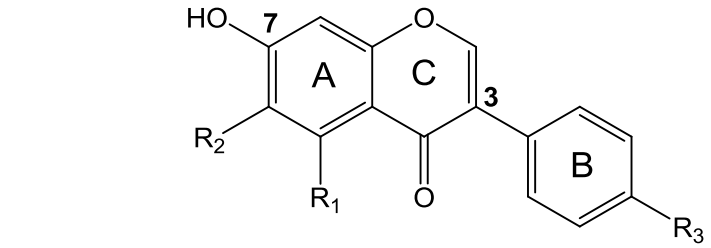
Chapter 5

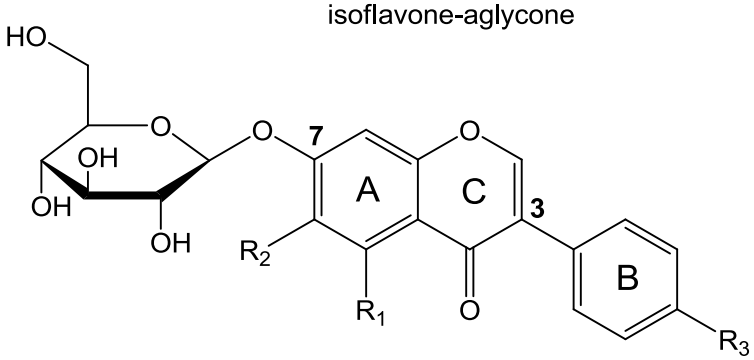
Identification of Potential Isoflavone Glycoside Sources

5.1. Introduction

Isoflavones are a structurally special group of flavonoids with the B-ring linked to the *C*-3 position of the C-ring. Isoflavones are known as phytoestrogens because they possess estrogen-like function, have attracted tremendous academic and commercial interest, especially in the last two decades. The structures of the most common isoflavones are shown in figure 5-1, illustrating the differences in the groups linked to the nuclear structure. If the OH group on the *C*-7 position reacts with one glucose molecule, it will become a 7-O-isoflavone glucoside (Wiseman, 2006; Cederroth & Nef, 2009). This is the most common glycoside form of isoflavones which exist naturally.



isoflavone-aglycone



isoflavone-glycoside

aglycone	glycoside	R_1	R_2	R_3
Daidzein	Daidzin (daidzein-7-O-glucoside)	H	H	OH
Genistein	Genistin (genistein-7-O-glucoside)	OH	H	OH
Glycitein	Glycitin (glycitein-7-O-glucoside)	H	OCH_3	OH
Formononetin	Sissotrin (formononetin-7-O-glucoside)	H	H	OCH_3
Biochanin A	Ononin (biochanin A-7-O-glucoside)	OH	H	OCH_3

Figure 5-1 Structures of common isoflavones

5.1.1. Distribution

Flavonoids are principally found in all types of higher plants. However, the distribution of isoflavones is largely restricted to the family *Leguminoseae*. Only few species have been reported as relatively rich in isoflavones, each with different isoflavone distribution patterns, which are summarized in figure 5-2 and table 5-1 (Cui, 2005; Marin *et al.*, 2005).

Figure 5-2 Plants reported rich in isoflavones. (For key, see table 5-1)

Table 5-1 Isoflavone contents of selected plants (Cui, 2005; Marin *et al.*, 2005)

Key (fig 5-2)	Plants (with *botanic name*)	Content of isoflavones
1	Alfalfa *(Medicago sativa)*	0.5-3.5%
2	Bean sprouts *(Vigna radiata)*	3.51mg/kg wet sample
3	Kudzu roots *(Pueraria lobata)*	0.95g/kg daidzein
4	Psoralea *(Psoralea corylifolia)*	2g/kg dry sample
5	Red clover *(Trifolium pratense)*	1.5-2.5%
6	Soy bean *(Glycine max)*	0.1-0.5%

Up to now, all of the materials reported to be rich in isoflavones are largely restricted to the subfamily *Faboideae,* family *Fabaceae*, order *Fabales,* class *Magnoliopsida*, division *Magnoliophyta*, kingdom *Plantae* (www.wikipedia.org/wiki/isoflavones, accessed 02/2007).

Alfalfa and red clover are flowering plants of the pea family cultivated as important forage crops and for increasing soil fertility due to their nitrogen fixing ability, and are native to Europe and the Middle East. Their tender shoots are eaten by man in some places as a leaf vegetable. Alfalfa and red clover have also been used as herbal medicines for over 1500 years (www.wikipedia.org/wiki/alfalfa, accessed 04/2007).

Psoralea and Kudzu are also used as herbal medicines, especially important in the Indian Ayurveda system of medicine and in Chinese medicine. *Psoralea* is a genus in the legume family (*Fabaceae*). Common names include tumble-weed and white tumble-weed. Most species are poisonous, but the starchy roots of *P. esculenta* and *P. hypogaea* are edible (www.wikipedia.org/wiki/psoralea, accessed 04/2007). Kudzu, *Pueraria lobata*, is one of about 20 species in the genus *Pueraria* in the pea family *Fabaceae*, subfamily *Faboideae*. It is native to the southeast of Asia. Kudzu root is a common food in the southeast of Asia, and its young leaves can be eaten not only by man, but also by animals and used as a soil fertility promoter (www.wikipedia.org/wiki/kudzu, accessed 04/2007).

Bean sprouts and soy beans are common foods in Asia. Normally there are two types of bean sprouts but mung bean sprouts are much more popular than soy bean sprouts.

Soy is the main protein source for many vegans and vegetarians. Tofu, a well-known processed soy bean product, is known as "meat for monks". There are thousands of processed soy bean foods available in China either fermented or non-fermented.

All of the above plants have been reported to be rich in isoflavones, and consequently have become research targets regarding human health especially the soy bean and red clover due to their ready availability (Radd & Setchell, 2003).

5.1.2. Isoflavone Pattern

Although some species of the subfamily *Faboideae* are rich in isoflavones, their isoflavone patterns are different, in other words, they contain different isoflavones. The following table shows the differences between soybean and red clover (Swinny & Markham, 2003; Visnevschi-Necrasov *et al.*, 2009), kudzu roots (Kirakosyan *et al.*, 2003a; Cherdshewasart *et al.*, 2007; Lau et *al.*, 2009), chick pea (Kuhnle *et al.*, 2009), and alfalfa (Mazur, 1998; Farag *et al.*, 2007).

Table 5-2 Isoflavone distribution pattern in different food plants

Material	Major isoflavones
Soy bean (*Glycine max*)	Daidzein, Genistein, Glycitein
Red clover (*Trifolium pratense*)	Daidzein, Genistein, Formononetin, Biochanin-A
Kudzu roots *(Pueraria lobata*)	Puerarin, Daidzein, Genistein
Chick pea (*Cicer arieyinum*)	Biochanin-A, Genistein
Alfalfa (*Medicago sativa*)	Formononetin, Coumestrol

5.1.3. The Factors Affecting Isoflavone Contents

There are many factors that can affect the isoflavone contents of plants, of which the most important are listed below.

5.1.3.1. Species and Cultivars

Undoubtedly, the genetic background is one of the most important factors affecting isoflavone content (Pietta & Mauri, 2001; Fleuriet & Macheix, 2003). The variation among species and cultivars leads to significant differences in both isoflavone contents and composition. As mentioned previously, the isoflavone contents and patterns differ in species although the distribution is mainly restricted to the legume family. Several groups have reported large differences in the contents of isoflavones in different species (for example, Sun & Ding, 1998; Cassidy *et al.*, 2000; Cherdshewasart *et al.,* 2007). In different varieties of soy bean, the contents of isoflavones could range from several hundreds of micrograms per gram to several thousands of micrograms per gram (Wang & Murphy, 1994a; Reinli & Block, 1996; Mazur, 1998).

5.1.3.2. Tissue Localization

The distribution of isoflavones is significantly different in different parts of the plants. In soy bean, most isoflavones exist in the cotyledon and hypocotyl, with a trace in the seed coat. 80-90% of the isoflavones are distributed in the cotyledon with a concentration of 0.1-0.3%, while in the hypocotyl the concentration of isoflavones can reach 1-2% (which makes up 10-20% of total isoflavone content although the hypocotyl makes up only 2% of soy seeds) (Cui, 2005; Marin *et al.* 2005). In kudzu, most isoflavones are found in the roots and seeds with traces in the leaf (Mazur & Adlercreutz, 1998; Kirakosyan *et al.*, 2003a).

5.1.3.3. Physiological Stage of Growth

Concentrations of flavonoids in a plant organ result from a balance between biosynthesis and further metabolism, including turnover and catabolism. Considerable variations are generally observed in the amount of flavonoids according to the physiological stage when plant organs are picked to be consumed or processed by humans. This may concern each type of organ (leaves, flowers, stalks, tubers, roots, etc.), and the most spectacular cases are those of fruits, as considerable variations in phenolic compounds occur during maturation (Fleuriet & Macheix, 2003). Flavonoids

have also been sometimes implicated in the control of plant growth, maturation and abscission.

Concentrations of soluble forms of flavonoid conjugates are generally highest in young fruits, with a maximum during the early weeks after blossoming and a rapid decrease during fruit development (Mayr *et al.*, 1995). Some scientists suggested dividing the life of a fruit into two main periods (Fleuriet & Macheix, 2003). In the first period, flavonoid derivatives accumulate in the fruit with a positive balance among *in situ* biosynthesis, migration and possible re-utilization, while in the second period, this balance becomes negative and the overall flavonoid levels in the fruit falls.

Raw materials in different physiological stages have different isoflavone contents (Dueñas *et al.*, 2009). Sun and Ding (1998) reported that in soy beans, the isoflavone contents decreased gradually during storage and increased gradually with soybean seed development. The content of isoflavones increased after blossoming for 1-2 months, and more than 76% of isoflavones can be accumulated in the last week of seed development, due to the accumulation of seeds and the loss of water content during seed maturation as well.

5.1.3.4. Environmental Factors

Secondary metabolism, and in particular phenolic metabolism, largely depends on external factors such as light, temperature, and various stresses (Fleuriet & Macheix, 2003; Lee *et al.*, 2007; Cherdshewasart *et al.*, 2007), which leads to the correlation between flavonoid contents and environmental factors.

Sun and Ding (1998) reported that there existed a positive correlation between isoflavone content in soy bean with latitude, but a negative correlation with longitude and also a positive correlation with elevation above sea level. Moreover, there was a negative correlation of isoflavone content with temperature and precipitation; a positive correlation with duration of sunshine. Light strongly stimulates the accumulation of isoflavones in soybean seedlings, especially in soybean leaves. However, the isoflavone contents tended to decline with higher soil moisture and higher fertility. Caldwell *et al.* (2005) also reported that increasing the temperature

from 18°C during seed development to 23°C decreased total isoflavone content by about 65% while a further 5°C increase to 28°C decreased the total isoflavone content by about 90%. They found that elevated CO_2 at elevated temperatures could partially reverse the effects of temperature on soybean seed isoflavone content.

The individual isoflavones often had different responses to the various growth conditions during seed maturation, modifying the proportions of the principal isoflavones. Therefore, subtle changes in certain environmental factors may change the isoflavone content of commercially grown soybean, altering the nutritional values of soy products (Caldwell *et al.*, 2005).

5.1.3.5. Food Processing

Food processing is an important factor which can affect not only the contents of total isoflavones in a food, but also the pattern of isoflavones before the food is consumed by humans (Sun & Ding, 1998). Food processing can cause significant amounts of isoflavone loss, processes which include:

- Soaking

  Soaking in water or basic solution is the first stage for production of some soy products like tofu, which may cause loss of up to 12% total isoflavones (Cui, 2005). Isoflavones were largely removed during water processing and also the pattern changed. After soaking, the contents of the acetyl form and the aglycone form increased accompanying the decreasing of the malonyl form of isoflavones because of the activity of endogenous enzyme β-glucosidase (Wardhani *et al.*, 2008).

- Heating

  Flavonoids have been reported to show a significant reduction during the thermal treatment, e.g. flavonols in onions (Price *et al.*, 1997) and broccoli (Price *et al.*, 1998a). In contrast, isoflavones are relatively stable molecules. Heating itself cannot change the contents of isoflavones but may change the pattern (Barnes *et al.*, 1994). As with soaking, heating can cause an increase of the acetyl form and the aglycone form resulting from the decrease of the malonyl form since thermal

treatment can stimulate the endogenous enzyme. Moreover, heating in water can significantly improve the isoflavone transferring into water leading to the loss of isoflavones (Sun & Ding, 1998).

- Solidifying/Gelatinizing

Solidifying can exclude isoflavones, so that they remain in the liquid phase, which may cause up to 44% isoflavone loss (Cui, 2005).

