Chapter 2

Materials and Methods

In this chapter standard materials and general methods used throughout the thesis are described in detail. Specific experimental details are described in the relevant methodology section of each chapter.

2.1. Chemicals

2.1.1. Flavonoids

The following flavonoids were purchased from Extrasynthese plc, Genay, France:

- apigenin (4',5,7-trihydroxyflavone; $C_{15}H_{10}O_5$; MW: 270.25; CAS number: 520-36-5)
- apigin (apigenin-7-O-glucoside; $C_{21}H_{20}O_{10}$; MW: 432.38; CAS number: 578-74-5)
- daidzein (4',7-dihydroxyisoflavone; $C_{15}H_{10}O_4$; MW: 254.25; CAS number: 486-66-8)
- daidzin (daidzein-7-O-glucoside; $C_{21}H_{20}O_9$; MW: 416.38; CAS number: 552-66-9)
- equol (4',7-dihydroxyisoflavan = 4',7-isoflavandiol; $C_{15}H_{14}O_3$; MW: 242.28; CAS number: 66036-38-2 / 531-95-3)
- genistein (4',5,7-trihydroxyisoflavone; $C_{15}H_{10}O_5$; MW: 270.25; CAS number: 446-72-0)
- genistin (genistein-7-O-glucoside; $C_{21}H_{20}O_{10}$; MW: 432.38; CAS number: 529-59-9)
- quercetin-3(-β-D)-glucoside (isoquercitin) ($C_{21}H_{20}O_{12}$; MW: 464.38; CAS number: 21637-25-2)

The following flavonoids were purchased from Sigma Chemicals plc, Poole, Dorset, UK:

- glycitein (4′,7-Dihydroxy-6-methoxyisoflavone; $C_{16}H_{12}O_5$; MW: 284.26; CAS number: 40957-83-3)

- naringenin (4',5,7-trihydroxyflavanone; $C_{15}H_{12}O_5$; MW: 272.27; CAS number: 67604-48-2)
- naringin (naringenin-7-O-rhamnoglucoside; $C_{27}H_{32}O_{14}$; MW: 580.53; CAS number: 10236-47-2)
- quercetin, dihydrate (3,3',4',5,7-pentahydroxyflavone; $C_{15}H_{10}O_7 \cdot H_2O$; MW: 338.27; CAS number: 6151-25-3)
- rutin (quercetin-3-rhamnoglucoside), trihydrate ($C_{27}H_{30}O_{16} \cdot 3H_2O$; MW: 664.56; CAS number: 250249-75-3)

The following flavonoids were purchased from PlantChem, Sandnes, Norway:

- quercetin-4'-O-glucoside ($C_{21}H_{20}O_{12}$; MW: 464.38; CAS number: 20229-56-5)
- quercetin-3,4'-O-diglucoside ($C_{27}H_{30}O_{17}$; MW: 626.38; CAS number: 29125-80-2)

Flavonoids were stored at room temperature unless manufacturers' labelling advised otherwise. Flavonoid standard solutions in general were prepared either in methanol, or methanol-water (50:50, V:V), or dimethyl sulfoxide (DMSO) according to solubility, and stored below 4°C.

2.1.2. Solvents

The following solvents were purchased from Fisher Chemicals plc, Loughborough, UK:

- acetone, analytical grade
- acetonitrile, HPLC grade
- dimethyl sulfoxide (DMSO), analytical grade
- ethanol, analytical grade
- ethyl acetate, analytical grade
- methanol, analytical grade
- methanol, HPLC grade

2.1.3. Buffer Salts, Acids and Bases

The following buffer salts and acids were purchased from Sigma Chemicals plc, Poole, Dorset, UK:

- L-ascorbic acid ($C_6H_8O_6$; MW: 176.12) 99%
- dibasic sodium phosphate (Na_2HPO_4; MW: 142.0)
- monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$; MW: 138.0)
- formic acid (HCOOH; MW:) 98-100%
- hydrochloric acid (HCl; MW: 36.46) min 37%
- sodium acetate ($CH_3COONa \cdot 3H_2O$; MW: 136.08)
- sodium bicarbonate ($NaHCO_3$; MW: 84.01)
- sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$; MW: 294.10)
- trifluoroacetic acid (CF_3COOH; MW: 114.02) minimum 98%

The following buffer salts, acids and bases were purchased from Fisher Chemicals plc, Loughborough, UK:

- citric acid ($C_6H_8O_7 \cdot H_2O$; MW: 210.14)
- glacial acetic acid (CH_3COOH; MW: 60.05)
- phosphoric acid (H_3PO_4; MW: 98) 85.87%
- sodium carbonate (Na_2CO_3; MW: 105.99)
- sodium chloride (NaCl; MW: 58.45)
- sodium hydroxide (NaOH; MW: 40.00)

2.1.4. Water

Deionized and purified water from a Millipore Direct-Q 5 purification system (Millipore, Watford, UK) was used throughout the study. In this system, tap water is pre-treated in a Proguard pre-treatment pack to deionise and remove chlorine, minerals and organics, then further purified and deionised by a Quantum polishing cartridge, finally filtered through a Millipak-40 0.22µm filter and stored in a 101 re-circulating tank (resistivity 18.2MΩ at 25°C).

