Countercurrent chromatography (CCC), CCC/MS and LC/MS techniques in studies of phytoestrogens in plant extracts and human urine.

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Countercurrent Chromatography (CCC), CCC/MS and LC/MS Techniques in Studies of Phytoestrogens in Plant Extracts and Human Urine

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Wales

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December 2003

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AKNOWLEDGEMENTS

Foremost, I would like to thank my supervisor Prof. Dai Games for introducing me into the field of mass spectrometry, and for providing me with the excellent opportunity to conduct the challenging research that I am very interested in. I would like to thank Dai and Marge Games for their assistance in the MSRU and for their contributions over my time in the MSRU. I would also like to thank Chinese government for the financial support in my study abroad.

I would like to take the opportunity to thank Prof Ian A Sutherland for giving me technical support in CCC and for his kindly help in amending my thesis. My thanks also extend to Dr Sue Plummer and Dr James Tang at the Cultech Limited, Swansea, for providing urine samples and standard materials. It was gratifying to work in collaboration with such a friendly organization.

I would also like to extend my gratitude to all the other members of staff at the Mass Spectrometry Research unit, for their much appreciated technical support and assistance: Brian Cooper, Mike Tunner, and Eileen Jones.

I would also like to thank my friends and fellow colleagues for their kindly help and companionship, in particular Jones, Masood, Jinglei, Swadipa and Supa.

I would like to thank my parents for their continual encouragement and support both in mentality and substance.

Finally, I would like to specially thank my husband and my dear daughter for their constant love and encouragement.

Lijuan Chen

SUMMARY

Phytoestrogens are members of classes of polyphenolic compounds synthesized by plants. They include isoflavones and other flavonoids, lignans, coumestanes and zearalenones. Photoestrogens are weak estrogen-like plant compounds that act similarly to the hormone estrogen, Over the last twenty years, interest in the physiological effects of phyto-estrogen containing plant substances has increased significantly, since it was demonstrated that the phytoestrogen components may play a potential role in the prevention and treatment of hormonally-dependent diseases, including breast and prostate cancer.

This thesis describes studies of High Speed Countercurrent Chromatography and combined Liquid Chromatography-Mass Spectrometry techniques in the separation, purification, identification and quantitation of phytoestrogens in plants and human fluid.

Chapter One provides a brief history and introduction to the principles and applications of high-speed countercurrent chromatography, liquid chromatography, mass spectrometry, and their combination of interfacing systems.

Chapter Two investigates the separation and purification the flavonoids from the ethyl acetate extract from the seeds of *O, indicum* using high-speed countercurrent chromatography. Five flavonoids are successfully separated and purified with high purities (above 92 %) and two components are for the first time separated and identified from the plant. Mass spectrometry, Ultraviolet and Nuclear Magnetic Resonance are used for the structural elucidations of flavonoids.

Chapter Three and Chapter Four compare different coil volumes of high-speed countercurrent chromatography instruments in the separation and purification of phytoestrogens. On line high speed countercurrent chromatography-mass spectrometry is studied for the separation and identification of flavones and isoflavones in plants.

Chapter five develops a simple and accurate method for the quantitative determination of phytoestrogens in soy food supplements using on line liquid chromatography-mass spectrometry. Nine isoflavones are determined and liquid chromatography-tandem mass spectrometry is also investigated to the structural elucidation of some unknown components.

Chapter Six develops a sensitive on line liquid chromatography-mass spectrometry method to determine the concentration of isoflavones and their major metabolites in human urine after consumption of soy-based supplements. The structural elucidations of some unknown metabolite isoflavones are also explored using liquid chromatography-tandem mass spectrometry.

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ABBREVIATIONS

α	Separation or Selectivity factor
ac	Alternating current
Abs	Absorbance
Ac	Ethyl acetate
amu	Atomic mass unit
API	Atmospheric Pressure Ionization
APCI	Atmospheric Pressure Chemical Ionization
AU	Absorption Unit
BAI	Baicalein
BDS	Base Deactivated Silica
°C	Degrees centigrade
CCC	Countercurrent chromatography
CE	Capillary Electrophoresis
CE/MS	Capillary electrophoresis/mass spectrometry
CI	Chemical Ionization
CZE	Capillary Zone Electrophoresis
CID	Collision induced dissociation
C_L	Analyte concentration in the lower phase
Cm	Analyte concentration in the mobile phase
cm	Centimeter
CPC	Coil Planet Centrifuge
CPC	Centrifugal Partition Chromatography
CRM	Consecutive reaction monitoring
Cs	Analyte concentration in the stationary phase
C_U	Analyte concentration in the upper phase
DAI	Daicalein
DCCC	Droplet countercurrent chromatography

dc	direct current
DES	*O*-Desmethylangolensin
DIH	Dihydrogenistein
DMSO	Dimethyl sulfoxide
E(or Et)	Ethyl acetate
EI	Electron Ionization
ELSD	Evaporative Light Scattering Detector
ESI	Electrospray Ionization
EtOAc	Ethyl acetate
FD	Field Desorption
FDA	Food and Drug Administration
FTIR	Fourier Transform Infra Red Spectrometry
g	gram
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
GEN	Genistein
GLC	Gas liquid chromatography
GLY	Glycitein
H	Hexane
hr	hour
HDES	Hydrodynamic Equilibrium System
HETP	Height Equivalent to Theoretical Plate
HP	Hewlett Packard
HPLC	High performance liquid chromatography
HSCCC	High speed countercurrent chromatography
HSES	Hydrostatic Equilibrium System
HPCL/APCI/MS	High performance liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry

HPLC/ESI/MS	High performance liquid chromatography/electrospray/mass spectrometry
HPLC/MS	High performance liquid chromatography/mass spectrometry
HPLC-UV	High performance liquid chromatography-ultraviolet
3'-HYD	3'-hydroxydaidzein
8-HYD	8-hydroxydaidzein
ID	Internal Diameter
IPA	Isopropyl Alcohol
IR	Infrared
IUPAC	International Union of Pure and Applied Chemistry
K′	Capacity or Retention Factor
K	Partition Coefficient
KeV	Kilo electron volts
kg	Kilogram
kV	Kilovolt
l	Litre
L	Length
LC	Liquid chromatography
LC/APCI/MS/MS	Liquid chromatography/atmospheric pressure chemical ionisation/mass spectrometry/mass spectrometry
LC/ESI/MS	Liquid chromatography/electrospray/mass spectrometry
LC/ESI/MS/MS	Liquid chromatography/electrospray/mass spectrometry/mass spectrometry
LLC	Liquid liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
LSC	Liquid-solid chromatography
LC-UV	Liquid chromatography-ultraviolet

M^+	Molecular ion
M(or Me)	Methanol
MS	Mass Spectrometry or Mass Spectrometer
m/z	mass to charge
mg	milligrams
min	minute
ml	millilitre
mm	millimetre
MRFA	L-methionyl-arginyl-phenylalanyl-alanine acetate
MRM	Multiple reaction ion monitoring
MS	Mass spectrometry
MS^1	Single-stage full scan mass spectrometry
MS^2 or MS/MS	Mass spectrometry-mass spectrometry (tandem mass spectrometry)
MS^n	Multiple-stage mass spectral analysis to the nth stage
ng	nanogram
nl	nanoliter
NMR	Nuclear magnetic resonance
NP-LC	normal phase-liquid chromatography
OD	Outer diameter
ODS	Octadecylsilane
PDA	Photodiode Array
PEG	Polyethylene glycol
pg	Picogram
psi	Pounds per square inch
R_F	Retention Factor
RLCCC	Rotation Locular Countercurrent Chromatography
RP	Reversed Phase

RPLC	Reversed Phase Liquid Chromatography
R_S	Resolution
SEC	Size exclusion chromatography
SFC	Supercritical Fluid Chromatography
SIM	Single Ion Monitoring
SPE	Solid phase extraction
SRM	Selected reaction monitoring
TIC	Total ion current
TLC	Thin layer chromatography
t_r	Retention time
W	Peak Width
W	Water
$W_{1/2}$	Peak Width at half height
V	Volts

CHAPTER 1

Introduction

1.1 INTRODUCTION TO CHROMATOGRAPHY

According to the definition of International Union of Pure and Applied Chemistry (IUPAC) [1], chromatography was named as " A method used primarily for the separation of components of a sample, in which the components may be distributed between two phases, one of which is stationary while the other moves. The stationary phase may be a solid, liquid supported on a solid, or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film and the mobile phase may be gaseous or liquid". The classification of chromatography is based upon the physical nature of the mobile phase: gas chromatography (GC), liquid chromatography (LC), or supercritical fluid chromatography (SFC). The chromatography process occurs as a result of repeated absorption/desorption steps during the movement of analytes along the stationary phase. The separation is based on the differences in distribution coefficients of the individual analytes in the sample.

1.1.1 Historical Developments of Chromatography

The technique of chromatography is considered as one of the most dynamic and versatile analytical methodologies. It has been widely used for the separation of analyte mixtures and the identification of their individual components. The concept of chromatography was introduced by the Russian botanist Tswett [2] in 1919 when he reported on the novel separation and isolation of various plant pigments by using a glass column packed with calcium carbonate. During the separation coloured bands relating to the differing plant components passed through the column and for this reason the process was termed, chromatography meaning colour writing. Unfortunately, his remarkable work was ignored by his contemporaries for nearly twenty years and regarded as being of little merit. Further development of chromatography occurred later in 1931 when Kuhn and his co-workers [3] applied Tswett's method to separate other plant pigments, including xanthophylls and carotenoids. At that time, this research generated renewed interest and recognition of chromatography as a valuable separation method. In the early 1940s Martin and

Synge[4] developed liquid-liquid partition chromatography in which the stationary phase was water retained on a solid support of silica gel. This technique had a large impact but further strengthened with the development of paper chromatography [5]. After 11 years later, James and Martin developed the technique of gas-liquid chromatography and demonstrated the integrated theory of this separation technique. Martin was award the Nobel Prize in Chemistry in 1952 because of his remarkable contribution to chromatography.

The development of chromatography could not be described as a smooth growth curve. It did not develop rapidly until in 1960s when high pressures and small particle sizes were employed. The high pressure and small particle size applied in a column provided much improved separation and the concept of the "height equivalent to a theoretical plate" was developed as a measure of column efficiency. These concepts were first applied to gas chromatography and later to liquid chromatography and it was at this time that the first high performance liquid chromatography experiments were carried out [6, 7]. After 40 years of rapid development, chromatography is now an extremely versatile and robust separation technique and is widely used in fields such as analysis of compounds in pharmaceuticals, food, medicine and biology and without doubt will continue to play an important role in a wide range of scientific studies.

1.1.2 Theory of Chromatography

In chromatography, the components of a sample mixture are separated by distribution between two immiscible phases, one of which is stationary (a solid or liquid) and the other mobile (a liquid, gas, or supercritical fluid). Separation occurs during chromatography due to the differential migration of the analytes in the column and they elute at different time intervals. The average rate of migration of an analyte through the column depends on the fraction of time spent in the stationary phase and on the affinity of the analyte to the stationary phase. Components that tend to reside in the mobile phase will move more quickly than those that prefer the stationary phase. The differential rate of migration of analytes results in the separation of the components as they pass through the column. Each analyte is distributed between the two phases according to its

distribution coefficient, K. The K was defined as

$$K=C_s/C_m \qquad 1.1$$

Where C_s is the molar concentration of the analyte in the stationary phase, and C_m is its molar concentration in the mobile phase. The various components must have different distribution coefficients if a sample mixture is to be separated. There are several important parameters to evaluate the separation and the efficiency.

1.1.2.1 The Chromatographic Retention

The retention time (t_r) is the time required for an analyte to move from its point of injection until its point of detection. Figure 1.1 shows the chromatographic separation of two compounds A and B.

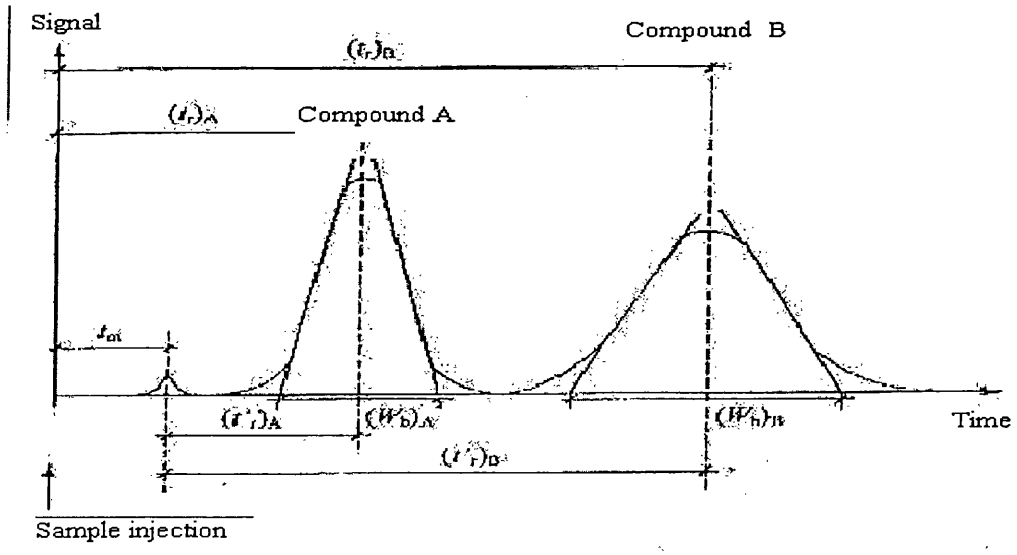


Figure 1.1 *The Chromatogram and its Characteristic Features*

1) The *dead time* (t_0) is the time taken for an analyte to pass through the column void volume (space occupied by the mobile phase). It is identical for all analytes in a given chromatographic system since they migrate with the same velocity (that of the mobile phase). The dead time can be obtained by measuring the time required for an unretained solute to pass through the system.

2) The *retention time* (t_r) is the time taken between the injection point and the peak maximum. Each solute has a characteristic retention time.

3) The *adjusted retention time* (t_r') is the time for which an analyte is retained, compared with that of an unretained compound and is therefore directly associated with the interaction of the analyte with the stationary phase.

$$t_r' = t_r - t_0 \qquad 1.2$$

1.1.2.2 The Capacity Factor (k')

The capacity factor is defined as the ratio of the amount of an analyte in the stationary and mobile phase respectively and can be calculated from the chromatogram by

$$k' = (t_r - t_0)/t_0 \qquad 1.3$$

It is the ratio of the adjusted retention time needed for a component to pass through the system, relative to the time for an unretained solute to pass through the same system. Since, it is independent of the mobile phase flow rate and the physical dimensions of the column, it can be used to compare retentions on different instruments.

1.1.2.3 The Selectivity Factor (α)

The selectivity factor is defined as the ratio of distribution coefficients of components A and B or the ratio of capacity factors of components A and B.

$$\alpha = K_B/K_A = \kappa'_B/\kappa'_A \qquad 1.4$$

The selectivity factor gives an indication of resolution between two or more species, prior to experimentation. If α=1, then no separation takes place, as the retention times are identical. Although the selectivity factor describes the separation of peaks it cannot give accurate information especially for LC systems since it does not take into account peak widths. A better measure of separation of two neighbouring peaks is provided by column resolution Rs. Which takes into account both retention difference and column efficiency.

1.1.2.4 Column Resolution (*Rs*)

The degree of separation or resolution (*Rs*) between two components in a chromatogram is determined from the differences in the corresponding retention times and baseline peak widths (W_b) or peak widths at half-height ($W_{1/2}$). For symmetrical peaks of Gaussian shape it can be defined as

$$Rs = \frac{2(t_{r2} - t_{r1})}{W_1 + W_2} = \frac{1.18(t_{r2} - t_{r1})}{W_{1/2}1 + W_{1/2}2} \qquad 1.5$$

Where t_{r2} and t_{r1} refer to the retention time values corresponding to components A and B, and W_1 and W_2 refer to the baseline peak widths corresponding to components A and B. For a more accurate value of Rs, it can also be calculated by the peak width at half-height ($W_{1/2}$). Generally, the peaks are not completely separated with a resolution of 1.0 but correspond to approximately 2 % peak overlap. Baseline resolution of peaks is considered to be achieved with a resolution of 1.5 and resolution in excess of 1.5 will result in longer separation times.

1.1.2.5 Separation Efficiency

Good separation efficiency means the attainment of sharp, symmetrical peaks, since this improves the potential for analyte separation and enhances detection. The chromatogram can be used to

characterise the separation efficiency by calculating the number of theoretical plates, or plate number, N, in the column. The most common approach assumes a gaussian peak:

$$N=16(t_r/w)^2 \qquad 1.6$$

$$N=5.54(t_r/w_{1/2})^2 \qquad 1.7$$

Theoretical plates are considered as a series of narrow discrete sections in a chromatographic column or layer and at each plate equilibration of an analyte between the two phases is assumed to occur. Movement of analyte and mobile phase is viewed as a series of transfers from one plate to the next. The number of equilibrations and thus the number of theoretical plates increases as a function of better packing, longer column length and optimum mobile phase flow rate conditions. The plate height H (HETP=height equivalent to theoretical plate) can be calculated by the plate number, N and the column length, L

$$H=L/N \qquad 1.8$$

Where H is the distance over which chromatographic equilibrium is achieved. This parameter allows intercolumn comparisons to be made.

1.1.3 Classification of Chromatography

Chromatographic separation can be classified in a number of ways according to stationary or mobile phase, separation process and mechanism. These will be briefly discussed.

1.1.3.1 Classification by mobile phase

Chromatography can be classified by the nature of the mobile phase i.e liquid chromatography (LC), gas chromatography (GC) and supercritical fluid chromatography (SFC) depending on whether the mobile phase is a liquid, gas or supercritical fluid, respectively. In liquid chromatography (LC), it can be further classified into ion exchange chromatography, size exclusion chromatography, reversed-phase and normal-phase chromatography. Reversed-phase and normal-phase chromatography are defined according to the polarity differences between the mobile phase and stationary phase. If the stationary phase is less polar than the mobile phase the system is defined as reversed-phase. In reversed-phase elution mode, polar molecules have less affinity to the stationary phase and will elute earlier. Conversely if the stationary phase is polar

and the mobile phase non-polar then the system is referred to as normal-phase. In this elution mode, the less polar molecules will elute earlier.

1.1.3.2 Classification by separation mechanism

This classification is based on the interaction between sample components and the two phases. It is considered as the most accurate and common basis for classification. The forces involved in these interactions are usually weak intermolecular forces such as van der Waals or hydrogen bonding. The chromatography can be classified into a number of types, which include: adsorption, partition, bonded phase, ion exchange, size-exclusion, affinity and countercurrent chromatography. The relevant principles involved in the chromatographic techniques studied in this project will be discussed in the sections on high performance liquid chromatography (HPLC) and countercurrent chromatography (CCC).

1.1.3.3 Classification by different purpose and fields

A) Analytical chromatography

This chromatography is widely used in the analytical laboratory and developed for the separation and determination of all kinds of complex sample. The column diameter and length for these purposes are small and a small amount of sample is injected.

B) (Semi) preparative chromatography

This chromatography can be further classified into preparative in the laboratory and scale up preparative chromatography. It is primarily used for the separation and purification components with high purity such as standard materials and protein purification. The column size and length are both big and a large amount of sample can be injected in one step.

1.1.4 High Performance Liquid Chromatography

High performance liquid chromatography has been developed extensively since reduced particle size of the stationary phase and increased pressure were used to improve the separation efficiency. Reverse phase HPLC has become the most popular method of separation currently used as it offers efficient separation with good resolutions in a short time. Its application almost occupies 80 % in total chromatography techniques. In reverse phase HPLC, most columns are strong

hydrophobic silica packing materials modified by some functional groups such as octyl(C_8), octadecyl(C_{18}), phenyl(C_6H_5) and amino(NH_2). Of the reverse phase stationary phases, the chemically bonded octadecylsilane(ODS) packing is the most commonly used and was also chosen in our study. ODS packing silica has OH groups on its surface which are reacted with an octadecyl group to give a strong non-polar stationary phase. Figure 1.2 shows their reaction. The mobile phases used in reverse phase HPLC generally are a mixture of water or an aqueous buffer and a water miscible solvent (e.g. methanol or acetonitrile).

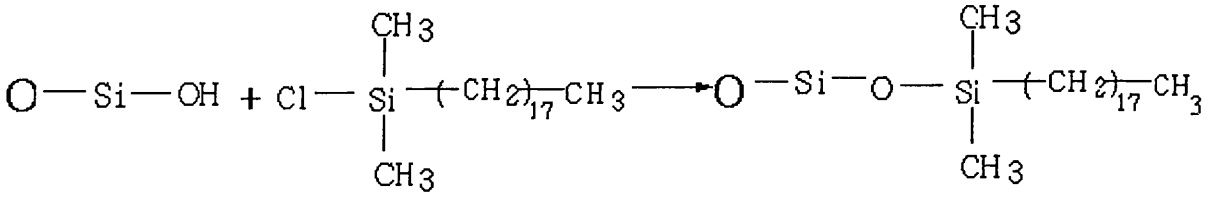


Figure 1.2 *Formation of C_{18} reverse phase stationary phase*

The basic chromatograph consists of a solvent reservoir, a solvent system (or pump), a sample injection device, a column, detector and a data system (Figure 1.3)

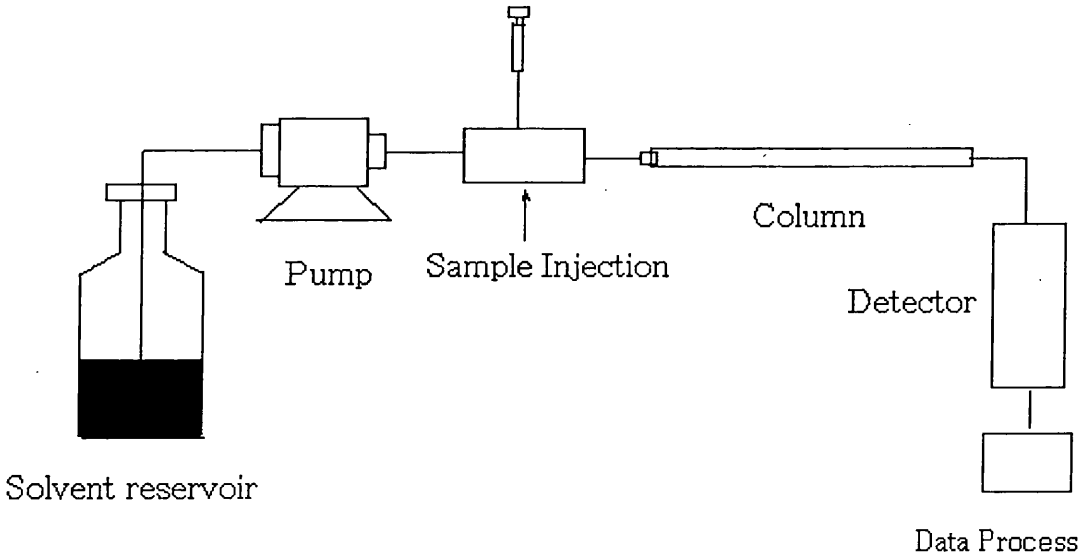


Figure 1.3 *Schematic diagram of the basic HPLC system*

The mobile phase in the reservoir is first degassed to prevent air bubble formation in the pump heads and in the column, which can reduce the efficiency and produce serious noise. Degassing can be performed by purging the solvent with helium, by ultrasonic treatment, by vacuum filtration or by applying a vacuum above the stirred solvent for about 30 minutes. A pump system is used to deliver solvent systems into the column and there are a number of different types of LC pumps available. The most widely used high-pressure pump for LC is a reciprocating pump for their overall performance and reliability. These pumps use a piston or a diaphragm to displace solvent from small chambers, via check valves, out of the pump. The pumps may have one, two or three but the most common is the dual head-reciprocating pump. The flow rate is controlled by the frequency of the piston movement or by the length of the piston stroke. Sample introduction can be performed manually via a hand-held micro syringe or automatically. The sample is introduced from a vial held in a sample tray and then separated by the column.

1.1.5 Detectors in chromatography

Many detectors are available for detection of analytes after chromatographic separation. The most commonly used is the ultraviolet detector, this detector allows detection of the components of a mixture and can also give concentrations of them. But this method relies upon the presence of an ultraviolet light absorbing chromophores. The diode array detector allows the detection of the light absorbance at a number of wavelengths and also allows the production of the absorbance spectra of the eluted components. Other detectors include refractive index detectors, fluorescence detectors, electrochemical detectors, radioactivity detectors and recently on line LC/mass spectrometry.

1.2 COUNTERCURRENT CHROMATOGRAPHY (HSCCC)

1.2.1 Brief history of CCC

The partition of solutes between immiscible solvent phases is an ideal method for separation and purification of natural products. By eliminating the various complications that arise from the interaction between solute molecules and the solid support matrix, this method can yield high purity fractions with high sample recovery rate and high reproducibility. HSCCC, being a

support-free liquid chromatography, which eliminates complications such as irreversible adsorption onto the solid support, tailing of the solute peaks, etc, has been extensively used for the separation and purification of natural products. Countercurrent chromatography began in the mid-1960s. In its early stages of development, the method was hindered by a limited mobile phase flow rate since the application of higher flow rates resulted in excessive loss of the stationary phase from the column. So it was quickly replaced by liquid chromatography due to its inherit disadvantages such as its time consuming operation and bulky fragile instrumentation.

The first widely distributed commercial model of droplet CCC [8] was produced by Ito and his co-workers. This droplet CCC uses unit gravity to move the droplets of the mobile phase through the column of the stationary phase in a tubular space. This simple CCC system produced a partition efficiency of 900 theoretical plates in the dinitrophenyl (DNP) amino acid separation, but it required a long separation time of 70 hours. This apparatus produced unfavourable impressions of this technique as being time-consuming and inefficient. Despite the successive development of centrifugal CCC using a variety of coil planet centrifuge systems, this problem persisted until the late 1970s when a new CCC system was developed. This new CCC technique was named high-speed countercurrent chromatography (HSCCC). HSCCC utilized a combination of a particular type of planetary motion and coaxial orientation of the coiled column to generate a rapid movement of the mobile phase through a bulk of stationary phase under an efficient mixing of the two phases, thereby achieving both high partition efficiency and an excellent retention of the stationary phase. This method opened a rich domain of commercialization of the instruments in the world. Further studies revealed that the method could be applied to dual CCC where two immiscible solvent phases can truly undergo countercurrent movement through the coiled column. This method has been applied not only to liquid-liquid dual CCC but also to foam separation, which utilizes the countercurrent movement of the foam and liquid phases through a long coiled column [9,10]. It has recently been found that a new type of the coil planetary centrifuge (CPC) system provides stable retention of the stationary phase for the viscous aqueous-aqueous polymer phase systems, which can be applied to separation of macromolecules and cell particles [11]. Most recently, a new technique called pH-zone-refining CCC has been developed. This method has been successfully applied to a variety of organic acids and bases, including amino acids and peptides, dyes, alkaloids, and some optical isomers [12]. During the past thirty years remarkable progress has been made in the HSCCC technique, Now, HSCCC has widely used for application to the separation of natural products [13,14,15], rare earth [16] protein [17] and chiral drug separation [18].

1.2.2 Principle of high-speed countercurrent chromatography

1.2.2.1 Two-phase Distribution in a Rotating Coil in Unit Gravity

There are two basic systems of CCC: the hydrostatic equilibrium system (HSES) and the hydrodynamic equilibrium system (HDES). Examples of hydrostatic CCC systems are droplet CCC [19,], rotation locular CCC [20] and centrifugal partition chromatography (CPC)[21]. However, these techniques are considered to be inefficient in terms of resolution and separation time. In the second system, the HDES, the column (coiled tube) rotates along its own axis (radius r) and is subject to a constant gravitational field. When the coil containing two immiscible solvent phases is rotated around its horizontally placed axis, the two solvent phases are evenly distributed along the length of the coil with the heavier phase on the head side and the lighter phase on the tail side. When the rotation speed is increased, the heavier phase entirely occupies the head side and the other phase is on the tail side. This hydrodynamic equilibrium condition provides a great advantage in performing CCC, because the system permits retention of a large amount of stationary phase in the coil. Figure 1.4 illustrates the Archimedean screw effect using a coiled tube slowly rotating in a unit gravitational field. All coils are sealed at both ends before being rotated.