- Fermenting

Fermenting may not change the total content of isoflavones in soy food but may change the pattern. As mentioned in chapter 1, most flavonoids exist as the glucoside forms naturally. However, large amounts of the aglycone form can be found in fermented food and in some products nearly 100% of isoflavone exists as the aglycone form due to the hydrolases from microbial sources (Wang & Murphy, 1994b; McDonald *et al.*, 1998; Chun *et al.*, 2008; Haron *et al.*, 2009).

5.1.4. Determining Isoflavone Contents

Regarding flavonoid analysis, the majority of published work now refers to qualitative and quantitative applications of high performance liquid chromatography (HPLC). Flavonoids can be separated, quantified, and identified in one operation by coupling HPLC to ultraviolet (UV), mass, or nuclear magnetic resonance (NMR) detectors. Recently, the technique of capillary electrophoresis (CE) has been gaining attention (Marston & Hostettmann, 2006).

One feature used for flavonoid detection is the presence of the phenyl ring. This chromophore is UV active and enables flavonoids to be easily detected. Their UV spectra are particularly informative, meaning that minor differences in structure are often seen as significant differences in their UV spectra, providing considerable structural information that can rapidly distinguish the type of phenol and the oxidation pattern. Modern instrumental techniques enable us to gain much information regarding the mass and UV-VIS spectra of individual components in a complex mixture. For the purposes of analysis, the flavonoids can be basically classified into

three types: flavonoid glycosides, non-polar flavonoids (aglycones, methylated, or alkylated flavonoids), and anthocyanins. Each type requires a different analytical technique (Bloor, 2001).

Preparative separation of flavonoids is still a challenge. There is no general, simple, straightforward strategy for the isolation of natural products, even if certain compounds are readily accessible by modern chromatographic techniques. Each particular separation problem has to be considered on its own and a suitable procedure has to be developed. A number of techniques have been used for the preparative separation of flavonoids. The choice of methods and strategies varies from research group to research group and depends often on the class of flavonoid studied (Swinny & Markham, 2003).

However, analytical separations of flavonoids are now routine. In quantitative measurements, the amounts of the individual components within a particular class of constituent need to be determined. Nowadays, this can easily be achieved through the use of GC, HPLC, and hyphenated techniques (Marston & Hostettmann, 2006).

5.1.4.1. Sample Preparation

Principally, the analysis of flavonoids should include extraction, separation (isolation), purification and determination, etc. However, due to the application of HPLC which can be used for either separation or determination, there may be no clear border between those steps. The analysis may simply be divided into sample preparation and sample determination. Sample preparation can include extraction, initial separation or purification until ready for HPLC injection.

Because flavonoids (particularly glycosides) can be degraded by enzyme action when collected plant material is fresh or non-dried, it is thus advisable to use dry, lyophilized, or frozen samples. When dry plant material is used, it is generally ground into a powder. For extraction, the solvent is chosen as a function of the type of flavonoid required. Polarity is an important consideration here. Less polar flavonoids (e.g., isoflavones, flavanones, methylated flavones, and flavonols) are extracted with chloroform, dichloromethane, diethyl ether, or ethyl acetate, while flavonoid

glycosides and more polar aglycones are extracted with alcohols or alcohol-water mixtures. Glycosides have increased water solubility and aqueous alcoholic solutions are suitable. The bulk of extractions of flavonoid-containing material are still performed by simple direct solvent extraction (Marston & Hostettmann, 2006).

Powdered plant material can also be extracted in a Soxhlet apparatus, first with hexane, for example, to remove lipids and then with ethyl acetate or ethanol to obtain phenolics. This approach is not suitable for heat-sensitive compounds.

A convenient and frequently used procedure is sequential solvent extraction. A first step, with dichloromethane, for example, will extract flavonoid aglycones and less polar material. A subsequent step with an alcohol will extract flavonoid glycosides and polar constituents.

Extraction is typically performed with magnetic stirring or shaking but other methods have recently been introduced to increase the efficiency and speed of the extraction procedure, such as pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), ultrasound-assisted extraction, and microwave-assisted extraction (MAE) (Marston & Hostettmann, 2006).

5.1.4.2. HPLC Determination

The method of choice for the qualitative and quantitative analysis of flavonoids is HPLC. Since its introduction in the 1970s, HPLC has been used for all classes of flavonoids and hundreds of applications have been published. The most frequently used detection method for HPLC is UV spectrophotometry. Routine detection in HPLC is typically based on measurement of UV absorption, or visible absorption in the case of anthocyanins (Marston & Hostettmann, 2006). No single wavelength is ideal for all classes of flavonoids since they display absorbance maxima at distinctly different wavelengths. The most commonly used wavelength for routine detection has been 280 nm, which represents a suitable compromise.

With the introduction of diode-array technology in the 1980s, a further dimension is now possible because coupled LC-UV with diode array detection (DAD) allows the

chromatographic elute to be scanned for UV-visible spectral data, which are stored and can later be compared with a library for peak identification. This increases the power of HPLC analysis because with the information from the UV spectrum, it may be possible to identify the compound subclass or perhaps even the compound itself. UV spectral data of 175 flavonoids in several solvents can be found, for example, in a book by Mabry *et al.* (1970). LC-UV with DAD enables simultaneous recording of chromatograms at different wavelengths. This improves the possibilities of quantification because detection can be performed at the wavelength maximum of the compound in question. These are typically to be found at 270 and 330 to 365nm for flavones and flavonols, at 290nm for flavanones, at 236 or 260nm for isoflavones, at 340 to 360nm for chalcones, at 280nm for dihydrochalcones, at 502 or 520nm for anthocyanins, and at 210 or 280nm for catechins (Merken & Beecher, 2000).

Peak purity can also be determined. The spectra of eluting peaks obtained at the apex and both inflexion points of the peak can be compared in order to obtain a measure of the purity of the particular component of the sample.

5.2. Aims of Chapter

The aim of this chapter was to obtain and analyse some natural materials which are reported to contain isoflavones, especially daidzin and genistin, and to investigate to what extent these materials contain isoflavones, particularly related to highest levels.

5.3. Materials and Methods

5.3.1. Sources of Plant Materials

The plant materials were obtained as described in chapter 2.

5.3.2. Acid and Base

- 1M hydrochloric acid: 8.28ml of hydrochloric acid (min 37%) was diluted by water and made up to a final volume of 100ml
- 6M sodium hydroxide: 4.8g of sodium hydroxide was dissolved in water and made up to a final volume of 20ml

5.3.3. Sample Preparation

Dried samples were ground, put in air-tight glass bottles, sealed, labelled and stored at -20°C until taken for further analysis. Fresh samples needed to be freeze-dried as soon as possible after purchase. Fresh samples were separated, weighed, and homogenised with 200ml water by blending for 3mins. After measuring the volume, 50ml of the homogenate was transferred to a 250ml round bottom flask, frozen by a mixture of dry ice and acetone, then transferred to a Birchover Instruments Freeze Dryer for 5-18hr for freeze-drying. When using the Scanvac Coolsafe Freeze Dryer, 50ml homogenate was frozen in a -80ºC extra low freezer before freeze-drying.

After being freeze-dried, the sample was carefully removed and powdered with a pestle and mortar, weighed, put into air-tight glass bottles, sealed, labelled, and stored at -20°C until analysis.

5.3.4. Isoflavone Extraction

Powdered sample (1g or 0.5g) was weighed and put in a 50ml test tube. 20ml of 80% methanol was added. The test tube was vortexed for 1min and then incubated in a shaking water bath at 37°C for 2.0hr. The solution was then filtered through Whatman No. 40 filter paper. 80% methanol was used to wash the filter paper and make the final filtrate to 25ml. An aliquot of this solution was then filtered through a 0.2µm PTFE filter and analysed by HPLC. The resultant chromatograms were examined for evidence of flavonoid glucosides or aglycones.

For the low content materials, 10-20g of raw sample was weighed prior to extraction, and the final extracted solution might be concentrated by evaporation before HPLC injection, dependent on the concentration of isoflavones.

5.3.5. Enzymatic Hydrolysis

In order to identify HPLC peaks, especially if there were some unknown components and in the absence of purified standard, all samples were hydrolysed by enzyme after extraction. Aliquots (1.0ml or 0.5ml) of the extract solution from 5.3.4 were removed into 2ml micro-centrifuge tubes, evaporated with a rotary evaporator or with the Genevac to dryness. Then 600µl buffer (0.1M, pH 5.5) and 150µl apple seed extracts (described as 3.3.4.) were added and the tubes were incubated at 65°C for 2hr. After incubation, 750µl of methanol was added to stop the reaction. The reacted solution was then removed and filtered through a 0.2µm PTFE filter and analysed by HPLC. The retention time and spectral characteristics were examined for evidence of flavonoid aglycones.

5.3.6. Acidic Hydrolysis

For some components which could not be hydrolysed by β-glucosidase, acidic hydrolysis was carried out by adding 300µl 1M hydrochloric acid (HCl) into a micro-centrifuge tube containing dried residue from 0.5ml extract solution. After incubating

for 2hr in a boiling water bath, the solution was neutralized by 50µl 6M sodium hydroxide (NaOH), then 400µl buffer (pH5.5, 0.1M) was added to buffer the pH between 5 and 6, and then 750µl methanol was added to make the final volume 1.5ml. This solution can be injected onto the HPLC after filtration. This method was modified from the method described by Garrett *et al.* (1999).

5.3.7. Recovery

In order to test the efficiency of extraction, experiments were carried out by adding specific amounts of daidzin and genistin to the raw material immediately before extraction and calculating the percentage recovery. For extracting soy flour, purified standard daidzin and genistin were used. For extracting other materials, previously determined soy flour extracts were used (10g of soy flour was weighed accurately, and evaporated to 25ml after being extracted by 80% methanol. This solution was analysed for concentrations of daidzin and genistin in triplicate by HPLC).

5.4. Results and Discussion

The chromatograms of the tested materials are shown below followed by peak identification, isoflavone contents, and recovery (if applied). Peaks were identified on the following criteria: (i) comparison to behaviour of standards; (ii) characteristics of peak spectra; (iii) behaviour after enzymatic or acid hydrolysis; (iv) comparison to literature reports. Some compounds, for example, acetyl- and malonyl- form of soy isoflavones, were not available in standard form because these components are still not widely commercially available may be due to they are relatively unstable (Farag *et al.*, 2007; Rostagno *et al.*, 2009). As a result, enzymatic or acidic treatments in sample preparation for isoflavone determination have been widely used and the results have been broadly accepted, for example, Mazur & Adlercreutz (1998), Liggins *et al.* (2000), Kuhnle *et al.* (2009).

5.4.1. Soy Flour

Chromatograms of soy flour are showed in figure 5-3, including unhydrolysed and hydrolysed by apple seed extracts. The isoflavone contents of toasted soy flour were estimated in table 5-3 with the recoveries of daidzin and genistin.

