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Evaluation of a modified food frequency questionnaire to measure lignans in Australian men and women

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Evaluation of a Modified Food Frequency Questionnaire to Measure Lignans in Australian Men and Women

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ABSTRACT

Phytoestrogens are plant compounds that possess estrogenic and biological properties that have been postulated to protect against chronic diseases. Isoflavonoids and lignans are two main classes of phytoestrogen that have been investigated for their estrogenic efficacy and occurrence in the human diet. Isoflavonoids are found in soy and related products, whereas lignans are found in a wider range of plant-based foods, such as cereals, vegetables, fruits, legumes, nuts and seeds; and in beverages such as tea, coffee and wine. In Western populations with low dietary intake of soy products, compared to the Asian counterparts, lignans could be a more important and consistent source of phytoestrogens from the diet. Data for the phytoestrogen content in foods are now available, as more recent research has been conducted to quantify content in commonly consumed food choices in Western populations. The collation of these published values has led to the desire to adequately assess lignan intake.

The aims of the research were to evaluate the validity and reliability of a phytoestrogen food frequency questionnaire (FFQ) tool, with a reference method, the weighed food record (WFR), and urinary biomarkers, to measure phytoestrogen intake in the Australian context. The phytoestrogen FFQ was updated and refined to align with food groups and dietary patterns in the current Australian Dietary Guidelines, in particular to optimise measurement of lignans from the FFQ, and utilise current databases of lignan content available from direct measurement of lignans in foods. Intake level and contributing food sources of each class of phytoestrogen, and the associations between social and lifestyle characteristics and phytoestrogen intake and urinary biomarker were also explored.

This was a cross-sectional study that recruited 59 Australian men and women aged 18 to 67 years at Joondalup campus, Edith Cowan University. Intake of lignans, isoflavonoids and enterolignans from foods was assessed using the 277-item phytoestrogen FFQ and 3-day WFR, and excretion was assessed with urinary biomarkers. Published values of phytoestrogen content in foods were utilised to measure the intakes. Subjects collected three 24-hour urine

samples and phytoestrogen concentration was analysed using a liquid chromatography-mass spectrometry (LC-MS) technique for four lignan subclasses, five isoflavonoids and two enterolignans.

Statistical analyses were conducted using IBM SPSS for Windows (SPSS Inc., Version 22 Chicago, IL). Median intake comparisons were assessed with the Wilcoxon signed rank test. Associations between the intake and excretion measurements of two dietary assessment methods were assessed using Spearman's Rho correlations. Level of agreement between methods was assessed with cross-classification analysis and Bland Altman plots. A triangular comparison between the three methods was conducted with the Method of Triads (MOT) using the software R. The Mann-Whitney U test and Kruskal-Wallis one-way ANOVA were used to compare the median intakes and excretion across categories of social and lifestyle factors.

The FFQ had acceptable convergent validity for intake of total lignans and enterolignans when compared to a WFR, in terms of median intakes (lignans: 3914 versus 4302 μg/day, $p=0.09$; enterolignans: 54 versus 65 μg/day, $p=0.81$, respectively); and associations between the two methods (lignans $\rho=0.42$, $p<0.001$ and enterolignans $\rho=0.62$, $p<0.001$). Median intakes of total isoflavonoids from the FFQ and WFR were also comparable (3851 versus 2287 μg/day, $p=0.67$) with a significant association between the two tools ($\rho=0.36$, $p<0.001$). A 7% to 17% of subjects were misclassified into opposite quartiles for different phytoestrogen classes. The FFQ also had acceptable reliability when compared to a repeat FFQ, for all classes of phytoestrogens, with strong correlations between intakes ($\rho=0.78$ to $\rho=0.85$, $p<0.001$ for all) and minor misclassifications into opposite quartiles, between 2% to 5%. The FFQ had poor validity in relation to biomarkers of phytoestrogen excretion. The median intake and excretion of combined lignans and enterolignans were not significantly different (4051 versus 3104 μg/day, $p=0.22$, respectively), but there was no significant correlation between intake and excretion ($\rho=0.16$, NS). For total isoflavonoids, excretion was 10-fold lower than intake from the FFQ (268 versus 3851μg/day, respectively $p<0.001$) and there was no significant correlation between the two tools ($\rho=0.10$, NS). Misclassification into the

opposite quartile was also relatively high for both phytoestrogen classes (21% to 23%). Visual inspection of the Bland Altman graphs showed trends towards greater inconsistency at higher median intakes and urinary excretion. Higher validity coefficients (VCs) were observed for the FFQ in measuring total lignans (VC=0.57) or the FFQ and WFR for measuring combined lignans plus enterolignans (VC=0.79, 0.78, respectively).

Top contributing food sources of lignans were from the nuts and seeds group (30%), nonalcoholic beverages (19%), and breads and cereals (19%); for enterolignans, from dairy products (86%) followed by nonalcoholic beverages (11%). Soy and related food products were the major contributors (78%) to total isoflavones, followed by breads and cereal products (17%).

Female subjects who were Caucasian, were at, or had achieved university education level and took regular commercial dietary supplements, were more likely to have a higher lignan and enterolignan intake and excretion level than subjects with different characteristics.

Based on these findings, we conclude that the modified phytoestrogen FFQ is highly reliable. It would be a useful assessment tool for example to rank usual intake of phytoestrogen classes for individuals within a group, or quantify mean intakes between different population groups. It is not acceptably valid or accurate for estimation of individual phytoestrogen status, for example for use in experimental studies or to investigate associations with chronic diseases. The lack of associations between measurement of the FFQ and biomarkers could partly be due to limitations of the FFQ tool, such as recall bias or inaccuracies in the estimation of frequency of intake or portion sizes. They also suggest that urinary biomarkers alone are not sufficient for estimation of phytoestrogen status and that additional biomarkers obtained from faecal and plasma samples should be considered for a more complete picture of phytoestrogen status.

The declaration page
is not included in this version of the thesis

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KEY WORDS

Phytoestrogens
Lignans
Enterolignans
Isoflavonoids
Dietary intake
Food frequency questionnaire
Validation
Method of triads
Urinary excretion

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LIST OF ABBRVIATION TERMS AND ACRONYMS

FFQ	Food frequency questionnaire
WFR	Weighed food record
SECO	Secoisolariciresisol
MAT	Matairesinol
PINO	Pinoresinol
LARI	Lariciresinol
SYR	Syringaresinol
MED	Medioresinol
END	Enterodiol
ENL	Enterolactone
FSANZ	Food Standards Australia New Zealand
ABS	Australian Bureau of Statistics
NHMRC	National Health and Medical Research Council
USDA	United States Department of Agriculture
PAL	Physical activity level
UK	United Kingdom
USA	United States of America
BMI	Body mass index
MOT	Method of triads
VC	Validity coefficient
LC-MS	Liquid chromatography-mass spectrometry

CHAPTER ONE: INTRODUCTION

1.1 Background and conceptual framework

The consumption of plant-based diets, including a variety of vegetables, fruits, grains, legumes, and nuts and seeds, has been inversely associated with the progression of chronic diseases (World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR), 2007; National Health and Medical Research Council (NHMRC), 2013a; Mann, 2000). Optimising plant-based diets may increase the potential health benefits, which are attributed to vitamins, minerals, and more recently, a diverse array of bioactive constituents in plant foods (NHMRC, 2013a). Of these constituents, phytoestrogens have been thought to protect against chronic diseases, particularly certain types of cancer, due to their estrogenic properties and other biological activities (Adlercreutz, 2007; NHMRC, 2013a; WCRF/AICR, 2007).

The Australian Dietary Guidelines (the Guidelines) and the Australian Guide to Health Eating (AGHE) were developed to provide guidance on the types and amounts of foods from the five food groups to inform dietary patterns that promote health and wellbeing (NHMRC, 2013a, 2013b). The Guidelines encourage Australians to consume a variety of items from the five food groups, with emphasis on the consumption of plant-based foods, such as vegetables and legumes; fruits; grains, especially whole grains and high fibre cereals; and nuts and seeds. It is important to measure the dietary consumption of phytoestrogens in Australia, as the population is likely to follow dietary patterns that include variable amounts of plant-based foods in which phytoestrogens contribute to the perceived health benefits.

The current research focuses on the capability of a modified food frequency questionnaire (FFQ) to measure intake of two major classes of phytoestrogen, the lignans and isoflavonoids, in a sample of the Australian population. The validation of the food frequency questionnaire is the basis of this applied research. The conceptual framework that depicts the relationship

between the development and evaluation of the FFQ is shown in **Figure 1**. Central to the framework is the FFQ and the estimation of phytoestrogen status in relation to dietary intake and urinary excretion. The FFQ food list was reviewed and modified with respect to the content of lignans and isoflavones from published databases, to include a range of commonly-consumed foods based on dietary patterns described in the Australian Dietary Guidelines. Individual differences in colonic microflora could influence the bioavailability and hence metabolism and urinary excretion of phytoestrogens. Demographic, social and lifestyle characteristics such as gender, BMI, bowel habits, and supplements use are also associated determinants of status (Hanna, O'Neill, & Lyons-Wall, 2010). The repeatability of the FFQ was evaluated by administering the FFQ to subjects on two occasions one to two weeks apart; and the validity was evaluated by comparing intake from the FFQ with intake derived from a 3-day weighed food record (WFR) reference method, and with excretion of urinary biomarkers. Phytoestrogen status determined by both the dietary assessment tools and urinary biomarkers, is the outcome of this research that informs the bioavailability and metabolomics profiles of lignans and isoflavonoids. The Method of Triads was applied for a triangular comparison between the FFQ, the 3-day WFR and the urinary excretion of metabolites for estimating phytoestrogen status. The implication of the research is to investigate the potential applicability of phytoestrogen status as a biomarker of health and wellbeing.

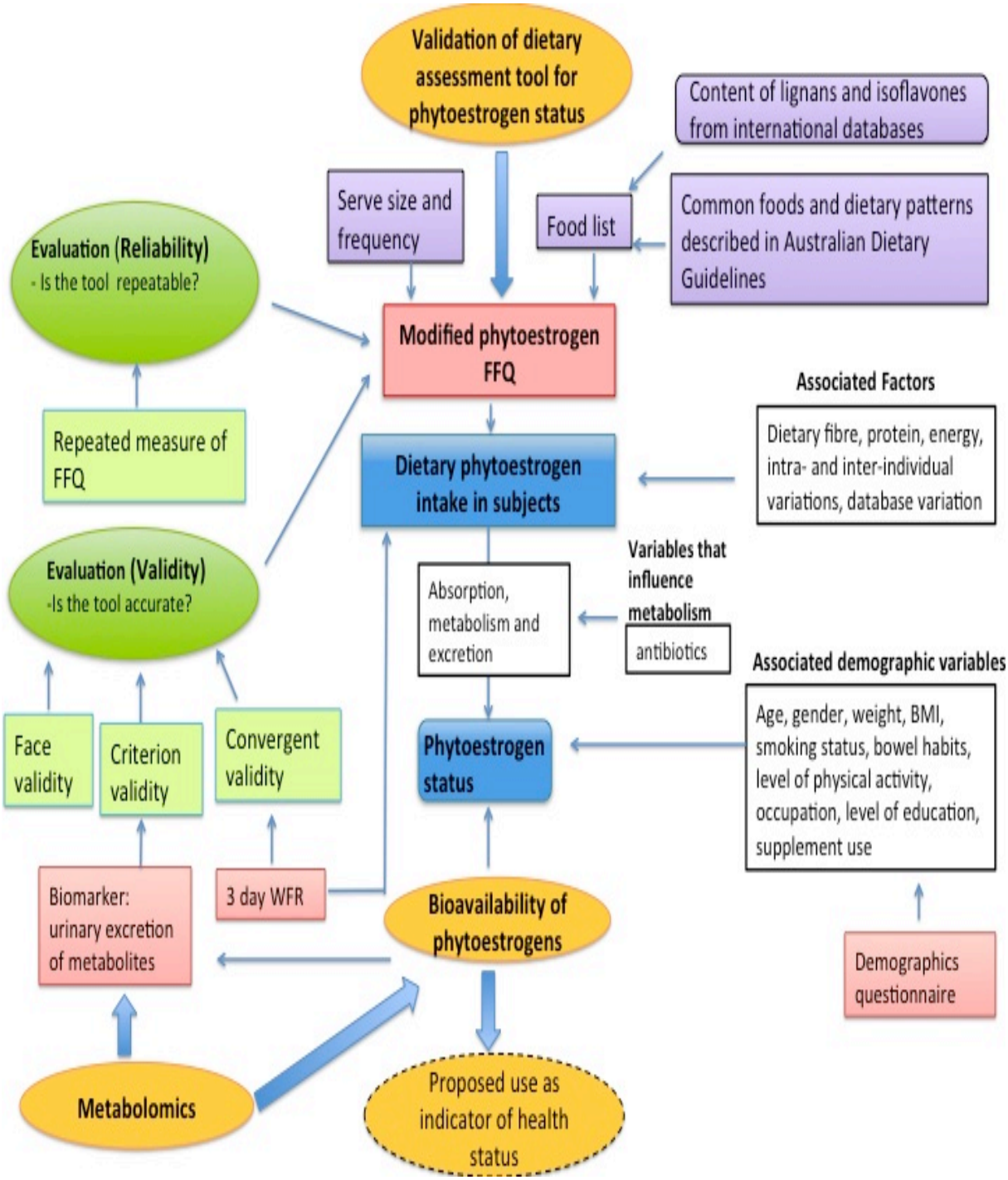


Figure 1. Conceptual framework for the evaluation of a modified FFQ, dietary phytoestrogen intake and status, and associated factors and variables.

The validation of a dietary assessment tool for estimating phytoestrogen status is the basis of this applied research. Methods are shown in green, tools are in red and phytoestrogen status is in blue. Phytoestrogen status determined by both the dietary assessment tools and urinary biomarker is the outcome of this research that informs the bioavailability and metabolomics profiles. If the FFQ is shown to a valid and reliable tool, it could be used to further investigate the role of dietary phytoestrogens in health and prevention of disease

1.2 Aims and hypotheses

This research was conducted in a sample of the Australian population. The aims were:

1. To modify and refine a FFQ to estimate phytoestrogen intake based on a previous questionnaire for use in an Australian population;
2. To estimate the validity of the FFQ by comparison with intake obtained from a 3-day WFR reference method and biomarkers of urinary phytoestrogen excretion;
3. To estimate the reliability of the FFQ by comparison with intake obtained from pre FFQ and post FFQ;
4. To estimate dietary phytoestrogen intake using the FFQ and WFR and evaluate the major contributing food sources;
5. To determine the social and lifestyle characteristics and variables associated with phytoestrogen intake and biomarkers.

The hypotheses being tested are:

1. There is no significant difference between average phytoestrogen intakes derived from the FFQ versus the 3-day WFR reference method.
2. There is no significant difference between the average phytoestrogen intake derived from the pre FFQ versus the post FFQ, administered one week apart.
3. There are significant positive associations between phytoestrogen intake derived from the pre FFQ and post FFQ, and FFQ and WFR; and between phytoestrogen intake estimated from the FFQ or WFR and urinary excretion of phytoestrogen metabolites.
4. There is acceptable agreement observed in Bland Altman plots between individual values for phytoestrogen intake derived from the FFQ and WFR, and pre FFQ and post FFQ; and between phytoestrogen intake estimated from the FFQ and urinary biomarkers.
5. Of the measures of assessment of phytoestrogen status, the FFQ has a higher validity coefficient (VC), calculated with the method of triads, than the WFR or biomarkers; the VCs are within the accepted theoretical range of 0 to 1.

1.3 Significance

There is a need for dietary tools to adequately assess phytoestrogen intake of different populations living in Western locations, including multiethnic groups such as those living in Australia today. Data for the phytoestrogen content in foods are now available, as more recent research has been conducted to quantify content in commonly consumed food choices in Western populations. The collation of these published values has led to the desire to adequately assess lignan and isoflavonoid intake. The public interest in following dietary patterns that promote health and wellbeing has driven the need to know more about the current intake level, and food sources and dietary patterns related to these bioactive compounds such as phytoestrogens. These compounds may play a role in the mechanism and physiological changes that lead to health benefits and wellbeing.

This study evaluates the validity and reliability of a modified FFQ tool to measure phytoestrogen intake in the Australian context. The contribution of these compounds and their association with chronic diseases has not been completely elucidated, indicating the importance of having valid and reliable tools with low burden for assessment of phytoestrogen intake and status. Once validated, the FFQ could be used as the tool to measure phytoestrogen intake in nutritional epidemiological studies to investigate the relationship between dietary phytoestrogen intake and chronic diseases in Australia. As there is currently no optimal intake level established for phytoestrogen, the findings of the research could also contribute to development of an optimal intake level for these bioactive constituents in dietary guidelines.

CHAPTER TWO: LITERATURE REVIEW

2.1 Phytoestrogens

Phytoestrogens are non-steroidal plant compounds that possess estrogenic properties due to structural similarity to mammalian estrogens (Adlercreutz, 2007; Dixon, 2004; Mostrom & Evans, 2011; Wiseman, 2012). The structural features of phytoestrogens, including the diphenolic ring and the position of the hydroxyl groups, are crucial for binding to the estrogen receptor (ER) to elicit estrogenic activity (Miksicek, 1995; Turner, Agatonovic-Kustrin, & Glass, 2007) (**Figure 2)**. Phytoestrogens act like selective estrogen receptor modulators (SERMS) to inhibit or stimulate estrogen-like actions that include modulation of enzyme activities, induction of gene expression and regulation of cell growth (Cassidy, Hanley, & Lamuela-Raventos, 2000; Kurzer & Xu, 1997; Mostrom & Evans, 2011). Scientific attention has focused on the two main classes of phytoestrogens, isoflavonoids and lignans for their estrogenic efficacy and occurrence in the human diet (Cassidy et al., 2000; Landete, 2012; Wang, 2002). Isoflavonoids are found in soy and related products, and the legume and bean family. Lignans are found in a wider range of plant-based foods, such as cereals, vegetables, fruits, legumes, nuts and seeds; and in beverages such as tea, coffee and wine (Adlercreutz, 2007; Cassidy et al., 2000; Dixon, 2004; Landete, 2012). In Western populations with low dietary intake of soy products, lignans could be a more important and consistent source of phytoestrogens from the diet (Hanna, 2006).

2.2 Subclasses of phytoestrogens– lignans and isoflavonoids

Secoisolariciresinol (SECO), matairesinol (MAT), pinoresinol (PINO), and lariciresinol (LARI) are the four major dietary plant lignans or lignan precursors, of particular interest in relation to their bioavailability for human absorption and degree of conversion into two major lignan metabolites or enterolignans, enterodiol (END) and enterolactone (ENL) (Heinonen et

al., 2001). Two other lignan precursors from rye, syringaresinol (SYR) and medioresinol (MED), have also been identified as major dietary determinants of urinary lignan concentrations in the Finnish population (Heinonen et al., 2001; Peñalvo, Haajanen, Botting, & Adlercreutz, 2005). The chemical structures of lignans are illustrated in **Figure 2b, c.**

Genistin, daidzin and glycitin are the major dietary isoflavonoids. They are hydrolysed and converted by colonic bacteria into metabolites, in particular genistein, daidzein and glycitein, that can be absorbed and excreted into the urine (King & Bursill, 1998; Watanabe et al., 1998).

2.3 Absorption and metabolism of lignans and isoflavonoids

Lignans and isoflavonoids show similar patterns of absorption and metabolism with production of their unique metabolites (Heinonen, Hoikkala, Wähälä, & Adlercreutz, 2003; Landete, 2012). Lignans are diphenolic compounds that occur in plants as inactive conjugated glycosides or glycones with sugar moieties attached (Dixon, 2004). The active aglycone is formed through the removal of the sugar moiety by intestinal microbial enzymes, β-glucosidases (Mostrom & Evans, 2011). The unconjugated lignans undergo biotransformation through a further series of metabolic reactions to form two metabolites, enterodiol (END) and enterolactone (ENL) (Landete, 2012). The active lignan aglycones and their metabolites possess estrogenic properties and can act either as an estrogen agonist or an antagonist (Dixon, 2004; Kurzer & Xu, 1997; Mostrom & Evans, 2011; Thompson & Ward, 2002).

Dietary lignans undergo metabolic transformations in the colon. Metabolites are absorbed from the gastrointestinal tract into the portal vein and transported to the liver where they are conjugated with glucuronate or sulphate. They may be secreted with bile into the gastrointestinal tract and undergo hepatic recirculation or distributed in the body and finally excreted into urine or faeces; lignans are also secreted into bodily fluids such as breast milk. The absorption and metabolism of lignan precursors is summarised in **Figure 3** (Cassidy et al.,

2000; Duncan, Phipps, & Kurzer, 2003; Heinonen et al., 2001; Landete, 2012; Mostrom & Evans, 2011; Wang, 2002). In brief, SECO diglucoside (SDG) is deglycosylated by intestinal glucosidases to form the aglycone SECO, which can be further metabolised to END through a series of demethylation and dehydroxylation reactions, or dehydrogenated to form ENL as the end product. Both END and ENL can be detected in urine and reflect dietary intake of SECO. In a study of lignan metabolism using an in-vitro incubation model, Heinonen et al. (2001) measured the degree of conversion of lignan precursor to enterolignans in a 24 hour incubation period and observed that 72% of added SECO, 55% of PINO, 100% of LARI and 4% of SYR were converted to both ENL and END, and 62% of MAT to ENL only (Bowey, Adlercreutz, & Rowland, 2003; Wang, 2002; Wang, Meselhy, Li, Qin, & Hattori, 2000).

The conjugated isoflavone glycosides, genistin, daidzin and glycitin, and methylated precusors formononetin and biochanin A, form the main sources of dietary isoflavonoids (Setchell, Brown, Desai, Zimmer-Nechemias, & et al., 2001). Similarly to lignans, the isoflavonoid glycosides require the hydrolysis of the sugar moiety by intestinal β-glucosidases to release the aglycones, genistein, daidzein and glycitein (Setchell et al., 2002). The aglycones can be absorbed into the portal vein or undergone further transformation to different metabolites. For example, daidzein can be metabolised to dihydrodaidzein, O-desmethylangolensin and equol; genistein can be metabolised to dihydrogenistein and 6’-hydroxy-O-desmethylangolensin (Heinonen et al., 2003); glycitein can be metabolised to dihydroglycitein, dihydro-6,7,4’-trihydroxyisoflavone and 5’-O-methyl-O-desmethylangolensin (Simons, Renouf, Hendrich, & Murphy, 2005) 8-hydroxyl-glycitein and 6-hydroxyl-daidzein (Rüfer, Maul, Donauer, Fabian, & Kulling, 2007) (**Figure 4).** The methylated precursors, formononetin and biochanin A can be metabolised to daidzein and genistein, respectively (Reinli & Block, 1996).

2.4 Biological activities of lignan metabolites

END and ENL possess biological functions and weak estrogenic properties like other

phytoestrogenic compounds (**Figure 2c)**. It has been postulated that high production of these metabolites results from a high dietary exposure and provides subsequent protection against hormonal-dependent cancers, particularly breast and prostate cancer (Adlercreutz, 2007; Wang, 2002). Structural differences amongst phytoestrogen compounds exhibit different selective binding affinity for variants of the estrogen receptor (ER). While certain phytoestrogens, like the soy isoflavonoids, genistein and coumestrol, have a higher binding affinity for ER beta (β) than ER alpha (α), ENL preferentially binds to ER (α) (Kuiper et al., 1997; Mueller, Simon, Chae, Metzler, & Korach, 2004). This implies that selective binding affinity may be an attribute that enables phytoestrogenic compounds, particularly END and ENL, to have biphasic estrogenic or antiestrogenic characteristics, that is, the phytoestrogens exhibit estrogenic activity at low concentration (0.1-10μM) but antiestrogenic at high concentration (>10μM) (Wang & Kurzer, 1997). The biphasic effect allows END and ENL to potentially regulate biological functions that are related to health. Upon binding to the ER, END and ENL might modulate ER protein metabolism and influence hormonal status of tissues, modify gene expression and the activity of enzymes. For instance, Adlercreutz et al. (1993) showed that ENL at high concentration can inhibit aromatase, which could reduce concentration of circulating estrogen; Chen et al. (2009) demonstrated that ENL inhibits cyclin D1 expression, which is responsible for proliferation and migration of prostate cancer cells. In addition to the discussed activities, END and ENL possess antioxidant activities as described by Hu, Yuan, and Kitts (2007), which could protect cells against DNA damage and lipid peroxidation.

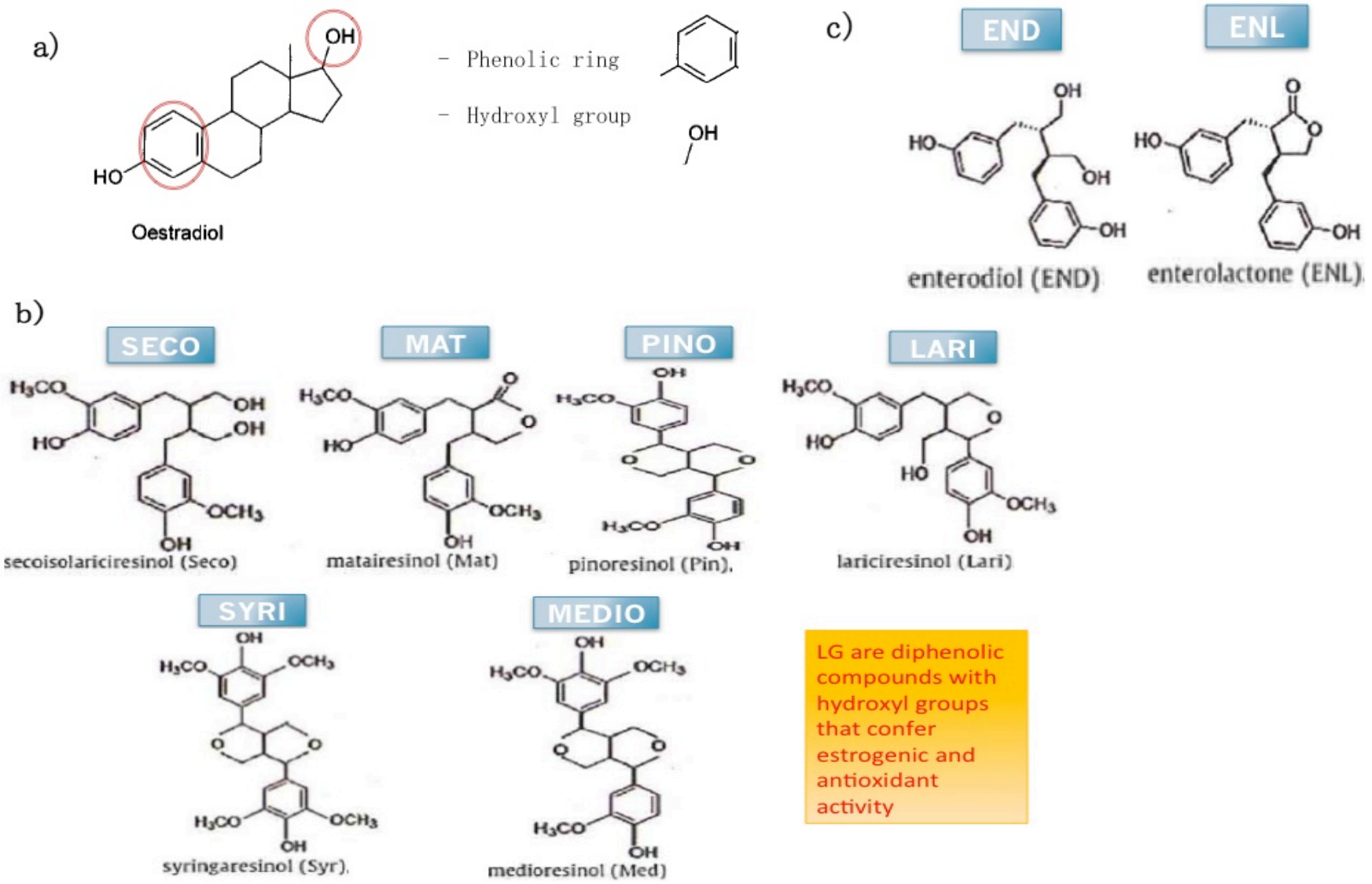


Figure 2. Chemical structures of a) mammalian estrogen, oestradiol; b) plant lignan precursors; and c) lignan metabolites and enterolignans.

(Images adapted and modified from Landete (2012) Hydroxyl groups and the diphenolic ring are structural features required for binding to the estrogen receptor.

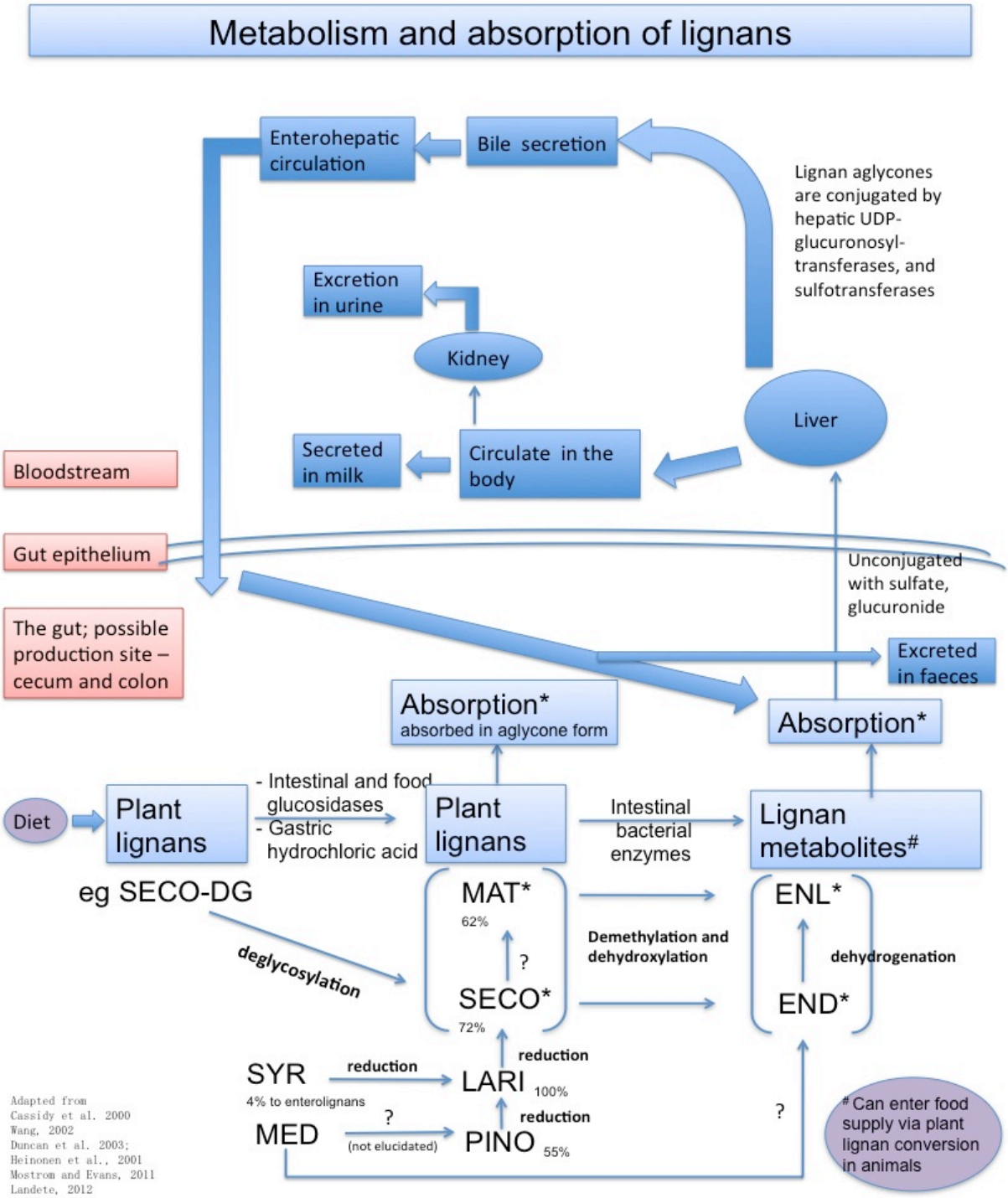


Figure 3. Metabolism of plant lignans and their metabolites.

(Adapted and modified from Cassidy et al. (2000); Duncan et al. (2003); Heinonen et al. (2001); Landete (2012); Mostrom and Evans (2011); Wang (2002))

Inactive plant lignans, or glycone, are obtained through the diet and are deglycosylated by intestinal bacterial enzymes to the active form or aglycone. Aglycone plant lignans can be readily absorbed but the majority are further metabolised into lignan metabolites, which are absorbed into bloodstream. Lignan metabolites circulate through the body and are secreted into body fluids or enter enterohepatic recirculation. They are finally excreted in urine.

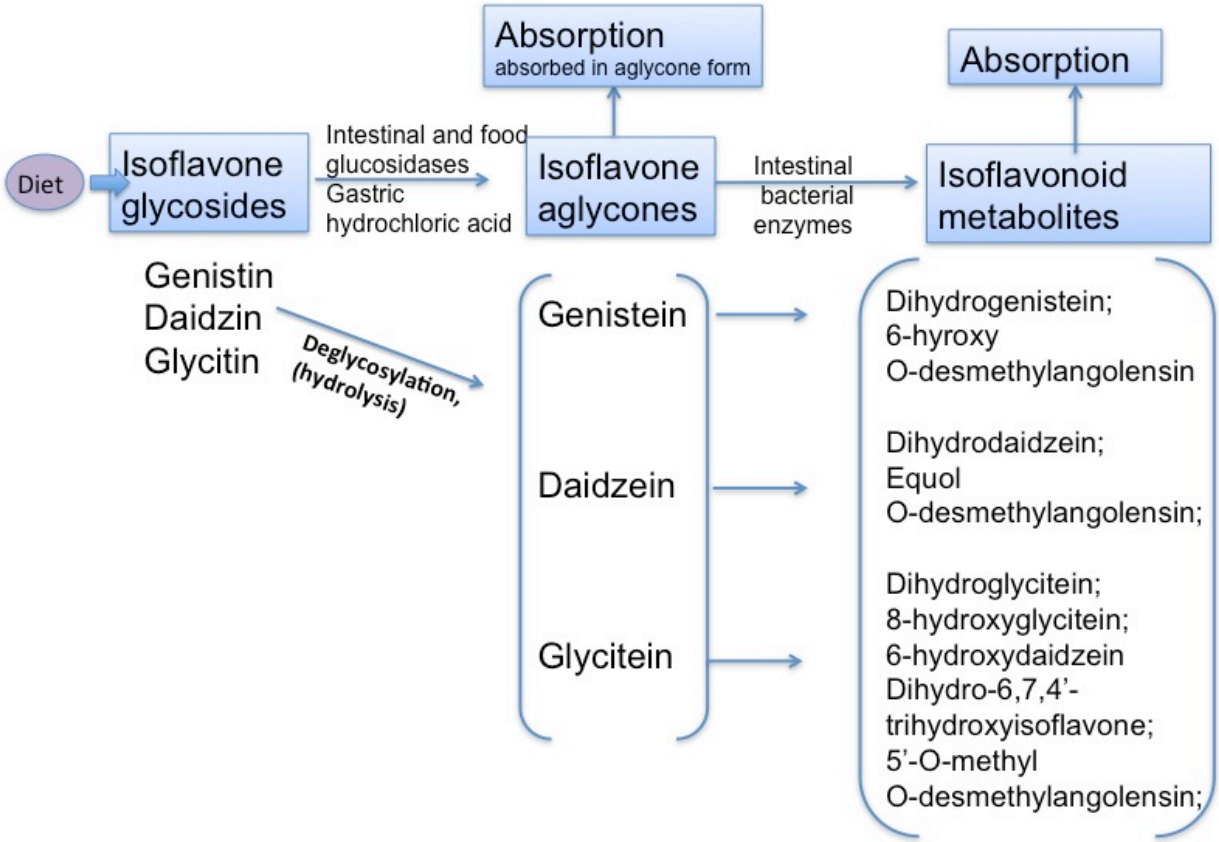


Figure 4. Metabolism of isoflavonoids.

(Adapted and modified from Cassidy et al. (2000); Duncan et al. (2003); Mostrom and Evans (2011)

Isoflavonoids and lignans share similar metabolic pathways. Isoflavonoids in the glycone form, are obtained through diet and are deglycosylated by intestinal bacterial enzymes to the active form or aglycone. The aglycones can be readily absorbed or further transformed into metabolites, which are absorbed into bloodstream. Metabolites circulate through the body and are secreted into body fluids or undergo enterohepatic recirculation, and finally excreted in urine

2.5 Epidemiological studies of lignans and isoflavones and risk of chronic diseases

Lignans and isoflavones are recognised as natural bioactive compounds with a spectrum of biological activities that could potentially influence progression of chronic diseases (Cunha, e Silva, Veneziani, Ambrosio, & Bastos, 2012; Vitale, Piazza, Melilli, Drago, & Salomone, 2013). Interest in lignans and isoflavones has been fueled by epidemiological research investigating the association between lignans and chronic diseases; particularly hormone-dependent cancers due to structural features of phytoestrogens being able to the bind to the estrogen receptor (ER) to elicit estrogenic activity (Turner et al., 2007) Epidemiological evidence for the effect of lignans and isoflavones on chronic diseases is briefly presented in this section of the literature.

Breast cancer

A case-control study conducted by McCann et al. (2004) examining lignan intake and breast cancer risk in pre- and post-menopausal women concluded that there was a significant 34% reduction of breast cancer risk in premenopausal women (OR: 0.66 95% CI 0.44 – 0.98) with the highest quartile intake of lignans of at least 673μg/d. Further, higher lignan intakes were found associated with improved survival among postmenopausal women with breast cancer (HR: 0.29, 95%CI 0.11-0.76) (McCann et al., 2010). However one limitation of these studies was that the results were not controlled for dietary confounding variables, for example, diets high in lignans and isoflavonoids are also high in other food components that have been investigated for potential disease prevention benefits. In a meta-analysis on epidemiological studies of lignans in relation to breast cancer risk, Buck, Zaineddin, Vrieling, Linseisen, and Chang-Claude (2010) found high lignan exposure was associated with a reduced risk of breast cancer in postmenopausal women (pooled RE:0.86, 0.77-0.94). These studies suggest that the protective effect may be more prominent in women with post-menopausal status, with reduction of estrogen production by the ovaries.

Isoflavone intake was inversely associated with risk of breast cancer for Japanese Brazilians

(OR: 0.25, 0.09-0.68), p<0.01) and non-Japanese Brazilians (OR: 0.56, 0.35-0.90) but these effects were not significantly associated with menopausal status (Iwasaki et al., 2009). In contrast, a study conducted by Morimoto, Maskarinec, et al. (2014) observed no significant associations between isoflavone intake and overall breast cancer risk in a multiethnic cohort (HR: 0.96, 0.85-1.08, p=0.40) although a trend of decreasing risk was observed with increasing intake in Latina, African American and Japanese American women. Similarly in Canadian women no associations were found between isoflavone intake and breast cancer risk among all women (OR: 1.06, 0.87-2.30), pre- (OR: 0.96, 0.69-1.33) or post- menopausal (OR: 1.09, 0.83-1.41) women (Cotterchio, Boucher, Kreiger, Mills, & Thompson, 2008). In a meta-analysis by Chen et al. (2014) isoflavone intake had a protective effect on both pre- (OR: 0.59, 0.48-0.69) and post- (OR: 0.59, 0.44-0.74) menopausal women in Asian countries but a marginal protective effect in Western countries (OR: 0.92, 0.83-1.00). These studies suggest that the protective effect of isoflavone intake does not differ by menopausal status. Living environment, location or a background diet traditionally high in soy intakes was a more important potential influence in the relationship between isoflavone intake and breast cancer risk, with the protective effect observed in Asian but not in Western women. Notably, these studies did not report intake of lignans, which are found broadly in plant-based foods, and in Western cultures could provide a more important source of phytoestrogens than isoflavones, which are mainly derived from soy and soy products.

Prostate cancer

A case-control study conducted by Heald, Ritchie, Bolton-Smith, Morton, and Alexander (2007) investigated serum phytoestrogens and risk of prostate cancer in a group of 916 Scottish men, and found a significant reduction of prostate cancer risk with increased level of serum ENL >28.9 nmol/l (OR: 0.40, 0.22-0.71), and with soy food consumption (OR: 0.52, 0.30-0.91), but not with serum daidzein, genistein and equol. However, conflicting evidence was observed in other case-control studies with no significant associations between risk of prostate cancer and circulating serum enterolactone (Kilkkinen et al., 2003; Stattin et al., 2002;

Ward et al., 2008), isoflavone intake (Hedelin, Klint, Chang, Bellocco, Johansson, Andersson, Heinonen, Adlercreutz, Adami, Grönberg, et al., 2006), or urinary isoflavonoids (Jackson, McFarlane-anderson, Simon, Bennett, & Walker, 2010); or an increased risk of prostate cancer associated with higher concentrations of urinary enterolactone (OR: 1.85, 1.01-3.44) and intake of phytoestrogens (OR: 1.41, 1.12-1.76) (Jackson et al., 2010; Ward & Kuhnle, 2010).