2.1.5. Preparation of Buffers

The buffers used in this study:

- 0.1M Citrate buffer, pH 3.5: 0.1M citric acid solution was added to 0.1M sodium citrate solution in the ratio 4:1 (V:V) and adjusted to pH 3.5
- 0.1M Acetate buffer, pH 4.5: 0.2M acetic acid solution was added to 0.2M sodium acetate solution in the ratio 14:11 (V:V), followed by dilution with an equivalent volume of water, and finally adjusted to pH 4.5
- 0.1M Acetate buffer, pH 5.5: 0.2M acetic acid solution was added to 0.2M sodium acetate solution in the ratio 3:22 (V:V), followed by dilution with an equivalent volume of water, and finally adjusted to pH 5.5
- 0.1M Phosphate buffer, pH 6.5: 0.2M monobasic sodium phosphate solution was added to 0.2M dibasic sodium phosphate solution in the ratio 17:8 (V:V), followed by dilution with an equivalent volume of water, and finally adjusted to pH 6.5
- 0.1M Phosphate buffer, pH 7.0: 0.2M monobasic sodium phosphate solution was added to 0.2M dibasic sodium phosphate solution in the ratio 31:69 (V:V), followed by dilution with an equivalent volume of water, and finally adjusted to pH 7.0
- 0.1M Phosphate buffer, pH 7.5: 0.2M monobasic sodium phosphate solution was added to 0.2M dibasic sodium phosphate solution in the ratio 4:21 (V:V), followed by dilution with an equivalent volume of water, and finally adjusted to pH 7.5
- 0.1M Carbonate buffer, pH 10.5: 0.2M sodium carbonate solution was added to 0.2M sodium bicarbonate solution in the ratio 4:1 (V:V), followed by dilution with an equivalent volume of water, and finally adjusted to pH 10.5

2.2. Foods

2.2.1. Food Materials for Enzyme Studies

The following foods were purchased from Julian Graves, Merrion Centre, Leeds, UK:

- Whole almonds (*Prunus dulcis*)
- Pumpkin seeds (*Cucurbita moschata/Cucurbita maxima*)
- Sunflower seeds (*Helianthus annuus*)

The following foods were purchased from Morrison's supermarket, Merrion Centre, Leeds, UK:

- Apples (*Malus domestica*)
- Honeydew melons (*Cucumis melo*)
- Oranges (*Citrus aurantium/Citrus sinensis*)
- Pineapples (*Ananas comosus*)
- Pomegranates (*Punica granatum*)
- Strawberries (*Fragaria vesca, ananassa*)
- Tomatoes (*Lycopersicum esculentum*)

2.2.2. Food Materials for Isoflavone Sources

The following foods were purchased from Out Of This World, New Market Street, Leeds, UK:

- Soy flour (*Glycine max*), toasted powder, Suna Organic

The following foods were purchased from Xing Fat Hong Chinese Supermarket, Templar Lane, Leeds, UK:

- Soy bean (*Glycine max*), dried
- Broad bean (*Vicia faba*), dried
- Broad bean (*Vicia faba*), salted

The following foods were purchased from Wing Fat Hong Chinese Supermarket, Faulkner Street, Manchester, UK:

- Kudzu roots (*Pueraria lobata*), dried sliced
- Kudzu starch (*Pueraria lobata*), fine powder

The following foods were purchased from Morrison's supermarket, Merrion Centre, Leeds, UK:

- Broad bean (*Vicia faba*), fresh nuts, frozen
- Soy bean (*Glycine max*), fresh nuts, frozen, Bird's Eye
- Mung bean (*Vigna radiata*), dried nuts

The following food materials were purchased from Tesco Metro, Bond Street, Leeds, UK:

- Bean sprouts (*Vigna radiata*), fresh
- Chick pea (*Cicer arietinum*), dried
- Passion fruits (*Passiflora edulis*), fresh

The following food material was collected at Aire riverside, Leeds, UK:

- Clover *(Trifolium pratense)*, tender leaf

2.3. Equipment

The following laboratory instruments were used throughout this project:

- Precisa 510 digital analytical balance (0.1-500g)
- Metler PC 2200 digital analytical balance (0.01-1g)
- Stanton Instruments Unimatic CL41 analogue analytical balance (0.001-0.1g)
- Clifton DU-8 ultrasonic water bath
- Grant OLS 200 shaking water bath
- Büchi Rotavapor-EL
- Birchover Instruments Freeze Dryer 3.5
- SanYo -80ºC extra low freezer

- Scanvac cool safe Freeze Dryer with Vacuubrand 2.5 rotary vane vacuum pump
- Genevac EZ-2 Series personal evaporator
- Moulinex Optiblend 2000 blender
- Human Instruments pH 210 bench pH meter with H1131B electrode
- Eppendorf 5415C desktop micro-centrifuge (rotor radius 7.3cm, with maximum force of 16000g on 24 (1.5-2.0ml) samples)
- Beckman J2-HS centrifuge
- Scotsman AF-80 ice-flaker
- Gallenkamp IH-150 incubator
- Fisherbrand whirlimixers
- Gallenkamp stirrer and hot plate
- Soccorex and Eppendorf automatic pipettes (10µl-5ml)
- Four timer digital clock

2.3.1. Spectrophotometer

A Cecil CE 2021 digital spectrophotometer equipped with a dual photodiode detector was used for some experiments in chapter 3 and chapter 4. This machine was capable of scanning wavelengths 190-900nm.

2.3.2. Other Consumables

The following disposable consumables were purchased from Plastibrand plc, Wertheim, Germany:

- Plastic cuvettes (1.5ml; 1.0cm)
- Micro centrifuge tubes (2.5ml, 5ml)
- Pipette tips (100µl, 1000µl, 5 ml)

2.4. High Performance Liquid Chromatography (HPLC)

To determine flavonoid content, high performance liquid chromatography (HPLC) was used as the main analytical tool in this project, with a C18 silica-based reverse phase column. A reversed-phase separation, used in the majority of applications, uses a non-polar stationary phase to retain non-polar components then uses polar solvents as the mobile phase for elution, and most commonly gradient elution, while in normal-phase chromatography the opposite situations apply. With a suitable choice of solvents and appropriate sequence gradient, reversed-phase HPLC is a sensitive and efficient technique for the analysis of organic compounds with widely varying polarities especially in separating and quantifying closed related compounds. Various types of detection methods are available including diode array, fluorescence, and electrochemical detection.