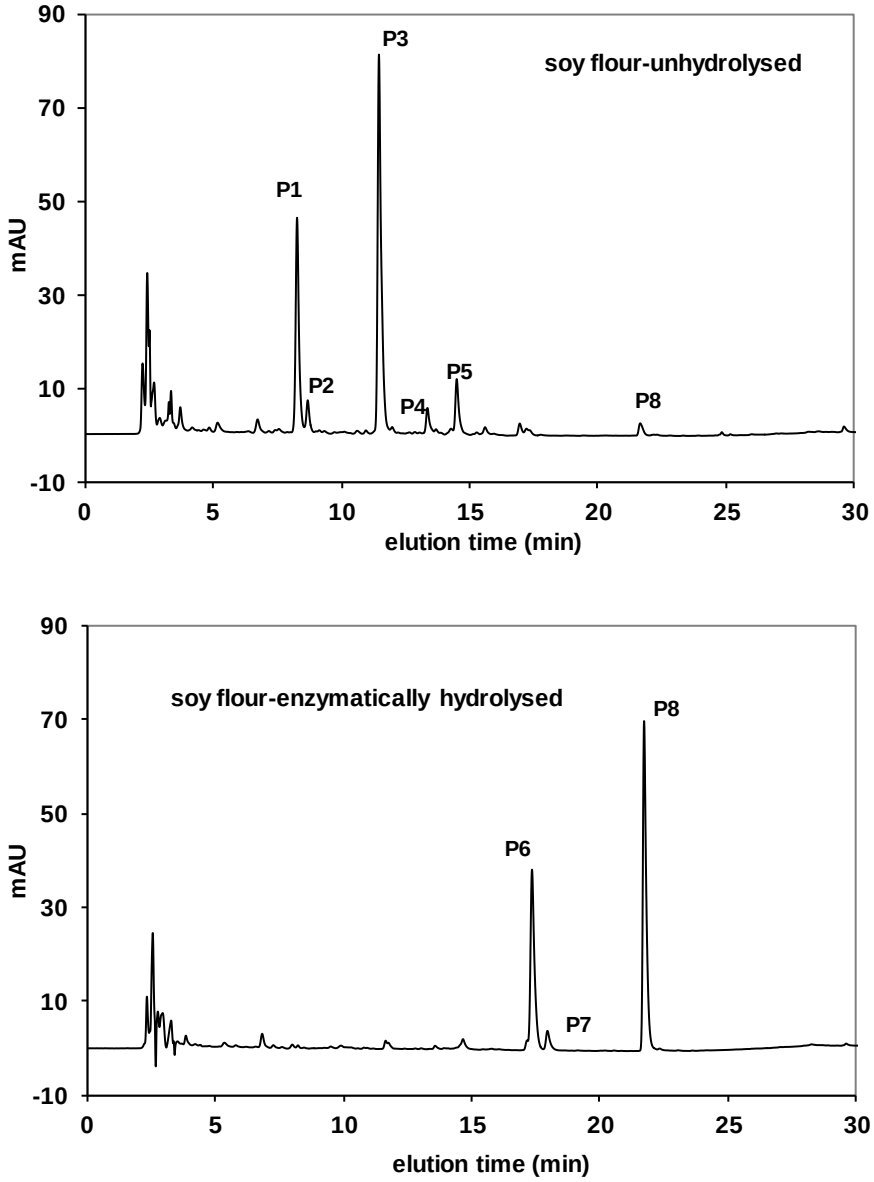


Figure 5-3 Chromatograms of toasted soy flour. Top: unhydrolysed; Bottom: hydrolysed by apple seed extracts. (For discussion of peaks, see text)

From the chromatograms in figure 5-3, peaks could be identified as: P1: daidzin; P2: glycitin; P3: genistin; P6: daidzein; P7: glycitein; P8: genistein. P4 and P5 are

probably acetyl- or malonyl- forms of daidzin or genistin; their spectra were very similar to daidzin and genistin, but could not be further identified due to lack of purified components.

In soy flour, isoflavones exist as glucoside forms which are mainly daidzin and genistin, and small amounts of glycitin, small amounts of acetyl or malonyl forms, and small amounts of aglycone forms. After hydrolysis, the peaks representing glucoside forms, which were P1, P2 and P3, disappeared or decreased sharply; the peaks representing acetyl or malonyl forms also decreased (P5) or disappeared (P4); while the peaks representing aglycone forms, which were P6 and P8, increased sharply; and P7, a new peak, appeared, which is probably glycitein, the aglycone form of glycitin.

The isoflavone contents of toasted soy flour were estimated in table 5-3 together with the recoveries of daidzin and genistin.

Table 5-3 Isoflavone contents in four samples of toasted soy flour (mg/100g)

	1	2	3	4	Average ± SD (mg/100g, n = 4)	Recovery ± SD (%, n = 4)
Daidzin	108.88	108.6	102.34	99.68	104.88 ± 4.60	95.05 ± 2.28
Genistin	125.82	128.63	130.28	129.63	128.59 ± 1.97	94.28 ± 1.63
Glycitin*	28.76	30.32	29.98	29.54	29.65 ± 0.68	--
Daidzein	ND	ND	ND	ND	--	--
Genistein	9.98	10.32	10.87	10.76	10.48 ± 0.42	--
Total**	168.45	173.32	170.26	166.88	170.68 ± 2.47	--

600µl of daidzin and genistin stock solutions (500µg/ml) were used for recovery determination

*calculated as daidzin equivalents

**calculated as aglycone equivalents after hydrolysis

SD: standard deviation; ND: not detected

5.4.2. Broad Bean

Broad bean has been reported to be rich in isoflavones especially daidzin (Mazur *et al.*, 1998). However, this experiment did not detect high concentrations of daidzin in the sample analysed. The isoflavone contents determined were very low as seen in figure 5-4.

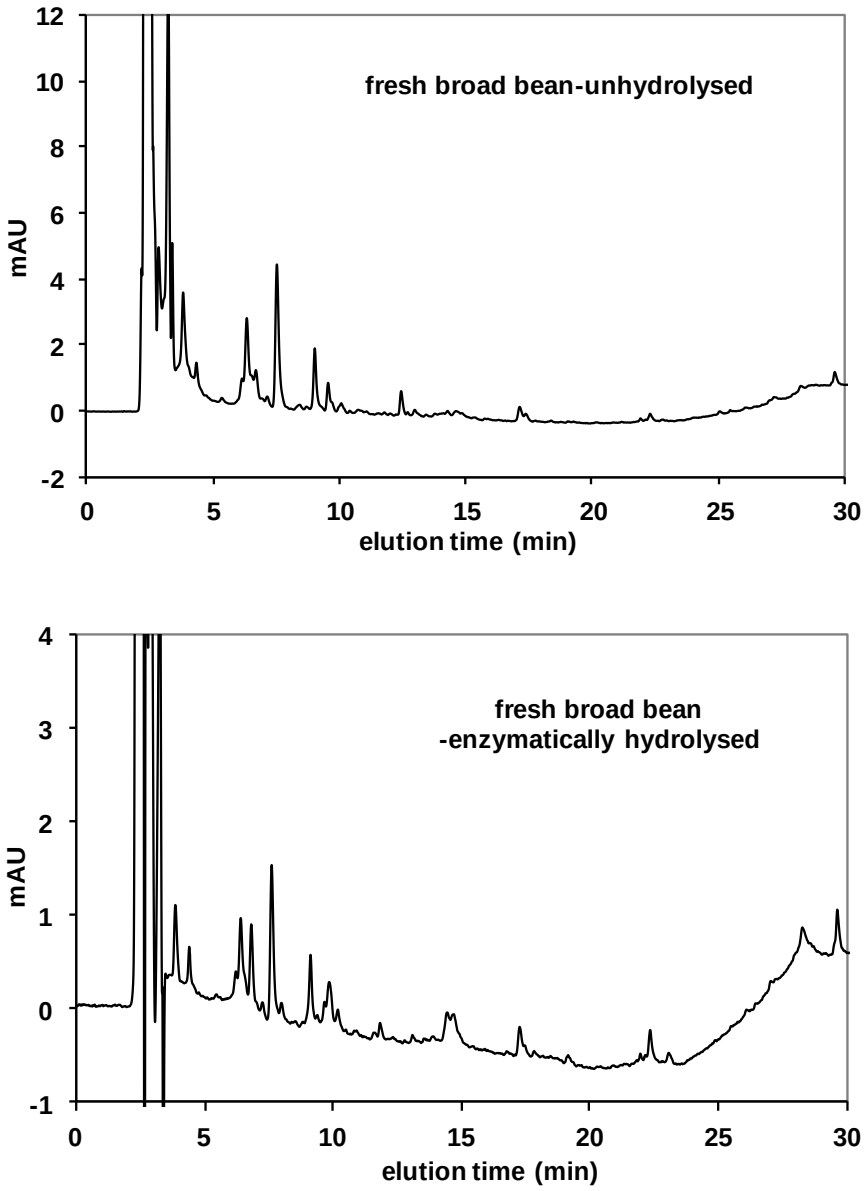


Figure 5-4 Chromatograms of broad bean (whole bean). Top: unhydrolysed; Bottom: hydrolysed by apple seed extracts. No peak can be identified as isoflavone.

5.4.3. Chickpea

Chick pea was found to contain certain levels of isoflavones, mainly genistein and biochanin-A. The chromatograms of chickpea are shown in figure 5-5.

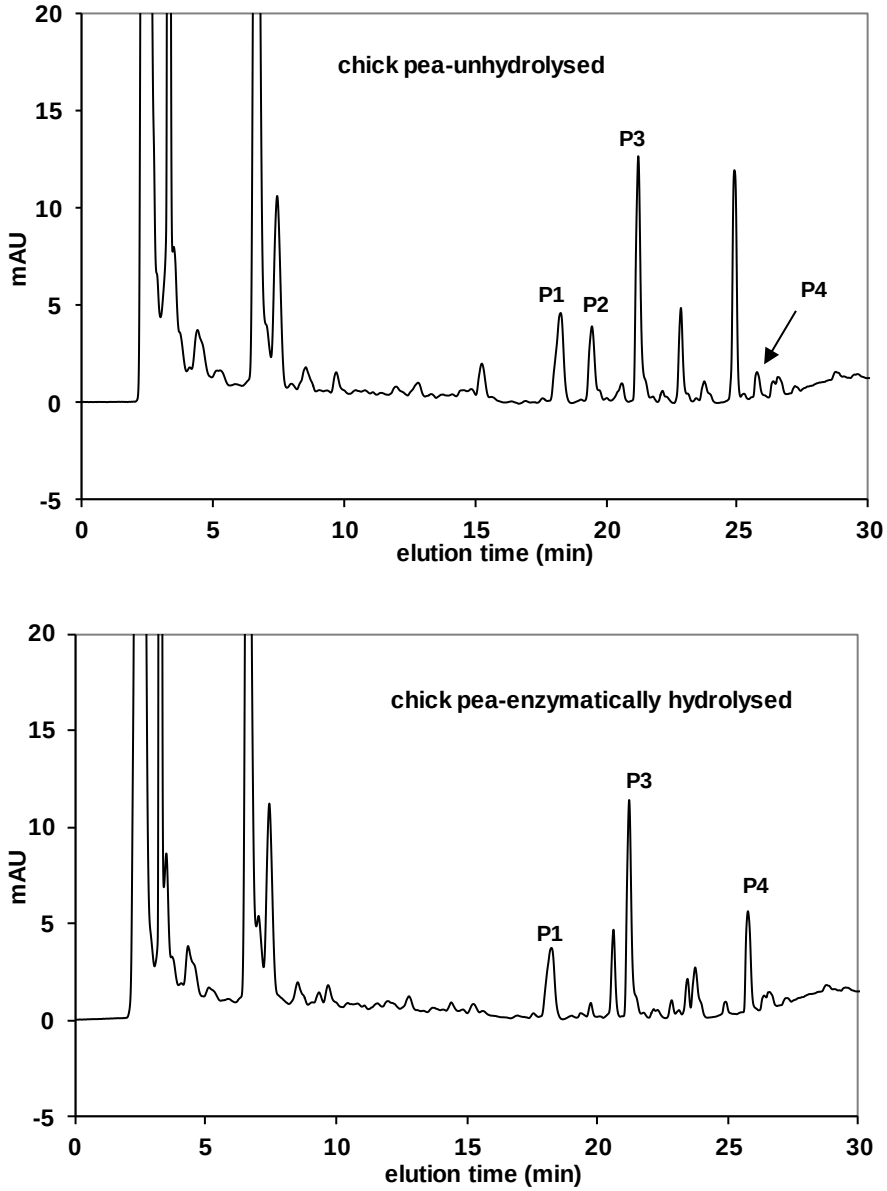


Figure 5-5 Chromatograms of chickpea. Top: unhydrolysed; Bottom: hydrolysed by apple seed extracts. (Peaks identified: P1: daidzein; P2 maybe ononin (biochanin-A-7-glucoside); P3: genistein; P4: biochanin-A)

5.4.4. Clover

Chromatograms of clover extracts are shown in figure 5-6, both of them were chromatograms of an hydrolysed sample but the different scales of the y axis emphasizing the presence of peaks other than formononetin and biochanin-A.