Other cancers and chronic diseases

The associations between lignans and isoflavones and other cancers and chronic diseases have also being investigated. Lignan and isoflavone intakes have been associated with decreased risks of adenocarcinoma of the oesophagus and gastroesophageal junction in Swedish women (Lin, Yngve, Lagergren, & Lu, 2012), endometrial and ovarian cancer in American and Australian women (Horn-Ross, John, Canchola, Stewart, & Lee, 2003; Neill et al., 2014), lung cancer (Schabath, Hernandez, Wu, Pillow, & Spitz, 2005) and oesophageal cancer in Chinese subjects (Tang et al., 2014).

A cross-sectional study conducted by Franco et al. (2005) investigated lignan intake and cognitive function in postmenopausal women and found that high intakes were associated with intact cognitive function in the older postmenopausal women. Another cross-sectional study exploring the phytoestrogen intake and cardiovascular disease risks conducted by Van der Schouw et al. (2005) concluded that the risk of coronary heart disease was lower with increasing lignan and isoflavone intake in Western women, particularly in current or past smokers. In contrast Talaei, Koh, van Dam, Yuan, and Pan (2014) found that intake of soy and isoflavones were not associated with the risk of cardiovascular disease mortality in Chinese Singaporean adults.

The studies presented in this section demonstrate epidemiological evidence to support an inverse association between lignan and/or isoflavones, and risk of chronic diseases, although some studies have found conflicting results. There were no systematic reviews or meta-analyses found on epidemiological studies on prostate and other cancers or chronic

diseases in relation to phytoestrogen intake. The potential health benefits and high prevalence of lignans in Western diets cannot be ignored and to our knowledge there are limited epidemiological studies in the Australian population. This highlights the need to develop a dietary assessment tool specifically to quantify intake of lignans and isoflavones in Australian context.

2.6 Food frequency questionnaire

The food frequency questionnaire (FFQ) is a dietary assessment tool used to assess habitual dietary intake by determining the frequency of consumption of a list of foods that are dietary contributors to that particular nutrient or dietary component, over a specified period of weeks, months or years (Willett, 1998). There are usually three components of a FFQ, a food list, a frequency, and a portion size response section, where the responders indicate how often and how much of each food is consumed.

The FFQ is a common method used in epidemiological studies with large population size because it can be self-administered, thus allowing the investigators to collect a substantial amount of dietary data in a relatively inexpensive manner. In addition, FFQs allow investigators to identify food patterns associated with certain nutrient intakes, to rank individuals by quantile of food or nutrient intakes and to examine the intake in relation to risk of chronic diseases (Bingham, 2007; Willett & Lenart, 1998). The ability of the FFQ to correctly reflect the intake of the population is determined by the discrimination between the variation in dietary patterns in different groups (Willett, 1998). Therefore, identifying dietary patterns and inclusion of commonly consumed food sources in the study population is fundamental to the design of a FFQ to successfully capture the dietary component from the appropriate food sources.

There are seven approaches used to evaluate a FFQ, as stated by Willett and Lenart (1998): (1) comparison of means, (2) proportion of total intake accounted for by foods included on the questionnaire, (3) reproducibility, (4) validity, (5) comparison with biochemical markers, (6) correlation with a physiological response and (7) the ability to predict disease. Of these, the

common methods used in validation studies of phytoestrogen FFQs in this literature review were the comparison of means or medians, and comparison of biochemical markers (Bhakta et al., 2005; French, Thompson, & Hawker, 2007; Hanna et al., 2005; Heald, Bolton-Smith, Ritchie, Morton, & Alexander, 2005; Horn-Ross et al., 2006; Huang et al., 2000; Lin, Wolk, et al., 2012; Tseng et al., 2008; Yamamoto, Sobue, Sasaki, Kobayashi, & et al., 2001), convergent validity in comparison with an independent reference method (Bhakta et al., 2005; Chan et al., 2008; Fernandez, Omar, & Husain, 2013; Hanna et al., 2005; Horn-Ross et al., 2006; Huang et al., 2000; Yamamoto et al., 2001), and reproducibility with the FFQ in repeated measures (Frankenfeld et al., 2003; Frankenfeld et al., 2002; French et al., 2007; Hanna et al., 2005; Horn-Ross et al., 2006). Four of the above approaches were implemented in this study to evaluate a specific FFQ in the Australian context; and the other three approaches in relation to measurement of total intake, physiological response and disease risk were not utilised, as they were not relevant for the current research.

2.7 Evaluation of food frequency questionnaires

2.7.1 Validity

2.7.1.1 Weighed food record – convergent validity

Food records are records of weighed or estimated amounts of food and beverages at the time of consumption for a specified number of days (Bingham, 2007). Multiple food records are often conducted to consider the daily variation in intake of individual subjects. Three to ten days of food records, with weekend days included, are usually required to represent individual usual intake of energy and macronutrients (Buzzard, 1998; Lee & Nieman, 2010).

Choosing an appropriate reference method is important for assessing the convergent validity of the FFQ, to compare the newly established instrument with other related instruments and verify that it measures the same construct (Thorkildsen, 2010). Since there is no perfect reference method in validation studies, the associated errors of the FFQ and the reference

method should be as independent and uncorrelated as possible (Nelson, 1997; Willett & Lenart, 1998). The FFQ and the diet history interview are both retrospective dietary tools and therefore share similar sources of errors that may lead to the underestimation of the correlation between the two methods (Willett and Lenart 1998). The 'ideal' reference method for validating a FFQ is a food record because the sources of errors of food records are reasonably uncorrelated to those of the FFQ (Willett & Lenart, 1998). The bias related to these errors impacts on the accuracy, completeness and representativeness of the dietary data collected, and therefore on the validity assessment of the two dietary tools. Since the bias associated with these sources of error cannot be eliminated, a good reference method should ideally share different sources of error. Therefore, the proposed reference method for validating the phytoestrogen FFQ in the current study was a weighed food record (WFR). Food scales and household measuring tools were provided to assist in portion size estimation to ensure accuracy and consistency of the dietary data obtained.

Subjects may subconsciously modify their usual food intake for ease of recording a food record, which will lead to inaccurate assessment, potentially undermining a correlation between the intake derived from the FFQ and the food record (Lee & Nieman, 2010; Willett & Lenart, 1998). Other sources of error are attributed to the food composition data used to measure the nutrient intakes (Willett & Lenart, 1998). Both FFQ and food record methods could under- or over-estimate the intake due to the natural variability of the dietary components in food, for instance, phytoestrogen content in plant foods can vary because of genetics, growth and environmental factors, and effects of harvesting and processing (Kuhnle, Dell'Aquila, Runswick, & Bingham, 2009; Lyons-Wall, 2007). Due to the natural variability of phytoestrogen content in foods and incompleteness of databases, especially for lignan intake, it is challenging to measure the exact 'true' dietary intake, even with a valid and reliable dietary assessment tool. For the current study, lignan and isoflavonoid intakes were estimated from the content currently available from published international databases.

2.7.1.2 Urinary biomarkers – the criterion validity

Willett and Lenart (1998) stated that biochemical indices could be an informative and objective comparison method when there is uncertainty regarding the estimated intakes and validity between the questionnaire and a reference dietary assessment method. The associated errors of biomedical indices are likely to be independent to those of the two dietary assessment methods, and are mainly attributed to subject variation in absorption and metabolism of the nutrients, and external errors related to laboratory measurement and bioavailability of the nutrient or constituent from food (Willett & Lenart, 1998).

Biological markers or biomarkers, in blood and urine are collected as a criterion to compare with the estimated intake derived from the dietary tool in validation and epidemiological studies (Borneman, 2010; Hunter, 1998). The biomarker must be sensitive to intake of the nutrient (Willett & Lenart, 1998). The time interval of biomarker collection is dependent on the pharmacokinetics of the target nutrient, the subjects' variability of the nutrient intake, and the research design (Bates, Thurnham, Bingham, Margetts, & Nelson, 1997; Kelemen, 2007; Samet & Alberg, 1998).

2.7.1.3 Strengths and limitations of the food frequency questionnaire, food record and urinary biomarker tools

The strengths and limitations of the food frequency questionnaire, food record and biomarker tools are summarised in **Table 1**. The sources of errors of a FFQ, WFR and urinary biomarkers are relatively unrelated to each other. For example, completion of a FFQ relies on the subject's memory to recall the correct food consumption and frequency of intake, whereas when completing a food record, the process of recording is open-ended and does not depend on the subject's memory because details about the food and drinks are recorded at the time of consumption. In addition, a food record does not restrict subject's choice on a fixed list of foods from a FFQ.

However, given that details about the subject's dietary intake are required to complete food records, the subject burden is relatively high and literacy and numeracy are required to

understand the process to accurately complete food records. Biomarkers can reflect true status assessed by the diet assessment method; nevertheless biomarkers are prone to be affected by systematic errors related to work in laboratory measurement.

For the current study the modified FFQ was administered for the previous month to capture usual intake over a feasible retrospective time period. The weighed food record was administered over a relatively short period of 3 days, to minimise the subject burden, and included 2 weekdays and 1 weekend day to optimise the chance of capturing usual intake. Data for urinary biomarkers were collected over a 3-day period to coincide with the intake from the food record and provide an estimate of usual excretion. Laboratory measurements of urinary biomarkers were based on standardised methods from the published literature.

Table 1. Limitations (sources of errors) and strengths of food frequency questionnaire (FFQ), weighed food record (WFR), and biomarker

(Buzzard, 1998; Hunter, 1998; Willett, 1998; Willett & Lenart, 1998)

FFQ		**WFR**		**Biomarker**	
Limitation	**Strength**	**Limitation**	**Strength**	**Limitation**	**Strength**
Reliance on subjects' memory on food consumed over the period of time	Relative low subject's burden compared to weighed record	High subject's numeracy and literacy requirement and burden to complete the recording - to record the weighed food consumptions and details like specific types/brands, amount, preparation method, ingredients used in recipe and dishes	Open-ended, do not depend on subjects' memory or restrict subject's choice on a fixed list of foods	External sources of errors: subject variation in metabolism of the nutrient, bioavailability of nutrient from food	Reflect true intake when it is time integrated to reflect nutrient intake during the period evaluated by the diet assessment methods being tested
Restrictions imposed by a fixed list of foods	Potential to reflect long-term habitual intake	Poor response rate* resulted from low subject motivation	Direct assessment of portion size by measuring the weight and size of the food and drinks consumed	Systematic errors associated with laboratory and analytical, techniques, contamination and stability of biomarker	Specific and sensitive to the intake of the nutrient
Interpretation of the questions and perception of serve sizes	Can be self-administered or completed with assistance of trained personnel	Day-to-day dietary variation, require multiple days of intake to account the variability of the subject's intake.	More detailed dietary information can be recorded and more accurate dietary analysis and assessment	Stress or inconvenience brought to subjects when collecting samples for the biomarkers	
Difficulty in choosing the correct/appropriate category of frequency of the food is consumed		Error associated with the measuring and recording process which leads to change in eating behaviour – the food record may not be representative of what subject normally eat (change in eating behavior)			

*Cole (1997) stated that "In nutritional studies involving the measurement of food intake over several days, the degree of non-response can be 30% or more" (p.67). A study conducted by Kolar et al. (2005) indicated that the response rate of a self-administrated 3-day food record is 68% (32% non-response rate) in one hundred 50-76 years men and women. Another study conducted by Dietrich et al. (2003) showed that the response rate of a 24-hour food record, which was designed to measure fruit and vegetable intake of primary school children, is 76% (24% non-response rate).

2.7.1.4 Method of Triads

The Method of Triads is a statistical model for a triangular comparison developed by Kaaks (1997) to assess the interrelationship between the dietary questionnaire or test method (Q), a detailed dietary assessment or reference method (R) and biochemical measurements or biomarkers (M) to the 'true' (T) intake of nutrients. The assumptions for this method to be valid are that the errors associated with the questionnaire, reference method and biomarker measurements are uncorrelated or independent, and the measurements Q, R and M are linearly related to the 'true' intake (T) (Kaaks, 1997; Willett & Lenart, 1998; Yokota, Miyazaki, & Ito, 2010). Ocké and Kaaks (1997) described this method as an approach to assess the agreement between the questionnaire (Q), reference method (R) and biomarker (M) measurements in relation to a nutrient status. Validity coefficients (VC) are calculated for the correlation between the measurement intakes of the methods and the hypothetical 'true' intake status. The VC is readily influenced by the strength of the correlation coefficient (*r*) because the VC is mathematically derived from the associations between the two individual assessment methods. The theoretically acceptable range of VC is between 0 and 1, although VC exceeding 1 is acceptable if the assumptions were not violated. A VC exceeding 1 is called Heywood case.

Several validation studies have applied the method of triads to assess three or more measurement methods for different nutrients (Andersen et al., 2005; Bhakta et al., 2005; Daures et al., 2000; Kabagambe et al., 2001; McNaughton, Marks, Gaffney, Williams, & Green, 2004; Ocké & Kaaks, 1997; Shai et al., 2005; Tan, Charlton, Tan, Ma, & Batterham, 2013). Studies conducted by Kabagambe et al. (2001), Daures et al. (2000), Shai et al. (2005) and Andersen et al. (2005) assessed more than three measurement methods. The four other studies by Ocké and Kaaks (1997), McNaughton et al. (2004) and Bhakta et al. (2005) Tan et al. (2013) assessed two dietary and one biochemical assessment method. Bhakta et al. (2005) was the first and only study to apply the method of triads to evaluate phytoestrogen status, using a specific FFQ, 24-hour recalls and plasma phytoestrogens.

2.7.2 Reliability

The reliability of the FFQ is the consistency of the measurement of the tool for the same subject at different occasions of administration (Cade, Burley, Warm, Thompson, & Margetts, 2004; Willett & Lenart, 1998). The reliability assessment is often a component of the evaluation of FFQ in validation studies (Cade, Thompson, Burley, & Warm, 2002; Willett & Lenart, 1998). Correlation coefficients are frequently used in assessing the reliability of the FFQ (Cade et al., 2002). Acceptable correlation coefficients commonly range from 0.5 to 0.7 and the correlation tends to be higher for administration of the FFQ with shorter periods of time, one month or less apart, when compared to longer periods, six months to one year apart, due to possible recall bias with shorter timeframe, or time or seasonal change in food intake with longer time periods (Cade et al., 2004; Cade et al., 2002; McLennan & Podger, 1995). Although correlation coefficients can be used to assess the validity and reliability of the FFQ, the Bland-Altman plot is a more robust method to assess the level of agreement between the intake estimated with two assessment methods or with the same method at different occasions (Altman & Bland, 1983; Bland & Altman, 1986). The plot is constructed by plotting the differences in intake against the mean intakes derived from the two points of measure. In the current study, reliability of the FFQ was tested by administering a repeat FFQ after a short period of time, 1 to 2 weeks, to minimise the possibility of seasonal changes in food intake.

2.8 Validation studies on phytoestrogen food frequency questionnaires

Of previous studies that have evaluated phytoestrogen food frequency questionnaires, ten studies measured isoflavones only, one study measured lignans only, and four studies measured both isoflavones and lignans (**Table 2**). This section addresses the research, published in 2000 and onwards, in the evaluation of these food frequency questionnaires in relation to convergent and/or criterion validity.

Convergent validity

Studies conducted by Bhakta et al. (2005) and Horn-Ross et al. (2006) assessed convergent validity of the phytoestrogen FFQ and demonstrated that their FFQs were moderately to highly correlated to the reference methods, with correlation coefficients ranging from r=0.50 to 0.79. For Bhakta et al. (2005); the correlations were significant for both lignans (r=0.70, $p<0.001$) and isoflavones (r=0.57, $p<0.001$), with higher correlations observed for lignans suggesting that the FFQ was valid for phytoestrogen intake, particularly lignans, when compared to the reference method.

Criterion validity

Criterion validity of the phytoestrogen FFQs was assessed in five studies, with Hanna et al. (2005), Horn-Ross et al. (2006) and French et al. (2007) using urinary phytoestrogen excretion, Bhakta et al. (2005) using plasma phytoestrogen levels, and Lin, Wolk, et al. (2012) using serum ENL levels. The associations between intake from the FFQ and excretion for lignans were weaker than those for isoflavones for Hanna et al. (2005) (lignans: r=0.138, $p>0.05$; isoflavones: r=0.207, $p\leq0.05$), and French et al. (2007) (lignans: r=0.46, p=0.02; isoflavones: r=0.64, $p<0.001$). Further comparison between lignan and isoflavone intakes and urinary excretion conducted by Horn-Ross et al. (2006) suggested that the both 24h dietary records (DR) and FFQ had a stronger correlation with measurement of urinary isoflavones (ρ=0.43-0.50) than lignans (ρ=0.16-0.21). Similar findings were observed by Bhakta et al. (2005) who compared intake from a FFQ with 24h dietary records (DRs), and with plasma concentrations, and found stronger correlations for isoflavones (r=0.21-0.43) than lignans (r=0.08-0.10). The study conducted by Lin, Wolk, et al. (2012) compared lignan intake from two different FFQs with serum ENL levels; tLin, Wolk, et al. (2012)Lin, Wolk, et al. (2012)Lin, Wolk, et al. (2012)Lin, Wolk, et al. (2012)Lin, Wolk, et al. (2012)Lin, Wolk, et al. (2012)Lin, Wolk, et al. (2012)hey found that the FFQ-97, with open-ended responses allowed more dietary information to be recorded in addition to the closed-ended frequency category response, was significantly correlated with serum ENL

levels (ρ=0.22, p=0.01) and concluded that FFQ-97 was a better tool for assessing lignan intake.

Notably, two studies have estimated phytoestrogen intake in Australian populations (Hanna et al., 2005; Lahmann et al., 2012). In one study, the FFQ was valid for measurement of isoflavonoids when tested against urinary biomarker excretion but had poor validity for measurement of lignans, possibly due to the inability of the FFQ to capture the range of food items consumed in varied diets; or due to use of an indirect method for estimating the lignan content in food items, which may not have accurately estimated intake (Hanna et al., 2005). The second study used a FFQ that was validated for energy and nutrient intake but not specifically for phytoestrogens (Lahmann et al., 2012).

This section informs the key results and study design from earlier research in the evaluation of the current FFQ for measuring phytoestrogen intake. While all studies provided comparisons of average intakes and correlations between tools at the group level, three studies further conducted Bland-Altman analyses to evaluate agreement at the individual level (Chan et al., 2008; Fernandez et al., 2013; Frankenfeld et al., 2003). Five studies conducted cross-classification analyses for level of agreement by calculating the percentage of subjects categorised into the same and opposite quantiles of isoflavone intake and biomarker concentration (Bhakta et al., 2005; Chan et al., 2008; Fernandez et al., 2013; French et al., 2007; Heald et al., 2005). Bhakta et al. (2005) additionally used the Method of Triads to evaluate the FFQ for phytoestrogen intake in a triangular comparison of the measurements from an FFQ, 24hDRs, and plasma phytoestrogens. These statistical analyses were employed to evaluate the modified FFQ in the current research. Open-ended questions were also incorporated into the modified FFQ in addition to the closed frequency questions, to elicit any further information on subjects' food intake.

Estimation of lignans

Earlier studies only measured two lignans, SECO and MAT, as more recently quantified

lignan precursors, LARI and PINO, were not available at the time. Lin, Wolk, et al. (2012) estimated intakes of LARI, PINO, MED and SYR in addition to SECO and MAT. For the current research, four primary lignan precursors, SECO, MAT, LARI and PINO were measured, as they are present in a wide range of food sources commonly consumed in the Australian population. From the validation studies conducted to evaluate isoflavone intake, two to eight isoflavone-rich food items listed in the FFQ were reported to be sufficient to capture variability in intake (Frankenfeld et al., 2003; Frankenfeld et al., 2002; Tseng et al., 2008; Verkasalo et al., 2001). As lignans are present in a wider range of food sources than isoflavones, the FFQ for the current research was modified to focus on enhancing lignan measurement by including a range of food sources for which lignan content were available. Subjects from the majority of validation studies were females, but male subjects and subjects of both gender were recruited by Heald et al. (2005) and Frankenfeld et al. (2002). Lignans are found ubiquitously in Western and non-Western dietary patterns and lignans are considered to be nutritionally important to the Australian community with diverse demographic backgrounds. Therefore both male and female subjects from various cultural backgrounds were recruited for the current research to reflect the diversity in dietary intake.

Verkasalo et al. (2001) recognised the importance of the timing of biomarker collection with respect to the administration of the dietary assessment tool, hence understanding the pharmacokinetics of phytoestrogens is essential for the study design. Huang et al. (2000) and Tseng et al. (2008) collected the first morning/overnight sample separately from the rest of the 24-hour sample and found that phytoestrogen concentrations in the first morning or overnight urine were highly correlated to concentrations in the 24-hour sample. In the current study, three consecutive 24 hour urine samples including one weekend and two weekdays, were collected in an attempt to capture usual intake of lignans and isoflavonoids for comparison with usual intake from the modified FFQ.

2.9 Phytoestrogen databases

Much of the pioneering work on direct analysis of lignans in foods has been done by Mazur and colleagues (Adlercreutz & Mazur, 1997; Mazur, 1998b; Mazur & Adlercreutz, 2000; Mazur & Adlercreutz, 1998; Mazur, Duke, Wähälä, Rasku, & Adlercreutz, 1998; Mazur et al., 1996; Mazur, Uehara, Wähälä, & Adlercreutz, 2000; Mazur et al., 1999; Mazur, Wähälä, Rasku, et al., 1998; Mazur, Wähälä, Wang, & Adlercreutz, 1998; Nilsson et al., 1997a, 1997b; Nurmi et al., 2003). In these studies, SECO and MAT were the only two lignans analysed. In the past decade, other researchers have discovered additional lignan precursors that are major determinants of dietary lignan intake and metabolism, including LARI, PINO, SYR and MED (Bonzanini, Bruni, Palla, Serlataite, & Caligiani, 2009; Durazzo, Zaccaria, Polito, Maiani, & Carcea, 2013; Milder, Arts, Putte, Venema, & Hollman, 2005; Moreno-Franco et al., 2011; Peñalvo, Adlercreutz, Uehara, Ristimaki, & Watanabe, 2008; Smeds et al., 2007; Smeds, Jauhiainen, Tuomola, & Peltonen-Sainio, 2009; Thompson, Boucher, Cotterchio, Kreiger, & Liu, 2007; Thompson, Boucher, Liu, Cotterchio, & Kreiger, 2006). SYR and MED have been analysed in cereals, such as rye, wheat, and in seeds like sesame seeds but have not yet been comprehensively quantified in other foods (Peñalvo et al., 2005; Smeds et al., 2007).

The prominent isoflavones in foods are daidzein, genistein and glycitein, however, the latter contributes only approximately 10% to the total isoflavones in soy (Rüfer et al., 2007; Simons et al., 2005). The U.S. Department of Agriculture has compiled a comprehensive database for isoflavone content in 557 foods from various articles containing analytical data for the two major isoflavones, daidzein and genistein, and their methylated precursors formononetin and biochanin A, and also glycitein (Bhagwat, Haytowitz, & Holden, 2008).

Lignans are present in a wide range of plant-based foods, including breads and cereals, vegetables, nuts, seeds and legumes, fruits, and beverages like tea, coffee and wine (Mazur, 1998b; Mazur & Adlercreutz, 1998; Mazur, Duke, et al., 1998; Mazur, Wähälä, Rasku, et al.,

1998; Milder, Arts, et al., 2005; Moreno-Franco et al., 2011; Nurmi et al., 2003; Peñalvo et al., 2008; Thompson et al., 2006). Oil seeds and nuts contain high amounts of lignans, with linseed (or flaxseed) being the richest source with approximately 3.4 mg/g (Horn-Ross et al., 2000; Meagher & Beecher, 2000; Pillow et al., 1999; Smeds et al., 2007; Thompson et al., 2006). Small but significant concentrations of lignan metabolites, especially ENL, have been detected in dairy products like cheese, milk and yoghurt (Antignac, Cariou, Le Bizec, & Andre, 2004; Kuhnle et al., 2008b). There are negligible quantities of lignan metabolites in meat and seafood (Kuhnle et al., 2008b).

Isoflavones are concentrated in soybeans, with approximately 2-4 mg isoflavones/g, and foods derived from soy, such as soymilk beverages, and foods fortified with and/or containing soy as an ingredient (Adlercreutz & Mazur, 1997; Bhagwat et al., 2008; Mazur, 1998b). Moderate amounts are present in other legumes such as beans and peas (0.03-0.08mg isoflavones/g), and small amounts in bread (0.02-0.03mg/g) and other food sources (<0.01mg isoflavones/g) namely fruits and vegetables, nuts and seeds, beverages, meat and dairy (Bhagwat et al., 2008; Kuhnle, Dell'Aquila, Aspinall, Runswick, Joosen, et al., 2009; Kuhnle et al., 2008a, 2008b; Kuhnle, Dell'Aquila, Aspinall, Runswick, Mulligan, et al., 2009; Mazur, Duke, et al., 1998; Mazur, Wähälä, Rasku, et al., 1998).

As no culturally-specific database is available for Australian foods, data on the lignan and isoflavone content of foods was sourced from published international values. Examining different lignan databases provided information on the range of lignan content from different food sources. This assisted in selecting appropriate food items to be included in the food list to ensure the design of the FFQ to be relevant to the target population. Foods rich in lignans, for example, linseed and related food items, soy and linseed bread were included. A range of commonly consumed items in Australia with moderate lignan content, including vegetables, fruits, nuts, seeds and legumes, grains and cereals, and beverages like tea, coffee and wine, and soy and related food products were also included.

2.10 Cross-sectional studies on phytoestrogen intake and food sources

Intake of phytoestrogens and contributing food sources

Previous studies have quantified intake of dietary phytoestrogens in different populations from various countries and regions with 24 cross-sectional studies conducted from 2005 to 2014. Five studies have estimated intakes of lignans, isoflavones and enterolignans; five estimated lignans and isoflavones; two estimated lignans and enterolignans; six studies have estimated only lignans and six studies estimated only isoflavones. The intakes and contributing food sources varied across different studies due to a number of factors such as dietary diversity and demographic differences in the population, and different phytoestrogen databases and dietary tools for assessment of intake (**Table 3**).

Dietary lignan intake varies with different dietary patterns. For instance, Milder, Feskens, et al. (2005) found that beverages like tea, coffee and wine, Brassica vegetables like cabbage, nuts and seeds, wheat bread and fruits were major lignan-contributing foods in the Dutch population, whereas in the Finnish population, the main contributors were rye products, berries, coffee, tea and whole grains (Nurmi, Mursu, Peñalvo, Poulsen, & Voutilainen, 2010). Similarly in Latvia, rye and seed bread, nuts and seeds and coffee and tea beverages were major contributing foods for lignans (Meija et al., 2013). Despite differences in dietary patterns, the average lignan intakes were comparable in the three studies, between 1.22 mg/d and 1.26 mg/d.

Differences in phytoestrogen intakes and food sources were reflected in regional dietary preference in The EPIC cohort study (Zamora-Ros, Knaze, et al., 2012; Zamora-Ros, Not, et al., 2012). Major food sources of lignans were fruits, vegetables and alcoholic beverages such as wine in Mediterranean (MED) countries, Greece, Spain, Italy and South of France; and non-alcoholic beverages like tea and coffee, vegetables, cereals in Non-Mediterranean (non-MED) countries, North-East and North-West of France, Germany, the Netherlands,

Denmark, Sweden and Norway, and U.K.. Major food sources of isoflavones were soy products, legumes and vegetables in MED countries and soy products, and non-alcoholic beverages such as soy beverages in non-MED countries. Dairy products contributed more than 90% of enterolignan intake in both MED and non-MED countries. Population groups of different ethnic backgrounds and dietary habits had different intake levels and contributing food sources of phytoestrogens. In the EPIC study, the Health conscious group in U.K. had the highest intake of lignans (2.15 mg/d) and isoflavones (12.1 mg/d) as the group was characterised by higher consumption of fruit, vegetables, legumes, nuts and seeds, cereal and soy products than other cohorts in the EPIC study (Zamora-Ros, Knaze, et al., 2012); corresponding intakes from Greece were 1.04 mg/d for lignans and 0.24 mg/d for isoflavones. Vegetables dishes, milk beverages and bread products were main food sources of phytoestrogens in the soya-consuming groups and bread products, breakfast cereals, and tea and coffee in the non soya-consuming groups from the EPIC-Norfolk cohort study (Mulligan et al., 2013).

Chinese and Japanese American women had relatively high intake of isoflavones (9.71 mg/d and 12.59 mg/d) compared to Caucasian and African American women (0.32 mg/d and 0.28 mg/d) although intakes of lignans were comparable (0.20 to 0.40 mg/d) (Huang et al., 2012). Main food sources for lignans were coffee, black tea, Chinese herbs in soup or tea and green tea amongst the multiethnic women. The main food sources for isoflavones were soymilk beverages in Caucasian, African American and Chinese Americans but soybean and related products for Japanese Americans. In contrast, Chinese living in China consumed vegetables namely soybean sprouts, Chinese lettuce, tofu and taro as their main sources of isoflavones, and carrot, Chinese lettuce, taro and white gourd as main sources for SECO (Hu et al., 2014).

The intake of lignans (0.14 mg/d, 0.18 mg/d) and isoflavones (0.32 mg/d, 0.37 mg/d) of South Asian residents and native British residents, respectively, living in U.K. were similar but with different contributing food sources (Bhakta et al., 2006). The South Asians had more

vegetables and vegetable dishes (38%) for isoflavones than the native British, who had only 2% contribution from vegetables. Breads provided the main source of lignans in both groups.

2.11 Pharmacokinetics of lignans and isoflavones

Based on pharmacokinetic studies, enterolignans, END and ENL appeared in blood 7-10 hours after a lignan supplement from flaxseed, and daidzein and genistein appeared 2 hours after an isoflavone supplement from soy meal, with the majority excreted in urine within 24 hours of the supplement (King & Bursill, 1998; Kuijsten, Arts, Vree, & Hollman, 2005; Watanabe et al., 1998). The delay of appearance reflected the time required for the bacterial conversion to metabolites in the colon prior to absorption. Plasma END reached its peak at 15 hours and ENL at 20 hours in Dutch subjects; daidzein and genistein reached at 6 hours in Japanese subjects and at 7-8 hours in Australian subjects. In some subjects, a second peak of enterolignans, predominantly ENL, and genistein were observed in plasma. This peak could be explained by the reabsorption via the gastrointestinal tract into bloodstream and redistribution of enterolignans and isoflavones through the enterohepatic circulation. Both enterolignans and isoflavones were continually excreted for the first two days before they declined on the third day. In pharmacokinetic studies conducted in human subjects, approximately 40% of enterolignans, 62% of daidzein and 22% of genistein in Australian subjects (King & Bursill, 1998), and 36% daidzein and 18% genistein in Japanese subjects (Watanabe et al., 1998) were accumulatively excreted through urine over the two to three days after intake of the flaxseed or soy supplement.

A positive dose-dependent relationship has been established between supplement consumption and urinary excretion in humans for both isoflavonoids (Morimoto, Beckford, Franke, & Maskarinec, 2014) and lignans (Hutchins, Martini, Olson, Thomas, & Slavin, 2000); and similarly in animal models (King, Broadbent, & Head, 1996; Rickard et al., 1996). Dietary lignan precursors, SECO, MAT and LARI have been detected in human urine as a

result of incomplete metabolism to enterolignans by intestinal microbes (Adlercreutz et al., 1995; Bannwart, Adlercreutz, Wähälä, Brunow, & Hase, 1989). Isoflavonoid metabolites, glycitein, formononetin, biochanin A and equol have also been reported in urine (Hanna et al., 2005).

These studies, demonstrate a positive dose-dependent relationship between intake and excretion, and justify the choice of urinary excretion of isoflavonoid and lignan metabolites as appropriate biomarkers of dietary intake for the current study. They further indicate that a variable proportion, between 18% to 62% for different metabolites, is excreted in urine within 1 to 3 days after ingestion of precursors in the diet.

2.12 Phytoestrogen status and demographic, social and lifestyle characteristics

Epidemiological studies have shown that higher intakes of phytoestrogens and phytoestrogen rich foods were associated with lower risks of breast and prostate cancers (Heald et al., 2007; Iwasaki et al., 2009; McCann et al., 2004; McCann et al., 2010). Demographic, social and lifestyle characteristics associated with the intake of phytoestrogen rich diets could contribute to these health outcomes. Factors related to the demographic, social and lifestyle status, for instance, age, gender, occupation, education, participation in physical activity and smoking, influence food choices and could therefore impact the observed relationships between phytoestrogen intake and risk of chronic diseases. This section is briefly presented with results from correlation, regression and quantile analysis of population-based cross-sectional, prospective cohort and case-control studies.

Milder et al. (2007) in a cross-sectional analysis of a Dutch population identified several factors, namely age, weight, current smoking status and frequency of defecation (all $p<0.001$), that explained 12.7% of the differences in plasma ENL, and age and use of antibiotics explained 2.1% of the difference in plasma END. Kilkkinen et al. (2001) found that constipation contributed to a 2.7% variance in serum ENL of Finnish men ($R^2=0.0268$), whereas BMI, smoking, age and constipation together contributed to a 14% variance in serum ENL in Finnish women. Rybak, Sternberg, and Pfeiffer (2013) observed that smoking

compared to non-smoking was associated with a 25% reduction in urinary O-desmethylangolensin (DMA) and ENL; a 25% increase in BMI was associated with a 21% decrease in urinary ENL; and increasing physical activity was associated with a greater than 6% increase in urinary ENL. The relationship between urinary phytoestrogens and socio-demographic and lifestyle variables was metabolite-specific, which further complicated the situation.

Johnsen et al. (2004) in a case-control study, found that smoking, more frequent bowel movements, and higher BMI were inversely correlated with concentrations of plasma ENL ($p<0.001$), while in a prospective cohort study, significant determinants of urinary lignan and isoflavone excretion for Chinese women were BMI, menopausal status, participation in exercise, and vitamin supplement use ($p<0.05$)(Wu et al., 2012). Morisset et al. (2009) in a cross-sectional analysis found that older Canadian women who consumed higher lignan intake and had higher plasma ENL tended to have lower BMI and total body fat mass ($p<0.05$). Older Australian women, who consumed soy and linseed, had higher levels of physical activity level, socio-economic position and education, were more likely to use supplements (all $p\leq0.001$) (Hanna et al., 2010). Using regression analysis, these determinants were inversely associated with urinary phytoestrogen metabolites, which suggested that older women with higher body weight and body fat composition may have lower dietary phytoestrogen intake, or different metabolism and distribution in body tissues, compared to women with the opposite characteristics.

Lampe (2003) and Lampe, Atkinson, and Hullar (2006) in a review of experimental studies in human subjects, suggested that gender-specific physiological differences in phytoestrogen absorption and metabolism i.e. gut transit time, and composition of intestinal microbiota may have influenced the bioavailability of phytoestrogen and consequently the level of biomarkers, i.e. women tend to have more phytoestrogen metabolite-producing bacterial communities than men (Clavel et al., 2005). The formation of lignan and isoflavone metabolites is facilitated by intestinal microbiota, and use of antibiotics that disrupt the composition and balance of intestinal microbiota have impacted on the bioavailability and metabolism of phytoestrogens.

For example, Kilkkinen et al. (2002) found that subjects who had used antibiotics up to 12-16 months prior to serum sampling had significantly lower serum ENL concentrations. Sutherland et al. (2012) found that the antimicrobial agent tetracycline inhibited the daidzein-metabolising bacteria; thus less daidzein metabolites were produced.

In summary demographic, social and lifestyle variables, such as BMI, bowel movement, smoking status, physical activity level, age and gender, supplement and antibiotics use are important determinants in dietary phytoestrogen intake and consequently biomarker levels. Information on these sociodemographic variables was obtained for the current study to explore possibly associations with phytoestrogen status, and recent intake of antibiotics was an exclusion criterion. In Australia, the population consists of different cultural and ethnic groups (Australian Bureau of Statistics, 2014). This would enrich the cultural diversity as different ethnic groups have distinctive dietary preferences. Therefore cultural background and ethnicity was also explored in relation to phytoestrogen intake and metabolites in the current research.

2.13 Concluding remarks

The literature review of the current thesis firstly covered the background knowledge of phytoestrogen absorption and metabolism, and epidemiological health outcomes of phytoestrogen consumption. Secondly it discussed the use of dietary tools and evaluation of the dietary tool with a reference method and biomarkers. Thirdly, it reviewed recent research conducted on validation and evaluation of FFQ to measure phytoestrogen, and pioneering and recent work on analysing phytoestrogen content in foods and establishment of phytoestrogen databases. Then it critically appraised studies that quantified intake of phytoestrogens and identified contributing food sources in different populations from various countries and background, with the use of different methodologies, dietary tools and phytoestrogen databases. Lastly, it described the pharmacokinetics of phytoestrogen metabolism and the influence of demographic, social and lifestyle factors associated with phytoestrogen status.

Much of the early research had focused on isoflavones, being one of the two main classes of

phytoestrogen. More recent research work has focused on lignans because they are more prevalent in dietary patterns followed by Western populations. Lignans and isoflavones being structurally alike, their pathway of absorption and metabolism is similar. The metabolism of phytoestrogens indicates that dietary precursors are actively converted to metabolites and excreted in urine in a dose-dependent manner. Measurement of metabolites in urine therefore provides a feasible method to assess phytoestrogen status and evaluate the validity of the dietary assessment tool. A range of sociodemographic and lifestyle factors can influence this association and therefore need to be considered.

Epidemiological research has demonstrated that phytoestrogens are positively associated with reduced risk of hormone dependent cancers, although not all studies are supportive and more evidence is required to draw conclusions regarding the relationship between phytoestrogens and risk of chronic diseases.

Results from validation studies showed that the FFQs were not consistently valid or reliable in measuring phytoestrogen intake, especially for lignans, possibly due to lack of specificity of the FFQ for measuring the range of lignan sources in a varied diet; or limitations of available databases for estimation of lignans. Most studies have been restricted to female subjects, therefore, in the current research, subjects of both genders from diverse backgrounds were recruited.

Previous cross-sectional studies have focused on phytoestrogen intake of populations from Scandinavian, European, American and Asian countries, with isoflavones being the focus in Asian countries and lignans and enterolignans being the focus in Western countries. Contributing food sources to phytoestrogens varied amongst countries, as populations had different food choices and cultural preferences. Limited data are available on phytoestrogen intake in the Australian multicultural context.

This review of the literature mapped the ground of knowledge on phytoestrogens, critically reviewed what has been done in this field of research, and informed the knowledge gap that should be addressed. For the current study an original phytoestrogen FFQ needs to be updated

and refined to align with food groups and dietary patterns in the current Australian Dietary Guidelines, in particular to optimise measurement of lignans from the FFQ, and utilising current databases of lignan content available from direct measurement of lignans in foods.

Table 2. Results of the evaluation of food frequency questionnaires in measuring phytoestrogen intake

Author (country)	FFQ: PEs measured, # of food items	Study sample	Convergent validity, Intake by reference method	Criterion validity, Excretion by biomarker	Reliability, Intake by FFQ2	Results and comments
Validation studies that evaluate for both lignan and isoflavone phytoestrogen						
Hanna (Australia) 2005	IFs (daidzein, genistein, glycitein); LGs (SECO, MAT) PEs (IFs+LGs), 112 items	141F (40-59y)	The questionnaire was previously validated with 7dWFRs, adopted and modified for PEs measurement	√ FFQ and 3x24h urine over 1week Total IFs (r=0.207, P≤0.05), Total LGs (r=0.138, P>0.05), Total PEs intake and urine IFs in (r=0.245, P≤0.01), Total PEs intake and urine LGs (r=0.199, P≤0.05), Total PEs intake and urine PEs(r=0.283, P≤0.01) Median excretion: IFs 0.418 (0.005-41), LGs 0.771 (0.004-11)	√ FFQ1 and FFQ2 after 6-10months IFs Intake (r=0.60, CI95% 0.23-0.81) LGs intake (r=0.36, CI95% 0.07-0.68) Intake not reported	**FFQ intake**: IFs median 0.00mg/d, LGs 1.61mg/d **Conclusion:** FFQ appeared more reliable for IFs than LGs**.** FFQ was not valid with urine LGs **Strength:** Australian population **Limitation**: female subjects; LGs intake measured based on LGs content from an indirect method
Bhakta (U.K.) 2005	IFs (genistein, daidzein), LGs (SECO, MAT), 277 items	58F (25-75y)	√ FFQ and 12x24hDRs for 1year IFs (r=0.57, P<0.001) LGs (r=0.70, P<0.001) IFs Mean: 405 µg/d, median:194µg/d LGs mean:141µg/d, median:110µg/d	√ FFQ and 4x2ml plasma every 3month for 1 year FFQ and plasma Genistein (r=0.21, P=0.12), plasma Daidzein (r=0.32, P=0.02), and plasma ENL (r=0.10, P=0.43) 24hDRs and plasma Genistein (r=0.43, P<0.001), plasma Daidzein (r=0.40, P<0.002), and plasma ENL (r=0.08, P=0.50) Mean excretion: genistein 18.3nmol/L, daidzein 7.8nmol/L, ENL 13.7nmol/L	×	**FFQ intake:** IFs mean 471µg/d, median 327µg/d, LGs mean 208µg/d, median 149µg/d **Conclusion:** FFQ appeared valid for LGs. FFQ had highest VC for LGs **Strength:** method of triads. Genistein,24hDrs(0.95),FFQ(0.46),Plasma(0.45).Daidzein,24hDRs(0.83),FFQ (0.67),Plasma(0.45).LGs,24hDRs(0.73);FFQ (0.91),Plasma(0.11), cross classification **Limitation:** female subjects; no reliability was assessed.

