-spray source are transferred to the orthogonal time-of-flight analyser via the two RF lenses. Ions are then focused onto the pusher by the acceleration, focus, steer and tube lenses. The pusher pulses a section of the beam towards the reflectron, which then reflects ions back to the detector. Ions with the

highest mass to charge ratio (m/z) have a longer flight time between the pusher and detector, so ions are separated in mass. TOF MS performs parallel detection of all masses within the spectrum simultaneously at very high sensitivity and acquisition rates, allowing each spectrum to be representative of the eluent composition at that point in time even with high chromatographic flow rates. Figure 4 shows a schematic of the ion optical system.

Figure 4: Ion optics



The TOF MS was operated in negative ion mode, with capillary voltage 2900V, extraction cone voltage 3V to minimise ion fragmentation with this low molecular weight sample, and sample cone voltage 25V - a value towards the lower end of the operating range since small molecules were being analysed (larger cone voltages increase ion fragmentation within the source). Source temperature was 100^{o}C and the RF lens was set to 250, since standard appropriate settings are 300 for compounds 200-500Da and 200 for compounds <200Da.

Compounds were quantified using a specific quantify method file created in MassLynx. Mass-to-charge ratios and retention times for the compounds of interest were specified and internal standards identified in the quantify method file. For unlabelled analytes, retention time was set equal to that of the relevant internal standard.

Chromatograms were smoothed by the Savitzky-Golay method, using a smoothing window size of 5 (selected by dragging across a peak at half height) and a magnitude of 2 smooths.

Peak detection and integration parameters were left at default values and each peak manually integrated after running the automated quantify method. Responses were based on peak area and a report of the peak areas for each compound in each sample was saved and printed after manual integration.

Analysis of data

The relative response ratio for each analyte was determined by;

Relative response ratio (RRR) = analyte peak area ÷ internal standard peak area

Calibration standards were constructed in MS Excel by plotting RRR for each analyte against the known mass of that analyte present in the standard vial. Linear regression was performed on these points and the equation of the line used to interpolate samples according to their RRR. Each batch of samples was quantified according to the linear regression of the calibration standards analysed on the same day, so each batch was calculated by a different equation of the form:

Genistein concentration in sample (ng/g) = $(mRRR + c) \div a$

Daidzein concentration in sample (ng/g) = $(m_1RRR_1 + c_1) \div a$

Where m = gradient of linear regression through genistein calibration points

m_1 = gradient of linear regression through daidzein calibration points

RRR = response ratio of genistein relative to $^{13}C_3$-genistein

RRR_1 = response ratio of daidzein relative to $^{13}C_3$-daidzein

c = intercept of genistein linear regression with y-axis

c_1 = intercept of daidzein linear regression with y-axis

a = weight of aliquot of urine/blood sample in grams

For 24-hour urine samples, the concentration of each isoflavone was multiplied by the total collection volume in millilitres to give the isoflavone excretion in ng/24hours.

Statistical analysis was performed using Microsoft Excel® and the results are presented in Chapter 4.

A number of procedures were employed to ensure the validity and repeatability of the results obtained by the methods outlined above. These procedures are described in Chapter 3.

ACCURACY, VALIDITY AND REPEATABILITY

Conducting the study

A number of measures were employed to ensure that data obtained during the study was accurate. At each visit, participants were asked whether they had remembered to take all of the study supplements at the correct time. Urine collections were validated by PABA recovery to ensure that incomplete urine collections were not taken to be reliable samples for isoflavone analysis. The 17 samples which were deemed to be invalid collections are shown in Appendix 13.

All data which were entered into electronic files during the study were later checked.

Dietary analysis:

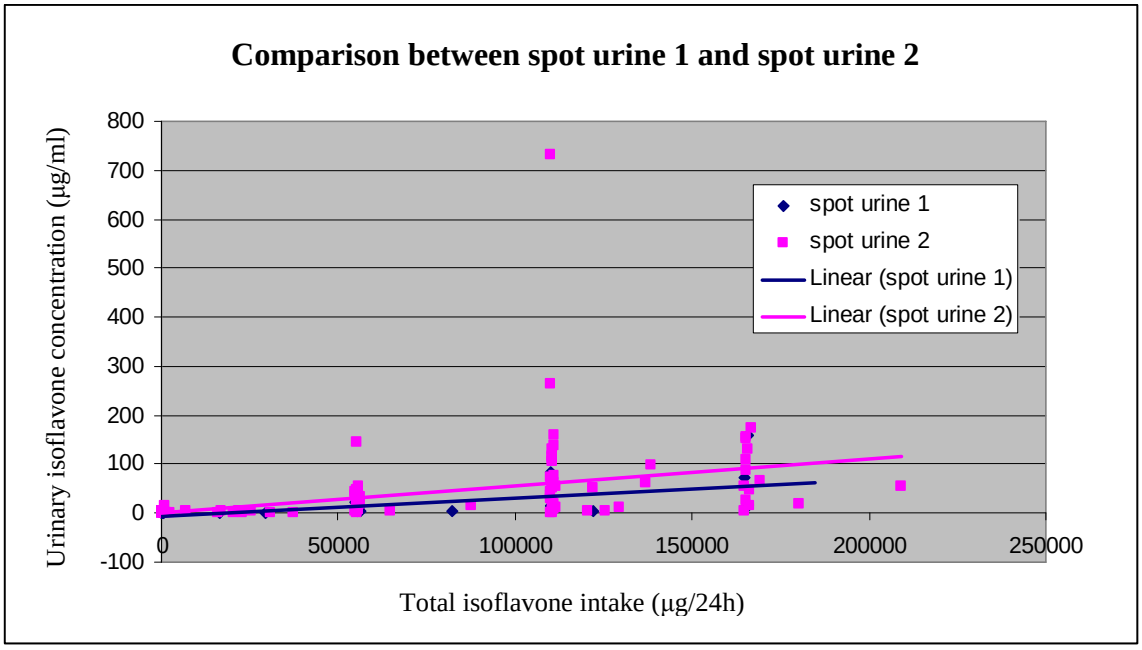
Dietary records were verified with volunteers to ensure that no meal had been omitted from the record and that portion sizes were recorded. To assist the volunteers with accurate recording, full instructions for recording portion sizes were included in every food diary. Daily caloric intake was compared with basal energy requirements and participants weighed at the beginning and end of the study to give an indication of whether normal, sufficient dietary intake had been maintained.

The database used to assign isoflavone values to foods has been validated for this purpose in a previous study using duplicate diet analysis (Ritchie *et al.*, 2005).

During each supplement phase, each participant provided two spot urine samples and two blood samples, one pair of samples after taking the supplement for three days (blood 1 and urine 1) and one pair of samples after taking the supplement for seven

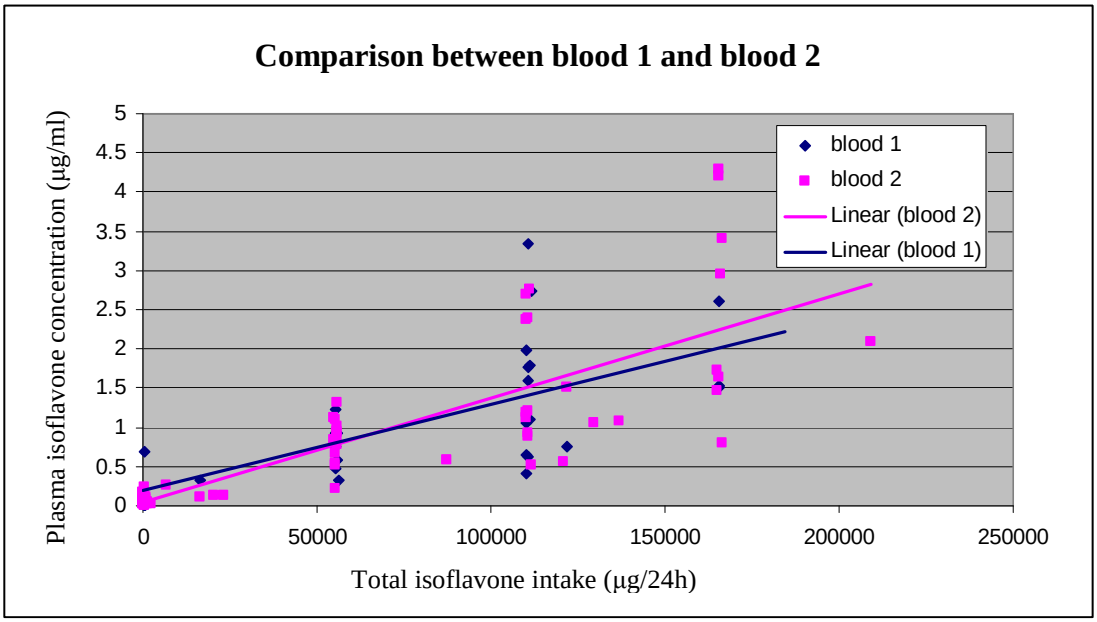
days (blood 2 and urine 2). Blood and urine 1 corresponded to a 24-hour recall of dietary intake while blood and urine 2 were taken during the two-day food diary period. Due to the expense of analysing the urine samples, only some of the urine 1 samples were analysed. Urine 2 during each phase was analysed for every participant. Eighteen of the urine 1 samples were compared with urine 2 samples for the same participant using a paired t-test. No significant difference was found between spot urine isoflavone concentration after taking the supplement for three days and urine concentration after taking the supplement for seven days ($p = 0.1995$). All of the spot urine 1 samples are compared with all of the spot urine 2 samples in Figure 5.

Figure 5:



It was not possible to obtain a blood sample from every participant at every visit due to difficulties with phlebotomy. Therefore, some of blood sample 1 and some of sample 2 were included in the analysis to provide one blood sample per sample phase, per participant. Blood 1 samples were compared with blood 2 samples using a homoscedastic t-test and no significant difference was found between plasma concentration of isoflavones after three days and after seven days of supplementation ($n = 75$; $p = 0.5811$). Pairs of blood samples where both blood 1 and blood 2 were obtained for the same participant were compared using a paired t-test, and again no significant differences were found ($n = 6$ pairs; $p = 0.7119$). All of the blood 1 samples are compared with all of the blood 2 samples in Figure 6.

Figure 6:

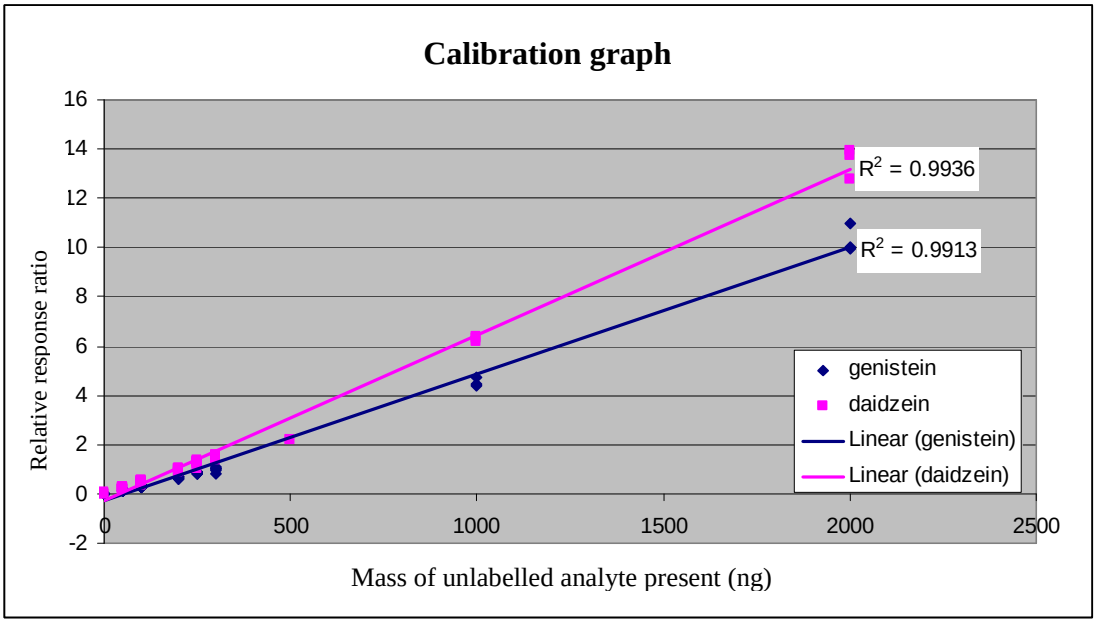


Analytical method:

Calibration

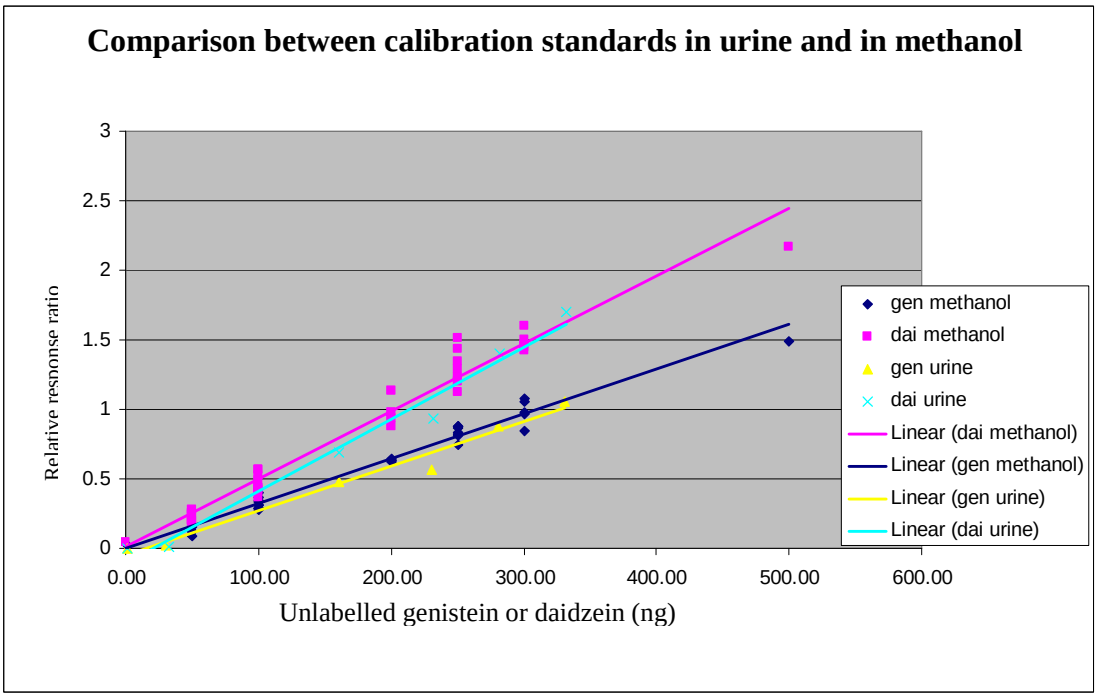
Calibration standards were prepared as previously described and included in every run. The concentration of isoflavones present in samples was calculated using the equation of the line of the calibration curve from that same run. This was carried out in order to control for variations in system performance from day to day. However, all calibration points were subsequently plotted on the same graph and shown to fall on the same line (Figure 7). Figure 7 demonstrates that response of the detector was linear in the range of relative response ratios 0 to 10, so the volume of urine/blood sample prepared was adjusted to give a relative response ratio within this range.

Figure 7:



The calibration curves for each run were made up in 50% v/v methanol:water for ease of preparation but were compared with a calibration curve made using a urine sample spiked with unlabelled isoflavones and prepared in an identical manner to the study samples. The resulting calibration curve was superimposed with the curves generated by calibration standards in 50% methanol.

Figure 8:



Limits of detection and quantification

The limit of detection was defined as the concentration of isoflavone which produced peaks with a signal-to-noise ratio (s/n) >2. A control urine sample divided into 250uL aliquots and the aliquots spiked with six different concentrations of genistein and daidzein and a constant amount of $^{13}C_3$ genistein and daidzein (250ng).

Signal-to-noise ratios for these spiked urine samples are given in Table 6.

Table 6: Signal-to-noise ratios

Concentration of added genistein/daidzein (ng/ml)	s/n genistein	s/n daidzein
5	2.17	0.75
10	3.40	2.12
15	3.10	2.17
20	2.78	2.00
25	3.25	3.44
50	2.89	3.67

Thus, the lower limit of detection (LOD) for genistein was defined as 5ng/ml and the lower limit of detection for daidzein was defined as 10ng/ml.

Percentage genistein and daidzein recovery close to the LOD were used to assess the lower limit of quantification and are shown in Table 7.

Table 7: Percentage recovery close to the limits of detection

Concentration of added genistein/daidzein (ng/ml)	% genistein recovered	% daidzein recovered
5	134	94
10	103	99
15	106	96
20	98	96
25	103	106
50	94	91

The lower limit of quantification (LOQ) was defined as the concentration, greater than the LOD, at which genistein or daidzein recovery was between 90%-110%. Thus, the LOQ for genistein was 10ng/ml for each analyte. This compared favourably with the limits of detection reported in other studies (Atkinson *et al.*, 2002; Bloedon *et al.*, 2002; Ritchie *et al.*, 2004). Since intake was compared with total genistein plus daidzein concentrations in blood and urine, the LOQ for genistein + daidzein was deemed to be 20ng/ml. Concentrations lower than 20ng/ml were averaged to 50% of the LOQ (ie 10ng/ml) for the purposes of calculating the correlation with intake.

The limit of detection of analytes from urine samples was extrapolated to blood samples. This was because insufficient plasma was obtained from any one individual to prepare a set of spiked samples as was carried out with urine samples.

Because the signal-to-noise ratio is the primary determinant of the lower limit of detection, the analytical method was designed to maximise the signal-to-noise ratio, thus lowering the limit of detection. The use of isotopically labelled internal standards allows individual mass chromatograms to be examined which dramatically reduces noise in itself. The extensive sample cleanup method was employed to minimise the 'background' in chromatograms. Other measures to reduce chromatographic noise were:

- placement of the column in a column heater at 23^{o}C to protect it from temperature fluctuations.
- using only HPLC-grade filtered and de-gassed solvents.
- mixing mobile phase B in the solvent reservoir rather than mixing online during isocratic elution.