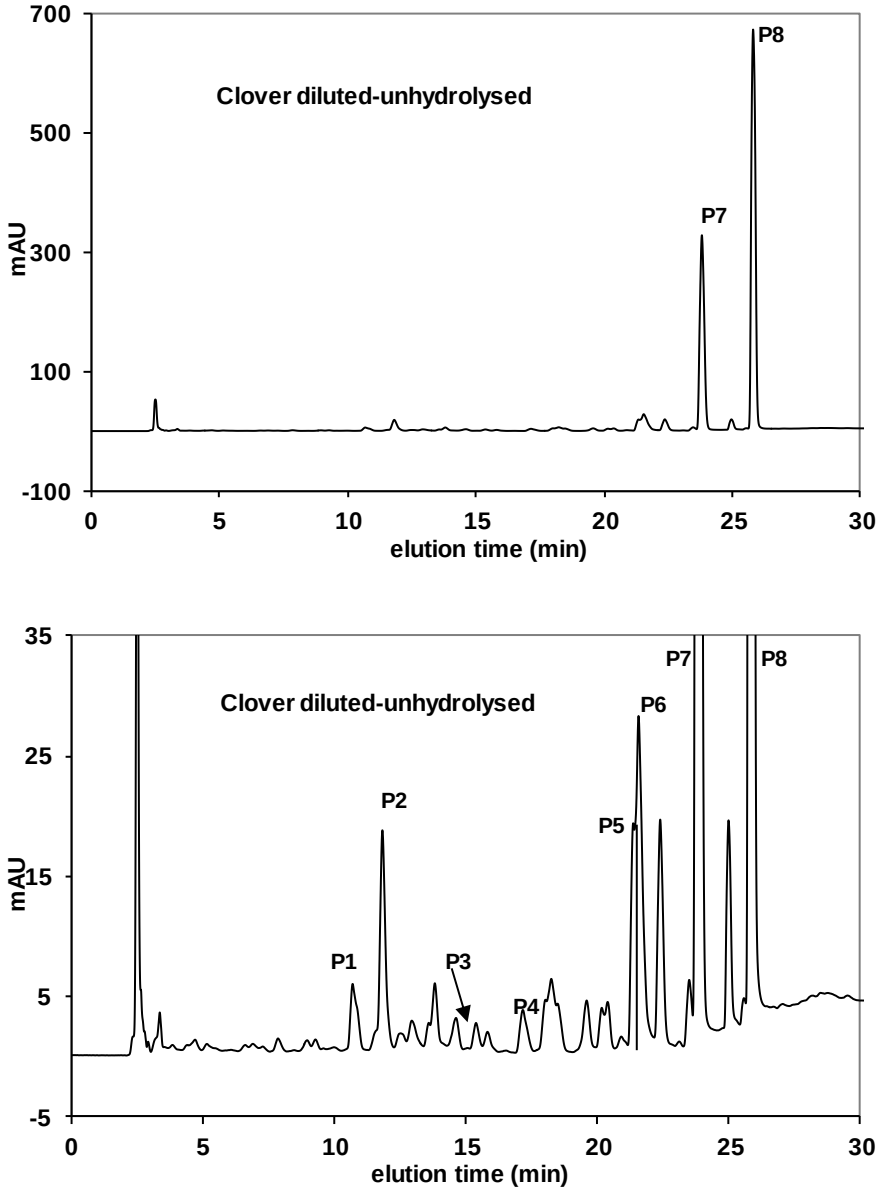


Figure 5-6 Chromatograms of clover. Top: original extract; Bottom: original extract but changing Y axis scale in order to show other peaks. Both chromatograms were of an unhydrolysed sample. (Peaks identified: P1: rutin; P2: quercetin-3-glucoside; P3: maybe sissotrin (formononetin-7-glucoside); P4: daidzein; P5: maybe ononin (biochanin-A-7-glucoside); P6: genistein; P7: formononetin; P8: biochanin-A)

The biggest two peaks observed in figure 5-6 were formononetin and biochanin-A, indicating that these two isoflavones were the predominant components in clover, greater in amount than daidzein and genistein. The UV spectra of formononetin and biochanin-A are shown in figure 5-7. However, their concentrations could not be determined due to the unavailability of standard.

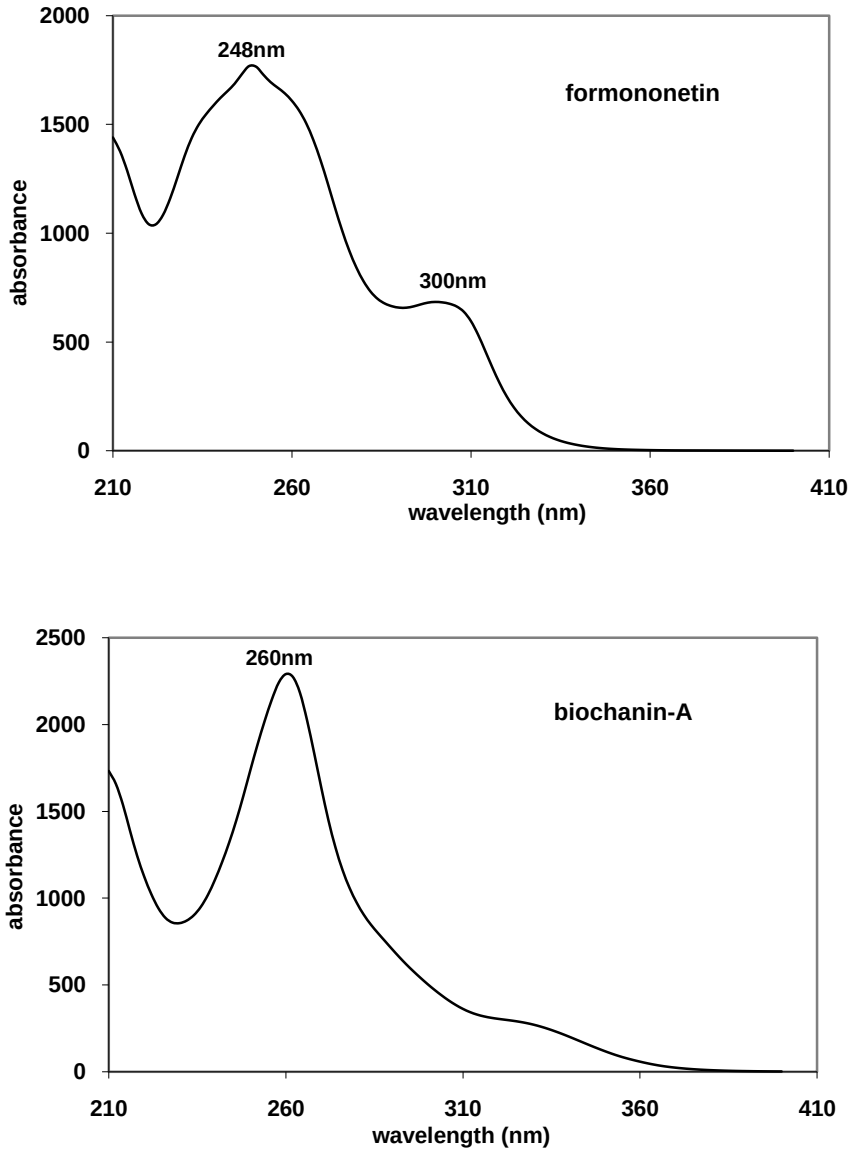


Figure 5-7 UV Spectra of formononetin and biochanin-A. Absorption maxima have been reported to be 248nm for formononetin, 300nm and 260nm for biochanin-A respectively (adapted from Mabry *et al.*, 1970).

5.4.5. Kudzu

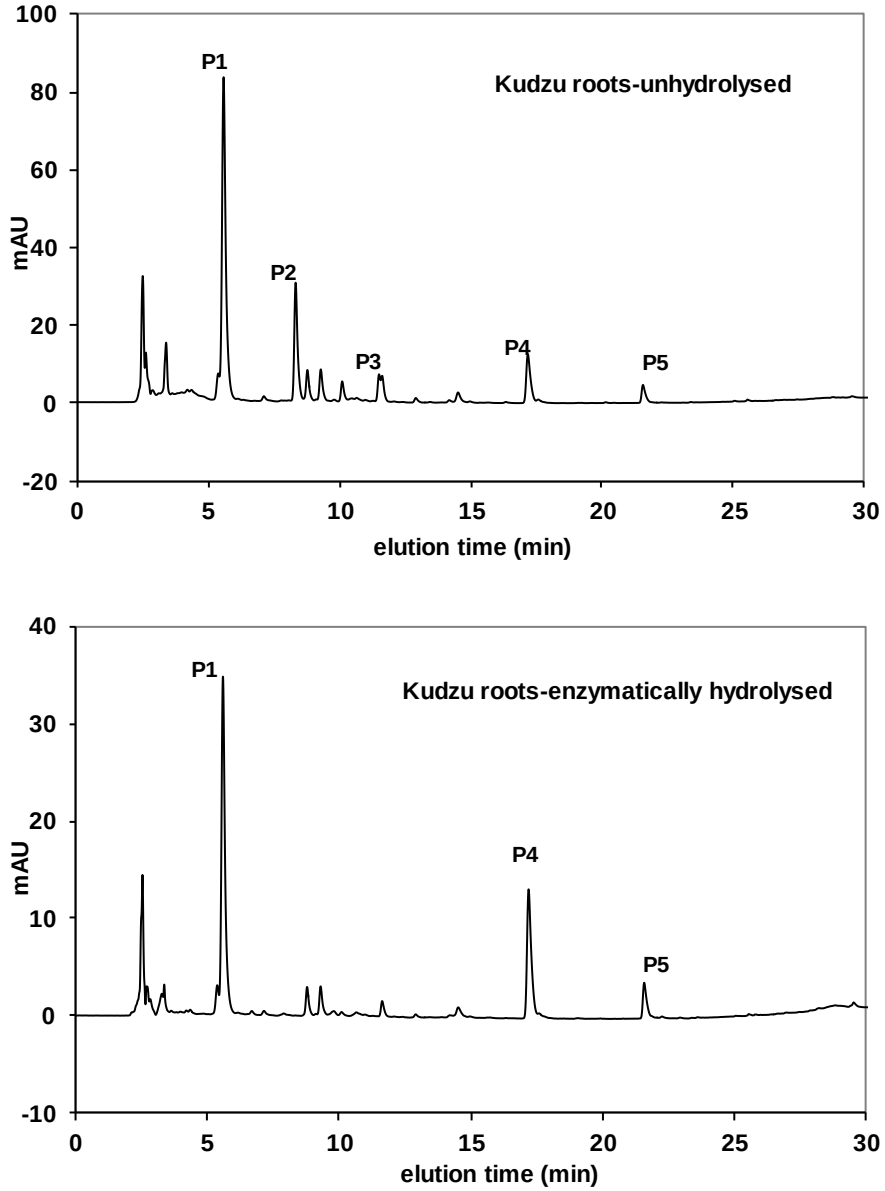


Figure 5-8 Chromatograms of kudzu roots. Top: unhydrolysed; Bottom: hydrolysed by apple seed extracts. (Peaks identified: P1: puerarin; P2: daidzin; P3: genistin; P4: daidzein; P5; genistein)

Kudzu (chromatograms showed in figure 5-8) shows interesting isoflavone contents. Puerarin is daidzein-8-C-glucoside, the other glucoside form of daidzein, which can also produce daidzein after being hydrolysed. The spectrum of puerarin is shown in figure 5-9. The isoflavone contents of kudzu were estimated in table 5-4, with the

content of puerarin calculated as daidzin equivalents. More isoflavones existed as the aglycone forms in kudzu than in soy.

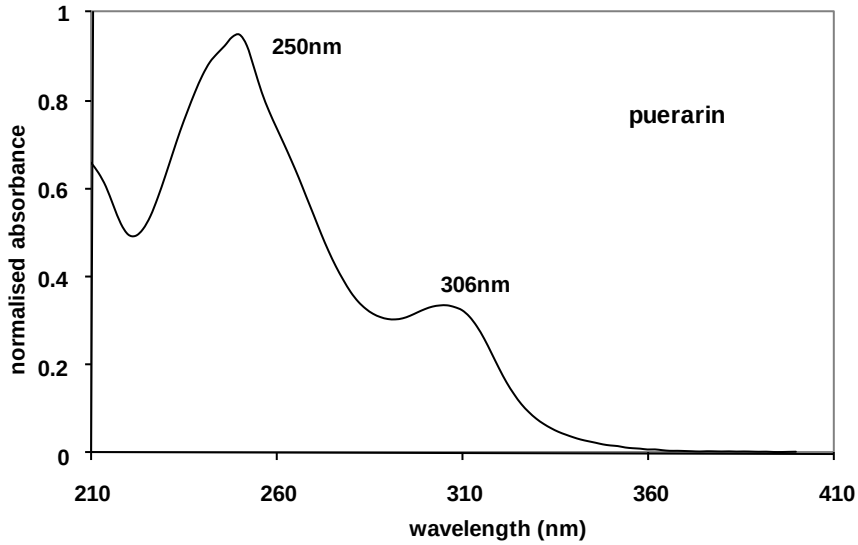


Figure 5-9 Spectrum of puerarin, highest absorbance appeared at 250nm and 306nm.