In Figure 1.4.A the coil is first filled with water, and air bubbles and glass beads are introduced into the coil. The air bubbles stay at the top of the coil and the glass beads stay at the bottom of the coil due to the effect of gravity while the Archimedean screw force drives both objects toward one end (left) of the coil as the coil is rotated slowly in the gravitational field. All objects present in the coil either lighter or heavier than water will move through the coil in the same direction toward one end. This end of the coil is conventionally called the "head" and the other end is the "tail".

Figure 1.4 B performs a similar experiment using two mutually equilibrated solvent phases. In the upper diagram, the coil is filled with the lighter phase and a small volume of the heavier phase is introduced at the tail. Similar to the figure 1.4 experiment, the droplets of heavier phase will stay at the bottom of the coil and move toward the head of the coil and in the lower diagram, a small amount of the lighter phase suspended in the heavier phase in the coil moves toward the head.

In Figure 1.4 C, nearly equal volumes of the lighter and heavier phases are introduced in the coil. The two phases are separated in each helical turn, the lighter phase in the upper portion and the heavier phase at the lower portion. The rotation forces both phases to competitively advance toward the head of the coil. When the two phases establish a hydrodynamic equilibrium from the head side of the coil where each phase occupies nearly equal space in each helical turn while any excess of either phase is pushed back toward the tail end of the coil. Once this hydrodynamic equilibrium is reached, further rotation of the coil results in mixing of the two solvent phases back and forth within each helical turn while the overall distribution of the two phases in the coil remains unaltered.

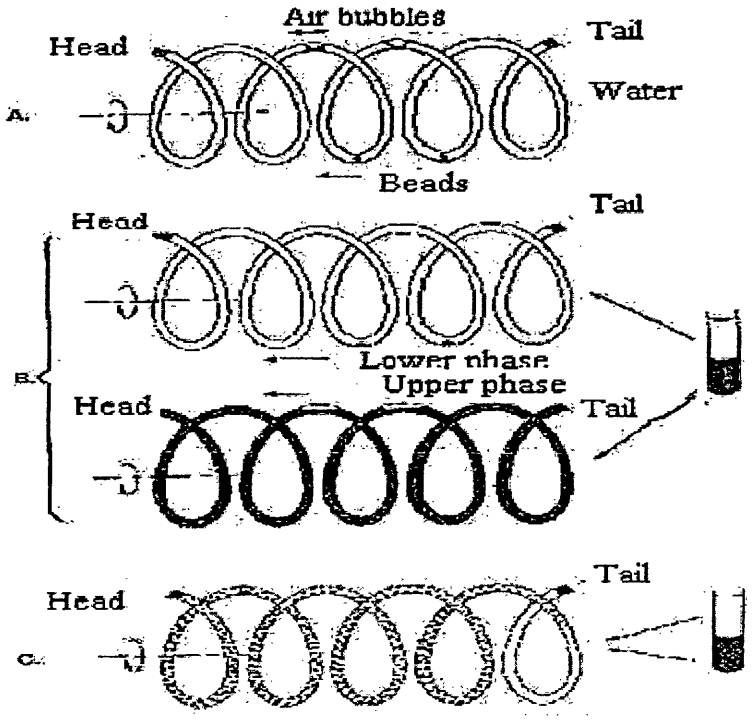


Figure 1.4 A-C *Motion of various objects in a slowly clockwise rotating coil* [22]

In CCC, the amount of the stationary phase retained in the coil is an important factor governing the resolution of solute peaks; the higher retention greatly improves peak resolution. Further studies revealed that the resolution of stationary phase is greatly altered by the rotational speed of the coil [23]. This hydrodynamic equilibrium condition provides a great advantage in performing CCC, because the system permits retention of a large amount of stationary phase in the coil if the lighter phase is eluted in a normal mode (head-to-tail direction) or the heavier phase in a reversed mode (tail-to-head direction).

The two-phase distribution in the rotating coil described above is a complex hydrodynamic phenomenon that has not been formulated mathematically. Some valued researches [22] were done to describe some relationship in the Flow-through coil Planet Centrifuge between the stationary phase distribution in the rotating coil and the acting force field at the various portions of the coil.

1.2.2.2 Flow-through coil Planet Centrifuge

In the 1970s, a series of planetary centrifuge systems had been developed for performing CCC based mainly on the hydrodynamic equilibrium. All of these centrifuge systems were equipped with a rotary seal-free flow-through device so that the mobile phase could be eluted through the rotating column without the conventional rotary seal. Developments of the HDES technology have provided fast and efficient instruments with various configurations depending on the relative of the two rotational axes and the ratio of the two radii to the two rotation speeds. Most of them have been studied by Ito and classified into I, L, J and X [22]. The well known and most commercialised is the type J high-speed countercurrent chromatography. The type J CCC planet centrifuge system has widely applied for the separation of natural and synthetic products.

1.2.2.3 The Brunel CCC

The Brunel CCC is a "J" type coil planet centrifuge (see Figure 1.5). It has two bobbins with two coils wound on each bobbin. It is designed around the twin coil planet centrifuge first proposed by Ito but has a novel flying lead arrangement that reduces wear. The coil is simply a continuous length of PTFE tubing spirally wound on a drum, with an inlet and an outlet lead. The B-values (the ratio of the planet radius to the orbital radius) for the Brunel CCC are in the range of 0.7 to 0.83 (see Figure 1.6). The coil volume varies between 50 and 250 ml depending on the model and the total volume of the pairs on each bobbin is identical. The coil is made of PTFE (3.3 mm OD. and 1.6 mm ID). The coil is mounted on an epi-cyclic coil planet centrifuge, which rotates the coil in planetary motion. In this way, a given point on the coil will experience a fluctuating force field from high "g" to a low "g". The effect of this is to produce zones of mixing and settling which travel from "tail" end to the "head" end of the coil, where the "head" is defined as the end where a bubble would travel under the action of the Archimedean screw effect. In this system, a sample

injected with the mobile phase will experience approximately 50000 mixing and settling steps an hour as it passes through the coil.

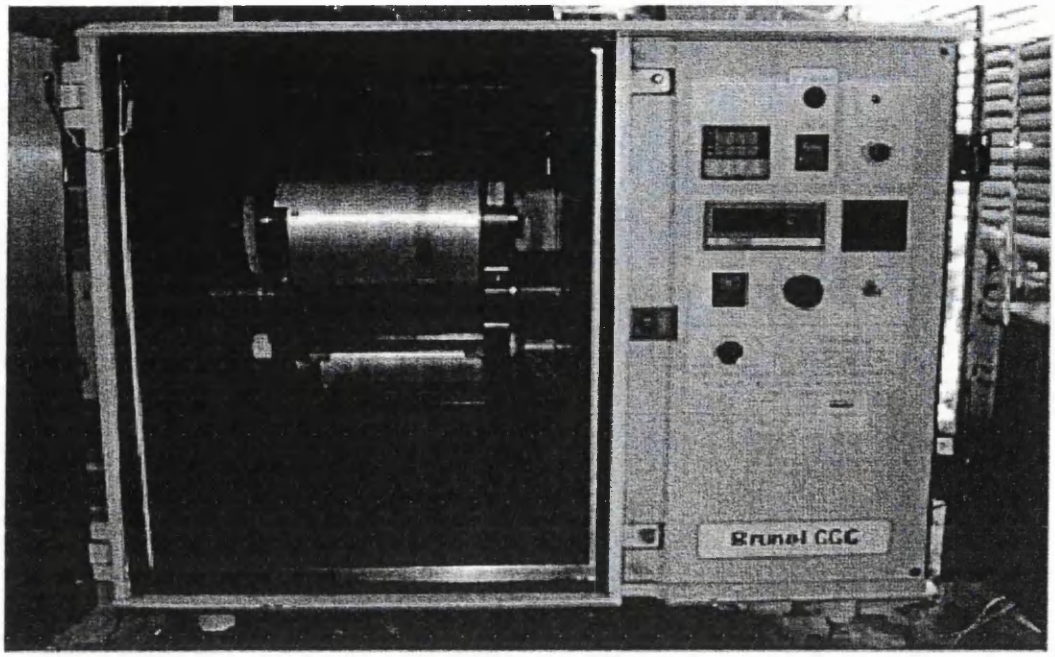
1.2.3 The operational procedure

The operation is very simple. First, two immiscible solvents are saturated in a separatory funnel by vigorous shaking. Either the upper or lower phase of the two-phase solvent system, which is intended to be the stationary phase, is pumped into the coil. After the coil is fully filled with the stationary phase, the mobile phase is then pumped in once the coil is rotating. Some of the stationary phase will be displaced. When hydrodynamic equilibrium is established, only the mobile phase exits the coil and the sample (which is ideally in a mixture of the two phases) is introduced via a sample loop at the inlet of the coil. The separated constituents are eluted in the order of the partition coefficient to be collected in a fraction collector.

1.2.4 Detectors for CCC

In the past on-line detection in HSCCC has been almost entirely performed with a UV-VIS absorbance monitor. On many occasions, this on-line monitoring in CCC is disturbed by carryover of the droplets of the stationary phase. In addition, the mobile phase is in a subtle equilibrium with the stationary phase and a slight change in temperature may cause cloudiness of the effluent or result in the bleeding of the stationary phase producing a chaotic emulsification in the flow stream. This emulsification can be minimised by post-column addition of a third solvent to coalesce the two immiscible liquids. However, this addition of solvent decreases the sensitivity and causes peak broading [24]. Evaporative light-scattering detectors (ELSD) can be useful for the detection of molecules without chromophore groups but compounds that have melting points equal to or lower than the ELSD nebulizer temperature may be difficult to detect and in addition the quality of solvents used in the separation must be carefully considered [25]. The coupling of HSCCC with mass spectrometry has been reported to avoid the solvent problems associated with conventional detectors [26]. Mass spectrometry offers universal detection with high sensitivity but is limited to use with analytical HSCCC and in addition the limited solvent selection must be considered for MS detector.

An alterative approach is to collect fractions at the outlet of the CCC and analyse by TLC, HPLC or LC/MS. This approach is a laborious and time-consuming process. Until now, there is no universal detection method used in CCC. Further research work on this field needs be done.



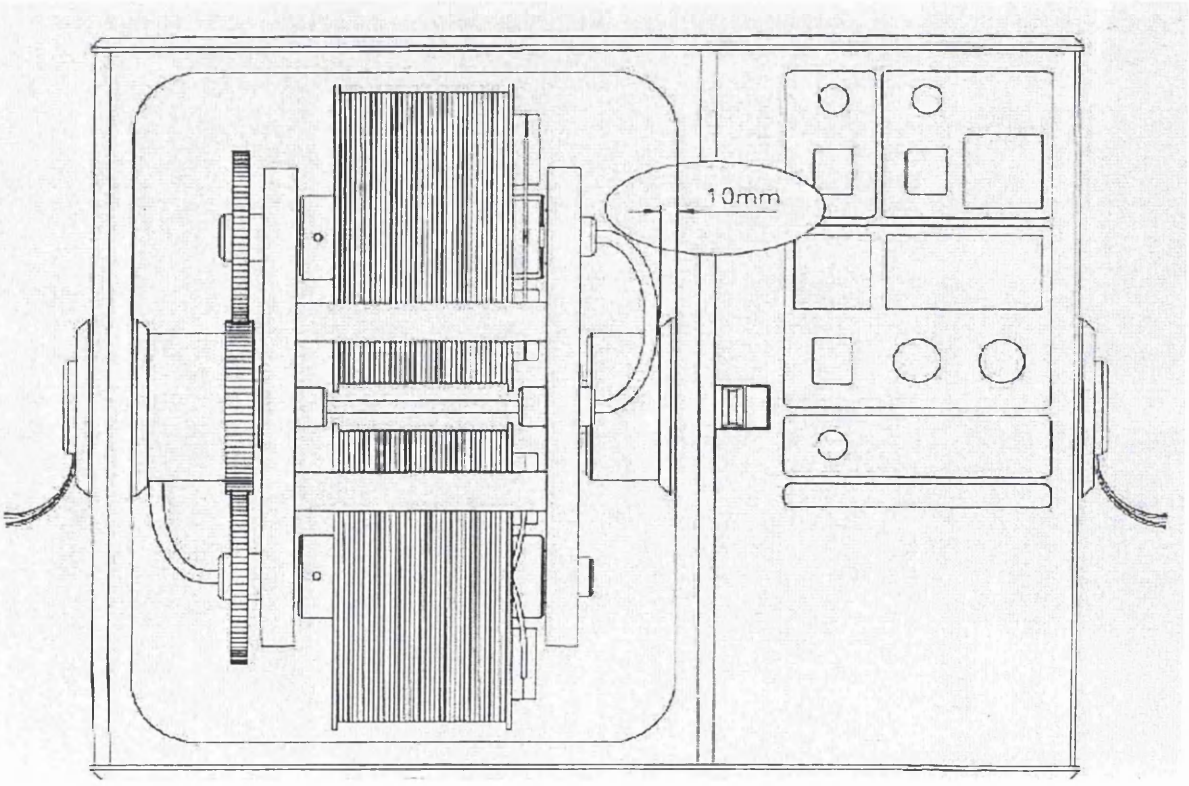
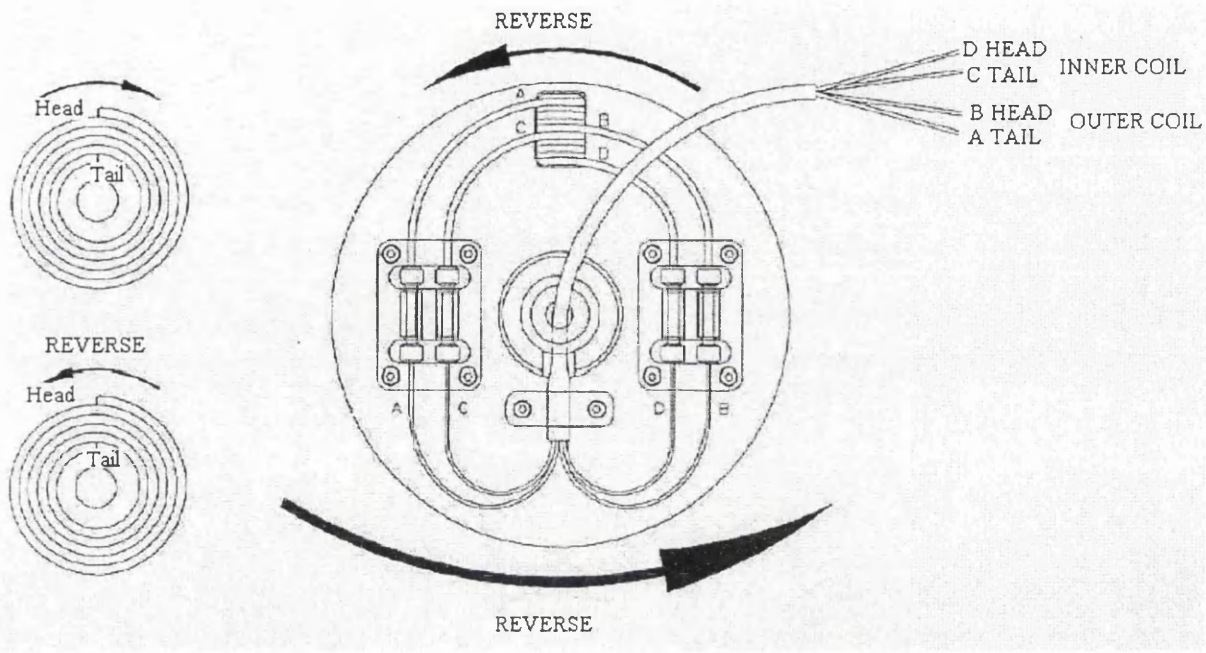


Figure 1.5 *The Brunel CCC instrument. Upper: Door open; Bottom: Structure inside*



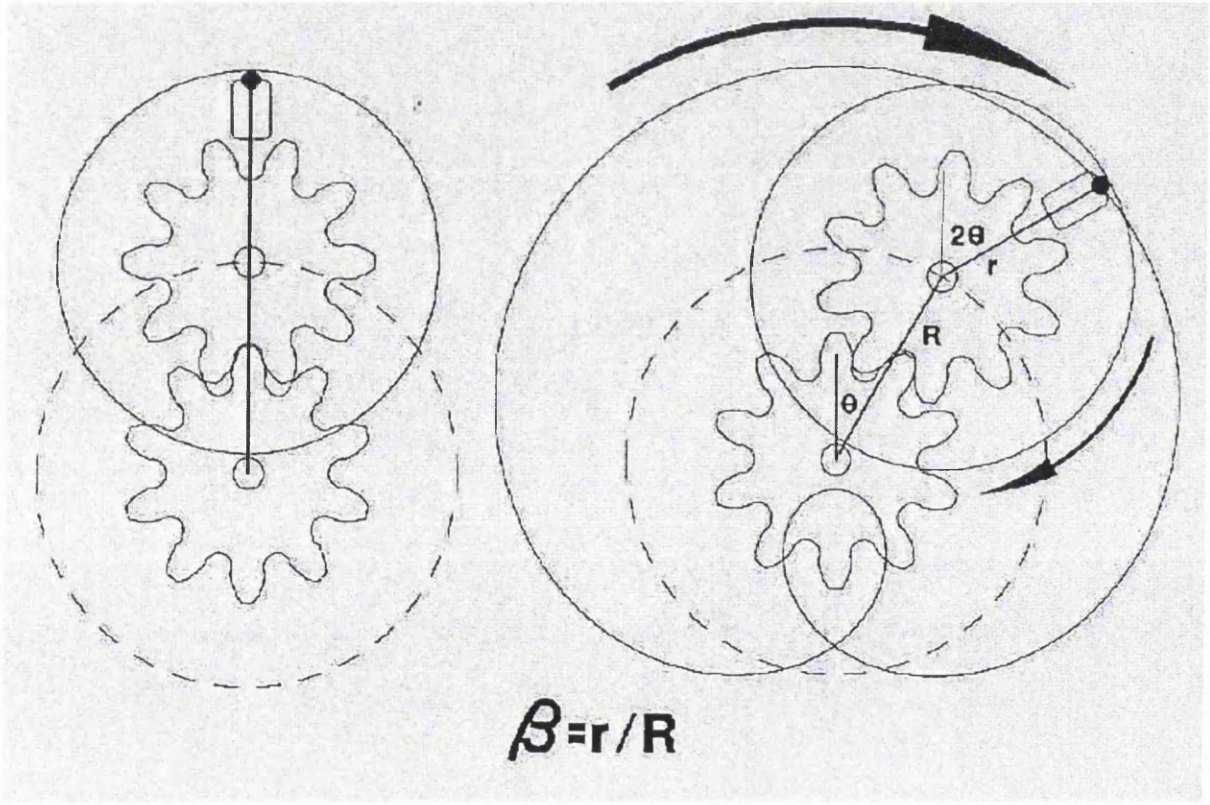


Figure 1.6 *Upper: The cross sectional view of PTFE wound on a drum; Bottom: The definition of β*

1.2.5 Selection of solvent system

Selection of a HSCCC solvent system means choosing a suitable column. In CCC, the selection of a suitable solvent system is the most important step. Successful separation in CCC requires a proper choice of a two-phase solvent system that provides a suitable range of partition coefficients (K) for the desired compounds. The most efficient solvent systems are those that yield K values of 0.2-5[27]. The analytes to be separated should have distribution ratios different from each other. In general, the two-phase solvent system should satisfy the following criteria:

A) The solvent system should provide nearly equal volumes of the upper and lower phases in order to avoid the waste of stationary phase.

B) The two –phase solvent system should yield a reasonably short settling time. In HSCCC, the settling time should be shorter than 30s for satisfactory retention of the stationary phase in the column.

C) The K value of the desired compound should be close to one, which gives a retention volume equal to the total column capacity. A lower value may result in poor resolution and a greater value may result in excessive band broadening.

1.2.5.1 TLC for selection of solvent system

An empirical approach for solvent selection is solvent screening by TLC. The organic layer of a two-phase system is used as the eluent and the R_f values are measured by both normal phase and reverse phase modes. The R_f values should lie between 0.3-0.7. This method is very simple and convenient, but sometimes is not very accurate, because TLC still involves both partition and adsorption mechanisms, whereas CCC is based on liquid-liquid partition phenomena.

A good method for solvent selection is a literature search for the suitable solvent system previously used for the similar compounds.

1.2.5.2 Direct Measurement of the partition coefficients (K)

The partition coefficient of a component can be determined by a simple test tube procedure: A known amount of the sample is thoroughly equilibrated with the two phase solvent system, 1-2 ml of each phase is mixed with a suitable solvent (such as water or methanol) and the absorbance is determined with a spectrophotometer. In addition to the ultraviolet (UV) and visible wavelengths,

fluorescence, radioactivity, enzymatic activity, and various types of bioassay can also be used for determination of the partition coefficients. When a pure compound is not available, the above method only gives an average K value of multiple components, which may not be useful for a practical separation. In this case, an aliquot of each phase is preequilibrated with the samples separately analysed by HPLC or thin-layer chromatography. From a pair of chromatograms obtained from each phase, the K value for each component is obtained by computing the ratio in height or area between the corresponding peaks.

1.2.5.3 Analytical CCC for rapid selection of solvent system

In recent years, analytical CCC had a rapid development because of its small solvent consumption and faster analysis time. In this study, analytical CCC was also used for rapid measurement of partition coefficients and selection of solvent system [28].

1.3 INTRODUCTION TO LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY (LC/MS)

1.3.1 Mass Spectrometry

A mass spectrometer is an analytical instrument that is capable of forming, separating, and detecting ions, either atomic or molecular based on their mass to charge ratio. The mass to charge ratio of an ion is often abbreviated as m/z and the resulting mass spectrum is a plot of the relative abundance of the generated ions as the function of the m/z ratio. Any mass spectrometer can be basically described as a set of functional modular components. These components include: sample inlet, ion source, mass analysis, ion detection and data handling. A mass spectrometer is a highly sophisticated and computerised instrument and necessary training must be experienced before operation.

1.3.2 Liquid Chromatography/Mass Spectrometry Interfacing

Liquid chromatography / mass spectrometry (LC/MS) is an analytical technique in which a (high performance) liquid chromatograph (LC) and a mass spectrometer (MS) are combined. The effluent from the LC is introduced into the mass spectrometer. The LC is the input device for the

mass spectrometer, and the mass spectrometer is the detector for the LC. An LC/MS interface is required to translate from the high-pressure environment of the LC to the very low-pressure environment of the mass spectrometer. The coupling of liquid chromatography/mass spectrometry provides sensitive, selective, rapid and information-rich analytical methodology in many fields. It has become a robust and routine analytical tool and offers the analytical chemist one of the most powerful analytical techniques of modern time. Many separation techniques such as gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE) can be coupled with mass spectrometry and these coupling techniques have been widely used in pharmaceutical, food and ecological fields. The direct on-line combination of a liquid chromatography and a mass spectrometer was considered for many years as incompatible, because the mass spectrometry requires a vacuum and the analytes of interest to be present in the gas phase whereas HPLC provides separation of involatile compounds in a liquid mobile phase.

Different methods were used to tackle these problems [29, 30]. Some of these coupling methods such as moving belt coupling or the particle beam interface are based on the selective vaporization of the elution solvent before the analyte enters the spectrometer source. Another method, such as continuous-flow fast atom bombardment, relies on reducing the flow of liquid that is introduced into the interface in order to obtain a flow that can be pumped directly into the source. Later a series of HPLC/MS coupling methods such as thermospray (TSP), electrospray, atmospheric pressure chemical ionisation (APCI) have been introduced, which of these the atmospheric pressure chemical ionisation can tolerate flow rates of about 1 ml/min without requiring a flow split.

1.3.2.1 Electrospray Ionisation

Electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) are two examples of atmospheric pressure ionisation sources. Both of them are soft ionisation techniques. Such sources ionise the sample at atmospheric pressure and then transfer the ions into the mass spectrometer. In electrospray, a sample solution from the HPLC column enters the ionisation source as a fine mist of droplets via a stainless steel needle, which has an accompanying flow of nitrogen gas surrounding it. An electrospray is produced by applying a strong electric field, under atmospheric pressure, to a liquid passing through a capillary tube with a weak flux. The electric field is obtained by applying a potential difference of 3-6 KV between this capillary and the

counter-electrode, producing electric fields of 10^6 Vm^{-1}. This field induces a charge accumulation at the liquid surface located at the end of the capillary, which will break to form highly charged droplets. A gas injected coaxially at a low flow rate allows dispersion of the spray to be limited in space. These droplets then pass either through a curtain of heated inert gas, most often nitrogen, or through a heated capillary to remove the last solvent molecules. The ESI sources can tolerate flows up to 0.2 ml min^{-1}, allowing an easy coupling to a classical HPLC system. The tolerated flow can go down to about 100 nl min^{-1} with the nano-electrospray version, allowing coupling with HPLC using small-bore or capillary columns. Electrospray ionisation is the softest ionisation technique currently available providing a sensitive means of analysing a wide range of polar molecules. ESI molecule ions from the compounds being studied are mainly in either a protonated, $[M+H]^+$ or deprotonated, $[M-H]^-$ ions.

1.3.2.2 Atmospheric Pressure Chemical Ionisation

Atmospheric pressure chemical ionisation, like electrospray ionisation, is a mass spectrometer ionisation source in which ionisation occurs not in a vacuum but at atmospheric pressure. In contrast to electrospray ionisation, in which the ionisation process occurs in solution phase, atmospheric pressure chemical ionisation is a gas-phase ionisation process whereby gas-phase molecules are isolated from the carrier solvent before ionisation. In the case of the Finnigan LCQ instrument, the liquid is eluted from an insert capillary, surrounded by a coaxial flow nitrogen nebulising gas and auxiliary gas (usually nitrogen) is flowed into the APCI nozzle. The combination of nebulising gas and heat nebulises the liquid into a fine mist. The droplets in the mist enter the vaporiser, where they are flash vaporised at temperatures up to 600°C. The sample vapour is then carried towards the corona discharge needle by the flow of the sheath and auxiliary gas. Ionisation is initiated when a high voltage, typically of between ± 3 to 5 kV (positive for positive ions and negative for negative ions), is applied to the tip of the corona needle. The corona needle is surrounded by a reagent and ion plasma. The sample vapour is then ionised by ion-molecule reactions with the reagent ions in the plasma. A schematic representation of the formation of a positive ion adduct within the Finnigan APCI source is depicted below:

Primary ion formation

$$e^- + N_2 \longrightarrow N_2^{+\cdot} + 2\,e^-$$

Secondary ion formation

$$N_2^{+\cdot} + H_2O \longrightarrow N_2 + H_2O^{+\cdot}$$

$$H_2O^{+\cdot} + H_2O \longrightarrow H_3O^+ + HO^{\cdot}$$

Proton transfer

$$H_3O^+ + M \longrightarrow (M + H)^+ + H_2O$$

Adduct ion formation

$$H_3O^+ + M \longrightarrow (M + H_3O)^+$$

In contrast to electrospray ionisation, where flow rates less than 1 μl /min are possible, APCI requires comparably high liquid flow rates from roughly 200 to 2000 μl/min because the ionisation mechanisms of APCI and electrospray are fundamentally different. However, the two methods have the potential to provide complimentary analyte characterization. To generalize, electrospray ionisation is more applicable to high molecular-weight compounds; as electrospray requires less heat and has the potential to produce multiply charged ions. Less polar molecules, such as steroids, generally ionise better by APCI.