- interspersing analytical samples with blank injections of 100% methanol to prevent build-up of contaminants on the column during the run.
- flushing of the column for one hour with 98% methanol at the end of each overnight run.

To obtain maximum signal, the injection volume was optimised to give large analyte peaks while still being less than half the injection volume required to produce symptoms of column overload (such as peak spreading, fronting or splitting) (Dolan, 2006).

Internal Standard:

The use of $^{13}C_3$-labelled internal standards enabled definitive identification of analytes by matching retention time. The internal standard (IS) also allowed correction for losses of analyte in sample preparation, since both analyte and IS behave identically and it can be presumed that losses of IS and unlabelled analyte will be proportionally identical, analytes are quantified relative to the internal standards. Percentage recovery of IS can be used to determine the degree of losses in sample preparation.

Intra-assay coefficient of variation

The intra-assay coefficient of variation (sometimes referred to as percent relative standard deviation or %RSD) is an expression of the degree of inaccuracy in measurement of samples, prepared in multiples and analysed in the same run on the same day. For bioanalytical LC-MS such as the determination of pharmacologically active substances in blood and urine, 15-20% imprecision and inaccuracy (ie coefficients of variation between 15 and 20) are generally accepted due to relatively low concentrations of the analytes and the large background from a biological matrix

(Dolan, 2006). Other authorities consider a coefficient of variation less than 10% to be preferable. The intra-assay coefficients of variation (CV) for this study are given in Table 8.

Table 8: Intra-assay coefficients of variation

Sample	Genistein + Daidzein (ng/ml)	Coefficient of variation (%)	Machine
IS6 baseline spot urine	3180.95 4449.69 3276.03 3662.13	13.72	SCRI (Scottish Crop Research Institute, Invergowrie)
IS72 110mg/d spot urine 1	14243.43 13784.34 13046.37 13315.16	3.36	SCRI
IS71 baseline spot urine	4364.57 4789.39	4.64	SCRI
IS70 55mg/d blood 2	530.90 519.92	1.05	SCRI
IS64 55mg/d blood 1	1466.30 1094.54 1122.22	13.77	SCRI
IS71 55mg/d 24hU (17/10/05)	7320.49 7026.57	2.03	SCRI
IS71 55mg/d 24hU (13/10/05)	6584.85 7114.09 6878.08 8291.57	8.98	SCRI
IS71 55mg/d 24hU (12/9/05)	6185.56 6586.34 7041.18	5.29	SCRI
IS71 55mg/d 24hU (9/6/06)	8197.83 9467.76	7.19	BMS (biomolecular sciences St Andrews)
IS66 55mg/d 24hU	6195.65 5964.54	1.90	BMS
IS71 55mg/d 24hU	8876.77 8368.27 8427.55	2.65	BMS
IS70 165mg/d 24hU	24205.48 22934.35 19615.61	8.70	BMS
IS24 110mg/d spot urine 2	163395.71 153763.58	3.04	BMS

The mean intra-assay coefficient of variation for samples run at SCRI was 6.61% (range 1.05-13.77%) and for samples run at BMS was 4.70% (range 1.90-8.70%).

Inter-assay coefficient of variation

The inter-assay coefficient of variation is an expression of the degree of inaccuracy in measurement of analytes when samples are prepared and analysed on different days by the same method. Again, a coefficient of variation between 15 and 20% is acceptable but less than 10% is preferable. In this study, the inter-assay coefficient of variation was assessed for each machine and the values for both machines combined to determine the percentage inaccuracy over the entire set of analyses. The results are given in Table 9.

Table 9: Inter-assay coefficients of variation

Sample	Genistein + Daidzein (ng/ml)	Coefficient of variation (%)	Machine
IS71 50mg/d 24hU	6185.56 6586.34 7041.18 6584.85 7114.09 6878.08 8291.57 7320.49 7026.57	7.98	SCRI
IS71 50mg/d 24hU	8967.77 9003.29 7997.99 8197.83 9467.76 8980.75 8713.85 8876.77 8368.27 8427.55	4.87	BMS
IS71 50mg/d 24hu	6185.56 6586.34 7041.18 6584.85 7114.09 6878.08 8291.57 7320.49 7026.57 8967.77 9003.29 7997.99 8197.83 9467.76 8980.75 8713.85 8876.77 8368.27 8427.55	12.41	SCRI and BMS

Thus, the degree of imprecision between assays at SCRI was 7.97% and at BMS was 4.87%. By analysing the same sample multiply on both machines it was demonstrated that inter-assay imprecision was 12.41%, which falls within the acceptable range defined above.

Inter- and intra-assay coefficients of variation observed were comparable with the precision seen in previous studies of isoflavone concentration in biological matrices (Atkinson *et al.*, 2002; Holder *et al.*, 1999; Hutchins *et al.*, 1995; Karr *et al.*, 1997; Watanabe *et al.*, 1998).

The methods employed in data collection for this study were therefore deemed to be sufficiently repeatable, valid and accurate to produce reliable results.

RESULTS

Introduction

The primary aim of this study was to validate 24-hour urine, timed plasma and spot urine samples as reliable measures of isoflavones intakes <165mg/d. A secondary aim was to determine whether plasma concentration or urinary excretion reaches a plateau at this level of intake.

Data from 31 participants (11 male, 20 female) were analysed. Scatter plots of the relationship between intake and each biomarker are presented for both non-transformed and log-transformed data. Relationships and agreement between the biomarkers are described. Percentage urinary recovery and percentage of dose appearing in plasma were plotted against dose to determine the linearity of dose response for this intake range. The effect of age and gender on biomarkers of intake was also explored.

Participant demographics

The mean age of participants was 29 years (range 18-60 years). Participant demographic data are shown in Table 10.

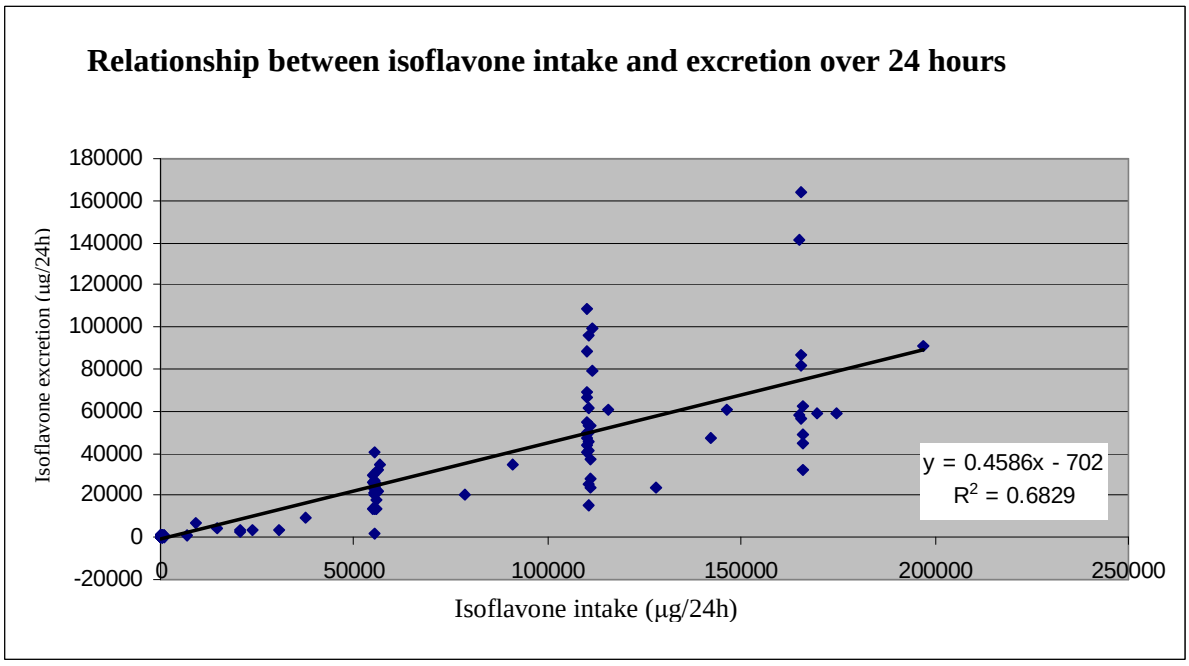
Table 10: Participant demographics

ID Code	Gender	Age	Height (m)	Weight at start (kg)	Weight at end (kg)	BMI at start
is 1	M	19	1.82	104	106	31.40
is 4	F	19	1.62	46	46	17.53
is 5	F	19	1.56	60	60	24.65
is 6	M	18	1.86	110	113	31.80
is 9	F	19	1.57	60	59	24.34
is 15	F	21	1.74	67	69	22.13
is 17	F	21	1.70	76	77	26.30
is 18	M	21	1.82	76	78	22.94
is 23	M	21	1.82	88	86	26.57
is 24	F	20	1.74	57	56	18.83
is 25	F	23	1.61	75	75	28.93
is 26	M	21	1.86	96	96	27.75
is 27	F	19	1.56	64	62	26.30
is 28	M	20	1.87	98	100	28.02
is 35	F	20	1.79	68	66	21.22
is 40	F	19	1.70	67	67	23.18
is 42	F	27	1.73	85	85	28.40
is 44	F	21	1.78	60	60	18.90
is 48	M	18	1.79	81	81	25.28
is 56	F	55	1.61	74	73	28.55
is 57	F	57	1.57	53	52	21.50
is 59	F	60	1.62	64	61	24.39
is 61	F	35	1.62	67	67	25.53
is 63	M	23	1.78	99	100	31.25
is 64	F	22	1.57	51	51	20.69
is 66	M	56	1.75	80	80	26.12
is 67	F	43	1.50	54	55	24.00
is 68	M	49	1.79	88	90	27.46
is 70	F	28	1.55	59	59	24.56
is 71	F	56	1.62	70	67	26.67
is 72	M	26	1.85	73	74	21.33

RELATIONSHIPS BETWEEN INTAKE AND BIOMARKERS

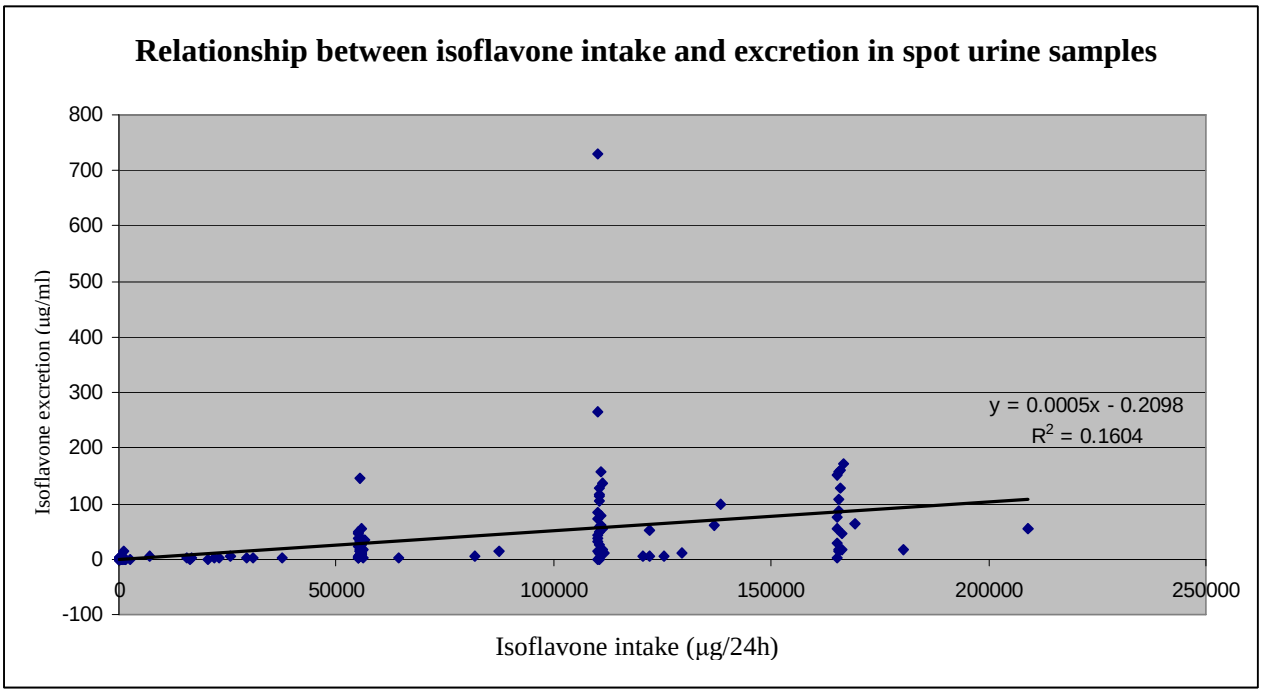
Each individual participant's dietary intake of isoflavones was added to his or her supplemental intake and scatter plots of total intake against plasma and urinary isoflavones were produced. Linear regression was performed on the scatter plots and the equations of these lines are displayed on Figures 9 to 12 along with Pearson's correlation coefficient (r^2).

Figure 9:



n = 109 samples from 31 volunteers

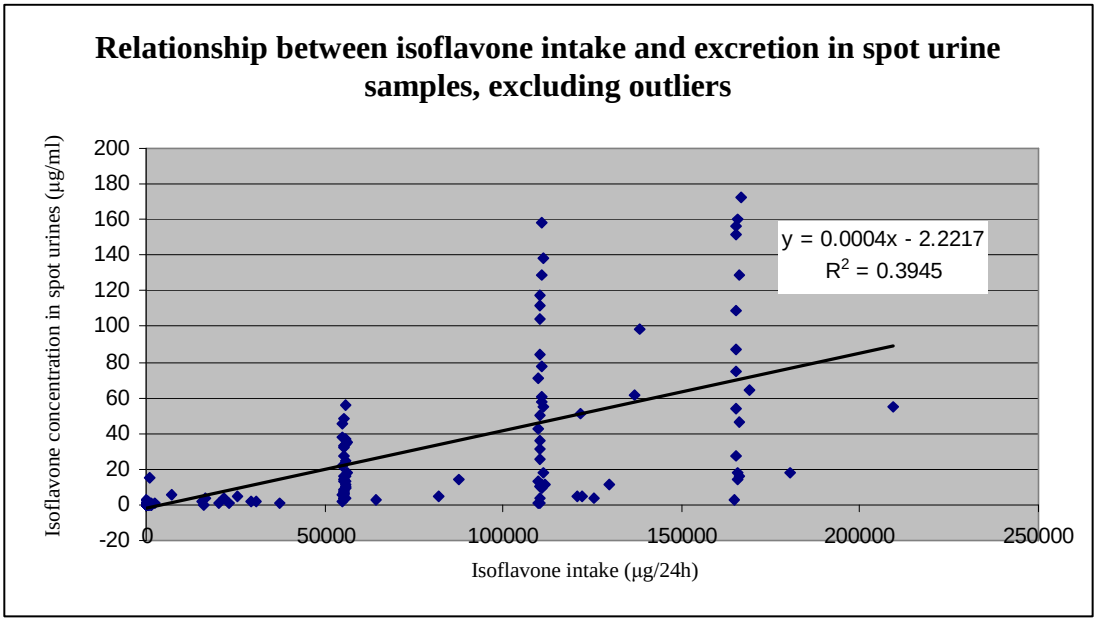
Figure 10:



n = 143 samples from 31 volunteers

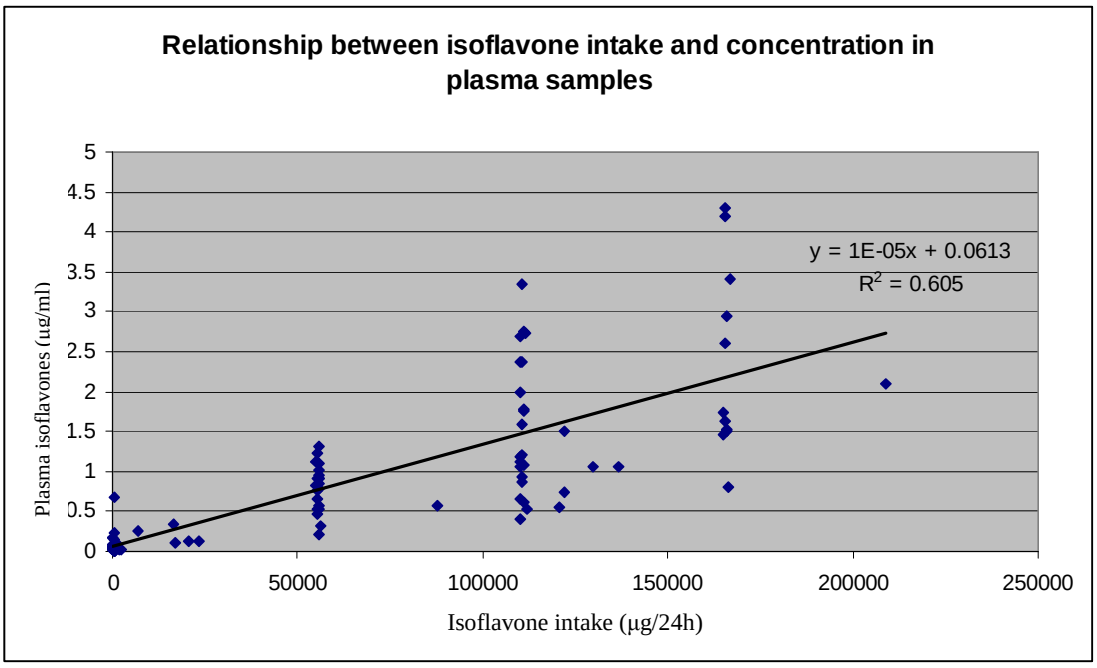
The three outliers with higher than expected isoflavone concentration in spot urine samples belonged to two female subjects who had a low fluid intake and passed small volumes of urine over 24 hours. These three spot urine samples were visibly darker and more concentrated than the other spot urine samples. Re-plotting the relationship between intake and spot urine concentration excluding these three samples gave the relationship shown in Figure 11.

