

## Chapter 6

# ***Preparation of A Novel Food Source of Isoflavone Glycosides and Aglycones for Possible Use in Human Studies of Isoflavone Absorption and Metabolism***

## 6.1. Introduction

### 6.1.1. Common Pathways of Absorption and Metabolism

Interpretation of the *in vivo* biological activity of flavonoids from *in vitro* data requires an understanding of their bioavailability, particularly absorption and metabolism. Although the apparent bioavailability of flavonoids is highly variable between types of flavonoid, the pathways involved in the absorption and metabolism are common to all flavonoids (Williamson, 2004).

Day *et al.* (2004) and Williamson (2004) have summarized the general pathways of flavonoid absorption and metabolism. The simplified routes of flavonoid metabolism in the human body are shown in figure 1-20. The key points are described in subsequent sections.

#### 6.1.1.1. Flavonoids reach the small intestine unchanged from the food

Flavonoid glycosides are stable to most normal cooking methods, stomach acid pH, and to secreted gastric enzymes. Usually intact flavonoid glycosides reach the small intestine following ingestion. Some flavonoid aglycones are absorbed in the rat stomach to a limited extent, glycosides are not (Piskula, 2000; Crespy *et al.*, 2002). However, a study carried out by Allred *et al.* (2001) demonstrated that conversion of genistin to its aglycone form genistein begins in the mouth and then continues in the small intestine.

#### 6.1.1.2. Glycosylated flavonoids must be deglycosylated before absorption

Deglycosylation is a prerequisite for subsequent conjugation by intestinal enzymes and transport to the serosal or mucosal sides. Isoflavone aglycone but not the glycoside form can be absorbed in the small intestine (Setchell *et al.*, 2001). The initial step in the absorption process for glycosylated flavonoids and isoflavonoids is

deglycosylation by lactase phlorizin hydrolase (LPH) (Day *et al.*, 2000b). The product of the deglycosylation reaction is a free aglycone that can then diffuse into epithelial cells either passively or by facilitated diffusion. An alternative absorptive mechanism involves transport of the flavonoid glycoside into the enterocyte in an intact form via the function of a sugar transporter, and then deglycosylation by cytosolic  $\beta$ -glucosidase (Day *et al.*, 1998; Day *et al.*, 2003). Both pathways of absorption give rise to intracellular aglycone.

#### **6.1.1.3. The small intestine is the major site of flavonoid conjugation**

Flavonoids less commonly undergo phase I metabolism (Day *et al.*, 2004). For phase II, the primary conjugations in flavonoid metabolism are the glucuronidation and sulfation of hydroxyl groups, and the methylation of catechol groups, although a proportion of some flavonoids escape intestinal conjugation under certain conditions (Williamson, 2004). Plasma conjugates of flavonoids are found not as glucosides but sulfated, glucuronidated, or methylated derivatives and are rarely substituted.

#### **6.1.1.4. Deconjugation and re-conjugation**

The liver receives flavonoids from the blood, including blood from the small intestine during first pass metabolism. The blood delivers flavonoids to tissues throughout the body (Donovan & Waterhouse, 2003). Flavonoids from the small intestine that reach the liver are almost entirely conjugates, especially of glucuronides. De-conjugation and re-conjugation can be catalysed by  $\beta$ -glucuronidase and sulfatase occurring in liver cells and other tissue cells, resulting in sulfates, methylates, and even aglycones found in some tissues (Williamson, 2004).

#### **6.1.1.5. Microbial metabolism in the colon**

Glycosylated flavonoids and bile-excreted flavonoid conjugates can be further metabolised by colon micro-flora enzymes which possess a very large capacity for de-conjugation, including de-glycosylation, de-glucuronidation, and de-sulfation, leading to production of aglycone and further metabolites (Williamson, 2004). This is especially important to isoflavones. Isoflavone aglycones can be absorbed

substantially in the colon since they are relatively resistant to degradation (Setchell & Cassidy, 1999). In addition, a biological activation of daidzein occurs in some individuals to equol, and this step is known to increase phytoestrogen activity (Setchell *et al.*, 2002a).

#### 6.1.1.6. Elimination

There are two routes for flavonoid elimination. In human urine, there is excretion of absorbed flavonoid after endogenous and microbial metabolism, while in human faeces, there is excretion of non-absorbed dietary flavonoid glycosides, microbial metabolites and biliary excreted metabolites (Day *et al.*, 2004).