Author (country)	FFQ: PEs measured, # of food items	Study sample	Convergent validity, Intake by reference method	Criterion validity, Excretion by biomarker	Reliability, Intake by FFQ2	Results and comments
Horn-Ross (U.S.) 2006	IFs (daidzein, genistein, formononetin, biochanin A), LGs (SECO, MAT) Coumestant (coumestrol), Block95 FFQ plus 32 items for PEs	175F (≤85y)	√ FFQ and 4x24DRs for 10 months (no p-values) FFQ1, IFs (ρ=0.70), LGs (ρ=0.74) FFQ2, IFs (ρ=0.72), LGs (ρ=0.76) IFs median1.6mg/d, LGs 161μg/d	√ FFQ and 2x24h urine (no p-values reported) FFQ and urine IFs (ρ=0.50), urine LGs (ρ=0.16) 24hDRs and urine IFs (ρ=0.43), urine LGs (ρ=0.21) Excretion not reported	√ FFQ1 and FFQ2 10 month apart (no p-values reported) IFs (ICC=0.77) LGs (ICC=0.80) SECO (ICC=0.81) MAT (ICC=0.67) IFs median 1.5mg/d, LGs 137μg/d	**FFQ intake:** Median IFs 1.5mg/d, LGs 138μg/d **Conclusion:** FFQ appeared more valid for IFs than LGs when comparing with 24hDRs and urine PEs, but it appeared more reliable for LGs than IFs **Strength**: large sample size **Limitation:** female subjects, no p-values reported for the significance of results
French, Thompson and Hawker (Canada) 2007 :	IFs (daidzein, genistein), LGs (SECO, MAT), 53 items	26F (25-42y)	×	√ FFQ and 1x24h urine FFQ1 and urine IFs (r = 0.54, P = 0.004), and urine LGs (r = 0.40, P = 0.045), FFQ2 and urine IFs (r = 0.64, P <0.001), and urine LGs (r = 0.46, P = 0.019) IFs mean 17.4umol/d, median 4.1umol/d, LGs mean 20.6umol/d, median 6.4umol/d	√ FFQ1 and FFQ2 one month apart IFs intake (r = 0.69, P < 0.001), LGs intake (r = 0.69, P<0.001) IFs mean 10.8mg/d, median 0mg/d, LGs mean 8.8mg/d, median 0.7mg/d	**FFQ intake:** IFs mean 13.7mg/d, median 15mg/d, LGs mean 13.8mg/d, median 3.8mg/d **Conclusion:** FFQ appeared valid for LGs and IFs when compared to urine PEs and equally reliable for LGs and IFs **Strength**: cross-classification **Limitation:** female subjects, no convergent validity and reliability, small sample size

Author (country)	FFQ: PEs measured, # of food items	Study sample	Convergent validity, Intake by reference method	Criterion validity, Excretion by biomarker	Reliability, Intake by FFQ2	Results and comments
Validation study that evaluate for lignans						
Lin (Sweden) 2012	LGs (SECO, MAT, LARI, PINO, MED and SYR), 67 items for FFQ-87, 93 items for FFQ-97	140F (55-75y)	×	√ FFQs and 1x serum 3 months apart FFQ-87 and serum ENL (ρ=0.09, P=0.47), FFQ-97 and serum ENL (ρ=0.22, P=0.01) ENL Mean 23.2nmol/l, median 20.8nmol/l,	×	**FFQ intake:** FFQ87 mean 1616µg/d, median 1552µg/d, FFQ97 mean 1516µg/d, median 1497µg/d **(**P=0.002) **Conclusion:** FFQ-97 was a better tool to measure LG status than FFQ-87. **Strength:** lignan specific, include more LG subclasses **Limitation:** not validation study because no reference method. Only comparing two FFQs with serum LGs to determine which FFQ is a better tool for LGs

Author (country)	FFQ: PEs measured, # of food items	Study sample	Convergent validity, Intake by reference method	Criterion validity, Excretion by biomarker	Reliability, Intake by FFQ2	Results and comments
Validation studies that evaluate for isoflavones						
Huang (U.S.) 2000	IFs (daidzein, genistein), Block 95 FFQ plus additional 58 items for PEs	69F. 51 Japanese (48.4) and 18 Caucasian (45.8)	√ FFQ and 4x48hDRs over 4weeks Daidzein (r=0.58, P<0.0001), Genistein (r=0.52, P<0.0004) (Mean, median) Japanese, daidzein 7336µg/d, 4402µg/d, genistein 11118µg/d,6887µg/d. Caucasian, daidzein 1036µg/d, genistein 2049µg/d	√ FFQ and 4x24h urine over 4 weeks Daidzein (r=0.49, p<0.0002, unadjusted), Genistein (r=0.30, p=0.0346, unadjusted) Average of 48hDRs and 4x24h urine) Daidzein (r=0.45, p<0.0027, unadjusted), Genistein (r=0.41, p=0.0057, unadjusted) First morning and 24h urine Daidzein (r=0.58), Genistein (r=0.50) Excretion not reported	×	**FFQ intake:** (mean, median) Japanese, daidzein 7580µg/d, 5680µg/d, genistein 11402µg/d, 8778µg/d, Caucasian, daidzein 736µg/d, 108µg/d, genistein, 1435µg/d, 210µg/d **Conclusion:** IFs Intake moderately correlated with that of 48DRs and excretion **Strength:** Intake by ethnicity. **Limitation**: female subjects; no reliability was assessed. Small sample size
Verkasalo (U.K.) 2001	IFs (daidzein, genistein), Total IFs (sum of daidzein, genistein, glycitein), FFQ from UK arm of EPID study plus 3 questions of soya intake	80F (20 in each 4 groups of soy intake level) (20-39y)	×	√ (FFQ and 1x plasma) (p<0.001) Daidzein (r=0.74), genistein (r=0.69), Total IFs intake and plasma daidzein (r=0.56), and plasma genistein (r=0.53) 7dFDs and 1x plasma Daidzein (r=0.79), Genistein (r=0.80), Total IFs intake and plasma daidzein (r=0.79) and plasma genistein (r=0.80) No overall excretion reported	×	**No overall FFQ intake reported** **Conclusion:** IFs intake can be estimated with either a FFQ or 7dFDs. Plasma can be biomarker of soy intake **Strength:** Small number of soy items sufficient to estimate intake and have good correlation with biomarker **Limitation**: no convergent validity and reliability assessed.

Author (country)	FFQ: PEs measured, # of food items	Study sample	Convergent validity, Intake by reference method	Criterion validity, Excretion by biomarker	Reliability, Intake by FFQ2	Results and comments
Yamamoto (Japan) 2001	IFs (daidzein, genistein), 8 items	247 subjects, 125F (55y) and 122M (56y)	√FFQ and 4x7dDRs over 1 year Daidzein (r=0.60, CI:0.5-0.69), Genistein (r=0.59, CI:0.5-0.68) Mean daidzein 14.5mg/d, genistein 23.4mg/d	√ FFQ and 1x serum, and 2x 24h urine serum daidzein (r=0.26, CI:0.13-0.39), genistein (r=0.22, CI: 0.08, 0.35), urine daidzein (r=0.40, CI:0.21-0.60), genistein (r=0.30, CI:0.08-0.51) 7dDRs and 1x serum, and 2x 24h urine serum daidzein (r=0.37, CI:0.25-0.49), genistein (r=0.36, CI:0.18, 0.55), urine daidzein (r=0.48, CI:0.31-0.64), genistein (r=0.36, CI:0.18-0.55) 1x serum and 2x 24h urine serum and urine daidzein (r=0.22, CI:0.01-0.42), genistein (r=0.32, CI:0.12-0.52) Mean serum daidzein 119.9nmol/l, genistein 475.3nmol/l, Mean urine daidzein 17µmol/d, genistein 14.2µmol/d	√ FFQ1 and FFQ2 one year apart r=0.75, CI: 0.68-0.82 r=0.72, CI: 0.61-0.83	**FFQ intake:** mean daidzein 18.3mg/d, genistein 31.4mg/d **Conclusion:** FFQ appeared to be a valid and reproducible in estimating IFs intake **Strength:** female and male subjects, large sample size, account for seasonal difference (repeated DRs for each season and repeated FFQ one year apart) **Limitation**: single urine and serum samples

Author (country)	FFQ: PEs measured, # of food items	Study sample	Convergent validity, Intake by reference method	Criterion validity, Excretion by biomarker	Reliability, Intake by FFQ2	Results and comments
Frankenfeld (U.S.) 2002	IFs (daidzein, genistein), 40 items for Soy FFQ, 122 items (2 for IFs) for WHI FFQ	77F and M (20-40y)	×	√ FFQs and 1x plasma Soy FFQ daidzein (r=0.45, CI:0. 25,0.61), genistein (r=0.53, CI:0. 35, 0.67) WHI FFQ daidzein intake (r=0.45, CI:0. 25,0.61), genistein intake (r=0.46, CI:0. 26, 0.62) Daidzein plasma concentration mean 8.8mmol/dl, median 4.7mmol/dl, genistein mean 16.9mmol/dl, median 12.8mmol/dl	√ Soy FFQ and WHI FFQ, completed at the same time -compare 2 different FFQs) (p<0.001) Daidzein (r=0.82), Genistein (r=0.83) WHI FFQ daidzein mean 5.6mg/wk median 2.1mg/wk, genistein mean 6.5mg/wk median 3mg/wk	**FFQ intake:** daidzein mean 9.7mg/wk, median 7.6mg/wk, genistein mean 11.9mg/wk, median 11.1mg/wk **Conclusion:** Soy FFQ appeared to be a valid estimate of soy intake **Strength:** female and male subjects **Limitation**: convenience sample, only compare the intake from two different FFQs (no reliability of one FFQ was assessed)
Frankenfeld (U.S.) 2003	IFs (daidzein, genistein), 20 items for soy FFQ, 122 items (2 for IFs) for comprehensive FFQ	96F postmeno pausal, (50-79y)	×	√ FFQs and 2x plasma one at each visit (p<0.001) Soy FFQ daidzein (r=37), genistein (r=0.43) Comprehensive FFQ daidzein (r=0.35), genistein intake (r=0.38) Daidzein plasma concentration (mean, median) 6.9mmol/dl,5mmol/dl, genistein12.2mmol/dl, 10.9mmol/dl	√ FFQs one week apart Diadzein and genistein (r=0.67, p<0.001) Comprehensive FFQ daidzein mean 2.2mg/wk, genistein mean 2.5mg/wk	**FFQ intake:** daidzein mean 6.2mg/wk, median 3.7mg/wk, genistein mean 8.1mg/wk, median 7.5mg/wk **Conclusion:** Soy FFQ and comprehensive FFQ appeared to be reliable and valid **Strength:** Bland Altman analysis (no plots) between the two FFQs **Limitation**: female subjects. No convergent validity assessed

Author (country)	FFQ: PEs measured, # of food items	Study sample	Convergent validity, Intake by reference method	Criterion validity, Excretion by biomarker	Reliability, Intake by FFQ2	Results and comments
Frankenfeld (China) 2004	IFs (daidzein, genistein), 107 items (6 soy items)	1823F (30-64y)	×	√ FFQ and 2x serum Significant trend of increasing serum daidzein and genistein level across increasing quartiles of isoflavone intake ($p<0.01$) No overall excretion reported	×	**No overall FFQ intake reported** **Conclusion:** FFQ provided ranking of IFs consumption and exposure **Strength:** rank intake for large sample size **Limitation**: Population not representative (female textile workers). No convergent validity, reliability and correlation were assessed.
Heald (Scotland) 2006	IFs (daidzein, genistein), 155 items	203M (50-74y)	Previously validated against with 4dWDRs for nutrients intake, but for PEs	√ SCG-FFQ and 1x serum 4-9 weeks apart daidzein (r=0.24, CI:0.11,0.36, p=0.001) genistein (r=0.26, CI:0.12,0.38, p<0.001) Total IFs (r=0.27, CI:0.13,0.39, p<0.001) Median daidzein 18nmol/l, genistein 33.79nmol/l, IFs 51.49nmol/l	×	**FFQ intake:** median IFs 1mg/d **Conclusion:** FFQ appeared to be valid for ranking intakes **Strength**: level of agreement (cross classification) analysis (categorised into same and opposite tertile of intake and serum concentration) **Limitation:** male subjects, no convergent validity and reliability were assessed

Author (country)	FFQ: PEs measured, # of food items	Study sample	Convergent validity, Intake by reference method	Criterion validity, Excretion by biomarker	Reliability, Intake by FFQ2	Results and comments
Tseng (U.S.) 2008	IFs (daidzein, genistein), 26 items for soy FFQ, 126 items for DAF	27F (30-50y)	×	√ Soy FFQ, DAF and 4x24h urine (no p-values reported) Soy FFQ daidzein (r=0.49), genistein (r=0.54) Diet assessment form (DAF) daidzein (r=0.54), genistein (r=0.33) 24h urines and overnight urine daidzein (r=0.84), genistein (r=0.93), glycitein (r=0.76), ODMA (r=0.94), equol (r=0.61) 24h excretion daidzein mean 323ng/mgC, median 110ng/mgC, genistein mean 136ng/mgC, median 67ng/mgC	×	**FFQ intake:** SFQ daidzein mean 2.9mg/d, median 0.015mg/d, genistein mean 3.9mg/d, median 0.013mg/d, DAF daidzein mean 2.1mg/d, median 0.032mg/d, genistein mean 2.2mg/d median 0.129mg/d **Conclusion:** Soy FFQ and DAF valid in ranking intake; overnight urine correlate well with 24h urine **Strength:** Comparison of excretion between first-morning and 24h urine (similar to Huang et al. 2000). **Limitation:** female subjects, small sample size, no p-values reported
Fernandez (Malaysia) 2013	If (genistein), 19 items	64 pregnant F	√ FFQ and 7dFDs Genistein (r=72, p<0.001) FD mean=9.9mg/d	√ FFQ and serum No coefficient and p-values presented Dose-response relation (p<0.01) between intake and serum level across quintile of intake: mean of serum level increases as quintile of intake increases Mean serum 55.9nmol/l	√ FFQ1 and FFQ2 three to four weeks apart No coefficient and p-values presented FFQ2 intake mean 13.3mg/d	**FFQ intake:** mean 13.7mg/d **Conclusion:** 33 items FFQ is valid in measuring genistein. **Strength:** Bland Altman plot and percentage agreement (cross-classification) **Limitation:** female subjects, small sample size, measure only genistein

Author (country)	FFQ: PEs measured, # of food items	Study sample	Convergent validity, Intake by reference method	Criterion validity, Excretion by biomarker	Reliability, Intake by FFQ2	Results and comments
Chan (Hong Kong) 2008	IF (combined total IFs), 47 items	145 Chinese mid-life women	√ FFQ and 23 day x 24h DRs throughout 1-year) IFs (ρ=0.53, p<0.001) Median intake 6.3mg/d (p<0.0057)	×	√ (FFQ1 and FFQ2 thirteen month apart) ICC=0.68 FFQ2 median 7.7mg/d (P=0.83)	**FFQ intake:** IFs mean 6.6mg/d **Conclusion:** soy FFQ is valid and reliable in measuring IFs. **Strength:** Bland Altman plot and percentage agreement (cross-classification) **Limitation:** female subjects

Key: FFQ = food frequency questionnaire, 24h-, 48h-,7d-DRs = 24-hour, 48-hour, 7-day dietary recalls/records, 7dWFRs = 7-day weighed food records, 4dWDRs=4-day weighed diet record, 7dFDs=7-day food diaries, DAF =Diet assessment form, LGs = lignans, IFs = isoflavones, PEs = phytoestrogens, ENL = enterolactone, VCs= validity coefficients, r = Spearman correlation coefficient, ρ = Pearson correlation coefficient, ICC = intraclass correlation coefficient, F=female, M=male

Table 3. Cross-sectional studies of lignan and isoflavone intake and food sources

Author Country	Population	Dietary tool	Intake μg/d, (mean and/or median)	Phytoestrogens measured; Database; Food sources (%)
Studies that measure lignan, isoflavone and enterolignan phytoestrogens				
Hedelin (2006) Sweden	1,130 male control, 1,499 case of prostate cancer, 35-65y	261-items validated FFQ for phytoestrogens	Mean (range), Median Control Total LGs 4,855 (292-106,958), 3,045 Total enterolignans 16.06 (0-167), 13.05 Total IFs 343 (1-18,975), 15 Case Total LGs 4,929 (310-73,754), 3,044 Total enterolignans 16.06 (0-102), 13.05 Total IFs 491 (0-37,760), 16	LGs: SECO, MAT, PINO, LARI, SYR, MED; Enterolignan; END, ENL; IFs: daidzein, genistein, formononetin, biochanin A, equol; Finnish, Swedish; N/A
Hedelin (2008) Sweden	45,448 female control, 1,014 case of breast cancer, 30-49y,	80-items self-administered validated FFQ	Mean±SD (range), Median Control Total plant LGs 1,763±886 (15-14,473), 1,632 Total enterolignans 11±9 (0-110), 9 Total IFs 73±166 (0-5,023), 7 Case Total plant LGs 1,784±887 (31-7,435), 1,639 Total enterolignans 10±8 (0-67), 8 Total IFs 78±181 (0.2-1,979), 7	LGs: SECO, MAT, PINO, LARI, SYR, MED; Enterolignan: END, ENL; IFs: daidzein, genistein, formononetin, biochanin A, equol; Finnish, Swedish; N/A

Author, Country	Population	Dietary tool	Intake μg/d, (mean and/or median)	Phytoestrogens measured; Database; Food sources (%)
Lahmann (2012) Australia	2,078 female control cohort from case-control gynaecological cancer study, 18-79y	135-items FFQ, modified and validated in Australia for energy and nutrients intake	Mean±SD, Median (IQR) Total LGs 693±296, 639 (323), Total enterolignans 22±12, 21(15) Total IFs 3,293±5,71, 1,286 (1,693)	LGs: SECO, MAT, PINO, LARI; Enterolignan: END, ENL; IFs: Equol, daidzein, genistein, glycitein, formononetin, biochanin A; U.K. (for SECO, MAT, and total enterolignans and IFs), Canadian (LARI, PINO); Low intake (≤1286μg/d): non-soya bread (23%), vegetables (22.5%), fruits (16%) High intake (≥1286μg/d): Soybean and linseed bread (22%), Soybean milk (19%), non-soya bread (12%)
Zamora-Ros (2012) European countries	36,037 adults from European Prospective Investigation into Cancer and Nutrition (EPIC) study, 35-74y	24 hour dietary records	Mean±SE Total LGs 1,037±30 (Greece) to 2,145±90 (U.K.) Total enterolignans 10±0 (Greece) to 18.5±0 (Sweden) Total IFs 236±140 (Greece) to 12,133±360 (U.K)	LGs: SECO, MAT, PINO, LARI; Enterolignan: END, ENL; IFs: daidzein, genistein, glycitein, formononetin, biochanin A; Dutch, U.K., Canadian; Mediterranean countries Total LGs: Fruits (31%), vegetables (26%), alcoholic beverages (11%) Non-Mediterranean countries Total LGs: Non-alcoholic beverages (25%), vegetables (23%), Cereals products (20%) Mediterranean countries Total IFs: Soy products (49%), legumes (9%), vegetables (7%) Non-Mediterranean countries Total IFs: Soy products (32%), sugar and confectionary (17%), non-alcoholic beverages (13%) Mediterranean countries Total enterolignans: dairy (92%), sugar and confectionary (2%), condiments and sauces (2%), meat (1%) Non-Mediterranean countries total enterolignans: dairy (90%), condiments and sauces (3%), sugar and confectionary (2.9%)

Author, Country	Population	Dietary tool	Intake μg/d, (mean and/or median)	Phytoestrogens measured; Database; Food sources (%)
Mulligan (2012) U.K.	20,437 adults from of EPIC Norfolk cohort, 40-79y	7 day food diaries	Mean±SD, Median (IQR) Total LGs, non soya-consuming (NSC) men 311±178, 273 (209-364), Total LGs, soya-consuming (SC) men 361±230, 309 (237-422) Total LGs, NSC women 251±141, 229 (180-293) Total LGs, SC women 318±212, 276 (215-351) Total enterolignan NSC men 19±9, 18 (13-24) Total enterolignan SC men 18±9, 17 (12-24) Total enterolignan NSC women 16±8, 16 (11-21) Total enterolignan SC women 16±8, 15 (10-20) Total IFs, NSC men 1,033±871, 858 (633-1,155) Total IFs, SC men 4,664±4,973, 2,413 (998-6,845) Total IFs, NSC women 734±597, 615 (457-819) Total IFs, SC women 5,055±6,054, 2,830 (779-6,938)	LGs: SECO, MAT, Shonanin; Enterolignan: END, ENL; IFs: daidzein, genistein, glycitein, formononetin, biochanin A; U.K.; NSC group (men and women); bread and bread rolls (43%), breakfast cereals (12%), tea and coffee (6%) SC group (men and women): vegetables dishes (41%), milk, soy/goat/sheep (25%), bread and bread rolls (11%)

Author, Country	Population	Dietary tool	Intake μg/d, (mean and/or median)	Phytoestrogens measured; Database; Food sources (%)
Studies that measure lignans and isoflavones				
Bhakta (2006) U.K.	221 South Asians residents in U.K. and 49 Native British residents, 25-75y	12 x 24-hour recalls for 1 year	Mean±SD, Median (IQR) Total IFs South Asians 322±877, 184 (121-278) Total LGs South Asians 137±82, 111 (77-182) Total IFs Native British 366±176, 334 (227-449) Total LGs Native British 183±132, 149 (87-229)	IFs: daidzein, genistein; LGs: SECO, MAT; U.K. (for IFs), Finnish (LGs) IFs - South Asians, breads (51%), vegetables/vegetable dishes (38%), milk/milk products (8%), flours, grains & cereals (2%) Native British, breads (93%), milk/milk products (3%), vegetables/vegetable dishes (2%), flours, grains. cereals (1%) LGs - South Asians, breads (70%), vegetables/vegetable dishes (12%), miscellaneous (7%), fruit and fruit juices (5%), flours, grains, cereals (3%) Native British, breads (60%), fruit and fruit juices (21%), miscellaneous (9%), vegetables/vegetables dishes (6%), alcohol (2%)
Cotterchio (2008) Canada	3,370 women	178-items FFQ	Median (IQR) Total LGs, 857 (305, 4,005), Total IFs, 230 (93, 842)	LGs: SECO, MAT, PINO, LARI; IFs: daidzein, genistein, glycitein, formononetin; Canadian; IFs, soybean (37%), soy milk (18%), tofu (13%) LGs, Flaxseed (88%), multigrain bread (6%), flax bread (3%)
Saleh (2011) Bangladesh	111 postmenopausal women	7-day FFQ	Mean±SD, Median (range) Total LGs, 7,320±3,280, 7,310 (5,530-9,830) Total IFs, 320±160, 320 (190-390)	LGs: SECO, MAT; IFs: daidzein, genistein, formononetin, biochanin A; Finnish (scoring PEs concentration); IFs, soybean oil (96%), Beans and peas (4%), cabbage, cauliflower LGs, beans and peas (6%), cabbage, cauliflower, carrot (5%), onion, garlic, pepper (3%)

Author, Country	Population	Dietary tool	Intake μg/d, (mean and/or median)	Phytoestrogens measured; Database; Food sources (%)
Huang (2012) U.S.	Multiethnic women from The Study of Women's Health Across the Nation (SWAN) midlife	137-items FFQ	Median (IQR) Total LGs, 191 (146) African American Total LGs, 250 (170) Caucasian Total LGs, 291 (248), English speaking Chinese Total LGs, 354 (232), Chinese speaking Chinese Total LGs, 233 (180), English speaking Japanese Total LGs, 371 (292), Japanese speaking Japanese Total IFs, 285 (545) African American Total IFs, 318 (699) Caucasian Total IFs, 5,487 (13,545) English speaking Chinese Total IFs, 9,714 (15,215) Chinese speaking Chinese Total IFs, 6,743 (9,334) English speaking Japanese Total IFs, 24,585 (21,704) Japanese speaking Japanese	LGs: SECO, MAT, PINO, LARI; IFs:daidzein, genistein, glycitein, formononetin; Canadian; LGs - African American: coffee (12-13%), black tea (7-16%), green salad (9-11%), Chinese herbs in soup/tea (5-12%) Caucasian: coffee (13-23%), black tea (6-20%), green salad (10-12%), broccoli (6-8%) English speaking Chinese: Chinese herbs in soup/tea (25%), black tea, (13%) green tea (8%), green leafy vegetables (6%). Chinese speaking Chinese: Chinese herbs in soup/tea (23%), black tea (16%), green leafy vegetables (11%), green tea (9%) English speaking Japanese: coffee (15%), green tea (12%), green salad (10%), black tea (9%). Japanese's speaking Japanese: green tea (20%), Chinese herbs in soup/tea (13%), black tea (11%), coffee (9%) IFs - African American: soymilk (76-86%), tofu (4-11%), donut (2-9%), meat substitute (6%) Caucasian: soymilk (54-86%), tofu (5-18%), meat substitute (5-8%), fresh green soybean (5-21%) English speaking Chinese: Soymilk(51%), tofu(21%), fresh green soybeans(10%), dry spiced tofu(9%). Chinese speaking Chinese: Soymilk(52%), tofu(27%), dry spiced tofu(5%), fermented soybean(3%) English speaking Japanese: Fresh green soybean(36%), tofu(21%), soymilk(15%), roasted soybean(13%). Japanese speaking Japanese: fermented soybean(30%), fresh green soybean(26%), tofu(21%), soymilk(8%)

Author, Country	Population	Dietary tool	Intake μg/d, (mean and/or median)	Phytoestrogens measured; Database; Food sources (%)
Hu (2014) China	1,000 subjects from 31 provinces or province-level administrative regions	Modified semi-quantitative food frequency questionnaire	Mean±SD, Median Total IFs 183.2±122.7μg/kg/day, 149.9μg/kg/day SECO 26.1±20.2μg/kg/day, 22.5μg/kg/day With average weight of Chinese population, 60kg Estimated Total IFs, 10,992μg/day, SECO, 1,566μg/day	Total IFs daidzein, genistein, glycitein, LGs, SECO; Chinese (on plant-derived foods) Total IFs Soy (22%), soybean sprouts (19%), Chinese lettuce (17%), silken bean curd (13%), taro (6%) SECO Carrot (40%), Chinese lettuce (10%), taro (9%), white gourd (6%)
Studies that measure lignans and enterolignans				
Touillaud (2007) France	58,049 women, 41-72y	208-items self-administered validated diet history questionnaire	Median (range) Total LGs 1,112 (0-5,702) Total enterolignans 768 (0-2,538)	Total LGs: SECO, MAT, PINO, LARI, Total enterolignans: END, ENL; Finnish, Dutch, French, Canadian (indirect analysis for content of lignan metabolites produced); Fruit (35%), vegetables (30%), tea (11%)
Peñalvo (2012) Spain	3,534 children and young adults, 2-24y	24 diet recalls over the year	Mean, Median (IQR) Total LGs, 1,080, 830 (0.49-1.33) ENL alone, 14, 13 (8-20) Mean SECO, MAT, PINO LARI, 786	Total LGs: SECO, MAT, PINO, LARI, SYR, MED, Enterolignan: ENL; Spanish; White bread (17%), olive oil (16%), virgin olive oil (11%)
Studies that measure lignans				
Milder (2005) The Netherlands	4,660 adults, 19-97y	2 day dietary record	Mean±SD (range) Total LGs 1,241±2,052 (43-77,584)	Total LGs SECO, MAT, PINO, LARI; Dutch; Beverages (37%), vegetables (24%), nuts and seeds (14%)

Author, Country	Population	Dietary tool	Intake μg/d, (mean and/or median)	Phytoestrogens measured; Database; Food sources (%)
Galvan-Portillo (2007) Mexico	50 women, 17-37y	54-items FFQ	Mean±SD 104, plant lignan mean	Total LGs, SECO, MAT, PINO, LARI; Dutch, Finnish; Pinto bean (76% to SECO), broccoli (20% to LARI, 10% to PINO), orange (55% to MAT)
Nurmi (2010) Finland	100 men	4 day food record	Mean±SD (range) Total LGs 1,224±539 (250-989)	Total LGs SECO, MAT, PINO, LARI; Dutch, Finnish; Rye products, berries, coffee, tea, wholegrains
Sohrab (2013) Iran	2,618 adults, 19-84y	168-items validated semi-quantitative FFQ	Median Total LGs, 200	Total LGs SECO, MAT, PINO, LARI; European Phenol-Explorer online database; Nuts (r=0.28), wholegrain (r=0.26), meat poultry and fish (r=0.10)
González (2013) Iran	304 institutionalised elderly adults	Specially designed FFQ for each 14 institution	Mean±SD Total LGs, 450±0.3	Total LGs, SECO, MAT, PINO, LARI; European Phenol-Explorer online database; Olive oil, white bread, red wine, wholemeal bread
Meija (2013) Latvia	172 men and 97 women, 40-75y	147-item, country specific food frequency questionnaire modified for lignan	Mean±SD, Median (IQR) Total LGs Men 5,151±6,364, 2,783 (1,266-6,815) Women 3,339±4,429, 1,823 (1,022-3,349)	Total LGs, SECO, MAT, PINO, LARI, SYR, MED; Canadian (for SECO, MAT, PINO, LARI), Latvian (all LGs, particular SYD and MED, for Latvian local breads); Food sources (only SECO, MAT, PINO, LARI) Men, cereals (rye and seed bread) (90%), nuts and seeds (flaxseed) (7%), beverages (coffee and tea) (1%), vegetables (cabbage) (1%) Women, cereals (rye and seed bread) (60%), nuts and seeds (35%), vegetables (2%), fruit and berries (2%)

Author, Country	Population	Dietary tool	Intake μg/d, (mean and/or median)	Phytoestrogens measured; Database; Food sources (%)
Studies that measure isoflavones				
Wong (2007) Hong Kong (H.K.) SAR, China	1,701 men with lower urinary tract symptoms, ≥65y	Modified 285-item Block FFQ with 10 commonly consumed soya items in H.K.	Mean±SD, Median (IQR) Total IFs 15,730±23,130, 11,470 (13,230)	Total IFs daidzein, genistein, glycitein; U.S. (Dwyer et al. 1994, Anderson 1997); N/A
Iwasaki (2009) Japan and Brazil	780 Japanese in Japan, 162 Japanese Brazilian in Brazil, 758 non-Japanese Brazilian in Brazil, case/control of breast cancer study, 20-74y	136-item semi-quantitative FFQ for Japan, 118-item version for Brazil	Mean Total IFs Cases Japanese in Japan 43,500 Japanese Brazilian in Brazil 16,500 Non-Japanese Brazilian in Brazil 1,100 Control Japanese in Japan 46,100 Japanese Brazilian in Brazil 24,900 Non-Japanese Brazilian in Brazil 4,400	Total IFs, daidzein, genistein Japanese (Kimira et al.1998, Arai et al. 2000); N/A
Zamora-Ros (2010) Spain	40,483 subjects, 15,446 men and 25,237 women, 35-64y	Computerised diet history questionnaire developed/ validated for EPIC Spain cohort	Mean±SD, Median Total IFs Men 110±440, 0 Women 40±160, 0 All 80±330, 0	Total IFs, daidzein, genistein, glycitein; USDA isoflavone database; Soybean sprouts (72%), beans (17%), chickpeas (8%), legumes (2%) soy sauce (0.1%)

Author, Country	Population	Dietary tool	Intake μg/d, (mean and/or median)	Phytoestrogens measured; Database; Food sources (%)
Lee (2013) Korea	8,502 subjects from 2008 Korea National Health and Nutrition Examination Survey (KNHANES) 2008, ≥2y	24-hour dietary recall	Mean±SE Total IFs, subjects meet *dietary recommendation 25,800±2,800 Total IFs subjects do not meet *dietary recommendation 5,400±500	Total IFs daidzein, genistein, glycitein; Korean (Food composition table supplied by Korea National Academy of Agricultural Science); Mandarin (67%), soybean curd (15%), soybeans (11%), orange (3%), soybean sprout (2%); *Dietary recommendation: 1 to 3 servings of fruit and 5 to 7 servings of vegetables
Tang (2013) China (North-West)	359 cases of oesophageal cancer, 380 controls, (mean±SD age 61±11)	Validated 137-item semi-quantitative FFQ	Mean±SD Total IFs Cases 16,500±29,900 Controls 27,100±36,500	Total IFs, daidzein, genistein, glycitein; USDA isoflavone database; N/A
Bai (2014) U.S.	17,900 subjects from NHANES III	24-hour dietary recall	Mean±SE Total IFs, 2,350±270	Total IFs, daidzein, genistein, glycitein; USDA isoflavone database; N/A

CHAPTER THREE: METHODS

3.1. Research design

This is a cross-sectional study that evaluated the validity and reliability of a modified phytoestrogen-specific dietary tool. The study was conducted to firstly, compare phytoestrogen intake derived from the modified dietary tool with intake derived from a reference dietary tool and with urinary excretion of phytoestrogen biomarkers; secondly, assess the dietary intake and food sources of phytoestrogens in a group of 59 Australians with the modified dietary tool, and thirdly explore the relationship between phytoestrogen intake and excretion, and selected social and lifestyle characteristics. An overview of the study design, including tools development, data collection and analysis is illustrated in **Figure 5.**

3.2. Tools and method development for data collection

This section describes the development of the tools and methods used for data collection. Databases for the lignan and isoflavone content from dietary sources were collated and utilised to measure intake of phytoestrogens from foods and beverages consumed by the research subjects. Phytoestrogen intake status was assessed with a previously-developed specific FFQ, which was reviewed, expanded and updated to measure phytoestrogen intake in the current Australian context. A 3-day weighed food record (WFR) was used as the reference method to collect details of foods and beverages consumed over three consecutive days. Phytoestrogen excretion status was assessed with urinary biomarkers of phytoestrogen metabolites. A questionnaire was designed to collect selected demographic and lifestyle characteristics which were shown to be associated with phytoestrogen status in previous research.

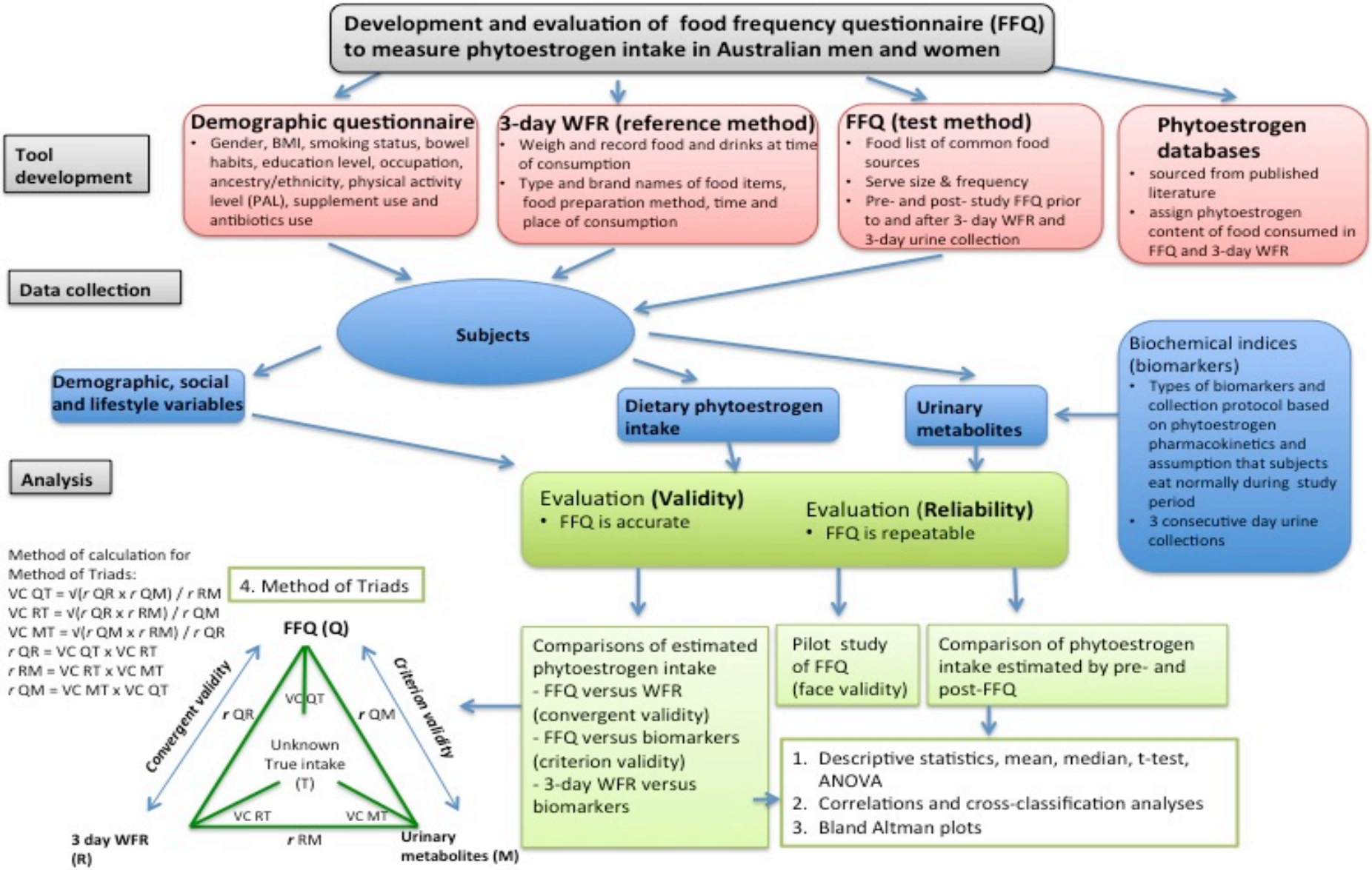


Figure 5. Overview of the study design

3.2.1. Dietary lignan and isoflavonoid database

There are no published data for the lignan content of Australian foods and therefore data were sourced from international published studies from The Netherlands, Finland, the U.S, and Canada (Blitz, Murphy, & Au, 2007; Mazur, 1998a; Mazur et al., 1999; Mazur, Wähälä, Rasku, et al., 1998; Meagher & Beecher, 2000; Milder, Arts, et al., 2005; Thompson et al., 2006). The database was compiled by a previous Master of Nutrition and Dietetics student at the University of Wollongong (2009) under the supervision of Associate Professor Philippa Lyons-Wall, and used with permission for the current study. This database was reviewed and updated by the candidate with more recently published lignan values and was used in the current study to quantify intake of the four primary dietary lignan precursors, SECO, MAT, LARI and PINO (Bonzanini et al., 2009; Clarke et al., 2013; Durazzo, Azzini, et al., 2009; Durazzo, Azzini, et al., 2013; Durazzo, Raguzzini, et al., 2009; Durazzo, Turfani, Azzini, Maiani, & Carcea, 2013; Durazzo, Zaccaria, et al., 2013; Kuhnle, Dell'Aquila, Aspinall, Runswick, Joosen, et al., 2009; Kuhnle et al., 2008a; Kuhnle, Dell'Aquila, Aspinall, Runswick, Mulligan, et al., 2009; Moreno-Franco et al., 2011; Nurmi et al., 2003; Peñalvo et al., 2008; Peñalvo et al., 2005; Smeds et al., 2007; Smeds et al., 2009).

Intake of two enterolignans, END and ENL was calculated using the one available database from the UK for animal-based foods (Kuhnle et al., 2008b). No plant-based foods contained END and ENL and no animal-based foods contained plant lignans.

Databases which provided the lignan content for all four dietary lignan precursors were preferentially selected for assigning lignan content to items for the current study. To account for the effect of the natural variability of phytoestrogens in foods, values for the same food item across multiple databases were averaged. The averaged values were used to assign lignan content to the food items in the phytoestrogen specific food frequency questionnaire and in the 3-day weighed food record. The final collated lignan database contained a total of over 1000 food and beverages items and included individual

foods, processed foods and mixed dishes from the major food groups: vegetables, fruits, fruit and vegetable juices; nuts and seeds, breads and cereals; dairy products and milk-like beverages; meats; also herbs and spices, tea and coffee, and alcoholic beverages. The collated database is available on request.

Intakes of five isoflavones, daidzein, genistein, glycitein, formononetin and biochanin A were quantified using the comprehensive United States Department of Agriculture isoflavone database (Release 2.0) (Bhagwat et al., 2008). The phytoestrogen values of the aglycone forms, based on wet weight, were used to quantify the dietary intake.

3.2.2. Phytoestrogen specific food frequency questionnaire

An existing 112 item phytoestrogen FFQ was developed by two Nutrition and Dietetics students at the University of Sydney under the supervision of Associate Professor Philippa Lyons-Wall. This FFQ was adopted by Hanna et al. (2005) in her doctoral research to measure phytoestrogens intake and is used with permission for the current study. The modification of this FFQ by the candidate for the current research was based on updating the content of lignans and isoflavones of items from published databases and increasing the number of items to include dietary patterns and typical serve sizes described in the Australian Dietary Guidelines (NHMRC, 2013a). Food sources rich in lignans and isoflavones i.e. flaxseed and soy and related food products, and commonly-consumed food sources of lignans, such as broccoli, pumpkin, oranges and berries, were included in the questionnaire. Food groups and common foods consumed in Australia were identified from the 1995 National Nutrition Survey conducted in a broad sample of 13,800 adults (McLennan & Podger, 1995) and from a secondary data analysis in 2010 of the baseline diet of 106 Australian men and women enrolled in a clinical trial, previously conducted by the candidate under the supervision of Associate Professor Philippa Lyons-Wall. The FFQ was designed to recall usual diet over the past month (4 weeks) and contained five columns. The ‘Food Item’ column contained the list of 277 foods and beverages in the FFQ. The ‘Brand’ and ‘Type’ column provided additional space for the

subjects to state further details about the food item, for example, the brand, the type or the colour. The ‘Standard serve size’ column stated the reference serve size of the food items taken from The Australian Dietary Guidelines (NHMRC, 2013a). Subjects used the standard serve size as a comparison to respond how much they usually consumed for that particular food item, which they entered in the ‘Portion size’ column. The ‘Frequency’ column allowed subjects to report how often they consumed each food item with reference to their portion size over the previous month. The questionnaire comprised 14 frequency categories: never/<1 per month, 1 per month, 2 per month, 3 per month, 1 per week, 2 per week, 3 per week, 4 per week, 5 per week, 6 per week, 1 per day, 2 per day, 3 per day and ≥4 per day (**Appendix A**).