1.3.3 The Finnigan MAT LCQ Ion Trap Mass Spectrometer [31]

In our studies, the above typed mass spectrometer was used. The various components of the Finnigan LCQ MS are illustrated in the block diagram Figure 1.7

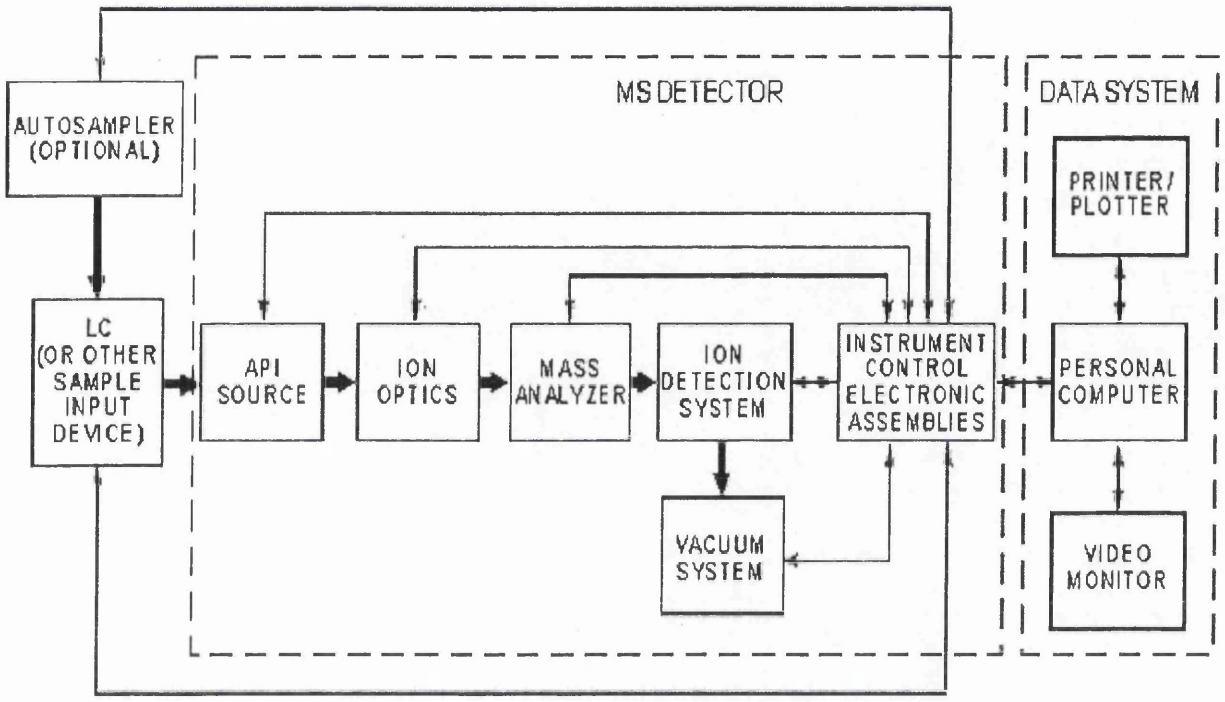


Figure 1.7 *Block diagram schematic of the LCQ system*

1.3.3.1 API source

The API source contains the API sources assembly (ESI or APCI) and API stack. In the API source, gas phase sample ions are formed from molecules that are contained in solution.

1.3.3.2 ESI probe Assembly

A diagrammatic representation of the ESI probe assembly of the LCQ ion trap is shown in Figure 1.8. The ESI probe assembly consists of the ESI flange and the ESI probe. The flow rates of 1μl/ml to 1 ml/min can be operated without the need of "splitting". The ESI flange holds the ESI probe in position next to the entrance of the heated capillary and also seals the atmospheric region of the API source.

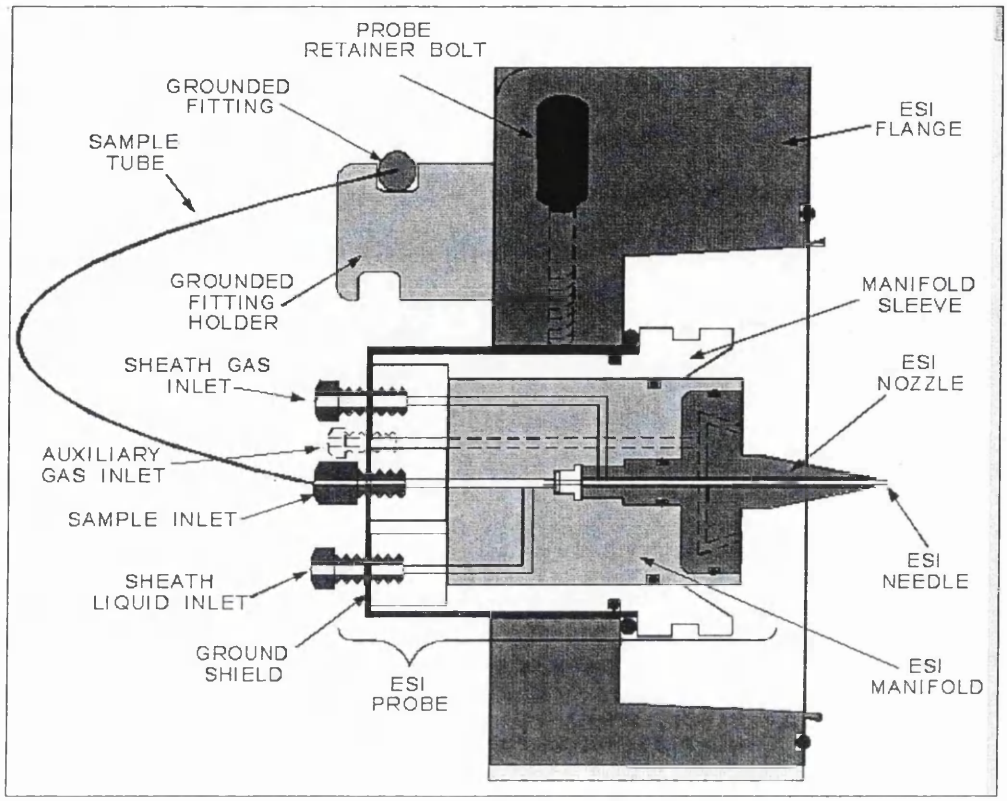


Figure 1.8 *The ESI probe assembly of the LCQ ion trap*

The liquid sample from a syringe or a HPLC column is introduced into the ESI probe through the sample inlet. The sheath gas (nitrogen) helps to nebulise the sample solution into a fine mist when the sample solution exits the ESI nozzle. A high voltage (± 3 to ± 5kV) is applied to the needle. The electric field in the air at the capillary tip is very high, and the solution leaves the electrospray needle as a fine mist of charged droplets. Nitrogen auxiliary gas, the outer coaxial gas, is also introduced through the ESI probe inlet, and assists in the nebulization and evaporation of the sample solution.

ESI as a soft ionisation technique has made significant contributions to modern mass spectrometry. Any polar or middle polar compound can be analysed by ESI. Some heat-labile or high molecular weight compounds, which were previous unsuitable for mass analysis, can be analysed by ESI. ESI has become particularly useful in the mass analysis of polar analyte such as biological polymers (proteins, peptides, glycoproteins and nucleotides), pharmaceuticals and their metabolites.

1.3.3.3 APCI probe Assembly

Figure 1.9 shows the APCI probe assembly. The APCI process is based on the classic ion-molecular or electron capture chemical ionisation reactions operating at atmospheric pressure. The sample solution enters the APCI nozzle through the sample inlet, and is nebulised into a fine mist by a coaxial stream of sheath and auxiliary gas. The droplets in the mist are flash vaporised at temperatures up to 600^0C and are ionised when a high voltage ((±3 to ± 5kV) is applied to the tip of the corona needle. The corona needle is surrounded by a reagent, ion plasma and the sample vapour is then ionised by ion-molecule reactions with the reagent ions in the plasma.

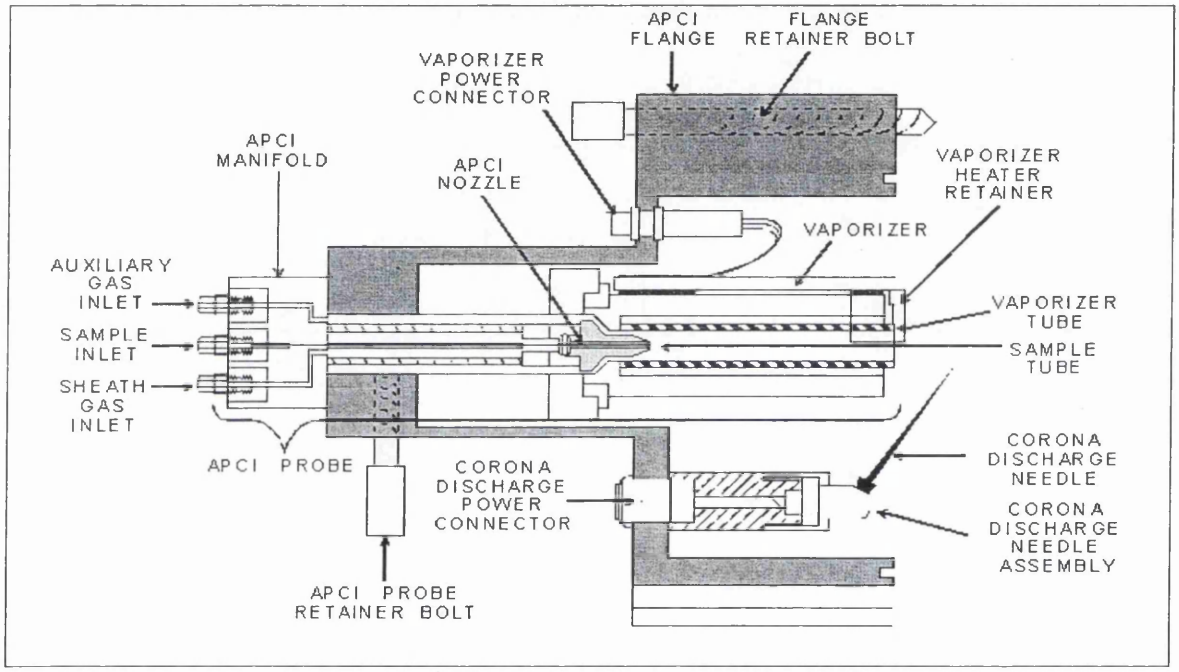


Figure 1.9 *The APCI probe of LCQ Ion Trap*

1.3.3.4 API Stack

The API stack consists of the spray shield, heated capillary, tube lens and skimmer (see Figure 1.10) .The heated capillary is a tube assembly that assists in desolvating ions that are produced by the APCI and ESI probe. A heater surrounds the hole and heats the capillary to temperatures up to 400°C. Ions in the gas or liquid phase are drawn into the heated capillary in the atmospheric-pressure region of the API source and are transferred to the capillary-skimmer region by a decreasing pressure gradient. A potential of typically ± 25V assists in repelling ions from the heated capillary to the skimmer. After exiting the heated capillary, the ions enter the tube lens,

which serves to focus the ions towards the skimmer opening into the mass analyser. It is optimised during the tuning process in order to maximise the ion current and therefore increases the sensitivity of target. This area of the capillary-skimmer region is the first area of first stage evacuation in the API source. The skimmer, held at ground potential, acts as a vacuum baffle between the higher-pressure capillary region (1 Torr) and the lower pressure first octopole region (10^{-3} Torr). The opening in the skimmer is offset with respect to the bore of the heated capillary to reduce the number of large charged particles that pass through the skimmer, which can then pass through the ion optics and the mass analyser and create noise.

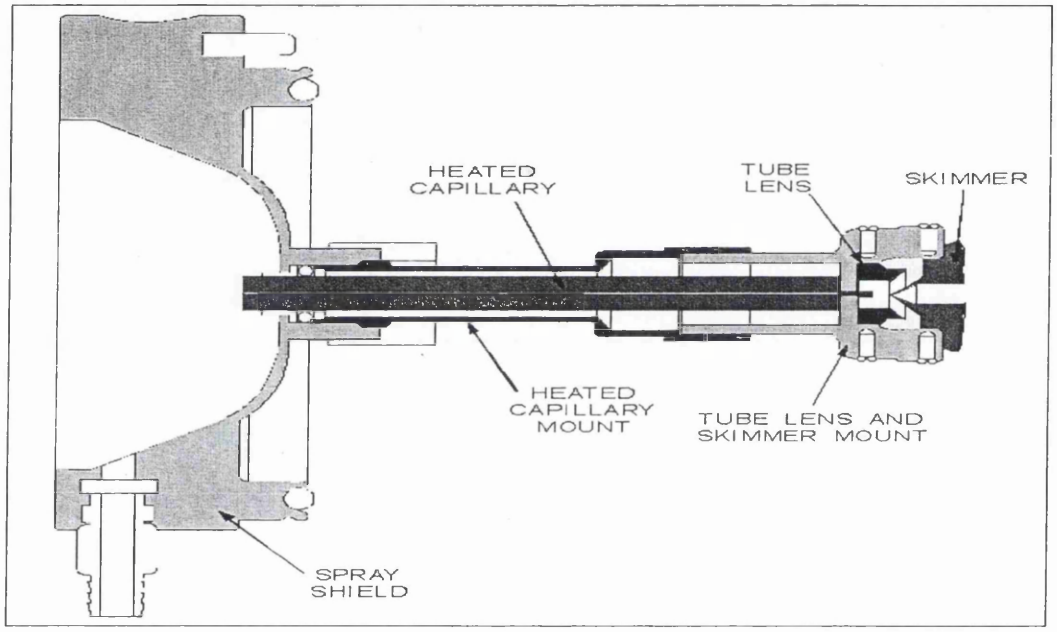


Figure 1.10 *Cross sectional view of the API stack in the LCQ system*

1.3.4 Ion Optics

The LCQ ion optics consists of two octapoles and an interoctapole (see Figure 1.11). The octapole is an orthogonal array of cylindrical rods that function as an ion transmission device. A RF voltage and a DC offset voltage applied to the rods create an electrostatic field that guides the ions along the axis of the octapole. The two octapoles lie in different pressure regions of the vacuum manifold and are separated by an interoctapole lens. The interoctapole function is to assist and gate ions, as well as serving as a gas baffle between the first octapole region and the analyser

region of the vacuum manifold. The potentials applied to the ion optics are also optimised during the tune process in order to maximise the ion current transmitted to the mass analyser cavity.

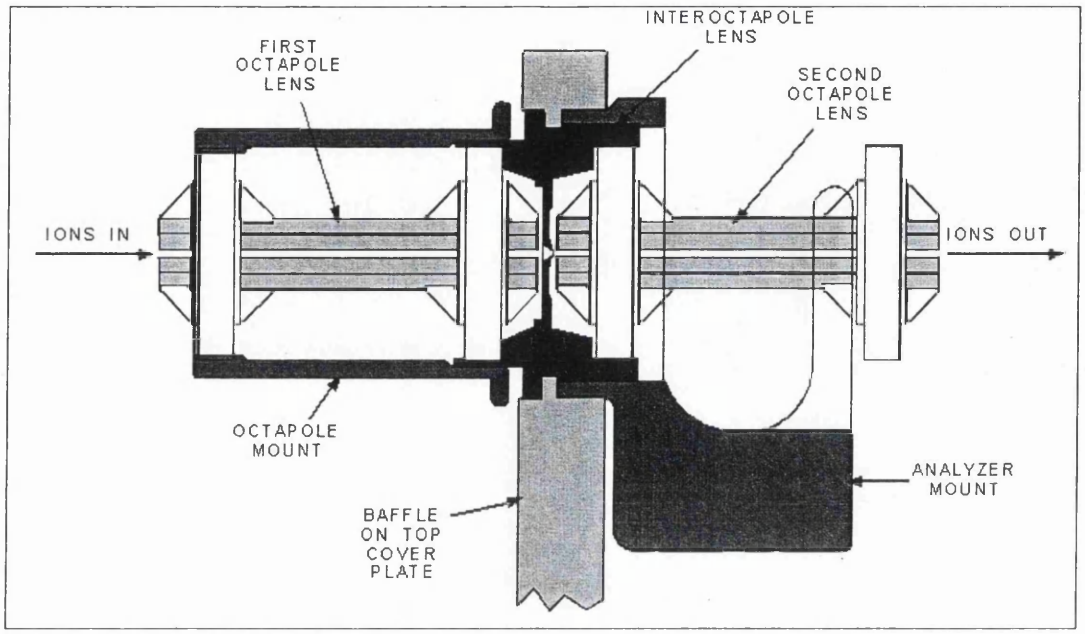


Figure 1.11 *Cross sectional view of the ion optics in the LCQ system*

1.3.5 The Quadrupole Ion Trap Mass Analyser

The LCQ MS detector is a quadrupole ion trap mass analyser. It consists of an entrance end-cap electrode, central ring electrode and an exit end-cap electrode (see Figure 1.12). They form a cavity and their functions include ion storage, ion isolation, collision-induced dissociation (CID for MS^n) and ion scan out processes.

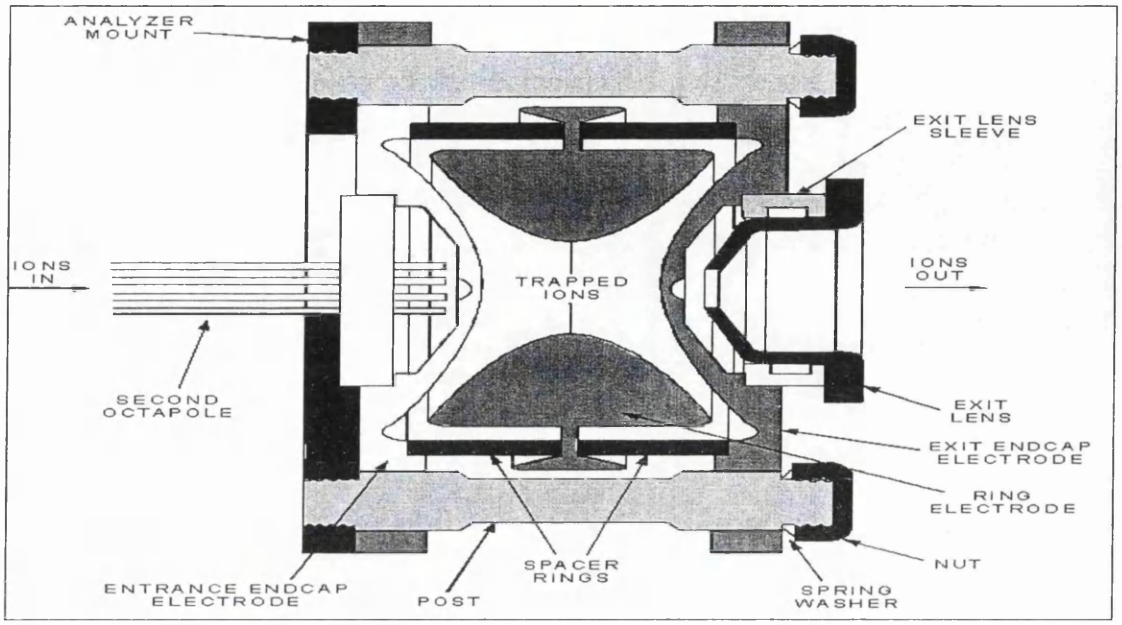


Figure 1.12 *Cross sectional view of the LCQ system mass analyser*

Both endcap electrodes have tiny apertures in their centres to permit ions to enter and exit the mass analyser cavity. The ring electrode is positioned symmetrically between the two-endcap electrodes. Various ac voltages (ring electrode RF voltage, waveform voltages, resonance excitation and ejection RF voltages) are applied to each of the three electrodes to trap and eject ions according to their mass-to-charge ratios. First, DC offset voltage is applied to the mass analyser electrode to draw ions in from the ion optics. If CID is chosen to analyse the sample, the entering ions collide with helium gas present inside the mass analyser cavity and slow them down so that they become trapped by the potential well created by the RF field being applied. The helium gas also serves to dampen the amplitude of the oscillations of the trapped ions, thereby focusing them into the centre of the cavity. Sensitivity and resolution are both enhanced due to these interactions with the helium gas. An RF voltage is applied to the central ring electrode to create a three-dimensional rotationally symmetrical quadrupole electric field. Ions of selected mass-to-charge ratios can be stored in this field in stable orbits and as the RF voltage is increased, the trajectories of the ions become unstable in order of increasing mass-to-charge ratio. Ions with unstable orbits are ejected from the mass analyser towards the ion detection system.

1.3.6 Ion Detection

The ion beam passes through the mass analyser and then is detected and transformed into a usable signal by a detector. The efficiency of an ion detection system is dependent upon its sensitivity, accuracy, resolution and response time. The LCQ system is equipped with an off-axis ion detection system (see Figure 1.13), which is located behind the mass analyser. It consists of a conversion dynode and a channel electron multiplier. It is capable of producing a high signal-to-noise ratio, and further permits voltage polarity interchange between the positive ion and negative ion mode of execution.

When positive ions strike the negative high-voltage conversion dynode, the conversion dynode converts ions from the mass analyser into secondary particles (negative ions and electrons). When negative ions strike the positive high-voltage conversion dynode, the secondary particles of interest are positive ions. The number of secondary particles is greater than that of ions striking the conversion dynode and the signal is amplified. These secondary particles are accelerated into the electron multiplier. They strike the cathode with sufficient energy to dislodge electrons as they collide with its curving inner wall. These electrons pass further into the electron multiplier, again striking the walls and finally result in a measurable current at the end of the electron multiplier. The current is amplified typically to the order of 10^5 –10^6 for a singly charged ion hitting the entrance of the electrode.

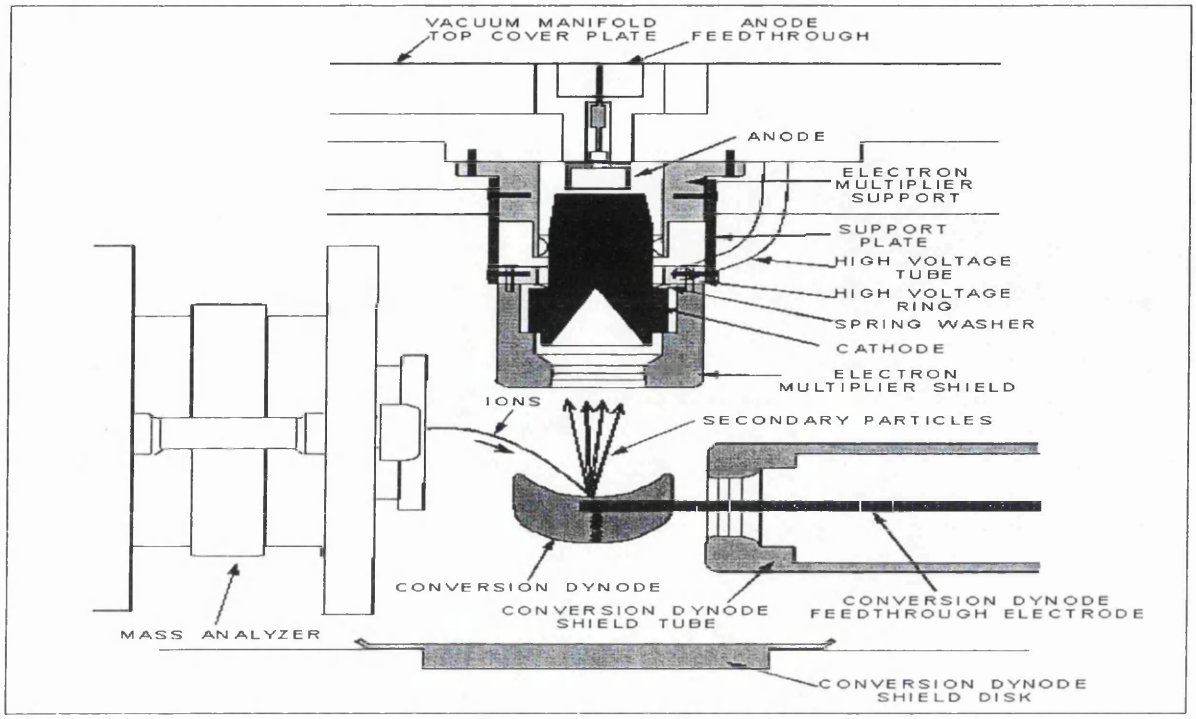


Figure 1.13 *Cross sectional view of the LCQ ion detection system*

1.3.7 Vacuum system in the LCQ System (see Figure 1.14)

In the LCQ system, the ion optics, mass analyser, ion detection system and the atmospheric pressure ionisation stack, are all enclosed in a vacuum manifold. The manifold is divided into three distinct chambers by two baffles. A Balzers® uno 030B rotary-vane pump is used to evacuate the capillary-skimmer region of the vacuum manifold. The pump sustains a minimum pressure of approximately 1 Torr.

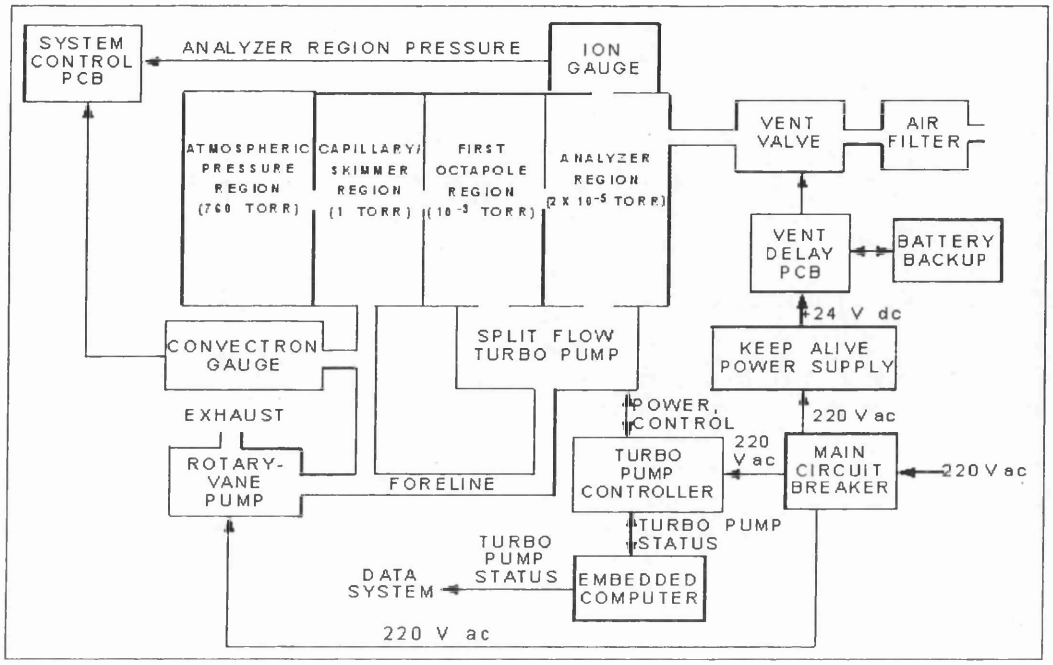


Figure 1.14 *Black diagram of the LCQ vacuum system*

The first octapole region and the analyser region of the vacuum manifold are evacuated by a Balzers-Pfeiffer® TMH 260-130 split-flow turbomolecular pump. The rotary vane pump establishes the vacuum necessary for the correct operation of the turbomolecular pump. Under standard operating conditions, the first octapole region is evacuated to 10^{-3} Torr by the interstage port of the turbomolecular pump, whilst a vacuum of 2×10^{-5} Torr is maintained in the analyser chamber by the high vacuum port of the turbomolecular pump. The rotary –vane pump should be periodically ballasted to ensure optimum performance. The pump oil requires changing periodically (normally once six months).

1.3.8 Calibration of the LCQ Ion Trap

Mass calibration of the instrument is very important and allows the MS detector to assign the correct mass values to the ion signals that it detects. The basic process of mass calibration involves the acquisition of a data file (mass spectrum) using standard mass calibration compounds. The data file is then compared with a mass calibration file of the same compound, which has the

correct mass assigned to each peak. Any difference between these two files is adjusted to bring the new data file into the line with the mass calibration file. This adjustment is then applied to all subsequent data files acquired. This process is called calibration. The LCQ ESI tuning and calibration solution contains caffeine, MRFA, Ultramark 1621 in 50:50 methanol: water containing 1 % acetic acid. Caffeine provides an ESI single charged peak at m/z =195.1. MRFA (L-methionyl-arginyl-phenylalanyl-alanine acetate) provides an ESI single charged peak at m/z=424.3 and a double charged peak at 262.2. Ultramark provides ESI single charged peaks at m/z 1022.0,1122.0,1222.0, 1322.0, 1422.0, 1522.0, 1622.0, 1722.0, 1822.0 and 1921.9.