### 6.1.2. Conjugates of Flavonoids

Conjugation is a common detoxification reaction which reduces the number of reactive hydroxyl groups, leading to increased solubility and molecular weight that is necessary for biliary or urinary excretion (Day *et al.*, 2004).

#### 6.1.2.1. Glucuronides

The metabolic fate in tissue of most ingested flavonoid glucosides is probably conjugation to glucuronic acid. Glucuronidation is catalysed by the enzyme UDP-glucuronosyltransferase (UDP-GT) which is present in metabolic tissues in many isoforms, and requires the co-factor UDP-glucuronic acid which is abundant within cells ensuring that the conjugation is unlikely to become saturated at high concentrations (Day *et al.*, 2004).

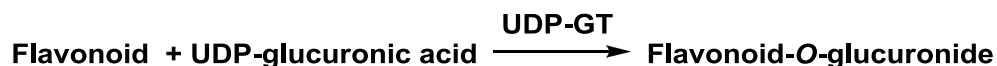


Figure 6-1 Glucuronidation of flavonoids by UDP-GT

### 6.1.2.2. Sulfates

Sulfation of flavonoids requires the enzyme sulfotransferase and the co-factor 3'-phosphoadenosine-5'-phosphosulphate (PAPS). The latter can be in limited supply in cells, thus sulfation tends to predominate only at low polyphenol concentrations (Day *et al.*, 2000a). However, as flavonoids from the diet are present at relatively low concentrations, the level of expression of conjugating enzymes in the small intestine may be the determining factor for which metabolites are formed rather than any saturation of individual conjugating enzymes (Day *et al.*, 2004).



Figure 6-2 Sulfation of flavonoids by sulfotransferase

### 6.1.2.3. Methylation

Methylation of catechol groups is also a common phase II reaction of flavonoids. Methylation is catalysed by catechol-*O*-methyltransferase and requires a catechol group as substrate and can occur for some flavonoids which possess a catechol group, like quercetin and catechins (O'Leary *et al.*, 2003; Ito *et al.*, 2005).

## 6.1.3. The Study of Bioavailability

### 6.1.3.1. Human Intervention Studies

Since epidemiological studies may indicate that flavonoids possess potential health benefits, large amounts of research regarding bioavailability have been carried out using different model systems, each having advantages and disadvantages (Day *et al.*, 2004).

In the simplest form of model, tissue homogenate extracts can be used to identify enzyme activity towards specific flavonoids. Kinetics, rates of reactions and

competition can be measured to determine likely metabolites (Hollman, 2001). Cell culture can provide additional evidence for the types of flavonoid metabolites that could be expected *in vivo*. However, depending on the cell type and passage number, enzyme expression may be significantly different to normal cells with living tissues (Hollman *et al.*, 1995).

Human intervention studies can provide an overview of the process of absorption and metabolism by determining pharmacokinetic parameters from plasma and urinary metabolites. After a dose of the compound of interest, information can be obtained about the sites and pattern of absorption, excretion and elimination, etc (Williamson, 2004), but these can be difficult to characterize due to lack of useful sample, or low levels of compound administered. Analytical techniques must be sensitive because physiological levels of metabolites may be very low and ethical considerations may limit the quantity and type of flavonoid dose given (Hendrich, 2002). This problem sometimes can be overcome by using animals, but animal study may not represent metabolism in humans adequately because metabolizing enzymes and intestinal microflora vary between mammalian species, although animal models may provide valid data. Furthermore, the metabolism of xenobiotics such as flavonoids is under a high degree of genetic control, hence there can be considerable variation between different groups of humans and even between individuals of the same group (Heim *et al.*, 2002). The so-called population of equol producers is a good example. Additionally, the diet itself may have a direct bearing on metabolism because certain dietary components can induce production of particular enzymes (de Pascual-Teresa *et al.*, 2006).

Human intervention studies always use oral administration of drugs or nutrients and the subsequent analysis of bio-fluids. Plasma and urine are the fluids most commonly analyzed, but others may be used, such as saliva or ileostomy fluid (Hollman *et al.*, 1995; Day & Williamson, 2003). With the aid of adequately sensitive analytical techniques a detailed pharmacokinetic picture of absorption, distribution and clearance can be built up using measurements of biomarkers with respect to time.