3.2.2.1 Pilot study of food frequency questionnaire

A pilot study of the FFQ was conducted to pretest the feasibility of the research tool (Persaud, 2010). A group of 10 people, known to the candidate and mainly from a nutrition and sport sciences background, were invited to complete the food frequency questionnaire and provided feedback based on a list of pilot questions, including the time it took to complete, the format and feasibility of the questionnaire, and improvement for the questionnaire from the respondent’s perspective **(Appendix B)**. On average, approximately 35 minutes was spent to complete the questionnaire. Most respondents believed that the format of the questionnaire was easy to understand and three suggested changes were made to improve the response for portion size in comparison to standard serve size or to answer portion size in exact amounts consumed or household measures. Half of the respondents thought that having visual aids for standard serve size and common household measures would be helpful in completing this questionnaire. Respondents also nominated commonly consumed food items to be considered on the questionnaire. Amendments were made to the FFQ according to this feedback. A food model booklet (Stamatis, 2000) was made available as a visual aid on request. The candidate embedded extra rows on the questionnaire for recruited subjects to record other foods and beverages that they consumed regularly but were not listed.

3.2.3. Demographic and lifestyle questionnaire

A questionnaire was designed to collect demographic and lifestyle information (**Appendix C**). The questions were based on those from the Australian Health Survey (ABS, 2013b), and previous published research (Hanna et al., 2010; Johnsen et al., 2004; Milder et al., 2007). Several social characteristics were found to be associated with lignan intake and/or biomarkers in the literature review. These variables included age, gender, height, weight and BMI, occupation, level of education, physical activity level, smoking status, bowel habits (frequency and movement) and commercial dietary supplemental use. The associations between ancestry/ethnicity and cultural background (language spoken at home, country of birth) and lignan status were also explored in the current study to address the multicultural nature of the sample group.

3.2.4. Three-day weighed food record

A 3-day weighed food record was chosen as the reference method for validating the phytoestrogen specific FFQ because the weighed food record has unrelated sources of errors from those of a food frequency questionnaire (Willett & Lenart, 1998). Subjects were provided with the weighed food record booklet to record and describe their food and beverages intake over 3 days. Subjects were required to record the amount, type and brands of the food and beverages consumed, the meal type, preparation method, and place of preparation and the time of consumption (**Appendix D**). Where subjects consumed a dish they prepared from a range of ingredients, they were required to record the recipe in the recipe section of booklet and to specify the amount consumed in the food booklet. Details of the ingredients and the amounts used in the recipes were important to identify ingredients that were rich in phytoestrogen content. A set of digital kitchen scales, which measured to the nearest 1g (Propert® 5 kg capacity, slimline stainless steel, Propert Houseware, NSW) and modular measuring cups and spoons (Décor Cook®, The Décor Corporation, VIC) were provided to each subject to standardise weighing and estimating the amount of their food and beverages intake.

3.2.5 Urine sample collection

A dose-dependent relationship between phytoestrogen consumption and urinary excretion has been reported in human intervention studies for both lignans (Hutchins, Martini, Olson, Thomas, & Slavin, 2000) and isoflavonoids (Morimoto, Beckford, Franke, & Maskarinec, 2014). This relationship demonstrates that the use of urinary biomarkers of phytoestrogen metabolites is an informative and objective method to test the criterion validity of the phytoestrogen questionnaire.

Subjects were asked to collect 24-h urine samples for three consecutive days. Each subject was provided with three 3 L bottles (SteriHealth, VIC) with ascorbic acid (1g per bottle) added as preservative (Sigma Aldrich, L-Ascorbic acid, NSW), funnels (SteriHealth, VIC) and specimen collectors (Victoria Healthcare Products, VIC). Subjects were given a urine collection protocol developed for this study and the procedure for urine collection was explained to subjects at their first visit to the study venue (**Appendix E**). On the day of collection, subjects discarded the first morning void and collected all urine for the remainder of the day, completing the collection with the first morning void on the next day. The candidate provided the subjects with advice on how to store the collected samples and agreed on an appropriate storage method during the urine collection period. An insulated cooler bag and three freezer blocks was provided to assist each subject with samples storage prior to delivery to the study venue. To verify the completeness of urine samples, subjects were asked to record on the bottle label whether each sample was either complete or incomplete, with the time of missed samples noted in a space provided (Murakami et al., 2008). Collections were repeated on the next two consecutive days.

Subjects delivered the three 24-hour urine collection to the study venue on completion. The samples were refrigerated and processed at the chemistry laboratory at Edith Cowan University within 48 hours of receipt. The total volume and total weight of each 24-hour urine sample was measured with graduated cylinders and a top pan balance **(Appendix F).** A set of five aliquots was prepared from each sample, and to each aliquot 10μL of 30%

sodium azide solution (0.3g/ml) was added as a bacteriostat **(Appendix G)**. Each aliquot was labeled and stored at -80°C until analysis.

Protocols for urine sample preparation and working with chemicals, and the Risk Assessment and Management Plan (RAMP) was developed with the Analytical Chemist to ensure the consistency of laboratory work, to assess the risk of the protocols and to outline any emergency response. The RAMP was assessed and approved by the School of Natural Sciences Safety Officer.

3.3. Subject recruitment

3.3.1 Selection criteria

3.3.1.1 Inclusion criteria

Subjects of both genders, aged between 18-70 years, from different cultural and social backgrounds were eligible to participate. These inclusion criteria were designed to capture the dietary diversity of phytoestrogen intake.

3.3.1.2 Exclusion criteria

Subjects were excluded from participating in the study if they were using or have used regular doses of antibiotics for at least one month over the past 6 to 12 months, have made substantial changes to their usual diet during the past month, have current gastrointestinal problems such as ulcerative colitis and Crohn's disease, or have undergone gastrointestinal surgery involving removal of the large intestine, or have had chronic or acute renal or liver disease in the previous 6-12 months (**Appendix H**).

3.3.2 Determination of an adequate sample size

The *a priori* estimated sample size to detect the significance was determined with a statistician using G*power (Version 3.1.3 for Mac OS X, Institut für Experimentelle Psychologie, Düsseldorf, Germany) for assessing the association between phytoestrogen intake and status measured by dietary methods and urinary biomarkers. The sample size

calculation indicated that 80 individuals were required to achieve a suggested moderate effect size (R^2 of 68%), based on correlations between the FFQ and reference methods from previous validation studies, and correlations between dietary methods and biomarkers (R^2 of 30%), with 80% power and a 5% significance level. This sample was within the range of the sample size recruited from previous validation studies (Bhakta et al., 2005; Hanna et al., 2005). The anticipated dropout rate was 10% resulting in a required sample size of 88 subjects.

3.3.3 Advertisement

A flyer was designed to advertise the study throughout the Edith Cowan University Joondalup campus across various facilities, including the library, student village and student guild, and online via the Graduate Research School Google group, Health and Wellness flyer, and Research news on Edith Cowan University portal (**Appendix I**).

3.3.4 Ethics

The study was granted ethical approval by the Human Research Ethics Committee, Edith Cowan University (Project number: 10415). All subjects provided informed written consent prior to starting the study and were given the contact details of the principal investigators and Ethics Officer in case of any concerns. Confidentiality of the data was strictly enforced. All questionnaires, food records and urine samples collected were stored securely. All data collected were entered into the candidate's computer and stored securely. Individual subjects were identified by study codes that matched his or her questionnaire, food record and urine samples. This study involved human subjects and was conducted following the principles in the National Statement on Ethical Conduct in Human Research, Edith Cowan University Policy for the Conduct of Ethical Human Research, and The Declaration of Helsinki.

3.3.5 Subjects

The subjects consisted of staff and students recruited at Edith Cowan University. Recruitment techniques included word of mouth and snowballing. Subjects who were in the study were asked to suggest the study to other interested individuals. **Figure 6** illustrates the process of subject recruitment from the start of the recruitment, screening, commencement and undertaking of the study, to the completion of the study.

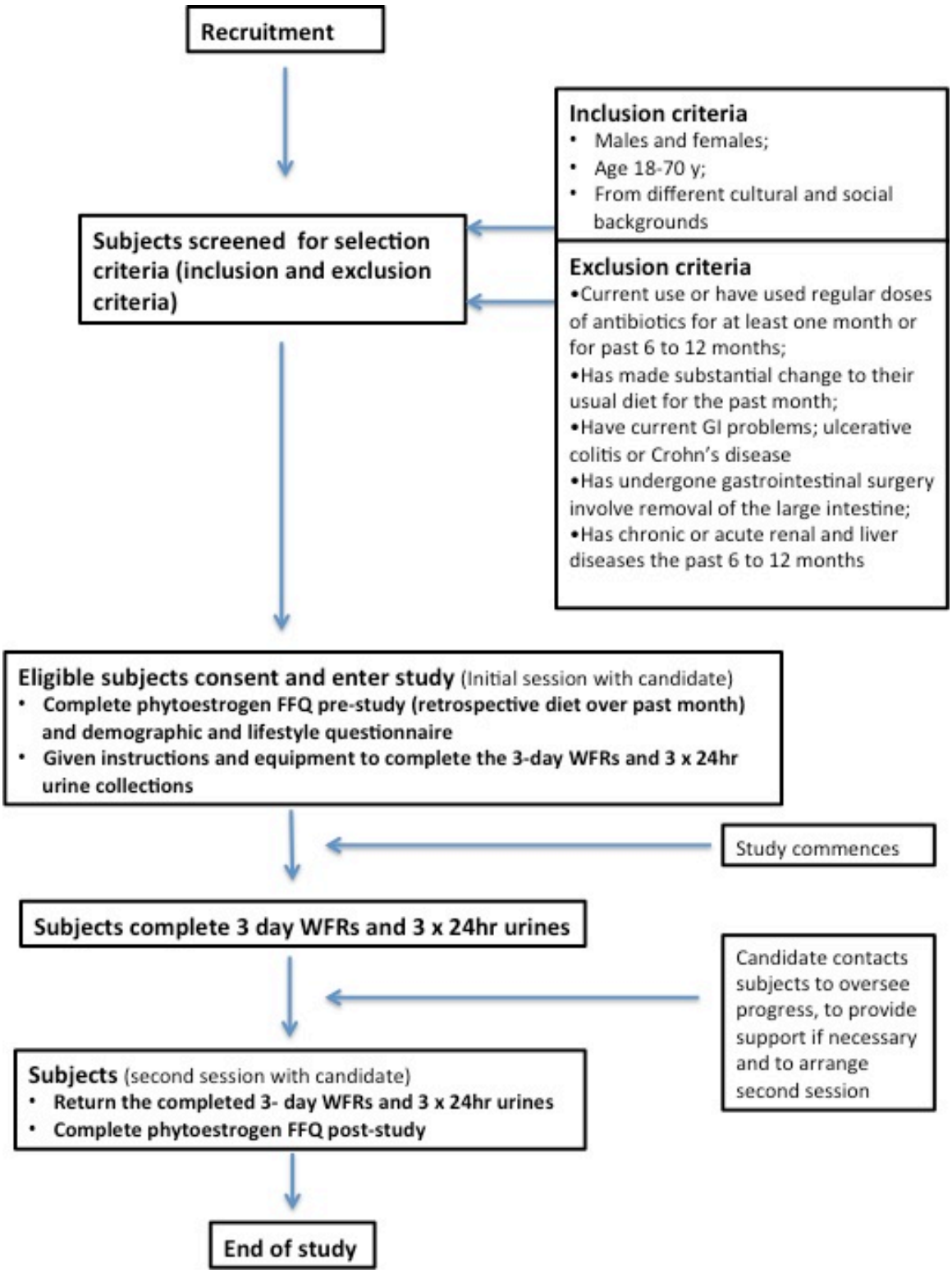


Figure 6. The process of subject recruitment from commencement, screening and selection, and participation, to the completion of the study

3.4. Data collection and analysis

Dietary intake was collected using the phytoestrogen FFQ and the 3-day WFR. Biomarkers of phytoestrogen metabolites were measured in the 24-hour urine samples, collected over three days to reflect subjects' habitual urinary excretion. Assuming that biomarker excretion was delayed after consumption of phytoestrogens in the diet, due to possible metabolic conversions in the colon prior to absorption, urinary excretion would more likely reflect intake on the previous day (King & Bursill, 1998; Kuijsten et al., 2005; Watanabe et al., 1998). Therefore urine collections were timed to start one day after commencement of the WFR.

The collection of the FFQ, 3-day WFR and urine samples is illustrated in **Figure 7.** In the first session, subjects completed the pre-study FFQ that reflected habitual diet over the past month, and the demographic and lifestyle questionnaire. They received instructions and the equipment for conducting a 3-day WFR and 3-day 24-hour urine collection **(Figure 8)**. For the current research, the minimum 3 days, including a weekend day, was required for the food record as it is sufficient to obtain usual dietary intake, and practical and feasible within the short data collection timeline (Buzzard, 1998; Lee & Nieman, 2010).

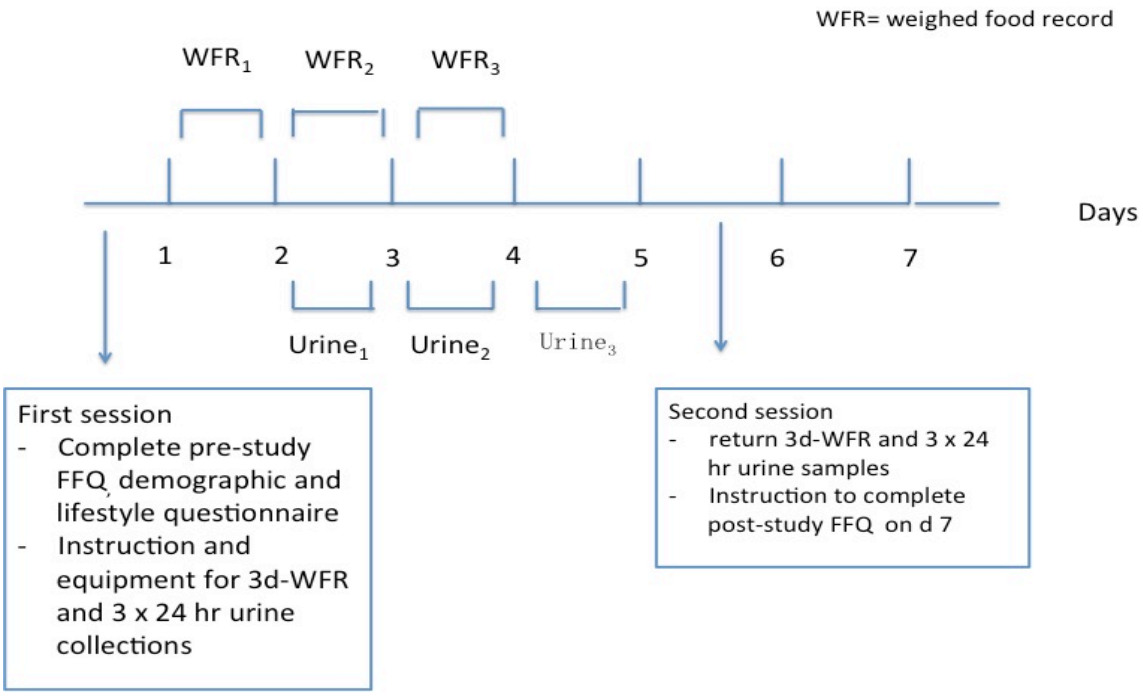


Figure 7. Data collection protocol

The study extended over a 7 day period with a 3 day WFR collected on days 1 to 3 and 24 hour urine samples on day 2 to 4. The FFQ was completed on two occasions at the beginning and end of the study period, coinciding with subject visits to the study venue.

Figure 8. Equipment provided to subjects to conduct the 3-day WFR and three 24-hour urine collections

Subjects returned the completed 3-day WFR and 3-day urine collection at the second visit to the study venue. The candidate reviewed all food records with the subjects to verify completeness and clarify ambiguous information. Subjects then completed the same FFQ (post-study FFQ).

3.4.1 Quality assurance and subject compliance

To ensure the quality of data collection, subjects were required to attend the first session at the study venue. The candidate explained each step of the data collection procedure and demonstrated the use of equipment. Subjects were encouraged to ask any questions that they had during the session to make sure they understood the process. The candidate discussed with the subjects their anticipated commence and finish date so a second session could be scheduled for collection of equipment, weighed food record and urine samples. Subjects were encouraged to contact the candidate anytime if they wished to have any questions answered or would like advice when they had events that affected their food recording and urine collection. A study calendar was provided to subjects as an outline of the steps they needed to complete during the study. Spaces were available on the calendar for subjects to record relevant information, for example, any events that affected the dietary recording and urine collection, e.g. sickness, menstruation for female subjects, travelling (**Appendix J**). Subjects who missed their urine sample or WFR recording were advised to immediately contact and discuss with the candidate to resolve the issue.

3.4.2 Assessment of energy and nutrient intake

The subjects' food intake from the WFRs was analysed for intake of energy, macronutrients and micronutrients using the AUSNUT (2007) database (FoodWorks® Professional Edition version 7, Xryis Software, QLD). Details of the type or brand and the preparation method were taken into account when choosing appropriate food items.

For food items not available in the AUSNUT database, a new food was constructed based on nutrient values of an existing similar food from other databases in FoodWorks®, or a new food was entered or edited with nutrient values from external sources, for example, the food label of the product or the USDA National Nutrient Database (U.S. Department of Agriculture, 2014). For subjects who provided detailed recipes in their WFRs, each ingredient and the quantity used for cooking the dish was entered into FoodWorks® to construct a new item.

3.4.3 Assessment of phytoestrogen intake

3.4.3.1 Analysis of phytoestrogen intake derived from food frequency questionnaire

A template based on the FFQ was constructed using a Microsoft Excel spreadsheet to calculate the phytoestrogen intake of usual diet over the past month. Values of phytoestrogen content (based on wet weight and edible portion) were selected from the dietary lignan and isoflavone databases and assigned to the listed 277 food and beverage items. The portion size in grams and the number of times (how often) this item was consumed was entered into portion size and frequency columns corresponding to that particular food item. The first step was to calculate the weight of the item consumed per day = portion of item consumed (g) x (number of times consumed per month/30) + portion of item consumed (g) x (number of times consumed per week/7) + portion of item consumed (g) x (number of times consumed per day). The second step was to measure each lignan, enterolignan and isoflavone intake, for example, SECO (μg) consumed = weight of item consumed per day (g) x SECO content (μg/100g). Total lignan intake (μg) was calculated as the sum of intake of SECO, MAT, PINO, LARI; total enterolignan intake (μg) was calculated as the sum of END and ENL; and total isoflavone intake (μg) was calculated as the sum of daidzein, genistein, glycitein, formononetin and biochanin A intakes.

Subjects recorded foods and beverages that were regularly consumed, but not listed, at the end of the questionnaire. These items were flagged and grouped together on a separate

spreadsheet and the foods were assigned with phytoestrogen content and added in to the totals.

3.4.3.2 Analysis of phytoestrogen intake derived from weighed food records

The data from the 3-day WFR analysed by FoodWorks®, were managed with Microsoft Access and exported to a Microsoft Excel spreadsheet to calculate phytoestrogen intake. Intake data were sorted by alphabetical order of food names to streamline the assigning process by giving the same phytoestrogen content values to all the same items at once. Phytoestrogen intake (μg) was calculated as: phytoestrogen content (μg/100g) x weight of item consumed (g). For recipes, the calculation of phytoestrogen content took into account the weight of the recipe consumed and content of phytoestrogen per serve.

For items where no phytoestrogen values were available from the database, the values were based on those from an appropriate item available within the same food group Phytoestrogen content of mixed foods (foods and dishes composed of two or more different components) was estimated based on the proportion of each ingredient specified by the subjects or common recipes. Where appropriate, the water content of the food item, particularly those with high water content e.g. fruits juice, and water-based recipes, soup, was identified with NUTTAB 2010 and the proportion of the solid content calculated, assuming phytoestrogens are only present in the solid part of the food matrices. Food items with no available values and no appropriate substitution were left unassigned and a phytoestrogen content of zero was assumed, for example, sugar, salt, confectionary, soft drinks, vodka, seafood like crab and octopus, spices like cinnamon and cumin.

3.4.4 Assessment of food sources contributing to lignan and isoflavone intake

Food items from the FFQ were listed according to food groups specified in the Australian Dietary Guidelines with nuts and seeds, and legumes and beans, as two separate groups, (NHMRC, 2013a) and two additional groups, non-alcoholic beverages including coffee,

tea and juices, and alcoholic beverages. Using the Australian Dietary Guidelines assured the food sources identified reflected the Australian dietary patterns. Total and individual phytoestrogen intake from each food group and from all foods was calculated, and the percentage food contribution was calculated by dividing total phytoestrogen intake of each food group with the total intake of all foods.

3.4.5 Assessment of urinary excretion of lignans and isoflavonoids

Chemicals

Methanol, acetonitrile and water were all LC-MS grade and supplied by Fisher Scientific. Pinoresinol, lariciresinol, secoisolariciresinol, enterodiol were supplied by PhytoLab (Vestenbergsgreuth, Germany). Matairesinol, enterolactone, daidzein, genistein, glycitein and equol were supplied by Cayman Chemical (Michigan, U.S.). LKT Laboratories (Minnesota, U.S.) supplied biochanin A and formononetin. Taxifolin was supplied by Sigma Aldrich (Castle Hill, NSW, Australia) and glucuronidase (activity > 100,000 U/mL) /sulfatase (> 20,000 U/mL) enzyme extracted from red abalone (Haliotis rufescens) was sourced from Kura Biotech (Puerto Varas, Chile). Formic acid and acetic acid were supplied by Fluka and Ajax Finechem Pty Ltd. respectively.

Sample preparation

The urine was thawed to room temperature and then centrifuged (10 min at 13.2 rpm, 23°C) (Eppendorf Centrifuge 5415R, Eppendorf, Germany). A 200 µL aliquot of the supernatant was transferred to a 2 mL Eppendorf tube before adding 240 µL of glucuronidase/sulfatase enzymes from Kura Biotec (Puerto Varas, Chile), 10 % (v/v) in sodium acetate (140 mM pH 4.8). Taxifolin (20 ng) was added as the internal standard (Fluka, Sigma Aldrich, Castle Hill, NSW, Australia). Enzyme and internal standard were mixed thoroughly with the supernatant (Boeco Vortex V1 Plus, Boeco, Germany).

The sample was then mixed gently and heated at 37 ^{o}C overnight (approximately 16 hrs) (Eppendorf thermomixer comfort, Eppendorf, Germany).

Methanol (20 µL) was added to each sample. The sample was then passed through a preconditioned solid phase extraction cartridge (Alltech 100 mg, C18). The loaded cartridge was washed with 90:10:0.1 water:methanol:acetic acid (1.0 mL) followed by acetonitrile (1.0 mL) to elute the lignans. The lignan extract was freeze dried and stored at -80 ^{o}C until analysed (Labconco FreeZone 2.5, Labconco, United States). The freeze dried sample was reconstituted in 90:10:0.1 water:methanol:formic acid (400 µL). Chloramphenicol (20 ng) was added to each sample and standard as an instrument check (Valentin-Blasini, Blount, Schurz Rogers, & Needham, 2000).

Liquid chromatography-mass spectrometry (LC-MS) analysis

Concentration of urinary phytoestrogen metabolites (ng/ml) was measured by an Analytical Chemist with a high resolution Time of Flight Mass Spectrometer (ABSCIEX 5600, AB SCIEX, Framingham, MA, United States) using electrospray ionisation in negative mode. This instrument was interfaced to a Shimadzu Nexera LC system (LC 30AD pump, SIL30AC autosampler and a CTO-20A column oven). The LC separation was carried out using a Grace Smart RP 18 column (50 mm x 2.1 mm x 3 mm) with a 10 µL injection loop. The flow rate was 0.5 mL/min. The mobile phase consisted of 0.1% v/v formic acid in water (A) and 0.1% v/v formic acid in acetonitrile (B) in a gradient starting with 10% B to reach 50% B after 7 min, 100% B after 9 min, held for 5 minutes and returning to 10% B for 5 min to equilibrate. The mass spectrometer was run in independent data acquisition (IDA) mode with enhancement to the precursor ions for the target analytes (secoisolariciresinol, matairesinol, pinoresinol, lariciresinol, enterodiol, enterolactone, daidzein, genistein, glycitein, equol, biochanin A and formononetin). The nebulizer gas, heater gas and curtain gas were run at 30, 45 and 50 psi respectively. The ion source voltage was 4500 V and the probe temperature was 550°C. Collected mass range was m/z 50-1000. MSMS data was collected with a collision energy of -35 V with a

spread of 15 V (Grace, Mistry, Carter, Leathem, & Teale, 2007).

Creatinine analysis

Twenty four-hour urinary creatinine concentrations were determined with Jaffé Creatinine method (Husdan & Rapoport, 1968) to assess completeness of the urine collection. Two ml urine samples without added sodium azide were centrifuged for 90 seconds at 130rpm (Eppendorf MiniSpin) and 200μL of sample was pipetted into a sample cup. The urine sample was diluted 1/20 on board the analyser for analysis (Abbott Architect c16000, Analytical Imprecision 5.4 mmol/L:CV_A: 2.0%, 12.2 mmol/L:CV_A:1.8 %). Creatinine reacts with picric acid in an alkaline medium to produce a coloured creatinine-picrate complex. The rate of formation of this complex, measured over a selected time interval at a primary wavelength of 500nm and secondary wavelength of 572 nm, is proportional to the concentration of creatinine in the sample (uncertainty of measurement, 0.22 mmol /L at 5.4 mmol/L ± 0.42 mmol/L at 12.2 mmol/L). Day-to-day variation in creatinine excretion of the three 24 hour urine samples of each subject was examined and compared with expected urinary creatinine excretion (creatinine index) to determine possible incompleteness of the 24 hour urine sample (Barr et al., 2005; De Keyzer et al., 2012; Dugdale, 2011).

3.4.6 Assessment of phytoestrogen status and social and lifestyle characteristics

Social and lifestyle characteristics data were collected with the demographic and lifestyle questionnaire. Height and weight were measured with subjects wearing light clothing and no shoes. Height was measured to the nearest 0.01cm and weight to the nearest 0.1kg with a digital measuring and weighing station (Seca 763, Seca GmbH & Co. KG., Hamburg, Germany) (Stewart, Marfell-Jones, Olds, & de Ridder, 2011). The height and weight were used to calculate body mass index (BMI): weight (kg)/square of height (m) (Diehl, 2011).

Subjects stated their occupation. All responses for the occupation were classified into one of the eight major groups according to the Australian and New Zealand Standard Classification of Occupation (ABS, 2013a). There were six categories of education level: University postgraduate, University undergraduate, Certificate or diploma, Year 12 Senior secondary school, Year 10 Junior secondary school and Other (e.g. primary education) (ABS, 2001). Subjects selected ethnic group that best described their and their biological parents ancestry/ethnicity and the language they speak the most at home. They indicated their country of birth and all responses were listed and classified into one of nine regions according to the Standard Australian Classification of Countries (ABS, 2011).

Physical activity was categorised at four different levels and subject selected the level that best described them: 1) Sedentary, move only for necessary chores/walking/other activities 1-2/week; 2) Light, walking/other activities >3/week; 3) Moderate, exercise to perspire 1-2 times/week, or moderate labour or physically demanding work; 4) High, heavy exercise or sport several times/week, heavy labour or physically demanding work (ABS, 2013b; Hanna et al., 2010). Subjects were asked to select the best option to describe his or her usual physical activity over the past month.

Smoking status was categorised as: never smoked, current smoker (daily, weekly or other frequency) and past smoker. Subjects described their bowel habits using frequency such as how many times per week and the movement such as ‘hard to pass’, ‘smooth and soft’ or ‘mushy and watery’ according to the Bristol stool chart (Lewis & Heaton, 1997). Subjects stated whether they used any commercial dietary supplements (yes or no), and provided the details of regular supplements they were taking.

3.4.7 Statistical analysis

Data entry and cleaning

Quality assurance procedures were implemented in data entry and analysis. All

questionnaires and WFRs were reviewed by the candidate to check for completeness and ensure that food weights and ingredients were recorded. Subjects were contacted by the candidate to clarify any missing or ambiguous details in the dietary, and demographic and lifestyle data.

The WFR data were checked for consistency. All WFRs and recipes were visually checked for accuracy by the candidate. All quantity and frequency numbers entered into the FFQ template were crosschecked with the hard copy of the FFQ. The candidate examined the nutrient analysis of 3-day WFR for extreme values that were not feasible and corrected for any errors. Data of phytoestrogen excretion were checked by the Analytical Chemist responsible for the LC-MS analysis. High and low outliers were identified by boxplots and histogram and checked they were correctly assessed for phytoestrogen intake and excretion. To calculate the average daily urinary excretion of phytoestrogens over the three days, the weighted average was calculated based on the relative urine volume for each day: average weighted phytoestrogen excretion (µg/day) = (volume (L) of day 1 /Total volume (L) of all 3 days) x (phytoestrogen in µg/day 1) + (Volume (L) of day 2 /Total volume (L) of all 3 days) x (phytoestrogen in µg/day 2) + (Volume (L) of day 3 /Total volume (L) of all 3 days) x (phytoestrogen in µg/day 3). The weighted averages for urinary excretion data were used in the statistical analysis.

The Mann-Whitney U test and Pearson's Chi-Square test of contingencies were conducted to test if the demographic and lifestyle characteristics of subjects who completed significantly differed from that of those who withdrew from the study.

Statistical analysis was conducted using Statistical Package for Social Sciences (SPSS for Windows, Version 22, 2014, Chicago, Illinois, USA).

Analysis for estimating dietary phytoestrogen intake

The normality of the phytoestrogen intakes from the FFQ and 3-day WFR were tested with the Shapiro-Wilk test. Transformation of natural log was applied to the data but the normality test indicated that not all data became normally distributed. Therefore, all data

were treated as non-normal distributions and non-parametric statistical tests were used throughout for median comparisons and correlations. For values of ENL, END and total enterolignan intake that were zero, a natural log transformation plus a constant was applied. Data were expressed as the mean±SD, median (IQR), and the minimum and maximum.

Analysis for assessing validity and reliability of FFQ

Non-parametric tests were applied to compare the median of FFQ with 3-day WFR for validity, and the median of pre FFQ and post FFQ for reliability. Comparison between phytoestrogen intake from the pre-FFQ and post-FFQ, the FFQ and 3-day WFR, and the FFQ or WFR and urinary biomarkers were conducted as follows. Associations were assessed with the Spearman's rank correlation coefficient on raw data. Cross-classifications (quartile agreements) were calculated by establishing the percentage of subjects in the same/exact, adjacent (±1 and/or ±2 quartiles) or extreme/opposite quartile; each set of intake and excretion data were divided into four equal groups with the three quartile points as the boundaries (Bhakta et al., 2005).

Bland Altman plots were used to assess the degree of agreement between methods and provide a graphical means to assess whether the two techniques or points of measure at different occasions were comparable for measuring phytoestrogens. Raw phytoestrogen intake data were used to construct Bland Altman plots (Altman & Bland, 1983; Myles & Cui, 2007).

Associations between phytoestrogen status and social and lifestyle characteristics

Social and lifestyle factors were organised in an Excel spreadsheet and imported to SPSS. As there were only one or two subjects in some categories, these factors were recategorised as follows. Occupation was coded into four categories: level 1 - Family duties and Students, level 2 - Clerical and administrative workers, level 3 - Community

and personal service workers and Technical and trade workers, level 4 - Professionals and Managers. Education was coded into two categories: level 1- Below university level (Other and Secondary education, and Certificate or diploma), level 2 – University level (University undergraduate and university postgraduate). Ancestry was coded into two categories: Caucasian and other ethnic groups which included South East Asian, South Asian, North East Asian, Middle Eastern, North Africa and Somalia Peninsular, Central/South America and Other and Mixed ancestry. Language spoken at home was coded into two categories with English and Other languages, including Cantonese, Arabic, Mandarin, Spanish, German, Hindi, Malay, Persian, Urdu, Afrikaans, Macedonia and Bahasa Indonesian. Country of birth was coded into four categories: group 1 - Oceania and Antarctica, group 2 - Europe, group 3 - North Africa and the Middle East, Sub-Saharan Africa and Americans, group 4 - Asia (South-East, North-East Asia, and Southern and Central Asia). Physical activity level (PAL) was coded into four (sedentary, light, moderate and high) and smoking status into three (never smoked, past and current smoker) categories, respectively. Bowel frequency was coded into two levels (1-7/week and >7/week) and two categories, mushy and watery/liquidy and smooth and easy to pass, and lumpy and hard to pass, for bowel movement (Lewis & Heaton, 1997).

The median ± IQR intake of total lignan, total enterolignan, total isoflavone and total phytoestrogens with respect to different categories of social and lifestyle characteristics were presented. The Mann Whitney U test was used to compare the difference in median intake with two levels or categories of the characteristic, namely, gender (male or female), education (below university or university level), ancestry (Caucasian or other ethnic groups) languages spoken at home (English or other languages), bowel frequency (1-7/week or >7/week), bowel movement (mushy/liquidy to smooth and easy to pass or hard to pass) and supplement use (yes or no). The median intake for the other characteristics with three or more levels or categories was assessed with Kruskal-Wallis One-way ANOVA.

Analysis of contributing food sources of phytoestrogens

Food items from the FFQ were listed in food groups specified in the Australian Dietary Guidelines with Nuts and seeds, and Legumes and beans as two separate groups. Three additional groups, Nonalcoholic beverages including coffee, tea and juices, Alcoholic beverages and Mixed food was added to reflect consumption of beverages and food composed of ingredients from two or more food groups. The FFQ template constructed for measuring phytoestrogen intake was used to evaluate the contributing food sources to dietary total lignans, total enterolignans and total isoflavones. Individual phytoestrogen intakes from each food group were calculated from the pre-FFQs. Total phytoestrogen intakes of all subjects were then calculated by summing individual phytoestrogens. The percentage contributions of each food group were calculated by dividing total phytoestrogen intake from that food group by total phytoestrogen intake in the whole group. The pre-study FFQ was selected to conduct this analysis to reduce possible bias and influence from completing the other dietary assessment tools.

Analysis for triangular comparison between FFQ, WFR, and urinary biomarker for phytoestrogen status

The Method of Triads (MOT) was used for the triangular comparison between the FFQ (Q), WFR (R) and urinary biomarkers (M) for assessing phytoestrogen status **(Figure 9)** (Kaaks, 1997). The analysis was conducted using software R (version 3.1.2, R Core Team, Vienna, Austria).

Scripts for performing analysis of correlations between the measurement methods (*r* QR, *r* QM, *r* RM), validity coefficients (VCs) between each measurement method and the true but unknown intake (VC QT, VC RT, VC MT), and the confidence intervals (CIs) for VCs using the bootstrapping method, were written by the Statistician on the R script interface. Bootstrapping is a random sampling technique with replacement from the research population; the data from the current research population were randomly

sampled for 1000 times (Kaaks, 1997; Ocké & Kaaks, 1997). A template that contained the dataset for intake and excretion measurements of Q, R and M from each subject, was constructed and linked to the scripts. Demographic and lifestyle continuous variables, such as age and BMI, and categorical variables, such as physical activity level, bowel movement, and language spoken at home were included in the MOT model.

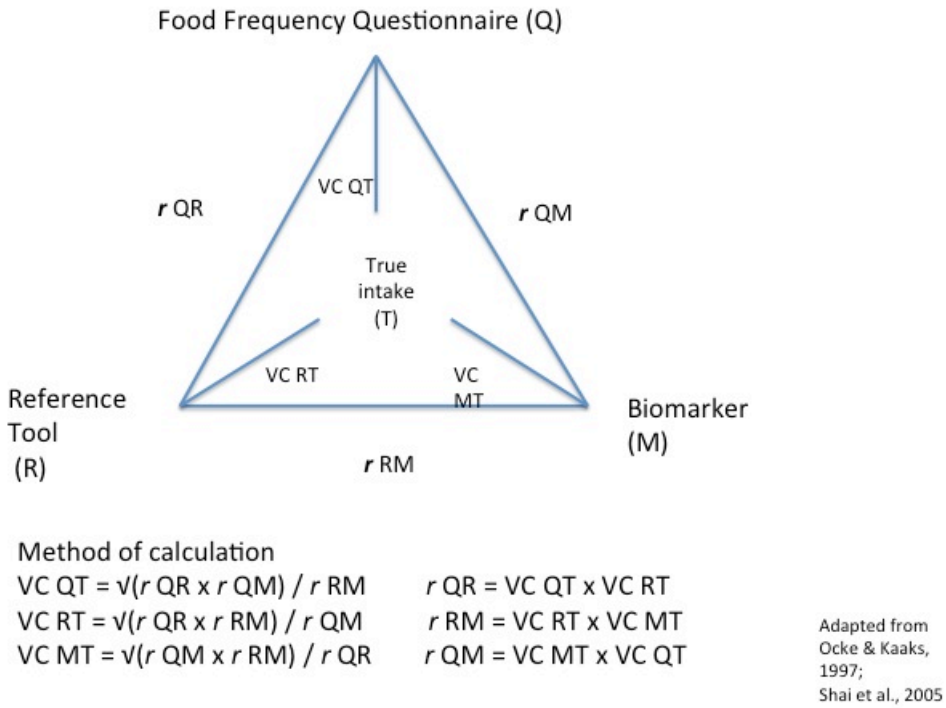


Figure 9. Graphical interpretation of the method of triads
(Adapted from (Ocké & Kaaks, 1997; Shai et al., 2005))
The method of triads is the triangular comparison between FFQ, reference method and biomarker. VCs are the associations between the measurement derived from the method and the unknown true intake. VCs are mathematically derived from correlations between the measurements from the two methods.

CHAPTER FOUR: RESULTS

4.1 Subjects

There were 131 individuals who expressed interest in the study during the four-month recruitment, of which 48 did not respond to the candidate's invitation, 10 declined, 2 were ineligible, and 71 consented to participate. Of these, 12 subjects withdrew and 59 completed the study **(Figure 10)**. The study was powered for a minimum of 80 subjects but due to the time constraints of subject recruitment, only 59 completed the study.

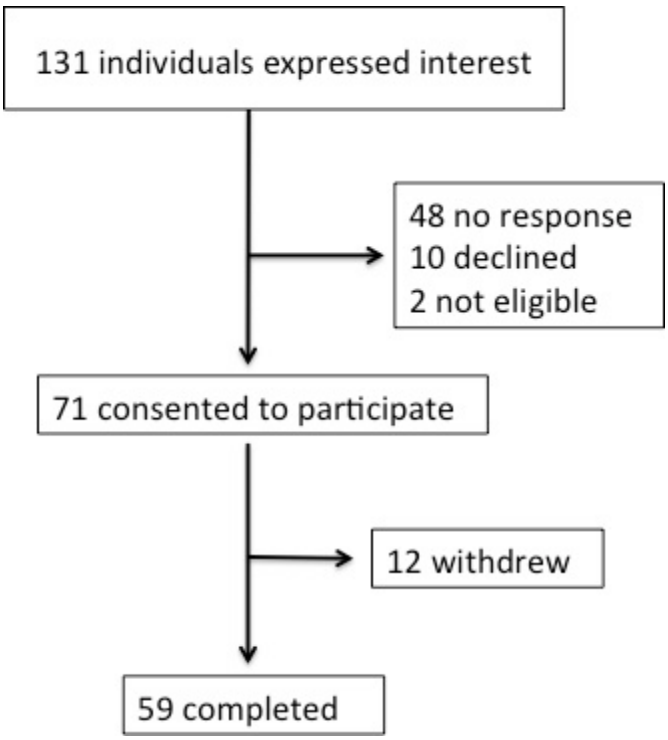


Figure 10. Flow chart of subject recruitment

The study population consisted of 40 females and 19 males aged 18 to 67 years who ranged in BMI from 17.9 to 54.0 kg/m^2. Almost half of the population (48%) were of normal weight (20.0-25.0 kg/m^2) and 44% were in the overweight/obese range (>25.01 kg/m^2). The demographic and lifestyle characteristics of recruited subjects according to their completed or

withdrawn status are outlined in **Table 4**. The Mann-Whitney U test and Pearson's Chi-Square test of contingencies indicated that completers were more likely to speak more than one language at home, be less physically active and less likely to use supplements than those who withdrew.