1.3.9 LCQ Ion Trap Scan Modes

1.3.9.1 Full scan Mode

During a full scan type with one stage of mass analysis (scan power = 1), the ions formed in the ion source are stored in the mass analyser. Then, these ions are sequentially scanned out of the mass analyzer to produce a full mass spectrum. Generally, single-stage, full scan experiments are used to determine the molecular weights of unknown compounds or of each component in a mixture of unknown compounds. The full scan type provides more information about an analyte than does selected ion monitoring (SIM). In the full scan mode the mass analyser is scanned from the first mass to the last mass in a given scan time. Full scan mode cannot give high sensitivity due to the long scan time in a range of masses.

1.3.9.2 Selected Ion Monitoring (SIM) Scan Mode

In the SIM scan type, the ions formed in the ion source are stored in the mass analyser, then, ions of interest for one or more mass-to-charge ratios are isolated and are scanned out of the mass analyser to produce a SIM mass spectrum (No data are acquired for other ions as they leave the mass analyser). Because only ions of selected mass-to-charge ratio are monitored, the selected ion monitoring scan type generally provides higher sensitivity than does the full scan type and is widely used for quantitation of known compounds.

1.3.9.3 Tandem Mass Spectrometry (MS^n)

Tandem mass spectrometry is any general method involving at least two stages of mass analysis. The first stage is to isolate a precursor ion and further undergoes a second stage or multistage to yield product ions and neutral fragments. Tandem mass spectrometry can provide more structure information and is very useful for structural elucidation of unknown components.

1.3.9.3.1 Collision induced dissociation (CID)

Collision induced dissociation (CID) can be performed in any of three places in the MS detector: ion source CID in the capillary-skimmer region, ion source CID in the octapole region, and mass analyser CID in the mass analyser. Ion source collision-induced dissociation (CID) is a technique for fragmenting ions in an atmospheric pressure ionisation (API) source. Either of two regions can be used for the ion source CID process: the capillary-skimmer region and the octapole region.

Capillary-skimmer region ion source CID: Ions emerge from the heated capillary and expand into the capillary-skimmer region of the API source with considerable translational kinetic energy. This kinetic energy is further increased by applying greater than usual voltages to the heated capillary and/or tube lens, which increases the difference in potential between these components and the skimmer (which is at ground potential). The voltages are increased sufficiently to impart enough kinetic energy to the ions so that, when they collide with solvent or air molecules (collision gas) present in the API source, the ions dissociate to form product ions.

Octapole region ion source CID: Ions pass through the skimmer of the API source into the ion optics region. They are accelerated by the difference in potential between the skimmer (which is at ground potential) and the first octapole. The octapole dc offset voltage is set such that it imparts enough kinetic energy to the ions so that, when they collide with solvent or air molecules (collision gas) present in the ion optics region, the ions dissociate to form product ions.

Mass analyser collision induced dissociation (CID): Application of resonance excitation RF voltage to the endcap electrodes enhances the motion of ions in the mass analyser in the axial direction and the ions gain kinetic energy. The amplitude of the resonance excitation RF voltage is sufficient to impart enough kinetic energy to the ions so that, when they collide with the helium amping gas present in the mass analyzer, the ions dissociate to form product ions.

Mass analysis occurs when the LCQ is operated in either MS/MS or MS^n scan power when a sufficient relative collision is applied. The collision energy is the translational kinetic energy (TKE) of a moving particle when it collides with a stationary target neutral species, such as a collision gas molecule. The amplitude of the resonance excitation RF voltage determines the TKE of ions in the mass analyser. The LCQ expresses collision energy in terms of relative collision energy (in percentage from 0 % to 100 %).

In the LCQ MS/MS a full scan type with two stages of mass analysis (scan power = 2) can be operated. In the first stage, the ions formed in the ion source are stored in the mass analyser, then ions of one mass-to-charge ratio (called the parent ions) are selected and all other ions are ejected from the mass analyser. The parent ions are excited so that they collide with background gas that is present in the mass analyser. The collisions of the parent ions cause them to fragment to produce one or more product ions (by mass analyser CID). Then the product ions are stored in the mass analyser (second stage) and are sequentially scanned out of the mass analyser to produce a full product ion mass spectrum.

In the LCQ MS^n a full scan type with three to ten stages of mass analysis (scan power = 3 to 10) can be operated. With three or more stages of mass analysis, the ions formed in the ion source are stored in the mass analyser (first stage). Ions of one mass-to-charge ratio are selected and all other ions are ejected from the mass analyser. The parent ions are excited so that they collide with background gas that is present in the mass analyser. The collisions of the parent ions cause them to fragment to produce one or more product ions (by mass analyser CID).

The product ions are stored in the mass analyser (second stage). Product ions of one mass-to-charge ratio are selected and all other ions are ejected from the mass analyser. The selected product ions now become the new parent ions for the next stage of mass analysis. The new parent ions are excited so that they collide with background gas. The collisions of the new parent ions cause them to fragment to produce one or more new product ions.

The new product ions are stored in the mass analyser (third stage). The process described in the previous paragraph is repeated up to seven more times until the final product ions of interest are produced. The final product ions are stored in the mass analyser (nth stage). Then, they are

sequentially scanned out of the mass analyser to produce a full final product ion mass spectrum.

1.3.9.3.2 Selected Reaction Monitoring (SRM) scan type

SRM scan type occurs when the LCQ is operated in either MS/MS or MS^n in which a particular reaction or set of reactions, such as the fragmentation of an ion or the loss of a neutral moiety, is monitored. In the SRM scan type, the ions formed in the ion source are stored in the mass analyser (first stage). Ions of one mass-to-charge ratio (called the parent ions) are selected and all other ions are ejected from the mass analyser. The parent ions are excited so that they collide with background gas that is present in the mass analyser. The collisions of the parent ions cause them to fragment to produce one or more product ions (by mass analyser CID). The product ions are stored in the mass analyser (second stage). Then, ions of interest for one or more mass-to-charge ratios are scanned out of the mass analyser to produce an SRM product ion mass spectrum. (No data are acquired for other ions as they leave the mass analyser). Like SIM, selected reaction monitoring allows for the very rapid analysis of trace components in complex mixtures. In SRM a limited number of product ions is monitored. A parent ion is selected; however, the entire mass spectrum of its product ions is not obtained. Rather, only one or a few selected product ions are monitored.

1.3.9.3.3 Consecutive Reaction Monitoring (CRM) scan type

CRM scan type occurs when the LCQ is operated in MS^n. In the CRM scan type, a scan type with three or more stages of mass analysis and in which a particular multi-step reaction path is monitored. In the CRM scan type, the ions formed in the ion source are stored in the mass analyser (first stage). Ions of one mass-to-charge ratio (called the parent ions) are selected and all other ions are ejected from the mass analyser. The parent ions are excited so that they collide with background gas that is present in the mass analyser. The collisions of the parent ions cause them to fragment to produce one or more product ions (by mass analyser CID). The product ions are stored in the mass analyser (second stage). Product ions of one mass-to-charge ratio are selected and all other ions are ejected from the mass analyser. The selected product ions now become the new parent ions for the next stage of mass analysis. The new parent ions are excited so that they collide with background gas. The collisions of the new parent ions cause them to fragment to produce one or more new product ions. The new product ions are stored in the mass

analyser (third stage). The process described earlier the previous paragraph is repeated up to seven more times until the final product ions of interest are produced. The final product ions are stored in the mass analyser (nth stage). Then, ions of interest for one or more mass-to-charge ratios are scanned out of the mass analyser to produce a CRM final product ion mass spectrum. (No data are acquired for other ions as they leave the mass analyser.)

1.3.10 Qualitative and Quantitative Analysis

The goal of quantitative analysis is to determinate the concentration of components in the sample. HPLC and mass spectrometry can perform the quantitative determination of components. Several factors must be evaluated when quantitative analysis is performed.

1.3.10.1 Specificity

The degree of specificity of quantitative analysis using mass spectrometry depends on how the spectrometer is used and even more on the signal that is used during the correlation. We can use the total ion current as a signal to determine the concentration of the compound as long as there is no interference with another substance. So on-line HPLC/mass spectrometry is a good method to perform the quantitative determination because all compounds are separated by HPLC with high purity. Many methods exist that improve the specificity of quantitative analysis of complex mixtures using mass spectrometry. These methods can be classified into either of two categories: those that act upon the sample and those that act on the spectrometer.

1.3.10.2 Sensitivity, Limit Of Detection (LOD) and Limit of Quantitation (LOQ)

Sensitivity in mass spectrometry is defined as the ratio of the ionic current change to the sample change in the source. It is important always that the relevant experimental conditions corresponding to sensitivity measurement should be stated. The limit of quantitation should be differentiated from the sensitivity. It is the smallest sample quantity that yields a signal that can be distinguished from the background noise (generally a signal equal to ten times the background noise). It should be noted that this minimum quantity is not enough to obtain an interpretable mass spectrum. The limit of detection depends on the abundance of the ionic species that is measured. The more abundant the measured ionic species with respect to all of the ions derived from the

analysed molecule, the higher is the limit of detection. Two important characteristics of a method are the limit of detection (LOD) and limit of quantitation (LOQ). They are considered to be an important part of any method validation. The limit of quantitation (LOQ) can be defined as the smallest concentration of analyte, which gives a response that can be accurately quantified. It is the concentration that can be quantitated reliably with a specified level of accuracy and precision. The limit of quantitation can be defined in either of three ways. One method uses a technique similar to that for LOD but requires a S/N ratio of at least 10. The second method is to define a certain level of precision and determine experimentally how large a peak needs to be for that level of precision. This can be accomplished by injecting sample concentrations that result in various S/N values and determining the precision from multiple injections of each sample concentration. A third method assumes that the baseline noise is approximated by a Guassian distribution with a width of 4 standard deviation units (SD) wide ($N' = 4\sigma$).

The third important feature in quantitation is the maximum level of quantitation, defined as the highest concentration that can reliably be determined. Often, this is determined by the limit of linearity of the detector (ie, when the detector no longer shows a linear response with specified increase in concentration). The maximum and minimum quantitable amounts will define the calibration range of the method.

1.3.10.3 External Standard Method

The role of a standard is to determine the mathematical relationship, in the concentration range to be measured, between the selected signal intensities and the mixture composition. A range of known amounts of external calibration standard is analysed. Without any modification of the analytical conditions, an equal volume of unknown sample is introduced into the mass spectrometer to be measured. The response values are plotted against the corresponding standard amounts to produce a calibration curve and the concentration of unknown sample can be calculated.

1.3.10.4 Internal Standard Method

This method is based on a comparison of the intensity of the signal corresponding to the product that has to be quantified with that of a reference compound called the internal standard. This

method allows the elimination of various error sources other than the minimal intrinsic error due to statistical reasons. Another component, which is not present in the sample but has very similar structure to the measured components, is added into the sample to be analysed. A calibration curve is produced by plotting the amount ratio against the signal response ratio. The analyte concentration can be determined from the calibration curve.

1.4 INTRODUCTION TO PHYTOESTROGENS

Bioactive compounds are extra nutritional constituents that typically occur in small quantities in foods, they are being intensively studied to evaluate their effects on health. Many epidemiological studies that have shown protective effects of plant-based diets on cardiovascular disease (CVD) and cancer. Many bioactive compounds have been discovered. These compounds vary widely in chemical structure and function and are grouped accordingly. Phenolic compounds, including their subcategory, flavonoids, are present in all plants and have been studied extensively in cereals, legumes, nuts, olive oil, vegetables, fruits, tea, and red wine. Many phenolic compounds have antioxidant properties, and some studies have demonstrated favourable effects on thrombosis and tumorogenesis and promotion.

Phytoestrogens are members of classes of polyphenolic compounds synthesized by plants. They include isoflavones and other flavonoids, lignans, coumestanes and zearalenones. Photoestrogens are found in plants and in many food supplements such as red clover, oilseeds, nuts and soybean. Phytoestrogens are estrogen-like plant compounds that act similarly to the hormone estrogen, although they are weaker than estrogen itself. In contrast to the wide-ranging presence of other flavonoids in higher plants, they are primarily confined to one group of plants, the sub-family of the Leguminosae. Various phytoestrogens are present in soy, but also in flaxseed oil, whole grains, fruits, and vegetables.

1.4.1 The relationship between Phytoestrogen Consumption and Cancer Prevention

Over the last twenty years, interest in the physiological effects of phyto-estrogen containing plant substances has increased significantly, since it was demonstrated that the diphenolic components may play a potential role in the prevention and treatment of hormonally-dependent diseases, including breast and prostate cancer. Epidemiological studies have proved to be a valuable tool in

this area of research, and have resulted in the exploration of the relationship between phyto-estrogen consumption and cancer prevention. Steroid-hormone dependent cancer, including those of the breast, prostate and colon, are leading causes of morbidity in western countries. In Asian countries, particularly rural areas, these diseases are relatively uncommon. The original associations between phytoestrogens and steroid hormone-dependent cancers were made through several ecological studies looking at the low incidence of breast, colon and prostate cancers in Asian countries compared to western ones. Soy consumption is very high in Asia compared to North American and Europe, and it was found that this food could be negatively correlated with cancer incidence. Since that time, studies on phytoestrogens increased dramatically. Messina et al have extensively reviewed the in vivo and in vitro data of soy, showing protective effects in both animal models and epidemiologic studies [32]. M.G.Hertog[33] et al studied 83 prostate cancer patients and found that after adjustments for total calories, greater consumption of most phytoestrogens, including isoflavones and other flavonoids, had a slightly protective effect on prostate cancer risk. In recent years, the potential health benefit of phytoestrogens has become increasingly recognized by many people. Our studies focus on two phytoestrogen sources: *Oroxylum indicum* and soy food.

1.4.2 Phytoestrogens-Chemistry and Metabolism

Plant foods contain at least twelve thousand natural chemicals produced for structural, hormonal, attractant and chemoprotective purposes. A remarkable diversity has been described for naturally occurring phytoestrogens, which share with steroidal oestrogens an ability to activate the oestrogen receptor and which have been shown to exert oestrogenic effects on the genital tract of female animals. The phytoestrogens include compounds in the large flavonoid family of plant secondary metabolites as well as plant lignans, coumestanes and zeralenones. The main known of phytoestrogens is the flavoonoids, isoflavones and coumestrol. Figure 1.15 shows their basic structures. The lignans secoisolariciresinol (SECO) and matairesinol(MAT), precursors of the hormone-like mammalian lignans, are of particular interest due to their abundance in the plants.

Flavonoids are normally classified into at least 10 chemical groups. Among them, flavones, flavonols, flavanols, flavanones, anthocyanins and isoflavones are particularly common in the diet. The most-studied members of these groups are included in Table [34].

Table Classification of flavonoids and their food sources

Subclass	Flavonoids	Food sources
Flavones	Apigenin, luteolin	Apple skin, celery
Flavonols	Quercetin, Kaempferol, myricetin	Onions,apples, tea
Flavanol	Catechin,epicatechin,epigallocatechin gallate	Tea
Flavanones	Hesperitin, naringenin	Citrus fruits, grapefruit
Anthocyanins	Cyanidim	Berries
Isoflavones	Genistein, daidzein	Soy, red clover

Isoflavonoids are a large and very distinctive subclass of the flavonoids. These compounds differ structurally from other classes of the flavonoids in having the phenyl ring (B-ring) attached at the 3-rather than at 2-position of the heterocyclic ring. Isoflavones constitute the largest group of natural isoflavonoids with about 364 aglycones reported. In this subclass the most investigated and interesting compounds with regard to oestrogenicity are genistein, daidzein, biochanin A and formonetin. Genistein is the most active principle with the highest binding affinity for the oestrogen receptor.

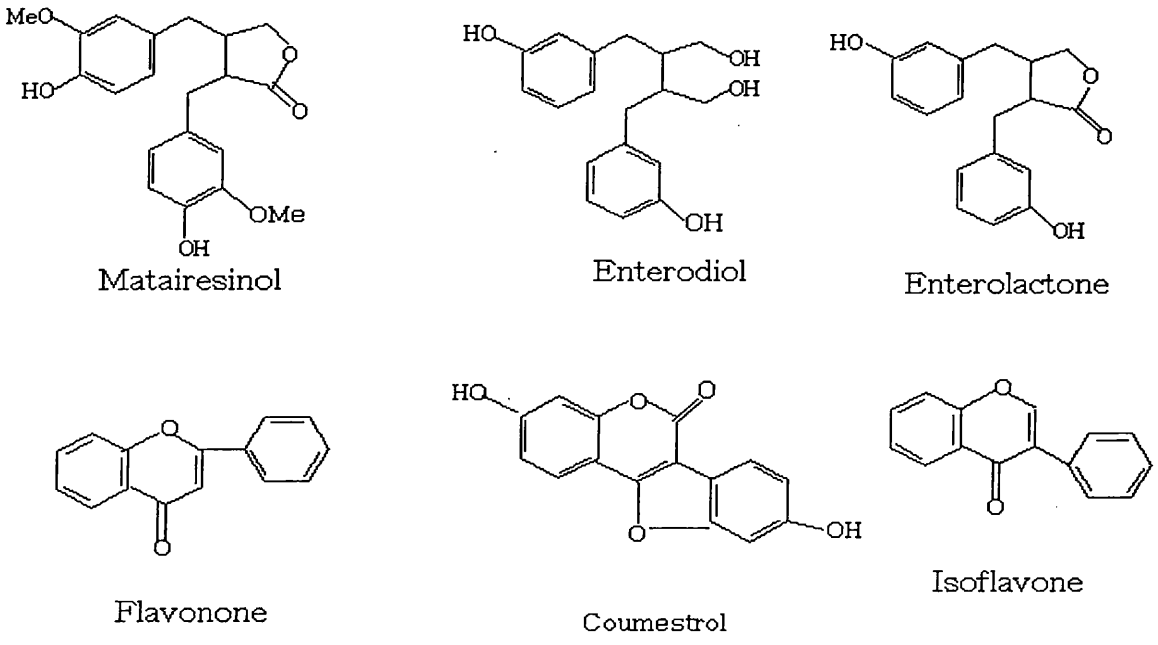


Figure 1.15 *Basic structures of Ligan, Coumestrol, Flavone and Isoflavone*

1.4.3 Introduction to *Oroxylum indicum*

Oroxylum indicum Vent (O indicum) is a deciduous tree (see Figure 1.16) belonging to the Bignoniaceae family, which is distributed in South Asia, Southeast Asia and China. It is usually utilized as a crude drug in Indian Ayurvedic medicine and Chinese medicine for curing stomach disorders, dysentery and rheumatic swelling. In Thailand, the fruits (called peh-gaa) and flowers of the plant are consumed as a common part of the diet in the north and northeastern areas. The seeds contain such flavonoids as chrysin (5,7-dihydroxyflavone), oroxylin A (5,7-dihydroxy-6-methoxyflavone), baicalein (5,6,7-trihydroxyflavone), baicalein glycosides, benzoic acid and fatty acids [35]. Wall et al [36] reported significant antimutagenic activity against 2-aminoanthracene in the ethanol-dichloromethane soluble fraction prepared from twigs and leaves of O. indicum. In recent studies, baicalein and chrysin have been reported to show anti-inflammatory, anti-allergic antioxidant and anticancer activities and baicalein showed a hypentensive effect.

1.4.4 Soy food and Red clover

Much of the clinical and epidemiological research regarding isoflavones is based on intake of soy foods containing soy protein. Soy protein specifically has been shown to significantly reduce menopausal symptoms such as "hot flushes," although not as much as conventional estrogens replacement therapy. Intake of soy protein has also been found to help reduce total cholesterol and LDL while increasing HDL ("good" cholesterol). In fact, the U.S. Food and Drug Administration allow products that contain at least 6.25 grams of soy protein per serving. Similarly, soy isoflavones when used as supplements to foods have been shown to maintain and even increase bone density. Soy isoflavones, which also happen to be antioxidants, may also help prevent breast and prostate cancer.

Other plants, such as kudzu and red clover, also contain isoflavones. There is little clinical research specifically with kudzu (Pueraria lobata) and it is technically difficult to measure some of its unique isoflavones. Studies of red clover isoflavones in reducing menopausal symptoms have suggested a beneficial effect but results have varied — similar to that found with soy isoflavones. Red clover may also help maintain the density of the bone in the lower spine in both menopausal and peri-menopausal women, although not post-menopause. Red clover isoflavones have not shown a beneficial effect on reducing cholesterol, although both red clover and soy isoflavones may improve the elasticity of blood vessels in menopausal and peri-menopausal women.

1.4.5 Common separation and purification methods for phytoestrogens

Among the different methods available for the separation and determination of phytoestrogens, chromatography including gel chromatography, classic column chromatography and modern high performance liquid chromatography are common techniques for these purposes. Generally, milligrams up to kilograms of pure components from natural plants can be obtained in a reasonable time at a reasonable cost. High-performance liquid chromatography (HPLC) is the current method of choice for analysis of samples and for small-scale purifications. While the analytical sector of this is well established, the preparative and process scale sector is not. HPLC equipment is expensive, the usage of solvent is high, and column variables can change with time and result in poor reproducibility.

1.4.6 Comparison of support-solid separation methods and HSCCC

The classic methods for separation bioactive compounds from natural products were combined with PPC (paper partition chromatography), thin-layer chromatography (TLC), and CLC. The development and application of such modern chromatographic techniques as gas chromatography (GC), high-performance liquid chromatography (HPLC), thin-layer scanning and electrophoresis as well as new types of high-efficiency chromatographic supports have significantly raised the technical level of isolation and have shortened the period of research projects. However, as far as the separation and purification for large scale up proposes, there are currently no high-resolution purification systems that can be scaled from analytical to full process scale while maintaining the same principle of purification. In addition, all of the above techniques need solid supports, which inevitably result in irreversible adsorption and contamination of fractions, and may even cause denaturation and deactivation.

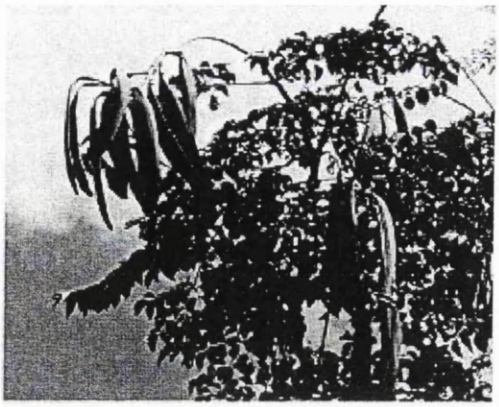


Figure 1.16 *Plants of Red Clover (left) and Oroxylum indicum (right)*

HSCCC and HPLC are similar in several aspects. Both are based on difference partition of separated samples in two phases. Both techniques separate solutes in a mixture and keep one phase stationary while a second phase moves through it. The major difference between them is the type of column used.

HSCCC is a support free separation technique, which means that both of the stationary and mobile phases are liquid. It has long been recognized as an effective means for purification of a wide variety of bioactive molecules and offers various advantages over conventional HPLC especially on the preparative scale.

1.5 AIMS

The objectives were to fully examine the utility of HSCCC for the separation and purification bioactive compounds from the seeds of O indicum. Our studies show that HSCCC is a powerful tool in obtaining high purity of compounds from natural products in preparative process scale sector. The second part of the studies was to develop analytical methods for the identification and quantitation of phytoestrogens in natural products and human fluid. In Chapter 2, HSCCC was used to separate and purify flavonoids of the extract from the seeds of O indicum. Five flavonoids were successfully separated and purified with high purities (above 92 %) and two components were for the first time separated and purified from the plant. Mass spectrometry, UV (Ultraviolet)

and NMR (Nuclear magnetic resonance) were used to the structure elucidation.

In Chapter 3 and Chapter 4, different coil volumes of HSCCC were compared in separation and purification of phytoestrogens and on line HSCCC/ mass spectrometry were studied for the separation and identification of flavones and isoflavones in plants.

In Chapter 5 and Chapter 6, quantitative determination of phytoestrogens in soy food supplement and human urine were studied. Sensitive LC/MS methods were developed to determine the concentration of isoflavones and their major metabolites.

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CHAPTER 2

Separation and Identification of Flavonoids in the Seeds of *O indicum* by HSCCC, LC/ESI/MS and LC/ESI/MS/MS

2.1 INTRODUCTION TO THE FLAVONOIDS

The use of medical plants for curing illnesses can be traced back to the millennia of the early civilizations in China, India and the Near East. Traditional Chinese medicine is an extremely rich summation of the experience acquired by the Chinese people in thousands of years of struggle against disease. It is an important part of the brilliant Chinese ancient culture and has played a tremendous role in safeguarding the health of the people.

The flavonoids, one of the most diverse and widespread groups of natural products [1, 2], occupy a prominent position among the natural phenols. Over 5000 different flavonoids have been described to date and they are classified into at least 10 chemical groups [3]. Among them, flavones, flavonols, flavanol, anthocyanins and isoflavones are particularly common in the diet. A number of flavonoids have been shown to suppress carcinogenesis in various animal models [4]. Some polyphenolic compounds have shown promising results related to the treatment of coronary heart disease, and as anticancer [5], anti-aging as well as anti-inflammatory agents [6, 7]. There is currently considerable interest in these compounds, as they appear to exert a beneficial effect on several key mechanisms involved in the pathogenesis of cancer. The antioxidant property of flavonoids was the first mechanism of action studied, in particular with regard to their protective role of being highly effective against cardiovascular disease. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including single oxygen and various free radicals [8], which are possibly involved in DNA damage and tumour promotion [9]. Flavonoids occur in a variety of structural forms. All contain fifteen carbon atoms in their parent nucleus and share the common structural feature of two phenyl rings linked by a three-carbon chain. The three-carbon chain may be formed into a third, five- or six-membered ring through oxygen on one of these phenyl rings generating a tricyclic system. Those possessing a six-membered heterocyclic ring are defined as flavonoids and isoflavonoids. Figure 2.1 shows their typical structures.

In tricyclic compounds of the flavonoid and isoflavonoid types, the rings are usually labelled A, B and C. Natural flavonoids and isoflavonoids are usually oxygenated and bear hydroxyl or methoxyl substituents. A large number of flavonoids and isoflavonoids occur as O-glycosides in which one or more of the hydroxyl groups of the flavonoid are bound to a sugar or sugars via an

acid labile hemiacetal bond. In flavonoid C-glycosides the sugar is C-linked and this linkage is acid resistant. Generally, the flavonoid C-glycosides are less reactive and more water-soluble.