### **6.1.3.2. Feeding samples**

Human intervention studies are always carried out through providing subjects with various sources of certain chemicals and collecting bio-fluid samples. Two types of oral administered food samples of flavonoids can be used, which are:

- Flavonoid supplements; including purified flavonoid isomers and plant extracts. For instance, soy protein extracts, any kind of tablets or capsules containing flavonoids. It is easy to control the amount of target compound at the required level, although it may not be in line with the natural situation, so it is useful for getting initial information on flavonoid bioavailability. However the metabolism conditions may also be simplified, so it may cause some error when the information is used to build up the whole picture especially for estimating health benefits. For example, some other components in human food, such as fibre or fat, may also affect absorption and metabolism. These factors cannot be reflected in the data obtained by using flavonoid supplements.
- Real food rich in flavonoids either modified or unmodified. These foods are much closer to the real human diet and the data obtained is relatively more relevant. However, under certain circumstances, in real human food target compounds may not reach a level high enough for analysis after ingestion and absorption. Sometimes the taste of food samples may not be accepted by subjects due to the food preparation method. For example, for testing isoflavone aglycone absorption, fermented foods used in many experiments were Miso and Tempeh. These fermented soy-based foods cannot be accepted by most western people because of the high content of salt. In East Asia, people do not consume them as a main course but as some kind of seasoning. Consequently by using these foods only, the amount of isoflavone aglycone consumed has to be restricted to a lower, acceptable level. On the other hand, in natural real foods, the components may be too complicated, and this may be a disadvantage for analyzing data for separating tested factors (individual components).

Some of the problems of supplements or foods may be overcome by the development of novel foods. A novel food should be: (a) accepted by all people, hence can be used to test the differences between populations and even individuals, (b) the components (including flavonoid and other components) in the final product should be able to be adjusted easily.

### **6.1.3.3. Pharmacokinetics**

Pharmacokinetic parameters are important in understanding the absorption and metabolism of flavonoids. The time to reach maximum plasma concentration (T<sub>max</sub>) is an indicator of the site of absorption, and typically small intestine uptake is represented by values of <3hr and the colon by values of 5 to 10hr, although this depends on the meal size and transit times. For flavonoid glycosides, the attached sugar is a major determinant of the T<sub>max</sub>. An attached glucose may lead to absorption in the small intestine, whereas an attached rhamnose may lead to absorption in the colon after microflora deconjugation. The half-life represents both the rate of appearance within and the clearance from the bloodstream and the time available for a biological effect to occur. For the half life, the flavonoid itself is the major determinant: quercetin > isoflavones > catechins (Williamson, 2004).

It should be noted that most pharmacokinetic values have been measured based on the determination of flavonoid aglycone after deconjugation. True pharmacokinetic parameters are for appearance of the administered compound only, but most flavonoids are conjugated with glucuronic acid, sulfate, or methyl groups making this impossible (Williamson, 2004). The short half life of flavonoids makes the plasma concentration difficult to use as a biomarker of long-term flavonoid levels in the diet, and it has been shown, for example, that the plasma concentrations of hesperetin and naringenin are poor biomarkers of intake.



## **6.2. Aims of Chapter**

The aims of this chapter were to develop two novel foods that were identical but which varied in the nature of the isoflavone content – i.e. high in isoflavone glycosides or high in isoflavone aglycones – that could be used in human feeding studies investigating isoflavone absorption and metabolism.

## **6.3. Materials and Methods**

### **6.3.1. Hydrolysis Efficiency of Apple Seed Extracts Towards Soy Flour**

In order to obtain optimum results for isoflavone hydrolysis in a real food, an initial experiment regarding the hydrolysis efficiency of apple seeds towards soy flour was carried out.

Apple seed samples (3×10g) were separately put in a Moulinex Optiblend 2000 blender for 5mins (using the small mixer) and milled into fine powder before transfer into 3 beakers, and mixed with 20g dried soy flour and 200ml water. These mixtures were covered by parafilm and incubated under different conditions, which were at room temperature (20°C) for 24hrs, at 37°C for 18hrs, and at 65°C for 2hrs.

Another 10g of apple seeds were used as control. After milling into a fine powder and transfer into a beaker, the apple seeds were mixed with 200ml water and boiled for 10 mins, and then mixed with 20g soy flour after cooling down.

After incubation, all 4 beakers were dried at 80°C in a water bath and then isoflavones were extracted and determined as described in 5.3.4.

### **6.3.2. Preparation of Soy Biscuits**

The recipe for producing the soy biscuits was originally downloaded from [www.recipe.com](http://www.recipe.com) and then developed. Both recipes were designed for 2 meals.