2.4.1. Merck-Hitachi HPLC System

A Merck-Hitachi HPLC system was used in chapter 3 and chapter 4, which was a reversed-phase Merck-Hitachi Lachrom L-series HPLC-DAD and included the following components:

- A L-7200 (80 vials) auto-sampler allowing automated and precise sample injections
- A D-7000 quaternary pump, for high-pressure pulse-free mixing and transfer of solvents through the system
- A C18 column packed with Prodigy 5µm ODS3 silica (100A), of 250mm length and 4.6 mm internal diameter (Phenomenex, Macclesfield, UK)
- A L-7450 diode array detector, with a D2 deterium lamp and a wavelength ranging from 220nm to 600nm

The system was controlled by a desktop computer running under Microsoft Windows 2000 via a GPIB connecter and operated with the D-7000 HSM software.

2.4.2. Agilent 1200 Series HPLC System

The experiments in chapter 5 & 6 were carried out by using a Agilent 1200 series HPLC system, which including:

- A G1321A fluorescence detector, provides quantitative data and fluorescence spectra from a single run and simultaneous multi-wavelength detection improves sensitivity and selectivity.
- A G1315B diode array detector, a dual-lamp design for highest sensitivity with a wavelength ranging 190nm to 950nm
- A G1316A Thermostatted column compartment, is built with two heat exchangers for solvent pre-heating, allowing precision heating temperature ranging 10°C to 80°C for reproducible retention times and peak areas
- A G1329A Thermostatted Autosampler, with a sample capacity of 100 samples. Variable volume injections in the 1 to 100ul range are programmable. Temperature controlled vial compartment ranging 4-40°C
- A G1330B ALS Thermostatted Autosampler temperature controller, temperature controlled ranging 4-40°C
- A G1322A vacuum degasser, built with four solvent channels with flow rate 1-10ml/min, capable of passing solvent through a membrane tube which is permeable to gas but not liquid to reduce baseline noise and quenching effects
- A G1311A quaternary pump, optimised to ensure virtually pulse-free and stable solvent flow with dual floating pistons, with four solvent inlets with built-in gradient control, with flow ranging 0.2-10ml/min and pH ranging 1-12.5
- A C18 column packed with Prodigy 5μm ODS3 silica (100A), of 250mm length and 4.6 mm internal diameter (Agilent, Cheshire, UK)

Data acquisition and analysis was managed by ChemStation software designed to run on a desktop computer with Microsoft Windows XP which was connected to the module by GPIB.

2.4.3. Consumables and Solvents

In order to achieve accurate, reproducible and high resolution analytical results, HPLC grade solvents were used in all HPLC analysis, which were purchased from Fisher Chemicals PLC and Sigma Chemicals PLC (see 2.1.2 and 2.1.3). Other consumables were purchased from Chromos Express PLC, Macclesfield, UK, which were:

- 2ml crimp top glass vials (12×32mm) associated with 11mm aluminium crimp caps with butyl/PTFE septa
- 0.2µm PTFE syringe filter of diameter 17mm

Glass vials were capped using a vial hand crimper to avoid evaporation of samples. All samples were filtered prior to injection to avoid blockage of auto-sampler needle by particle or residues.

In addition, the following disposable consumable was purchased from Terumo Corporation, Terumo Europe, N. V. 3001 Leuven, Belgium:

- Syringe (polypropylene, 3 part Luer fitting, 2ml)

The following disposable consumable was purchased from Becton Dickinson, Ireland:

- Syringe needle (Luer fitting, 21 gauge, 1.5in long)

2.5. HPLC Methods

2.5.1. Merck-Hitachi HPLC Method

This method was described by Rothwell (2005). The mobile phase consisted of solvent A (0.1% trifluoroacetic acid (TFA) in Milli-Q purified water) and solvent B (100% acetonitrile), and were degassed in an ultrasonic water bath for 10mins before use in order to minimise pressure fluctuations that could lead to baseline drift and inaccurate solvent mixing which could cause changes of retention times in the case of

the Merck-Hitachi model. After that, mobile phases were placed in glass bottles connected to the solvent lines. Air bubbles were purged from the solvent lines by opening the purge valve and pumping the mobile phases at a flow rate of 10 ml/min (ten times higher than analysis flow rate) until the lines were clear of air. Then the mobile phases were mixed with 83% TFA in water (solvent A) and 17% acetonitrile (solvent B) and the system was left to come to the correct pressure which would normally be around 130 bar. If the pressure was consistently high (above 170 bar), 100% acetonitrile (solvent B) was pumped through the column to remove residues. If the pressure was consistently low, the system was checked for leaks and the solvent lines checked for air bubbles and purged again if necessary. The column temperature was kept at 30 °C. Column performance decreases with time as signified by increased back-pressure and peak widths, in which case the column would be changed.

The solvent mixture was pumped through the system at a flow rate of 1 ml/min, using a 35min sequence gradient to separate compounds by polarity. For water-soluble compounds, for example ascorbic acid, was eluted almost immediately after the solvent front, flavonoid glycosides eluted typically between 6-12mins, and flavonoid aglycones usually between 15-24mins.

The gradient elution started at 17% of solvent B for 2 min, increased to 25% at 7min, 35% at 15min, 50% at 20 min and finally reached 90% at 25min and held this ratio for further 3mins before returned to the starting mixture (17% solvent B) for a 6mins re-equilibrium post run (see figure 2-1). In order to minimise shifts in retention times and peak areas, solvent A (0.1% TFA in water) was changed at least every three days, solvent B (acetonitrile) was changed at least every two months.