Table 4. Demographic and lifestyle characteristics of recruited subjects (n=71) according to completed or withdrew status

Variables[a]	Number (%) of Subjects who completed (n=59)	Number (%) of Subjects who withdrew (n=12)	p-value
Gender			
Male	19 (32)	4 (33)	
Female	40 (68)	8 (67)	0.593
Education level achieved			
University postgraduate	24 (40.7)	5 (41.7)	
University undergraduate	22 (37.3)	5 (41.7)	
Certificate/diploma	11 (18.6)	0 (0)	
Secondary school	2(3.4)	2(16.7)	0.290
Occupation			
Family duties	3(5.1)	1(8.3)	
Students	34(57.6)	5(41.7)	
Clerical and administrative workers	2(3.4)	2(16.7)	
Community and personal service workers	5(8.5)	0(0)	
Technician and trade workers	1(1.7)	0(0)	
Managers and Professionals (in Education, Health, Legal and social welfare, and Information and organisation)	14(23.7)	4(33.3)	0.691
Ancestry			
Caucasian	38(64.4)	9(75)	
South East Asian	6(10.2)	0(0)	
South Asian	3(5.1)	0(0)	
North East Asian	5(8.5)	0(0)	
Middle Eastern, North Africa, Somalia Peninsular	2(3.4)	0(0)	
Central/South America	2(3.4)	1(8.3)	
Other and Mixed ancestry (Caucasian & Central and West Africa; Caucasian & Indigenous Australian; Caucasian & Central/South American; Caucasian & Asian)	3(5.1)	2(16.7)	0.435

Variables[a]	Number (%) of Subjects who completed (n=59)	Number (%) of Subjects who withdrew (n=12)	p-value
Language spoken at home			
English	38(64.4)	12(100)	
Other languages (Cantonese, Arabic, Mandarin, Spanish, German, Hindi, Malay, Persian, Urdu, Afrikaans, Macedonian, Bahasa Indonesian)	21(35.6)	0(0)	0.014
Region of Country of birth			
Oceania and Antarctica	29(49.2)	7(58.3)	
Europe (North-West Europe, Southern and Eastern Europe)	10(16.9)	4(33.3)	
North Africa and the Middle East	2(3.4)	0(0)	
Asia (South-East Asia, North-East Asia, Southern and Central Asia)	14 (23.7)	0(0)	
Americans	2(3.4)	1(8.3)	
Sub-Saharan Africa	2(3.4)	0(0)	0.330
Physical Activity Level (PAL)			
Sedentary	6(10)	1(8.3)	
Light	18(30)	1(8.3)	
Moderate	21(35)	3(25)	
High	15(25)	7(58.3)	0.047
Smoking status			
Never smoked	38(64.4)	7(58.3)	
Past smoker	14(23.7)	3(25)	
Current smoker	7(11.9)	2(16.7)	0.884
Bowel frequency			
1-3/week	3(5.1)	1(8.3)	
4-7/week	21(35.6)	5(41.7)	
>7/week	35(59.3)	6(50)	0.526
Bowel movement			
Mushy and watery/liquidy	2(3.4)	0(0)	
Smooth and easy to pass	53(89.8)	10(83.3)	
Lumpy, Hard to pass	4(6.8)	2(16.7)	0.448
Commercial supplement use			
No	32(54.2)	2(16.7)	
Yes	27(45.8)	10(83.3)	0.018

[a] Chi-square test of contingencies was used for Gender, Ancestry, Language spoken at home, Region of country of birth, Smoking status, Bowel movement and Commercial supplement use. Mann-Whitney U test was used for Education level achieved, Occupation, Physical activity level and Bowel frequency.

4.2 Intake and excretion of phytoestrogens

4.2.1 Intake and excretion of lignans and enterolignans

Median, range of intakes of lignans and enterolignans measured by the pre- and post-FFQ and WFR, and excretion of corresponding urinary metabolites (biomarkers) are shown in **Table 5**. Urinary PINO and LARI were not detected, as the mass spectrometer used was unable to ionise the analytes.

There were wide inter-individual ranges of intake observed for lignans from all dietary assessment methods, with smallest variation (20-fold) in LARI from the pre- and post-FFQs and highest variation (500-fold) in PINO from the WFR. Similar wide inter-individual variation was observed in urinary excretion of metabolites, with smallest variation (40-fold) in SECO and highest variation (500-fold) in ENL. All data were markedly skewed towards higher values.

The majority of total lignan intake was contributed by SECO, from 45% to 71% across dietary assessment methods; followed by PINO (14% to 30%), LARI (10% to 20%), and MAT (4% from all methods). SECO contributed to the majority of lignan excretion (53.5%) followed by MAT (46.5%).

ENL consistently contributed more than 99% to total enterolignan intake with less than 1% from END. The proportion of END increased to 15% in urinary excretion (**Table 5**).

4.2.2 Comparison of intake and excretion of lignans and enterolignans

Due to the non-normal distribution of these variables, non-parametric measures, the Wilcoxon signed rank test to compare median intakes, and Spearman's Rho correlations were used for all comparisons.

The reliability of the FFQ for lignans and enterolignans was examined by comparing intakes from the pre and post FFQ **(Table 5).** Median intakes were significantly different for

individual lignans ($p<0.05$ to 0.002), total lignans ($p<0.001$) and combined total lignans and enterolignans ($p<0.05$); but not for ENL and END. There were highly significant associations between individual lignans and enterolignans and combined total lignans and enterolignans (r_s =0.71 to 0.86, $p<0.001$ for all).

The convergent validity of the FFQ for lignans and enterolignans was examined by comparing intakes from the pre FFQ and WFR (**Table 5**). The median intakes of individual lignans and enterolignans and combined total lignan and enterolignan by pre FFQ and WFR were not significantly different ($p>0.05$), except for LARI ($p=0.03$). Moderately significant associations were observed between individual lignans and enterolignans, and total lignans and enterolignans (r_s =0.32-0.62, $p<0.05$), except for END.

The criterion validity of the FFQ was examined by comparing lignan and enterolignan intakes from the pre-FFQ with urinary excretion of corresponding biomarkers; intake from the WFR was also compared with biomarker excretion. Because of the known interconversions of metabolites after ingestion in the diet, comparisons of intake and excretion were based on the combined value for all lignans plus enterolignans; individual associations were not examined (**Table 5**). There were no significant differences between median combined intake of total lignans and enterolignans from the pre-FFQ or WFR and median biomarker excretion; there were no significant associations between intake and excretion.

Table 5. Median daily intake and excretion of lignans and enterolignans - comparisons between pre-FFQ, post-FFQ, 3-day WFR (n=59) and urinary biomarkers (BM) (n=53)

Tools / **(µg/day)**	**Pre FFQ**	**Post FFQ**	**WFR**	**BM†**	**Reliability** (pre-post FFQ)	**Validity** (pre FFQ-WFR)	**Validity** (pre FFQ-BM‡	**Validity** (WFR-BM)‡
Median (Q1-Q3)					**Wilcoxon signed ranked test,** Z statistics, (p-value) for median			
SECO	2625 (1166, 4806)	2204 (958,4157)	1813 (837, 3903)	809 (419, 991)	-2.57 **(p=0.010)**	-1.62 (p=0.105)	-	-
MAT	163 (84, 252)	125 (75, 229)	183 (77, 381)	336 (211, 540)	-3.09 **(p=0.002)**	1.37 (p=0.169)	-	-
PINO	431 (266, 929)	391 (214, 638)	562 (211, 1546)	-	-2.40 **(p=0.017)**	0.99 (p=0.324)	-	-
LARI	518 (320, 685)	466 (226, 658)	636 (283, 1234)	-	-1.98 **(p=0.048)**	2.21 **(p=0.027)**	-	-
Total lignans	3914 (1965, 7317)	3839 (1795, 5845)	4302 (1920, 6354)	1118 (776, 1530)	-3.53 **(p<0.001)**	0.09 (p=0.929)	5.17 (**p<0.001**) (a)	5.66 (**p<0.001**) (a)
ENL	54 (22, 104)	52 (21, 101)	64 (34, 82)	1583 (589, 3151)	-1.62 (p=0.106)	-0.24 (p=0.813)	-	-
END	0.31 (0.14, 0.60)	0.35 (0.18, 0.68)	0.32 (0.05, 0.83)	175 (81, 344)	1.36 (p=0.176)	0.75 (p=0.453)	-	-
Total enterolignans	54 (23, 104)	53 (22, 102)	65 (35, 83)	1837 (740, 3856)	-1.41 (p=0.158)	-0.24 (p=0.807)	-6.33 (**p<0.001**)	-6.33 (**p<0.001**)
Total lignans and enterolignans±	4051 (1995, 7945)	3930 (1851, 6058)	4446 (1877, 6959)	3104 (1685, 5725)	-3.65 (**p<0.001**)	0.32 (p=0.751)	1.24 (p=0.217)	1.16 (p=0.244)
Min, Max					**Spearman's Rho correlation,** (95% CI)			
SECO	161, 43455	78, 15391	328, 14673	74, 3220	0.81** (0.69, 0.88)	0.45** (0.22, 0.64)	-	-
MAT	21, 1765	18, 1713	23, 1682	19, 5269	0.82** (0.71,0.89)	0.45** (0.21,0.63)	-	-
PINO	59, 6612	27, 3203	41, 22560	-	0.71** (0.56,0.82)	0.32* (0.07, 0.53)	-	-
LARI	96, 2248	72, 1688	112, 9014	-	0.79** (0.66,0.87)	0.39** (0.15,0.59)	-	-
Total lignans	377, 46919	195, 18426	587, 47213	93, 6884	0.85** (0.76,0.91)	0.42** (0.18,0.61)	0.15 (-0.13, 0.40)(a)	0.24 (-0.03, 0.48)(a)
ENL	0, 416	0, 237	0, 257	54, 25657	0.86** (0.79, 0.90)	0.63**(0.44,0.76)	-	-
END	0, 2.34	0, 3.73	0, 4.16	16, 6491	0.75** (0.62,0.85)	0.15 (-0.11,0.39)	-	-
Total lignans and enterolignans±	389, 46963	203, 21618	670, 536273	480, 35549	0.86** (0.77, 0.91)	0.44** (0.21, 0.63)	0.16 (-0.12, 0.41)	0.14 (-0.14, 0.40)

Table 5. continued

Median daily intake and excretion of lignans and enterolignans

Tools (µg/day)	Pre FFQ	Post FFQ	WFR	BM†
Percentage (%) contribution				
SECO	71.15% (1st)	69.92% (1st)	45.33% (1st)	53.5%
MAT	3.73% (4th)	4.29% (4th)	4.46% (4th)	46.5%
PINO	14.70% (2nd)	13.78% (2nd)	30.35% (2nd)	-
LARI	10.42% (3rd)	12.01% (3rd)	19.86% (3rd)	-
ENL	99.42%	99.22%	99.10%	84.6%
END	0.58%	0.78%	0.90%	15.4%

**p<0.001*p=0.02 (a) comparison between intake of SECO, MAT, PINO, LARI and excretion of SECO, MAT

† the dash line'-' for the BM column indicated that PINO and LARI were not detected in the LC-MS and subsequent analysis was not applicable

‡ the dash line '-' indicated that the comparison was not reported for between individual lignan intake and excretion due to inter-conversion from individual to enterolignan

± combined total intake of lignans (SECO, MAT, PINO, LARI) and enterolignans (ENL, END)

4.2.3 Intake and excretion of isoflavonoids

Median (IQR) and range of intakes of isoflavonoids measured by pre FFQ, post FFQ and WFR, and excretion of corresponding urinary metabolites (biomarkers) are shown in **Table 6**. Urinary biochanin A was not detected, as the mass spectrometer was unable to ionise the analyte.

Inter-individual variation in isoflavonoid intakes were overall greater than those for lignans and enterolignans, with 300- to 6,000-fold variation observed for major isoflavonoids, daidzein and genistein, and up to 8,000-fold for glycitein, formononetin and biochanin A, where intake from individual subjects was very low, less than 1 μg/d. Data were notably skewed towards higher values.

The majority of total isoflavonoid intake was contributed by daidzein (37% across all dietary intake methods) and genistein (3% to 47%), with smaller contributions from glycitein (5% to 9%), formononetin (0.3% to 3%) and biochanin A (1% to 7%). Daidzein contributed to the majority of isoflavonoid excretion (61%), followed by genistein (33%).

4.2.4 Comparison of intake and excretion of isoflavonoids

Due to the non-normal distribution of these variables, non-parametric measures, the Wilcoxon signed rank test to compare medians, and Spearman's Rho correlations, were used for all comparisons.

The reliability of the FFQ for isoflavonoids was examined by comparing intakes from the pre and post FFQs. Median intakes were not significantly different for individual or total isoflavonoids **(Table 6).** There were highly significant associations between individual isoflavonoids and total isoflavonoids (r_s =0.76 to0.87, $p<0.001$ for all).

The convergent validity of the FFQ for isoflavonoids was examined by comparing intakes from the pre FFQ and WFR reference method **(Table 6)**. The median intakes of individual and total isoflavonoids were not significantly different. Moderately significant associations were observed between individual or total isoflavonoids (r_s =0.36 to 0.46, $p<0.001$ for all, except

formononetin r_s =0.26, p<0.05).

The criterion validity of the FFQ was examined by comparing isoflavonoid intakes from the pre-FFQ with urinary excretion of corresponding biomarkers; intake from the WFR was also compared with biomarker excretion (**Table 6**). Median intakes for individual and total isoflavonoids from the FFQ and WFR were significantly different to median biomarker excretion (p<0.001 for all). There were significant associations between intake from the WRF and biomarker excretion for daidzein (r_s =0.46, p<0.001), glycitein (r_s =0.30, p<0.05) and total isoflavonoids (r_s =0.28, p<0.05) but not for genistein or formononetin; there were no significant associations between intake from the FFQ and biomarker excretion.

4.2.5 Intake and excretion of total phytoestrogens

Total phytoestrogen intake varied from 150-fold in the post-FFQ to 400-fold in the WFR. Total isoflavones contributed 45% to 69% of total phytoestrogen intake obtained from the three dietary tools and to 80% of urinary biomarker excretion (**Table 7**).

4.2.6 Comparison of intake and excretion of total phytoestrogens

There were significant associations between phytoestrogen intakes from the pre- and post FFQs (r_s =0.78, p<0.001) and pre-FFQ and WFR (r_s =0.33, p<0.05), but not between the FFQ or WFR and urinary biomarker excretion. (**Table 7**)

Table 6. Median daily intake and excretion of isoflavonoids (µg/day) -comparisons between pre-FFQ, post-FFQ, 3-day WFR, (n=59) and urinary biomarkers (n=53) (BM)

Tools / **(µg/day)**	**Pre FFQ**	**Post FFQ**	**WFR**	**BM†**	**Reliability** (pre-post FFQ)	**Validity** (pre FFQ-WFR)	**Validity** (pre FFQ-BM)†	**Validity** (WFR-BM)†
Median (Q1-Q3)					**Wilcoxon signed ranked test,** Z statistics, (p-value) for median			
Daidzein	1213 (269,4673)	608 (200,5294)	695 (203,3558)	161 (40, 569)	-1.28 (p=0.199)	-0.16 (p=0.874)	4.81 (**p<0.001**)	4.37 (**p<0.001**)
Genistein	1321 (243,5245)	1006 (218,5429)	721(280,4424)	91 (35, 489)	-1.01 (p=0.312)	-0.41 (p=0.684)	5.62 (**p<0.001**)	5.39 (**p<0.001**)
Glycitein	198 (36,1046)	145 (31,1328)	87 (26,1060)	18 (5, 55)	-1.19 (p=0.233)	-0.15 (p=0.880)	5.38 (**p<0.001**)	5.23 (**p<0.001**)
Formononetin	108 (26,367)	67 (21,372)	55 (21,378)	1.9 (1.4, 2.5)	-1.18 (p=0.239)	-0.52 (p=0.602)	6.03 (**p<0.001**)	6.08 (**p<0.001**)
Biochanin A	97 (29,252)	103 (24,256)	57 (22,175)	-	-0.94 (p=0.345)	-0.78 (p=0.437)	-	-
Total isoflavonoids	3851 (920,11857)	2667 (795,12823)	2287 (847,11129)	268 (96, 1388)	-0.69 (p=0.487)	0.42 (p=0.673)	5.75 (**p<0.001**)(b)	5.63 (**p<0.001**) (b)
Min, Max					**Spearman's Rho correlation,** (95% CI)			
Daidzein	41, 124990	36, 22508	40, 89183	8, 7780	0.77***(0.63,0.85)	0.46***(0.23,0.64)	0.18 (-0.10, 0.43)	0.46*** (0.22, 0.65)
Genistein	52,164962	41, 30551	60, 121264	10, 3554	0.76***(0.63,0.85)	0.44***(0.21,0.63)	0.03 (-0.24, 0.30)	0.23 (-0.04, 0.47)
Glycitein	9, 36727	6, 8669	2, 15807	0.9, 692.2	0.80***(0.69,0.88)	0.45***(0.22,0.63)	0.22 (-0.05, 0.46)	0.30* (0.03, 0.53)
Formononetin	5, 1994	1, 2661	2, 1981	0.5, 68.2	0.83***(0.73,0.90)	0.26*(0.01,0.49)	0.14 (-0.14, 0.40)	0.02 (-0.25, 0.29)
Biochanin A	6, 1098	1, 1200	2, 15918	-	0.87***(0.79,0.92)	0.38***(0.14,0.58)	-	-
Total isoflavonoids	202, 326708	122, 57996	208, 226389	20, 10293	0.78***(0.66,0.86)	0.36***(0.12,0.57)	0.10 (-0.18, 0.36)(b)	0.28** (0.01, 0.51)(b)

Table 6. continued

Median daily intake and excretion of isoflavonoids

Tools / **(µg/day)**	**Pre FFQ**	**Post FFQ**	**WFR**	**BM†**	**Reliability** (pre-post FFQ)	**Validity** (pre FFQ-WFR)	**Validity** (pre FFQ-BM)†	**Validity** (WFR-BM)†
Percentage (%) contribution (a)								
Daidzein	37.87% (2nd)	37.94% (2nd)	37.24% (2nd)	61.4% (1st)				
Genistein	48.74% (1st)	47.16% (1st)	44.47% (1st)	33.3% (2nd)				
Glycitein	9.85% (3rd)	9.33% (3rd)	8.77% (3rd)	5.0% (3rd)				
Formononetin	2.09% (4th)	3.22% (4th)	2.37% (5th)	0.3% (4th)				
Biochanin A	1.46% (5th)	2.35% (5th)	7.15% (4th)	-				

***p<0.001 **p=0.044 *p=0.03

(a) total isoflavone biomarker = daidzein, genistein, glycitein and formononetin

(b) comparison between intake of daidzein, genistein, glycitein, formononetin and biochanin A and excretion (excluding biochanin A, not detected)

Table 7. Median daily intake and excretion of total phytoestrogens (µg/day) - comparisons between the pre-FFQ, post-FFQ, 3-day WFR (n=59), and urinary biomarker (n=53) (BM)

Tools (µg/day)	**Pre FFQ**	**Post FFQ**	**WFR**	**BM**	**Reliability** (pre-post FFQ)	**Validity** (pre FFQ-WFR)	**Validity** (pre FFQ-BM	**Validity** (WFR-BM)
Median (Q1-Q3)					**Wilcoxon signed ranked test,** Z statistics, (p-value) for median			
Total phytoestrogens	9633 (4663, 21329)	8592 (3863, 19259)	7775 (4774, 28596)	3924 (2205, 7575)	-2.05 (**p=0.041**)	1.05 (p=0.294)	4.27 (**p<0.001**)	4.40 (**p<0.001**)
Min, Max					**Spearman's Rho Correlation,** (95% CI)			
Total phytoestrogens	1331, 333011	440, 65893	1233, 543004	522, 38567	0.78** (0.66, 0.87)	0.33* (0.08, 0.54)	0.12 (-0.16, 0.38)	0.002 (-0.27, 0.27)
Percentage (%) contribution (a)								
Total isoflavones	68.9%	65.6%	44.9%	20.8%				
Total lignans and enterolignans	31.1%	34.4%	55.1%	79.2%				

**p<0.001 *0.011

(a) contribution to total phytoestrogen

4.3 Cross classification of phytoestrogen intake

Dietary intake and urinary excretion data for individual phytoestrogens were categorised into quartiles and a comparison of ranks was conducted between tools to further evaluate the validity and reliability of the modified FFQ (**Table 8**).

The reliability of the FFQ was examined by comparing intakes from the pre- and post- FFQ; 64% to 73% of total lignans, enterolignans or isoflavones were correctly classified into the same quartiles, with 23.7% to 32.2%, correctly classified into adjacent quartiles.

The convergent validity was examined by comparing intakes from the pre-FFQ and WFR; 32% to 47% of total lignans, enterolignans or isoflavones were correctly classified into the same quartile, with 45.8% to 52.5%, correctly classified into adjacent quartiles.

The criterion validity was examined by comparing intakes from the pre-FFQ with biomarker excretion; 24.5% to 26.4% of total lignans, enterolignans or isoflavones were correctly classified into the same quartile, with 47.2% to 54.7% correctly classified into adjacent quartiles; cross classification of ranks between the WFR and biomarker excretion was comparable.

More than 90% were ranked in either the same or adjacent quartile when comparing the pre-FFQ and post-FFQ, indicating the FFQ was a highly reliable tool for ranking intake in the same, ±1 or ±2 quartiles. More than 80% were ranked in either the same or adjacent quartile when comparing the FFQ with WFR indicating that the FFQ was a highly valid tool for ranking intake. More than 70% were ranked in either the same or adjacent quartile when comparing the FFQ with the urinary biomarker indicating moderate criterion validity (Fernandez et al., 2013).

Table 8. Cross classification comparison between quartiles of phytoestrogen intake and excretion for pre FFQ, post FFQ, WFR (n=59) and urinary biomarkers (n=46)

	Pre FFQ and Post FFQ			**Pre FFQ and WFR**			**Pre FFQ and Biomarker†**			**WFR and Biomarker†**		
Phytoestrogens	Same quartiles	Adjacent quartiles	Opposite quartiles	Same quartiles	Adjacent quartiles	Opposite quartiles	Same quartiles	Adjacent quartiles	Opposite quartiles	Same quartiles	Adjacent quartiles	Opposite quartiles
SECO	62.7%	33.9%	3.4%	32.2%	52.5%	15.3%	28.3%	54.7%	17.0%	26.4%	58.5%	15.1%
MAT	64.4%	28.8%	6.8%	33.9%	52.5%	13.6%	24.5%	54.7%	20.8%	24.5%	52.8%	22.6%
PINO	57.6%	39.0%	3.4%	23.7%	55.9%	20.3%	-	-	-	-	-	-
LARI	59.3%	37.3%	3.4%	35.6%	50.8%	13.6%	-	-	-	-	-	-
Total lignan	71.2%	23.7%	5.1&	32.2%	52.5%	15.3%	26.4%	54.7%	18.9%	30.2%	58.5%	11.3%
ENL	71.2%	27.1%	1.7%	50.8%	40.7%	8.5%	28.3%	47.2%	24.5%	26.4%	49.1%	24.5%
END	66.1%	30.5%	3.4%	25.4%	54.2%	20.3%	26.4%	43.4%	30.2%	22.6%	49.1%	28.3%
Total enterolignan	72.9%	25.4%	1.7%	47.5%	45.8%	6.8%	24.5%	47.2%	28.3%	28.3%	47.2%	24.5%
Daidzein	64.4%	32.2%	3.4%	39.0%	49.2%	11.9%	26.4%	52.8%	20.8%	41.5%	47.2%	11.3%
Genistein	66.1%	28.8%	5.1%	45.8%	42.4%	11.9%	22.6%	54.8%	22.6%	24.5%	58.5%	17.0%
Glycitein	72.9%	22.0%	5.1%	33.9%	54.2%	11.9%	43.4%	35.8%	20.8%	33.9%	47.2%	18.9%
Formononetin	64.4%	33.9%	1.7%	30.5%	55.9%	13.6%	35.8%	43.4%	20.8%	24.5%	49.1%	26.4%
Biochanin A	67.8%	28.8%	3.4%	39.0%	45.8%	15.3%	-	-	-	-	-	-
Total isoflavone	64.4%	32.2%	3.4%	37.3%	45.8%	16.9%	26.4%	52.8%	20.8%	37.7%	45.3%	17%
Total Lignan and Total Enterolignan	71.2%	23.7%	5.1%	33.9%	50.8%	15.3%	28.3%	49.1%	22.6%	24.5%	60.4%	15.1%
Total Phytoestrogens‡	71.2%	25.4%	3.4%	35.6%	49.2%	15.3%	26.4%	54.7%	18.9%	26.4%	47.2%	26.4%

†the dash line '-'indicated that the PINO, LARI and biochanin A were not detected in the LC-MS and the analysis cannot be conducted

‡ total phytoestrogens = total lignans + total enterolignans + total isoflavonoids

4.4 Bland Altman plots of assessing the validity of food frequency questionnaire with weighed food record

Bland Altman plots for comparing consistency of individual and total phytoestrogen intakes estimated by pre FFQ and WFR are shown in **Figures 11-14**.

The mean bias between intakes, and limits of agreement for phytoestrogen intakes measured from the pre-FFQ and WFR are shown in **Table 9**. The mean bias for individual phytoestrogens measured by the two tools, ranged from 1256 µg/day for SECO to –1075 µg/day for PINO; the FFQ overestimated intake compared to the WFR for all individual phytoestrogens except SECO, ENL, daidzein and genistein.

The Bland Altman plots showed a similar trend towards greater bias at higher mean phytoestrogen intakes (**Figure 11-14**), suggesting poorer consistency of intake between the FFQ and reference method for those subjects reporting higher phytoestrogen intakes.

Table 9. Bias and 95% limits of agreement for measures of phytoestrogen by pre FFQ compared with the WFR

Phytoestrogens	n	Mean difference/Bias *	95% CI	Limits of agreement†
Lignans				
SECO	58	1256	-383, 2895	-11212, 13724
MAT	58	-67	-161, 27	-784, 650
PINO	58	-1075	-2249, 98	-10001, 7850
LARI	58	-658	-1125, -190	-4217, 2902
Total lignan	58	-544	-3248, 2160	-21109, 20021
Enterolignans				
ENL	59	4	-11, 19	-115, 123
END	59	-0.2	-0.4, 0	-2.1, 1.7
Total enterolignan	59	3.8	-11, 19	-115, 123
Isoflavones				
Daidzein	59	522	-4505, 5550	-38061, 39105
Genistein	59	1106	-5498, 7710	-49578, 51790
Glycitein	59	-251	-1108, 1610	-10180, 10682
Formononetin	59	-10	-157, 137	-1142, 1122
Biochanin A	59	-697	-1396, 1.2	-6059, 4664
Total isoflavone	59	1172	-11811, 14154	-98460, 100803
Total lignan and enterolignan	59	-9217	-26794, 8360	-178626, 125680
Total phytoestrogens	59	-8045	-30272, 14181	-178626, 162535

*Bias is the difference (pre FFQ -WFR) between the raw values of intake phytoestrogens estimated from the pre FFQ and WFR. This is the absolute difference between the raw mean intakes estimated from the pre FFQ and WFR.

† 95% Limits of agreement (mean difference±2SD)

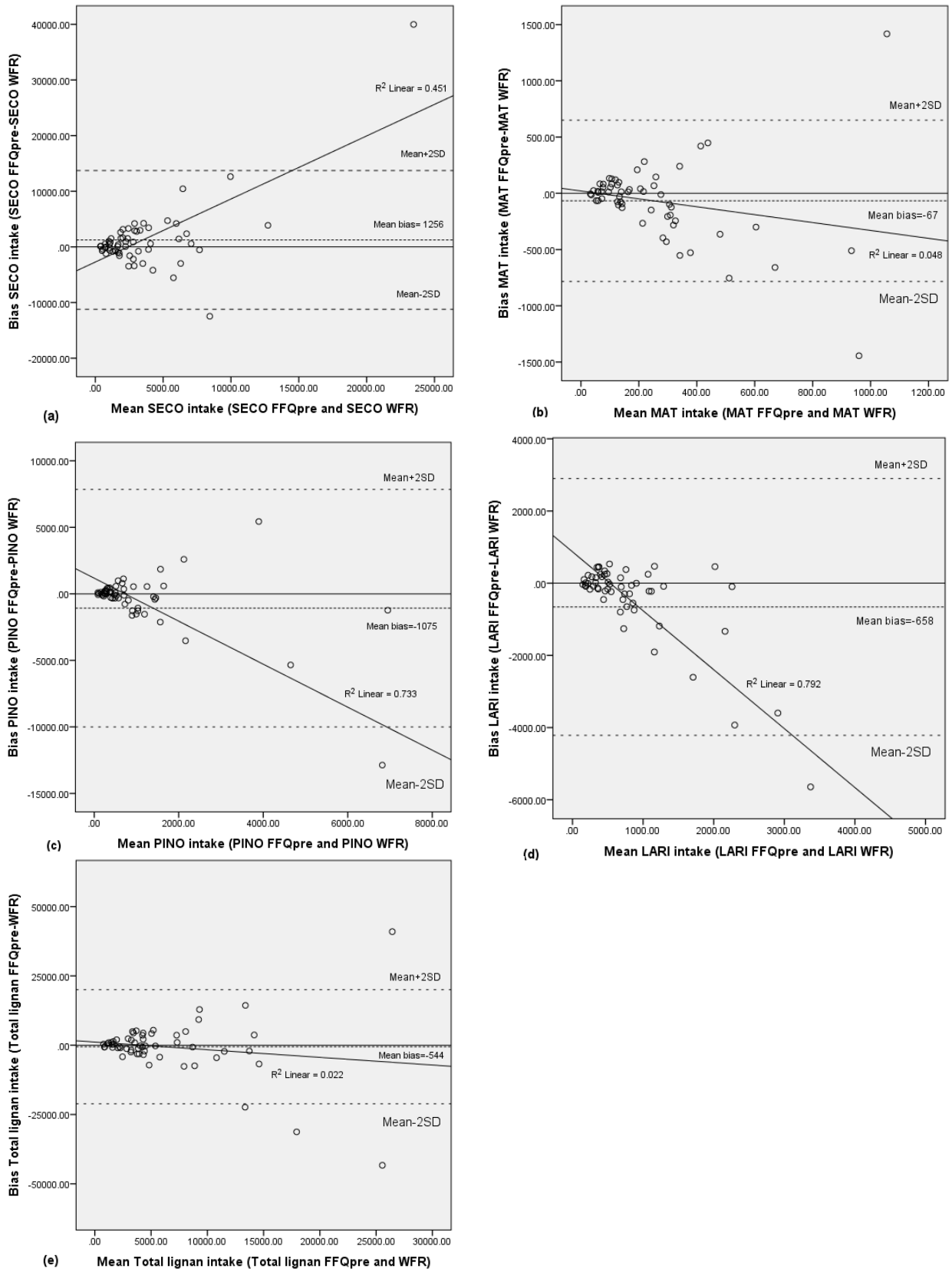


Figure 11. Bland Altman plots of estimated intake of (a) SECO, (b) MAT, (c) PINO, (d) LARI, (e) total lignan by pre FFQ and average of 3-day WFR.

The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD of the mean difference)

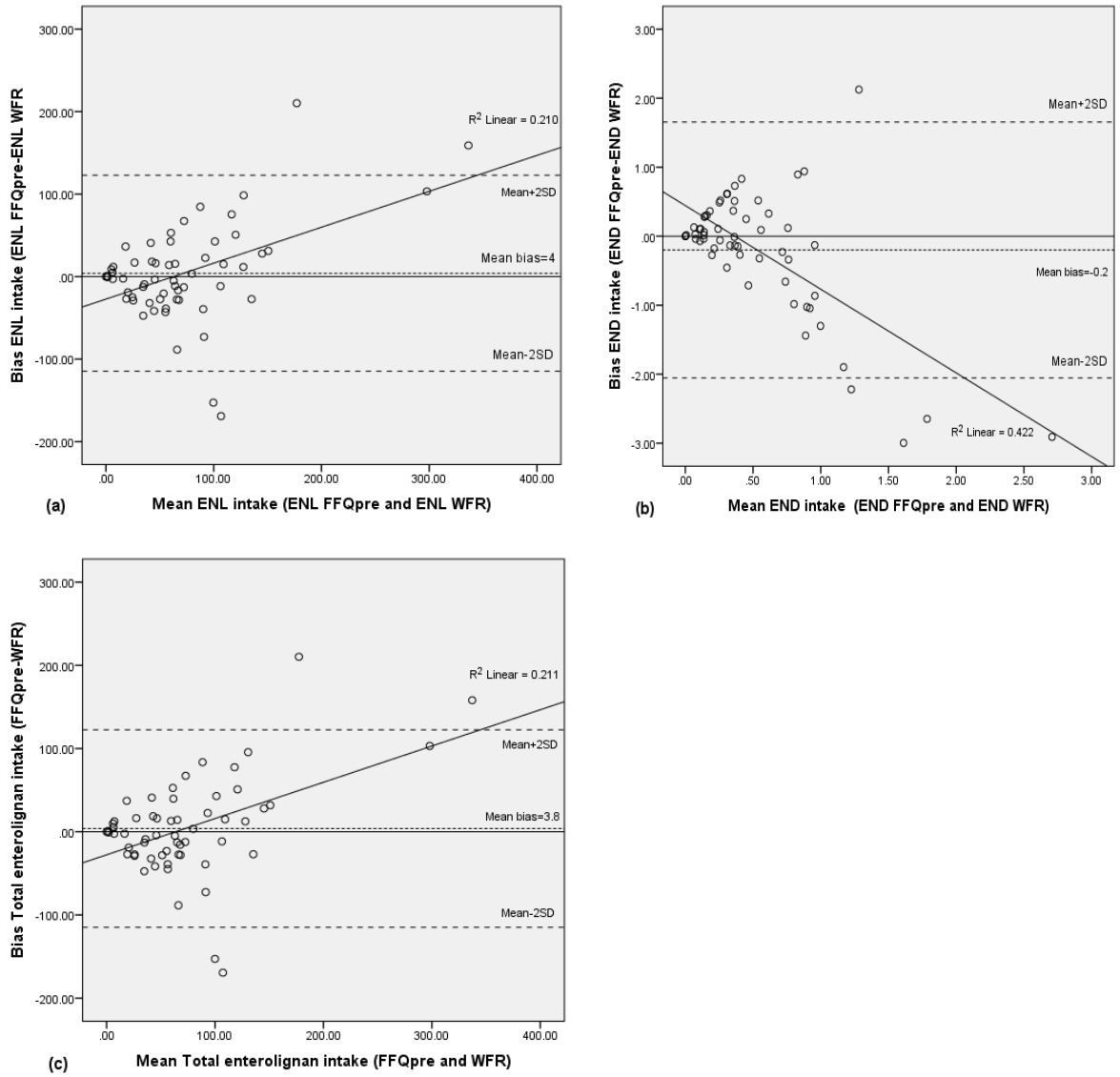


Figure 12. Bland Altman plots of estimated intake of (a) ENL, (b) END, (c) total enterolignan by pre FFQ and average of 3-day WFR.

The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD of the mean difference)

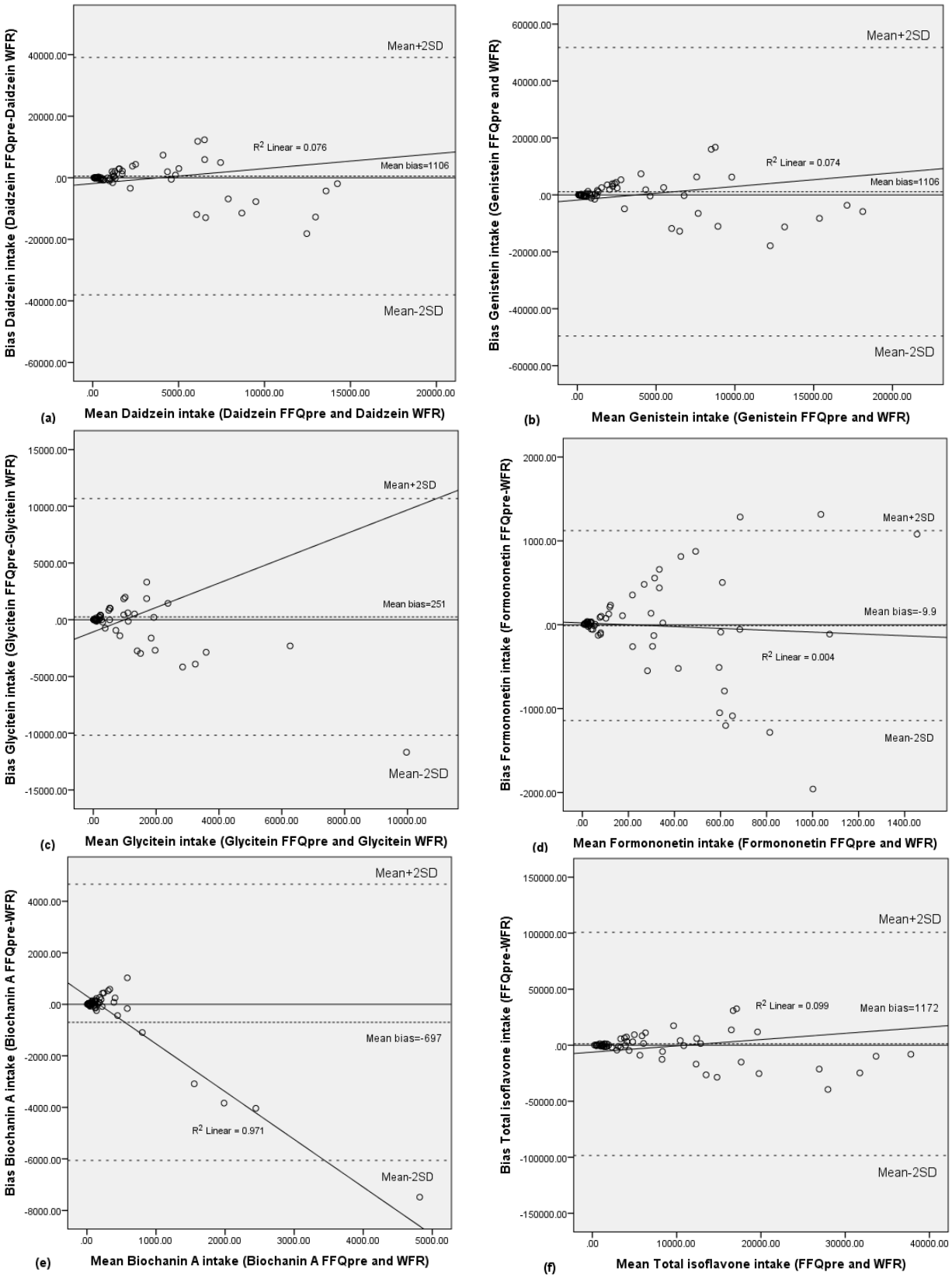


Figure 13. Bland Altman plots of estimated intake of (a) daidzein, (b) genistein, (c) glycitein, (d)formononetin, (e) biochanin A, (f) total isoflavone by pre FFQ and average of 3-day WFR.

The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD of the mean difference)

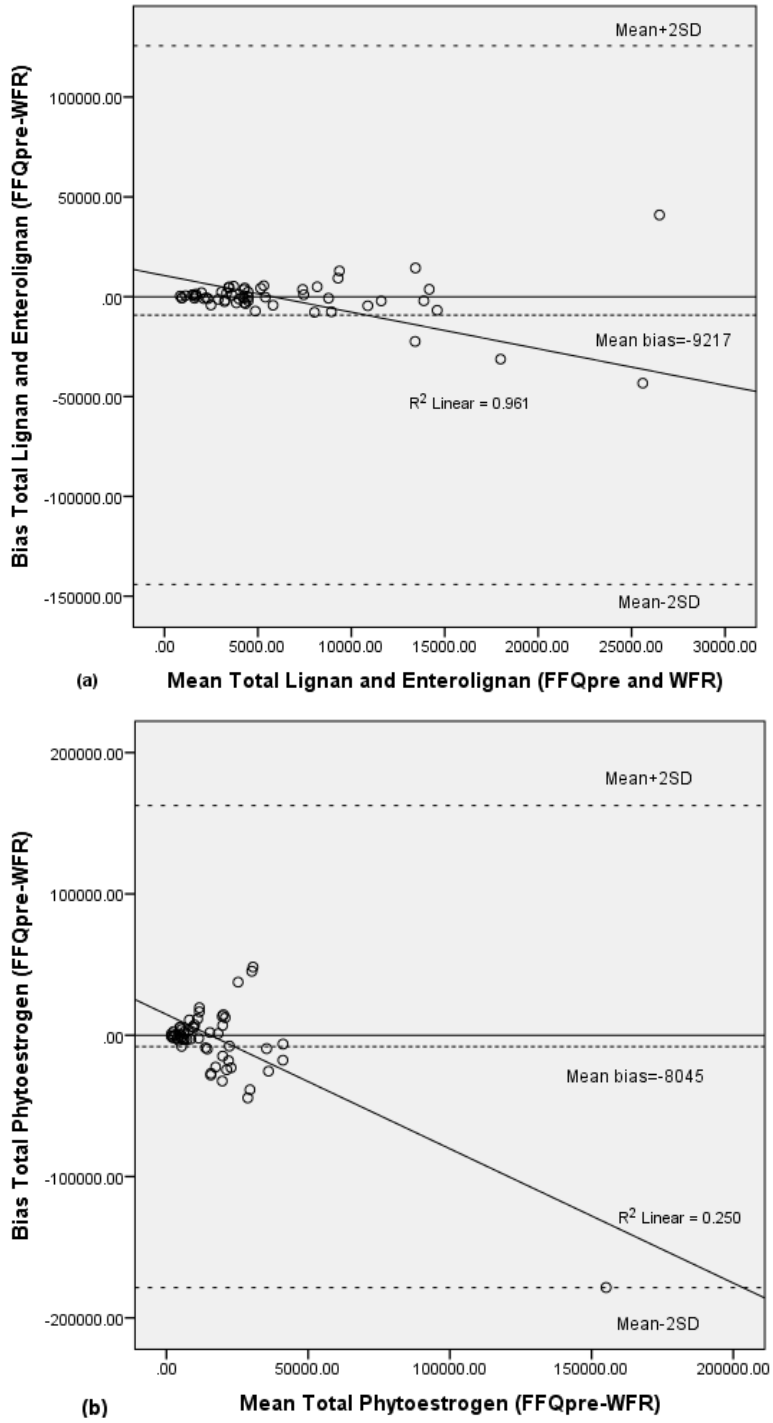


Figure 14. Bland Altman plots of estimated intake of (a) total lignan (SECO, MAT, PINO, LARI) and enterolignan (END, ENL) (b) Total phytoestrogen (total lignan, total enterolignan and total isoflavone) by pre FFQ and average of 3-day WFR.