Table 5-4 Isoflavone contents in three samples of kudzu (mg/100g)

	1	2	3	Average ± SD (mg/100g, n = 3)	Recovery ± SD (%, n = 3)
Daidzin	64.38	63.62	66.73	64.91 ± 1.63	94.09 ± 2.17
Genistin	8.36	7.75	7.92	8.01 ± 0.32	93.41 ± 1.84
Puerarin*	202.80	208.82	205.33	205.65 ± 3.03	--
Daidzein	12.37	11.82	12.06	12.08 ± 0.28	--
Genistein	22.68	20.84	24.52	22.68 ± 1.84	--
Total	310.59	312.85	316.56	313.33 ± 3.02	--

800µl of soy flour extract solution was used for recovery determination

*calculated as daidzin equivalents

SD: standard deviation

5.4.6. Mung Bean

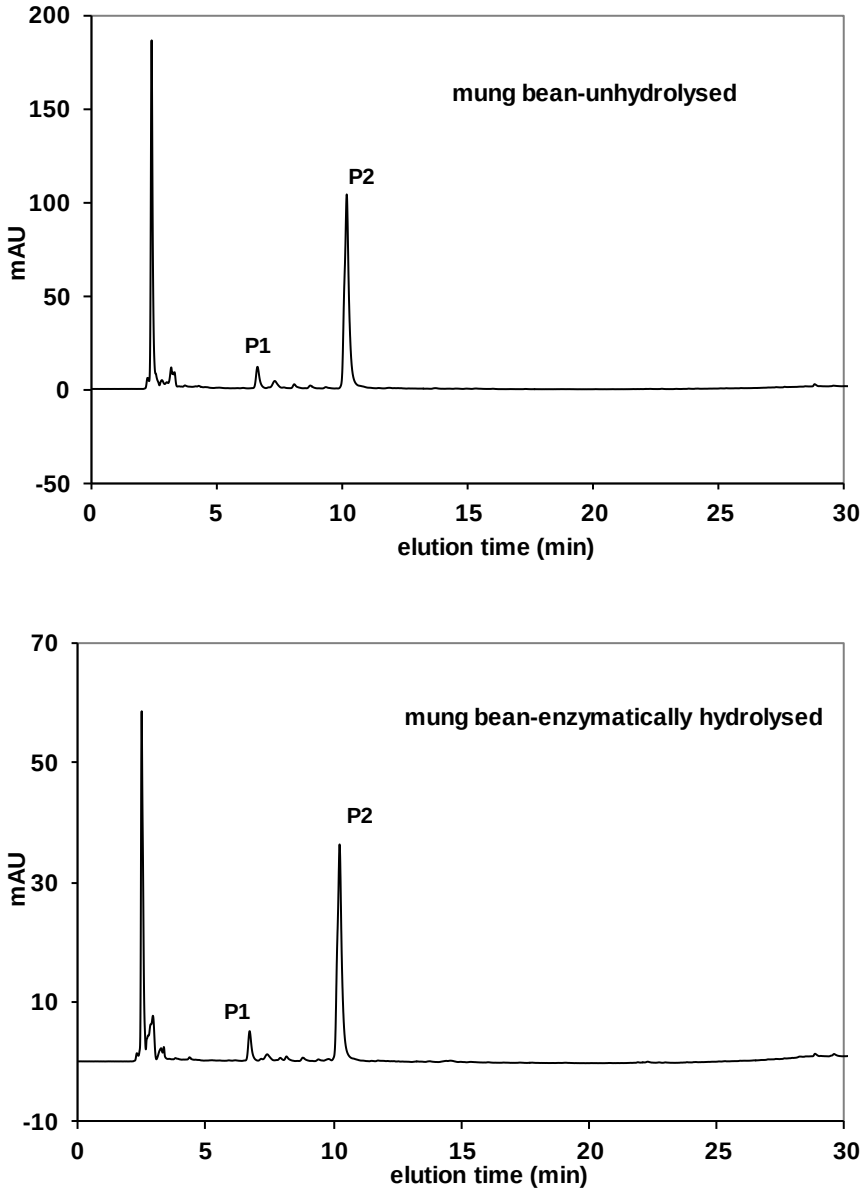


Figure 5-10 Chromatograms of dried mung bean extracts. Top: unhydrolysed; Bottom: hydrolysed by apple seed extracts. No peak can be identified as isoflavone. For discussion of P1 and P2, see text.

No peak could be identified as isoflavone in this mung bean extracts (chromatograms shown in figure 5-10), but the UV spectra of peaks, shown in figure 5-11, were characteristic of a flavonoid and P2 might be some form of apigenin. (see figure 2-6) based on similarity of the spectra.

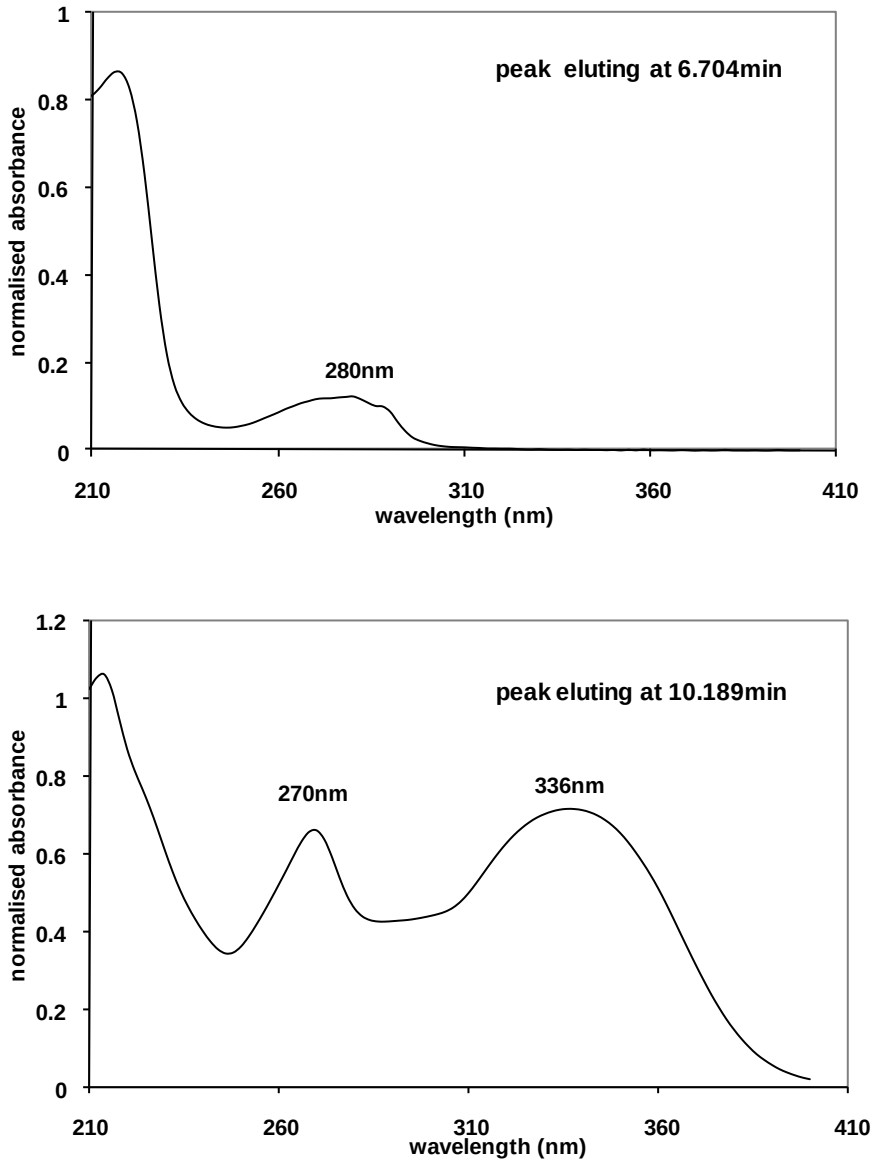


Figure 5-11 Spectra of the peaks in mung bean extracts. Absorbance maxima appeared at 280nm for peak 1 (eluting at 6.704min), 270nm and 336nm for peak 2 (eluting at 10.189min).

Mung bean has also been reported to be rich in phytoestrogens (Mazur, 1998; Mazur *et al.*, 1998), but it looks like there were no isoflavones in the extracts produced here.

However, in bean sprouts, which are the tender sprouts of mung bean, the isoflavone contents, especially genistin, were much higher than in dried mung bean. The chromatograms are shown in figure 5-12.

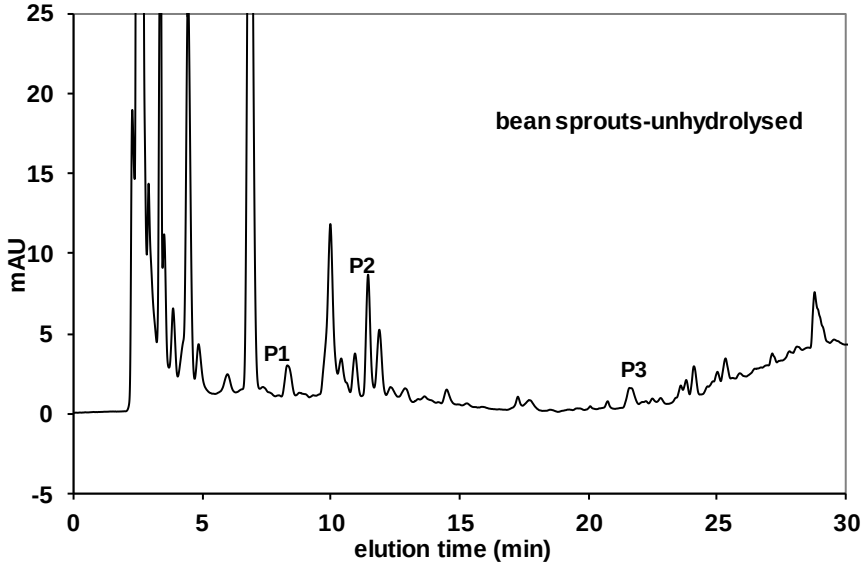


Figure 5-12 Chromatogram of mung bean sprout extracts. (Peaks identified: P1: daidzin; P2: genistin; P3: genistein)

5.4.7. Passion Fruits

Passion fruit is not a member of the legume family, but isoflavones, especially daidzin, were found in both flesh and shell although at low levels (figure 5-13).

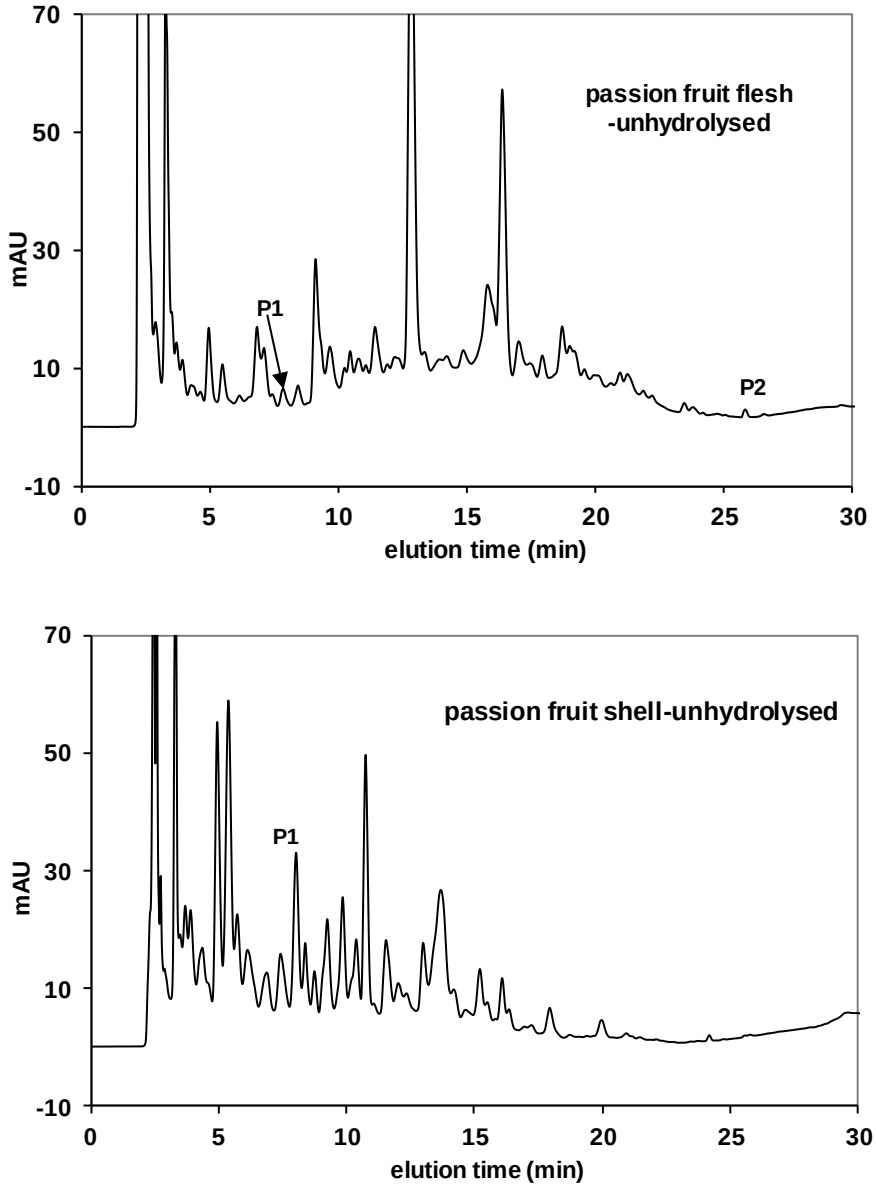


Figure 5-13 Chromatograms of passion fruit extracts. Top: passion fruit flesh; Bottom: passion fruit shell. (Peaks identified: P1: daidzin; P2 may be biochanin-A but was not further identified)