The injection needle was set at a draw speed of 200µm/min and injection volume was usually 10µl unless otherwise stated. The autosampler needle and injection pore were automatically washed five times before every run with 50% methanol to remove the residue from the previous injection.

Absorbance was zeroed automatically before injection and was recorded at all wavelengths ranging from 220 to 600nm. Chromatograms were monitored at 270, 280,

290 and 370nm. According to their UV absorption spectra, the monitoring wavelength was 270nm for daidzein, genistein, and their glycosides, 370nm for quercetin and their glycosides. The detector conditions were: spectral bandwidth 4 nm; acquisition rate of spectra (spectra interval) 800 msec.

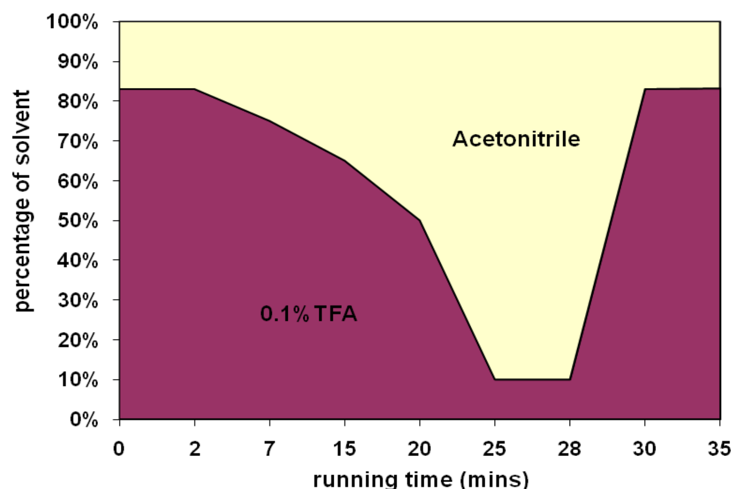

Figure 2-1 Solvent gradient over the course of a run for the Merck-Hitachi Lachrom L-series model

2.5.2. Agilent 1200 HPLC Method

The Agilent 1200 series model was converted from Merck-Hitachi method. The mobile phases were solvent A (0.1% TFA in water) and solvent B (100% acetonitrile) with similar gradient elution. The mobile phases were placed in capped glass bottles with the solvent lines immersed and the solvents were degassed in the vacuum degasser before priming the pump by opening the prime valve on the binary pump. The system was run for 5mins at a flow rate of 5 ml/min until the solvent lines were free of air. Once the pump was properly primed the priming valve was closed and the mobile phases were pumped through the column for 10mins for equilibraton. Then the mobile phases were mixed with 83% TFA in water (solvent A) and 17% acetonitrile (solvent B) at a flow rate of 1.0ml/min and the system was left to reach the pressure higher than 300bar, using an 35mins gradient elution starting at 17% solvent B (acetonitrile) for 2min, increasing to 25% at 7min, 35% at 15min, 50% at 20min, and finally 90% at 25min and hold this ratio for further 3mins before returning to the

initial conditions (17% solvent B) again for a further 7mins re-equilibrium post run (see figure 2-2). The column temperature was kept at 30ºC and the maximum column pressure was set at 600bar. If the pressure was over 600bar, an error message was generated in the user interface and the system was shut down.

The default draw speed for the autosampler was set at 100µl/min and the sample injection volume was 10µl otherwise stated. The autosampler uses a flow-through design to assure washing of the needle (50% methanol) outside in order to minimise carry-over. Before the start of the injection, and during analysis, the 6-port injection valve is in the mainpass position where the solvent flows through the autosampler ensuring that the injection needle is flushed during the run. During the sampling sequence, the injection valve switches to the bypass position and the solvent from the pump enters the valve and flows directly to the column. When the needle is lowered into the needle seat, the injection valve switches back to the main-pass position, flushing the sample onto the column.

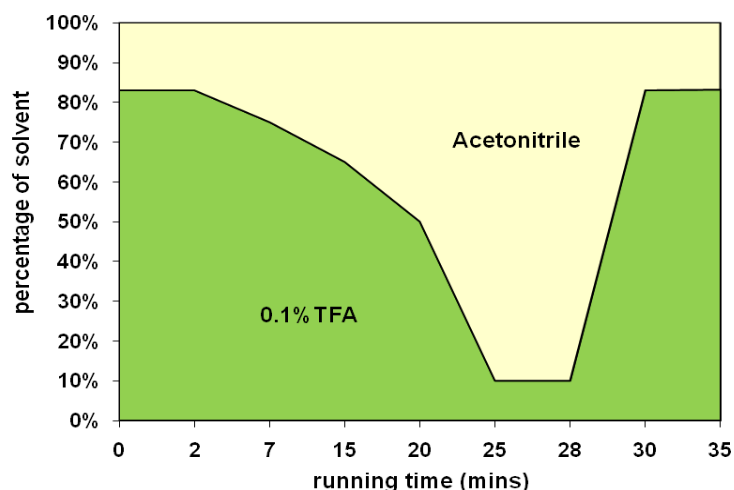

Figure 2-2 Solvent gradient over the course of a run for the Agilent 1200 series model

Absorbance was recorded at all wavelengths ranging 190 to 950 nm. Chromatograms were observed at 260, 270, 280, 290 and 370 nm, and the monitoring wavelength was 270nm for daidzein, genistein, and their glycosides, 370nm for quercetin and its glycosides. The detector's spectral bandwidth was 1nm. Normally flavonoid

glycosides were eluted between 6-12mins, and flavonoid aglycones were eluted between 15-24mins.