The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD of the mean difference)

4.5 Bland Altman plots of assessing the validity of food frequency questionnaire with biomarkers

Bland Altman plots for comparing consistency of intake and excretion of total lignans, enterolignans and isoflavonoids estimated by pre FFQ, and urinary biomarker excretion are shown in **Figures 15-18**. The comparisons of individual phytoestrogens are not shown.

The mean bias between intake from the pre-FFQ and biomarker excretion, and limits of agreement are shown in **Table 10.** The mean bias ranged from -3119 µg/day for total enterolignans to 13179 µg/day for total isoflavonoids; the FFQ overestimated compared to urinary excretion for all phytoestrogen classes except total enterolignans.

Bland Altman plots (**Figures 15 to 18**) demonstrated a trend towards larger bias at higher mean estimates of intake and excretion of the phytoestrogen classes. This trend is similar to those observed for individual phytoestrogens in **Figures 11 to 14**, indicating poorer consistency of measurement between intake and excretion at higher intakes.

Table 10. Bias and 95% limits of agreement for measures of phytoestrogen by pre FFQ compared with the Biomarker (BM)

Phytoestrogens	n	Mean difference/Bias *	95% CI	Limits of agreement†
Total lignan	53	4188	2128, 6249	-10762, 19139
Total enterolignan	53	-3119	-4517, -1722	-13259, 7020
Total isoflavone	53	13179	761, 25598	-76926, 103285
Total lignan and enterolignan	53	1069	-1421, 3559	-16999, 19136
Total phytoestrogens	53	14248	1513, 26983	-78156, 106652

*Bias is the difference (pre FFQ -BM) between the raw values of intake phytoestrogens estimated from the pre FFQ and excretion estimated from BM. This is the absolute difference between the raw mean intakes estimated from the pre FFQ and excretion from BM.

† 95% Limits of agreement (mean difference±2SD)

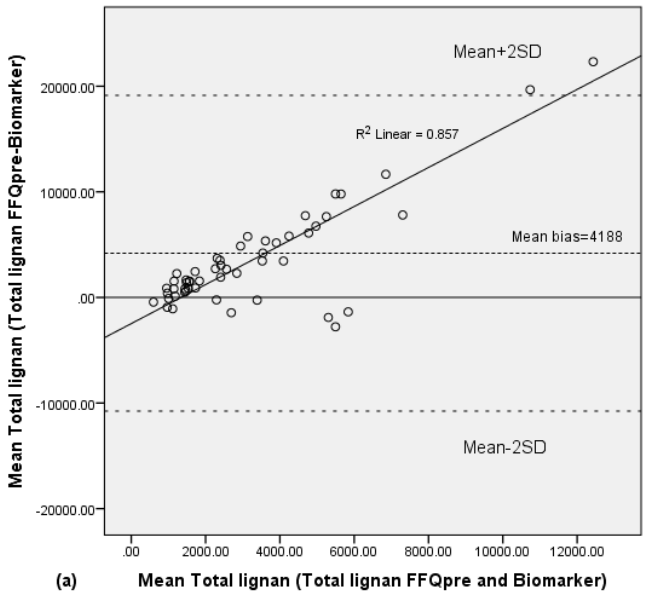


Figure 15. Bland Altman plots of estimated intake of (a) total lignans by pre FFQ (SECO, MAT, PINO, LARI) and average of urinary biomarkers (SECO, MAT).
The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD ± mean difference)

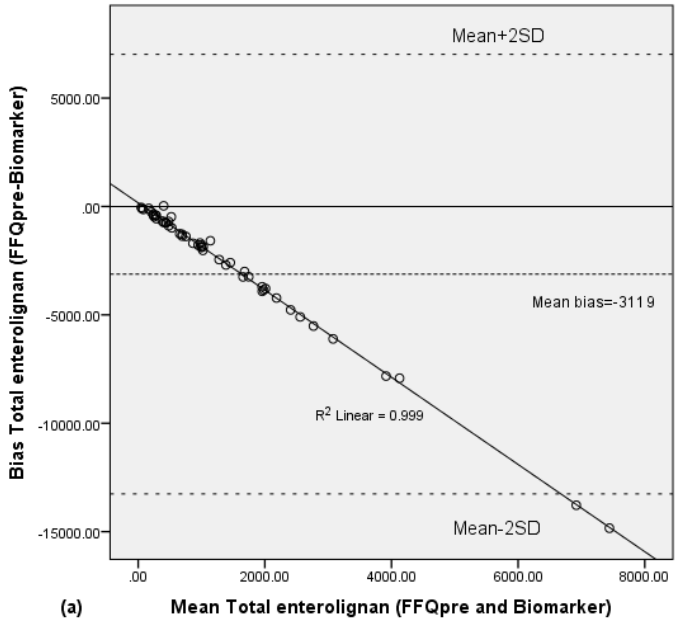


Figure 16. Bland Altman plots of estimated intake of (a) total enterolignan (END, ENL) by pre FFQ and average of urinary biomarkers.
The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD ± mean difference)

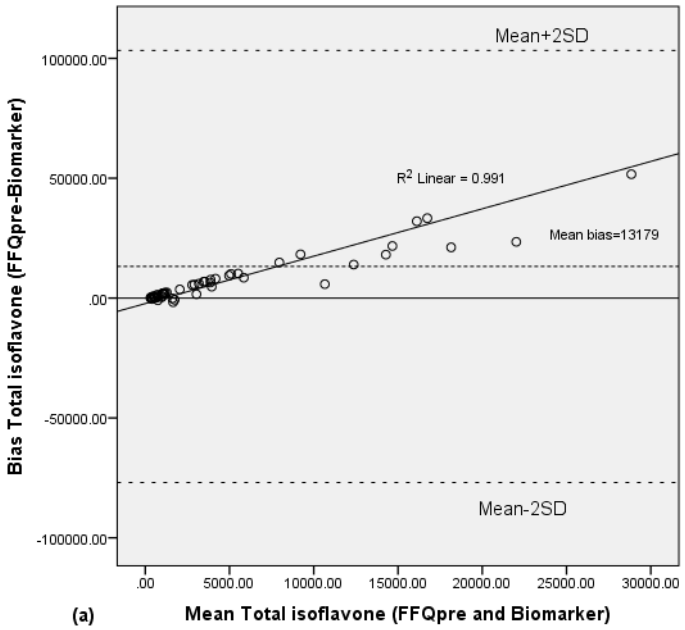
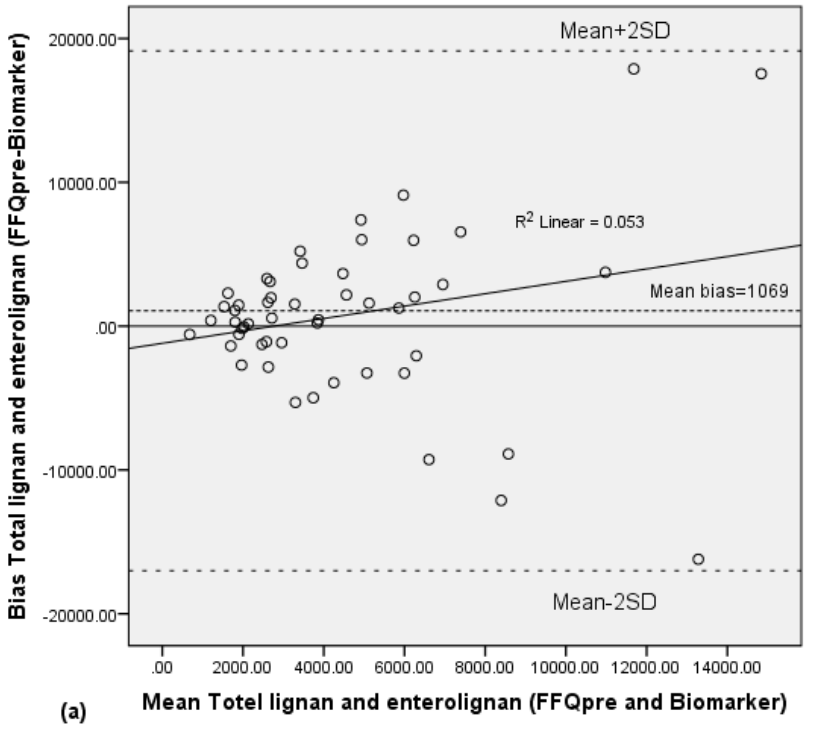


Figure 17. Bland Altman plots of intake of (a) total isoflavone intake by pre FFQ (daidzein, genistein, glycitein, formononetin, biochanin A) and average of urinary biomarkers (daidzein, genistein, glycitein, formononetin). The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD ± mean difference)



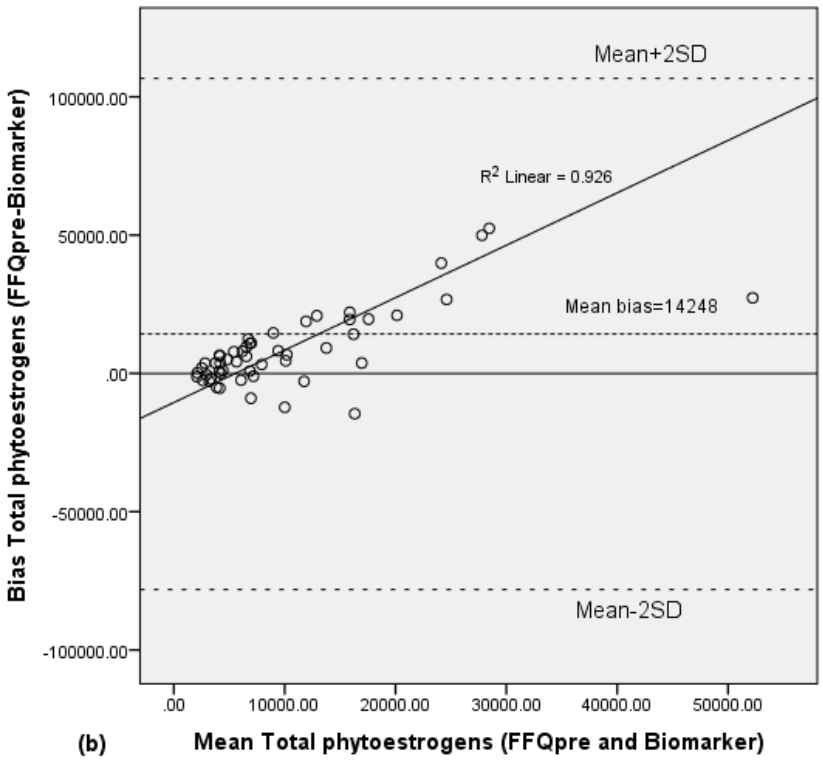


Figure 18. Bland Altman plots of estimated intake of (a) total lignan (SECO, MAT, PINO, LARI) and enterolignan (END, ENL) (b) total phytoestrogen (lignan, enterolignans and isoflavones) by pre FFQ and average of urinary biomarkers.

The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD of the mean difference).

4.6 Bland Altman plots of assessing the reliability of FFQ

Bland Altman plots for comparing consistency of phytoestrogen intakes estimated by the pre FFQ and post FFQ are shown in **Figures 19 to 22**. The mean bias between the intakes and limits of agreement for phytoestrogen intake measured by the FFQ administered 1 to 2 weeks apart are shown in **Table 11**. The mean bias for individual phytoestrogens ranged from -9.2 µg/day for biochanin A to 2496 µg/day for genistein. The pre-FFQ overestimated intake compared to the post-FFQ for all individual phytoestrogens except END and biochanin A. The Bland Altman plots show a trend towards greater bias at higher mean phytoestrogen intakes suggesting poorer consistency of intake between repeat FFQs for those subjects with higher phytoestrogen intakes (**Figures 19-22).**

Table 11. Bias and 95% limits of agreement for measures of phytoestrogen by pre FFQ compared with the post FFQ

Phytoestrogens	n	Mean difference/Bias*	95% CI	Limits of agreement†
Lignans				
SECO	58	1119	-294, 2552	-9693, 11951
MAT	58	32	12, 52	-123, 187
PINO	58	262	-34, 557	-1985, 2508
LARI	58	89	-8, 186	-650, 828
Total lignan	58	1512	112, 2911	-9136, 12159
Enterolignans				
ENL	59	8.9	-3.2, 21.1	-85, 103
END	59	-0.1	-0.19, 0.04	-0.97, 0.81
Total enterolignan	59	8.9	-3.3, 21.1	-85, 103
Isoflavones				
Daidzein	59	1823	-2212, 5858	-29142, 32789
Genistein	59	2496	-2788, 7780	-38057, 43048
Glycitein	59	522	-663, 1707	-8573, 9617
Formononetin	59	1.2	-98, 100	-757, 759
Biochanin A	59	-9.2	-51, 33	-330, 311
Total isoflavone	59	4833	-5670, 15336	-75774, 85439
Total lignan and enterolignan	59	1529	155, 2903	-9015, 12074
Total phytoestrogens	59	6362	-4371, 17095	-76008, 88732

*Bias is the difference (pre FFQ-post FFQ) between the raw values of intake phytoestrogens estimated from the pre FFQ and post FFQ. This is the absolute difference between the raw mean intakes estimated from the pre FFQ and post FFQ. † 95% Limits of agreement (mean difference±2SD)

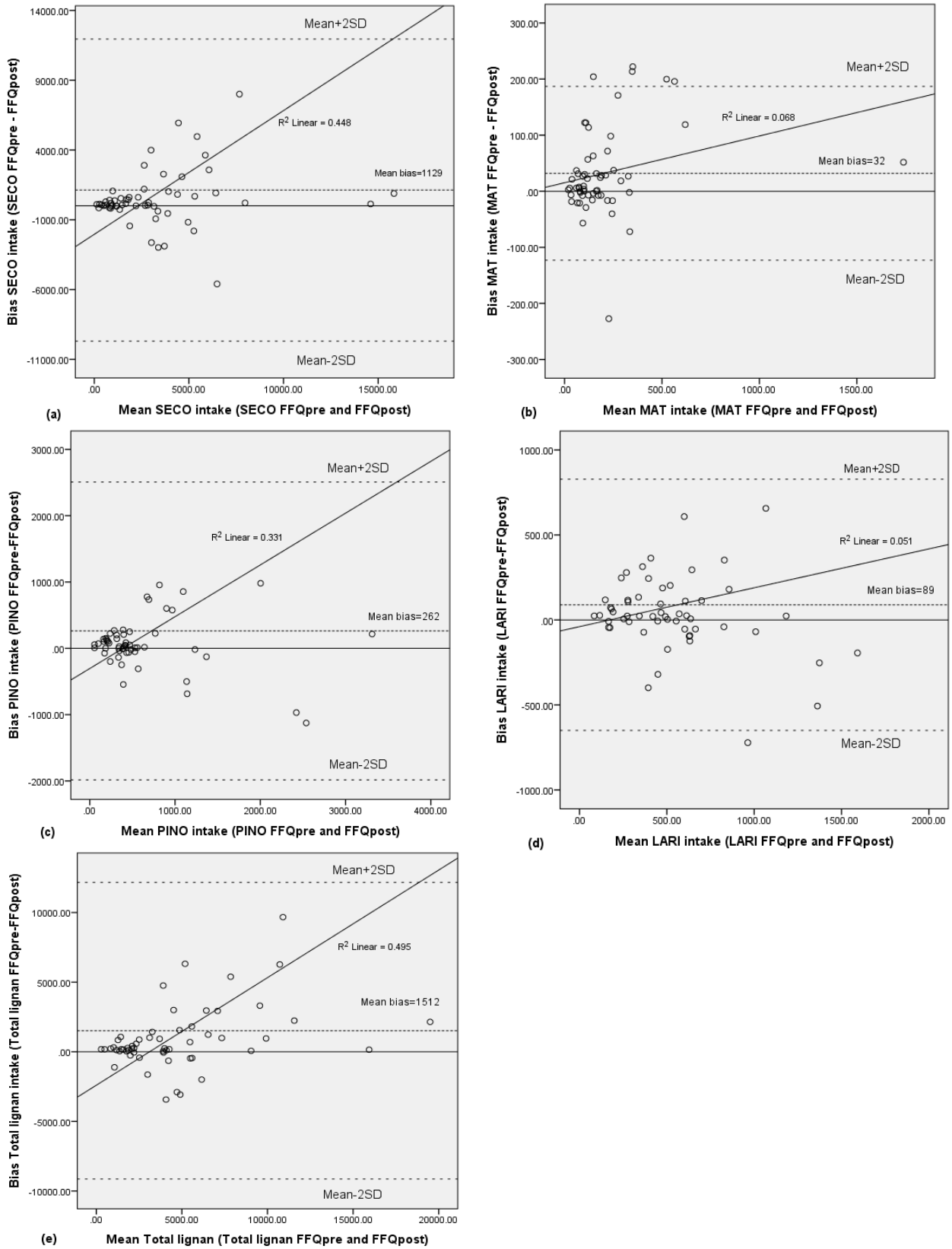


Figure 19. Bland Altman plots of estimated intake of (a) SECO, (b) MAT, (c) PINO, (d) LARI, (e) total lignan by pre FFQ and post FFQ. The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD of the mean difference)

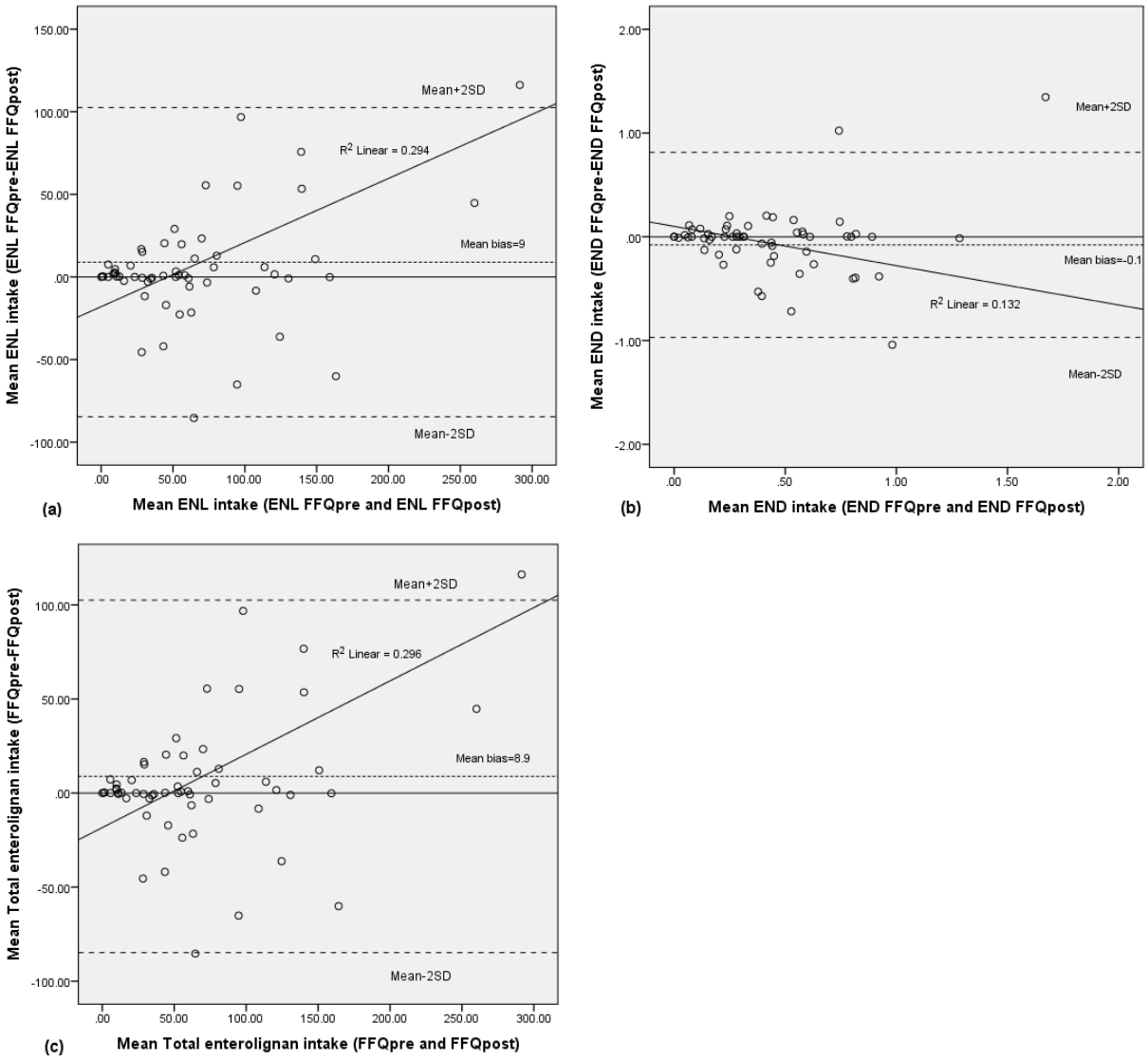


Figure 20. Bland Altman plots of estimated intake of (a) ENL, (b) END, (c) total enterolignan by pre FFQ and post FFQ.

The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD of the mean difference)

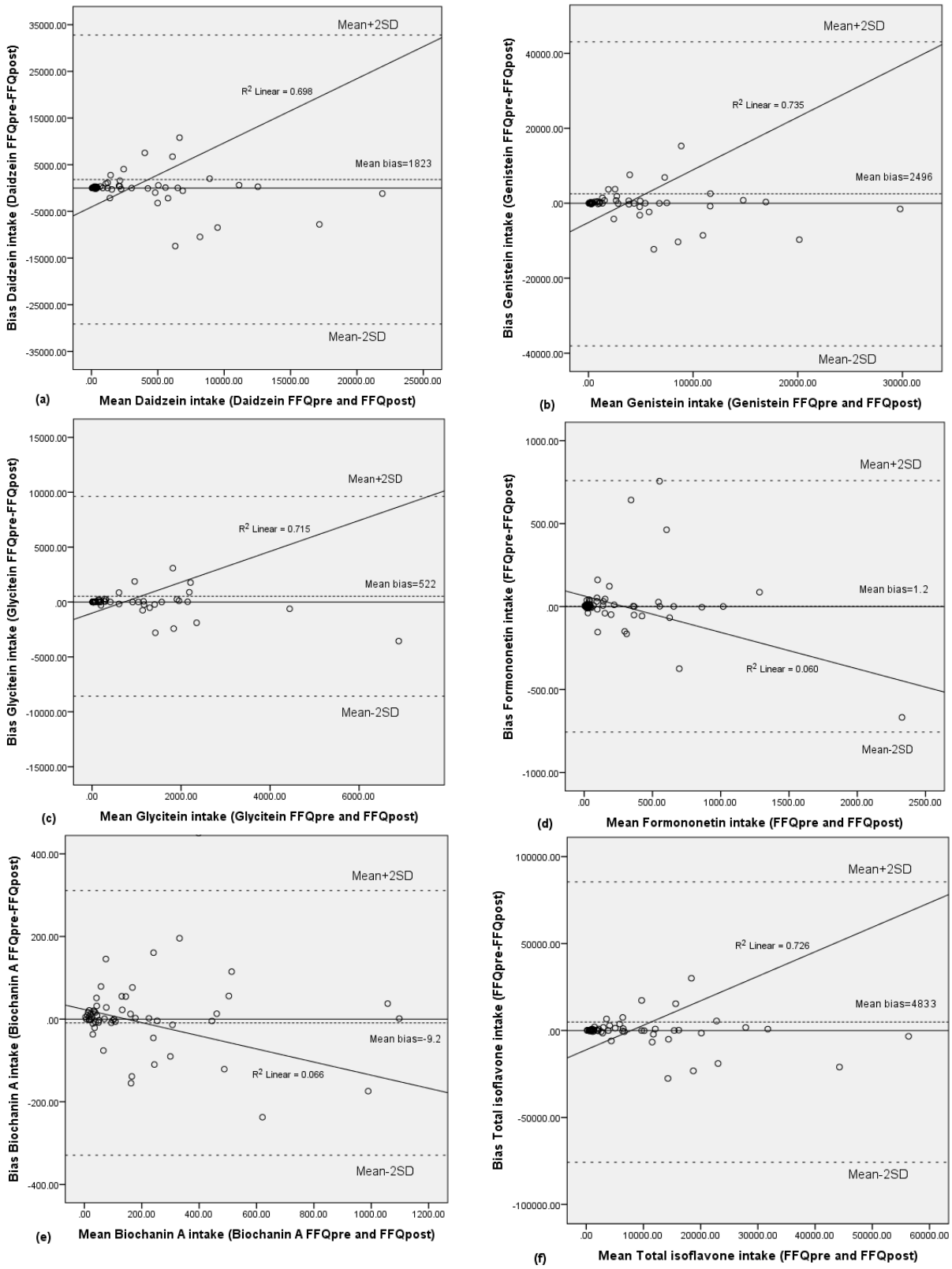


Figure 21. Bland Altman plots of estimated intake of (a)daidzein (b)genistein (c)glycitein (d)formononetin (e)biochanin A (f)total isoflavone by pre FFQ and post FFQ.

The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD ±the mean difference)

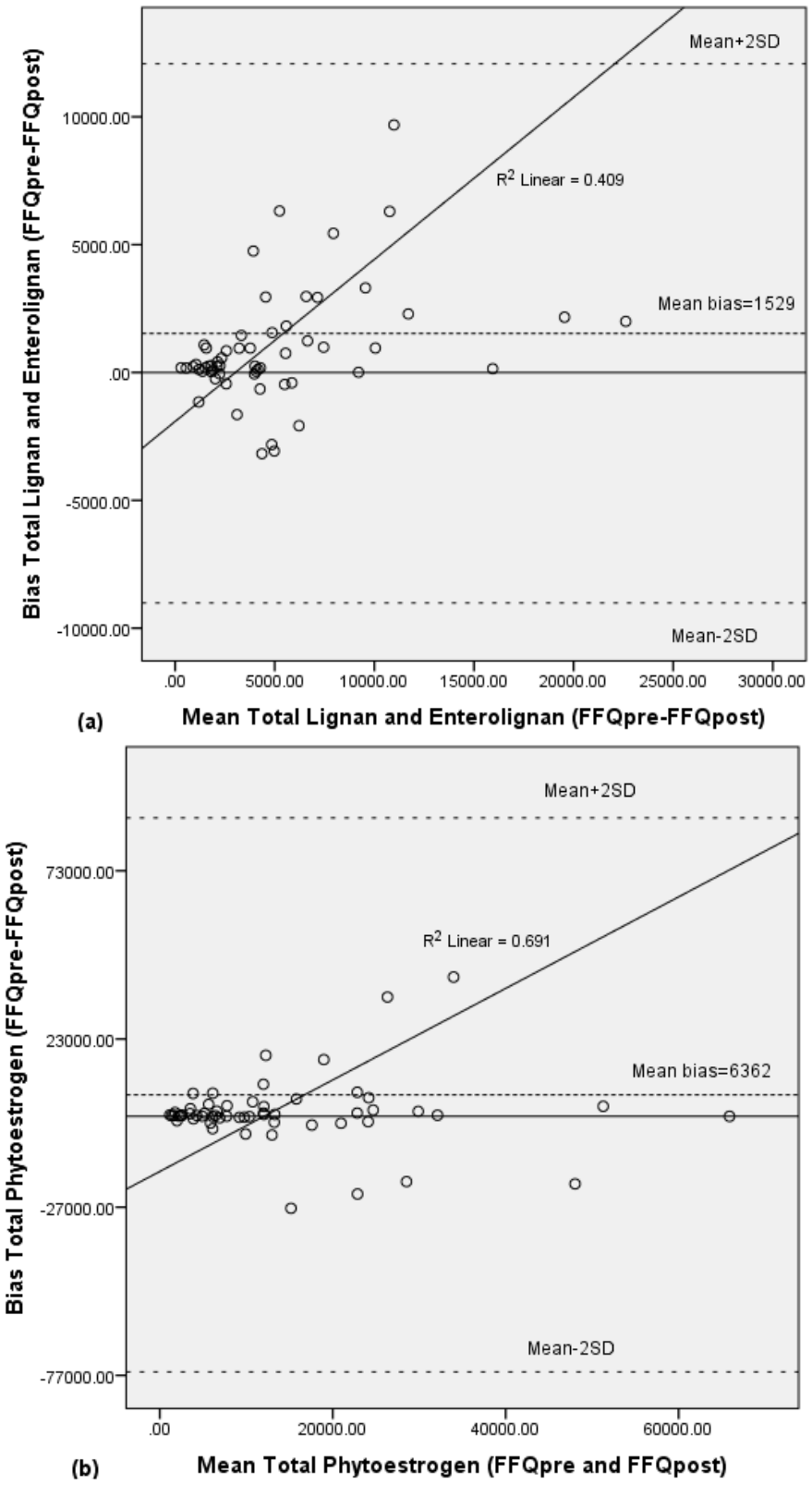


Figure 22. Bland Altman plots of estimated intake of (a) total lignan (SECO, MAT, PINO, LARI) and enterolignan (END, ENL) (b) total phytoestrogen (total lignan, total enterolignan and total isoflavone) by pre FFQ and post FFQ. The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD of the mean difference)

4.7 Bland Altman plots for weighed-food record and urinary biomarkers

Bland Altman plots for comparing consistency of total phytoestrogen intake and excretion for total lignans, enterolignans and isoflavonoids, estimated by the WFR and urinary biomarkers are shown in **Figures 23-26**. The mean bias between the intakes and excretion of phytoestrogen classes ranged from -3120 µg/day, for total enterolignans, to 14230 µg/day for total lignans (**Table 12**); dietary intake estimated by the WFR was greater than urinary biomarker excretion for total lignans and isoflavonoids, but urinary excretion of total enterolignans exceeded enterolignan intake. The Bland Altman plots showed the bias of the estimates between WFR and biomarkers, (**Figure 23-26**), was larger when the mean estimates of intake and excretion were higher. This trend is similar to what was observed between FFQ and biomarker in Figures 15-18 and between estimates of intake between FFQ and WFR in Figures 11-14.

Table 12. Bias and 95% limits of agreement for measures of phytoestrogen by WFR compared with the Biomarker

Phytoestrogens	n	Mean difference/Bias*	95% CI	Limits of agreement†
Total lignans	53	14230	-5928, 34388	-132037, 160497
Total enterolignans	53	-3120	-4515, -1726	-13239, 6999
Total isoflavones	53	11703	2922, 20485	-52016, 75423
Total lignans and enterolignans	53	11110	-9021, 31240	-134958, 157177
Total phytoestrogens	53	22813	1303, 44323	-133264, 178890

* Bias is the difference (WFR-BM) between the raw values of intake phytoestrogens estimated from the WFR and excretion from BM. This is the absolute difference between the raw mean intakes estimated from the WFR and excretion from BM.

† 95% Limits of agreement (mean difference±2SD

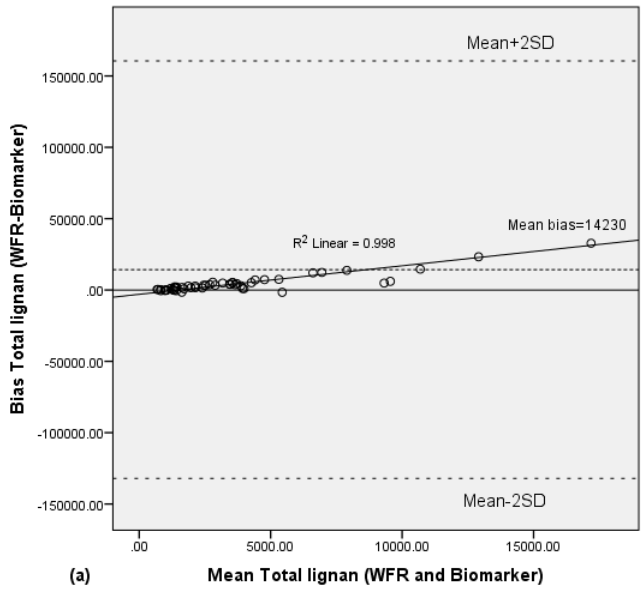


Figure 23. Bland Altman plots of estimated intake of (a) total lignan (SECO, MAT, PINO, LARI) by average of 3-day WFR and average of urinary biomarker (SECO, MAT).

The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD ± mean difference)

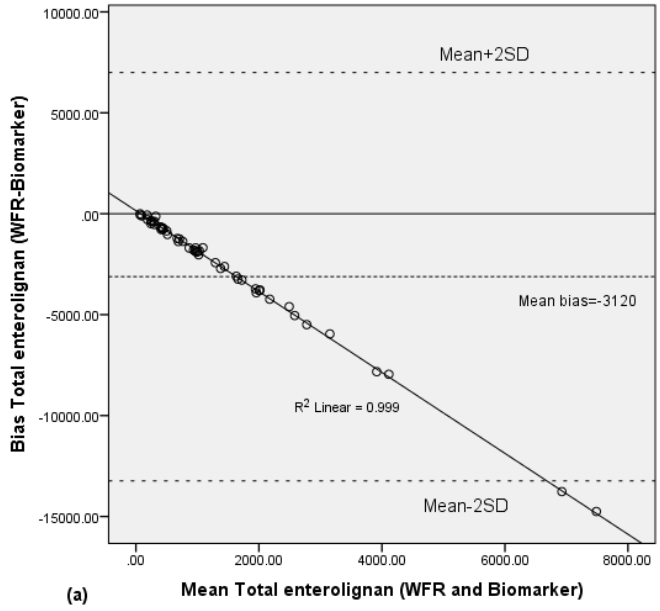


Figure 24. Bland Altman plots of estimated intake of (a) total enterolignan (END, ENL) by average of 3-day WFR and average of urinary biomarker (END, ENL).

The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD ± mean difference)

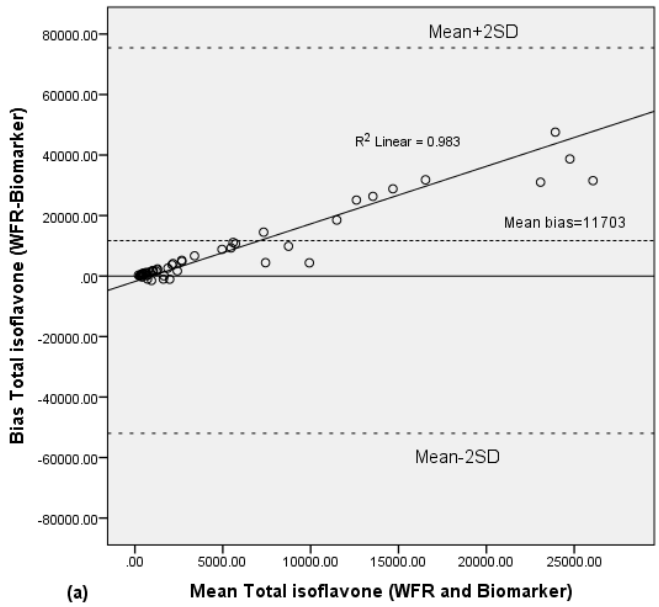


Figure 25. Bland Altman plots of estimated intake of (a) total isoflavone (daidzein, genistein, glycitein, formononetin, biochanin A) by average of 3-day WFR and average of urinary biomarker (daidzein, genistein, glycitein, formononetin).

The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD ±the mean difference)

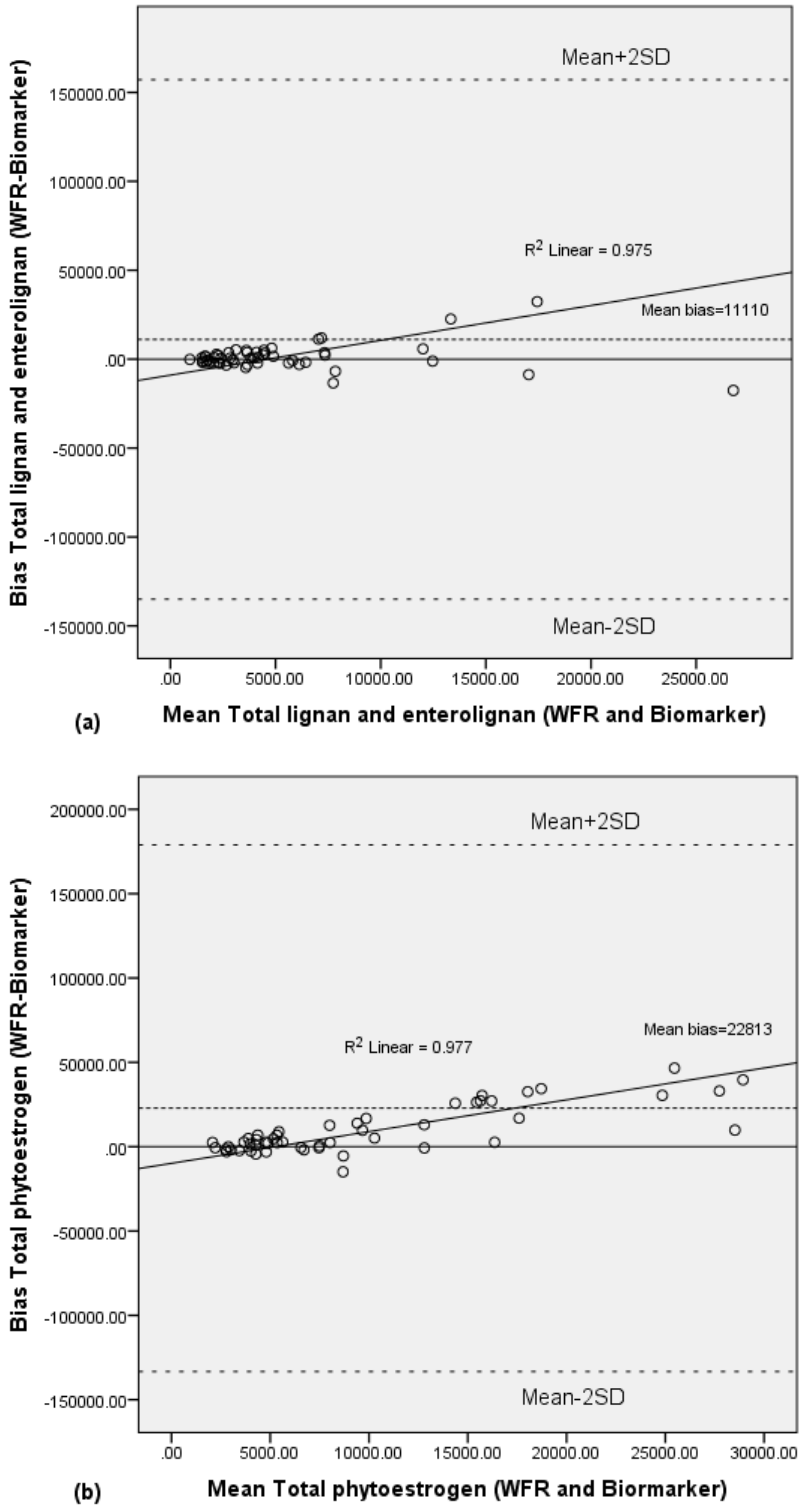


Figure 26. Bland Altman plots of estimated intake of (a) total lignan (SECO, MAT, PINO, LARI) and enterolignan (END, ENL) (b) total phytoestrogens (total lignans, total enterolignans and total isoflavones) by average of 3-day WFR and average of urinary biomarkers.
The central dashed line represents the mean difference (bias). The upper and lower dashed lines represent the 95% limits of agreement (2SD of the mean difference)

4.8 Intake and excretion of phytoestrogens and demographic and lifestyle characteristics

The associations between demographic and lifestyle characteristics and median intakes of total lignans, enterolignans and isoflavones from the FFQ and WFR are shown in **Tables 13 and 14,** respectively. Associations with median biomarker excretion are shown in **Table 15**. The Mann-Whitney U test and Kruskal-Wallis one-way ANOVA were used to compare the median intakes across categories of these demographic and lifestyle factors. A Bonferroni adjustment was made for multiple comparisons, with a probability of $P<0.0125$ taken as statistically significant.

Based on the FFQ, total lignan intake was higher in subjects with Caucasian ancestry, who spoke English at home and who used commercial supplements (all p=0.01); there was a trend towards higher intakes in females compared to males (p=0.03) and those born in Oceania, North Africa or Middle East (p=0.02). Total enterolignan intake was higher in subjects who used commercial supplements (p=0.01); there was a trend to higher intakes in subjects with university education (p=0.02) (**Table 13**). Based on the WFR, there were trends towards higher total lignan intake in subjects who spoke English at home and higher total enterolignan intake in those who used commercial supplements (both p=0.03). Phytoestrogen intake was significantly in subjects who were either sedentary or had high physical activity (both p=0.01) (**Table 14**).