Figure 11:



n = 140 samples from 31 volunteers

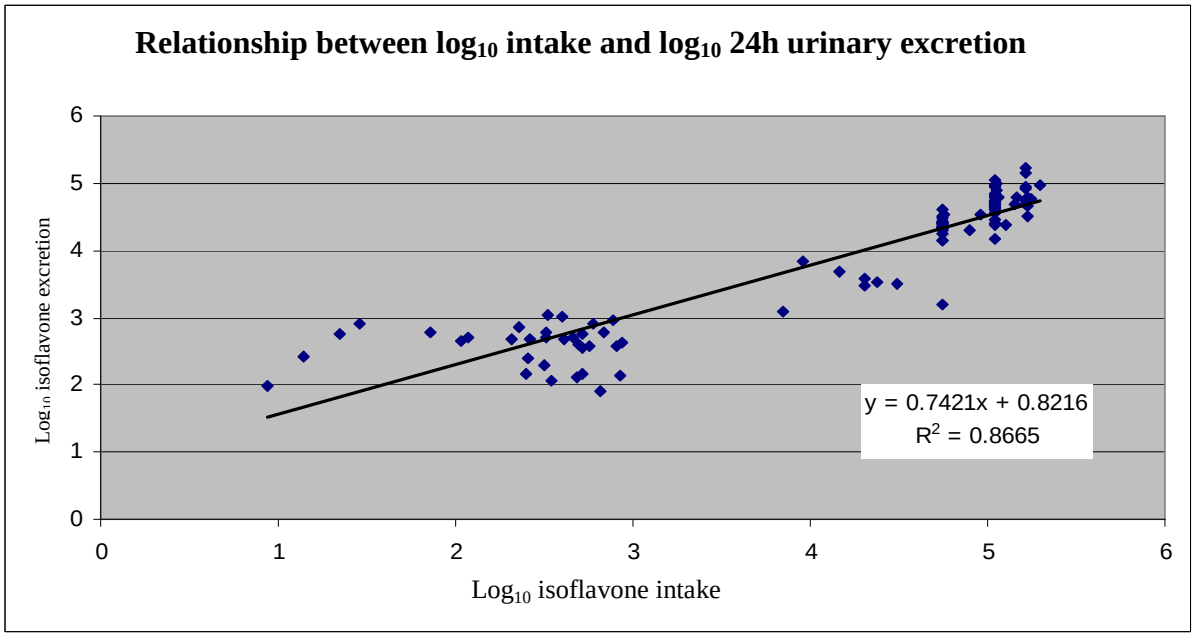
Figure 12:



n = 100 samples from 30 volunteers

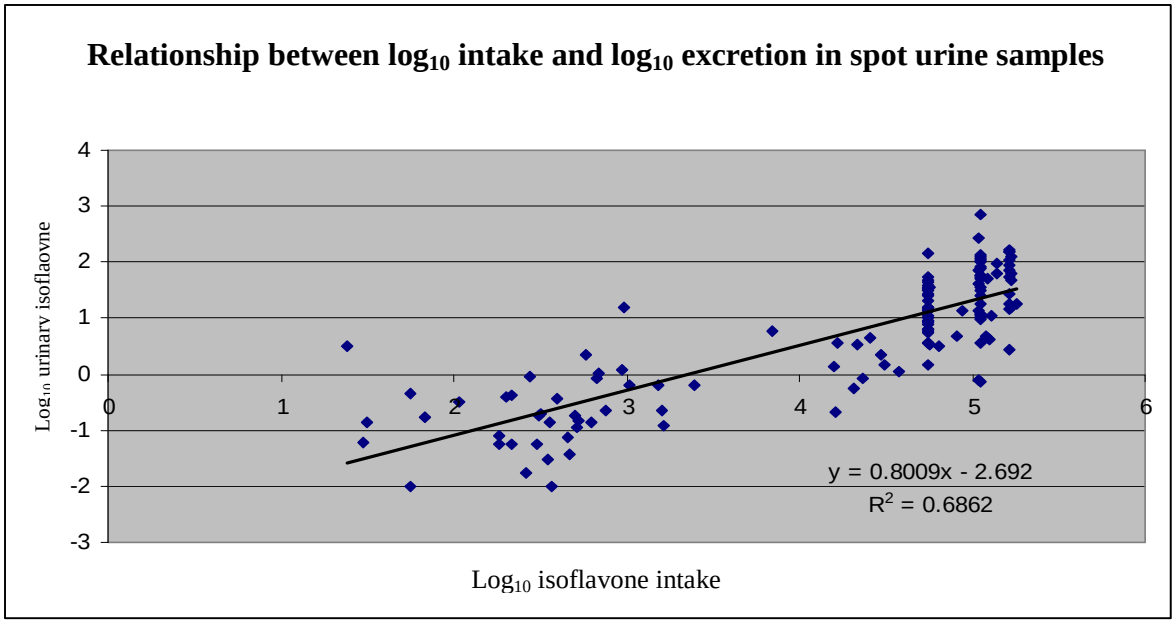
Because it was hypothesised that the relationship between intake and excretion would not be perfectly linear, data on both axes were log transformed (log_{10}) and plotted again in Figures 13 to 15 to clarify the relationship between intake and excretion.

Figure 13:



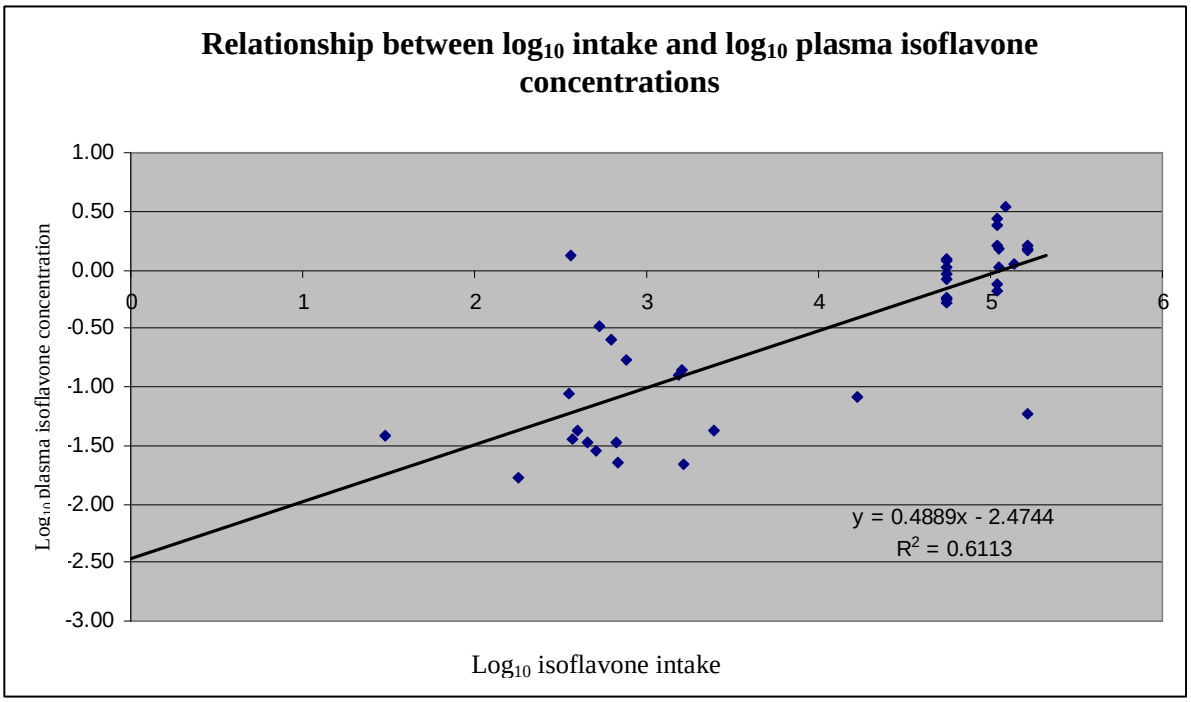
n = 109 samples from 31 volunteers; $p < 0.001$

Figure 14:



n = 143 samples from 31 volunteers; $p < 0.001$

Figure 15:



n = 100 samples from 30 volunteers; $p < 0.001$

Larger values of r^2 for log transformed data indicate that this model is a better fit. The equations of linear regressions on log-log graphs were therefore used to define the relationship between intake and excretion or plasma concentration of isoflavones.

For 24-hour urine samples:

$Y = mX + c$

$Y = 0.7421X + 0.8216$

Where $Y = \log_{10}$(isoflavone excretion in μg/24h) and isoflavone excretion = y

$X = \log_{10}$(isoflavone intake in μg/24h) and isoflavone intake = x

Therefore;

$\log y = 0.7421(\log x) + 0.8216$

c = 0.8216 which is a logarithm of a constant, k

$0.8216 = \log_{10} k$ $[c = \log k \equiv k = 10^c]$

$k = 10^{0.8216}$

$\log y = 0.7421(\log x) + \log 10^{0.8216}$

$\log y = 0.7421(\log x) + \log 6.63132$

$\log y = \log(x^{0.7421}) + \log 6.63132$ $[n(\log x) = \log x^n]$

$\log y = \log 6.63132(x^{0.7421})$ [log a + log b = log (ab)]

$y = 6.63132x^{0.7421}$

where y = isoflavone excretion in μg/24h

and x = isoflavone intake in μg/24h

For spot urine samples:

$Y = mX + c$

$Y = 0.8009X - 2.692$

Where $Y = \log_{10}$(isoflavone excretion in μg/ml) and isoflavone excretion = y

$X = \log_{10}$(isoflavone intake in μg/24h) and isoflavone intake = x

Therefore;

$\log y = 0.8009(\log x) - 2.692$

c = -2.692 which is a logarithm of a constant, k

$-2.692 = \log_{10} k$ [$c = \log k \equiv k = 10^c$]

$k = 10^{-2.692}$

$\log y = 0.8009(\log x) + \log 10^{-2.692}$

$\log y = 0.8009(\log x) + \log 0.002032357$

$\log y = \log(x^{0.8009}) + \log 0.002032357$ [$n(\log x) = \log x^n$]

$\log y = \log 0.002032357(x^{0.8009})$ [$\log a + \log b = \log (ab)$]

$y = (2.0324 \times 10^{-3})x^{0.8009}$

where y = isoflavone excretion in μg/ml

and x = isoflavone intake in μg/24h

The outliers in spot urine concentration were included when deriving this equation since it is to be expected that spot urine samples will vary in their absolute concentration and therefore also in their isoflavone concentration. It was considered that exclusion of these outliers would unjustifiably bias the equation derived.

For plasma samples:

$Y = mX + c$

$Y = 0.4889X - 2.4744$

Where $Y = \log_{10}$(plasma isoflavone in μg/ml) and plasma isoflavone = y

$X = \log_{10}$(isoflavone intake in μg/24h) and isoflavone intake = x

Therefore;

$\log y = 0.4889(\log x) - 2.4744$

c = -2.4744 which is a logarithm of a constant, k

$-2.4744 = \log_{10} k$ [$c = \log k \equiv k = 10^c$]

$k = 10^{-2.4744}$

$\log y = 0.4889(\log x) + \log 10^{-2.4744}$

$\log y = 0.4889(\log x) + \log 0.003354285$

$\log y = \log(x^{0.4889}) + \log 0.003354285$ [$n(\log x) = \log x^n$]

$\log y = \log 0.003354285(x^{0.4889})$ [$\log a + \log b = \log (ab)$]

$y = (3.3543 \times 10^{-3})x^{0.4889}$

where y = plasma isoflavone in μg/ml

and x = isoflavone intake in μg/24h

Statistical Significance

Statistical significance of the correlation between intake and biomarkers was assessed by a student's t-test using the following equation:

$$t = r \times \sqrt{\left[\frac{n-2}{1-r^2}\right]}$$

The value of p for each corresponding value of t was determined from t-tables.

Therefore, for the correlation between isoflavone intake and 24hU excretion:

$r^2 = 0.8665$; $n = 109$

$t = (\sqrt{0.8665}) \times \sqrt{(107 \div 0.1335)}$

$= 0.930859817 \times \sqrt{801.498127}$

$= 0.930859817 \times 28.31074226$

$t = 26.3533$

$p < 0.001$

For the correlation between isoflavone intake and spot urine isoflavone concentration:

$r^2 = 0.6862$; $n = 143$

$t = (\sqrt{0.6862}) \times \sqrt{(141 \div 0.3138)}$

$= 0.828371897 \times \sqrt{449.3307839}$

$= 0.828371897 \times 21.19742399$

$t = 17.5593$

$p < 0.001$

For the correlation between isoflavone intake and plasma isoflavone concentration:

$r^2 = 0.6113$; n = 100

$t = (\sqrt{0.6113}) \times \sqrt{(98 \div 0.3887)}$

$= 0.781856764 \times \sqrt{252.1224595}$

$= 0.781856764 \times 15.87836451$

$t = 12.4146$

$p < 0.001$

CALCULATING INTAKE FROM CONCENTRATION IN BIOLOGICAL FLUIDS

In this study, the independent variable was isoflavone intake, since this was manipulated by giving oral isoflavone supplements. However, biomarkers of isoflavone exposure may also be used to infer intake from a measured plasma concentration or measured excretion. Therefore, equations relating intake to biomarkers were re-arranged to allow intake to be calculated from a known concentration in biological fluids.

For 24-hour urines:

$y = 6.63132x^{0.7421}$

therefore $6.63132x^{0.7421} = y$

$x^{0.7421} = y \div 6.63132$

$x = \sqrt[0.7421]{(y \div 6.63132)}$

$\equiv \quad x = (y \div 6.63132)^{1.3475}$

where y = isoflavone excretion in μg/24h

and x = isoflavone intake in μg/24h

For spot urine samples:

$y = (2.0324x10^{-3})x^{0.8009}$

therefore $(2.0324x10^{-3})x^{0.8009} = y$

$x^{0.8009} = y \div 2.0324x10^{-3}$

$x = \sqrt[0.8009]{(y \div 2.0324x10^{-3})}$

$\equiv \quad x = (y \div 2.0324x10^{-3})^{1.2486}$

where y = isoflavone excretion in μg/ml

and x = isoflavone intake in μg/24h

For plasma samples:

$y = (3.3543x10^{-3})x^{0.4889}$

therefore $(3.3543x10^{-3})x^{0.4889} = y$

$x^{0.4889} = y \div 3.3543x10^{-3}$

$x = \sqrt[0.4889]{(y \div 3.3543x10^{-3})}$

$\equiv \quad x = (y \div 3.3543x10^{-3})^{2.0454}$

where y = plasma isoflavone in μg/ml

and x = isoflavone intake in μg/24h

The correlation between the three biomarkers of isoflavone exposure was also assessed and log-log graphs with correlation coefficients are shown in Figures 16 to 18. Outliers in spot urine concentration were included.

Figure 16:

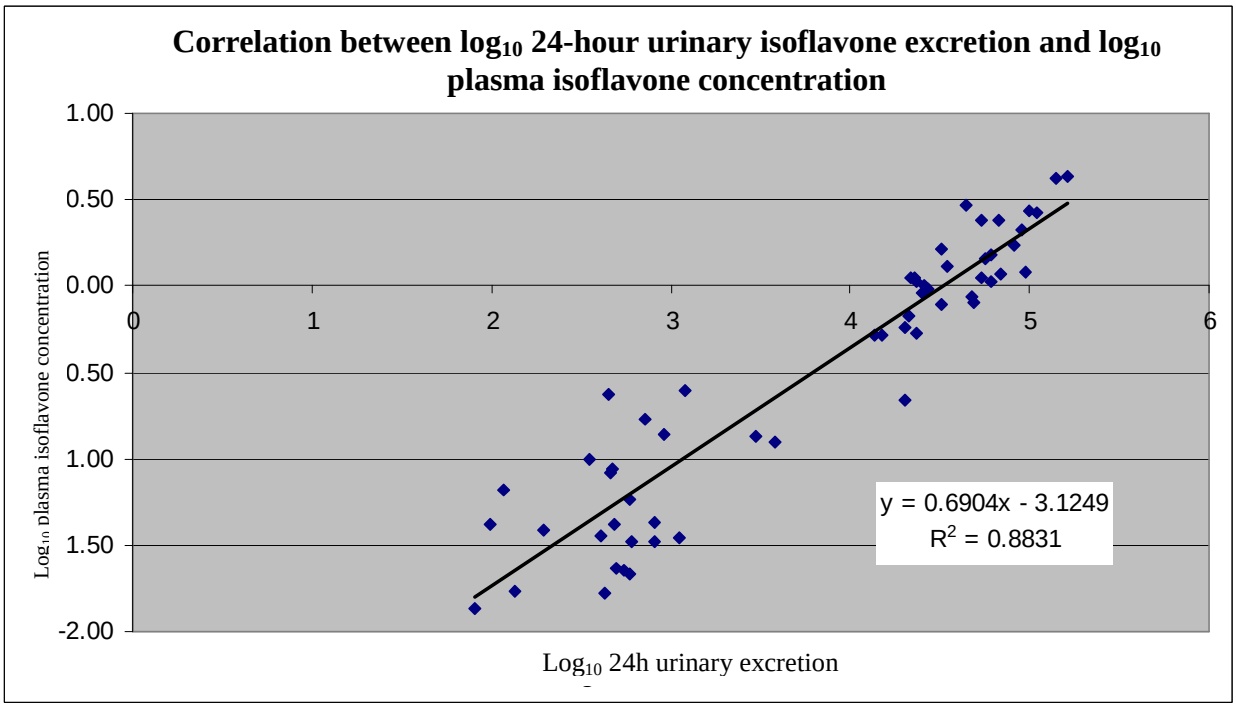


Figure 17:

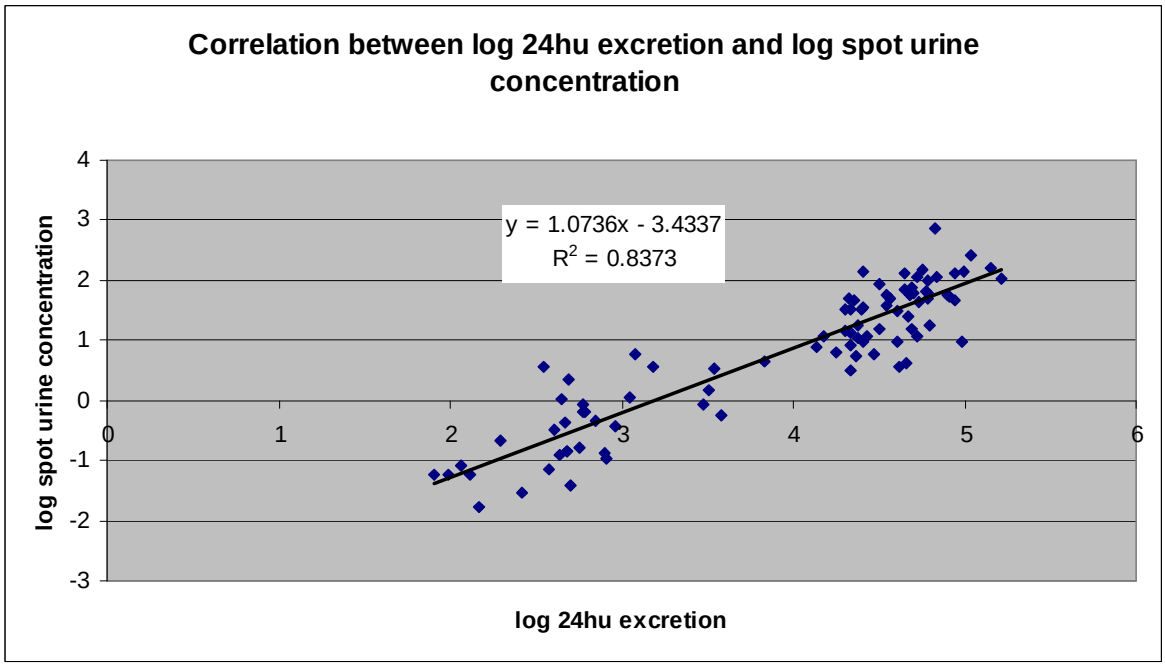
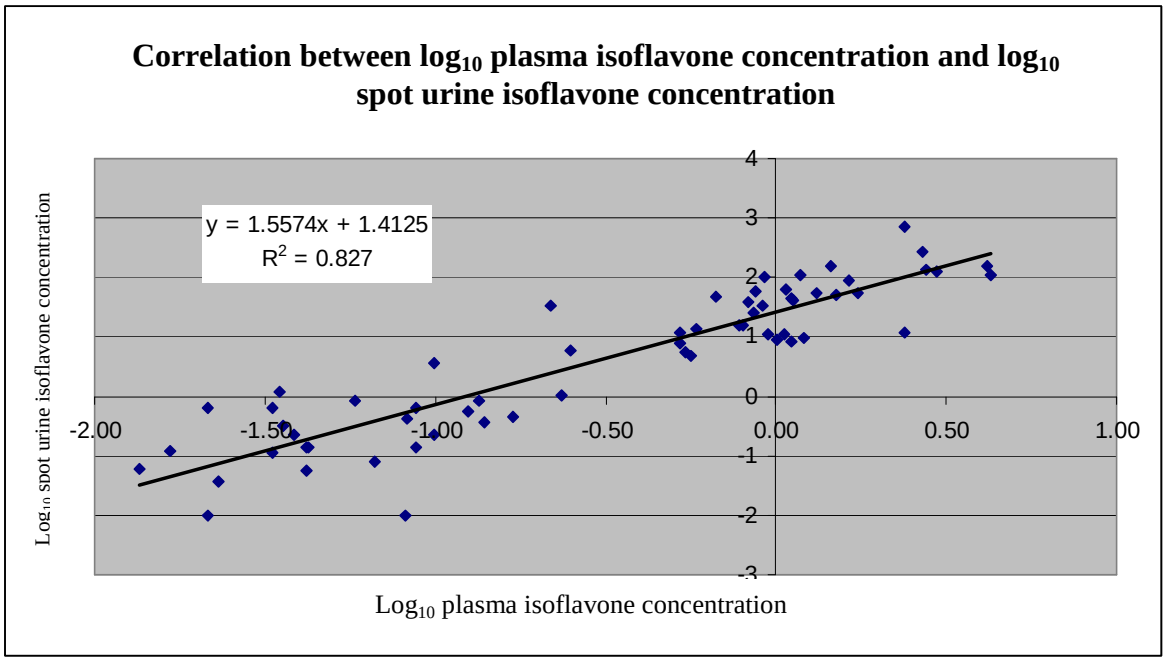


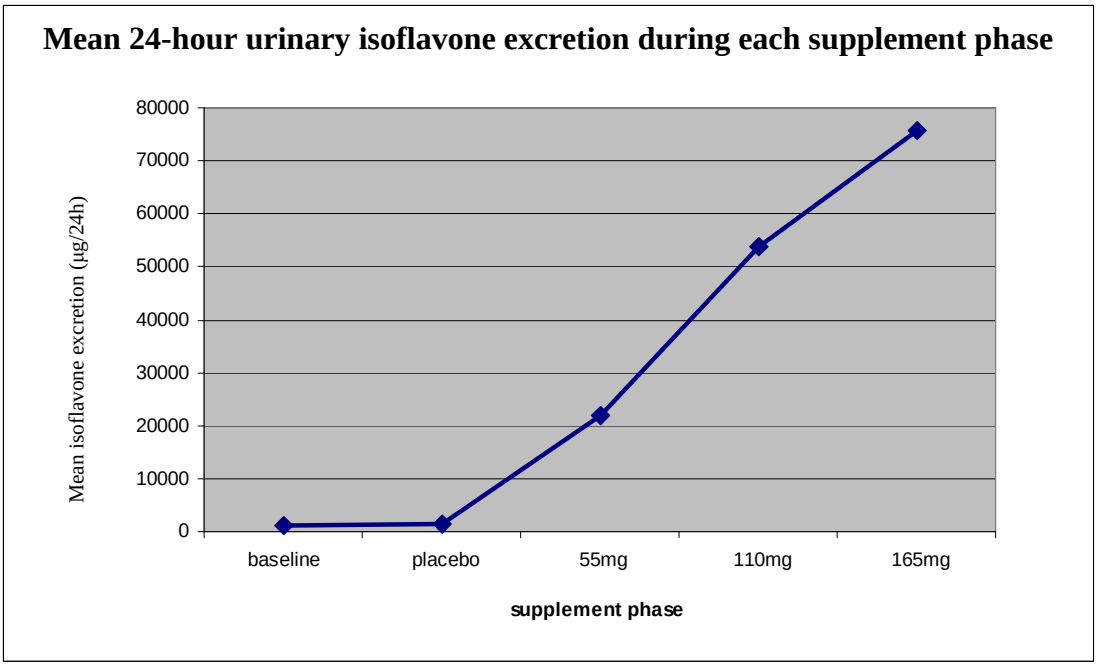
Figure 18:



LINEARITY OF DOSE RESPONSE

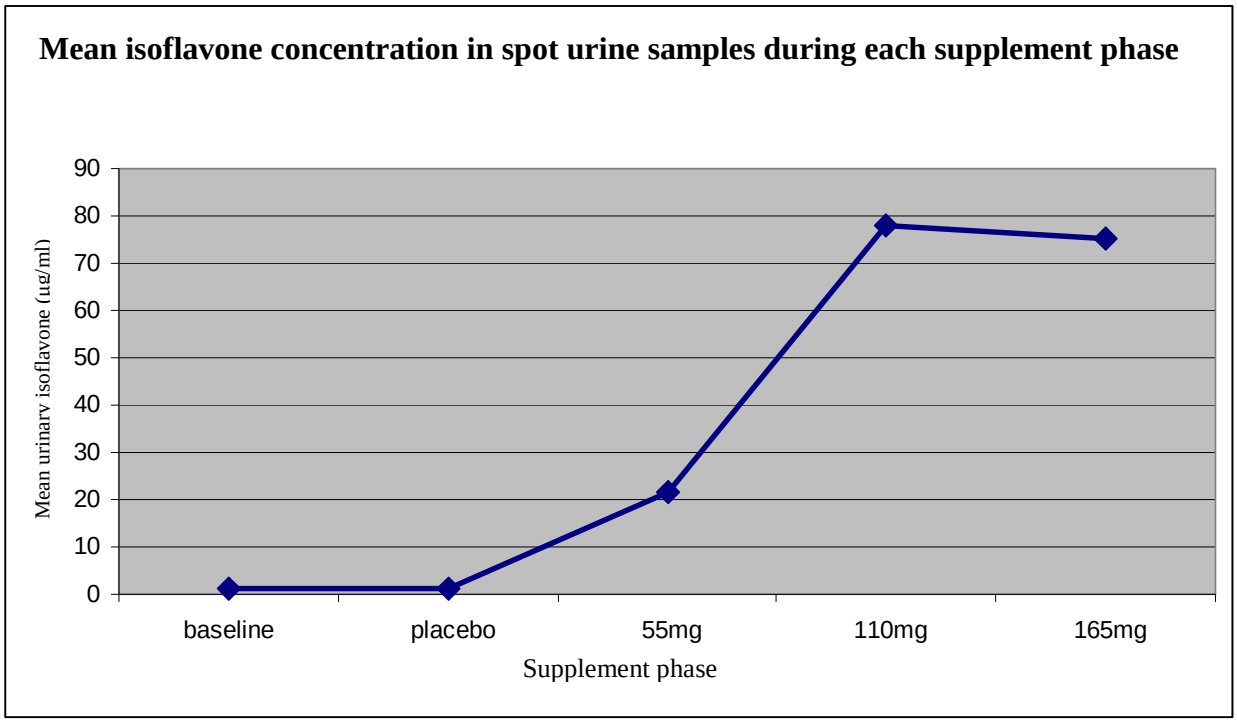
To determine whether plasma isoflavone concentrations or urinary isoflavone concentrations during isoflavone supplementation <165mg/d appeared linear, mean isoflavone excretion in 24-hour urine samples, spot urine samples and plasma samples taken during each intervention phase are depicted in Figures 19 to 22.

Figure 19:



24-hour urine samples show an almost linear relationship between supplement dose and excretion over 24hours

Figure 20:



Isoflavone concentration in spot urines apparently reached a maximum at an intake of 110mg/d but when the three outliers previously identified (IS64 during 55mg/d and 110mg/d phases; IS9 during 110mg/d phase) were excluded from the mean spot urine concentration, the relationship between isoflavone intake and spot urine isoflavone concentration is close to linear (Figure 21).

Figure 21:

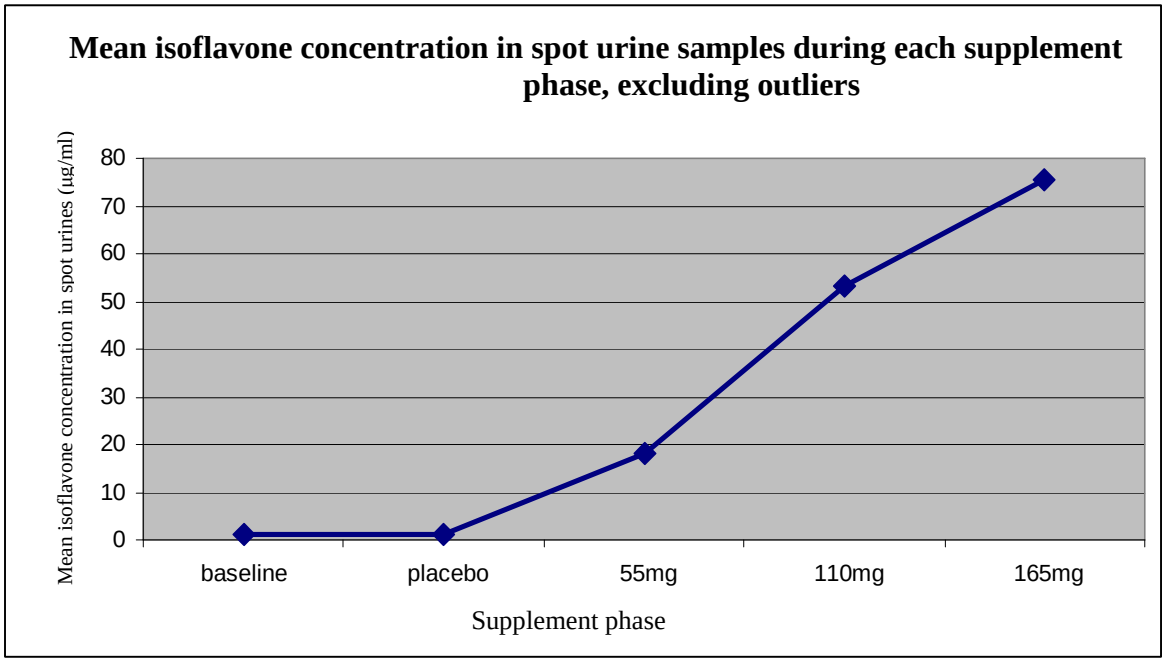
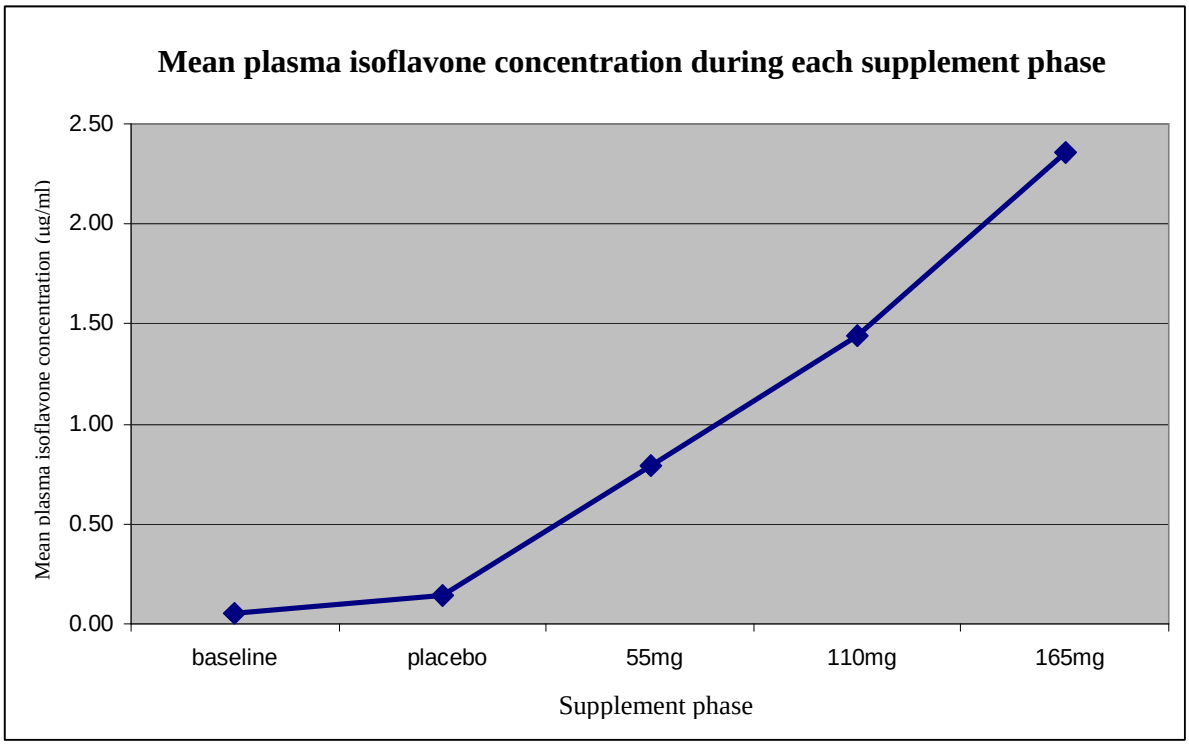


Figure 22:



The relationship between supplement dose and plasma isoflavone concentration is close to linear.

To further explore the relationship between increasing dose and the proportion of ingested isoflavones absorbed and excreted, percentage recovery of the ingested dose was plotted against oral intake in milligrams.

Percentage recovery in 24-hour urine samples was calculated using the equation derived from Figure 13 to calculate urinary excretion in 24 hours from a given intake:

$y = 6.63132x^{0.7421}$

where y = 24-hour urinary isoflavone excretion (ug/24h)

and x = isoflavone intake (ug/24h)

Percentage recovery was then defined as;

% recovery = 100(y ÷ x)

Similarly, plasma isoflavone concentration corresponding to a given intake was calculated from the equation:

$y = (3.3543x10^{-3})x^{0.4889}$

where y = plasma isoflavone in μg/ml

and x = isoflavone intake in μg/24h

and the percentage of the ingested dose appearing in plasma (per ml) was defined as;

% dose per ml plasma = 100(y ÷ x)

Percentage recovery for six values of x were calculated and plotted in Figures 23 and 24 which show a curvilinear relationship between ingested dose and percentage recovery/percentage of the dose appearing in plasma.