2.5.3. Equol Separating Method

In order to separate genistein and equol, which would be eluted together by using above HPLC methods (2.5.1. and 2.5.2.), another HPLC method was developed using the Agilent 1200 HPLC system, named the equol separating method. The equol separating method is similar to the Agilent 1200 method which was described in 2.5.2 but different solvents were used. Solvent A was 0.05% formic acid in water, solvent B was a mixture of acetonitrile, methanol and water (20/40/40; V/V/V). The solvent gradient of the equol separating method is shown in figure 2-3. The monitoring wavelength for daidzein, genistein, and their glycosides was 260nm, for equol it was 280nm.

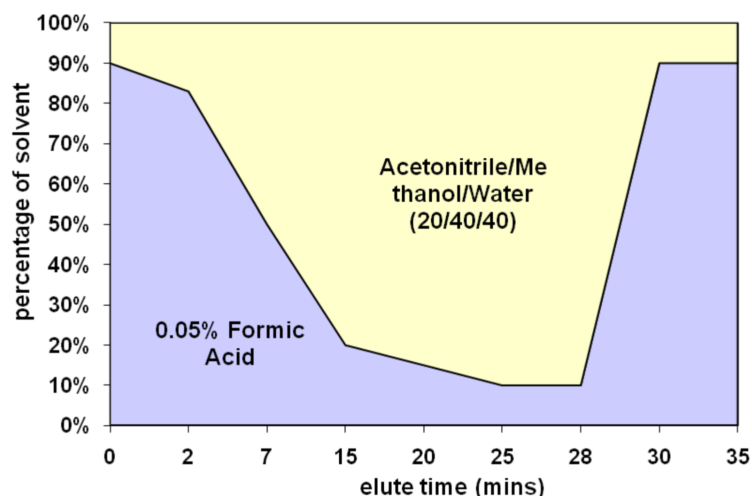

Figure 2-3 Solvent gradient for the equol separating method

2.6. Flavonoid Determination

2.6.1. Verifying Flavonoid Peaks

Identifying a mixture of flavonoids in a food sample can be difficult because the components are much more complicated than in a purified flavonoid sample. The following methods were used to identify flavonoids:

- Comparison of UV absorption spectra. In general, flavonoids show high and characteristic UV absorption because of the conjugated phenolic ring structure, which is also the principle of their UV protection function. Spectra are different between flavonoids but they always have two typical absorption bands, one is in the range of 250-300nm and the other is in the range of 350-400nm. Spectra for flavonoid standards in methanol were obtained using HPLC scanning all wavelengths between 200-600nm. The absorption spectra were used as a reference where the identity of flavonoids needed to be confirmed. However, identification of flavonoids by using spectra only maybe difficult because different flavonoid conjugates or members of a sub-class may have similar spectra and the nature of the solvent may cause shifts in the spectra.
- Comparison of retention times. Flavonoid identification was made by comparing the retention time of the eluted sample with the standard compounds.
- Spiking. The suspected flavonoid peak was verified by spiking the sample with a known amount of the flavonoid standard. An increase of this flavonoid peak area after spiking and the appearance of no other peaks confirm the identity of the flavonoid.

2.6.2. UV-VIS Spectra of Flavonoids

The UV absorption spectra of flavonoids tested were obtained by HPLC and used as reference. Figure 2-4 shows spectra for isoflavone glucosides, figure 2-5 isoflavone

aglycone and equol, figure 2-6 flavones, figure 2-7 flavonols and flavonol glucosides, and figure 2-8 flavonones.

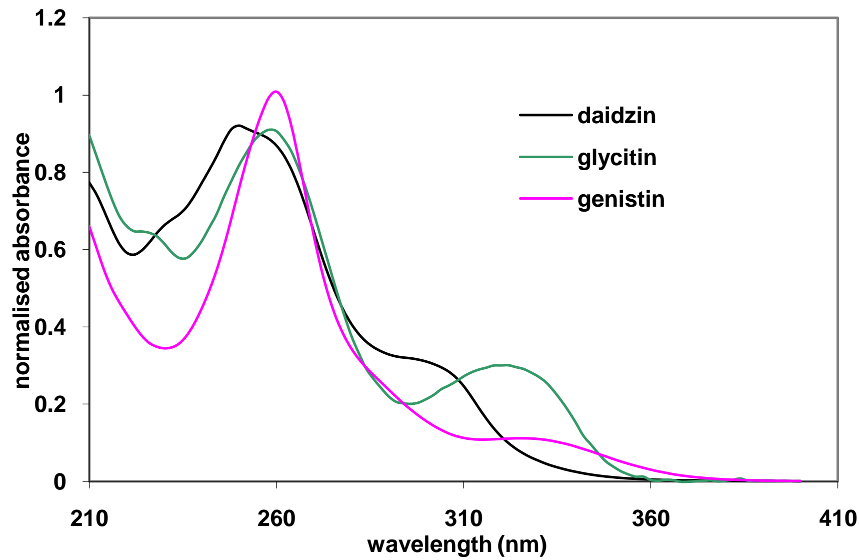

Figure 2-4 UV-VIS absorption spectra of tested isoflavone glucosides in 50% methanol