Based on the urinary biomarkers, there were no significant associations for total lignan or total isoflavone intakes. There were trends towards higher enterolignan excretion in females compared to males, in subjects with education to secondary school, certificate or diploma (both p=0.03), and in subjects from Oceania (p=0.04) (**Table 15**). Commercial supplements consumed by subjects included multivitamins, calcium, iron, fish oil, protein powder. Isoflavonoid or lignan dietary supplements such as LSA (linseed, soy, almond) were coded as food items.

Table 13. **Average intake of lignans, enterolignans, isoflavones (µg/day) from pre-food frequency questionnaire (FFQ pre) according to subjects' characteristics (n=59)**

Subject characteristics	Total lignans Median±IQR	Total enterolignans Median±IQR	Total isoflavones Median±IQR	Total phytoestrogens Median+IQR
Gender				
Male (n=19)	2356±2663	54±83	2453±8806	6678±10786
Female (n=40)	4660±6735	54±81	4684±12260	10828±17064
p-value†	**0.03**	0.75	0.48	0.12
BMI(kg/M^2)				
<20.00 (n=5)	2942±4310	61±211	832±10582	6064±11713
20.00-25.00 (n=28)	3895±3762	48±81	2216±9109	8398±13686
25.01-30.01 (n=15)	4148±8576	44±110	7102±14858	12905±27614
>30.01 (n=11)	4108±11931	59±64	6202±11771	12299±23708
p-value‡	0.74	0.66	0.45	0.49
Age (y)				
18-28 (n=26)	3102±4820	53±67	1971±7937	7325±13826
29-39 (n=16)	3895±4066	44±83	6257±5873	9842±7164
40-50 (n=9)	3984±4983	66±93	11857±27316	19921±28639
>50 (n=8)	9809±9733	57±124	9540±27171	18596±33138
p-value‡	0.08	0.94	0.34	0.19
Occupation				
Family duties (n=3) and Students (n=34)	3894±6853	52±75	2453±10407	9553±14836
Clerical and administrative workers (n=12)	4894±5444	76±107	6656±10421	11845±14362
Community and personal service (n=2) & Technician and trade workers (n=2)	3941±2557	56±35	11602±20726	16165±22081
Professionals (n=5) & Managers (n=1)	3143±13101	63±202	4273±15787	8914±40140
p-value‡	0.94	0.85	0.80	0.98
Education level achieved				
Below university (Other (n=1), Secondary school (n=1), Certificate/diploma (n=11)	5254±9271	15±57	7102±25839	18365±36049
University (undergraduate (n=22, postgraduate (n=24)	3757±5195	59±82	2518±9002	9452±12740
p-value†	0.53	**0.02**	0.49	0.42

Table 13 continued Subject characteristic	Total lignans Median±IQR	Total enterolignans Median±IQR	Total isoflavones Median±IQR	Total phytoestrogens Median+IQR
Ancestry				
Caucasian (n=38)	5264±8158	55±83	2352±12354	10757±21450
Other ethnic groups (n=21)[1]	2356±2608	37±91	5732±9600	7303±11404
p-value†	**0.01**	0.73	0.67	0.23
Language spoken at home				
English (n=38)	4156±8156	57±84	3217±12354	11811±21009
Other languages (n=21)[2]	2345±3707	37±91	3853±9974	7303±13608
p-value†	**0.01**	0.54	0.94	0.16
Region of country of birth				
Oceania and Antarctica (n=29)	5640±8544	59±97	1992±10462	11322±19906
North-West (n=9) & Southern and Eastern Europe (n=1)	3939±6117	57±50	2299±16692	7936±27408
North Africa and the Middle East (n=2), Americans (n=2), Sub-Saharan Africa (n=2)	5596±4623	69±160	7598±9613	11840±10843
Asia (n=14)[3]	2182±2229	29±49	5967±12533	7300±14059
p-value‡	**0.02**	0.44	0.74	0.91
Physical activity level (PAL)				
Sedentary (n=5)	4108±9009	13±22	16263±17243	26918±16872
Light (n=18）	3884±5731	61±106	5624±6875	10477±11824
Moderate (n=21)	3897±3639	54±75	1950±8595	6678±11279
High (n=15)	4164±9030	55±70	3853±10474	12368±31924
p-value‡	0.64	0.13	0.06	0.053
Smoking status				
Never smoked (n=38)	3491±3736	52±78	4684±9940	8560±13680
Past smoker (n=14)	7098±14890	49±108	8924±31712	22765±47001
Current smoker (n=7)	4148±8705	71±102	1544±3358	9028±10146
p-value‡	0.08	0.54	0.17	0.08

Table 13 continued Subject characteristic	Total lignans Median±IQR	Total enterolignans Median±IQR	Total isoflavones Median±IQR	Total phytoestrogens Median+IQR
Bowel frequency				
1-7/week (n=24)	3152±3762	36±61	4792±11015	7387±14220
>7/week (n=35)	4108±6889	55±92	2584±10937	12299±20687
p-value†	0.06	0.15	0.88	0.17
Bowel movement				
Mushy and watery (n=2) & Smooth and soft to pass (n=53)	3897±5865	54±82	2584±11610	9633±18639
Hard to pass (n=4)	4652±8033	54±117	4683±4872	10175±11717
P-value†	0.55	0.74	0.94	0.85
Commercial supplements use				
No (n=32)	2644±3434	36±51	2382±14764	7278±17800
Yes (n=27)	5640±8595	71±120	5732±9229	12299±15973
P-value†	**0.01**	**0.01**	0.52	0.10

*Mann-Whitney U test

** Kruskal-Wallis One-Way ANOVA

[1] South East Asian (n=6), South Asian (n=3), North East Asian (n=5), Middle Eastern, North Africa, Somalia Peninsular (n=2), Central/South America (n=2), Other (n=1) & Mixed ancestry (n=2)

[2] Cantonese, Arabic, Mandarin, Spanish, German, Hindi, Malay, Persian, Urdu, Afrikaans, Macedonian, Bahasa Indonesian) [3](South-East (n=6), North-East (n=5), Southern and Central (n=3)

Table 14. Average intake of lignans, enterolignans, isoflavones from 3-day weighed food record (WFR) according to subjects' characteristics (n=59)

Subject characteristics	Total lignans Median±IQR	Total enterolignans Median±IQR	Total isoflavones Median±IQR	Total phytoestrogens Median+IQR
Gender				
Male (n=19)	4460±4547	65±43	3171±8770	7775±24986
Female (n=40)	4302±6228	62±59	2069±12438	8137±23522
p-value†	0.81	0.74	0.91	0.99
BMI(kg/M^2)				
<20.00 (n=5)	4160±17955	79±139	931±4248	4630±21840
20.00-25.00 (n=28)	4284±7337	69±50	2809±12172	9009±31556
25.01-30.01 (n=15)	4543±4185	65±93	5277±27635	9860±24552
>30.01 (n=11)	3104±3555	53±40	1251±7804	6341±8049
p-value‡	0.99	0.65	0.14	0.30
Age (y)				
18-28 (n=26)	3791±7231	62±51	2809±12524	8506±25734
29-39 (n=16)	4548±8956	66±69	2063±4836	8117±28086
40-50 (n=9)	3104±3674	78±61	1748±17001	8781±18157
>50 (n=8)	5555±7353	55±81	1596±11030	7000±18830
p-value‡	0.49	0.92	0.44	0.97
Occupation				
Family duties (n=3) and Students (n=34)	4543±8953	53±50	2448±9476	8781±21185
Clerical & administrative workers (n=12)	2292±4074	60±96	1102±7507	4799±11979
Community and personal service (n=2) &Technician and trade workers (n=2)	3829±7729	79±30	15195±34408	23134±38137
Professionals (n=5) & Managers (n=1)	4831±4133	73±105	12966±30147	19242±28317
P-value‡	0.55	0.25	0.16	0.18
Education level achieved				
Below university (Other (n=1), Secondary school (n=1), Certificate/diploma (n=11)	4554±8497	40±68	11129±34173	17647±36894
University (undergraduate (n=22, postgraduate (n=24)	4134±4411	66±58	2284±8575	7603±16531
p-value†	0.77	0.25	0.24	0.25

Table 14 continued Subject characteristics	Total lignans Median±IQR	Total enterolignans Median±IQR	Total isoflavones Median±IQR	Total phytoestrogens Median+IQR
Ancestry				
Caucasian (n=38)	4499±6149	69±57	3426±14968	11030±24919
Other ethnic groups (n=21)[1]	3473±4587	58±55	2010±3963	7714±13114
p-value†	0.25	0.37	0.18	0.13
Language spoken at home				
English (n=38)	4972±5878	53±63	3426±14971	8506±25475
Other languages (n=21)[2]	2485±4003	39±61	1565±7322	5985±13203
p-value†	**0.03**	0.15	0.23	0.08
Region of country of birth				
Oceania and Antarctica (n=29)	4612±6262	58±71	2280±16827	7492±25384
North-West (n=9) & Southern and Eastern Europe (n=1)	4276±6285	73±45	4340±12950	14577±27402
North Africa and the Middle East (n=2), Americans (n=2), Sub-Saharan Africa (n=2)	2292±9863	74±38	1705±19509	8826±36564
Asia (n=14)[3]	4008±5365	39±60	1775±6225	8278±10988
p-value‡	0.46	0.37	0.72	0.71
Physical activity level (PAL)				
Sedentary (n=5)	5510±10868	8±103	13670±31778	17647±31510
Light (n=18）	2898±3869	39±83	1221±1874	5971±4262
Moderate (n=21)	4160±5929	65±37	4236±11878	12822±23798
High (n=15)	5502±15512	79±55	3596±23915	19278±37868
p-value‡	0.36	0.09	0.08	**0.01**
Smoking status				
Never smoked (n=38)	4501±5019	65±44	2809±8851	9009±12930
Past smoker (n=14)	4951±5314	53±75	1630±27454	6249±42342
Current smoker (n=7)	1766±11815	82±69	14572±28859	27240±32199
p-value‡	0.36	0.47	0.85	0.99

Table 14 continued Subject characteristics	Total lignans Median±IQR	Total enterolignans Median±IQR	Total isoflavones Median±IQR	Total phytoestrogens Median+IQR
Bowel frequency				
1-7/week (n=24)	2875±4280	42±52	1934±11002	7874±12781
>7/week (n=35)	4554±9262	78±62	3171±10259	7775±23802
p-value†	0.06	0.07	0.64	0.17
Bowel movement				
Mushy and watery (n=2) & Smooth and soft to pass (n=53)	4160±4053	58±48	2010±9283	7492±14648
Hard to pass (n=4)	13679±24396	111±52	15940±25020	33881±7626
p-value†	0.10	0.07	0.09	**0.03**
Commercial supplements use				
No (n=32)	4493±6687	58±60	1976±12172	9009±23244
Yes (n=27)	4160±3877	76±87	3171±10313	7492±26296
p-value†	0.91	**0.03**	0.69	0.98

†Mann-Whitney U test

‡ Kruskal-Wallis One-Way ANOVA

[1] South East Asian (n=6), South Asian (n=3), North East Asian (n=5), Middle Eastern, North Africa, Somalia Peninsular (n=2), Central/South America (n=2), Other (n=1) & Mixed ancestry (n=2)

[2] Cantonese, Arabic, Mandarin, Spanish, German, Hindi, Malay, Persian, Urdu, Afrikaans, Macedonian, Bahasa Indonesian) [3](South-East (n=6), North-East (n=5), Southern and Central (n=3)

Table 15. Average excretion of lignans, enterolignans and isoflavones from urinary biomarkers according to subjects' characteristics (n=53)

Subject characteristic	Total lignans Median±IQR	Total enterolignans Median±IQR	Total isoflavones Median±IQR	Total phytoestrogens Median+IQR
Gender				
Male (n=18)	1253±684	1178±1903	274±582	3554±5091
Female (n=35)	932±794	1952±3991	259±1569	4190±6828
p-value†	0.14	**0.03**	0.56	0.24
BMI(kg/M^2)				
<20.00 (n=5)	895±581	1930±1752	252±561	2531±2221
20.00-25.00 (n=25)	1228±1332	2734±3668	573±2007	6363±5555
25.01-30.01 (n=14)	976±734	642±1822	152±517	2567±4497
>30.01 (n=9)	1318±586	1837±1872	208±391	3449±4099
p-value‡	0.42	0.054	0.12	0.07
Age (y)				
18-28 (n=23)	1052±887	1942±3083	392±2001	4606±4523
29-39 (n=15)	1228±621	1887±3489	259±1139	3540±5333
40-50 (n=8)	1313±1668	1052±2722	454±4104	5320±8237
>50 (n=7)	1019±674	1934±3706	81±161	3449±6325
p-value‡	0.93	0.49	0.11	0.75
Occupation				
Family duties (n=3) and Students (n=29)	1028±767	1579±2556	226±313	3178±4698
Clerical and administrative workers (n=11)	1341±954	3279±3425	1203±1138	4606±4282
Community and personal service & (n=2), Technician and trade workers (n=2)	1461±4130	2219±10674	3722±4730	8427±15661
Professionals (n=5) & Managers (n=1)	676±900	1002±2880	309±2968	3063±6264
p-value‡	0.09	0.47	0.06	0.12
Education level achieved				
Below university (Other (n=1), Secondary school (n=1), Certificate/diploma (n=10)	1036±902	3322±5824	416±2315	5710±8151
University (undergraduate (n=19, postgraduate (n=22)	1170±738	1445±2361	259±1292	3782±4520
p-value†	0.72	**0.03**	0.64	0.17

Table 15 continued Subject characteristics	Total lignans Median±IQR	Total enterolignans Median±IQR	Total isoflavones Median±IQR	Total phytoestrogens Median+IQR
Ancestry				
Caucasian (n=33)	1118±698	1934±3116	273±1503	3897±5425
Other ethnic groups (n=20)[1]	1122±973	1411±2419	256±954	4123±5449
p-value†	0.66	0.19	0.78	0.49
Language spoken at home				
English (n=34)	1146±830	1985±3341	321±1491	4237±5539
Other languages (n=19)[2]	1036±696	1378±1191	248±1107	3782±4546
p-value†	0.53	0.34	0.74	0.45
Region of country of birth				
Oceania and Antarctica (n=26)	1146±762	2966±2812	390±1506	4445±4520
North-West (n=7) & Southern and Eastern Europe (n=1)	1258±913	769±1952	266±3841	3220±8067
North Africa and the Middle East (n=2), Americans (n=1), Sub-Saharan Africa (n=2)	1118±1186	927±2330	268±1136	2181±4289
Asia (n=14)[3]	1045±752	1201±1528	213±1751	3227±6394
p-value‡	0.96	**0.04**	0.98	0.58
Physical activity level (PAL)				
Sedentary (n=4)	1417±4968	3274±5356	5691±6628	11406±8578
Light (n=17）	932±587	1887±2952	199±354	3449±3006
Moderate (n=19)	1118±665	1332±2305	273±1574	3540±6019
High (n=13)	1464±1711	1952±3355	413±1785	6314±5285
p-value‡	0.42	0.71	0.09	0.07
Smoking status				
Never smoked (n=35)	1118±857	1942±3047	274±1462	4285±5088
Past smoker (n=12)	1220±889	1828±3474	146±470	3317±7574
Current smoker (n=6)	850±1901	1631±13333	918±1593	3490±16318
p-value‡	0.71	0.85	0.15	0.96

Table 15 continued Subject characteristics	Total lignans Median±IQR	Total enterolignans Median±IQR	Total isoflavones Median±IQR	Total phytoestrogens Median+IQR
Bowel frequency				
1-7/week (n=21)	903±914	1445±3576	413±1338	4284±4554
>7/week (n=32)	1199±730	1862±2914	260±1298	3910±5555
p-value†	0.29	0.86	0.65	0.72
Bowel movement				
Mushy and watery (n=2) & Smooth and soft to pass (n=47)	1123±753	1837±2820	261±1292	3924±4783
Hard to pass (n=4)	913±4472	4289±12713	450±1722	5453±18415
p-value†	0.81	0.71	0.86	0.83
Commercial supplements use				
No (n=30)	1087±924	1824±3040	265±2035	4445±5529
Yes (n=23)	1118±794	1837±3340	274±749	3540±4637
p-value†	0.57	0.86	0.47	0.46

†Mann-Whitney U test

‡Kruskal-Wallis One-Way ANOVA

[1] South East Asian (n=6), South Asian (n=3), North East Asian (n=5), Middle Eastern, North Africa, Somalia Peninsular (n=2), Central/South America (n=1), Other (n=1) & Mixed ancestry (n=2)

[2] Cantonese, Arabic, Mandarin, Spanish, German, Hindi, Malay, Persian, Urdu, Afrikaans, Macedonian, Bahasa Indonesian) [3](South-East (n=6), North-East (n=5), Southern and Central (n=3)

4.9 Food sources contributing to lignans, enterolignans and isoflavones

The contributing food sources to dietary total lignans, enterolignans and isoflavones, are shown in **Figure 27 to 29**. They were based on usual dietary patterns over the previous month as recorded in the pre FFQ.

The top three contributing food sources of lignans were from the nuts and seeds group (30%), such as linseed and cashews; nonalcoholic beverages such as coffee, tea and plant-based milk-like beverages (19%); and breads and cereals including multigrain bread (19%). The least important contributing food group was meat, poultry, seafood and eggs (**Figure 27)**.

The majority of dietary enterolignans (86%) were from dairy products, namely milk, yoghurt and cheese from animal origin, followed by nonalcoholic beverages (11%). Dairy products, e.g. milk, were used in coffee and tea, and specialty coffees, such as like latte and cappuccino, were commonly consumed in this research population. Plant-based food groups, including nuts and seeds, breads and cereals, vegetables, fruits, legumes, and alcoholic beverages do not contain enterolignans and therefore contributed none to dietary intake (**Figure 28**).

Legumes, including soy and related food products and beans and related food products were the major contributors (78%) to total isoflavones; breads and cereal products was the second highest contributor (17%) (**Figure 29**).

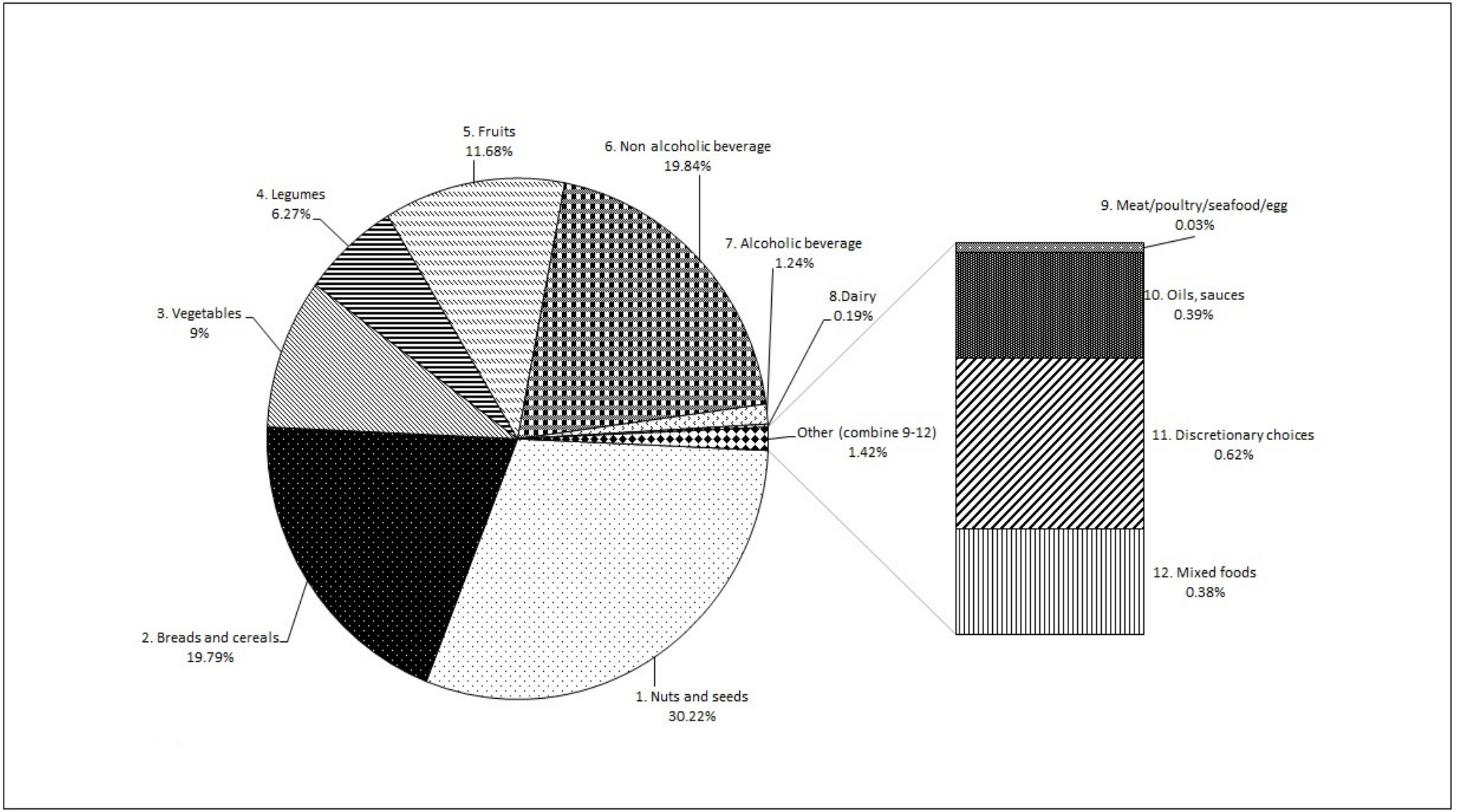


Figure 27. Contributing food sources to dietary lignans estimated in usual diet over 1 month from food frequency questionnaire (n=59)

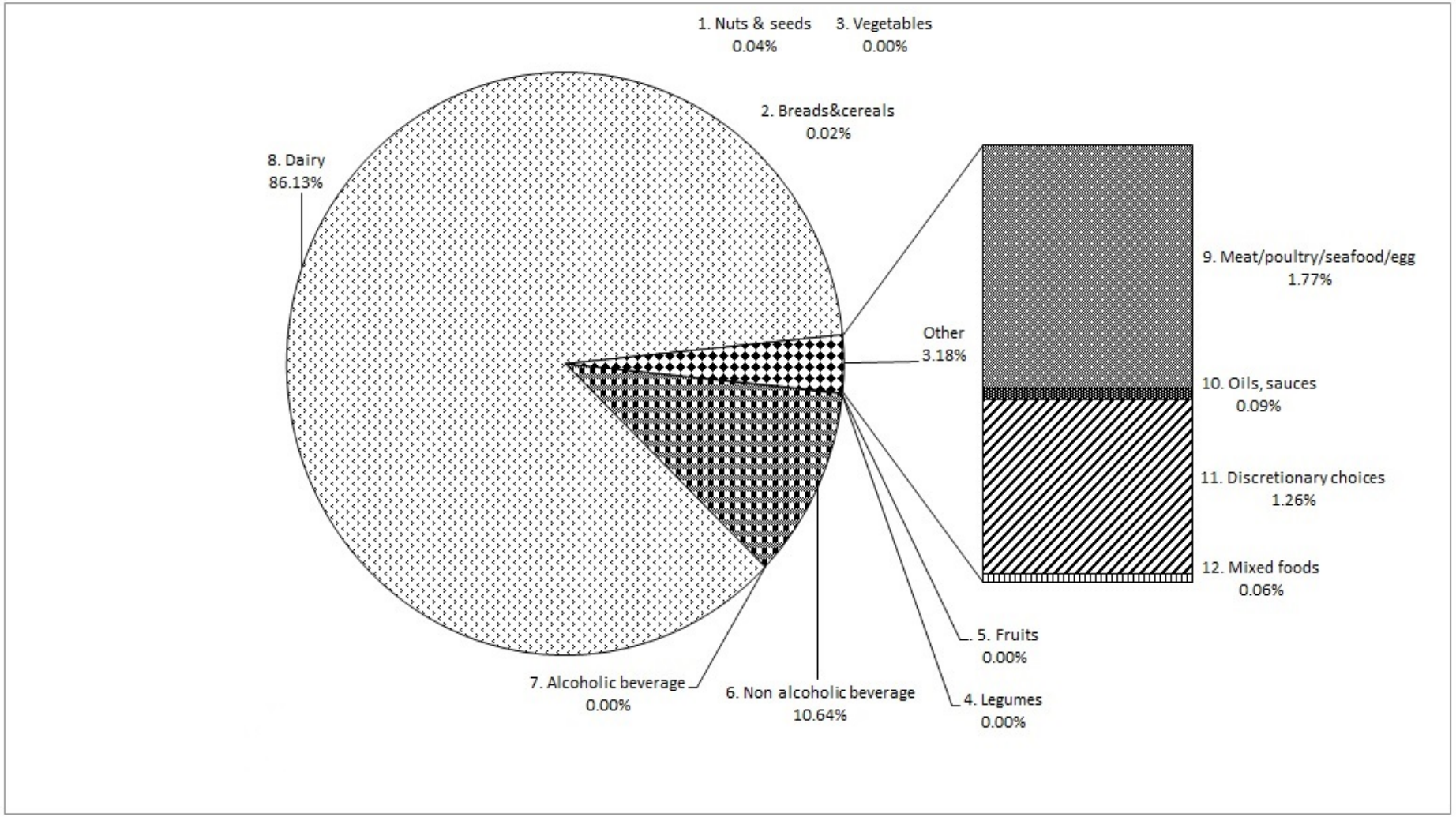


Figure 28. Contributing food sources to dietary enterolignans estimated in usual diet over 1 month from food frequency questionnaire (n=59)

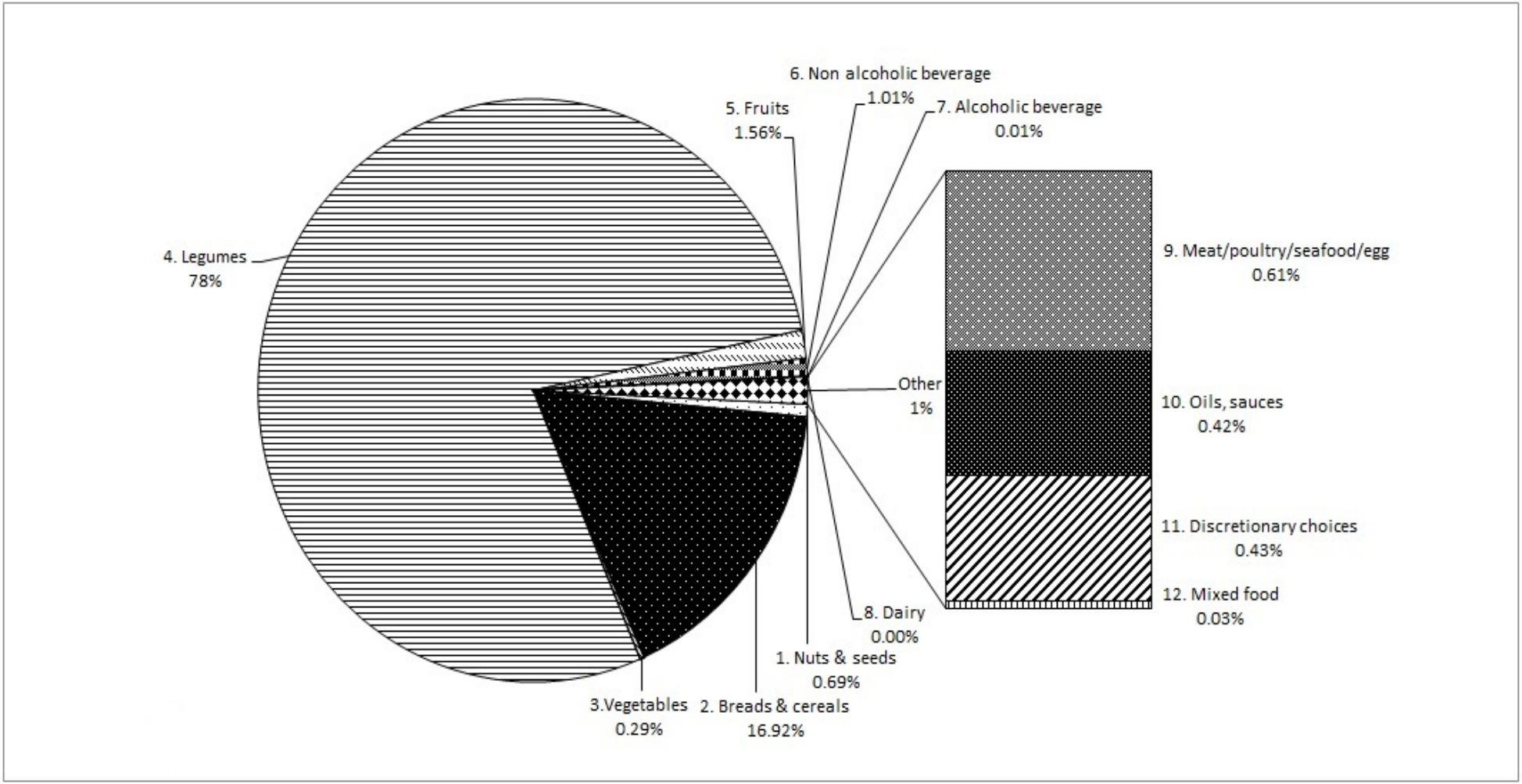


Figure 29. Contributing food sources to dietary isoflavones estimated in usual diet over 1 month from food frequency questionnaire (n=59)

4.10 Triangular comparison of FFQ, WFR and biomarker

The triangular comparison between three measurement methods, namely the FFQ, WFR and biomarkers for each phytoestrogen was conducted with the Method of Triads (**Figure 9**). The validity coefficients (VC) between FFQ (Q) and true intake (T), between WFR (R) and (T), and between the biomarker (M) and (T) are reported in **Table 16**. Spearman correlations were selected due to the non-parametric nature of the data.

All VCs were estimated based on intake and excretion of each measurement with adjustment for demographic and lifestyle variables. These variables were selected based on results in **Section 4.8**. Variables were included in the MOT model if the median measurements were found to be significantly different across levels or categories of that variable for at least two methods, for example, the intake classified by gender was found to be significantly different in both FFQ and WFR. Comparison between enterolignan intake and excretion was not conducted due to inter-conversion from dietary intake of lignans to excretion in the form of enterolignans.

The correlations between FFQ and WFR (rQR) were moderate but between biomarker and WFR (rRM) or FFQ (rQM) were low. Heywood cases, that is the value exceeding 1, appeared on most VCs for (R) and (T) on each comparison except for that of total lignans and enterolignans (VC=0.78) and total phytoestrogen (VC=0.33) (**Table 16**). VCs for (Q) and (T) ranged between 0.10 and 0.79, and for (M) and (T) ranged between 0.13 and 0.56, except for the two Heywood cases for MAT and total phytoestrogen. Total lignans and enterolignans had the highest VC in the WFR (0.78) and FFQ (0.79), and daidzein in the biomarkers (0.56). The VCs for formononetin and total phytoestrogen were based on Spearman's correlations unadjusted for demographic and lifestyle variables, since the VCs when adjusted could not be calculated due to an odd number of negative correlations, i.e. one or all three negative correlation coefficients.

Table 16. Validity coefficients for triangular comparison between FFQ, WFR and biomarker adjusted for demographic and lifestyle characteristics

	Correlation coefficients†			Validity Coefficients (VC)‡ (95% CI)		
	rQR±	rRM±	rQM±	WFR (R) &True intake (T)±	FFQ (Q) & (T)±	Biomarker (M) & (T)±
SECO	0.44	0.21	0.05	2.07 (0.44, 12.80)	0.38 (0.13, 2.41)	0.13 (0.02, 0.55)
MAT	0.44	0.10	0.16	13.68 (0.41, 16.53)	0.10 (0.14, 4.54)	1.65 (0.02, 1.29)
Total Lignan	0.41	0.24	0.16	1.26 (0.51, 10.02)	0.57 (0.09, 2.03)	0.28 (0.05, 1.33)
Daidzein	0.52	0.45	0.24	2.50 (1.28, 19.09)	0.43 (0.08, 1.71)	0.56 (0.10, 2.37)
Genistein	0.51	0.22	0.07	3.34 (0.58, 21.39)	0.33 (0.07, 2.36)	0.23 (0.03, 1.61)
Glycitein	0.51	0.26	0.27	1.37 (0.63, 9.02)	0.58 (0.14, 2.58)	0.48 (0.07, 1.67)
Formononetin^	0.18	-0.11	-0.13	1.45 (0.16, 15.88)	0.33 (0.06, 4.03)	0.44 (0.02, 2.47)
Total Isoflavone	0.42	0.26	0.15	2.16 (0.65, 14.57)	0.42 (0.09, 2.18)	0.37 (0.05, 1.84)
Total Lignan and Enterolignan	0.41	0.10	0.13	0.78 (0.21, 4.07)	0.79 (0.15, 2.71)	0.16 (0.02, 0.58)
Total Phytoestrogen*	0.08	0.25	0.07	0.33 (0.03, 4.09)	0.26 (0.03, 2.97)	1.68 (0.23, 12.86)

†Spearman correlation coefficients

‡VCs were based on Spearman correlation adjusted for demographic and lifestyle variables. Lignans, SECO, MAT, total lignan, and total lignan and enterolignan were adjusted for age, BMI and language spoken at home (English or non-English), isoflavones, daidzein, genistein, glycitein, formononetin, total isoflavone were adjusted for age and BMI. Total phytoestrogens were adjusted for physical activity level (sedentary, light, moderate, high) and bowel movement (mushy/watery and smooth to pass or hard to pass)

± Q=food frequency questionnaire (FFQ), R=weighed food record (WFR), M=biomarker, T=true intake

^ VCs were based on Spearman correlation unadjusted for demographic and lifestyle variables due to validity coefficients not calculated or adjusted for Spearman correlation when there is a negative correlation between the two measures

CHAPTER FIVE: DISCUSSION

5.1 Research findings

The current research focused on evaluation of the validity and reliability of a modified FFQ specially designed to measure intake of phytoestrogens in a group of 59 men and women living in Perth, Western Australia. Three classes of phytoestrogens were measured: lignans, enterolignans and isoflavonoids.

An original 112-item FFQ was customised by expanding the food list to include 277 items, to align with food groups and items provided in the most recent Australian Dietary Guidelines (NHMRC, 2013a); selected ethnic items commonly-available in the Australian market, were also included. Estimation of the lignan content of foods from the FFQ was also modified. There is currently no available comprehensive database for estimating dietary lignans and in the original 112-item FFQ, lignan content was based on an indirect in-vitro colonic fermentation method providing estimated availability from foods of two plant lignans (Thompson, Robb, Serraino, & Cheung, 1991). For the current FFQ, estimates were based on more recent data for direct measurement of four lignans in plant-based foods, previously compiled by our research group from a range of international published values. The FFQ was also expanded to include food sources of enterolignans, which are obtained exclusively from animal products such as dairy foods; these are derived from consumption of plant lignans by the animal and interconversion to enterolignans that then are absorbed and secreted into milk. The purpose of modifying the FFQ was an attempt to improve performance as a potential tool for measuring phytoestrogen intake, especially lignans, which had been a limitation with the original questionnaire. The modified FFQ was designed to capture phytoestrogen intake over the previous month.

The validation involved the use of a reference dietary assessment tool, namely WFR, and

urinary biomarkers of phytoestrogen excretion. The measurement derived from the FFQ was compared with the WFR and biomarkers. The reliability of the FFQ was tested by comparing the intake of pre FFQ with that of the post FFQ, administered one week apart.

5.1.1 Evaluation of the FFQ

Convergent validity and reliability

Convergent validity was evaluated by comparing usual daily intake of phytoestrogens from the FFQ with usual intake from a 3-day WFR reference method collected over consecutive days to include two weekdays and one weekend day. In support of our first and third hypotheses, the FFQ had acceptable convergent validity for intake of total lignans and enterolignans when compared to a WFR, in terms of median intakes (lignans: 3.91 versus 4.30 mg/day, $p=0.09$; and enterolignans: 0.054 versus 0.065 mg/day, $p=0.81$, respectively); and associations between the two methods (lignans $\rho=0.42$, $p<0.001$; and enterolignans $\rho=0.62$, $p<0.001$) (**Table 5**). Median intakes of total isoflavonoids from the FFQ and WFR were also comparable (3.85 versus 2.29 mg/day, $p=0.67$) with a significant association between the two tools ($\rho=0.36$, $p<0.001$), although weaker than for total lignans (**Table 6**). In addition to validation as a continuous measure, the FFQ was examined by cross-classifications to determine whether it could accurately rank subjects into quartiles of intake assessed by the WFR. With a total of 7% to 17% of subjects misclassified into opposite quartiles for different phytoestrogen classes, the results further indicate that the FFQ does accurately classify subjects assessed by the WFR (**Table 8**).

In support of our second hypothesis, the FFQ also had acceptable reliability when compared to a repeat FFQ, for all classes of phytoestrogens, with strong correlations between intakes ($\rho=0.78$ to $\rho=0.85$, $p<0.001$ for all) (**Tables 5, 6**) and minor misclassifications into opposite quartiles, between 2% to 5% (**Table 8**).

Contrary to our fourth hypothesis, when the FFQ was assessed for agreement between individual values in the WFR, the mean bias was moderately high for all phytoestrogen classes (-0.54 mg/day for total lignans, +0.004 mg/day for enterolignans and +1.17

mg/day for isoflavonoids); and visual inspection of the Bland Altman graphs showed trends towards greater inconsistency at higher median intakes (**Figures 11e, 12c, 13f**).

Criterion validity

Criterion validity was evaluated by comparing usual intake of four lignans and five isoflavonoids from the FFQ with usual excretion of their biomarkers in three 24-hour urine samples. Extensive interconversions of dietary lignans occur in the colon, with the majority of dietary lignans converted to enterolignans prior to absorption and excretion (**Figure 3**). For example in the current study, intake of enterolignans was low at 0.054 mg/day while urinary excretion was 30-fold higher at 1.84 mg/day. For this reason the assessment of criterion validity was conducted using one combined value for lignans plus enterolignans. Although median intake and excretion were not significantly different (4.05 versus 3.10 mg/day, p=0.22, respectively), there was no significant correlation between intake and excretion (ρ=0.16, NS) (**Table 5**). For total isoflavonoids, excretion was 10-fold lower than intake from the FFQ (0.27 versus 3.85 mg/day, respectively p<0.001) and there was no significant correlation between the two tools (ρ=0.10, NS). Misclassification into the opposite quartile was also relatively high for both phytoestrogen classes (21% to 23%) (**Table 8**). When usual intake from the 3-day WFR was compared with 3-day urinary excretion collected over the same time period, the associations became stronger and significant for isoflavonoids (ρ=0.28, p<0.05) but not for lignans and enterolignans (ρ=0.14, NS). Despite previous studies reporting significant dose-dependent relationships between intake and excretion of phytoestrogens and their respective metabolites (French et al., 2007; Hanna et al., 2005; Huang et al., 2000; Yamamoto et al., 2001), there were no significant associations in the current data, indicating poor validity for the modified FFQ in relation to biomarkers of phytoestrogen excretion.

Based on these findings, we conclude that the modified phytoestrogen FFQ is highly

reliable. It would be a useful assessment tool for example to rank usual intake of phytoestrogen classes for individuals within a group, or quantify mean intakes between different population groups. It is not acceptably valid or accurate for estimation of individual phytoestrogen status, for example for use in experimental studies or to investigate associations with chronic diseases.

Reliability and validity data for individual phytoestrogens showed similar trends to data for phytoestrogen classes with highly significant positive associations between intakes from the repeat FFQs, moderately significant associations between intakes from the FFQ and WFR, but weak and non-significant associations between intake from the FFQ and excretion of respective urinary biomarkers.

Comparison of results with previous studies

Previous reliability studies using repeat FFQs administered from 1 month to 10 months apart, have found moderate to strong associations between intake of isoflavones (r=0.60 to r=0.77) and lignans (r=0.36 to r=0.80) (French et al., 2007; Hanna et al., 2005; Horn-Ross et al., 2006). Our associations of between ρ= 0.69 to 0.85 are comparable or higher, possibly due to the shorter time frame of 1 week between repeat questionnaires, which would have avoided possible seasonal changes that could contribute to variability in previous studies with longer time intervals.

Contrary to the current findings of weak non-significant associations between intake from the FFQ and excretion of urinary isoflavonoids (ρ=0.10) or lignans (ρ=0.16), previous studies have reported significant moderate associations for isoflavonoids (r=0.21 to 0.54) but smaller or non-significant associations for lignans (r=0.14 to 0.40) (French et al., 2007; Hanna et al., 2005; Horn-Ross et al., 2006). Reasons for the discrepancy in our study could be the lack of excretion data for PINO and LARI, due to failure of ionisation of metabolites during the LC-MS analysis procedure (Grace et al., 2007; Valentin-Blasini

et al., 2000), which would have resulted in underestimation of total lignan excretion, and potential recall bias when subjects were recalling frequency and portion size in completing the FFQ. Previous studies did not attempt to measure intake or excretion of these two lignans yet they were significant dietary contributors in our study, providing about 25% of total lignan intake. Other factors that could affect bioavailability and hence excretion of the phytoestrogens, include variations in individual gastrointestinal transit time, efficiency of conversion of metabolites in the colon, and the degree of enterohepatic recycling (Clavel et al., 2005; Lampe, 2003; Lampe et al., 2006; Landete, 2012). Collection of faecal samples and measurement of faecal losses in future studies could help identify these sources of variability.