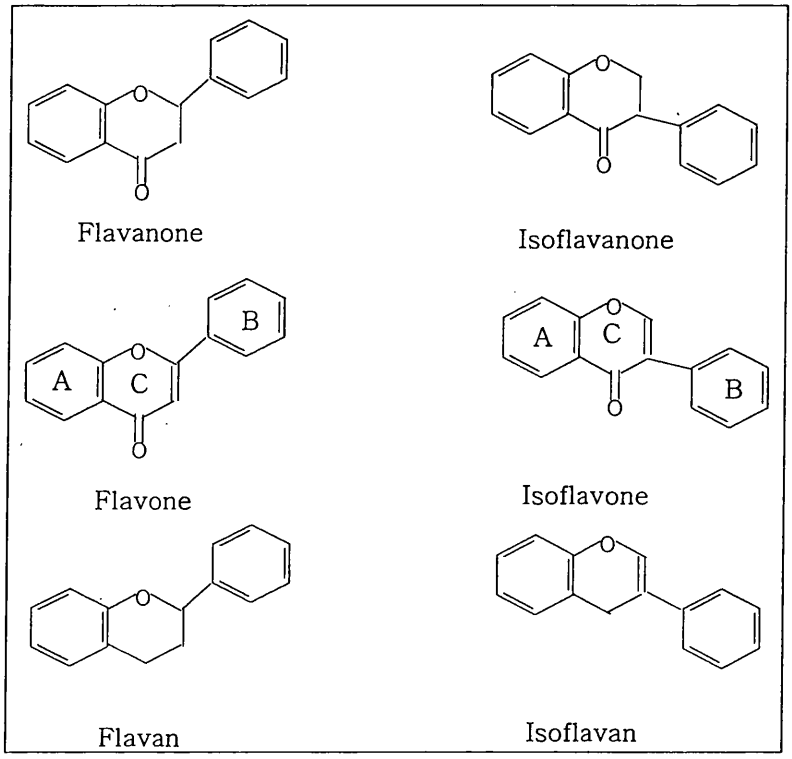


Figure 2.1 *Basic structures of flavonoids and isoflavonoids*

2.2 BACKGROUND TO OROXYLUM INDICUM

Oroxylum indicum (O indicum) is a small to medium sized tree found in China and India. Its stem bark possesses anti-inflammatory activity [10]. This plant is used to treat smallpox, fever, spleen complaints, dropsy and anaemia [11]. The seeds of O indicum are known as the crude drug 'Mu Hu Die' in China and have been used as an analgesic and anti-inflammatory agent for the treatment of cough, bronchitis and other diseases. Flavones [12, 13], sterols and prunetin [14] have been reported in different parts of the tree. In the seeds of O indicum, amounts of bioactive flavonoids such as

baicalein, baicalin, chrysin, apigenin have been identified [15]. In recent studies, baicalein and chrysin have been reported to show anti-inflammatory, anti-allergic [16], antioxidant and anticancer activities and baicalein showed a hypertensive effect [17, 18, 19]. A recent clinical test has identified that the extracts of its seeds can result in body weight loss and reduce postprandial blood-glucose. Surprisingly, there are few papers reported of the research on *O indicum*. Most of research reported on flavonoids focus on known plants such as ligan [20] and gingko [21]. In fact, *O, indicum* contains similar important flavonoids to gingko and its price is far cheaper than that of gingko. As far as the importance of bioactive constituents is concerned, more attention should be paid to the activity by scientific research.

2.3 AIMS

In order to take traditional Chinese medicines to a higher level and utilize them on a large scale, it is both necessary and interesting to find the bioactive components in the traditional drugs by modern scientific techniques and to use them as lead compounds for new drug design. In this chapter, our aims are to separate and purify known and unknown bioactive flavonoids from the seeds of *O indicum* by HSCCC and use the results to screen bioactive compounds in order to service new drug design.

2.4 METHODS FOR SEPARATION AND PURIFICATION OF FLAVONOIDS

The classic methods for separation of flavonoids from medicinal plant were combined with PPC (paper partition chromatography), thin-layer chromatography (TLC)[22] and CLC [23]. The development and application of modern chromatographic techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC)[24] and thin-layer scanning have greatly shortened the period of research projects. However, these techniques need solid support and have the following limitations: 1). The preparative separation and purification of flavonoids from plant materials by these methods usually required multiple chromatographic steps on silica gel, polyamide column [25, 26] and inevitably will lead to irreversible adsorption and contamination of fractions on to the solid stationary phase and may even cause denaturation and deactivation. 2). Currently chromatographic techniques are mainly used for microanalysis and structure

determination and they are limited in application to preparative work. 3). The important need for isolation and micro and semi preparative purification in the study of phytochemistry could not be met by classical column chromatography or preparative TLC because they are manual techniques and show poor reproducibility. Modern semi preparative and preparative HPLC give excellent separation and purification results, but they are also greatly limited in their use not only because they are expensive, but also because they consume a great amount of solvent and have strict requirements for the pre-treatment of samples. More importantly, the separations of polyphenolic natural products are difficult because of "peak tailing" and irreversibly adsorption on silica gel in RP-HPLC.

2.5 HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY FOR THE SEPARATION AND PURIFICATION OF FLAVONOIDS

HSCCC, being a support-free liquid chromatography, which eliminates complications such as irreversible adsorption onto the solid support, tailing of the solute peaks, etc, has been extensively used for the separation and purification of natural products. The basic principles of HSCCC as described in the W. D. Conway book [27] in Chapter 1. HSCCC is an important tool in the search for new bioactive constituents and has advantages over HPLC in follow areas: 1). HSCCC is a support-free liquid chromatography and any constituents can be recovered by pumping out the stationary phase; 2). Any decomposition can be minimized and bioactivity can be conserved. This advantage is very useful for screening bioactive constituents; 3). Running costs are low (only solvents are needed); 4). Separation procedures can be compatible with aqueous and non-aqueous solvent systems. This is the reason why CCC has been recognized as a most valuable technique for the isolation and purification of bioactive components from natural products. CCC has separated a large number of polar natural products; many of them are polyphenolics [28, 29, 30, 31]. Flavonoids are well suited and shown to be separated and purified by this method [32, 33, 34, 35].

In this chapter, HSCCC was applied to the separation and purification of five flavonoid components from the seeds of *O indicum* followed by the identification of these using LC/MS electrospray ionisation and NMR.

2.6 EXPERIMENTAL

2.6.1 Chemicals

Solvents hexane, methanol, water, 1-butanol, and ethyl acetate were of AnalR grade. All these solvents were purchased from Fischer chemicals (Loughborough, UK). All solvents were degassed prior to use. Gases used included oxygen free nitrogen (OFN), helium and air, which were purchased from BOC Ltd. (Surrey, UK). Standard materials genistein, chrysin were purchased from SIGMA

2.6.2 Selection of solvent system

Selection of HSCCC solvent systems means choosing a suitable column. In CCC, the selection of a suitable solvent system is the most important step [20]. Successful separation in CCC requires a proper choice of a two-phase solvent system that provides a suitable range of partition coefficients (K) for the desired compounds. The partition coefficient is usually expressed as $K = C_U / C_L$ or Cs / Cm, where C_U indicates solute concentration in the upper phase and C_L the concentration in the lower phase. Similarly, Cs indicates the solute concentration in the stationary phase and Cm the concentration in the mobile phase. Generally speaking, the most suitable range of *K* values for CCC is 0.5 - 2, where either upper or lower phase can be used as the mobile phase. The parameter Cs / Cm < 0.5 would result in a loss of peak resolution while Cs / Cm > 1 would require a long retention time with excessive sample broadening. A good method for solvent selection is a literature search for the most suitable solvent system previously used for similar compounds. If the nature of the compound is unknown or previous data for the similar compounds are not available, Table 2.1 [27] can give a good direction for choosing a solvent system. In Table 2.1, the search for a solvent system may begin with the chloroform-methanol-water with a ratio of 2: 1: 1. If the partition coefficient *K* (C_U / C_L) falls somewhere between 0.2 and 5 in this solvent system, the desirable K value may be obtained by further modifying the volume ratio of each component, substituting acetic acid for methanol, or partially replacing chloroform by carbon tetrachloride (CCl_4) or methylene chloride. If the sample is more unevenly partitioned into one of the phases, the chloroform solvent systems may be inadequate, and the search must be directed to other solvent systems that provide broader ranges of hydrophobicity and polarity such as hexane - ethyl acetate – methanol - water.

When the sample is mostly distributed into the lower nonaqueous phase of the chloroform solvent

system, a slightly more hydrophobic solvent system of n-hexane-EtOAc-MeOH-H_2O = 1: 1: 1: 1 (where Et = C_2H_5, Ac = CH_3CO and Me = CH_3) should be tested, as indicated by an upward arrow in the table 2.1. Further search should be directed upward, if the sample is still largely distributed into the upper nonaqueous phase, or downward if the sample is more concentrated in the lower aqueous phase. If the continued search leads to the top solvent system of hexane – methanol - water (2: 1: 1) and still indicates the need for a more hydrophobic solvent system, the solvent composition can be further modified by reducing the volume of water and or replacing methanol by ethanol.

On the other hand, if the sample is mostly distributed in the upper aqueous phase of the chloroform solvent system, the search should be directed in the opposite direction, toward the polar solvent systems as indicated in the downward arrow. If the most polar solvent system of n-butanol-water listed at the bottom of the table still partitions the sample largely into the lower aqueous phase, the n-butanol solvent system may be modified by adding a small amount of an acid and/ or salt. The most commonly used among these modified solvent systems are n-butanol - acetic acid - water (4: 1: 5), n-butanol - trifluoroacetic acid - water (1: 0.001-0.001: 1), and n-butanol - 0.2 M ammonium acetate (1: 1), all of which have been extensively used for the separation of peptides [37]. Among those, n-butanol - acetic acid - water (4: 1: 5) requires the application of a reversed elution mode for the multiplayer coil in the type J HSCCC centrifuge.

Table 2.1 *Guide for the selection of the suitable two-phase solvent system*

	Hexane	Ethyl acetate	Methanol	n-Butanol	Water
	10	0	5	0	5
	9	1	5	0	5
↑	8	2	5	0	5
	7	3	5	0	5
	6	4	5	0	5
	5	5	5	0	5
↓	4	5	4	0	5
$CHCl_3$ –MeOH-H_2O (2:1:1)	3	5	3	0	5
	2	5	2	0	5
↑	1	5	1	0	5
	0	5	0	0	5
↓	0	4	0	1	5
	0	3	0	2	5
	0	2	0	3	5
	0	1	0	4	5
	0	0	0	5	5

Another consideration for the selection of solvent systems is pH value, especially for the separation of the ionic compounds; the pH of the solvent system may often become a critical factor in peak resolution. At the pH near pKa, these molecules are present in two forms, ionised and unionised, each having its own partition coefficient: Since these two species constantly interchange to maintain their equilibrium state during separation, they tend to form a broad peak. This can be prevented by choosing a pH for the solvent remote from the pKa of the compounds.

The partition coefficient (K) of a component can be determined by a simple test tube procedure: A known amount of the sample is thoroughly equilibrated with the two-phase solvent system, 1-2 ml of each phase is mixed with a suitable solvent (such as water or methanol) and the absorbance is determined with a spectrophotometer [36]. In addition to the ultraviolet (UV) and visible wavelengths, fluorescence, radioactivity, enzymatic activity, and various types of bioassay can also be used for determination of the partition coefficients. When a pure compound is not available, the above method only gives an average K value of multiple components, which may not be useful for a practical separation. In this case, an aliquot of each phase pre-equilibrated with the samples is separately analysed by HPLC or thin-layer chromatography. From a pair of

chromatograms obtained from each phase, the K value for each component is obtained by computing the ratio in height or area between the corresponding peaks [37].

2.6.2.1 TLC for selection of solvent system

An empirical approach for a solvent selection is solvent screening by TLC. The organic layer of a two-phase system is used as the eluent and the R_F values are measured by both normal phase and reverse phase modes. The R_F values should lie between 0.3 - 0.7. This method is very simple and convenient, but sometimes is not very accurate, because TLC still involves both partition and adsorption mechanisms, whereas CCC is based on liquid-liquid partition phenomena.

2.6.2.2 Analytical CCC for rapid selection of solvent system

In recent years, analytical CCC has had a rapid development because of its small solvent consumption and analysis time. In our study, analytical CCC was also used for rapid measurement of partition coefficients and selection of solvent systems [38].

2.6.3 Measurement of percentage retention of stationary phase (S_F)

2.6.3.1 Measurement of extra volume ($V_{\epsilon XT}$) of HSCCC

According to the theory of HSCCC [39, 40], the volume of a stationary phase retained in the coil plays an important factor in getting high-resolution efficiency. The more the volume of a stationary phase retains in the coil, the higher the resolution efficiency is. The volume of a stationary phase (V_S) retained in the coil system can be expressed as a fraction (S_F) or percentage retention of stationary phase as follows:

$$S_F = V_S / V_C \text{ or } Vs / V_C \times 100\ \% \qquad 2.1$$

Total system volume $(V_{SV}) = V_C + V_{in} + V_{out} = V_S + V_m + V_{in} + V_{out.}$ 2.2

V_m - the active mobile phase volume in the coil, V_{in} - the volume in the inlet leads

V_{out} - the volume in the outlet leads, V_c – the coil volume

Extra Coil Volume ($V_{\epsilon XT}$) is defined as the volume in the inlet and outlet leads and the coil feed

pipes. The volume of the coil feed pipes is usually very small. $V_{\epsilon XT}$ can be expressed as

$$V_{\epsilon XT} = V_{dis} - V_m \qquad 2.3$$

V_{dis} – the volume of mobile phase, which displaces a fixed amount of stationary phase from the coil. Wood has observed that there is a relationship between the stationary phase eluted and the square root of mobile phase flow, which can be expressed as equation [41]:

$$V_{dis} = V_{\epsilon XT} + AQ_m^{1/2} \qquad 2.4$$

Where Q_m = flow rate of mobile phase.

It is obtained by incrementally increasing the mobile phase flow until a new equilibrium is reached and plotting the new eluted volume of stationary phase against the square root of the flow. The intercept at $Q_m = 0$ gives the value of $V_{\epsilon XT}$ and any value above this gives the volume of mobile phase in the coil at any given flow in excess of this value. In our case, the $V_{\epsilon XT}$ was measured by this method.

The volume of mobile phase in the coil (V_m) can be calculated by subtracting the extra-coil volume from the displaced volume of stationary phase in Equation 2.3. Then the volume of stationary phase (V_s) can be calculated if the coil volume (V_c) is known accurately:

$$V_s = V_c - V_m \qquad 2.5$$

.V_{sv} is usually measured by determining the increase in weight when the column is filled with water and then blowing it out using a regulated N_2 supply. So, if the $V_{\epsilon XT}$ can be measured, it is possible to get a value for the retained volume of stationary phase (V_s) by subtracting the measured volume (V_{dis}) from the system volume (V_{sv})

$$V_s = V_{sv} - (V_m + V_{\epsilon XT}) = V_{sv} - V_{dis} \qquad 2.6$$

Du et al [42] observed that plotting the retention of stationary phase against the square root of the mobile phase flow resulted in a linear plot; the linear regression equation obtained from such a plot can be used to predict how retention changes with rotation speed. This is done as follow:

$$S_F = 100 - B\,F^{1/2} \qquad 2.7$$

Where the gradient B is a non-numeric constant with dimensions. It is known, from Wood's modelling the coil planet centrifuge as a constant pressure pump and analysis of flow by HAGEN-Poiseuille[40] that:

$$B = 100/V_c \times (8\pi\mu_m L^3/\Delta P)^{1/2} \qquad 2.8$$

μ_m is the viscosity of the mobile phase, L the length of the coil and ΔP the pressure drop across the coil. Wood has also shown that the pressure drop across the coil is related to the density difference of the phases ($\Delta\rho$), the acceleration field (ω^2) and the length (L) of the tubing, as follows:

$$\Delta P = KR\Delta\rho\,\omega^2 L \qquad 2.9$$

2.6.3.2 Measurement of S_F

According to the equations: $V_m = V_{dis} - V_{\epsilon XT}$, $V_s = V_{sv} - V_{dis}$ and $V_c = V_s + V_m$

$$\text{Retention } (S_F\,\%) = \frac{V_s}{V_c} * 100 = \frac{V_{sv} - V_e}{V_c} * 100 = \frac{V_c - V_m}{V_c} * 100 \qquad 2.10$$

2.6.4 Preparation of the two-phase solvent systems and sample solution

The sample solution is usually prepared by dissolving the sample mixture in the solvent to be used for separation. When the sample amount is small and the target component has a low K value, the sample may be dissolved in the stationary phase. However, it is better to dissolve the sample in a solvent mixture consisting of equal volumes of both upper and lower phases for the following reasons: if a large amount of sample is dissolved in a one phase solvent, the physical properties of the two-phase solvent system is altered and in the extreme case the solvent forms a single phase. Injection of this sample into the separation column would result in a detrimental loss of the stationary phase and bad resolution would be observed. Also, when the sample mixture contains multiple components with a wide range of polarity, the use of the two-phase system can minimize

the volume of the sample solution, hence improving the peak resolution.

In contrast to HPLC, HSCCC permits relatively large amounts of sample injection without seriously affecting the peak resolution. Generally, the volume of sample is one-tenth of the capacity of column volume [27]. Too much volume of injection of sample will result in the loss of the retention of the stationary phase.

2.6.5 Extraction of sample

Generally, methanol, ethyl acetate and butanol were used in the extraction of different flavonoids. The extract solvent depends on the polarities of flavonoids. In the seeds of O indicum, the flavonoids can be classified into two groups, one is aglycone flavonoid and the other is glycoside flavonoid. The glucoside flavonoids are more polar than aglycone flavonoids because of the sugar moiety in the former case. Hence, different polarity solvents were used to extract the two groups. In the extract of ethyl acetate, the major aglycone flavonoids were obtained. When a more polar solvent system such as 2-butanol was used to extract the seeds, major glycoside flavonoids were extracted from the seeds of O indicum. In this study, three different extraction methods were employed to get different components and were then subjected to the separation by HSCCC.

2.6.5.1 Extraction method 1: Extract of flavonoids from the seeds of *O indicum* by ethyl acetate

100 g of *O indicum* plant was treated with liquid nitrogen in order to freeze the seeds of *O indicum,* and then the seeds of O indicum were ground to powder. 50 g of the powder of seeds were refluxed for 5 hours in 90 % methanol, the extract was then filtered and evaporated .The residue was redissolved in 200 ml water and extracted three times with ethyl acetate. The extract of ethyl acetate was evaporated to dryness and yielded 6.5 g of a yellow powder and the layer of water was extracted by method 2 (see below). The extract from ethyl acetate was labelled as sample 1, in this extract; three major components were found and labelled compounds 2, 3 and 4.

2.6.5.2 Extraction method 2: Extract of glucoside flavonoids from the seeds of *O indicum*

The layer of water from extraction method 1, which contains more polar components, was

extracted three times with butanol. After evaporation, deep yellow crystals were obtained. It was labelled sample 2

2.6.5.3 Extraction method 3: 2-butanol extract of flavonoids from the seeds of *O indicum*

250 g of seeds of *O indicum* were extracted for three days with 600 ml of 70 % methanol; the extracts were then filtered and evaporated. The residue was redissolved in 200 ml H_2O and extracted three times with 250 ml 2-butanol. The 2-butanol layer was evaporated with a rotary evaporator at a temperature of 30°C. Yellow powder (25 g of complex mixture) was obtained. It was labelled sample 3. This mixture was directly subjected to HSCCC. From this extract, five components were obtained and they were labelled compounds A, B, C, D and E. The HSCCC fractionations of these extracts are discussed in sections 2.7, 2.8 and 2.9.

2.7 RESULTS AND DISCUSSION

2.7.1 HSCCC separation procedure

Countercurrent chromatography separation is usually performed with a standard procedure as follows: The column is first filled with the stationary phase followed by injection of the sample solution through the sample port. Then, the mobile phase is pumped into the column in an appropriate elution mode while the apparatus is rotated at the optimum revolution speed. Normally, the HSCCC permits a choice of two elution modes; reverse phase where the mobile phase is the lower aqueous phase, is head-to-tail mode. This usually produces better resolution by retaining a larger volume of the stationary phase in the column. Alternative in normal phase, the mobile phase is the upper organic phase, where elution is from tail to head. In this mode, there is a natural Archimedes pumping action in the direction tail to head, so this elution mode may produce a reversed pressure gradient in the column.

In this study, an experimental prototype J-type coil planet centrifuge high-speed preparative instrument was provided by Brunel Institute for Bioengineering, Brunel University. This machine was equipped with a HP1100 pump, a UV spectrophotometer detector and a sample collector. A manual sample injection valve was used to introduce the samples into the column. The CCC has four coils from analytical to preparative scale that are wound tightly on two separate bobbins on

one rotor; each bobbin contains two concentrically wound coils of PTFE tubing with a total volume of 490 ml. In this study, columns of volume 49.9 ml, 169.9 ml and 227.1 ml were used. The ß range was at 0.7 to 0.83. When one column was used, the other three coils were filled with methanol-water = 50: 50 to maintain balance. A series of biphasic mixtures were prepared and thoroughly equilibrated in a separation funnel by repeated vigorous shaking and degassing at room temperature; then 20 mg of the sample dissolved in 1 ml mobile phase was loaded. The coils were rotated at a constant speed of 800 rpm, and the system purged with one of the biphasic phase up to the head or tail of the coil. The mobile phase was then pumped into the coil from head (centre) to tail (periphery) at a constant flow rate by HP1100 pump (Hewlett Packard) and the resulting effluent was collected at the tail in a graduated burette. The equilibration point of the system was determined when no more stationary phase was eluted (hydrodynamic equilibration). The retention volume of the system could then be calculated by subtracting the volume of stationary phase eluted at the end of the equilibration process and the extra coil volume from the total volume of the system on the Wood plot equation [40]. The effluent from the tail end of the column was continuously monitored with a UV detector and the retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column after the separation was completed. Peak fractions were collected into test tubes with a sampler controller (Gilson, France) for analysis by HPLC.

2.7.2 HSCCC analyses sample 1 from the extract method 1

2.7.2.1 HPLC analyses sample 1 from the extract method 1

In the extract method 1, HPLC analysis [see Figure 2.2] shows that the sample 1 contained four constituents 1-4. Three major components existed in sample 1 and another minor component was also obtained.

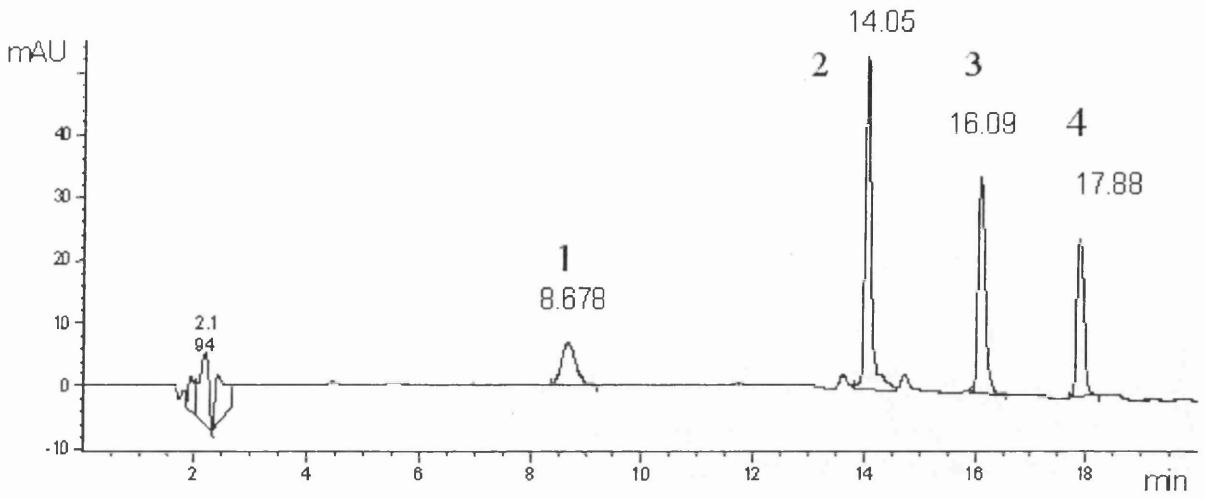


Figure 2.2 *HPLC chromatogram of sample 1 from the extract method 1*

Experimental conditions: A cosmosil C_{18} RP column (150 × 4.6 mm I.D) was employed (Phenomenex) at a temperature of 30 °C, flow rate of 1.0 ml /min and wavelength of 275.5 nm. A gradient elution of formic acid 0.2 % (A) and 100 % acetonitrile (B) was employed: 0 - 10 min 80 % A and 20 % B; 10 - 12 min 55 % A, 45 % B; 12 - 20 min 20 % A, 80 % B.

2.7.2.2 Measurement of partition coefficient (K) of components in sample 1 in hexane-ethyl acetate-methanol-water (H-E-M-W) solvent system.

A HPLC method was used to measure the K values of components in sample 1. HPLC analysis showed that three major constituents and one minor component existed in the ethyl acetate extract of the seeds. A H-E-M-W solvent system is a medium polar solvent composition. Shake tests showed the three major components could partition into two layers in this solvent system. Hence, determination of K values of the three major components was used for choosing a suitable composition of this solvent system. Approx, 1 mg of the test sample was added to a 10 ml test tube to which 2 ml each of pre-equilibrated two-phase solvent system was added. The test tube was stoppered and shaken vigorously for 1 min to thoroughly equilibrate the sample between the two phases. Then, equal volumes (about 100 μ L) of the upper and lower phases were diluted with methanol to 1 ml and analysed by HPLC, respectively. The K value of each component was determined by computing the ratio in height or area between the corresponding peaks. Table 2.2

shows their K values.

Table 2.2 *K values for the addition of methanol to the H-E-M-W solvent system*

* H-E-M-W (Ratio of volume)	% MOH v/v	$K_{component\ 2}$	$K_{component\ 3}$	$K_{component\ 4}$
1: 1.2: 0.4: 1	0.111	0.54	5.4	6.40
1: 1.2: 1: 1	0.238	0.58	2.7	4.20
1: 1.2: 1.2: 1	0.273	0.65	0.98	3.00
1: 1.2: 1.6: 1	0.333	0.60	0.60	0.7
1: 1.2: 2: 1	0.385	0.75	0.20	0.17
1: 1.2: 2.4: 1	0.429	1.4	0.07	0.17
1: 1.2: 3: 1	0.484	2.00	0.06	0.07

*H=hexane, E=ethyl acetate, M=methanol, W=water.

K was defined as the ratio of peak areas of corresponding component in the upper phase over lower phase by HPLC analysis.

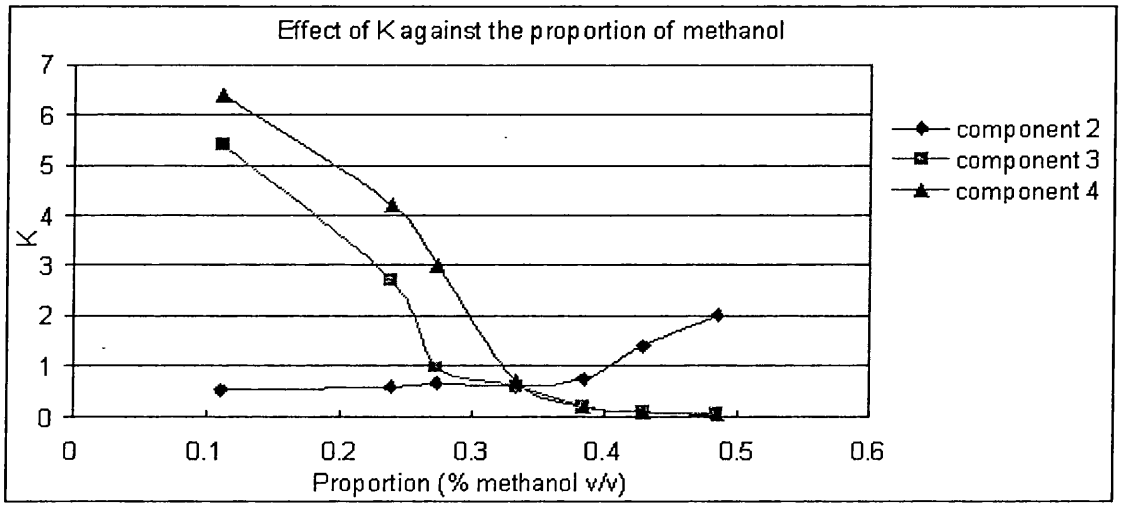


Figure 2.3 *The effect of the proportion of methanol on the K values for components 2, 3 and 4*

When increasing the ratio of methanol, component 2 partitions into upper phase (organic phase) and components 3 and 4 partition into lower phase (aqueous phase). These results show that component 2 is more hydrophobic than components 3 and 4. If the aqueous phase was chosen to be the mobile phase in CCC, the component 2 would be eluted first, and then component 3. The

last component to be eluted would be component 4. When the ratio of hexane-methanol-water = 1:1.2:1 remains constant and the ratio of ethyl acetate is varied, the partition coefficients will change as well (Table 2.3).