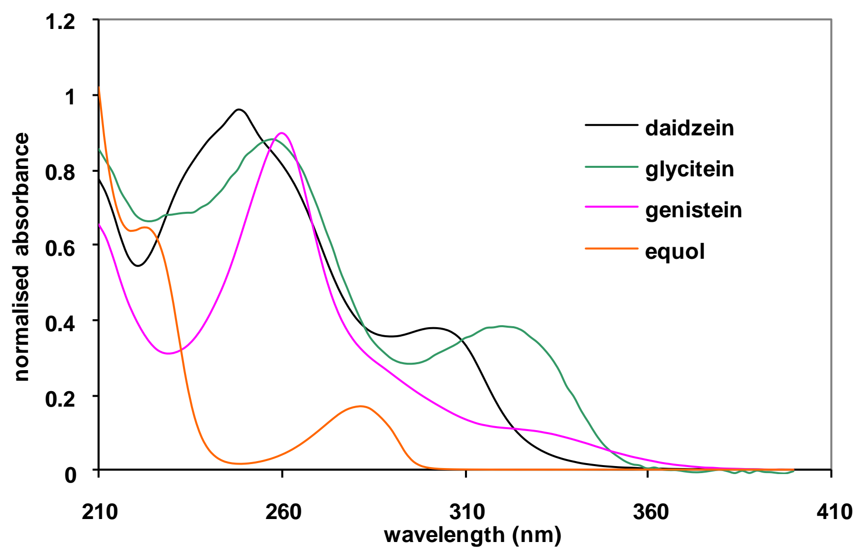

Figure 2-5 UV-VIS absorption spectra of tested isoflavone aglycones and equol in 50% methanol

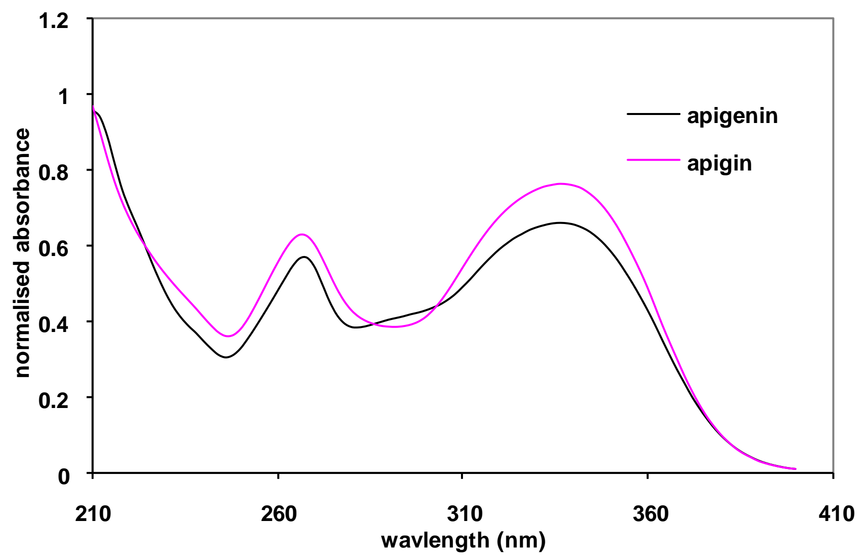

Figure 2-6 UV-VIS absorption spectra of tested flavones in 50% methanol

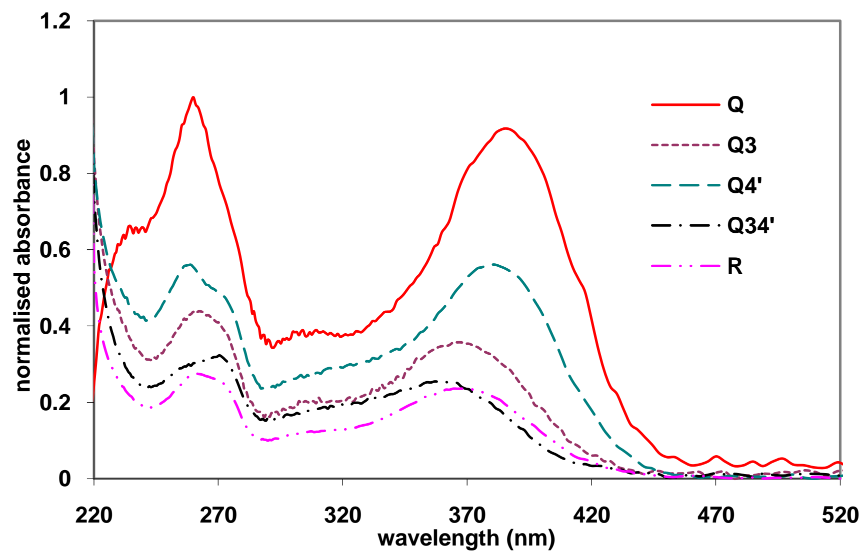

Figure 2-7 UV-VIS absorption spectra of tested flavonols in 50% methanol (abbreviations: Q: quercetin; Q3: quercetin-3-glucoside; Q4′: quercetin-4′-glucoside; Q34′: quercetin-3, 4′-diglucoside; R: rutin)

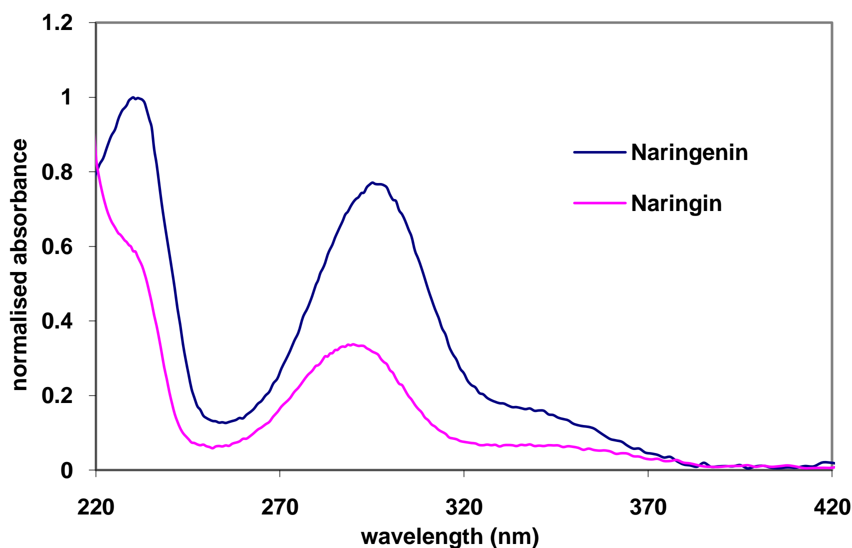

Figure 2-8 UV-VIS absorption spectra of tested flavonones in 50% methanol

2.6.3. Standard Curves for Flavonoids

In order to determine flavonoid contents in unknown samples, a series of standard curves were prepared.