#### **6.3.2.1. Baking Powder/Soy Flour Biscuit**

- Preparation of soy flour: 30g of apple seeds were put in a Moulinex Optiblend 2000 blender (use small mixer), milled for at least 5mins in order to obtain a fine powder, and mixed with 600ml water and boiled for 10mins. The mixture was then added to 60g soy flour. This cocktail then was put in a water bath at 80°C until completely dried.
- Preheat the oven to 230°C and grease an 8-inch square baking pan.
- Put 140g wheat flour, previously dried soy flour, 15g baking powder and 7g salt into a large mixing bowl. Add 75g vegetable shortening and toss it to coat with the flour. Break the chunks into 5 or 6 smaller chunks and start rubbing the flour and shortening together with fingers and letting the mixture fall back into the bowl. When the mixture looks like irregular lumps it has been mixed enough.
- Add 250ml of whole milk all at once and stir to moisten all the flour and shortening. Dust a surface with flour, turn the dough onto the surface and knead the dough ten times.
- Pat the dough into a circle about 1/2 to 3/4 of an inch thick. Cut the biscuits with a two-inch cookie cutter and place on the prepared pan with a little space between the biscuits.
- Bake 15-18 minutes or until the tops are lightly browned.

#### **6.3.2.2. Baking Powder/Hydrolysed Soy Flour Biscuit**

- Hydrolysis of soy flour: 30g of apple seeds were put in a Moulinex Optiblend 2000 blender (use small mixer), milled at least 5mins in order to obtain a fine powder, and then mixed with 60g soy flour and 600ml water. This cocktail

was incubated in a water bath at 37°C for 18hr. After being hydrolysed, the soy flour was dried in water bath at 80°C.

- Then the hydrolysed soy flour was used to make hydrolysed soy flour biscuits. The procedure was the same as in 6.3.2.1 with hydrolysed soy flour taking the place of dried soy flour.

The biscuits made by recipe 6.3.2.1., which contained soy isoflavones mainly as glycoside forms, were denoted as Food A; while the biscuits made by recipe 6.3.2.2., which contained soy isoflavones mainly as free aglycone forms, were denoted as Food B.

### **6.3.3. Determination of Isoflavones in Biscuits**

A biscuit was taken and milled. The fine powder was weighed (2g) and put in a 50ml glass tube. 20ml of 80% methanol was added, and then the tube was sealed by parafilm, vortexed for 1min, and incubated in a shaking water bath at 37°C for 2hr. Then the extract solution was filtered through Whatman No. 40 filter paper. 80% methanol was used to wash the tube and filter paper and made the final volume of filtrate 25ml. Aliquots of this solution were used for HPLC analysis after being filtered through a 0.2µm PTFE filter.

## 6.4. Results

### 6.4.1. Hydrolysis Efficiency of Apple Seed Extracts Towards Soy Flour Isoflavones

There was no significant difference between the hydrolysis percentages for isoflavones in soy flour under the different conditions tested. The results (table 6-1) show that soy isoflavones were hydrolysed completely under all 3 conditions. So any of the 3 hydrolysis conditions can be used for the soy flour hydrolysis in biscuit-making. Incubation at 37°C for 18hr were the conditions used.

Table 6-1 Results for hydrolysis efficiency of apple seed extracts towards soy flour isoflavones

Peak identified	20°C, 24hr	37°C, 18hr	65°C, 2hr	Control
Daidzin	-	-	-	++
Glycitin	-	-	-	++
Genistin	-	-	-	++
Daidzein	++	++	++	-
Glycitein	++	++	++	-
Genistein	++	++	++	+

- : No peak;

+: Peak with small area;

++: Big peak with good resolution

### 6.4.2. Isoflavone Contents in Biscuits

The chromatograms of soy biscuits (both hydrolysed and unhydrolysed) are shown in figure 6-3. Estimated isoflavone contents are shown in table 6-2.