Figure 23:

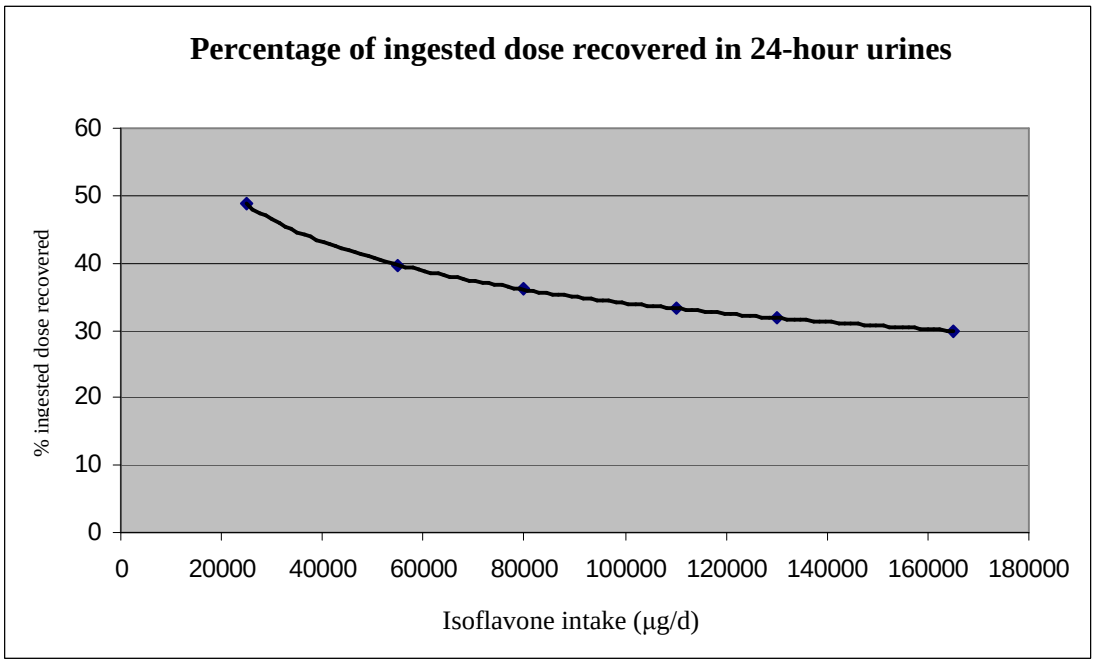
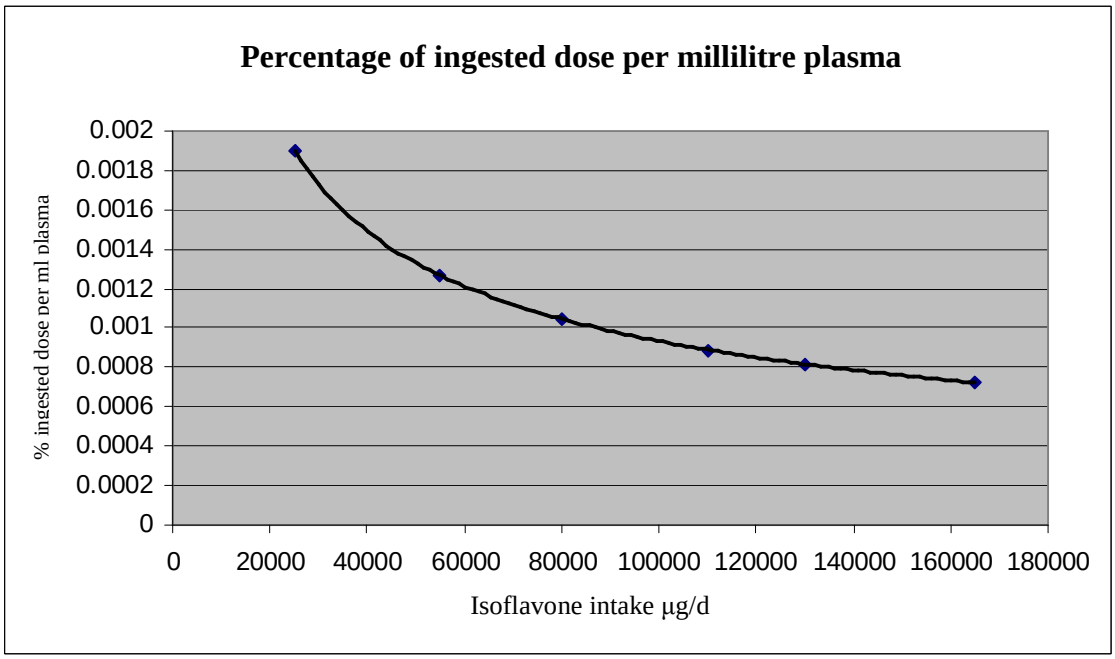


Figure 24:



EFFECT OF AGE ON THE RELATIONSHIPS BETWEEN INTAKE AND EXCRETION AND PLASMA CONCENTRATION

No effect of the participant's age on plasma concentration of isoflavones or urinary isoflavone excretion over 24 hours was evident, as depicted in Figures 25 and 26.

Figure 25:

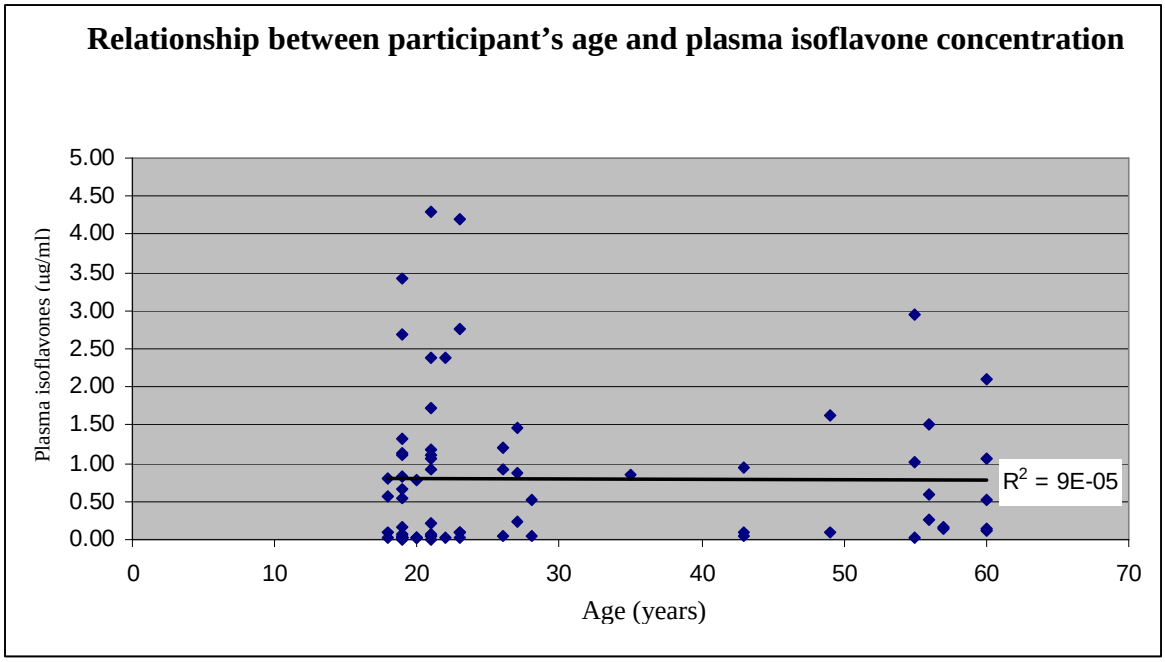
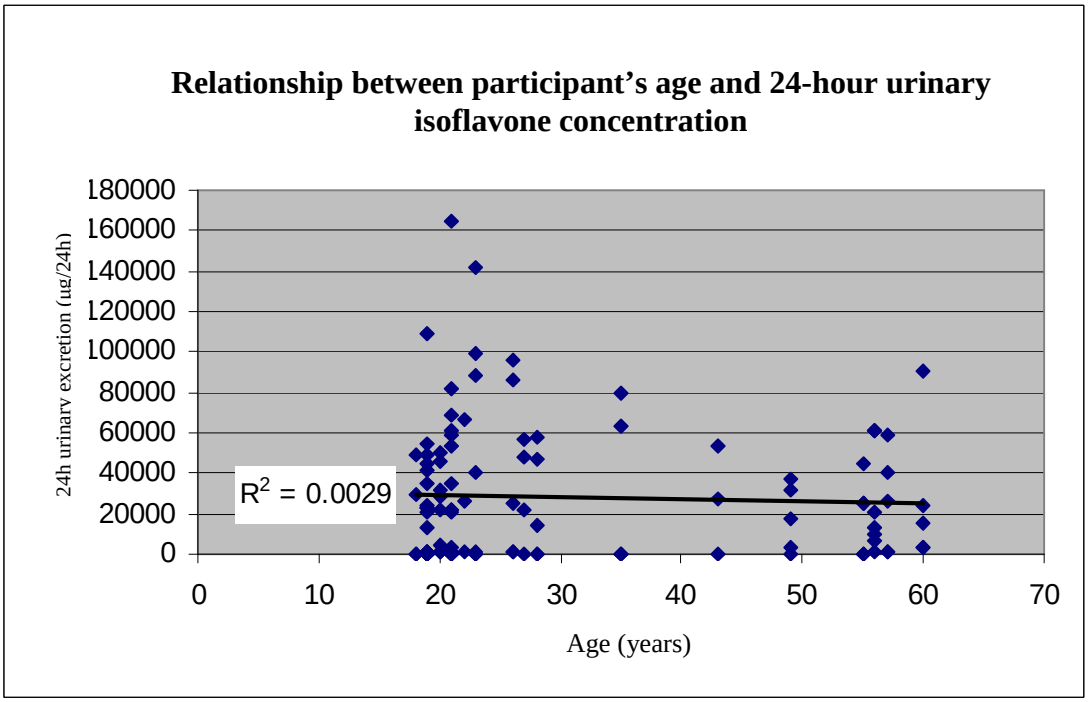


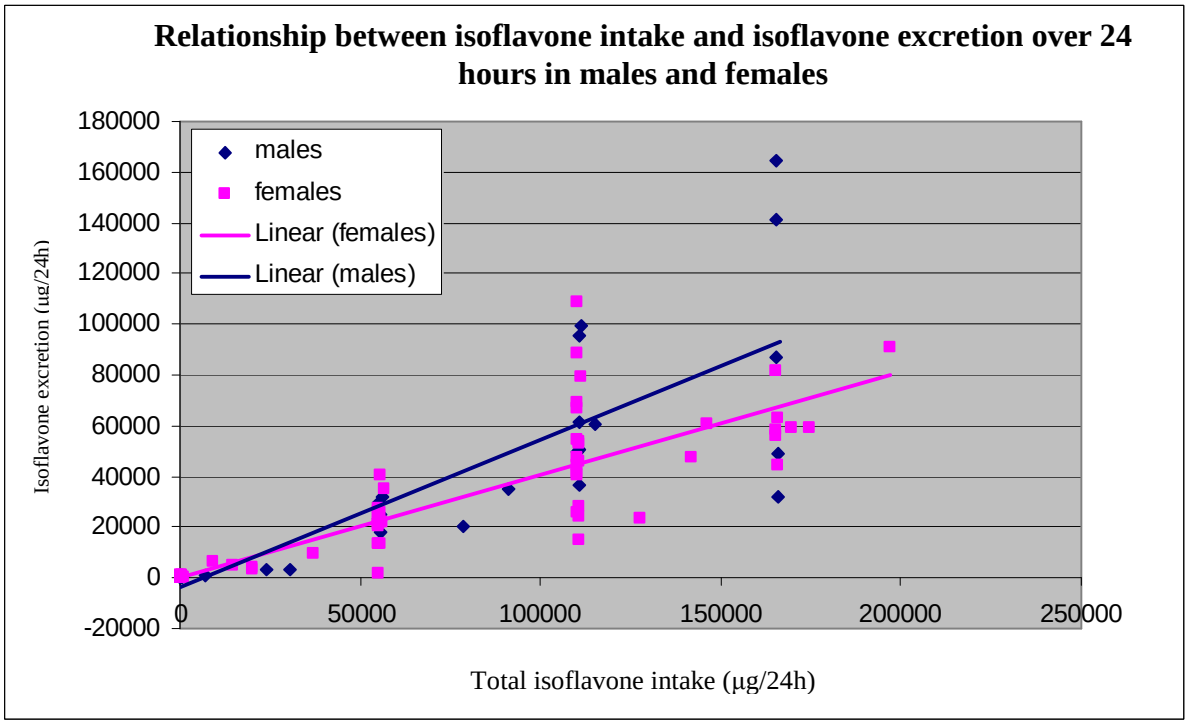
Figure 26:


Relationship between participant's age and 24-hour urinary isoflavone concentration
24h urinary excretion (ug/24h)
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0
10
20
30
40
50
60
70
Age (years)
R2 = 0.0029

EFFECT OF GENDER ON THE RELATIONSHIP BETWEEN INTAKE AND EXCRETION AND PLASMA CONCENTRATION

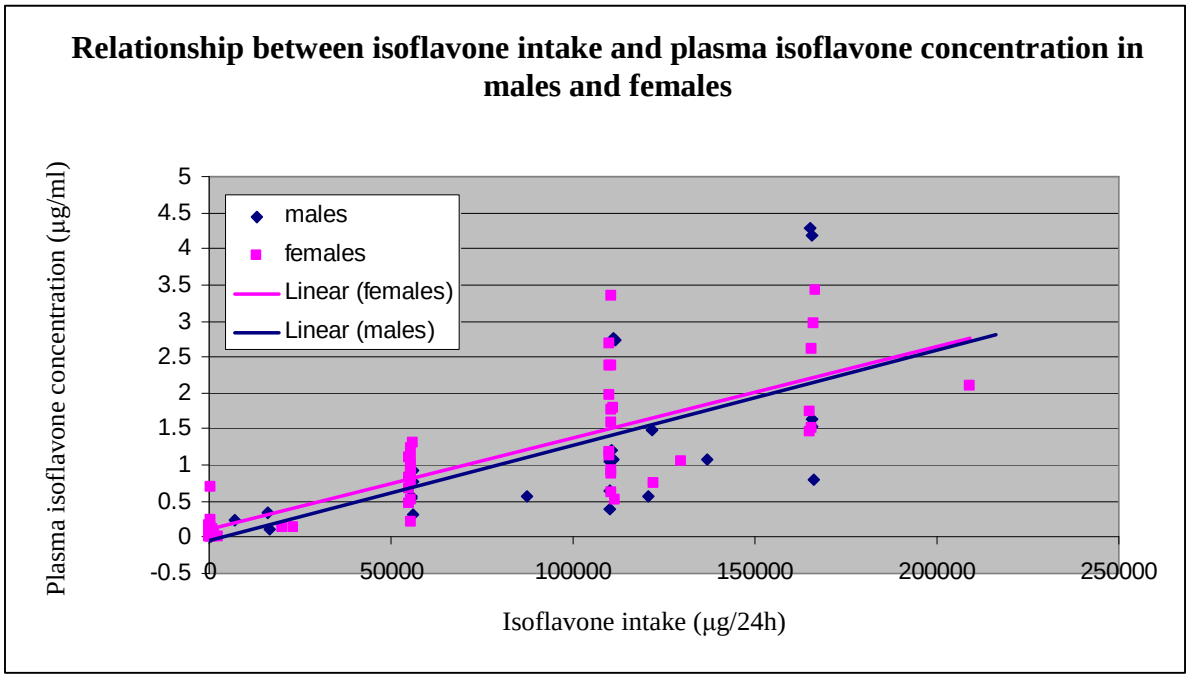
No significant difference in 24-hour urinary isoflavone excretion was found between males and females over all phases of supplementation ($p = 0.23$). The relationship between isoflavone intake and 24-hour urinary isoflavone excretion in the two groups is shown in Figure 27.

Figure 27:



No difference was found in the relationship between isoflavone intake and plasma isoflavone concentration between males and females (Figure 28).

Figure 28:


Relationship between isoflavone intake and plasma isoflavone concentration in males and females
Plasma isoflavone concentration (µg/ml)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
0
50000
100000
150000
200000
250000
males
females
Linear (females)
Linear (males)
Isoflavone intake (µg/24h)

EFFECT OF BMI ON THE RELATIONSHIPS BETWEEN INTAKE AND EXCRETION AND PLASMA CONCENTRATION

No effect of the participant's BMI on plasma isoflavone concentration or on urinary isoflavone excretion over 24 hours was evident, as depicted in Figures 29 and 30.