Table.2.3 *K values for the addition of ethyl acetate to the H-E-M-W solvent system*

H-E-M-H	% E(v/v)	Kcomponent 2	Kcomponent 3	Kcomponent 4
1:0.4:1.2:1	0.111	0.023	0.12	1.181
1:0.8:1.2:1	0.200	0.04	0.335	2.028
1:1.2:1.2:1	0.273	0.06	0.652	3.068
1:1.6:1.2:1	0.333	0.114	1.578	4.786
1:2:1.2:1	0.385	0.178	1.54	3.459
1:3:1.2:1	0.484	0.287	3.42	6.462

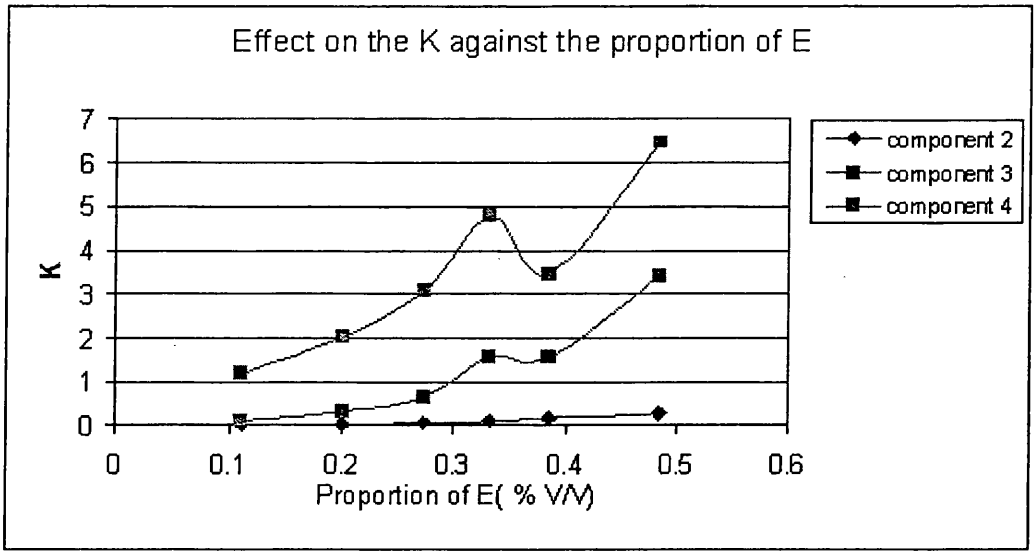


Figure 2.4 *The effect of the proportion of ethyl acetate on the k values for components 2, 3 and 4*

When the ratio of ethyl acetate was increased, all components partitioned into the organic phase. If the organic phase was chosen to be the mobile phase (normal phase), increasing the ratio of ethyl acetate will result in fast elution of all components. According to the literature, the best K values for good resolution are between 0.5 to 2.0. When the ratio of solvent system was 1:2:1.2:1, all constituents 1-4 had different K values and can be eluted in relative short time. Hence, the ratio of ethyl acetate for separation of these compounds should be not more than 2.0.

2.7.2.3 HSCCC separation of sample 1from the extraction method 1

The partition coefficients showed that the H-E-M-W solvent system provided promising results for a successful separation of the extraction of the plant sample. The experiments that follow describe the optimization procedures, which included different flow rate, rotation speed, isocratic and gradient elution and led to the final separation achieved for the extract sample 1 from extract method 1.

2.7.2.3.1 Separation of sample 1 with H-E-M-W solvent system in normal phase isocratic elution

Similar to HPLC, HSCCC can also be classified into normal phase and reversed phase elution modes. Normal phase elution is defined as the less polar phase is being used as the mobile phase

and reverse phase defined as the more polar phase being used as the mobile phase. Water is usually defined as the most polar solvent system commonly, hence the aqueous, often the lower, phase is the most polar phase. Most phase systems will operate in normal phase mode when the lower phase is the stationary phase and in reversed phase when the upper phase is the stationary phase. Generally in CCC, we label normal phase mode as tail to head (T-H) and reverse phase mode as head to tail (H-T)[27]. The solvent system H-E-M-W was found to be a good solvent system for the isolation and purification of the flavonoids from natural products.

Figures 2.5. A, B, C and D show the separations of sample 1 by normal phase isocratic elution when the ethyl acetate ratios were varied. In all these solvent systems, the stationary phase retention was more than 80 %, and the partition coefficients indicated that components 3 and 4 were the first eluted when the concentration of ethyl acetate was high. The components 1 and 2 would be last eluted in the normal phase mode.

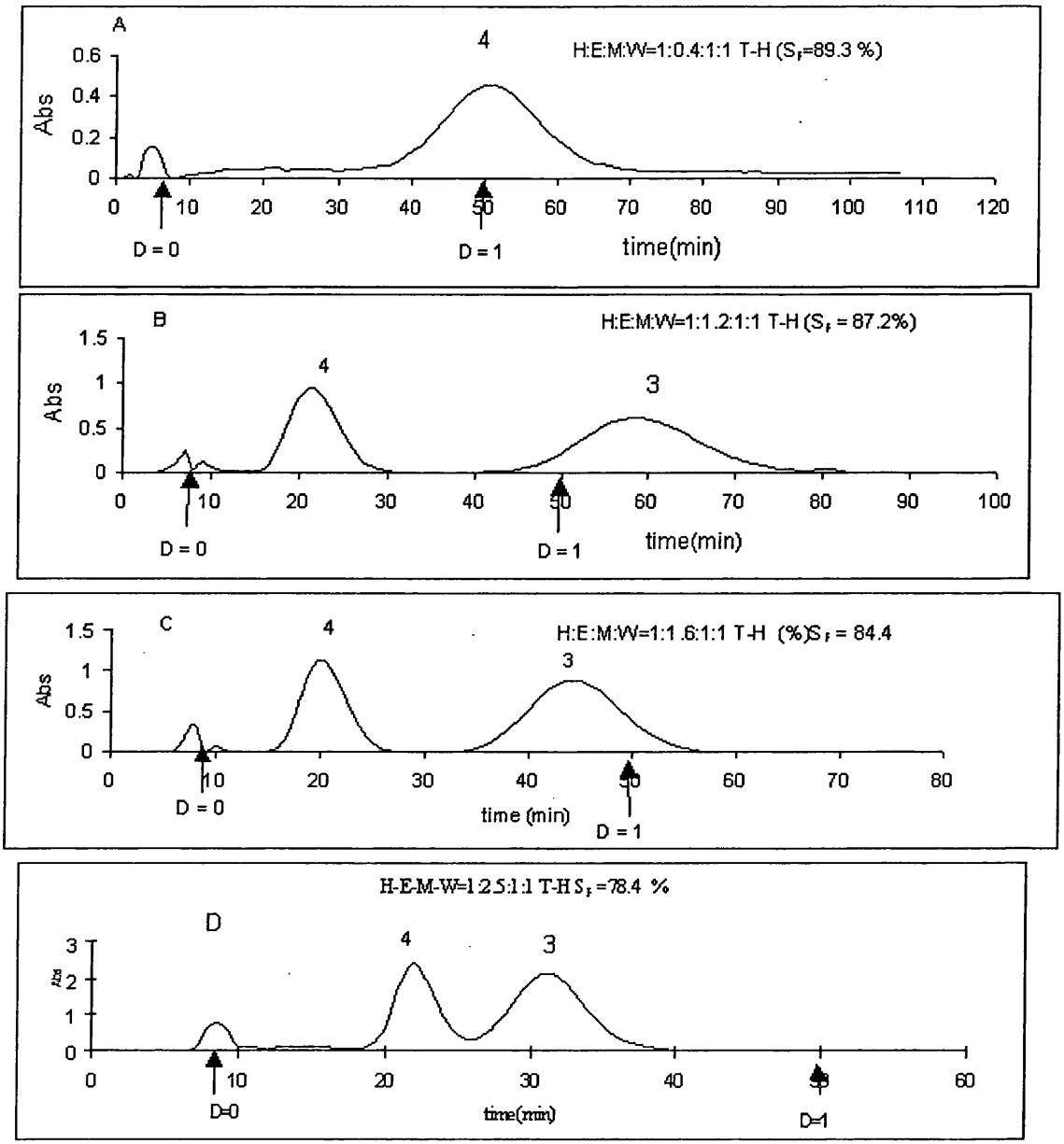


Figure 2.5 *Chromatogram in different ratios of ethyl acetate by HSCCC in normal phase*

Experimental conditions: Coil volume: 49.9 ml; Flow rate: 1 ml / min; Direction of motor: Reverse; Speed of rotation: 800 rpm; Oven temperature: 30°C; Sample injection volume: 0.5 ml; Sample concentration: 20 µg / ml; Stationary phase: Aqueous phase; Mobile phase: Organic phase.

Figure 2.5 A shows that only component 4 eluted when the concentration of ethyl acetate was 0.4. Figures 2.5 B and C show the effect of ethyl acetate in normal phase CCC for the separation of extraction of sample 1.When increasing the ratio of ethyl acetate to 1.2, components 4 and 3 can be separated completely, but the third component 2 is still retained in the stationary phase because of low partition coefficients even when increasing the ratio of ethyl acetate to 2.5 (Fig 2.5 D).

The H -E-M-W solvent system can only give good resolution of two components 4 and 3 for sample 1 in normal phase isocratic elution, but HPLC analyses showed the two components from the fractions are very pure. Another two components 1 and 2 were still retained in the stationary phase. When changing the elution mode into reversed phase, components 1 and 2 were eluted together from the stationary phase.

2.7.2.3.2 Application of H-E-M-W solvent system in normal phase gradient elution for sample 1

The ease of optimisation with gradient HPLC compared to sequential isocratic optimisation suggested that this was the route to take with CCC. Indeed, gradient CCC (either linear or step) is possible and is likely to have significant potential. The importance of gradient separation is the ability to cover the entire polarity range in one single run and shorten the elution time. This method was also used in the separation of sample. Figures 2.6 A) and B) show the gradient elution of sample from extraction method 1 when H-E-M-W=1: 0.4: 1: 1 to 1: 2.5: 1: 1 and H-E-M-W=1: 0.4: 1: 1 to 1: 3: 1: 1 were used.

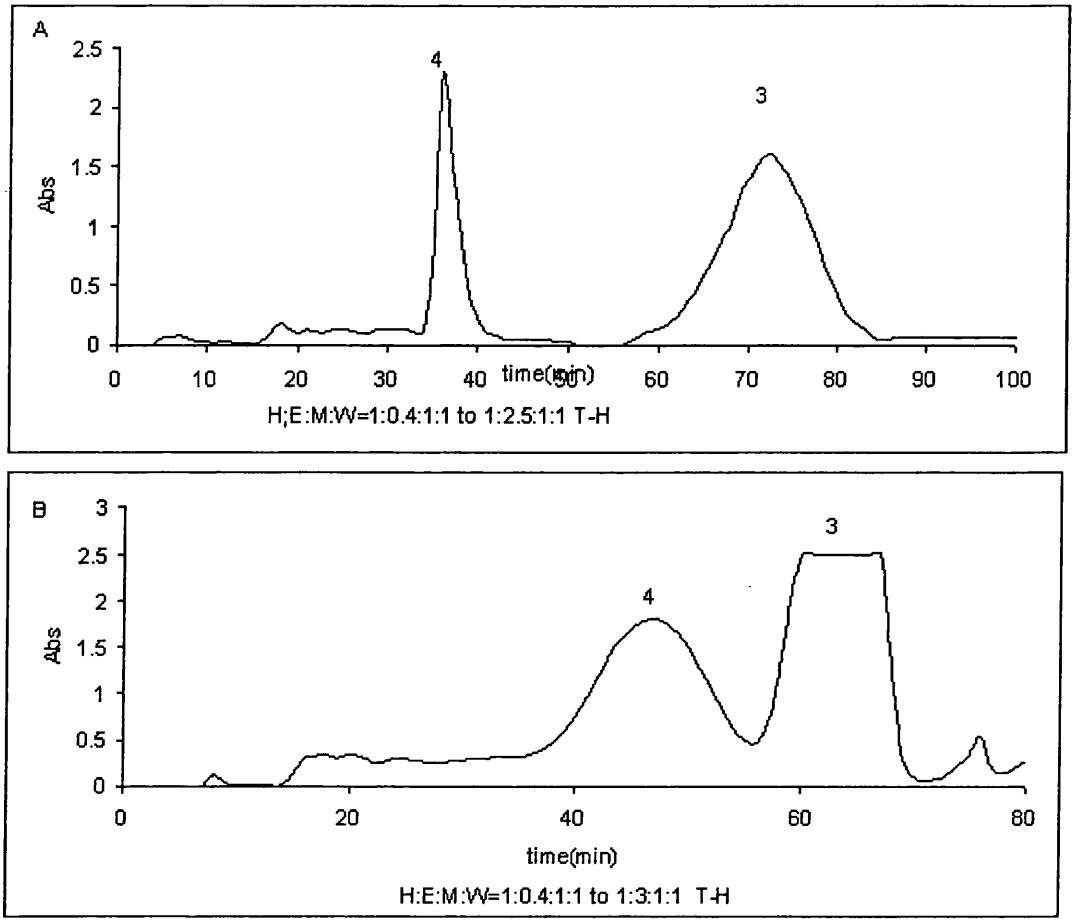


Figure 2.6 *Chromatogram of CCC with ethyl acetate gradient elution*

HSCCC experimental conditions see Figure 2.5

Gradient programme: 0 – 20 min 100 % A; 20 – 60 min 100 % A to 40 % A and 0 % B to 60 % B; 60 – 80 min 60 % B to 100 % B; Stop rotation at 80 min and keep the 100 % B isocratic elution about 50 minutes. A represents the lower phase in H-E-M-W=1: 0.4: 1: 1 and B represents the lower phase in H-E-M-W= 1: 2.5: 1: 1 or 1: 3: 1: 1.

The gradient elution can shorten the elution time, but without any improvement in the resolution, and another drawback of gradient elution is that the gradient elution will result in the loss of stationary phase during the gradient elution. In Figure 2.6 B after 60 minutes, an amount of stationary phase was washed off. In Figure 2.6 A about 5 ml stationary phases was washed off. The experiments show that the ratio of ethyl acetate has a big effect on the loss of stationary phase. In gradient elution, when the ethyl acetate is maintained in the ratio of 2.5, no large amount of stationary phase was washed off.

2.7.2.3.3 Application of H-E-M-W solvent system in reverse phase isocratic elution for sample 1

In this reverse phase elution mode, the organic phase was chosen to be the stationary phase and aqueous phase to be the mobile phase. According to the partition coefficients in shake tests, polar compounds will first be eluted out when increasing the concentration of ethyl acetate. Figures 2.7 A and B show that the reverse phase is better than normal phase and three compounds can be easily eluted. When increasing the ratio of ethyl acetate, the elution time of the three components can be shortened. When the ratio of H-E-M-W =1: 2: 1: 1 was chosen, the three components can be eluted in 100 minutes.

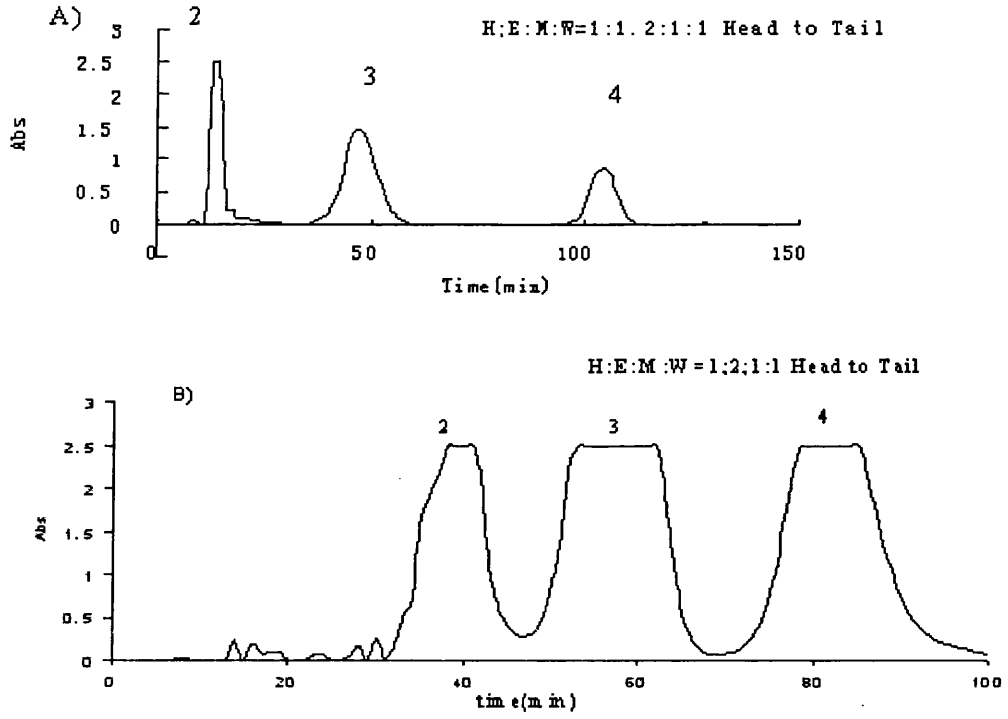


Figure 2.7 *HSCCC Chromatograms of sample 1 with reverse phase elution mode.*

Experimental conditions: Coil volume: 49.9 ml; Stationary phase: Organic phase; Mobile phase: Aqueous phase; Rotation speed: 800 rpm; Flow rate: 1.0 ml / min; Sample volume: 0 5 ml; Sample concentration: 20 μg / ml; Direction of motor: Reverse.

Figures 2.7 A and B show that the reverse phase isocratic elution had better elution efficiency and less consumption of organic phase than normal phase because the aqueous phase was chosen to be mobile phase.

2.7.2.3.4 Methanol gradient elution to the separation of sample 1 in H-E-M-W solvent system

When the ratio of methanol was increased, more polar compounds were eluted quickly. In an isocratic elution study, the ratio of methanol was increased to 3.0 and no stationary phase was washed off. When the methanol gradient elution was used to the separation of sample 1, three components can also be resolved. Figures 2.8 A and B show methanol gradients for the separation

of sample 1.

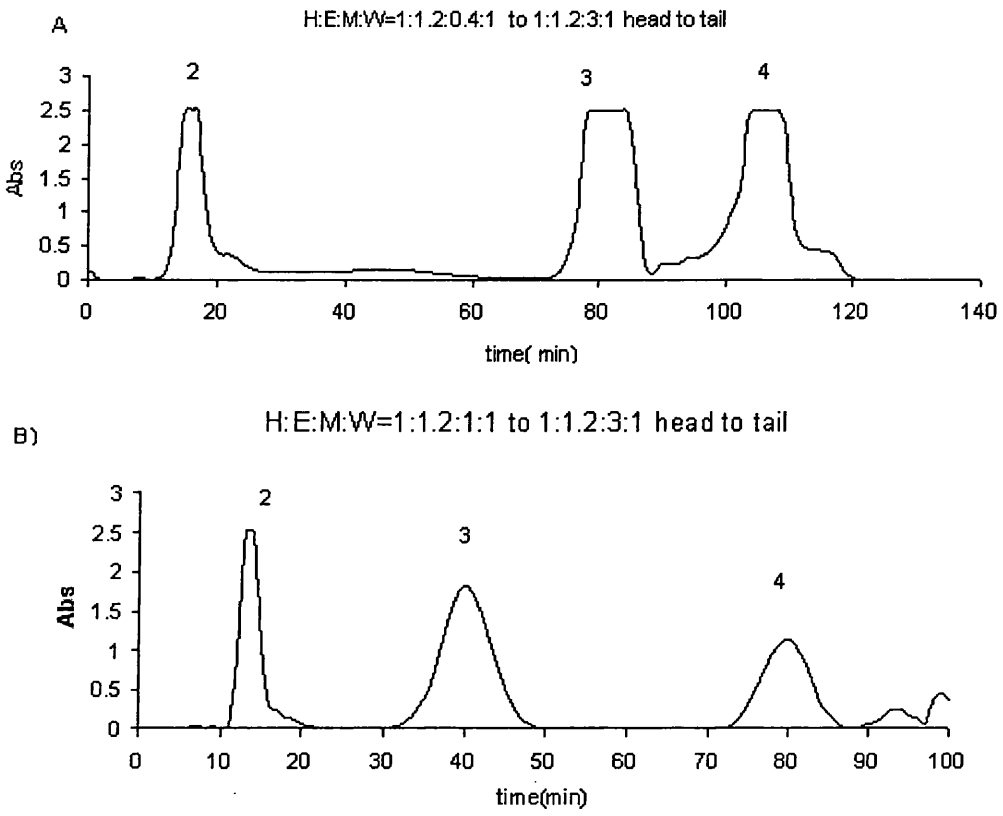


Figure 2.8 *HSCCC Chromatograms in different gradient elution programmes*

(HSCCC conditions see Figure 2.6). Stationary phase: Organic phase; Mobile phase: Aqueous phase; in the gradient programme, A represents the lower phase in H-E-M-W=1: 1.2: 0.4: 1 and B represents the lower phase in H-E-M-W=1:1.2:3:1 solvent system.

A) Min	%A	%B		B) Min	%A	%B	
0	100	0		0	100	0	
20	100	0		20	100	0	
60	40	60		60	40	60	
80	40	60	(stop rotation)	80	0	100	
150	0	100		150	0	100	(stop rotation)

Figures 2.8 A and B indicate that methanol gradient elution has good resolution for the components 2, 3 and 4 in the sample 1. In the gradient elution process, no stationary phase loss was observed. When the ratio of methanol was increased, the elution time of the sample can be shortened without loss of resolution. When the ratio of ethyl acetate was increased to 2, good resolution was also obtained. Figure 2.9 shows the methanol gradient when the ratio of ethyl acetate was increased to 2.

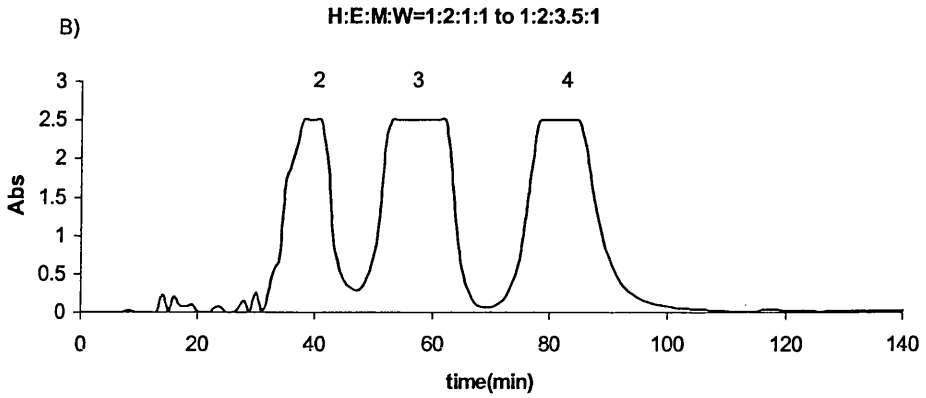


Figure 2.9 *Chromatogram in methanol gradient elution (Gradient programme see Figure 2.8B and other HSCCC experimental conditions see Figure 2.6)*

2.7.2.4 The effect of varying the flow rate on the separation of sample 1 by HSCCC in gradient elution

When the flow rate of elution was changed, the elution time was shortened, however, too high a flow rate could result in the loss both of resolution and stationary phase. When the flow rate was increased to 2.5 ml /min, the elution would be finished in 50 minutes. Figure 2.10 shows the effect of varying the flow rate on the separation time of the sample 1.

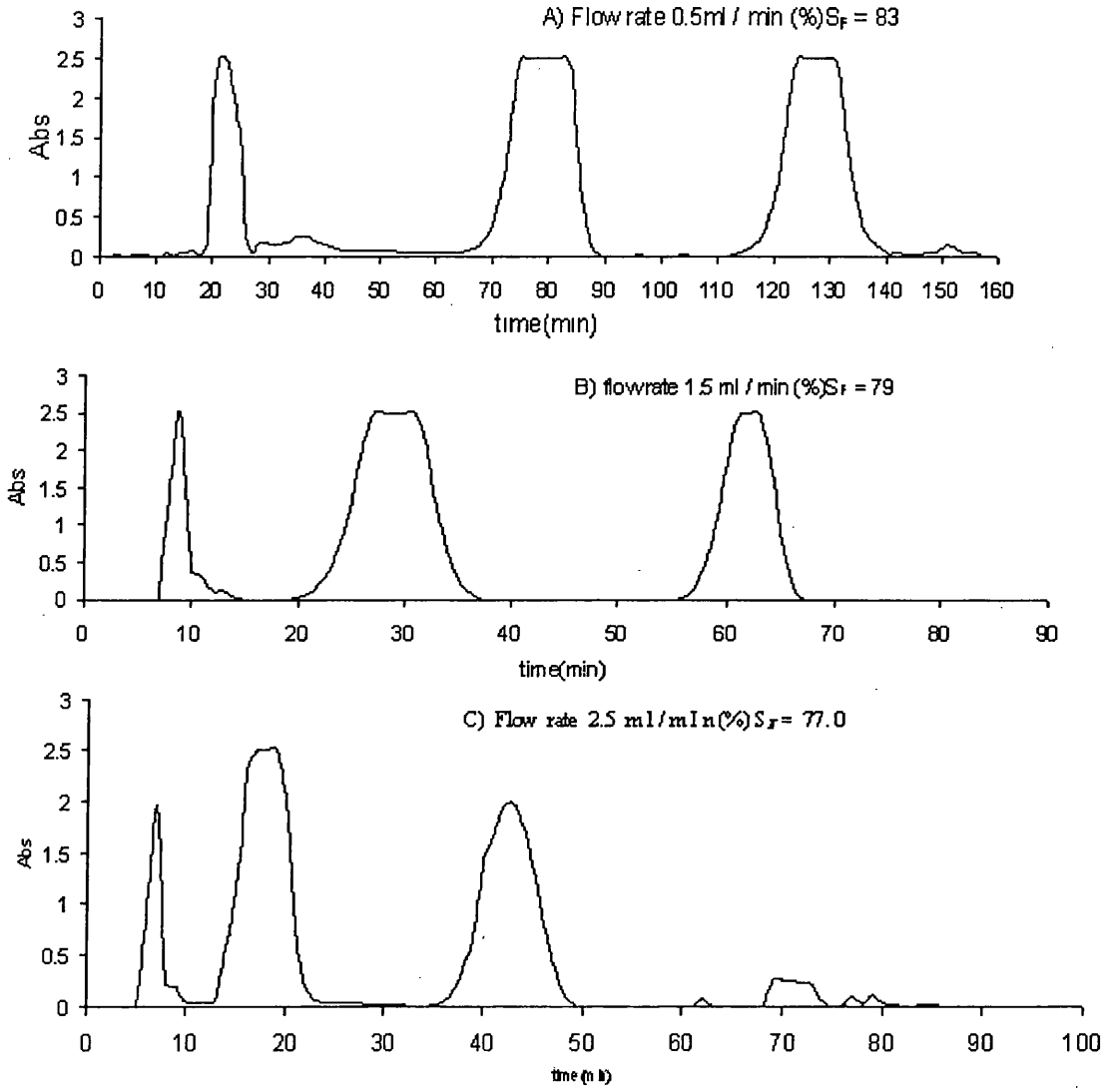


Figure 2.10 *Chromatograms with different flow rates by gradient HSCCC separation*

The H-E-M-W solvent system is a good solvent system for the separation of the ethyl acetate extract from the seeds of O, indicum. The isocratic elution of this solvent system at a ratio of 1:2:1:1 and gradient elution at 1: 1.2: 1: 1 to 1: 1.2: 3: 1 can get good resolution for the separation of this extract. After the CCC separation, three components were separated and collected, these fractions were subjected to HPLC analyses. Fractions 3 and 4 yielded 95 % and 98 % pure components 3 and 4. Fraction 2 only yielded an 85 % pure component 2 and another 15 % impurity component 1. Figure 2.11 shows the results for components 3 and 4 analysed by HPLC analyses.