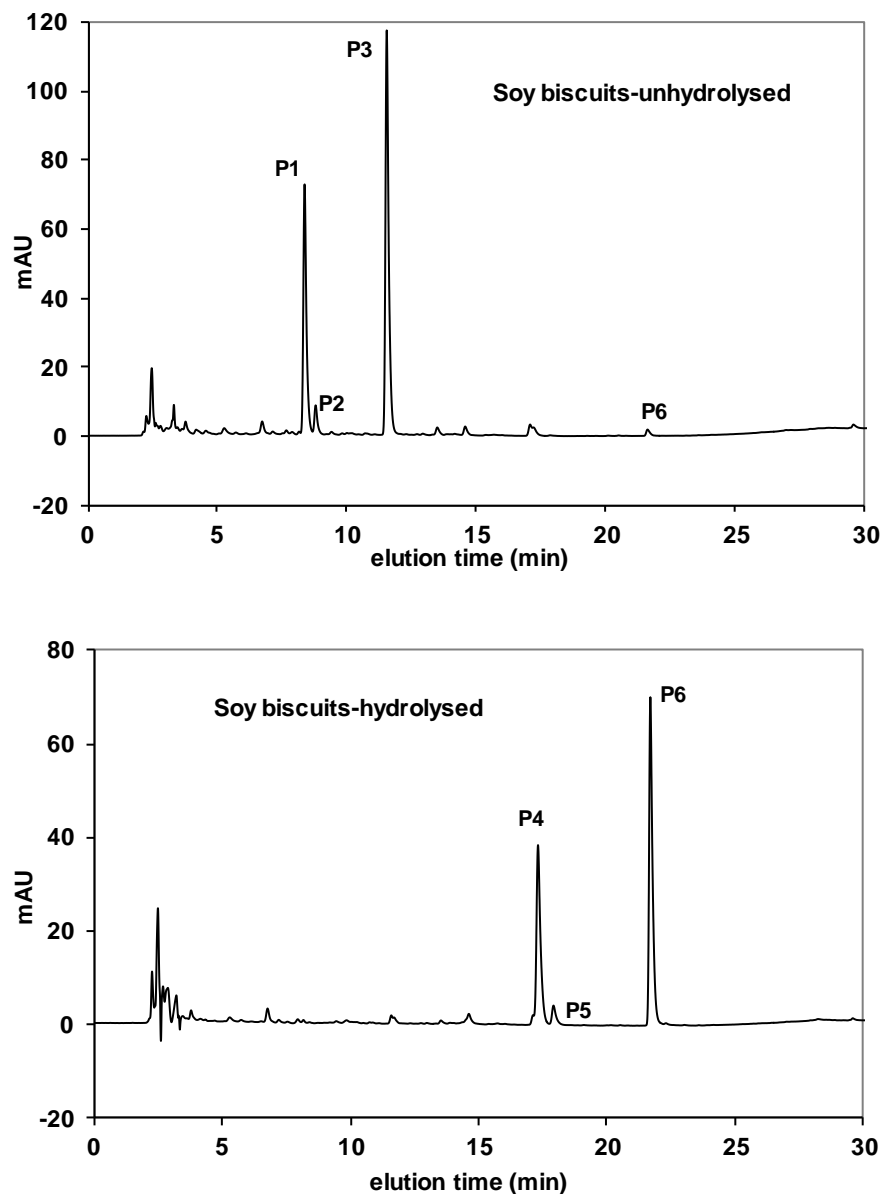


Figure 6-3 Chromatograms of isoflavones in biscuits (Top: Using unhydrolysed soy flour; Bottom: Using soy flour pre-heated with apple seed extracts). Peak identification: P1: daidzin; P2: glycitin; P3: genistin; P4: daidzein; P5: glycitein; P6: genistein. The isoflavone contents of the biscuits are shown in table 6-2.

Table 6-2 Isoflavone contents of biscuits (mg/biscuit meal)

Isoflavone	Unhydrolysed Soy Biscuits Mean $\pm$ SD	Hydrolysed Soy Biscuits Mean $\pm$ SD
Daidzin	31.86 $\pm$ 1.0	ND
Genistin	39.66 $\pm$ 1.1	ND
Glycitin*	6.02 $\pm$ 0.27	ND
Daidzein	ND	18.30 $\pm$ 1.3
Genistein	3.29 $\pm$ 0.31	26.60 $\pm$ 1.0
Glycitein**	ND	3.62 $\pm$ 0.17
Total***	50.6 $\pm$ 0.6	48.5 $\pm$ 1.1

ND: not detected;

\* calculated as daidzin equivalents;

\*\* calculated as daidzein equivalents;

\*\*\* calculated as aglycone equivalents after hydrolysis;

1 biscuit meal is equal to approximately 120g biscuits.

## 6.5. Discussion

The significance of the biscuits prepared as described is that glycosides or aglycones of the isoflavones can be fed and the consequences of isoflavone absorption patterns can be investigated, without worrying about differential effects of the food matrix. Absorption and metabolism of isoflavones is subject to considerable inter-individual variation.

### 6.5.1. Contradictory Results of Bioavailability of Isoflavones

As mentioned previously, bioavailability is critical and must always be considered in research on phytochemicals such as isoflavones (Holst & Williamson, 2004). In last two decades, many researchers have focused their interest on this area, however, whilst much information has been obtained more confusion also comes out due to the contradictory results.