Stock solutions of the flavonoid glycosides diadzein-7-glucoside (diadzin), and genistein-7-glucoside (genistin), flavonoid aglycones diadzein, equol and genistein, were used. Flavonoid standards (0µl, 25µl, 50µl, 75µl, 100µl, 125µl, 150µl) were pipetted into micro-centrifuge tubes and diluted by 50% methanol to make the final volume 1500µl, and then filtered through 0.20µm PTFE filters into 2ml HPLC vials for HPLC analysis. Then the resulting peak areas were plotted against amount of flavonoid. The X axis was the amount of flavonoids in the HPLC vials. The R^2 value showed that the linearity was very good. Note that the data for equol was obtained at 280nm; for other flavonoids 260nm was used. Figure 2-9 – figure 2-12 show the standard curves for daidzein, genistein, daidzin and genistin respectively obtained by the Agilent 1200 method; figure 2-13 and figure 2-14 show the standard curves for equol and genistein obtained by the equol separating method.

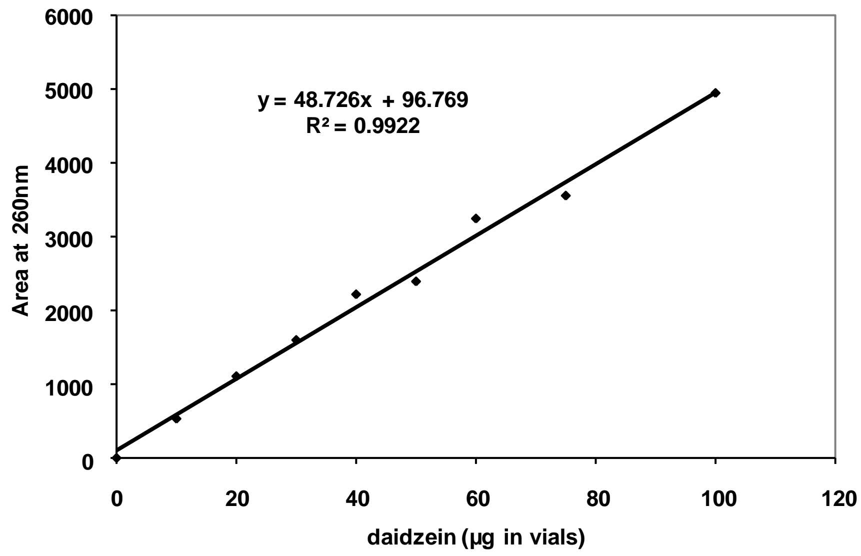

Figure 2-9 Standard curve for daidzein by the Agilent 1200 method

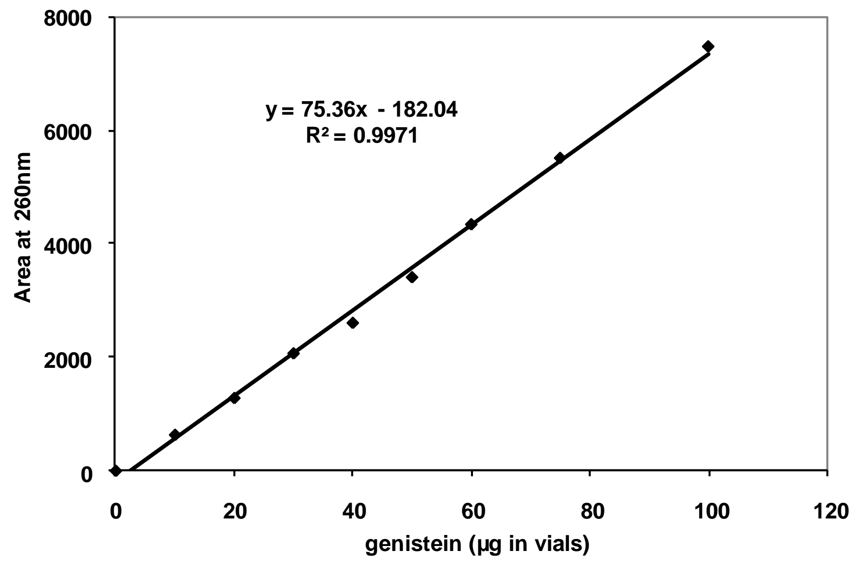

Figure 2-10 Standard curve for genistein by the Agilent 1200 method

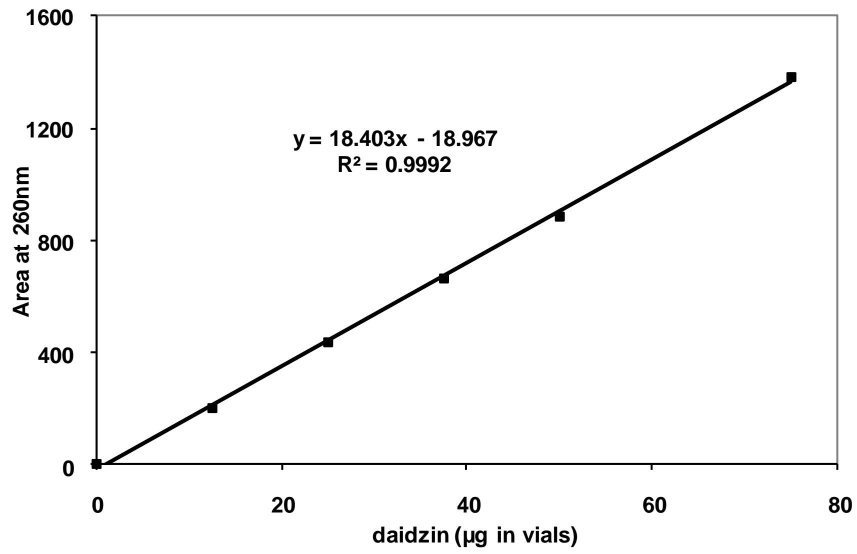

Figure 2-11 Standard curve for daidzin by the Agilent 1200 method

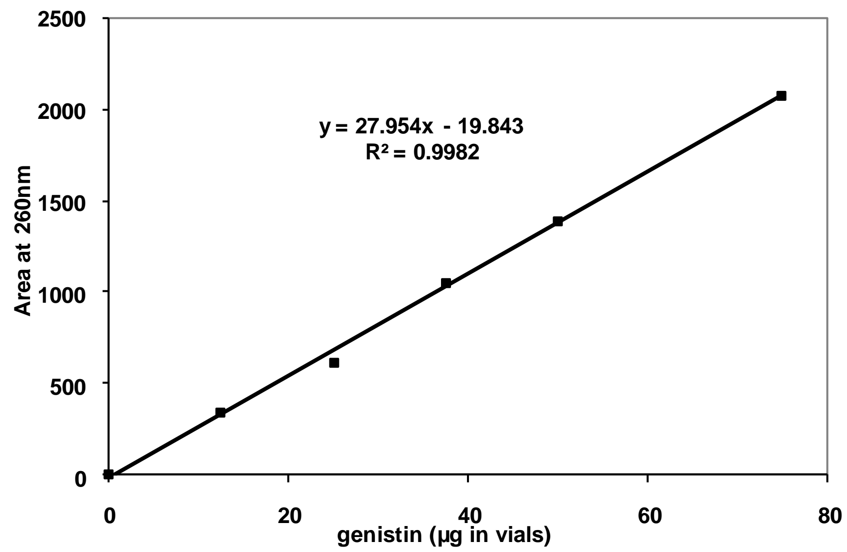

Figure 2-12 Standard curve for genistin by the Agilent 1200 method

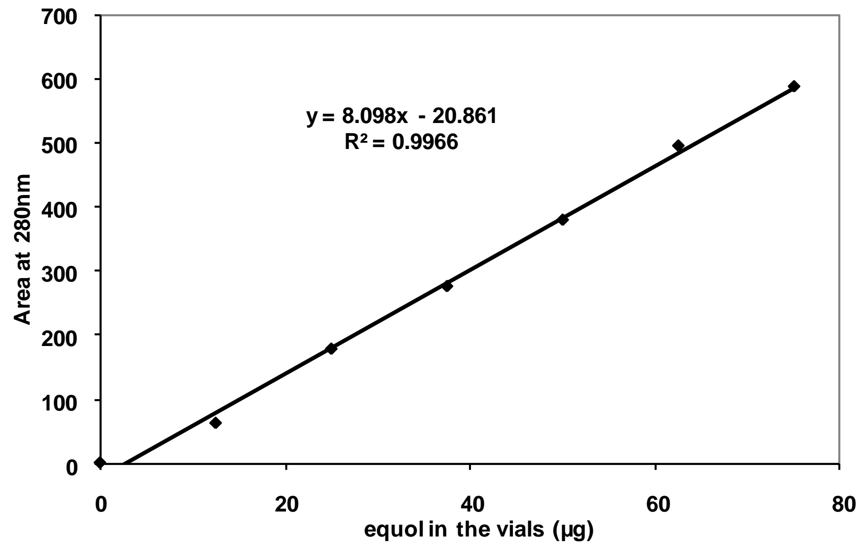

Figure 2-13 Standard curve for equol obtained by the equol separating method

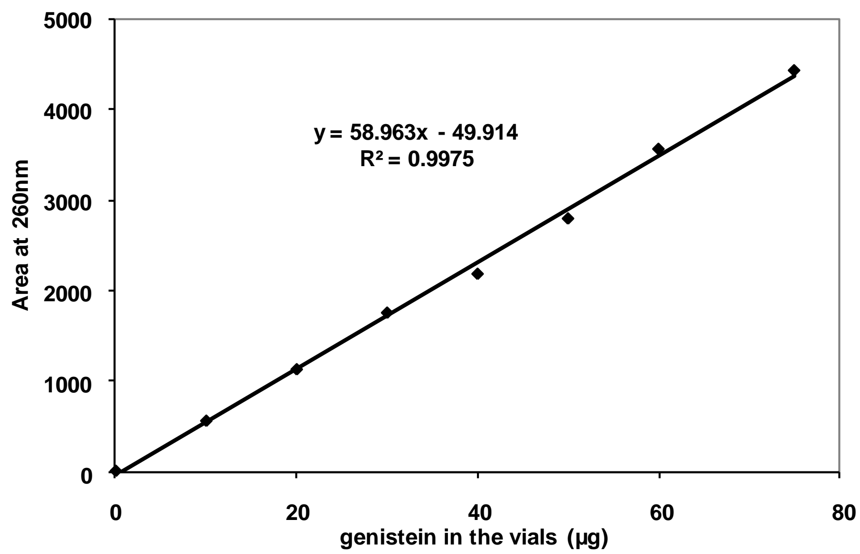

Figure 2-14 Standard curve for genistein obtained by the equol separating method