Figure 29:

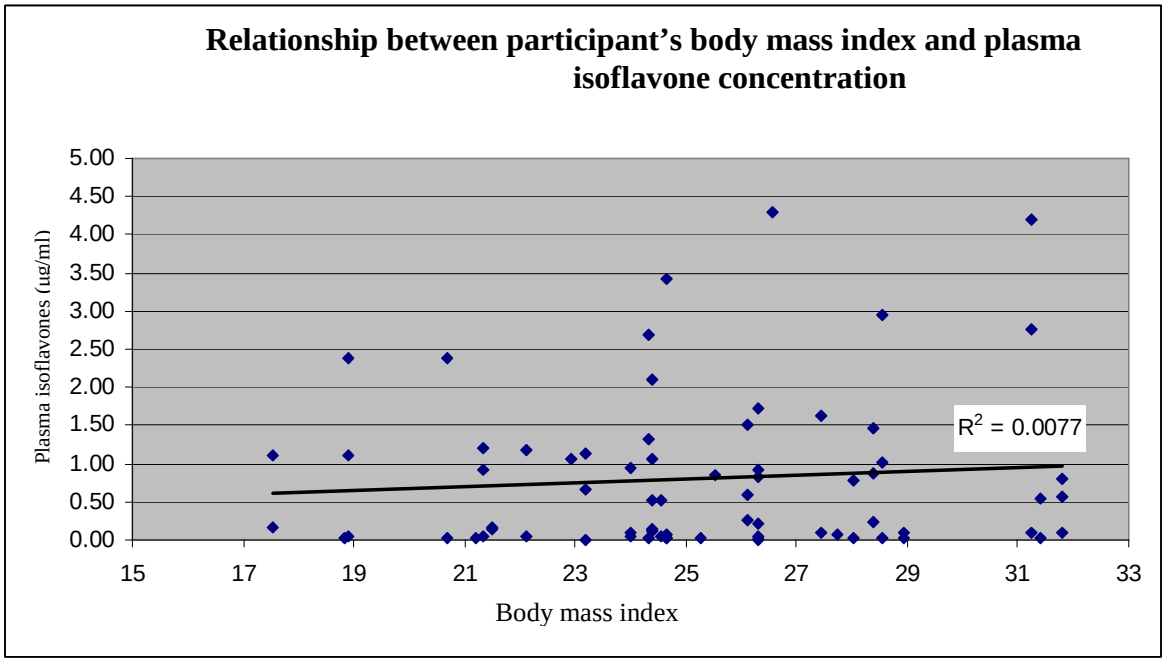
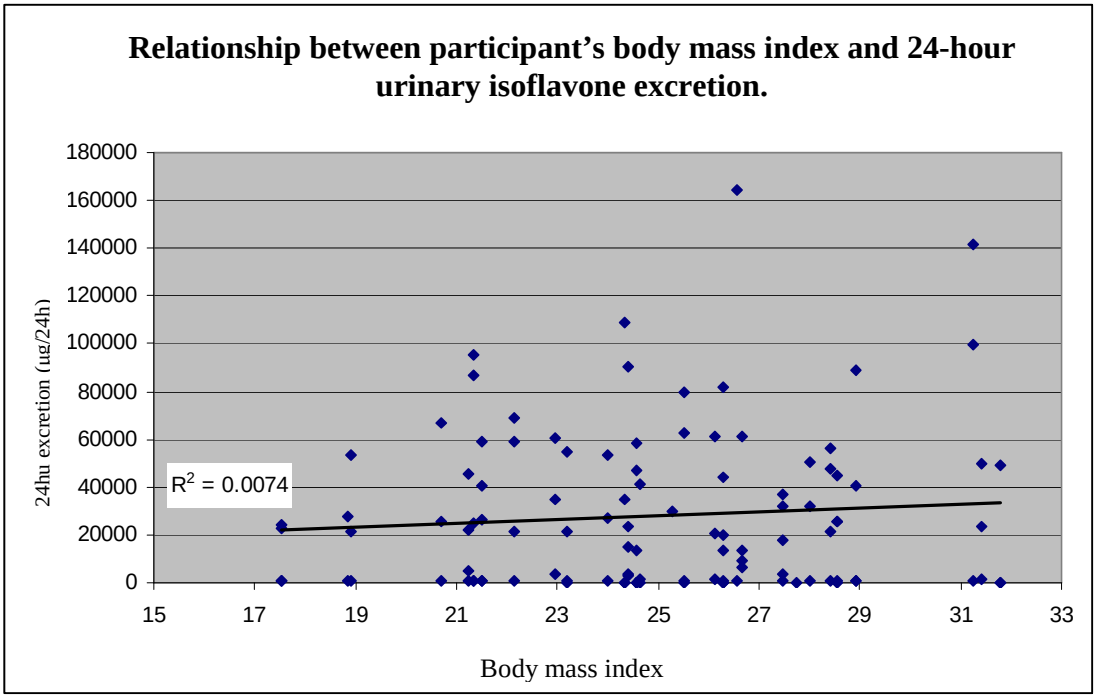


Figure 30:


Relationship between participant's body mass index and 24-hour urinary isoflavone excretion.
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
24hu excretion (ug/24h)
R2 = 0.0074
15
17
19
21
23
25
27
29
31
33
Body mass index

DISCUSSION

24-hour urine, plasma and spot urine samples as biomarkers of isoflavone intake

Strong, statistically significant correlations were found between; a) isoflavone intake and urinary isoflavone excretion over 24 hours; b) isoflavone intake and isoflavone concentration in spot urine samples; c) isoflavone intake and plasma isoflavone concentration.

No previous study has validated isoflavone biomarkers for this intake range. Correlations observed in this study between intake and biomarkers were comparable to or stronger than those reported by most other biomarker validation studies discussed in Chapter 1 (Grace *et al.*, 2004; Maskarinec *et al.*, 1998; Ritchie *et al.*, 2004; Ritchie *et al.*, 2004a; Ritchie *et al.*, 2004b; Takashima *et al.*, 2004; Verkasalo *et al.*, 2001).

This demonstrates that these markers are appropriate for use in intervention or epidemiological studies as measures of isoflavone intake or compliance with isoflavone supplementation. A further application might be in the relation of isoflavone concentrations in *in vitro* studies to the human oral intake required to achieve a similar circulating concentration.

Agreement between the biomarkers

In this study, strong positive correlations were found between biomarkers of isoflavone intake over the range of 0-165mg/d total isoflavones: 24hU isoflavones with plasma isoflavones ($r^2 = 0.88$), 24hU with spot urine samples ($r^2 = 0.84$) and spot urine with plasma samples ($r^2 = 0.83$). This is comparable to or stronger than correlations reported in other studies of isoflavone biomarkers, for example, in a large population-based study (Grace *et al.* 2004) correlations (r^2) between serum and spot urine isoflavones were 0.74 before adjustment for urinary creatinine and 0.85 after creatinine adjustment.

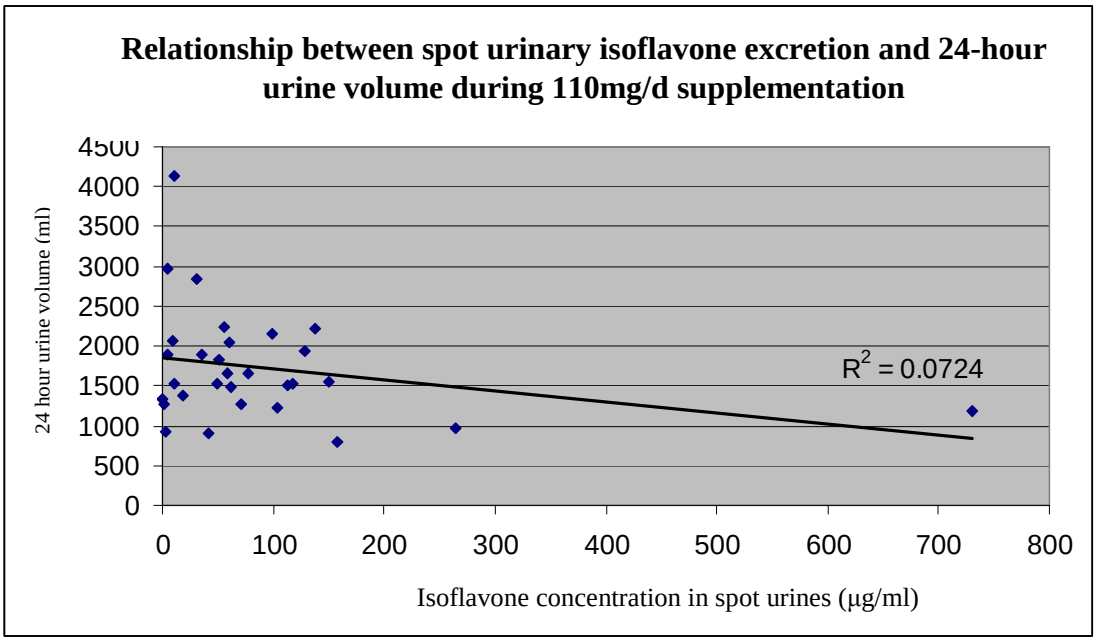
Setchell *et al*. (2003a) also found highly significant though considerably less strong correlations between biomarkers (maximum plasma concentrations of isoflavones (C_{max}) with urinary isoflavone concentration and total urinary output of isoflavones) in a study of $^{13}C_3$-labelled genistein and daidzein administered to 16 premenopausal women. R^2 values were as follows: between plasma daidzein C_{max} and total urinary daidzein 0.0558; between plasma genistein C_{max} and total urinary genistein 0.4211; between plasma daidzein C_{max} and urinary daidzein concentration 0.085; and between plasma genistein C_{max} and urinary genistein concentration 0.286. Though the scatter of points was wide, all correlations were highly significant.

Outliers in spot urine isoflavone concentration

Isoflavone concentration in spot urine samples taken 5-7 hours after supplementation correlated with isoflavone intake. However, outliers in spot urine concentration skewed the mean spot urine isoflavone intake during 110mg/d supplementation to produce an apparent plateau in urine concentration at high intakes that was not evident with 24-hour urine collections. This effect disappeared when two outliers – both noticeably dark urine samples - were excluded. For this reason, the relationship between spot urine concentration and the volume of the 24-hour urine collection from the previous 24 hours (as an approximation or surrogate measure of urine concentration on the day of spot urine sampling) was examined.

However, no correlation was found between 24-hour urine volume and isoflavone concentration in spot urine samples on the following day, even when outliers were included (Figure 31).

Figure 31:



One method of correcting for the concentration of spot urine samples when assessing the excretion of a single metabolite is to measure creatinine concentration in the spot urine sample. Urinary creatinine is then compared to the mean creatinine excretion per day for an individual of the same age, gender and bodyweight and a correction factor applied to the concentration of the metabolite of interest. This method has been applied in some (Grace *et al.* 2004) but not all (Ritchie *et al.* 2004a) studies of urinary isoflavones.

The results of this study indicate that even without creatinine adjustment, variation in isoflavone concentration in spot urine samples is sufficiently related to isoflavone intake to be used as a biomarker of intake. However, correcting for urinary creatinine would be likely to produce a more robust biomarker and would avoid the need to exclude very concentrated urine samples. Grace *et al.* (2004) corrected PE concentration in spot urine samples for creatinine and found that this produced stronger correlations with plasma PE concentrations. However, as discussed previously, correlations between plasma and urinary isoflavones in this study were robust by comparison with other published studies.

Percentage urinary recovery

The relationship between isoflavone intake and urinary excretion over 24 hours derived from the results of this study indicates that percentage recovery over 24 hours will range from approximately 30% at intakes of 160mg/d to approximately 40% at intakes of 25mg/d. There are a number of reasons why the ingested dose is not completely recovered in 24-hour urine collections. Firstly, a small proportion of the ingested dose is excreted in faeces, either without being absorbed or after hepatic conjugation and biliary excretion (Adlercreutz & Mazur, 1997; Setchell *et al.*, 2003b). Secondly, some of the ingested isoflavones are metabolised to other compounds before excretion. Genistein and daidzein may be metabolised to 3'7-dihydroxyisoflavan, dihydrodaidzein, O-DMA, equol, methylequol and other compounds (Adlercreutz & Mazur, 1997; Chang & Nair, 1995; Setchell *et al.*, 2003b). Thirdly, urinary excretion of isoflavones from a single dose is not complete within 24 hours but may also continue into the second and third day after ingestion (Setchell *et al.*, 2003a).

Bloedon *et al.* recovered 27-42% of ingested dose in urine during 24h after isoflavone ingestion, using doses of 2, 4, 8 and 16mg/kg bodyweight.
Setchell *et al.* (2003a) recovered an average of 30% of 13C daidzein and 9% of 13C genistein in urine over 24h. Most of the recovered isotopes were excreted in the first 24h after administration with a small proportion excreted on the second day and excretion on the third and fourth days comparable to that before dosing. The cumulative percentage recovery of genistein at 0.4mg/kg was 8.9% and at 0.8mg/kg was 8.3%. For daidzein, cumulative percentage recovery at 0.4mg/kg was 34.5% and

at 0.8mg/kg was 25.6%. However, no conclusions regarding the non-linearity of relationship between dose and percentage recovery can be reached from two different doses.

Limits of isoflavone uptake and excretion: the 'plateau effect'

In this study, curvilinear relationships were demonstrated between isoflavone dose and subsequent plasma isoflavone concentration and between isoflavone dose and percentage urinary isoflavone recovery over 24 hours. This has not been previously demonstrated although some data have suggested such a relationship (Setchell *et al.* 2003b).

Setchell *et al.* (2003a) reported that the bioavailability of genistein and daidzein did not increase linearly between doses of 0.4 and 0.8mg/kg bodyweight (equivalent to 29.2 and 58.4mg/d for a 73kg person). Bioavailability as measured by AUC_{inf} for daidzein was 5.15 for the lower dose and 8.70 for the higher dose, while for genistein it was 6.33 and 9.77, respectively.

Bloedon *et al.* (2002) noted some decrement in the percentage of the dose excreted in 24 hours at the higher doses they administered (doses were 2, 4, 8 and 16mg oral isoflavones per kg bodyweight). Assuming a similar mean weight to participants in the present study (73kg), this would equate to doses of 146, 292, 584 and 1168mg/d. However, this same study reported a linear relationship ($r^2 > 0.92$) between maximum plasma concentrations of total genistein and total daidzein and between the area under the plasma concentration-time curve (AUC) ($r^2>0.98$) for total genistein and daidzein

with increasing dose. They therefore propose that decrement in percentage urinary recovery over 24 hours was due to the dose not being fully excreted within 24 hours.

Izumi *et al.* (2000) showed that very high plasma concentrations of isoflavones can be attained after pharmacologic doses. Pharmacologic doses may override any rate-limiting step, a common phenomenon that has previously been demonstrated for vitamin E (Traber *et al*. 1998 cited in Setchell *et al*. 2003a).

The study by Bloedon *et al.* (2002) used two different isoflavone formulations, one 100% unconjugated and one 70% unconjugated, in contrast to the isoflavone glycosides administered in this study and present in the soy nuts administered by Setchell *et al*. (2003b). The mechanism of isoflavone absorption is not well elucidated but since isoflavones are more slowly absorbed when conjugated to glucose (Kano *et al.*, 2006), it is reasonable to speculate that the rate of isoflavone absorption might be limited at these larger doses, resulting in incomplete absorption of the isoflavone dose from glycosides before normal intestinal transit has removed the compounds from sites where they can be absorbed. Gut transit time is already known to be a significant determinant of isoflavone bioavailability (Hendrich, 2002). However, dose-response also tended towards curvilinearity in a study which used three ascending doses of isolated, $^{13}C_3$-labelled genistein and daidzein (Setchell *et al.* 2003b). Whether isoflavones are ingested with or without other foods may also be significant.

Further studies of the relationship between isoflavone formulation and percentage urinary recovery at increasing doses, or between isoflavone dose and faecal isoflavone recovery, could address this question.

If the proportion of the ingested dose absorbed is less for a larger dose, then studies or therapeutic regimens designed to produce high plasma concentrations of isoflavones would be more precise if supplements are administered in divided doses.

The effect of gender and body mass index

The effect of gender

No significant difference was found between the sexes in 24-hour urinary isoflavone excretion or plasma isoflavone concentration during this study.

Published data on the effect of gender on isoflavone bioavailability and excretion are contradictory. A 10-week soy feeding study in 76 healthy adults (Wiseman *et al.*, 2004) found no significant interaction of gender with plasma, urinary or faecal isoflavone concentrations with the exception of significantly higher plasma O-DMA concentrations in men than in women.

Bloedon *et al.* (2002) found significantly higher urinary excretion of total genistein and daidzein over 24 hours in women than in men, but only when the isoflavone formulation was 100% unconjugated, not when a 70% unconjugated formulation was administered.

Faughnan *et al.* (2004) reported significantly higher urinary genistein recovery in women after consumption of soy milk compared with textured vegetable protein, while in men no difference in urinary genistein was detected between the different soy foods. They did not find any effect of gender upon urinary daidzein excretion.

Zhang *et al.* (1999) found that bioavailability of daidzein and genistein based on plasma concentrations were similar in men and women although mean 48-hour urinary excretion of these isoflavonoids was significantly different.

Lu and Anderson (1998) found that women initially excreted more isoflavones in urine than men after soy milk supplementation, but that during the course of their 4-week study a progressive decrease in urinary excretion of genistein and daidzein was observed in women but not in men. This may explain the lack of any apparent gender difference in the present study. Since each participant completed three or four weeks of isoflavone supplementation (albeit separated by wash-out phases) and the order of supplements was randomised, any tendency for women to excrete more isoflavones in urine in response to supplementation might have been obscured by the duration of supplementation. The same would apply to the study by Wiseman *et al.* (2004).

The effect of body mass index

If isoflavones, due to their lipophilicity, are able to accumulate in adipose tissue then the ratio of lean to fatty tissue in an individual might affect the plasma concentrations or rate of excretion of these compounds. Therefore, the relationship between participants' body mass index (BMI) and plasma concentrations and 24-hour urinary excretion of isoflavones was tested. No correlation was found between BMI and plasma isoflavone concentration or between BMI and 24-hour urinary isoflavone excretion. The range of BMI among the study participants was 17.53-31.80 (mean 25.05), demonstrating that the biomarkers are valid over a wide range of body mass index.

Timing of spot urine and blood samples

Blood samples and spot urine samples were taken between 5 and 7 hours after ingestion of the supplement. This time interval was chosen because the peak concentration of isoflavones in plasma occurs at approximately 5-7 hours after ingestion (Bloedon *et al.* 2002; Setchell *et al.* 2003a, 2003b).

The shape of plasma appearance and disappearance curves for isoflavones are consistent within and between individuals and at different doses (Setchell *et al*. 2003a). Thus, provided that all participants were sampled at the same interval after supplement ingestion a reasonably strong correlation would have been found between intake and plasma concentration or excretion. However, the timing of samples after isoflavone ingestion will profoundly affect the plasma concentration that is associated with a particular intake.

When applying biomarkers in intervention studies where supplements are given once daily, the equations derived from this study to relate intake to excretion up to 165mg/d can be most accurately applied if samples are taken 5-7 hours after supplement ingestion. Divided doses of isoflavones throughout the day have been less studied, but if three doses per day were taken and the plasma curves superimposed then the diurnal variations in plasma and urine isoflavone concentrations will be less great and the timing of blood and urine sampling becomes less important. The 'divided doses' pattern of isoflavone ingestion is common when isoflavone-rich foods are integrated into the habitual diet, and Grace *et al.* (2004) validated untimed spot urine and plasma samples as biomarkers of low (<1.5mg/d) dietary intakes of PE consumed as part of the habitual diet throughout the day.

LIMITATIONS

Blinding:

No manufacturer could be found who would produce the three different potencies of isoflavone supplement required for this study. While this range of doses is available over-the-counter in a range of formulations, each individual manufacturer only produces one potency. In order to keep the tablet formulation constant in each phase of the study, the higher doses were achieved by giving two or three tablets at once. This compromised the blinding of the study, as participants might easily have guessed that taking more tablets equated to a higher dose phase. However, since pharmacokinetic parameters not symptoms or subjective measures were used to assess outcome, imperfect blinding was deemed to be less important than using supplements with the same formulation for each phase.

Creatinine adjustment:

Given that the problems of correlating spot urine to blood are found in samples of very concentrated urine (dark colour, small volume, unexpectedly high isoflavone content), creatinine adjustment would have been likely to produce a more robust correlation between intake and spot urine isoflavone concentration.