Izumi *et al.* (2000) investigated the human plasma isoflavone concentrations of 8 Japanese (4 males and 4 females, 31-58 years old) after feeding tablets containing low, medium and high soy isoflavone contents as either aglycone or glycoside form by HPLC. They found that the highest isoflavone concentrations in bloods were reached earlier and were many times higher after ingestion of aglycones rather than glycosides, and genistein concentrations in blood were significantly higher than those of daidzein when the intake of those two isoflavones were the same. Thus they made the conclusion that the isoflavone aglycones were absorbed faster and in greater amounts than were their glucoside forms in humans.

Setchell *et al.* (2001) determined the pharmacokinetic parameters of isoflavones by GC-MS and compared plasma kinetics of pure daidzein, genistein and their  $\beta$ -glycosides administered as a single-bolus dose (50mg in tablets) to 19 healthy women in the UK. Their results show that the highest concentrations appeared later after ingestion of glycosides compared to ingestion of aglycones although Tmax values were longer than reported by Izumi *et al.* (2000), in agreement with the residence time needed for hydrolytic cleavage of the glycoside moiety for bioavailability. They also

found that plasma genistein concentrations were consistently higher than daidzein and the area under the curve (AUC) of genistein (4.54  $\mu\text{g}/(\text{mL h})$ ) was much greater than that of daidzein (2.94  $\mu\text{g}/(\text{mL h})$ ). However, their results showed that the bioavailability of the isoflavones is greater when ingested as glycosides rather than as aglycones as measured by the AUC of the plasma levels.

Richelle *et al.* (2002) investigated the bioavailability of isoflavones using enzymatically-treated soy milk ingested by 6 postmenopausal European women. Zubik & Meydani (2003) investigated the bioavailability of daidzein and genistein in 15 American women aged 39-53 years with typical American dietary habits after ingestion of the aglycone or glucoside forms of isoflavone tablets. Pharmacokinetic parameters including  $T_{\text{max}}$ ,  $C_{\text{max}}$ , AUC, etc. were determined by HPLC. Both studies found that the bioavailability of genistein and daidzein was not different when ingestion was as either aglycone or glucoside, which makes this issue more confused.

In Australia, Tsangalis *et al.* (2005) investigated this topic by bifidobacteria-fermented soy milk ingested by 16 healthy postmenopausal women at three isoflavone levels with analysis of 24 hrs urine samples by HPLC. In Hawaii, Maskarinec *et al.* (2008) tested this issue by feeding soy milk and miso soup (traditional Japanese fermented soy food) to subjects and analysing urine samples by LC-MS. The subjects they chose were 21 Japanese-American females with at least half Japanese ancestry because they were expected to have had previous soya food exposure and a more comparable gut flora than subjects with different ancestries (Song *et al.*, 2006). However, both of these two studies concluded that there were no significant differences between bioavailability of isoflavone aglycones and glycosides.

In 2006, 2 studies (Cassidy *et al.*, 2006; Kano *et al.*, 2006) from the UK and Japan were published, which supported Izumi's finding. Cassidy *et al.* (2006) examined the effect of age, gender, and the food matrix on the bioavailability of isoflavones for both the aglycone and glucoside forms from 3 different soy foods, soy milk, textured vegetable protein, and tempeh ingested by 21 premenopausal women, 17 postmenopausal women, and 21 men recruited in Surrey, UK. Blood and urine samples were analysed by GC-MS. They found that consumption of tempeh (mainly



aglycone forms) resulted in higher serum peak levels of soy isoflavones and the associated AUC compared with textured vegetable protein (predominantly glucoside forms). Kano *et al.* (2006) in Japan examined both blood and urine samples by LC-MS from 12 Japanese subjects (9 men and 3 women, age 25-51 years) who had consumed 3 kinds of soymilk: untreated soymilk,  $\beta$ -glucosidase treated soymilk, and fermented soymilk. After the ingestion of soymilk, the total concentration of isoflavones in serum rose slowly and reached a maximum of  $0.94 \pm 0.39 \mu\text{mol/L}$  at  $6.0 \pm 1.2 \text{hr}$  and  $\beta$ -glucosidase-treated soymilk and fermented soymilk increased the serum isoflavone concentration significantly more quickly with maximum concentrations at 1.0hr of  $1.75 \pm 0.33 \mu\text{mol/L}$  and  $2.05 \pm 0.32 \mu\text{mol/L}$ , respectively. The urinary excretion of isoflavones after ingestion of these aglycone-enriched preparations was significantly greater than after consumption of untreated soymilk. They concluded that the isoflavone aglycones of soymilk were absorbed faster and in greater amounts than their glucosides.