5.4.8. Soy Nuts-Dried

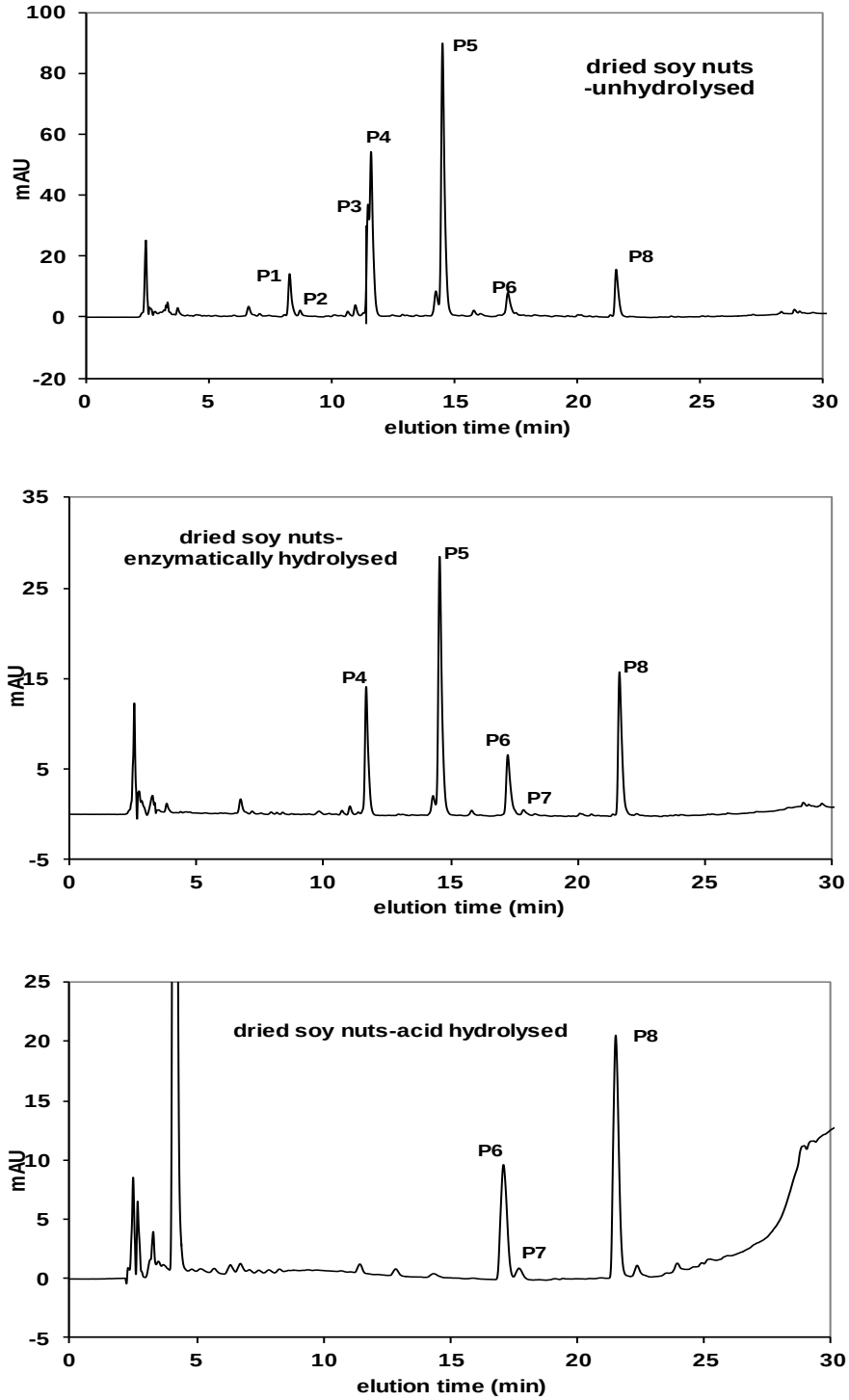
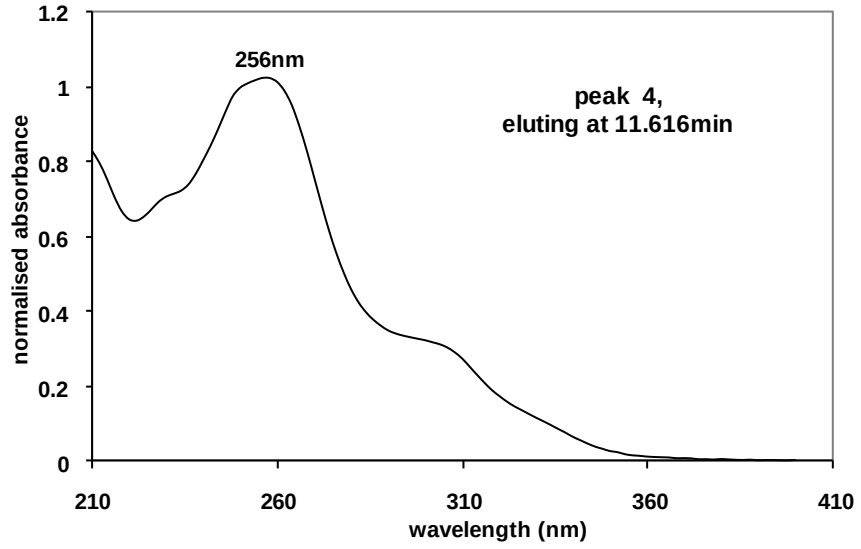


Figure 5-14 Chromatograms of dried soy nuts. Top: unhydrolysed; Middle: hydrolysed by apple seed extracts; Bottom: hydrolysed by 1M hydrochloric acid. Peaks identified: P1: daidzin; P2; glycitin: P3: genistin; P4: ?; P5: ?; P6: daidzein; P7: glycitein; P8: genistein.

Chromatograms of dried soy nuts are shown in figure 5-14, including unhydrolysed sample and hydrolysed by enzyme and acid. Peak 4 and peak 5 in figure 5-14 may also be some kind of derivatives of daidzein and genistein because their spectra were very similar to daidzin and genistin (see figure 5-15) but could not be absolutely identified due to lack of availability of standards. These two peaks disappeared after being hydrolysed with acid but not after enzymatic hydrolysis, so can be calculated as aglycone equivalents. The contents of isoflavones in dried soy nuts have been estimated in table 5-5 with the recoveries of daidzin and genistin, and the total contents of isoflavones have been calculated as aglycone equivalents after hydrolysis.



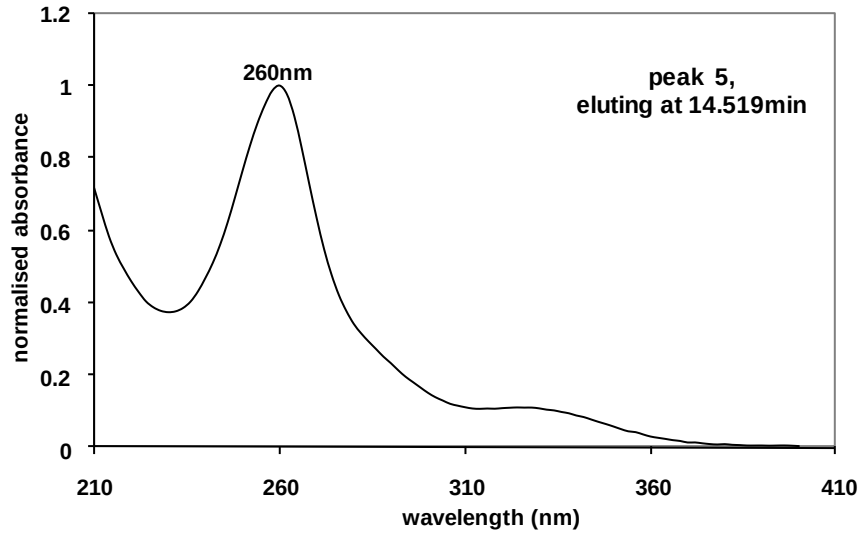


Figure 5-15 Spectra of P4 and P5 from HPLC of dried soy nuts. The highest absorbance appeared at 256nm and 260nm respectively.

Table 5-5 Isoflavone contents of three samples of dried soy nuts (mg/100g)

	1	2	3	Average ± SD (mg/100g, n = 3)	Recovery ± SD (%, n = 3)
Daidzin	35.77	33.48	34.05	34.43 ± 1.19	92.75 ± 1.05
Genistin	36.82	37.74	33.89	36.15 ± 2.02	92.07 ± 1.38
Glycitin*	4.43	4.87	5.21	4.84 ± 0.40	--
Daidzein	8.37	7.62	8.06	8.02 ± 0.38	--
Genistein	26.68	22.84	24.52	24.68 ± 1.93	--
Total**	201.72	197.13	200.88	199.91 ± 2.45	--

800µl of soy flour extract solution was used for recovery determination

*calculated as daidzin equivalents

**calculated as aglycone equivalents after hydrolysis

SD: standard deviation

5.4.9. Soy Nuts-Fresh

Fresh soy nuts were separated into different tissues. Chromatograms of soy nut coat and soy nut hypocotyls are showed in figure 5-16 to compare the differences in peak height. In fresh soy nuts, isoflavones mainly existed as acetyl or malonyl forms, so their concentration can only be calculated as aglycone equivalents after being hydrolysed. The contents of isoflavones from different soy nut tissues are shown in table 5-6.

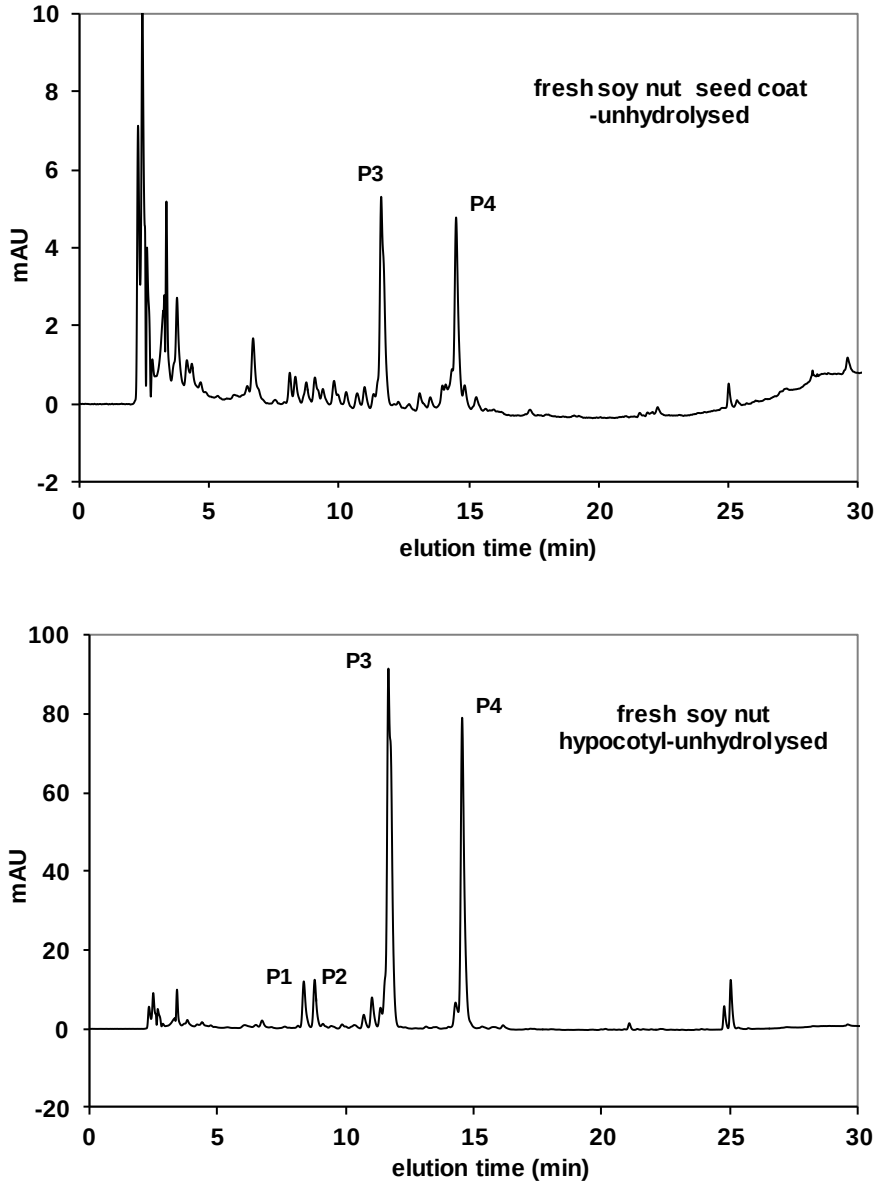


Figure 5-16 Chromatograms of the different fresh soy nut tissues compared. Top: soy nuts seed coat; Bottom: soy nuts hypocotyl. Both extracts are unhydrolysed. Note the difference of the Y axis. Peaks identified: P1: daidzin; P2; glycitin; P3 was the peak P4 in dried soy nuts; P4 was the peak 5 in dried soy nuts. The peaks after 25mins were so-called “ghost peaks”.