Controlling extraneous variables

Isoflavone pharmacokinetics might have been affected by other components of participants' diets or other supplements and medications they consumed during the study. In any study with free-living participants, compliance can only be monitored by verbal checks, the timing of supplements and samples can be approximately but not precisely controlled and there is no control over physical activity, diet and fluid consumption. However, since this study was intended to provide a tool for clinical and observational studies, the fact that such naturally-occurring variables were present increases the generalisability of the work to other free-living human studies, even at the expense of internal validity.

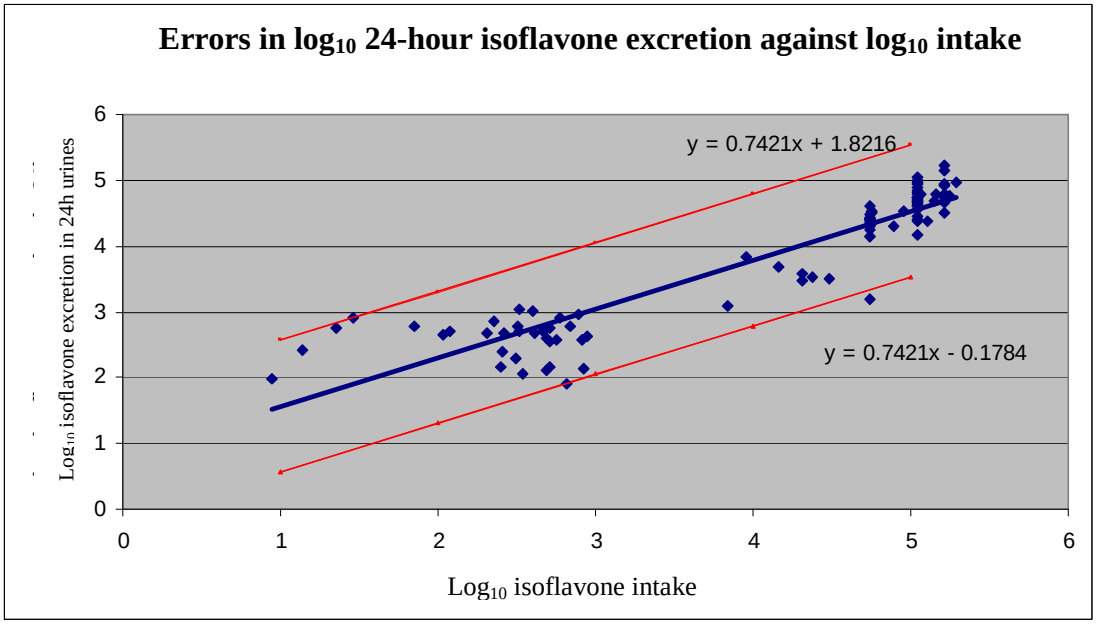
Interpretation of log-log graphs

Log-log graphs were used to clarify the relationship between intake and isoflavones in biological fluids. A larger value of r^2 (Pearson's correlation coefficient) for the linear regression lines on log-log graphs demonstrated that this model fitted the data better. The same treatment of data has been applied in other studies of isoflavone biomarkers (Arai *et al.*, 2000; Atkinson *et al.*, 2002; Frankenfeld *et al.*, 2003; Frankenfeld *et al.*, 2002; Grace *et al.*, 2004; Ritchie *et al.*, 2004a; Ritchie *et al.*, 2004b) and pharmacokinetics (Setchell *et al.* 2003b).

However, examining the log-log graphs alone masks the spread of data points in the original data set. For example, Figure 9 shows the relationship between isoflavone intake and 24-hour urinary excretion. It can be seen from the graph that the spread of data points becomes wider with increasing intake. When the data are transformed using $\log_{10}$ (Figure 13), this spread of data at high intakes is no longer evident.

Margins of error on log-log graphs of the relationship between isoflavone intake and biomarkers appear small. For example, Figure 32 is a scatter plot of log_{10} isoflavone intake against log_{10} isoflavone excretion in 24hour urines with the margins of error encompassing the spread of data points shown in red. On this graph a y-axis error of +/- 1 includes most of the outliers at all levels of intake.

Figure 32:



The equation of the upper error line is;

log_{10} intake = 0.7421(log_{10}24hU excretion) + 1.8216

and the equation of the lower error line is;

log_{10} intake = 0.7421(log_{10}24hU excretion) - 0.1784

By computing these equations to give the relationship between intake and excretion, as was performed for the equation of the linear regression on the scatter plot, it can be determined that the equation of the upper error line relates to;

$y = 66.31302x^{0.7421}$

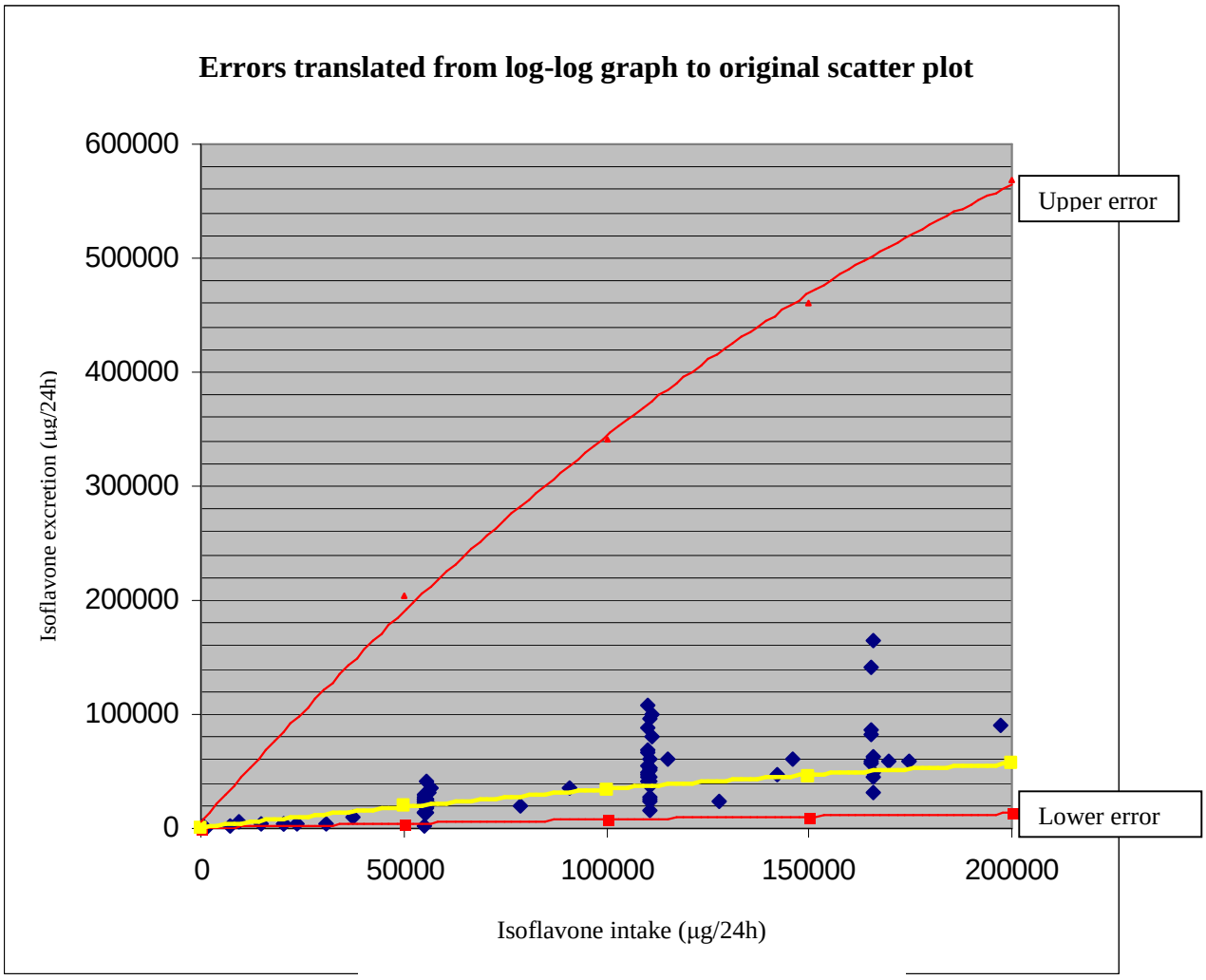
and the equation of the lower error line relates to;

$y = 1.507995x^{0.7421}$

Figure 33 shows these error margins derived from the graph of log(intake) against log(24h urinary excretion) in terms of intake (μg/24h) and excretion (μg/24h). The original data points before log transformation (in blue) and the relationship between intake and 24h urinary excretion derived from Figure 13 (in yellow) are also plotted.

Figure 33 demonstrates the width of the possible errors incurred by using an equation derived from a log-log graph to predict intake from excretion (or *vice versa*) and demonstrates the increased potential for error at higher intakes that is incurred by using this model.

Figure 33:



The above exercise also explains the spread of data points observed on the original scatter plot of intake (µg/24h) against excretion (µg/24h) (Figure 9) and justifies using logarithms of both axes to confirm the relationship between the two variables. This exercise illustrates why the relationships between isoflavone intake and biomarkers must be interpreted with caution.

CONCLUSIONS

The aims of this study were to validate 24-hour urine, timed plasma and timed spot urine samples as reliable measures of isoflavones intakes up to 165mg/d and to determine whether plasma concentration or urinary excretion reaches a plateau at this level of intake.

From the results presented it can be concluded that all the above biomarkers are valid for this intake range and can be applied in epidemiological or intervention studies as measures of isoflavone intake or compliance with isoflavone supplementation. A further application might be in assisting extrapolation of *in vitro* findings to corresponding human intakes based upon the concentration at which effects are manifest *in vitro.*

A curvilinear relationship was defined between a) isoflavone dose and bioavailability in plasma and b) isoflavone dose and urinary excretion over 24 hours.

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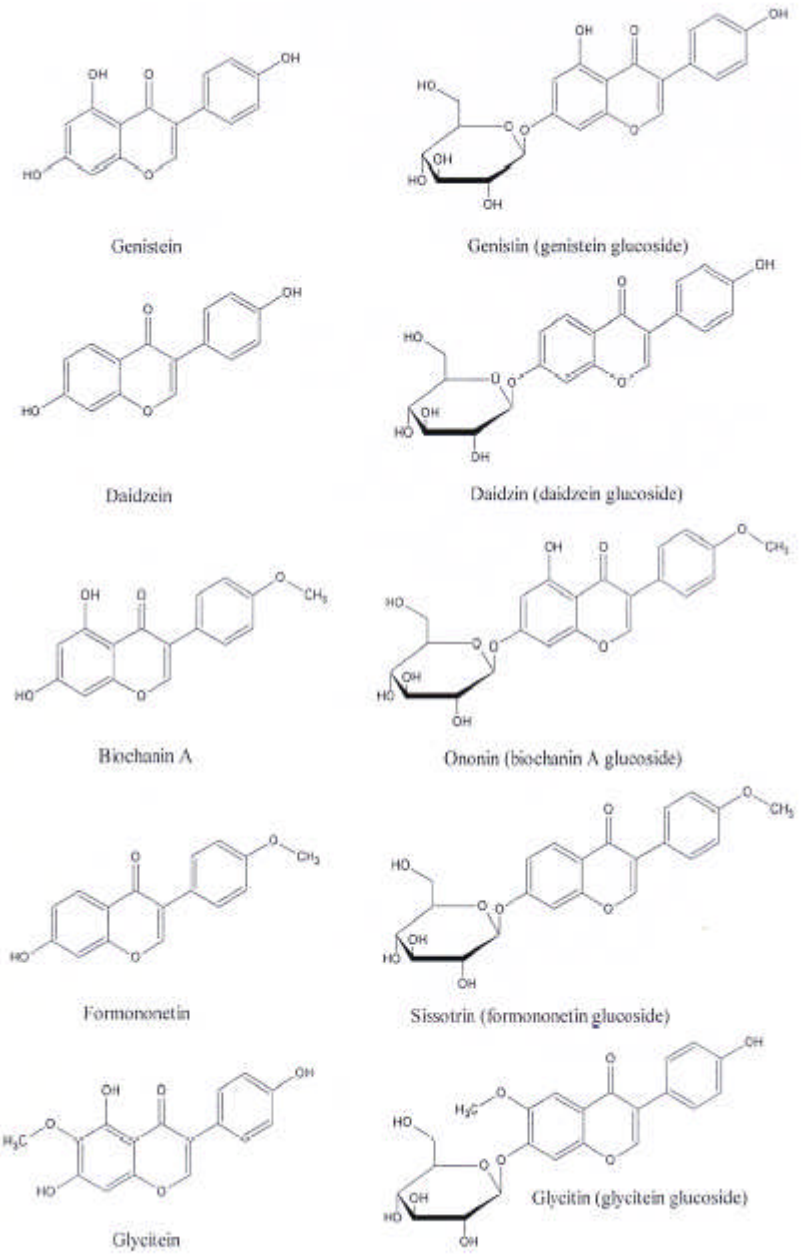
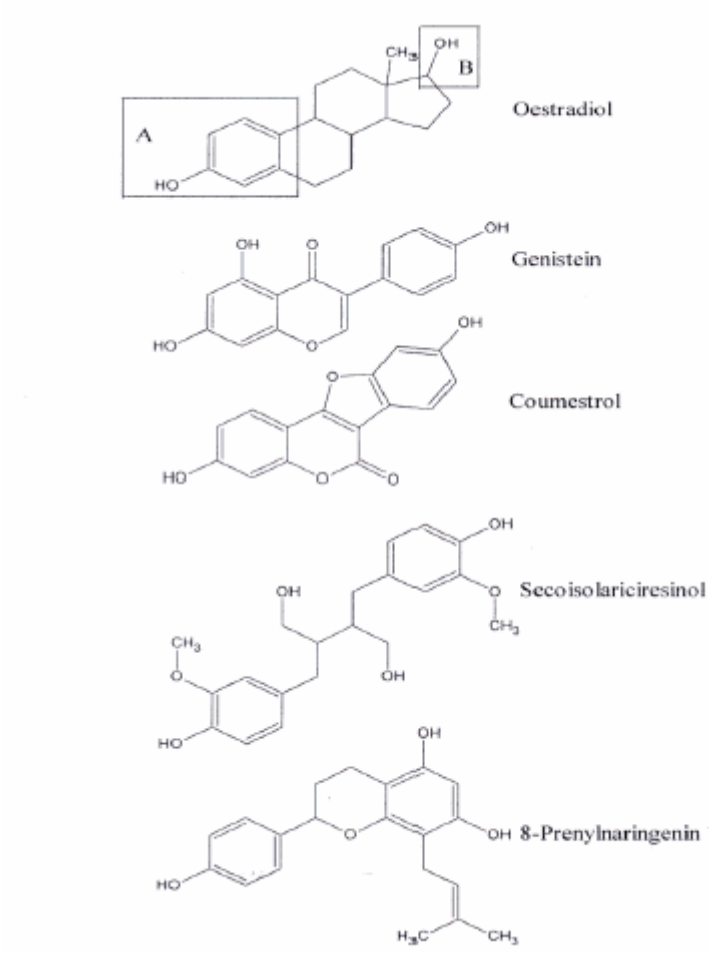
Zhang, Y., Wang, G.-J., Song, T., Murphy, P. & Hencrich, S. (1999). Urinary disposition of the soybean isoflavones daidzein, genistein and glycitein differs among humans with moderate fecal isoflavone degradation activity. *Journal of Nutrition*, **129,** 957-962.

Appendix 1: Isoflavones and their glycosides. (Reproduced from COT 2003b)

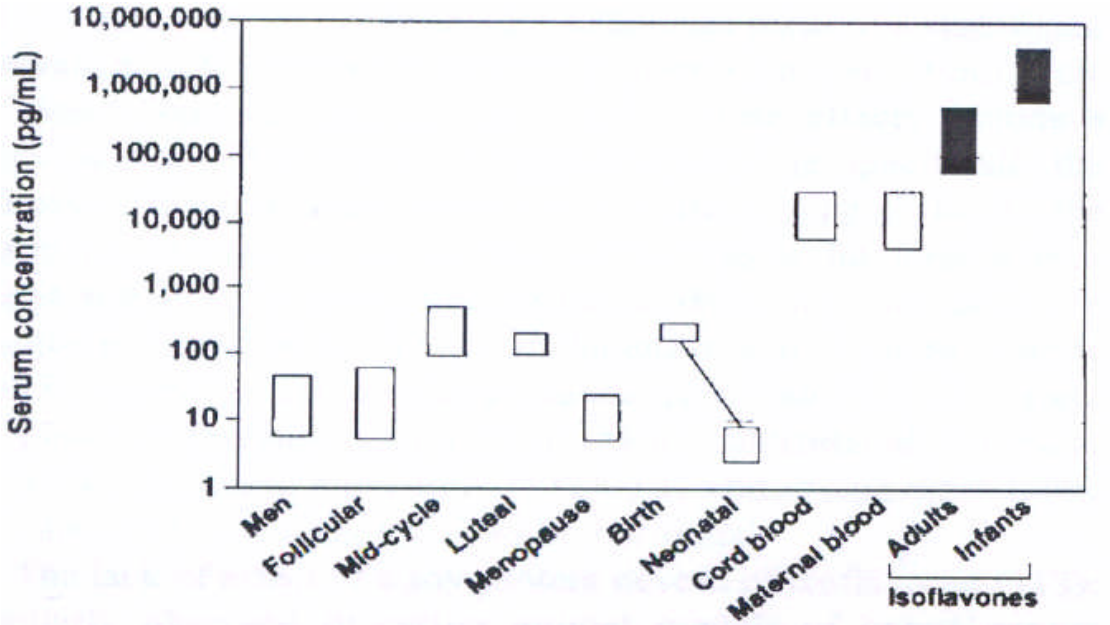


Appendix 2: Structural similarity of PE to oestradiol (reproduced from COT 2003b)



Appendix 3: Disparity in serum concentration between PE and oestradiol (reproduced from Setchell 1998)

Oestradiol is shown as unfilled boxes (□), isoflavones as black boxes (■). Data are shown on a log scale.