Unfortunately, another investigation carried out in Germany by Rüfer *et al.* (2008) supported the observation of Setchell *et al.* (2001). 7 Healthy young German men (22-30 years) were challenged with pure daidzein and daidzin capsules (1 mg aglycone equivalent /kg BW). Blood samples (up to 48hrs) and urine samples (up to 24 hrs) were collected and the concentrations of daidzein and its major bacterial and oxidative metabolites analysed by GC-MS and the pharmacokinetics were assessed. They found that the AUC and  $C_{\text{max}}$  in plasma, and the cumulative recovery in urine after administration of glucoside were 3-6 times greater than after the ingestion of aglycone, and all other quantified metabolites exhibited 2-12 times greater AUC,  $C_{\text{max}}$ , and urinary recoveries after consumption of glucoside. Thus the conclusion was that daidzin (glucoside form) exhibited a greater bioavailability than its aglycone daidzein.

More recently, Okabe *et al.* (2011) reported the soy aglycones were absorbed faster and in greater amounts than those of glycosides, leaving this topic as an enigma. 11 Healthy postmenopausal Japanese women ingested either an aglycone-rich fermented soy powder (Fsoy) and glucoside-rich non-fermented soy powder (Soy) dissolved in hot water. Blood samples (up to 24 hr) and urine samples (up to 48hr) were collected and analysed by LC-MS. They found that the Fsoy group showed significantly higher

maximum concentration ( $C_{max}$ :  $2.79 \pm 0.13$  vs  $1.74 \pm 0.13 \mu\text{mol L}^{-1}$ ) and area under the curve ( $AUC_{0-24h}$ :  $23.78 \pm 2.41$  vs  $19.95 \pm 2.03 \mu\text{mol day L}^{-1}$ ) and lower maximum concentration time ( $T_{max}$ :  $1.00 \pm 0.00$  vs  $5.00 \pm 0.67h$ ) compared with the Soy group. The cumulative urinary excretion of total isoflavones after 2h was significantly higher in the Fsoy group than in the Soy group and individual isoflavones (daidzein, genistein and glycitein) showed similar trends to total isoflavones, while equol did not differ between the two groups. They believed these results demonstrated that the isoflavone aglycones were absorbed faster and in greater amounts than those of glucosides in postmenopausal Japanese women.

In general, most of the research agrees with (i) aglycones appearing and reaching highest concentration in plasma and urine earlier than glycosides; (ii) the  $C_{max}$  of genistein in plasma being greater than that of daidzein while urine excretion of daidzein is greater than genistein. However, which form has greater bioavailability, aglycone or glucoside? This is still an enigma.

### **6.5.2. Isoflavone Contents in the Biscuits**

The soy flour biscuits produced contained 70g wheat flour, 30g soy flour, and 35g butter per meal. The taste seemed to be acceptable as either a breakfast or snack by most people, even those who don't normally like soy food very much.

In the batch of soy flour used, the isoflavone contents were relatively low compared with previous reports (Wang & Murphy, 1994a; Wang & Murphy, 1994b; Mazur, 1998), although the glycitin/glycitein content was relatively high. Variability between batches of flour for isoflavone content is well known. For different batches of soy flour, the amount of soy flour added in biscuits can be easily adjusted, thus the isoflavone contents in the biscuits can be targetted to any desirable amount, low, medium, or high, as experimental material to meet different requirements. The total isoflavone contents (including daidzein, glycitein, and genistein) were 50.6mg (aglycone equivalents) glycosides for the non-hydrolysed material (Food A) and 48.5mg aglycones for the hydrolysed material (Food B).

The enzyme selected performed very efficiently at de-glycosylation so that the food produced contained relatively “pure” aglycone, while other similar research (Richelle *et al.*, 2002; Tsangalis *et al.*, 2005; Cassidy *et al.*, 2006) used mixtures of aglycone and glycoside, although there was a higher proportion of aglycone compared to natural soy products without hydrolysis. As apple seeds are a kind of food waste (in juice production, for example), this may be a cheap source of the enzyme  $\beta$ -D-glucosidase compared with other commercial sources such as almond.