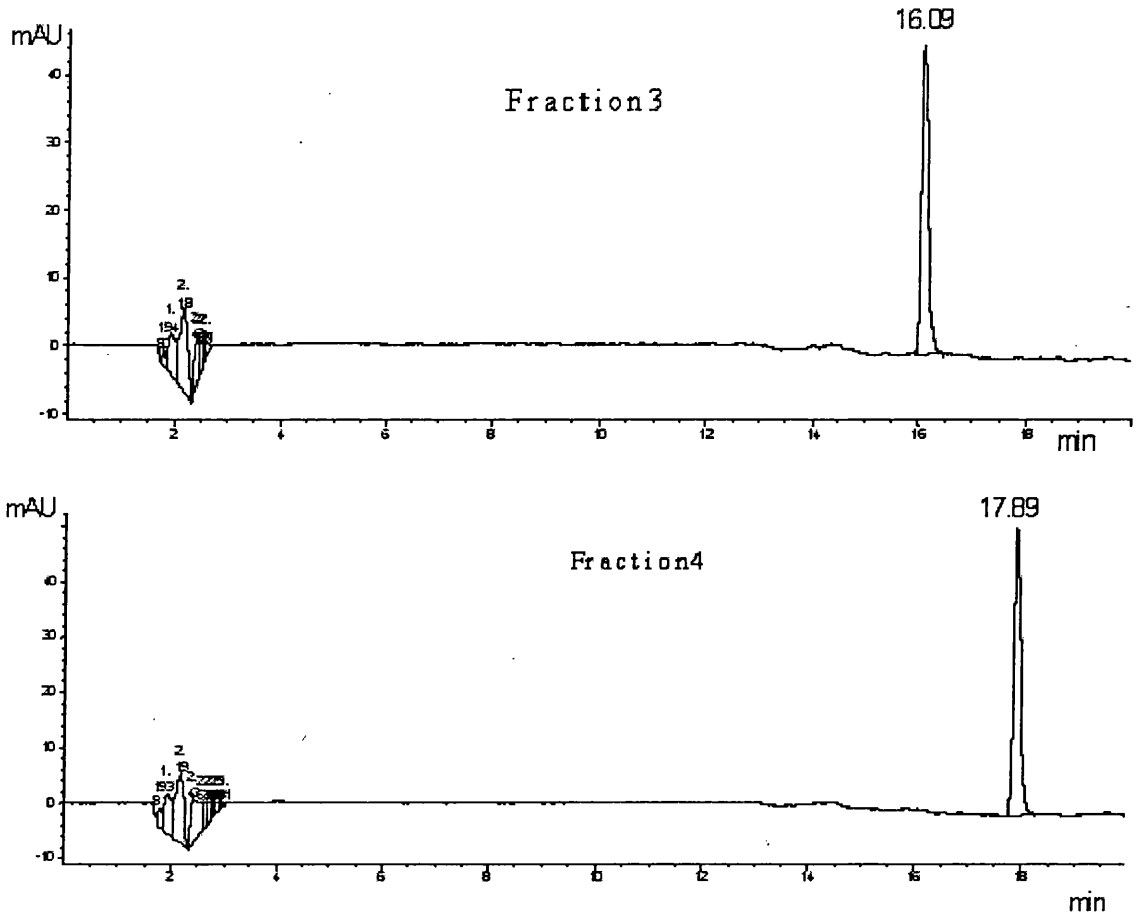


Figure 2.11 *Chromatograms of fractions 3 and 4 by HPLC analyses*

HPLC analysis conditions see Figure 2.2

2.7.2.5 Second separation of fraction 2 by HSCCC with a modified H-E-M-W solvent system

In order to further purify fraction 2, a modified solvent system (H-E-M-W)=0.2: 1.6: 0.2: 1.6 was employed and the purity of compound 2 was improved, a 75 % purity minor component I was obtained. After separating the fraction 2 by HSCCC, the purity of component 2 was greatly improved and a minor component 1 was also obtained. Figure 2.12 shows that a modified ratio of the H-E-M-W solvent system was used to separate fraction 2. Figure 2.13 shows the chromatogram of peak 2 by HPLC analysis.

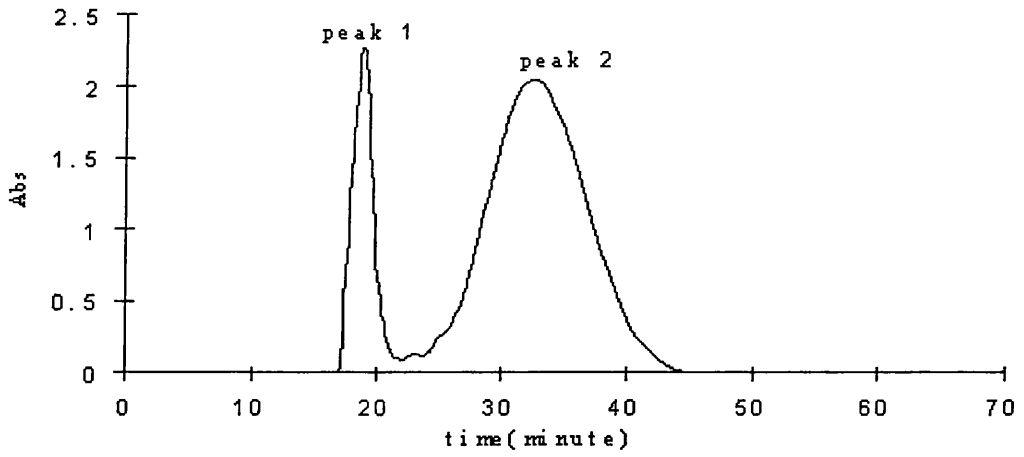


Figure 2.12 *Second step separation of fraction 2 by HSCCC*

Experimental conditions: Solvent system: H-E-M-W=0.2: 1.6: 0.2: 1.6; Coil volume: 49.9 ml; Stationary phase: Organic phase; Mobile phase: Aqueous phase; Rotation speed: 800 rpm; Flow rate: 1.0 ml / min; Sample: Fraction 2; Sample volume: 0 5 ml; Sample concentration: 20 μg / ml; Direction of motor: Reverse; Stationary retention: (%) S_F= 75.

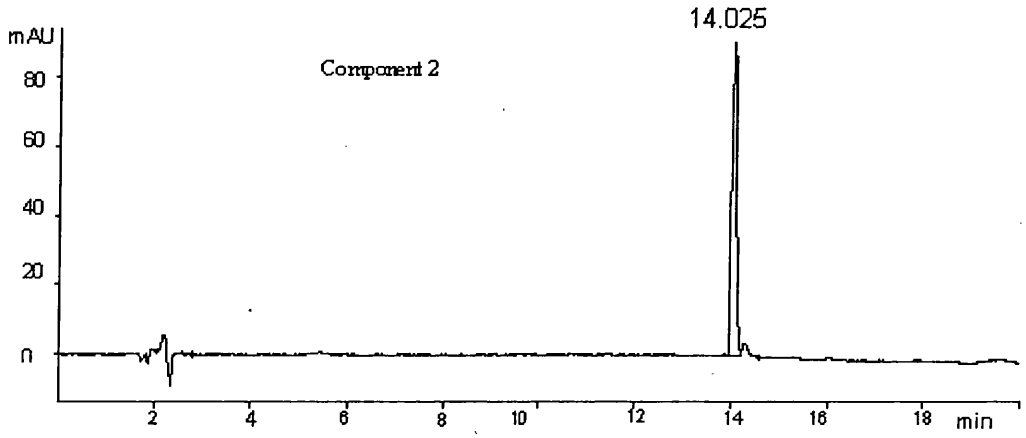


Figure 2.13 *HPLC analysis of the peak 2 after second separation by HSCCC*

HPLC analysis conditions see Figure 2.2

Conclusions: The results of HSCCC separation show that H-E-M-W is a good solvent system for the separation of sample 1 from extract method 1. Both isocratic elution at a ratio of 1: 2: 1: 1 and

gradient elution at 1: 1.2: 1: 1 to 1: 1.2: 3: 1 could resolve three compounds from this extract. Peaks 3 and 4 yielded 95 % and 98 % pure components. Peak 2 only yielded an 85 % pure component 2 and another 15 % impurity component 1. After a modified solvent system (H-E-M-W) 0.2: 1.6: 0.2: 1.6 was employed, the purity of peak 2 was improved to 98 %. A 75 % purity minor component 1 was obtained.

2.8 HSCCC SEPARATION OF SAMPLE 2 FROM THE EXTRACT METHOD 2

2.8.1 HPLC analysis of flavonoids sample 2 from the extract method 2

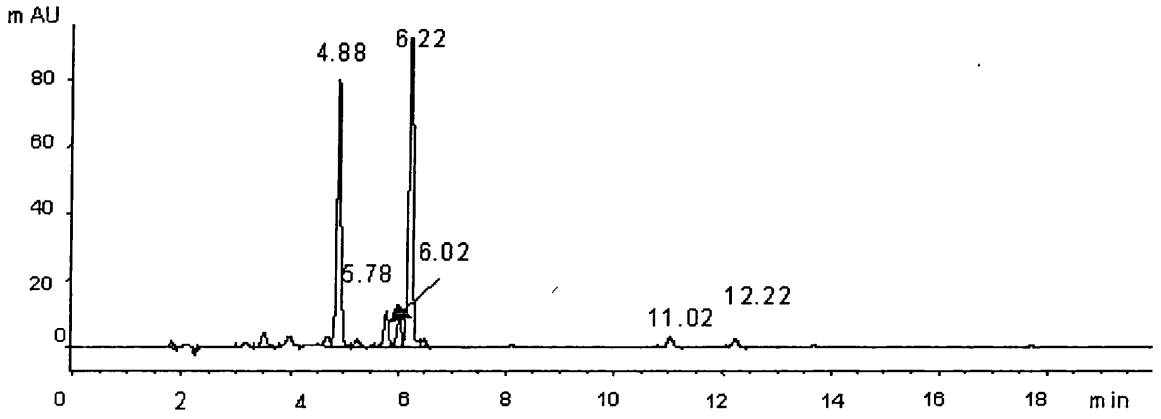


Figure 2.14 *HPLC chromatogram of sample 2 from extract method 2*

Experimental conditions: A cosmosil C_{18} RP column (150 x 4.6 mm I.D, Phenomenex) was used. Gradient elution: 0 -15 minutes 0.2 % formic acid from 80 % to 35 % and acetonitrile from 20 % to 65 %; 15-20 minutes 0.2 formic acid from 35 % to 10 % and acetonitrile from 65 % to 90 %; temperature 35°C; Flow rate 1.0 ml/ min; λ=275.5 nm.

2.8.2 HSCCC separation sample 2 from the extract method 2

Sample 2 from extract method 2 contained a lot of minor components [Figure 2.14] because their polarities are greater than aglycone flavonoids. In reverse phase elution mode for CCC, more polar compounds would be eluted first in the separation procedure. According to their partition

coefficients, a decreased ratio of methanol should be used for the separation of the flavonoid glucoside in a H-E-M-W solvent system.

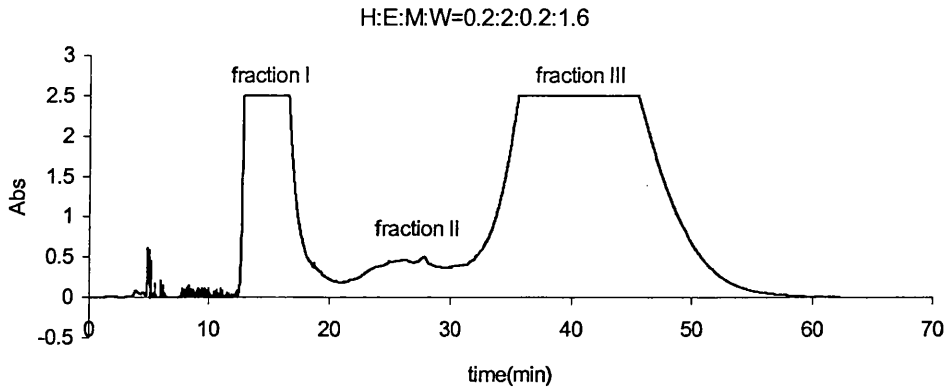


Figure 2.15 *HSCCC separation of sample 2 from the extract method 2*

Experimental conditions: Coil volume: 49.9 ml; Stationary phase: Organic phase; Mobile phase: Aqueous phase; Rotation speed: 800 rpm, Flow rate: 1.0 ml /min; Stationary retention S_F = 75.2 %; Sample concentration 15 mg / ml of 2-butanol extract.

Figure 2.15 shows that a ratio of H-E-M-W=0.2: 2.0: 0.2: 1.6 is suitable for the separation of sample 2 from extraction method 2 as long as the ratio of methanol is low. The reverse phase elution mode was chosen to effect the separation. If the normal phase elution mode was performed, all these components would be retained in the stationary phase. So, in this study, only the reverse phase elution mode was used for the separation of sample 2 from extraction method 2. Fraction II contained a very minor component in sample 2. After HSCCC separation, this minor component was enriched. Fractions I, II and III were subjected to HPLC analyses, their results are shown in Figure 2.16.

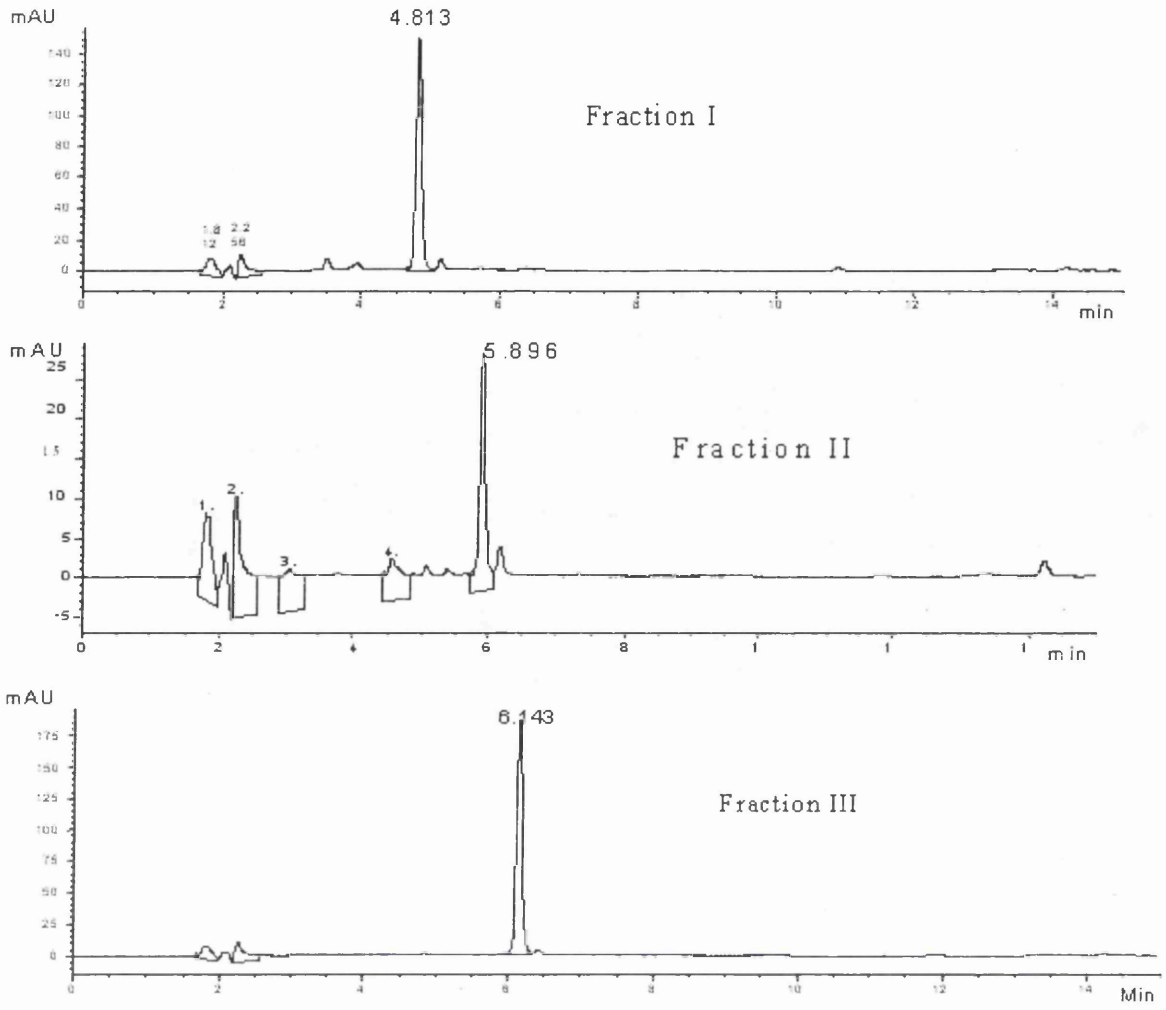


Figure 2.16 *HPLC separation of fractions from sample 2 after HSCCC*

HPLC experimental conditions see Figure 2.14

The purities of fractions I and III were 94 % and 98 %, respectively. Another small component II could be further purified. These results show that this solvent could also resolve the two major components in the sample 2 from extract method 2.

2.8.3 The effect of flow rate on the separation time

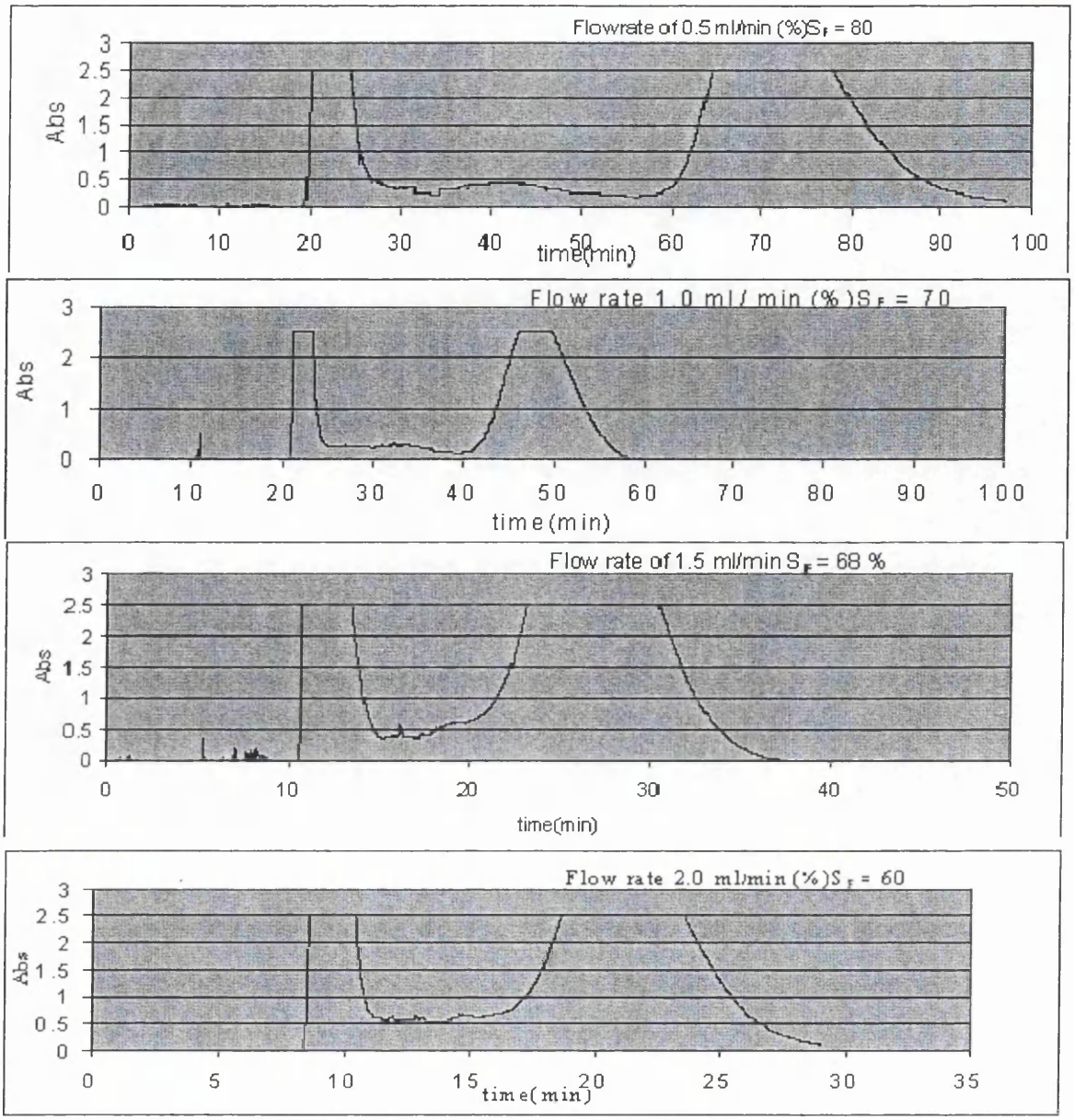


Figure 2.17 *The effect of flow rates on the separation of sample 2*

When varying the flow rate, the elution time was shortened greatly (Fig 2.17). When the flow rate was increased to 2.0 ml/min, the elution time was shortened to less than 30 minutes. However, fraction II coeluted with fraction III. So, a flow rate of 1.0 ml /min was used to separate sample 2. Thus it can be concluded that this solvent system can resolve two major components in the sample 2 from the extract method 2.

2.9 HSCCC SEPARATION OF SAMPLE 2 FROM EXTRACTION METHOD 2 WITH CHLOROFORM-METHANOL-WATER

H-E-M-W is a good solvent system for the separation and purification of flavonoid aglycones, but this solvent system could not separate sample 3 from extract method 3. In sample 3, flavonoids aglycone and glucoside coexist in the extract and cover a wide range of polarities. H-E-M-W solvent systems generally are suitable for the separation of hydrophobic components. Flavonoid glycosides are more hydrophilic than flavonoid aglycones, hence, another solvent system was chosen to separate sample 3 from extract method 3. Chloroform-methanol-water solvent system had been widely used for the separation of flavonoids, however, in recent years, this solvent system was avoided due to environment considerations.

In this study, chloroform-methanol-water was explored because aqueous phase was used as the mobile phase and chloroform was retained in the coil, avoiding introduction into the environment. This solvent system was successfully used in the separation of flavonoid aglycones and glycoside in a single run.

2.9.1 Measurement of K values in Chloroform-methanol-water solvent system for components in sample 2

High performance liquid chromatography was also used to determine the K values (Table 2.5) as described in section 2.7.2.2.

Table 2.5 *K values in chloroform-methanol-water solvent system when the proportion of methanol was varied for fractions I, II and III*

Proportion of methanol	K for fraction I	K for fraction II	K for fraction III
6: 10: 5	2.60	1.93	1.06
7: 10: 5	2.87	2.01	1.03
7.5: 10: 5	3.28	2.21	0.99
8: 10: 5	3.55	2.26	1.00
8.5:10: 5	3.93	2.47	1.04
9.0: 10: 5	4.45	2.48	0.91
10: 10: 5	4.89	2.51	0.89

2.9.2 HSCCC separation sample 2 by chloroform-methanol-water solvent system in reverse phase

In sample 2, more polar compounds existed in the extraction. According to their partition coefficients, an increased ratio of methanol should be used in the chloroform-methanol-water system. Figure 2.18 shows the separation of sample 2 in different ratios of the chloroform-methanol-water solvent system.

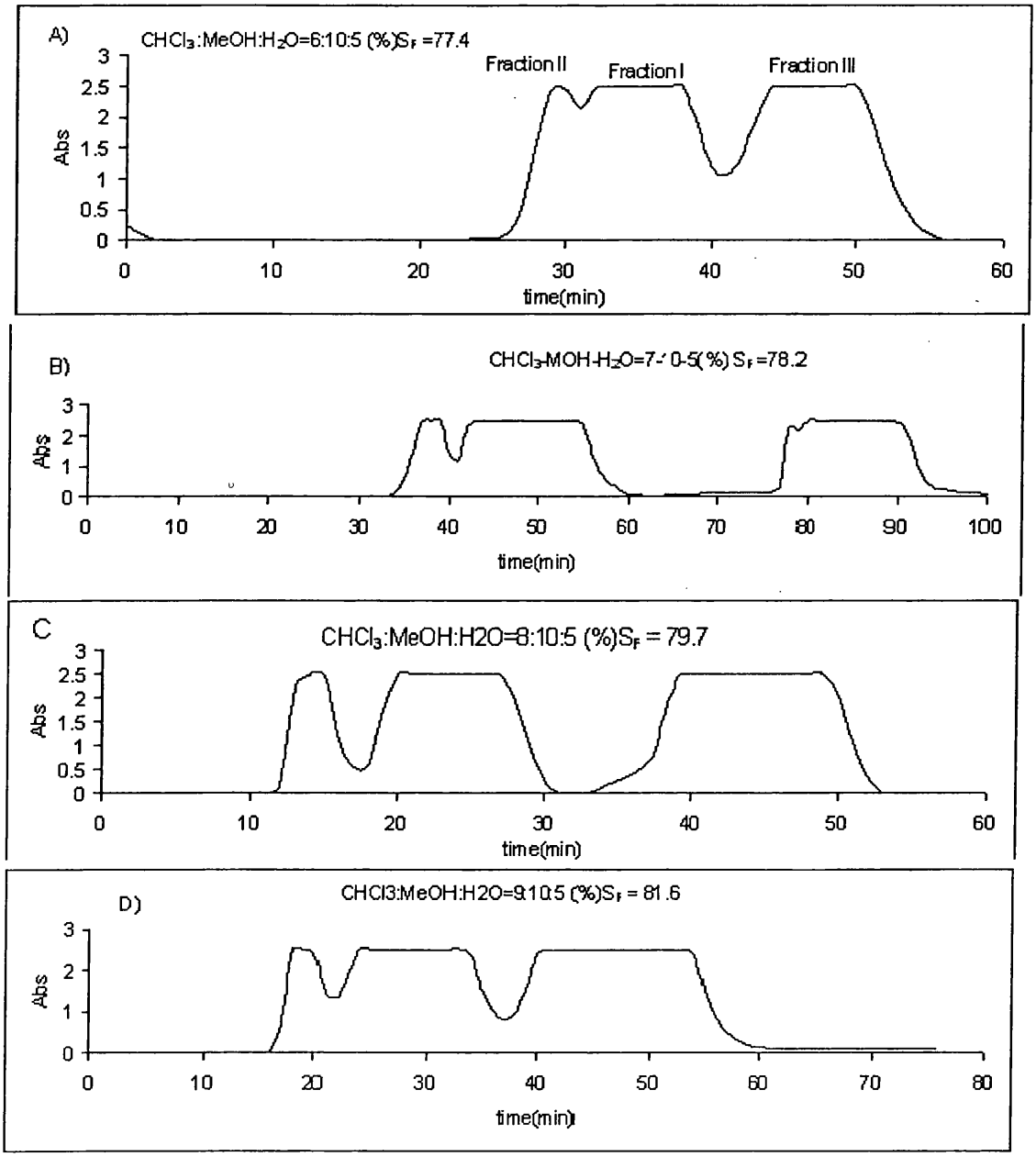


Figure 2.18 HSCCC *separation of sample 2 in different ratio of chloroform*

Experimental conditions: Isocratic elution; Head to tail; Coil volume: 49.9 ml; Sample injection volume 1.0 ml (20 mg / ml sample) Stationary phase: Upper phase; Mobile phase, Lower phase; Stop rotation at 200 minutes; Rotation speed: 800 rpm.

When varying the ratio of chloroform, different resolutions were obtained. Figure 2.18 shows that the ratio of 8:10:5 in chloroform: methanol: water was the best for the separation of the sample 2. Compared with H-E-M-W solvent system, chloroform-methanol-water system gave much better resolution. More importantly, a minor compound 2 from fraction II, which is difficult to separate by HPLC, was greatly enriched. It is very useful to get a larger amount of component 2 and identify its structure.