Cross classification analyses

In the current study, cross classification analyses using quartiles of intake, indicated that 58-73% between the two repeat FFQs, 24-51% of intakes between the FFQ and WFR, and 25-43% between the FFQ and urinary biomarkers, were classified into the same quartiles.

Ideally a large proportion of subjects classified in the same quartile affirms the tool's accuracy to rank individuals according to their phytoestrogen intake, but the proportion classified in the adjacent quartiles also suggests that the tool is effective in ranking intakes. Bhakta et al. (2005) conducted a validation study with 58 English women and compared dietary intake obtained by a FFQ with intake from twelve 24h dietary recalls collected over a year as the reference standard. Their results showed that 53% of subjects were classified into adjacent quartiles for total lignans and isoflavones, which is comparable to the 51% and 46% for total lignans and isoflavones, respectively, reported in our validation study. This proportion of cases should be included in considering the effectiveness of the tool in ranking intake. Eighty percent of cases or more in the same and adjacent quartiles is considered an excellent tool, and between 60-70% is a good tool (Fernandez et al., 2013). Using these criteria, the current FFQ is regarded as an excellent

tool in ranking intake against the reference method; and a good tool for ranking intake against biomarkers, with above 70% classified in the same or adjacent quartiles.

Individual agreement from Bland Altman plots

The Bland Altman plot is a method to assess the degree of agreement between two tools. The plots provide a graphical means to observe for systematic differences, including mean difference, limits of agreement and proportional bias between the two methods. The mean difference between the measurements of the FFQ and WFR represents the systematic error or bias of the tools, the limits of agreement show how far apart the measurements are by the two tools, and the proportional bias at low and high intake levels informs the trends in variation of phytoestrogen intake within the sample.

In the current study, the limits of agreement were relatively wide in all classes of phytoestrogens for evaluation of both reliability and validity of the FFQ, and proportional bias existed with greater inconsistency at higher than lower mean phytoestrogen intakes. Bland Altman analyses conducted by previous studies that examined intake of isoflavones have shown similar results, with the bias increasing with increasing intake between a FFQ and 24h dietary recalls (Chan et al., 2008) and between repeat measures of a FFQ administered 1 week apart (Frankenfeld et al., 2003).

In order to evaluate the clinical relevance of this variation, it is important to identify what this difference and variation represent with regard to dietary intake. The mean bias between the FFQ and WFR was 0.54 mg/day for total lignans, 0.004 mg/day for total enterolignans and 1.17 mg/day for total isoflavones, and is equivalent to consuming 0.16g of flaxseed, 17g or 1 tablespoon of milk and 4g or a teaspoon of tofu per day, respectively. The median intakes of 4.23 mg/day for total lignans, 0.062 mg/day for total enterolignans and 4.36 mg/day for total isoflavones that differentiates low intakes from high intakes, are equivalent to having 1.2 g or ¼ of teaspoon of flaxseed, 1 cup of milk, and 15 g or 1 tablespoon of tofu per day, respectively. Phytoestrogens, especially lignans, are present

in a wide range of foods but are concentrated in only limited food groups, namely nuts and seeds (especially flaxseeds) for lignans, dairy products of animal origin for enterolignans, and soy and related products for isoflavones. These food groups aligned to the top contributing food sources of the sample in the current study (**Figure 27-29)**. The consumption or non-consumption of foods from these food groups, both between subjects or day-to day within subjects, would result in sporadic intakes of the phytoestrogen classes and contribute to the observed high variation in the current study.

Triangular comparison with Method of Triads

The current research is only the second study to conduct a triangular comparison of three methods, the FFQ, WFR reference method, and biomarkers using the Method of Triads (MOT) to evaluate the performance of each method for determining phytoestrogen status (Bhakta et al., 2005). The validity coefficient (VC) indicates the correlation of the intake by one method with the true but unknown intake, and therefore is an indication of the degree of agreement between the methods in assessing phytoestrogen status in the research population.

Since the introduction of MOT by Kaaks (1997), validation studies have widely adopted this method when assessing three or more methods for nutrient status. To date, only Bhakta et al. (2005) has utilised the MOT for phytoestrogen status in a study comparing an FFQ, 24h recalls and plasma biomarkers in South Asian women in the UK. They reported the highest VC for 24h recalls in measuring genistein (0.95) and daidzein (0.83), the highest VC for the FFQ in measuring total lignans (0.91), and the poorest VC (0.11-0.45) for biomarkers in measuring genistein, daidzein and total lignans. This supports the current research in which higher VCs were observed for the FFQ in measuring total lignans (VC=0.57) or the FFQ and WFR for measuring combined lignans plus enterolignans (VC=0.79, 0.78, respectively) (**Table 16**). In addition, the VCs for the current research were adjusted for demographic and lifestyle variables and the VCs

improved slightly (data not shown).

The results for individual phytoestrogens were complicated by Heywood cases, defined as VCs or CIs exceeding 1. For example, VCs and CIs exceeded 1 for all variables measured by the WFR except combined total lignans and enterolignans (**Table 16**). VCs are mathematically based on the correlation coefficients (**Figure 9** in Methods). To have a high VC, that is within the acceptable range of 0 to 1, values of all correlation coefficients between the methods are required to be at a relatively equal level. In **Table 16**, coefficients were moderate for FFQ and WFR, but low for the urinary biomarkers. This suggests that the biomarker is not a good indicator of the true intake. The appearance of Heywood cases could be the result of random sampling variation or may result from the violation of the assumption of MOT, that the errors of the three methods are uncorrelated and that a linear relationship of measurements exists between the methods. In this regard, the WFR was chosen because it shared uncorrelated sources of error with FFQ (**Table 1)**. For the WFR, consumption was recorded for two weekdays and one weekend day using precise kitchen scales and measuring cups and spoons to assist in portion size estimation; the FFQ required a recall of past consumption on a regular frequency basis over the previous month. As the FFQ and WFR are two distinct tools requiring responses in different ways, the errors from each tool are likely to be unrelated.

Yokota et al. (2010) suggested that a larger sample size could reduce the chances of Heywood cases for the VCs. They also highlighted recent guidelines for consideration of sample sizes with at least 50 subjects for studies with a biomarker as a reference methods. There were 53 subjects with complete data for the FFQ, WFR and biomarkers. Therefore a further increase in the sample size could potentially improve the VCs.

5.1.2 Phytoestrogen intake and excretion

In comparison to previous studies conducted in Western countries, median intake of lignans in the current study (3.91mg/day) was three times higher than that by Lahmann et

al. (2012) in Australia, but similar to that found by Hedelin, Klint, Chang, Bellocco, Johansson, Andersson, Heinonen, Adlercreutz, Adami, Gronberg, et al. (2006) in Sweden (3.05mg/day), and considerably lower than that consumed in Eastern countries by Bangladeshi women (7.32 mg/day) (Saleh et al., 2011). Enterolignan intakes in the current study (0.056 mg/day) were also three fold higher than intakes reported by Lahmann et al. (2012) (0.022 mg/day). Isoflavones in the current study (3.85 mg/day) were considerably lower than amounts consumed in a Chinese population (11.5 mg/day) (Wong, Lau, Leung, Leung, & Woo, 2007).

Median urinary excretion in the current study of lignans (-0.12mg/day), enterolignans (1.84mg/day), and isoflavones (0.27mg/day) was consistently higher than the median excretion in an Australian group (lignans 0.01mg/day, enterolignans 0.22mg/day, isoflavones 0.07mg/day) (Hanna et al., 2005) and in Canada (enterolignans 837µg/day, isoflavones 759µg/day) (French et al., 2007).

Differences may be explained by the design of the FFQ, for example, the number of items included in the food list was longer in the current study than previous study by Hanna et al. (2005); use of different sources of published phytoestrogen databases for measuring intake; as well as different dietary preferences, for example high isoflavone intake related to high consumption of soy products as staple items in the Chinese population (**Table 3**).

5.1.3 Contributing food sources to phytoestrogen intake

The major contributing food sources to lignans in the current research were similar to other previous studies conducted in Western countries, with flaxseed, breads and cereals, beverages such as tea and coffee, vegetables, and fruits being the most common sources in the current research (**Figure 27)**. Other ethnic groups such as Chinese populations, consumed more legumes and beans, vegetables, and tea, than bread and cereals (Hu et al., 2014).

Enterolignan intake is more likely to be measured in Western countries because of frequent consumption of dairy products and other foods of animal origin. Foods of dairy origin, as expected, contributed to 80-90% of enterolignan intake in both the current research (**Figure 28)** and previous research (Zamora-Ros, Knaze, et al., 2012). Specialty milk beverages such as latte and cappuccino were included in the current FFQ, and subjects consumed these non-alcoholic beverages on a frequent basis.

Food sources contributing to isoflavone intake were soybeans, legumes and soy products including soymilk and tofu. Bread and cereals was the second highest contributor to isoflavones in the current research since manufacturers in Australia use soy flour as an ingredient in bread making (Lahmann et al., 2012). Popular items advertised for health, such as soy and linseed bread, are available in the consumer market and were consumed by subjects in the current research (**Figure 29**). In other ethnic groups, isoflavone intake was derived from less refined sources, legumes and beans, vegetables such as soybean sprouts and cabbage, and fruits (Bhakta et al., 2006; Huang et al., 2012; Saleh et al., 2011).

5.1.4 Intake of phytoestrogen and demographic and lifestyle characteristics

In the current study, an attempt was made to recruit subjects from diverse backgrounds to capture cultural diversity in an Australian group. While 64% of subjects were of Caucasian ancestry and spoke English at home, 36% spoke another language at home, including subjects from South East, South and North Asia, North Africa and South America; 68% of subjects were females and 32% were males. The majority was recruited from around the university and were university educated (78%); and 46% used commercial dietary supplements.

Female subjects who were Caucasian, were at, or had achieved university education level and took regular commercial dietary supplements, were more likely to have a higher lignan and enterolignan intake than subjects with different characteristics (**Table 13**). This

was also reflected in the urinary excretion of enterolignan metabolites in those subjects with the same characteristics (**Table 15**). The food choices were plausibly the reason for the higher intakes of lignans and enterolignans in these groups. For example, subjects who were of other ethnic background, spoke languages other than English at home and were born outside of Australia had varying intake level and variety of food choices from vegetables, fruit and cereal products, such as white rice and noodles, which contain low to moderate lignan content (**Section 2.9**). Use of commercial supplements could be an attribute of those subjects who were health conscious, and hence more inclined to select foods that were nutrient dense such as wholegrain cereals and specialty seed breads, promoted for health benefits and also rich in lignans. Subjects did not consume isoflavonoid and lignan dietary supplements. Our findings support those in a previous study conducted by Hanna et al. (2010), who investigated the intake of phytoesetrogens and associated lifestyle factors in Australian older women (aged 40-80 years), and reported higher education level and supplement use in the group of soy and linseed consumers with at least one serve of soy and/or linseed item.

5.2 Limitations

One limitation of the current research is that urinary biomarker excretion was not corrected for creatinine concentration, to indicate possible incompleteness of the 24-h urine collections. Although creatinine concentrations were analysed, the correction of phytoestrogen concentration was not conducted due to time constraints of thesis submission. In the current research, subjects were encouraged to state the completeness of the collection in a designated area on each urine bottle for any collection they had missed. Twenty-three out of the 59 subjects stated that they had incomplete urine samples at some time during their 3-day 24-hour collections. This could have added to the variability in the urinary biomarker data and indicates the need for a follow up analysis of data, after assessing samples for completeness and excluding any samples with implausibly low

creatinine concentrations.

Another limitation is related to the intrinsic properties of phytoestrogens. Phytoestrogen content naturally varies in foods as a result of genetic factors and the growing environment, and effects of harvesting and processing (Kuhnle, Dell'Aquila, Runswick, et al., 2009). Therefore the estimated intake may not be reflected in what was actually present in the food consumed. Possible hidden sources of soy isoflavones in the food supply, especially in processed foods, and foods with no database content of lignan, would result in underestimation of the intake of lignans and isoflavones. This would impact on the assessment of the FFQ validity with respect to the urinary biomarkers. For the current research, phytoestrogen contents reported across multiple databases for the same food item were averaged to minimise the effect of the natural variability of phytoestrogens. Food items with missing lignan content were substituted with the content of an appropriate food item in the phytoestrogen intake calculation. Other limitations that could impact on the assessment of FFQ validity, include subjects' day-to-day variation in intake, particularly when subjects had sporadic intake of phytoestrogen rich food on one day but not on the other day (See Bland Altman plots 4.4 and 4.7). Potential recall or social desirability bias when subjects were completing the FFQ, inaccuracies in estimation of frequency of intake or portion sizes and inter-individual variation in phytoestrogen absorption, metabolism and excretion could also have contributed to the variation in intake and excretion, and hence the evaluation of the FFQ.

While the current research included males and females from a range of cultural backgrounds, it is not widely representative as subjects were recruited from a university community rather than the general population. The anticipated number of subjects in the current study was not achieved, due to time constraints of subject recruitment, which could have reduced the likelihood of observing significant associations within the dataset.

5.3 Recommendations and conclusions

The evaluation of the phytoestrogen food frequency questionnaire assisted in establishing a valid and reliable dietary tool in measuring phytoestrogen status. The database used for the current study to estimate intake was updated with content for lignans and enterolignans from international published data. Even with actual estimates of phytoestrogen content in food from the database and a FFQ well designed for capturing the dietary intake, the bioavailability would be affected by natural variability of phytoestrogens in foods. Therefore analyses are required for the phytoestrogen content of foods grown and consumed in Australia, collated to develop a database culturally specific to the Australian context.

The purpose of the FFQ in the current research was to quantify intake at the individual and population level. While the FFQ was acceptably valid for measuring intake at the group or population level, Bland Altman plots indicated wide individual variability and greater variation of individual measurements between two methods at higher median intakes. One reason for the variability is the sporadic nature of phytoestrogens, which are concentrated in a few very rich food sources. Consuming as little as 1.2g of flaxseed, 1 cup of milk or 15g of tofu a day would significantly increase the variation in the intake. Using this as a reference, future researchers could screen consumers and non-consumers of phytoestrogen rich foods with preliminary questions about regular consumption of these typical foods before completing the dietary assessment tool.

The 3-day WFR was chosen as a reference method to evaluate convergent validity of the FFQ. However, the WFR only represented a snapshot of dietary consumption over 3 days and the observed sporadic nature of phytoestrogen intake over this relatively short time period, both between and within subjects, impacted considerably on the estimate of average daily intake. The FFQ was designed to survey usual diet over the past month and

hence average daily intake would be less impacted by these sporadic intakes. Therefore based on the study design and the interpretation of the results for the current research, it is more appropriate to use the FFQ for status of usual phytoestrogen intake.

Food metabolomics is a branch of the nutrition research that focuses on the digestion and biotransformation of foods and constituents and the identification of associated metabolites. These metabolites include biomarkers that measure dietary exposure. The current research involved the use of biomarkers to exemplify the exposure and bioavailability of phytoestrogens. The excretion of metabolites and the population intake level are of particular importance as these could relate food consumption over time with development of diseases. An optimal intake level and metabolomics profile could be derived from epidemiological studies of phytoestrogen status in relation to the status of disease or health and wellbeing.

Metabolites acquire a range of routes for their excretion, and after absorption, they can circulate in the plasma, and be secreted into bodily fluids, such as breast milk, and finally excreted in urine and faeces. Urinary biomarkers were adopted in the current research and although dose-dependent relationships have been observed between intake and excretion in previous studies after phytoestrogen supplementation (Hutchins et al., 2000; Morimoto, Beckford, et al., 2014), our cross-sectional data do not support a strong association. The complexity of metabolic pathways is an obstacle in nutrition research involving the use of biomarkers. A recommendation for future research is that the urinary biomarkers alone are not sufficient to indicate phytoestrogen status, and additional use of faecal and plasma collections would provide a more complete profile of phytoestrogen status. The effect of lifestyle factors and dietary components on the gastrointestinal environment should also be explored to address the inter- and intra-individual variation in biomarker excretion.

In conclusion, the results of the current research suggest that the modified phytoestrogen

FFQ is valid with respect to WFR and reliable for the majority of the phytoestrogens. The lack of associations between measurement of the FFQ and biomarkers, suggest that urinary biomarkers alone are not sufficient for estimation of phytoestrogen status. Addition of other biomarkers utilising faecal and plasma samples should be considered for a more complete assessment of phytoestrogen status.

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Appendix A. Modified food frequency questionnaire (compete version is available on request)

Food Frequency Questionnaire

Study title: Dietary intake study to validate a dietary assessment tool and Health Index

Thank you for taking the time to complete this questionnaire. It will give us important information about what foods and drink you have eaten and drunk over the past month. You will complete this questionnaire twice, one week apart.

The results of the questionnaire are entirely confidential:

- only the researchers will see your completed questionnaire
- your results will be combined with others to summarise the overall picture of food habits
- no individuals will be identified in the analysis or in any report

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Subject ID # _____

Questionnaire 1 2 (Please circle)

Date: _____

Contact details: _____

Instruction to complete this questionnaire

- Please recall your **usual diet** over the **past month** (4 weeks) when answering this questionnaire
- State the **brand, the type and/or the ingredients** where stated required and applicable
- The standard serve size is only for reference and does not indicate what you should be eating. This is what you should use to estimate your portion size you usually have
- With reference to **the standard serve size,** specify **how much** you would **usually** eat or drink the food item by indicating a **number** and a **measurement unit** in the **Portion size column** (e.g. 1/2 slice, 60 gram or put 0 or leave it blank if you select 'Never/seldom/<1 per month' in the Frequency column)
- State the **number of times (how often)** in the **Frequency column (put an 'X')** that you ate or drank that food item in the previous month with reference to the portion size you normally have
- Please attempt to **give answer to all food items**, do not leave blank even if you did not eat or drink that particular food item or you do not regularly consumed that food, just put an **'X' in the 'never/seldom' response column**

Examples of measurement units: gram (g), cup, tablespoon (tbsp), teaspoon (tsp), nut, piece, slice, small, medium, large
Abbreviation: dia. (diameter)

Example: this person had **2 slices** of multi-grain bread **3 times per week**, had **1/2 cup** of sliced carrots **twice per week** and no cashew.

Food Item	**Brand, type and/or ingredients (if applicable)**	**Standard serve size**	**Portion size & Measurement unit**	**Frequency** - How many times did you consume each item?												
				Never /<1 per month	Times per month			Times per week						Times per day			
					1	2	3	1	2	3	4	5	6	1	2	3	≥4
Multi-grain bread	Wheatbelt Multigrain chia & Quinoa	1 slice (40g)	2 Slice(s)							X							
Carrots, cooked	N/A	½ cup (75g)	1/2 Cup						X								
Cashew	N/A	¼ cup (36g)		X													

Question 1. Do you consume seeds on their own, i.e. not incorporated into food such as in bread?
Yes ➔ Answer *1.1 Seeds (page 1)* and Continue to 1.2 No ➔ Skip to answer *1.2 Nuts (page 1)* and then Continue to 1.3

Food Item	Brand, type and/or ingredients (if applicable)	Standard serve size	Portion size & Measurement unit	Frequency - How many times did you consume each item?													
				Never /<1 per month	Times per month			Times per week						Times per day			
					1	2	3	1	2	3	4	5	6	1	2	3	≥4
1. Nuts and Seeds (page 1 -2)																	
1.1 Seeds																	
Flaxseeds (linseeds)		¼ cup (30g);1 tbsp/tsp															
Sesame seeds (or ground)		¼ cup (35g)															
Sunflower seeds		¼ cup (38g)															
Poppy seeds		¼ cup (35g)															
1.2 Nuts																	
Cashews		¼ cup (36g);10 nuts															
Chestnuts		¼ cup (38g);10 kernals															
Peanuts		¼ cup (39g);32 nuts															
Hazelnuts		¼ cup (32g);10 nuts															
Pistachio		¼ cup (31g);30 nuts															
Walnuts		¼ cup (31g);10 halves nut															
Almonds		¼ cup (30g);1tbsp/tsp															
Brazil nuts		¼ cup (35g);10 nuts															
Pecans		¼ cup (29g);10nuts half															
Pine nuts		¼ cup (45g);1tbsp/tsp															
Coconut, fresh or desiccated		¼cup(30g);piece 5x5x1cm															
1.3 Nuts and seeds products																	
Peanut butter		1 tablespoon (25g)															
Tahini (ground sesame seeds)		1 tablespoon (20g)															
Hazelnut spread (e.g. Nuttela)		1 tablespoon (19g)															
Other nut spreads/paste	**Specify type:**	1 tablespoon (20g)															
LSA (Linseed, sunflower, Almond) sprinkles		1 tablespoon (12g)															

Appendix B. Pilot study questions

Name:_____

Face validity and pilot test questions for the food frequency questionnaire

Please spend some time to answer the following questions and express your opinion about the food frequency questionnaire after you have completed it.
Your response and opinion will be considered to make the food frequency questionnaire a better tool for everyone else to complete within a reasonable time.

1. How long did it take you to complete this questionnaire?

2. Do you find the format of the questionnaire easy to understand, i.e. portion size estimation, frequency of consumption? If not, please be specific of how it can be improved.

3. Do you require visual aids for any Standard serve size to assist your portion size estimation? i.e. a photo of how a medium apple looks like, a photo of a tablespoon.

4. Are there any food items that you normally consume but were not included in the questionnaire? Please name them to be considered in the questionnaire.

5. Are there any comments in regards to improving the questionnaire?

6. Would you prefer to do the questionnaire online? i.e. Qualtrics, PDF version with fillable space or Microsoft Word version ?

Thank you so much for your time to test this questionnaire and your comments!

Appendix C. Demographic and lifestyle questionnaire

Demographic and lifestyle questionnaire

Date:(DD/MM/YY)_____

Subject ID # _____

Contact details:_____

Date of birth:(DD/MM/YYYY)_____

1. **Gender**
   ☐ **Male** ☐ **Female**

2. **What is your age: _____**

3. **Weight:_____ Height:_____**
   BMI:_____

4. **What is your occupation, please specify:**
   _____

5. **What is your highest achieved or current level of education or qualification**
   ☐ **University postgraduate (Master degree, PhD)**
   ☐ **University undergraduate (Bachelor degree)**
   ☐ **Certificate/Diploma (TAFE/technical college/business college)**
   ☐ **Secondary school, Year 12**
   ☐ **Secondary school, Year 10**
   ☐ **Other, please specify: _____**

6. **Ancestry/ethnicity**
Please describe which group(s) best defines the ancestry/ethnicity (based on a mixture of culture, religion, skin colour and language) of you and your biological parents. You may choose more than one group for each person.

A. **Caucasian –** Australian/NZ (Anglo European), Europe (includes Russia Central and West Asia) & North Mediterranean, America, Canada, South Africa & Zimbabwe
B. **Indigenous Australian –** Aboriginal, Torres Strait Islands
C. **Pacific Islander –** New Zealand Maori or Pacific Islands, Hawaii, New Guinea
D. **South-East Asia –** Brunei, Cambodia, Indonesia, Laos, Malaysia, Myanmar/Burma, Philippines, Singapore, Thailand, Vietnam
E. **South Asian –** Afghanistan, Bangladesh, Bhutan, India, Maldives, Nepal, Pakistan, Sri Lanka
F. **North East Asia –** China, Hong Kong, Japan, Korea, Macau, Taiwan
G. **North Asia –** Mongolia, Siberia
H. **Middle Eastern, North Africa, Somalia Peninsular –** Algeria, Bahrain, Djibouti, Eritrea, Ethiopia, Egypt, Israel, Iran, Iraq, Jordan, Kuwait, Lebanon, Libya, Oman, Palestinian Territories turkey, Turkish Cypriots, Qatar Saudi Arabia, Somalia, Syria, Tunisia, United Arab Emirates, Yemen
I. **Sub-Saharan Africa –** indigenous African, African American
J. **Central/South America –** Central/South America
K. **Other**
L. **Don't know**

	Your biological father	**Your biological mother**	**You**
A. Caucasian	☐	☐	☐
B. Indigenous Australian	☐	☐	☐
C. Pacific Islander	☐	☐	☐
D. South-East Asian	☐	☐	☐
E. South Asian	☐	☐	☐
F. North East Asian	☐	☐	☐
G. North Asian	☐	☐	☐
H. Middle Eastern, North Africa, Somali Peninsular	☐	☐	☐
I. Sub-Saharan African	☐	☐	☐
J. Central/South American	☐	☐	☐
K. Other (please specify below)	☐	☐	☐
L. Don't know	☐	☐	☐

If Other, please specify for each:

Your Biological father:

_____

Your Biological mother:

_____

You: _____

7. **Which language do you mainly speak at home** *(if more than one language, indicate the one that is spoken most often)*
   - ☐ **English**
   - ☐ **Italian**
   - ☐ **Greek**
   - ☐ **Cantonese**
   - ☐ **Arabic**
   - ☐ **Mandarin**
   - ☐ **Vietnamese**
   - ☐ **Spanish**
   - ☐ **German**
   - ☐ **Hindi**
   - ☐ **Other, please specify: _____**

8. **What is your country of birth, please specify:**

   _____

   If you were not born in Australia, please specify your date of arrival to Australia:

   _____DD/MM/YYYY

9. **What is your physical activity level**
   - ☐ **Sedentary, move only for necessary chores/walking/other activities 1-2/week**
   - ☐ **Light, walking/other activities >3/week**
   - ☐ **Moderate, exercise to perspire 1-2 times/week, or moderate labour or physically demanding work**
   - ☐ **High, heavy exercise or sport several times/week, heavy labour or physically demanding work**

10. Your smoking status:

- ☐ **Never smoked**
- ☐ **Current smoker, daily, please specify how many times you smoke daily: __**
- ☐ **Current smoker, weekly, please specify how many times you smoke weekly:**
- ☐ **Current smoker, other, please specify the frequency you smoke: _____**
- ☐ **Past smoker**

11. Your bowel habits:

11.1 How often do you have bowel movement?

- ☐ **>7/week**
- ☐ **4-7/week**
- ☐ **1-3/week**
- ☐ **<1/week**

11.2 What is the best way to describe your bowel movement?

- ☐ **Hard to pass**
- ☐ **Smooth and soft to pass**
- ☐ **Mushy and watery**

12. Supplemental use

12.1 Do you take any commercial supplement?

☐ **Yes** ☐ **No**

12.2 How many regular supplements do you take? E.g. vitamins, fish oil, flaxseed etc.

- ☐ **None**
- ☐ **1**
- ☐ **2**
- ☐ **≥3**

Regular: supplements taken consistently in the past month, e.g. 1 times per week, daily

12.3 Please specify the type, brand name, dosage and frequency of each supplement you take: *(For the Frequency, please respond on either 'times per week ' or 'times per day')*

(TURN to the back page)

Type of supplement	Frequency (how often)		Description (e.g. brand name)	Dose (e.g. 500mg, 2 capsules or 2 teaspoons)
	Times per week	Times per day		
Example: Fish oil	3 times		Blackmores Omega Daily concentrated fish oil, double strength	1034mg omega -3 triglycerides-fish 1 capsule

Appendix D. Three-day weighed food record

3-Day Weighed Food Record

Subject ID # _____

Contact details: _____

PLEASE write food record on two weekdays and one weekend

Instruction to Complete the Food Record

Food records allow detailed description of all food and beverages consumed over a period of multiple days. The food records provide a way to validate the data supplied from the Food Frequency Questionnaire. Data from the food records will be analysed statistically. It is very important that as much detail regarding the food and drinks you consumed, including the meal you consumed for, the amount you consumed, the place of consumption and preparation is written down.

- Please eat as you usually eat
- Record everything you eat and drink, including snacks
- Complete the Meal, time, and place prepared and consumed columns for each meal or snack. Please write in name of restaurant if you know it.
- Start each new day on a new page
- You do not have to use the whole booklet; just record what you eat and drink on the day of recording.
- Please write clearly

How to Record Each Food

- Describe each food and beverages in detail, as best you can. See example below

INCLUDE	FOR EXAMPLE
How prepared	Fried, grilled, stir fried, roast, barbeque, pam fry
Added fat	Cook with oil, butter
Brand name and type	Wheatbelt Bakehouse Magnificent Multigrain with added Chia and quinoa
Portion size	1/2 cup, 2 slices, 1tbsp

- Describe each ingredient in a mixed dish in the food record, or use Recipes section on page 34. See example below

Spaghetti Bolognese	11/2 cups cooked spaghetti, 125g lean beef mince, 1/4 large onion, sliced, 1 clove garlic, 100g crushed tomato, sliced mushrooms, 2tbsp salt reduced tomato paste
Pizza	3/4 cup flour, 1tbsp vegetable oil, 40g cheese, 38g tomato, 20g mushroom, capsicum and onion, 75g pineapple, 40g chicken

- Ideally weigh the amount of food before eating and record any leftovers with the provided kitchen scale. Estimate the amount of each ingredient with measuring tools. Demonstration on how to weigh food and drinks with the scale will be provided. Weights of food can be calculated by weighing total and subtracting utensil weight

Accuracy and detail is essential. This includes obtaining information such as:

- Dairy foods – varying fat contents, calcium enriched
- Bread – type, e.g. white, multigrain, wholemeal, fruit/raisin, sour dough
- Method of preparing food = e.g. boiled, baked, pan fried, stir fried, steamed, roast
- Amount and type of fat used in cooking – olive, canola, sesame, butter
- Use of fortified foods – e.g. calcium enriched, fortified cereals wit nuts and seeds
- Brand names whatever possible – for bread, cereals

An Example:

Food Record:

Day: _____1_____ Date: _21 (DD)_ /_8 (MM)_ / _2013_(YYYY)_

Scale and measuring tools used? ☐ Yes ☐ No

Meal B=Breakfast L=Lunch D=Dinner S=Snacks	**Time**	**Place prepared and consumed** H=Home R=Restaurant O=Other, please specify	**Food and Beverages** (Please describe Brand name, cooking method used, type i.e. low fat, and any additional information)	**Amount and unit of consumption**, (e.g. Tbsp, Tsp, Oz., Pieces, Slice, Cup, gram, handful, glass, etc.)
B	8am	H	Homemade breakfast cereal:	1 bowl
			Milk, reduced fat, Harvey Fresh	200ml
			Corn flakes, Freedom Foods	1/4 cup
			Wheat germ powder	2 tsp
			Almonds	6 nuts
			Pistachio	4 nuts
			Dried cranberry	5 berries
			White chocolate chips	8 chips
			Oat, minute, McKenzie's	3/4 cup
			All microwaved	

Food Record:

Day: _____ **Date: ____ /____ / _____**

Scale and measuring tools used? ☐ **Yes** ☐ **No**

Meal B=Breakfast L=Lunch D=Dinner S=Snacks	**Time**	**Place prepared and consumed** H=Home R=Restaurant O=Other, please specify	**Food and Beverages** (Please describe Brand name, cooking method used, type i.e. low fat, and any additional information)	**Amount and unit of consumption**, (e.g. Tbsp, Tsp, Oz., Pieces, Slice, Cup, gram, handful, glass, etc.)

Appendix E. Urine collection protocol

Urine collection protocol for subjects

- It is **important** that you **drink normally** during the day. This means do not drink more or less fluids than you usually would during a normal day.
- For accurate evaluation of tests of how much metabolites are excreted on your 24-hour urine samples, it is **important** that you **make the effort to collect every drop of urine during each 24-hour period**.
- It is important that you will need to produce **at least 100ml of urine per 24 hour**

You will need:

- 3x3-litre urine collection bottles, with vitamin C added as preservative (supplied in 1st session)
- A pen, pencil or marker
- Funnel and Collection 'hat' (supplied in 1st session)
- Cooler bag, ice pack (supplied in 1st session)
- Watch/clock

1. You will be given equipment, 3x3-litre urine collection bottles, funnel, collection container/hat, cooler bag and ice pack for urine collection in the 1st session. Discuss with Winnie if you think you will need more collection bottles.

2. **RECORD** information, such as the start and complete date and time of urine collection, and sample complete or incomplete on the label of the bottles. If any sample is missed, record the time of the missed sample on the calendar provided.

3. **BEGIN** the urine collection, after you have **DISCARDED** the first urine sample you made in the morning or day after you wake up for the **day ONE only**. Record the time on the front of your bottle. You will collect **all samples** for the **day TWO and day THREE.**

4. **COLLECT** all urine for the remainder of the day, finishing with the last sample before rest. Write the finished time on the bottle.

5. Use the collection hat to assist the collection. Place the clean collection hat between the toilets bowl top and lid to hold it in place. Make sure the curved part of the hat is lined up with the front edge of the toilet bowl. You should urinate in the collection hat. If it is convenient, you can instead urinate directly into the bottles with the funnel.

6. Remove the red cap off the bottle; insert the funnel into the bottle. Transfer the urine to the bottle through the funnel without splashing or spilling. The collection 'hat' has a pour spout which makes it easy to pour the urine into the collection bottles. Close the cap when you finish transferring the urine sample into the bottle.
   Note: It is best to do the transferring immediately after urinating. If you get up during the night but do not pour the urine into the bottle immediately, please do so as soon as possible the next morning.

7. **Please store the bottles in a cool place**. If it is okay for you, you may store in the fridge. If not, for example, in bathroom or air conditioned environment, during the day of collection or in the cooler bag provided with ice pack. Remember to cap the bottle and close it tightly.

8. When you finish all 3x24-hour urine samples, put them all in the cooler bag with ice pack, along with other given equipment and ready to return them to Winnie. If you know you will be on ECU Joondalup campus after you have finished one 24-hour urine collection, you may bring the completed 24-hour urine bottle from the previous day to Winnie at the time of your convenience. Please contact Winnie to do so. Alternatively, you may choose to bring all the bottles along with other equipment and paperwork, i.e. Food record, calendar, food frequency questionnaire, to the 2nd session.

Note:
If you need to have a bowel movement, any urine passed with the bowel movement should be collected. Try not to include faeces with the urine collection. If faeces does get mixed in, do not try to remove the faeces from the urine collection bottle.

For female subject, please do not collect samples during menstrual cycle.

The vitamin C powder inside the bottles is added as preservative. You will not be in direct contact with the powder. Please leave them as it is and the powder is not harmful.

Appendix F. Laboratory protocol for measuring weight and volume of urine

Protocol for measuring total weight and volume of urine samples

You will need:
-24 hour urine sample
-top pan balance
-graduated cylinder, beaker

- Record subject name and subject ID in the 24-hour urine sample page of the Lignan project laboratory manual
- To obtain the weight of the 24-hour urine sample, weigh the urine bottle and the urine sample with the top pan balance, record the total weight (g) of bottle and urine in the laboratory manual
- Decant the urine into the graduated cylinder to measure the total volume (L) for each day sample and record its volume in the laboratory manual
- Weigh the empty bottle and subtract its weight from the total weight of the bottle and urine to obtain the weight of the urine
- Record the weight (g) and volume (L) of each sample in the 24-hour urine samples page of the manual (Day 1, 2 or 3), indicate the completeness of each sample, the date of sample return and the date of sodium azide added
- Rinse out the graduated cylinder after measurement of each day sample to minimise cross contamination

Appendix G. Laboratory protocol for urine sample aliquoting

Protocol for sample aliquoting and aliquot storage

You will need:
-24 hour urine samples
-sodium azide solution
-pipette that measure 10ml, 10μL and pipette tip
-dropper
-10ml centrifuge tubes, 2ml eppendorf
-beaker
-label, pen and plastic bag

- Create new entries of the urine aliquots in the Aliquot page of the Lignan project laboratory manual. Assign a subject ID to new subject. Assign a sample ID to each aliquot, record the content of each aliquot and create comment and note columns about what these samples, how they will be used for and how they will be treated
- Rinse all glassware with water and urine sample before use
- After measuring the total volume for each 24-hour urine sample with the graduated cylinder, decant urine into a 100ml beaker
- Adjust the pipette to measure 10ml. Insert the appropriate pipette tip that can transfer the volume of 10ml. Use this 10ml pipette to measure one 10ml aliquot from each day sample and transfer into 10ml polypropylene centrifuge tubes. Label them with their subject and sample IDs (refer to Lignan project laboratory manual). These aliquots are sent to PathWest for creatinine analysis
- Use a dropper or the pipette to measure two approximately 2ml urine into 2ml centrifuge tube from each day sample, you will have a total of six 2ml centrifuge tubes per subject. Close the lid. Label them with their unique sample IDs. These centrifuge tubes are reserved for Dr. Mary Boyce to develop urinary creatinine analysis method using capillary electrophoresis.
- Top up the beaker with urine if necessary

- Prepare a sodium azide solution as per the Protocol for preparing the sodium azide solution.
- Use the 10ml pipette to measure three 10ml aliquots from each day sample into 10ml polypropylene centrifuge tubes. You will have a total of twelve aliquots per subject, plus the first one prepared without sodium azide added for each day sample. Each tube is to be labeled with the type of sample (i.e. 24-hour urine), subject and sample ID, initial of person and the date of aliquoting
- Adjust a different pipette that can measure 10μL and insert a pipette tip that can transfer this volume. Use this pipette to measure 10μL sodium azide solution and add to the other three 10ml aliquots (Except for the ones that will be sent to PathWest for creatinine analysis).
- Mix the sodium azide by slowly inverting each tube 4 to 5 times.
- Pour the unused volume of urine from the cylinder to the sink or decant back to the urine bottles (if needed), rinse the sink with shower, and clean the cylinder after aliquoting each day sample. Dispose the pipette tip and dropper after aliquoting each day sample and change to a new one before pipetting new day sample
- You will have a total of twelve 10ml aliquots (four per each of three days sample) and six 2ml centrifuge tubes (two per each of three days sample) per subject. Ensure the lids are closed firmly
- Place the tubes for each subject in the rack. Put the rack with sample tubes in the plastic bag, with a paper label in the bag. The label should contain information of the name of the study (LIGNAN study), name of supervisor (Dr. Mary Boyce), date to discard (2018)
- Store the plastic bag with rack of sample tubes in the -80°C freezer (19.206)

Appendix H. Screening questions

Screening questions

These questions will be asked to screen potential participants.
All answer must be No in order for a participant to be eligible to participate

1. Are you currently taking any antibiotics?
   Yes, exclude the subject. No, ask Question 2

2. Have you taken any antibiotics for the past 6 to 12 months?
   Yes, exclude the subject No, ask Question 3

3. Did you make substantial changes to your usual diet for the past 3 months?
   Yes, exclude the subject No, ask Question 4

4. Do you have any currently diagnosed gastrointestinal problems, such as ulcerative colitis, or Crohn's disease?
   Yes, exclude the subject No, ask Question 5

5. Have you had gastric surgery that remove all or part of your large intestine?
   Yes, exclude the subject No, ask Question 6

6. Do you have chronic renal or liver diseases? Or have had acute renal or liver diseases in the previous 6 to 12 months?

Yes, exclude the subject. No, subject is eligible to participate

Appendix I. Study flyer

Participants invited to a dietary intake study for Health Index

Researcher Winnie Li from the Dietetics and Nutrition Research Group, Edith Cowan University, is looking for **80 male and female volunteers** from **different demographic and cultural backgrounds** and with **diverse eating habits** to participate a research study to validate a food frequency questionnaire and to evaluate a Health Index of diet.

The study involves attending *two sessions (approx. 30-40 minutes each)* to:

① Have your height and body mass measured,

② Complete a demographic and lifestyle questionnaire,

③ Complete the modified food frequency questionnaire,

and spending 4 days to

④ Complete a 3-day weighed food record during the time between the two sessions,

⑤ Collect 24-hour urine samples for 3 days during the time between the two sessions

★You will receive nutritional intake, feedback and an analysis of the Health Index for your diet, and a certificate of appreciation on request★

For more information or to participate this research study, please contact:

Winnie Li

Appendix J. Study calendar

Date: __/__/__		**Day1 THURSDAY or SUNDAY** Date:___/___/__	**Day 2 FRIDAY or MONDAY** Date: __/___/__	**Day 3 SATURDAY or TUESDAY** Date: ___/___/__	**Day 4 SUNDAY or WEDNESDAY** Date: __/___/___		Date:__/__/___
Session 1 (Attend at ECU)							**Session 2**
Demographic questionnaire **(Red)**	Food frequency questionnaire 1 **(Blue)**	Food record Day 1 **(Green)** *Complete?*	Food record Day 2 **(Green)** *Complete?*	Food record Day 3 **(Green)** *Complete?*		Food frequency questionnaire 2 **(Blue)**	Winnie reviews Food record **(green)**, Food frequency questionnaire **(Blue)** and urine samples **(3 bottles)** with you
Collect equipment and paperwork for food record and urine collection	***– About your usual diet for the past 4 weeks***		****Let go of the first urine on** Urine Day 1 *Complete?* if *incomplete,* please specify **Time missed:** _____am/pm **Volume of urine missed:** _____(ml)	****Collect all urine on** Urine Day 2 *Complete?* if *incomplete,* please specify **Time missed:** _____am/pm **Volume of urine missed:** _____(ml)	****Collect all urine on** Urine Day 3 *Complete?* if *incomplete,* please specify **Time missed:** _____am/pm **Volume of urine missed:** _____(ml)	***-Again about your usual diet for the past 4 weeks with the food intakes of the days you were doing the food record and urine sample collection***	Please return equipment (scales, cups and spoons, ice blocks, cooler bags) to Winnie

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