### 6.5.3. Soy Food Samples Used in Human Studies

Soy has been used frequently as natural food in human intervention studies on isoflavones. Soy milk is the most commonly applied followed by toasted soy nuts, soy protein or tofu, while other studies used other food preparations including cookies, chocolate/cereal bars (de Pascual-Teresa *et al.*, 2006; Song *et al.*, 2006), yoghurt (Mathey *et al.*, 2006), etc. Usually in these foods, soy isoflavones are presented as their natural glycosylated forms, i.e. glucosides and may be accompanied with certain proportions of acetyl or malonyl forms. Only the intake amount is adjusted by food preparation.

When aglycone forms of isoflavone are required specially in order to investigate the difference between aglycone and glycoside, enzymatic treatment need to be involved. There are two ways to prepare the experiment foods: choosing ready-made fermented soy foods, such as miso, tempeh (Cassidy *et al.*, 2006; Maskarinec *et al.*, 2008; Okabe *et al.*, 2011), or fermenting soy milk (Richelle *et al.*, 2002; Tsangalis *et al.*, 2005; Kano *et al.*, 2006).

de Pascual-Teresa *et al.* (2006) found that peak concentrations of genistein were attained earlier in serum following consumption of a liquid matrix rather than a solid matrix, accompanied by a lower urinary recovery. Cassidy *et al.* (2006) also observed that soy milk was absorbed faster and peak levels of isoflavones were attained earlier than with the other (solid) soy foods. So there would be differences between different food samples, say liquid or solid, although Xu *et al.* (2000) showed bioavailability was not affected by background diet or food sources.

For fermented soy milk, the enzymes used are commercially-available  $\beta$ -D-glucosidase (Richelle *et al.*, 2002; Kano *et al.*, 2006) or other materials that possess  $\beta$ -D-glucosidase activity, like active yeast (Kano *et al.*, 2006), probiotic biofidobacteria (Tsangalis *et al.*, 2005; Otieno *et al.*, 2006). However, some samples were not treated by high temperature after incubation with chosen enzyme materials (Richelle *et al.*, 2002; Tsangalis *et al.*, 2005; Kano *et al.*, 2006), thus the final foods consumed by subjects might contain materials with enzyme bioactivities, like live bacteria. Commercially-available fermented foods, usually active yeast is used, and the final products should be sterilized before sale. Kano *et al.* (2006) suggested that live bacteria with probiotic properties would affect intestinal flora, and thus would affect isoflavone metabolism even though unable to produce equol. This may be a reason to explain the contradictory results and needs to be considered in the future. Enzyme-treated soy biscuits were produced for the first time to manifest the real diet situation and, fortunately, the soy biscuits produced in this chapter had been baked at 230°C for more than 15mins and the enzyme activities were surely destroyed.

## 6.6. Conclusions

A novel, natural-style food, soy-based biscuits, has been developed which could be acceptable by many people and consumed as either a breakfast or snack. The biscuits could be used in a human intervention study to investigate the absorption and metabolism features of different forms of soy isoflavone *in vivo* via urine analysis in the future, given that the contents of isoflavone glycosides and aglycones can be experimentally manipulated.

In these foods, soy isoflavones exist as either glycoside forms or aglycone forms, have been adjusted by using apple seeds as a novel  $\beta$ -glucosidase enzyme source. The soy isoflavone concentrations of the biscuits were:

- In food A (unhydrolysed soy flour biscuits), there were  $31.86 \pm 1.0$  mg/ 100g daidzin,  $39.66 \pm 1.1$  mg/100g genistin,  $6.02 \pm 0.27$  mg/100g glycitin and  $3.29 \pm 0.31$  mg/100g genistein, making the total isoflavone  $50.6 \pm 0.6$  mg/100g as aglycone equivalents.
- In food B (hydrolysed soy flour biscuits), there were  $18.30 \pm 1.3$  mg/ 100g daidzein,  $26.60 \pm 1.0$  mg/100g genistein, and  $3.62 \pm 0.17$  mg/100g glycitein, making the total isoflavone  $48.5 \pm 1.1$  mg/100g as aglycone forms.

Apple seeds are a good source of  $\beta$ -D-glucosidase, and can be used to develop the novel food for isoflavone research, at any desirable isoflavone levels, with a more acceptable taste, and less cost. It may also be a cheap, valuable ingredient to improve food value by promoting absorption of isoflavones.

There is considerable inter-individual variation in the absorption and metabolism of isoflavones, in particular, the ability to produce equol from daidzein may have significant health impact. The biscuits could make a significant contribution to the study of human metabolism of isoflavones. Greater delivery of bioactive dietary compounds may have health benefits.