Table 5-6 Isoflavone contents in fresh soy nuts (mg/100g wet sample, n=3)

	Coat	Cotyledon	Hypocotyl	Whole nuts
	Average ± SD	Average ± SD	Average ± SD	Average ± SD
Daidzin	ND	ND	50.66 ± 2.21	4.82 ± 0.83
Recovery(%)	95.63 ± 3.98	96.34 ± 3.42	94.68 ± 3.64	93.72 ± 3.82
Genistin	ND	ND	ND	ND
Recovery(%)	92.87 ± 3.62	97.84 ± 4.21	93.26 ± 4.43	94.44 ± 4.08
Glycitin*	ND	ND	50.52 ± 2.04	ND
Daidzein	ND	ND	ND	ND
Genistein	ND	ND	ND	ND
Total**	46.32 ± 1.92	117.67 ± 3.33	914.91 ± 5.01	123.28 ± 4.24

800μl of soy flour extract solution was used for recovery determination

*calculated as daidzin equivalents

**calculated as aglycone equivalents after hydrolysis

SD: standard deviation; ND: not detected

5.5. General Discussion

5.5.1. The Method of Determination

As mentioned previously, isoflavone determination can be simply divided into sample preparation and sample analysis. The aim of sample preparation is to separate isoflavones from raw materials and other components by extraction into another solvent. Concentration and purification procedures may then be carried out. Some researchers have investigated optimum conditions for flavonoid extraction (Barnes *et al.*, 1994; Liggins *et al.*, 1998; Calabrò *et al.*, 2004; Chang *et al.*, 2004; Cho *et al.*, 2009; Rostagno *et al.*, 2009; Visnevschi-Necrasov *et al.*, 2009) and found that the best results could obtained by soaking samples in 70-80% methanol or ethanol and shaking at 37°C for about 2hrs. Under these conditions, isoflavones can be extracted from natural materials in their original forms since aqueous solvent can separate isoflavones with other components with best resolution, especially from other large molecular weight compounds; and this temperature is a balance point of extract velocity and protecting not heat-resistant forms of isoflavones. In the present study, 80% methanol was chosen and good recoveries were obtained (see table 5-3, table 5-4, table 5-5 and table 5-6.). Results show that this method can extract both polar isoflavones, like daidzin and puerarin, which are hydrophilic isoflavone glycosides eluted quite early; and non-polar isoflavones, such as formononetin and biochanin-A, which are hydrophobic isoflavone aglycones eluted later than daidzein and which are the naturally-existing isoflavone forms in red clover. The extraction recovery of these two isoflavones weren't determined. As all materials tested contained high levels of isoflavones, extra concentration procedures were unnecessary.

HPLC is nowadays the analysis method used predominantly in isoflavone determination, and is frequently connected with other detectors, including HPLC-UV Spectrophotometry, HPLC-Mass Spectrometry, HPLC-Nuclear Magnetic Resonance, etc (Marston & Hostettmann, 2006). Reversed-phase HPLC-UV spectrophotometry with Diode Array Detector (DAD) is used most popularly, while HPLC-MS and HPLC-NMR are also used frequently especially for determining unfamiliar

components. The usage of HPLC in determining isoflavones was reviewed by Merken & Beecher (2000).

Besides HPLC, other determination methods have been created and provided satisfactory results for specified samples, such as Capillary Electrophoresis (CE) (Tomás-Barberán, 1995; Urbánek *et al.*, 2002), Enzyme-Linked ImmunoSorbent Assay (ELISA) (Ambler & Peters, 1984; Mathey *et al.*, 2006). Usually such methods are very useful in dealing with large numbers of samples.

CE is an analytical technique providing high separation efficiency and short run times but exhibiting much lower sensitivity, and less reproducible quantitative data (Marston & Hostettmann, 2006). Several modes of CE are available, but the simplest and most versatile is Capillary Zone Electrophoresis (CZE), in which the separation is based on differences in the charge-to-mass ratio and analytes migrate into discrete zones at different velocities (Urbánek *et al.*, 2002). Anions and cations are separated in CZE by electrophoretic migration and electro-osmotic flow (EOF), while neutral species co-elute with the EOF. Compared with HPLC, CE can provide an alternative analytical method when higher efficiency or higher resolution is required (Tomás-Barberán, 1995).

A novel nonisotopic microtitration plate assay based on the human estrogen receptor has been developed by Garrett *et al.* (1999) to screen soy-based and soy-containing foods for their phytoestrogen content. The validation of the assay for use with food extracts has been demonstrated by investigation of recoveries after acidic and enzymatic hydrolysis, by investigation of matrix effects, and by comparison of results with HPLC analysis. Phytoestrogen levels in soy products analysed ranged between 520 and 1872μg of genistein equivalent/g of soy flour, 5-282μg/g of soy concentrates, 503-1292μg/g of soy-protein isolates, and 108-226μg/g of soy-based infant formulas. Comparison of results for 12 samples analysed both by the receptor assay and by HPLC showed good correlation ($r^2 = 0.905$). This assay, with sensitivity of 3.4μg/g, and 14 samples/plate analysed in 4hr following hydrolysis, provide a rapid and simple analysis method for screening phytoestrogen-containing foods.

5.5.2. Soy Isoflavones

5.5.2.1. Tissue location of soy isoflavones

It has been reported that in soy bean seeds, the distribution of isoflavone is dependent on the tissue location (Cui, 2005; Cho *et al.*, 2009). Most isoflavones are found in the cotyledon and hypocotyl and a trace in the seed coat. The following diagram shows an idea of the position of cotyledon and hypocotyls.

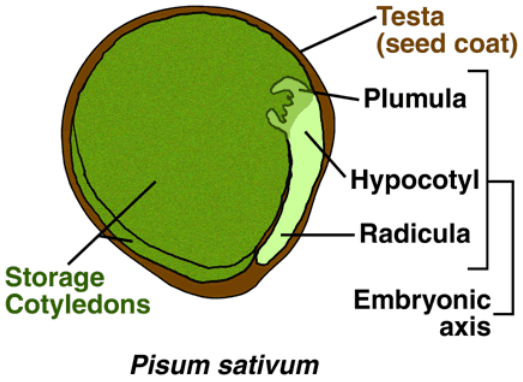


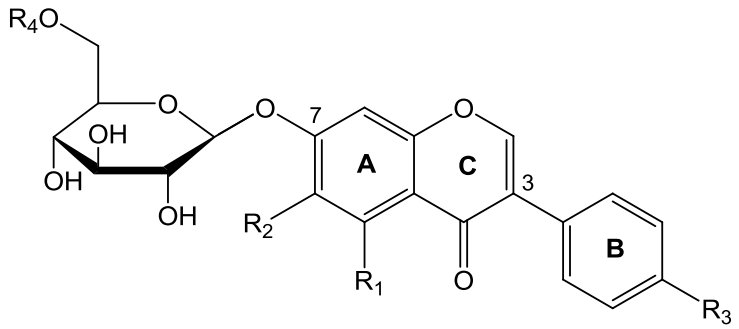
Figure 5-17 Diagram of pea (*Pisum sativum*) seed germination shows an idea of the position of cotyledon and hypocotyls (adapted from http://www.seedbiology.de/hormones.asp, accessed 09/2010)

In this study, fresh soy nuts were separated into different tissues, i.e. seed coat, seed cotyledon, seed hypocotyl and whole soy nuts. They were found to show huge differences in the isoflavone contents (see table 5-6). Hypocotyls have the highest isoflavone concentrations while the seed coats have the lowest. Cotyledon and whole soy nuts have the intermediate concentrations. That individual isoflavone glycitin could only be detected in the hypocotyl may be due to the lower contents of glycitin in other tissues.

5.5.2.2. Conjugate forms of soy isoflavones

Soy isoflavones have been reported to exist as 12 conjugate forms including 3 aglycone forms (Klejdus *et al.*, 2005). The 9 conjugated forms are the β-glucoside forms of daidzein, genistein and glycitein; the acetyl forms of daidzein, genistein and

glycitein; and the malonyl forms of daidzein, genistein and glycitein. The difference is the sugar moiety linked with the OH group on the 6″ position of the glucose residue of the β-glucoside form. The structure is shown in figure 5-18 (Barnes *et al.*, 1994; Ismail & Hayes, 2005).



R4 = H: isoflavone glucosides

R4 = COCH3: 6″-*O*-acetyl-isoflavone

R4 = COCH2COOH: 6″-*O*-malony-isoflavone

Figure 5-18 Conjugate forms of soy isoflavones

Naturally, in soy bean raw materials, most of the isoflavones exist as malonyl forms, reaching 60% (Barnes *et al.*, 1994). However, the malonyl forms are relatively unstable, and are very easily hydrolysed into acetyl forms and subsequently β-glycoside forms once heated. But conversion into aglycone forms is harder and needs the presence of exogenous enzyme (Cui, 2005). In some soy products which have been thermally treated, the isoflavones being determined may not be in the malonyl form any more, but are seen as acetyl forms and even glucoside forms only.

In soy flour, isoflavones mainly existed as glucoside forms, i.e. daidzin, genistin, and glycitin, indicating that most of the malonyl forms and acetyl forms had been hydrolysed into glucoside forms when being toasted. In soy nuts, no matter whether dried or fresh, there were large amounts of acetyl and malonyl forms of isoflavone. The difference in the isoflavone content results reflects the efficiency of the extraction method, and therefore the real situation in the plant material analysed.

Since β-glucosidase cannot hydrolyse the peak 4 and peak 5 in soy nuts significantly (see figure 5-14), and peaks 4 and 5 were high indicating there were large amounts of

these components existing in both fresh and dried soy nuts, acidic hydrolysis was carried out in order to calculate the total amount of isoflavones.

5.5.2.3. The elution order of 12 soy isoflavones

The principle of HPLC is eluting components according to their polarity. So the elution time may be different between different HPLC methods, but the elution order which correlates to polarity order should be similar and can be used as reference.

According to the HPLC method of the Japanese Association of Soy Isoflavones, the elution order of the 12 soy isoflavones should be: daidzin, glycitin, genistin, malonyldaidzin, malonylglycitin, acetyldaidzin, acetylglycitin, malonylgenistin, daidzein, glycitein, acetlygenistein, genistein (Cui, 2005).

5.5.3. Broad Beans

Broad beans have been reported to be rich in isoflavones (Mazur *et al.*, 1998). However, surprisingly, the isoflavone contents detected in the extracts (both fresh broad beans and salted broad beans) were very much lower than expected.

5.5.4. Chickpea Isoflavones

The isoflavone pattern of chick pea (*Cicer arietinum*) was different to soy. Besides daidzin and genistein, there was a large amount of biochanin-A although genistein was represented by the biggest peak. In chickpea, genistein and daidzein existed as aglycone forms while biochanin-A existed mainly as glycoside forms. This indicated that chickpea was a good source of biochanin-A and genistein in the diet because it is readily available in most food stores and supermarkets.

Chickpea is one of the earliest cultivated vegetables, rich in protein, carbohydrate, zinc, and folate, grown in the Mediterranean, western Asia, Indian and Australia, one of the most popular vegetarian foods in India, Pakistan, Bangladesh and the UK. (www.wikipedia.org/wiki/chickpea, accessed 03/2007). Mature chickpea can be

boiled, fried, stewed, fermented and processed in many ways. Figure 5-19 shows a picture of chickpea seeds.