Appendix 4: Isoflavone Tablet Specification

Manufacturer:	Custom Pharmaceuticals
Supplied by:	Lamberts Healthcare Ltd
Tablet Physical Properties	
Shape	Ovaloid
Mean Weight	1030mg
Colour	White
Quantity per carton	30 Tablets

FORMULATION

Tablet Content	Quantity Per Tablet	**Unit**
RM/12/031 Microcrystalline Cellulose	428.000	mg
RM/13/022 Soya Isoflavone Extract (40%) *Glycine max*	250.000	mg
RM/03/191 Calcium Carbonate	210.000	mg
RM/15/169 Pregelatinised Maize Starch	71.000	mg
RM/01/002 Croscarmellose Sodium	30.000	mg
RM/18/041 Stearic Acid Vegetable	15.000	mg
RM/12/011 Magnesium Stearate	5.000	mg
RM/01/007 Silicon Dioxide Colloidal	1.000	mg
CORE WEIGHT	**1010.000**	**mg**
Coating		
RM/12/039 Hypromellose 5cps	17.450	mg
(RM/12/150) Mastercote White FA0961G solids	10.100	mg
RM/07/008 Glycerol Vegetable	2.760	mg
COATING WEIGHT	**30.31**	**mg**
TABLET WEIGHT	**1040.310**	**mg**

FINISHED PRODUCT SPECIFICATION

Tablet Appearance	White, ovaloid, film coated tablet
Odour	Little or none
Uniformity of Weight	Complies with EP Specification
Average Weight (cores)	1010mg (limits 990 – 1030mg)
Average Weight (coated)	1030mg (limits 1000 – 1060mg)
Length (cores)	19.6mm (limits 19.0 – 20.2mm)
Width (cores)	8.45mm (limits 8.2 – 8.7mm)
Thickness (cores)	7.2mm (limits 7.0 – 7.4mm)
Hardness (cores)	Not less than 20kp
Friability (cores)	Not more than 0.3%
Disintegration (cores)	Not more than 30 minutes
Disintegration (coated)	Not more than 60 minutes

NOTE

1. Soya Isoflavone Extract (40%) – Genistin 48mg, Glycitein 5mg, Daidzin 41mg

A Certificate of analysis should be supplied with each batch.

This specification should be read in conjunction with our general purchasing conditions for tablets, and supercedes all previous issues.

Appendix 4: Placebo Tablet Specification

NOVOGEN
new generation pharmaceuticals

Product Specification

PRODUCT:	P081 Placebo
APPEARANCE:	An 11mm biconvex tablet. Coating colour WHITE.
THEORETICAL WEIGHT:	Coated 618mg
WEIGHT RANGE:	Coated 600 to 640mg
DISINTEGRATION:	Not more than 30 minutes
THICKNESS:	Coated 4.5 to 5.5mm

INGREDIENTS	Test Ref.	mg/Tablet
Note: Placebo, no active required		
EXCIPIENTS		
d-alpha TOCOPHEROL	S/S	0.42
beta TOCOPHEROL	S/S	0.03
gamma TOCOPHEROL	S/S	1.86
delta TOCOPHEROL	S/S	0.69
on a carrier of ACACIA GUM	S/S	3.0
CELLULOSE MICROCRYSTALLINE	BP93	63.78
CALCIUM HYDROGEN PHOSPHATE	BP93	519.34
SOY POLYSACCHARIDE	S/S	1.81
MAGNESIUM STEARATE	BP93	9.05
SILICA-COLLOIDAL ANHYDROUS	USP95	3.02
		603mg
COATING		
OPADRY OY29020 CLEAR	S/S	5.0
OPADRY – COLOUR WHITE Y-I-7000	S/S	10.0
CARNAUBA WAX	BP93	0.03

APPROVED BY QA SUPERVISOR:

APPROVED BY PRODUCTION DIRECTOR:

02/03/99 ACERO

Appendix 5: Recruitment poster

Healthy volunteers required for a study in nutrition

We are looking to recruit healthy men and women to take part in a study of compounds in the food which you eat regularly and in a nutritional supplement made from soya beans.

We wish to measure the levels of certain plant compounds in your urine and blood and see how this changes when you take a supplement.

The study will involve three weeks of supplementation and we will ask you for blood and urine samples twice during each of these periods.

For more information and to volunteer
please contact:

Dr Margaret Ritchie
Research and Teaching Fellow
Cancer Biology Group
Bute Medical School
Tel 01334 463 534

Miss Jay Mackinnon, ljm62@st-andrews.ac.uk
PhD student
Cancer Biology Group
Bute Medical School
Tel 01334 463 534

Appendix 6: Pre-enrolment telephone interview schedule

Comparison of Plasma and Urine Concentrations of Phyto-oestrogens Following Dietary Isoflavone Supplementation

PROCEDURE FOR THE INITIAL TELEPHONE SCREENING OF VOLUNTEERS

Time _____

Date _____

1. Thank-you for telephoning
2. Where did you hear about the study?
   _____
3. First of all, can I ask you a few questions just to see if the study is suitable for you, and then, if it appears that it is, we can arrange an appointment with myself so that you can discuss the details with me and find out if you wish to take part in the study. I will post out a volunteer information sheet to you which you may read over before you come for your appointment.
4. Full name
   _____
5. Age _____ Date of Birth _____
6. Contact phone number (home) _____
   (work) _____
7. What is your home address?

   Postcode:
8. Sex: male / female
9. Smoking habits? Current smoker / ex-smoker / non-smoker
10. Do you have any health problems?
    Yes / No
11. Are you currently taking any medication?
    _____
    _____

12. Have you taken any antibiotics within the last six weeks?
    Yes / No

13. Do you take any dietary supplements?
    Yes / No

14. If yes, what are they?
    _____
    _____

15. What is the name and address of your GP?

16. Are you resident within the St Andrews area and able to participate within the next two to six weeks?
    Yes / No

17. We will be asking for blood samples, urine samples, and 24-hour urine collections. Are you willing to do this?
    Yes / No

18. You will also be asked to complete food diaries. Are you willing to do this?
    Yes / No

19. Are you currently participating in any other research study?
    Yes / No

20. Are you currently in employment? (question asked for scheduling purposes)
    Full time _____
    Part time _____
    Not employed _____

If no points which indicate that the trial is unsuitable for the volunteer, then proceed to arrange an appointment, preferably within the next two to six weeks. Appointment should be booked and the time recorded in the study diary. Interview times are between 10.30am and 1.30pm on Monday, Wednesday, Thursday and Friday. Three people can be booked into one appointment time and the appointment should last for about 20 minutes.

Emphasise that an appointment does not mean commitment to the study, it provides an opportunity to get more information so that they can make up their minds after they have a chance to ask questions and discuss it in detail.

Send out an appointment card and letter with the volunteer information sheets and directions to the Bute Medical Building.

Appendix 7: Participant information sheet

Information for Participants

Comparison of Plasma and Urine Concentrations of Phyto-oestrogens Following Dietary Isoflavone Supplementation.

We invite you to take part in a research project. We believe it to be of potential importance. However, before you decide whether or not you wish to participate, we need to be sure that you understand firstly why you are doing it, and secondly, what it would involve if you agreed. We are therefore providing you with the following information. Read it carefully and be sure to ask any questions you have. We will do our best to explain and to provide any information you may ask for now or later.

Reason for the study

Foods contain a number of different nutrients such as fats, carbohydrates, vitamins, minerals and other substances. Some of these substances are called plant oestrogens. Plant oestrogens such as isoflavones are naturally occurring chemicals in soya beans and other foods. There have been a number of studies investigating their role in the prevention of several diseases such as cancer or heart disease. In order to assess the amounts of these compounds in your diet, we measure them in blood and urine.

This study aims to find several important pieces of information: how much of these plant oestrogens is present in your blood after you eat them and how much is excreted in your urine. This information could lead to a better understanding of what happens to these compounds in the body and help us to provide better nutritional advice in the future.

What the study will involve

We would like to ask healthy volunteers to help with a study which will last for six weeks, and will measure the levels of plant oestrogens in blood and urine samples when you are taking a supplement and when you are not.
If you agree to take part in the study, you will be asked to read and sign the local Ethics Committee standard consent form.

At the initial interview and discussion appointment you will be given a food diary to fill in for 7 days. You will also be given a measuring jug and urine container so that you can collect all the urine you pass over a 24-hour period, and tablets to take during this collection period which will allow us to check whether your urine collection is complete.

Visit 1:
We will collect your 24-hour urine sample and food diary from you, and you will be asked to provide another small urine sample and a small blood sample (10ml, which is about 2 teaspoonfuls). We will use these to measure the amount of isoflavones which are present before you supplement your diet.
You will be provided with 7 days supply of the supplement, which you should take at the same time each day. The time of day will depend on when you can visit us, and we will agree this together.

Interval Visits:
You will be asked to attend interval visits three days after Visit 1, ten days after Visit 2 and ten days after Visit 3, just to provide a small urine sample and blood sample and to tell us about the foods you have eaten over the previous 24 hours. This allows us to see how the foods you eat are having an effect on the plant oestrogens in your blood and urine.

Visit 2, Visit 3 and Visit 4:
Visit 2 will be one week after Visit 1, Visit 3 will be two weeks after Visit 2 and Visit 4 will be two weeks after Visit 3.
The day before each visit, we would like you to collect all the urine you pass over a 24 hour period and to take tablets as you did the first time you made a 24-hour urine collection, to allow us to make sure it is complete. On this day and on the day of your visit, we would also like you to keep a record of all that you eat and drink.
We will collect your dietary record and your 24-hour urine collection from you at the visit, and take another small blood sample and small urine sample. We will give you another 7 days supply of supplements, which you should begin to take **one week after** your visit. For the week following each study visit you will not need to take any supplements.
Visit 4 will be your last visit, so you will not be given any more supplements at this visit.

Finding out the study's results

Results will be published in scientific journals. We will send you a brief summary when the final results of the study are available. If you would like to receive them, we will also send you a summary of your own nutrient intakes estimated from your food diaries.
All the information you give us will be treated as confidential and used solely for the purposes of the research study. Individual participants will not be identifiable in any report about the study.

You are under no obligation to take part in this study. If you do decide to take part, you are free to withdraw at any time without giving a reason and without it affecting your future medical care. Please feel free to ask any questions you may have about the study (the contact number is below)

Dr Margaret R. Ritchie 01334 463 534
Research Fellow and Teaching Fellow
Bute Medical School, University of St Andrews

Appendix 8: Consent form (printed on Bute Medical School letterhead)

Comparison of Plasma and Urine Concentrations of Phyto-oestrogens Following Dietary Isoflavone Supplementation

Consent Form

The volunteer should complete this form herself/himself

	Please circle as appropriate
Have you read the volunteer information sheet?	**Yes / No**
Have you received enough information about the study?	**Yes / No**
Have you had the opportunity to ask questions and discuss the study?	**Yes / No**
Have you received satisfactory answers to all of your questions?	**Yes / No**
Who have you spoken to? …….....	
Do you understand that you are free to withdraw from the study;	
At any time?	**Yes / No**
Without having to give a reason?	**Yes / No**
Without this affecting your future medical care?	**Yes / No**
Do you agree to take part in this study?	**Yes / No**

Volunteer's signature……. Date……

Name (please print)……..

Contact telephone numbers:
Home……..…… Mobile……..
Email address: ……..

Researcher's signature…… Date……...

Appendix 9: Letter to general practitioners

[printed on Bute Medical School letterhead]
West Burn Lane
St Andrews
KY16 9TS

[date]

[practice address]

Dear Doctors,

Re: [participant name and address]
DOB:

Your patient, [name], has agreed to participate in a study investigating the relationship between soy isoflavone supplementation, concentrations of isoflavones in plasma and isoflavone excretion in urine.

S/he will be asked to complete a diet diary and to provide urine and blood samples, and will be randomised to receive isoflavone tablets containing 50mg or 100mg of isoflavones or placebo in a crossover design, as detailed in the enclosed information sheet.

If you have any questions about this study or feel, for any reason, that your patient should not take part, please contact Dr Margaret Ritchie at the Bute Medical School, University of St Andrews, tel. 01334 463 534, email mrr3@st-andrews.ac.uk

Yours sincerely,

Dr Margaret R. Ritchie
Research Fellow and Teaching Fellow

Appendix 10: Dietary recording instructions

Cancer Biology Group, Bute Medical School, University of St Andrews, KY16 9TS

Comparison of Plasma and Urine Concentrations of Phyto-oestrogens Following Dietary Isoflavone Supplementation

FOOD RECORD BOOK

7-DAY RECORD

Name:_____

***Please bring this Record with you when you attend the Study Visit at The Bute Medical School, University of St Andrews.**

Instructions for recording your food and drink

Amount/portion. Describe each item in **spoonfuls,** or other **household measures,** or as **small, medium or large portions**

Breads and Rolls:

Description Give type of bread or rolls (white, brown, wholemeal etc.).
Size of portion. Record the number of slices of bread and whether thick, average or thin sliced. For rolls record size. Note size of left over portion.

Fat Spreads

Description Give type of fat spread (Olivio, Tesco Low-fat spread, Flora, Flora-light, Flora-Extra Rich, Safeways sunflower etc), or state `unknown'.
If spreading on bread/rolls record whether thin, thick or average
Size of portion. Record the amount (1 curl or 1 teaspoon) added to potatoes/vegetables or used in frying etc. Record the amount of any left over portion.

Cooking Fats and Oils

Description Type and brand (hard vegetable oil, lard, Tesco vegetable oil, Soya, Sunflower or Olive oil etc.).
Amount of oil used i.e. number of tablespoons or teaspoons. If used for frying, state whether shallow or deep frying.

Sandwich or Roll Fillings

Description Give as complete details as possible: cheese, ham, beef, tuna etc. + salad, mayonnaise, pickles. (NB remember type of bread). Portion size of each individual item in the sandwich , if possible.
Record the amount of any uneaten portion.

Milk: On Cereals, in Hot Drinks, in Cold Drinks, used in home cooking

Description Give type (full, half or low fat, condensed, dried skimmed etc.).
Amount of milk used e.g. glassful, cupful, tablespoons.
Record the amount of any left over portion

Meat and Fish

Description Give type (and cut of meat), and method of cooking, and if +skin and fat eaten. *Remember details of fat/oils used*
Size of portion. Record amount of any uneaten portion.

Vegetables

Description Give type, whether fresh or tinned, and the method of cooking, or if eaten raw. *Remember details of fat/oils used*
Size of portion. Record amount of any uneaten portion. (of each vegetable).

Fruits

Description Give type, if raw, tinned or cooked, and whether skin eaten.
Size of portion. Record amount of any uneaten portion.

Home-Cooked Dishes

Description Give name of dish and main ingredients used. *Remember details of fat/oils used.* Record amount of each ingredient used and the method of cooking. Record the amount/size of the portion taken. Record the amount of any uneaten portion.

Ready-prepared/Bought Dishes

Description Give the full information provided on the packet (or stick ingredients and nutrition label into food record book).
Size of portion. Give the size/amount of the portion taken. Record the amount of any uneaten food

Eating Out

Description Describe all food items as fully as possible, and method of cooking if known. Give type of shop/restaurant (chip shop, indian etc).
Amount/portion. Describe each item in spoonfuls, or other household measures, or as small, medium or large portions. Record the amount of any uneaten food

Cereals, Biscuits, Sweets and all other Foods

Description: Give Brand Name and type in as much detail as possible.
Size of portion. Record the amount of any uneaten portion.

Non-alcoholic Drinks

Description: Give type, and additions of milk, sugar etc. to hot drinks. Say whether squash, fizzy, diet or natural fruit juices.
Size of portion e.g. average glass, carton, tumbler, wine glass etc.
Record the amount of any portion which is not drunk.

Alcoholic Drinks

Description Give type (heavy, lager, red/white wine, whisky etc.) and mixers.
Amount / portion e.g. pint, half pint, bottle, small can, large can or record as single pub measures.

Foods for which you regularly use only 1 type and brand

If, for example, you **always** use semi-skimmed milk at home, or always cook with a blended vegetable oil, you may give these details below, to avoid repeating the information each time you eat it.

Type of milk ALWAYS
used:_____
Type of fat spread ALWAYS
used:_____
Type of _____ALWAYS
used:_____
Type of _____ALWAYS
used:_____
Type of _____ALWAYS
used:_____

Please remember **that if you change from your usual type half way through completing your Food Record, or eat differently when away from home, you must give the details in the record.**

Appendix 11: 24-hour urine collection instructions

Comparison of Plasma and Urine Concentrations of Phyto-oestrogens Following Dietary Isoflavone Supplementation

24-HOUR URINE COLLECTION INSTRUCTION SHEET

Name _____ ID no. _____

Tel no (home) _____
(work)_____

Date of start: day _____ date _____

When you get up on _____ (day), DISCARD your first urine specimen. Please fill in the time you did this
_____

From then on put every specimen (every drop!) into the bottles provided.

Please remember to take PABA tablets with breakfast, lunch and dinner and note the time
Breakfast _____
Lunch _____
Dinner _____

Please note: it is very important that you take the last PABA tablet before 8pm

Put your last specimen in the bottles at about the same time as you did on the previous morning (at the start of the collection). Please fill in the day, date and time below.

Day _____ Date _____ Time _____

Please remember to pass urine before passing a stool, otherwise you may lose some urine.

Did you lose any specimens?
_____

Did you have any problems?
_____

Please return urine collections to _____
on _____

Thank-you for your co-operation. If there are any problems with this collection, may we ask you to repeat it?

Appendix 12: Invalid 24-hour urine collections:

Participant ID	Supplement phase	% PABA recovery
IS 23	Baseline	73
IS 23	55mg/d	20
IS 48	Baseline	78
IS 48	110mg/d	38
IS 63	Baseline	5
IS 63	Placebo	80
IS 63	55mg/d	82
IS 26	110mg/d	72
IS 26	55mg/d	34
IS 26	165mg/d	40
IS 6	110mg/d	77
IS 17	110mg/d	34
IS 24	55mg/d	70
IS 1	165mg/d	56
IS 5	165mg/d	66
IS 61	55mg/d	84
IS 66	Placebo	80