Figure 5-19 White and green chickpeas (adapted from www.wikipedia.org/wiki/chickpea, accessed 02/2008)

5.5.5. Clover

Red clover (*Trifolium pratense*), is a well-known forage crop and is reported to be rich in isoflavones. The isoflavones and phytoestrogens from red clover have been used to treat the symptoms of the menopause. Women who are pregnant or breastfeeding should avoid ingesting red clover (www.wikipedia.org/wiki/redclover, accessed 03/2007). It has also been reported that red clover can be used for many therapeutic purposes (Mu *et al.*, 2009).

The isoflavone pattern of red clover is shown in table 5-2. Besides daidzin, genistein, there are large amounts of formononetin and biochanin-A. The results did show there were large amounts of biochanin-A and formononetin in clover, not presented as glycoside forms, but instead, as aglycone forms, and the concentrations of these compounds were much higher than that of chickpea. The isoflavone forms differ to the research carried out by Wu *et al.* (2003) which showed that isoflavones existed in red clover predominantly as malonyl formononetin glucoside and malonyl biochanin-

A glucoside although certain levels of free aglycones existed. This could be explained either by differences in extraction method or differences between red clover samples.

In this case, the tender leaf of clover was collected before flowering, so it might include some other species of clover, like white clover (*Trifolium repens*) or alsike clover (*Trifolium hybridum*), since they always grow in the wild together, and the only significant difference between red clover and white clover is the colour of their flowers. Red clover has red flowers, white clover has white flowers, while the flower of alsike clover has pale pink or whitish coloring on the head but its leaf is unmarked. Figure 5-20 shows the similarity of 3 types of clovers before flowering.

Figure 5-20 Similarity of 3 species of clovers before flowering (adapted from www.uwyo.edu/Plants/Forages/3clovers.jpg, accessed 02/2008)

Results showed that the collected sample contained large amounts of formononetin and biochanin-A as free aglycone forms although white clover and alsike clover were previously reported to contain negligible levels of those two isoflavones (Wu *et al.*, 2003).

5.5.6. Kudzu Isoflavones

In kudzu (*Pueraria lobata*), which has a special isoflavone pattern, the biggest peak appeared at 5.545mins (see figure 5-8, P1), which has been confirmed as puerarin (Setchell *et al.*, 2001; Kirakosyan *et al.*, 2003a; Lau *et al.*, 2009).

Puerarin is daidzein-8-C-glucoside, also a glucoside form of daidzein (Pei *et al.*, 1999; Lau *et al.*, 2009). The structure of puerarin is shown in figure 5-21. In principle, after hydrolysing kudzu extract solution, the increased area of daidzein included not only that produced from daidzin, but also produced from puerarin. However, this hydrolysis reaction could not be carried out either by enzymatic catalysis or by acidic hydrolysis described in 5.3.6., because the *C*- type of glycoside of flavonoid cannot be either enzymatically hydrolysed or by acid (Mabry *et al.*, 1970). In order to hydrolyse this type of glycosylated flavonoid, another more drastic hydrolysis methods should be applied, such as the $FeCl_3$ hydrolysis method described by Mabry *et al.* (1970).

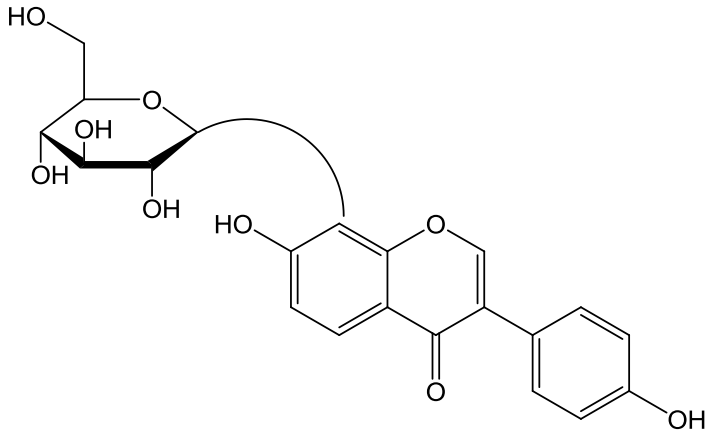


Figure 5-21 Structure of puerarin

Puerarin content in kudzu was calculated from the standard curve of daidzin due to the lack of purified puerarin standard. Thus isoflavone content was estimated although this might not be precise. Puerarin was an important isoflavone predominantly in kudzu.

5.5.7. Mung Bean

Mung bean (*Vigna radiata*), is very popular in south Asia and east Asia, and its sprouts have been reported to be rich in isoflavones (Kuhnle *et al.*, 2009). However, for Mung bean itself, the isoflavone content was very low and the main flavonoids observed in extracts weren't isoflavones.

However, in mung bean sprouts, which are simply called "bean sprouts" and easily available in most UK supermarkets, there were certain amounts of daidzin and genistin. This might also confirm that the isoflavone content is affected by the physiological stage of the plant (see 5.1.3.3).

5.5.8. Passion Fruits

Passion fruits (*Passiflora edulis*) is not a member of the family *Leguminoseae*, but belongs to the genus *Passiflora*, family *Passifloraceae*, order *Malpighiales*, class *Magoliopsida* (www.wikipedia.org/wiki/passionfruit, accessed 02/2008). Interestingly, it was found that there was a significant amount of daidzin in passion fruit, which means that humans might be able to supplement isoflavones from outside the *Leguminoseae* family.

Passion fruit is a plant cultivated commercially in frost-free areas for its fruit, native to South America and then widely spread (www.wikipedia.org/wiki/passionfruit, accessed 02/2008). The passion fruit is round to oval, yellow or dark purple at maturity, with a soft to firm, juicy interior filled with numerous seeds, and the fruit can be grown to eat or for its juice, which is often added to other fruit juices to enhance aroma. Nowadays it is easily found in supermarkets in the UK. Figure 5-22 shows the fruit, the flower, and the tree of passion fruit.

Figure 5- 22 Pictures of passion fruits (passion fruit; flower of passion fruit; passion fruit on the tree). (Adapted from www.wikipedia.org/wiki/passionfruit, accessed 03/2008)

5.5.9. Other Food Materials

In this study, up to 8 species and 20 samples have been analysed and most of them belong to the legume family (*Fabaceae* family) except passion fruit. The individual isoflavones analysed were puerarin, daidzin, glycitin, genistin, formononetin, and biochanin-A, in the order of their HPLC retention time, but only daidzin and genistin were quantified precisely. The results show that soy is a good source of daidzin and genistin, kudzu is good source of puerarin, red clover and chickpea are good sources of formononetin and biochanin-A. Some of the results were in line with the literature, some of them were not, even considering the factors which affect the isoflavone contents, for example the content of isoflavone in broad bean. This needs more confirmation in the future to make final conclusions.

Besides the materials have been tested, there are other materials reported rich in isoflavones, and some of them may also be available as human food. Determination of their isoflavone contents and pattern can provide more information for human isoflavone dietary supplementation.

First of all, some raw materials are worth trying, including alfalfa, psoralea, lupine, peanut, etc. Alfalfa (*Medicago sativa* L.) is an important forage crop and soil fertility promoter, native to Europe. Alfalfa seeds are readily found in local food stores especially those selling organic or health food. Interestingly, alfalfa was also reported as a source of β-D-glucosidase (Robinson, 1966) like soy bean (Hsieh & Graham, 2001; Suzuki *et al.*, 2006) and chickpea (Hinderer *et al.*, 1986). So it may become both a source of β-D-glucosidase and a source of isoflavones. Psoralea (*Psoralea corylifolia*), normally its seeds, is used as a Chinese herb and can be found in Chinese medical clinics or pharmacies either in China or in UK, and which has been claimed to contain the highest levels of daidzein and genistein (Shinde *et al.*, 2010). Lupine (*Lupinus spp*), a beautiful flowering plant, native to North America and then spread throughout the world, is also cultivated as forage and grain legumes, can bought from the internet and found as a processed food, for example, as pickled seeds, in the UK. Lupine has also been reported as containing high levels of genistein (Dueñas *et al.*, 2009). Peanut (*Arachis hypogaea*), a well-known nut, rich in protein, fat, starch, and other nutrients, is very popular in the UK, and has also been reported to contain certain levels of isoflavone especially genistein. Although the level of isoflavones in peanut may be lower than the other materials mentioned here (Mazur & Adlercreutz, 1998; Liggins *et al*., 2000), however, it may be the most available source. Additionally, some berries such as currants and raisins have also been reported to contain certain levels of daidzin and genistin (Mazur, 1998; Liggins *et al*., 2000) and may be worthy of analysis since they are not members of the Legume family.

Besides raw food materials, other plant tissues from the above species may also be worthy of analysis, including their leaf, stem, and sprouts, for instance, sprouts of alfalfa and spouts of lupine can also be found in food stores.

5.5.10. Summary of Isoflavone Contents

Thus, isoflavone contents in different plant tissues from different plant materials are summarized in table 5-7.

Table 5- 7 Summary of isoflavone contents in tested materials

Plant material		Isoflavones identified	Levels (mg/100g)	Comments
Broad bean-dried	Dried	ND	--	Lower than literature
	Salted	ND	--	
Broad bean-fresh	Coat	ND	--	Lower than literature
	Cotyledon	ND	--	
	Hypocotyl	ND	--	
	Whole nuts	ND	--	
Chick pea	Dried	Daidzein, ononin, genistein, biochanin-A	Not estimated	
Clover	Tender leaf	Sissotrin, daidzein, ononin, genistein, formononetin, biochanin-A	Not estimated	More in aglycone forms
Kudzu	Sliced & dried	Puerarin*	205.65 ± 3.03	Puerarin is predominant isoflavone as reported.
		Daidzin	64.91 ± 1.63	
		Genistin	8.01 ± 0.32	
		Daidzein	12.08 ± 0.28	
		Genistein	22.68 ± 1.84	
		Total	313.33 ± 3.02	
	Starch	ND	--	
Mung bean	Dried	ND	--	Reflected the difference in physiological stage
	Sprouts	Daidzin, genistin, genistein	Not estimated	
Passion fruit	Flesh	Daidzin, biochanin-A ?	Not estimated	Not legume family
	Shell	Daidzin	Not estimated	

Soy flour	Toasted	Daidzin	104.88 ± 4.60	
		Genistin	128.59 ± 1.97	
		Glycitin*	29.65 ± 0.68	
		Genistein	10.48 ± 0.42	
		Total**	170.68 ± 2.47	
Soy nuts-dried	Dried	Daidzin	34.43 ± 1.19	
		Genistin	36.15 ± 2.02	
		Glycitin*	4.84 ± 0.40	
		Daidzein	8.02 ± 0.38	
		Genistein	24.68 ± 1.93	
		Total**	199.91 ± 2.45	
Soy nuts-fresh	Coat	Daidzin/genistin-acetyl/malonyl form	Not estimated	Reflected the different distribution in plant tissues
		Total**	46.32 ± 1.92	
	Cotyledon	Daidzin/genistin-acetyl/malonyl form	Not estimated	
		Total**	117.67 ± 3.33	
	Hypocotyl	Daidzin	50.66 ± 2.21	
		Glycitin *	50.52 ± 2.04	
		Daidzin/genistin-acetyl/malonyl form	Not estimated	
		Total **	914.91 ± 5.01	
	Whole nuts	Daidzin	4.82 ± 0.83	
		Daidzin/genistin-acetyl/malonyl form	Not estimated	
		Total **	123.28 ± 4.24	

*calculated as daidzin equivalents

**calculated as aglycone equivalents after hydrolysis

ND: not detected

5.6. Conclusions

- It was confirmed that there are large amount of isoflavones in soy flour. The isoflavone pattern was daidzin, genistin, glycitin, plus small amounts of the acetyl forms of daidzin and genistin, and small amounts of the aglycone forms of daidzein and genistein. The contents were high enough and the pattern was simple enough to make this material suitable to be chosen for feeding experiments.

- Besides soy flour, some other materials were analysed and their isoflavone contents and pattern were determined, which included broad bean, chickpea, clover (tender leaf), kudzu, mung bean (dried bean and bean sprouts), passion fruit, and soy bean (dried and fresh).

- Amongst the tested materials, soy bean is the best source for daidzin and genistin (glucoside forms), kudzu is the best source for puerarin, while clover is the best source for formononetin and biochanin-A as the aglycone forms.

- Passion fruit may be an interesting non-legume source of isoflavones worthy of further study.

- Contrary to literature reports, broad beans and mung beans were poor sources of isoflavones, while mung bean sprouts contained isoflavones at relatively high levels.

- Less-well studied isoflavones such as biochanin-A, formononetin, puerarin and the malonyl and the acetyl conjugates merit further study on absorption, metabolism and bioactivity.