2.9.3 HPLC analyses of the fractions from the CCC separations

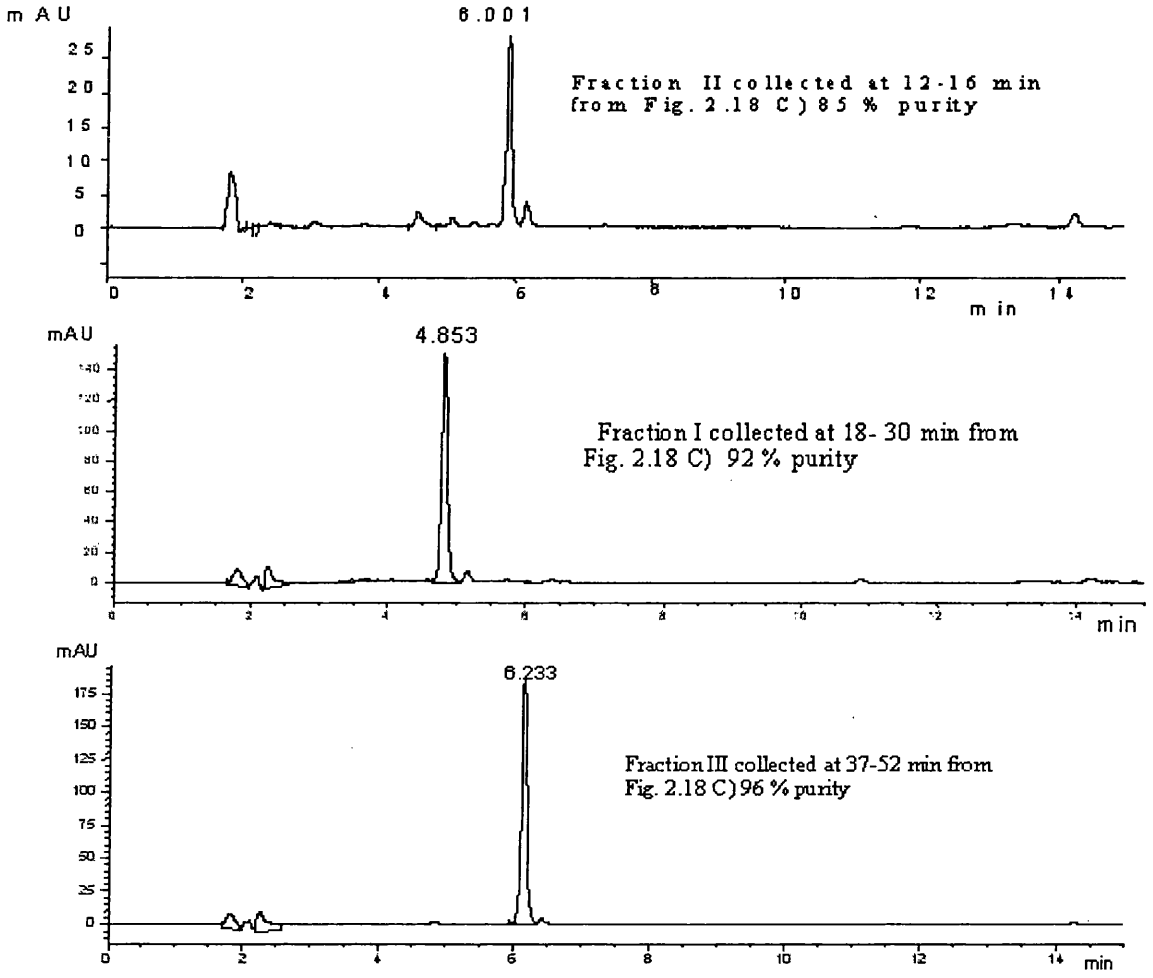


Figure 2.19 *HPLC analyses three fractions from sample 2 by HSCCC*

Fraction II collected from 12- 16 minutes contained one major component and its purity is about 85 %. It is a minor component in sample 2 from extract method 2. This component was very difficult to separate by HPLC (see Figure 2.14). However, this minor compound was greatly enriched after CCC separation. This result shows that HSCCC is a very powerful technique for the separation and purification of unknown components.

Fractions I and III were collected at 18-30 and 39-52 minutes, respectively. HPLC analysis showed that fraction I has the same retention time as component 1 from sample 1. Hence, fraction I and component 3 are the same substances. Fraction III has the same retention time as component 4 from sample 2 and they are same substance. The two fractions I and III are very pure components. After separation by HSCCC, three fractions were evaporated to dryness and redissolved into methanol for further analysed by HPLC/MS, UV and NMR in order to identify their structure.

2.10 HSCCC SEPARATION OF SAMPLE 3 FROM EXTRACTION METHOD 3 WITH CHLOROFORM-METHANOL-WATER

2.10.1 HPLC separation of sample 3 from extraction method 3

In sample 3 from extract method 3, four major components and some minor components were observed in HPLC analyses. These components were labelled A, B, C, D, E and F.

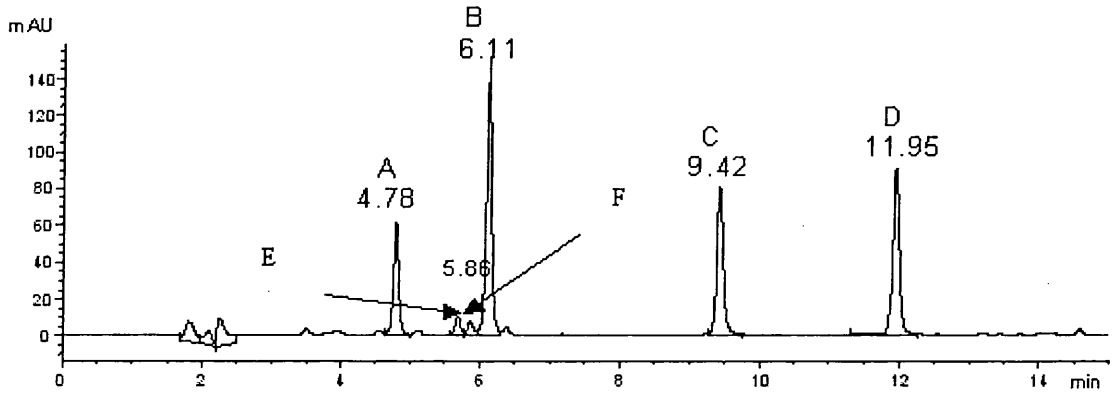


Figure 2.20 *Chromatograms of sample 3 from extraction method 3 by HPLC analyses*

Chromatographic conditions see Figure 2.14

Figure 2.20 shows that components E and F are minor components. Their retention times are very close and they are difficult to be resolved by HPLC.

2.10.2 HSCCC separation of the sample 3 from method 3 in reverse phase

Figure 2.20 shows that six components existed in sample 3 from extraction method 3. The retention times of components E and F were very close and were difficult to separate them even by preparative HPLC. Surprisingly, HSCCC could easily resolve this minor component E

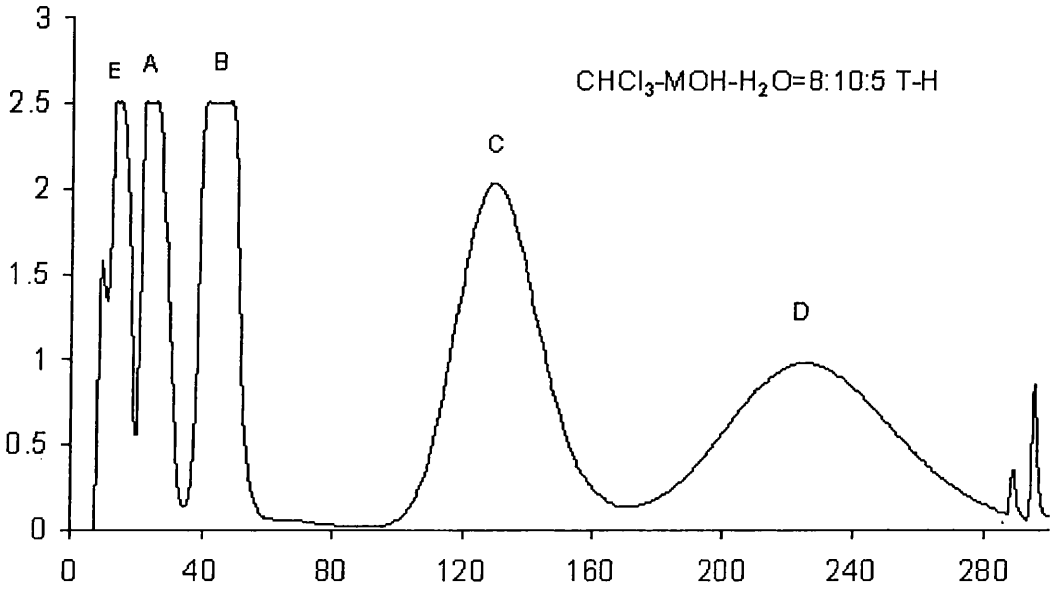


Figure 2.21 *HSCCC separation of sample from the extraction method 3*

HSCCC experimental conditions: Isocratic elution; Tail to head; Coil volume: 49.9 ml; Sample Injection volume 1.0 ml (20 mg/ml sample) Stationary phase: Lower phase, stationary retention (%) S_F =70; Mobile phase, Upper phase; Stop rotation at 300 minutes; Rotation speed: 800 rpm.

Figure 2.21 shows the separation result from the reverse phase elution mode. A minor component E could be enriched. These peak fractions were subjected to HPLC analysis and their results are showed in Figure 2.22

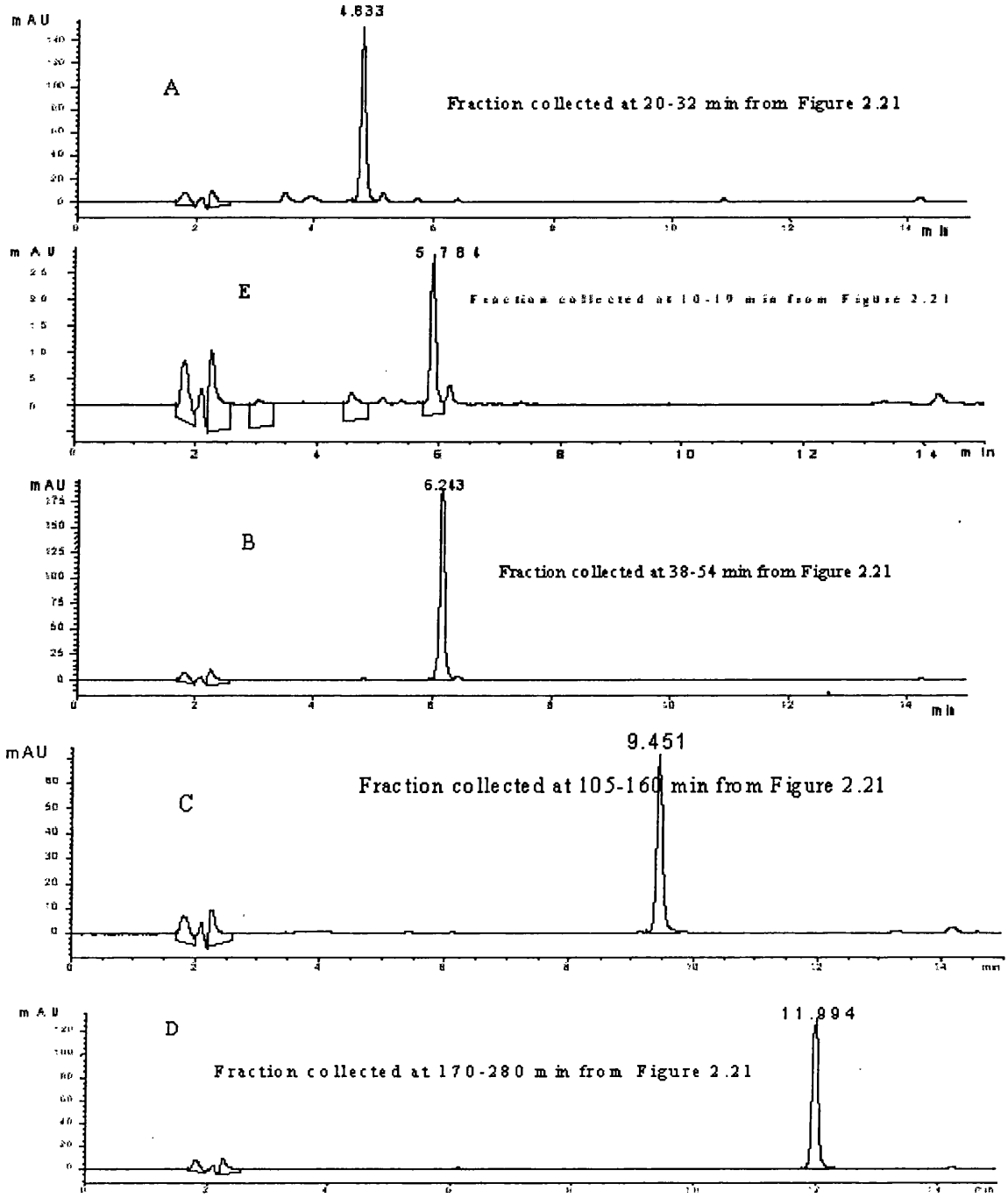


Figure 2.22 *HPLC analyses of all fractions of sample 3 after HSCCC separation*

(Chromatographic conditions see Figure 2.14)

However, after increasing the flow rate, components E, A and B could not be resolved very well. Figure 2.23 shows the separation results when changing the flow rate to 2.0 ml /min.

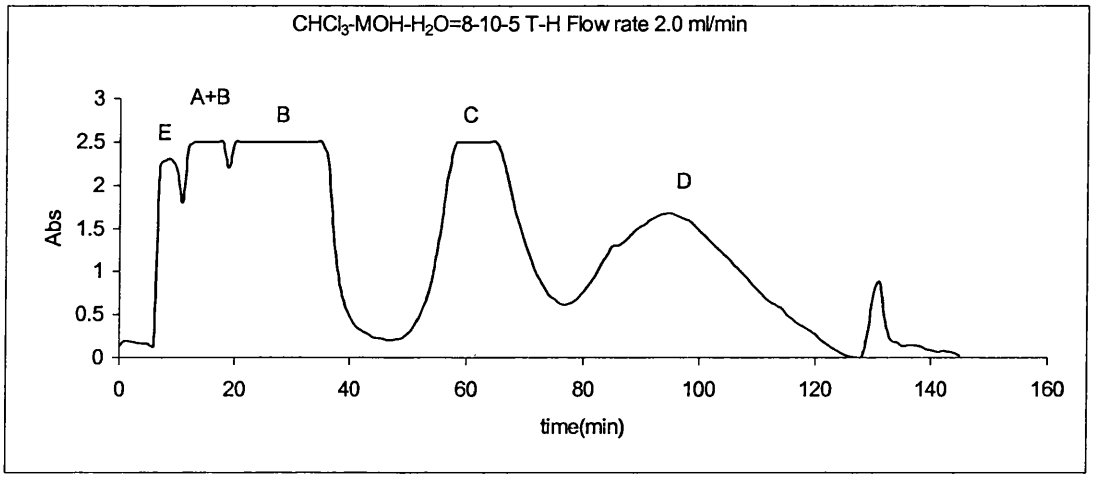


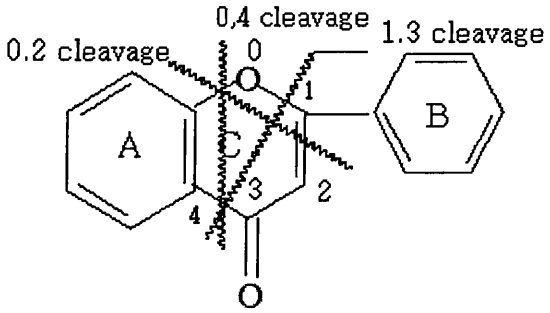
Figure 2.23 *Effect of the flow rate of elution on the separation of sample 3 by HSCCC*

Both H-E-M-W and chloroform-methanol-water solvent systems could be used for the separation and purification of the flavonoid aglycones and glycosides. The chloroform-methanol-water is a more suitable solvent system to the separation of flavonoid glycosides. Recently, this solvent system has not been widely used because of the environmental considerations. However, when the upper phase was used as mobile phase, not too much chloroform was consumed. In this case, this solvent system could be used.

From the solvent system of chloroform-methanol-water, five unknown compounds were obtained. Their purities were 96 %, 90 %, 85 %, 95 % and 98 %, respectively. LC/MS and NMR were used to identify all of these compounds.

2.11 IDENTIFICATION OF 5 COMPONENTS FROM SAMPLE 1, SAMPLE2 AND SAMPLE 3 AFTER HSCCC SEPARATION BY HPLC – ESIMS AND ESI MS/MS

Mass spectrometry has been successfully employed for the structure identification of flavonoids. Characteristic fragments in the mass spectra of flavonoids originate by fission of the $M^{+\cdot}$ ion into A- and B-ring derived fragments. These fragmentations usually involve one of the two competing pathways, I (Retro-Diels Alder) and II [43]. The aglycone type determines the dominant pathway.



Scheme *1* *Retro-Diels Alder*

In crude plant extracts, flavonoids are often present as O- or C-glycosides [30]. The O-glycosides have sugar components bonded to a hydroxyl group of the aglycone, whereas the C-glycosides have sugar components bonded to a carbon of the flavonoid aglycone, generally at position 6, 7 and 8. Contrary to flavonoid C-glycosides, the mass spectra of O-glycosides generate abundant aglycone ions by loss of a neutral mass of sugar and C-glycosides did not generate abundant alycone ions but instead gave characteristic ions of the fragmentation of the C-glycoside unit itself [31]. So, it is easy to differentiate between them by MS/MS spectra. Hence, ESI-MS and ESI-MS/MS can provide important structure information for flavonoids and can be of particular value in the determination of the nature and site of attachment of the sugar in O-glycosides.

2.11.1 Experimental

2.11.1.1. Instrumentation

HPLC system: Two LC systems were used, one was a Hewlett Packard 1050 LC system for determination of retention time of all components from samples 1, 2 and 3 and the other was a Hewlett Packard 1100 LC interfaced to a mass spectrometer. The UV detector was used at a

wavelength of 275.5 nm. The column used was Cosmosil C_{18} RP column (150 × 4.6 mm I.D Phenomenex). Mass spectrometer: A Finnigan Mat LCQ-Ion trap mass spectrometer was used for the identification of fractions from the HSCCC separation.

2.11.1.2 Calibration and tuning

Calibration on the Finnigan ESI source (LCQ) was carried out in the electrospray mode using the LCQ Tune solution. Tuning and calibration on the ESI system was performed using a solution containing 0.1 mg of caffeine, 25 nmol of MRFA and 0.05 % of Ultramark 1621 in 5 ml methanol-acetonitrile-water (25:50:25) containing 1.0 % acetic acid. Tuning and calibration were carried out under the Tune Plus window while infusing a tuning solution at a rate of 0.3 μl /min. The following masses 195, 524, 922, 1022, 1122, 1222, 1322, 1422, 1522, 1622, 1722, 1822, and 1922 were observed.

2.11. 2 Optimization of ESI parameters and chromatographic conditions

A Cosmosil C_{18} RP column (150 × 4.6mm I.D) was employed (Phenomenex) with temperature of 30°C, flow rate of 1.0 ml /min and wavelength of 275.5 nm. A gradient elution of formic acid 0.2 % (A) and 100 % acetonitrile (B) was employed: 0~10 min 80 % A and 20 % B; 10~12 min 55 % A, 45 % B; 12~20 min 20 % A, 80 % B.

A Hewlett Packard 1100 HPLC system was interfaced to a LCQ Ion Trap Mass Spectrometer (Finnigan MAT, San Jose, CA.USA). The ESI conditions were: Sheath gas flow rate: 60 arbitrary; Auxiliary gas flow rate: 10 arbitrary; Capillary temperature 220°C; Capillary voltage 10.0 V and tube lens offset 5.0 V.

2.11.3 Results and discussions

2.11.3.1 Identification of components 2, 3 and 4 from the sample 1 with extract method 1

Three fractions were eluted from sample 1 with H-E-M-W=1:1.2:1:1 in reverse phase elution [see Figure 2.7]. They were labelled components 2, 3 and 4. These fractions were identified by LC/MS and LC/MS/MS.

2.11.3.1.1 Identification of component 2 from sample 1 with HPLC/ESI/MS

Positive ESI and negative ESI could be used for the ionization of component 2 in sample 1. Figure 2.24 shows the chromatogram of component 2 of sample 1 with on-line positive HPLC/ESI/MS analysis.

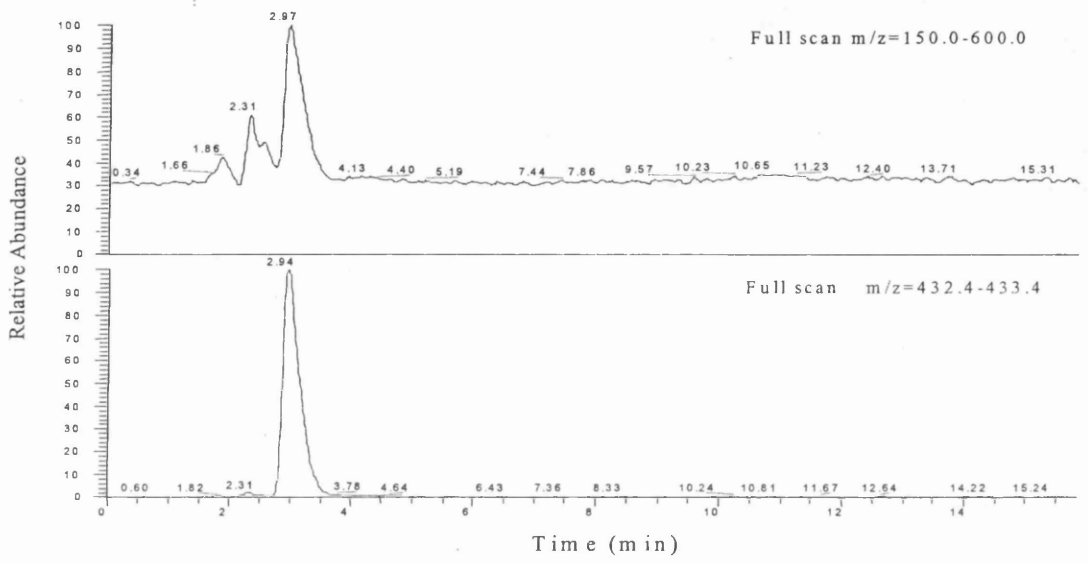


Figure 2.24 *Positive LC/ESI/MS chromatogram of component 2 of sample 1*

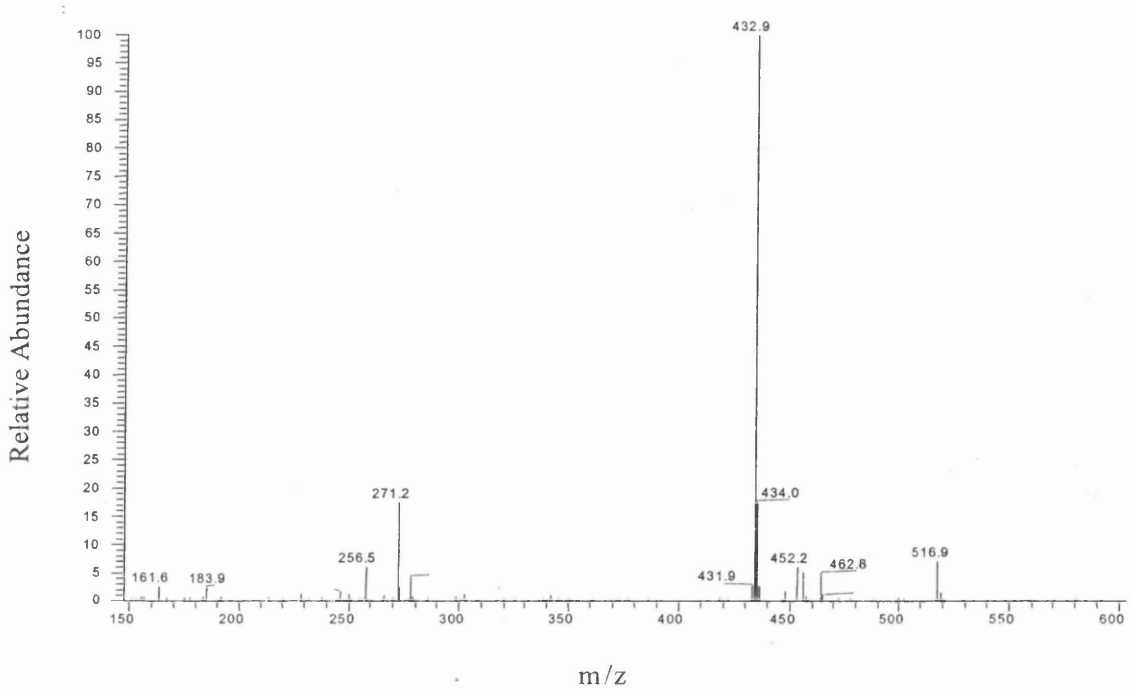


Figure 2.25 *Positive ESI mass spectrum of component 2 from the sample 1 after HSCCC*

Positive LC/ESI/MS full scan mass spectrum only gave a few fragment ions of the component 2. In order to get more structural information of the component 2, positive LC/ESI/MS/MS was used to identify the structure of component 2.

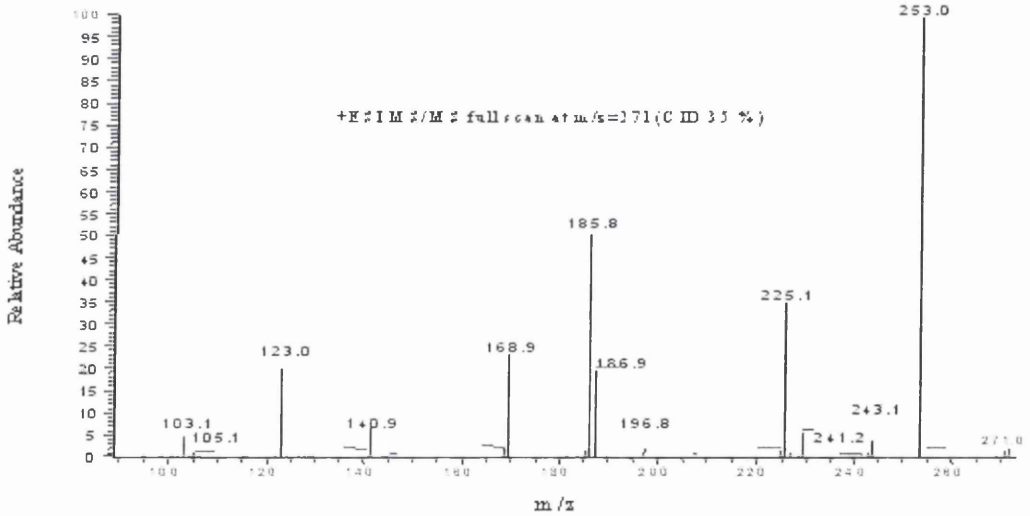


Figure 2.26 *Positive ESI/MS^2 mass spectrum of product ions at m/z=271 of component 2*

** CID=collision induced dissociation*

The mass spectrum of component 2 gave strong protonated molecule $[M+H]^+$ at m/z 433 and product ion of m/z 271 by loss of a neutral fragment of mass 162 Da in the +ESI full scan. This is not enough to identify the structure of component 2. The MS/MS spectrum of the ion at m/z 271 derived from the $[M+1]^+$ ion of component 2 exhibits five main diagnostic fragment ions at m/z = 253, 225, 169, 140.7 and 103. Fragment at m/z=169 shows the substitution of the A ring by 3 OH groups and fragment at m/z=103 shows no OH group in the B ring for component 2. Fragments of m/z=253 and m/z=225 represent typical loss of CO. Compound 2 was refluxed for 2 hrs at 90°C in 1.2 mol / ml HCl (methanol) solution and further evaporated to dryness using a rotary evaporator at a 35°C water bath. The residue was dissolved in methanol and then subjected to LC/MS/MS analysis. The hydrolyzed compound 2 gave the same retention time as that of standard baicalein. In order to further identify the structure of component 2, negative ESI/MS and ESI/MS^2 were also explored.

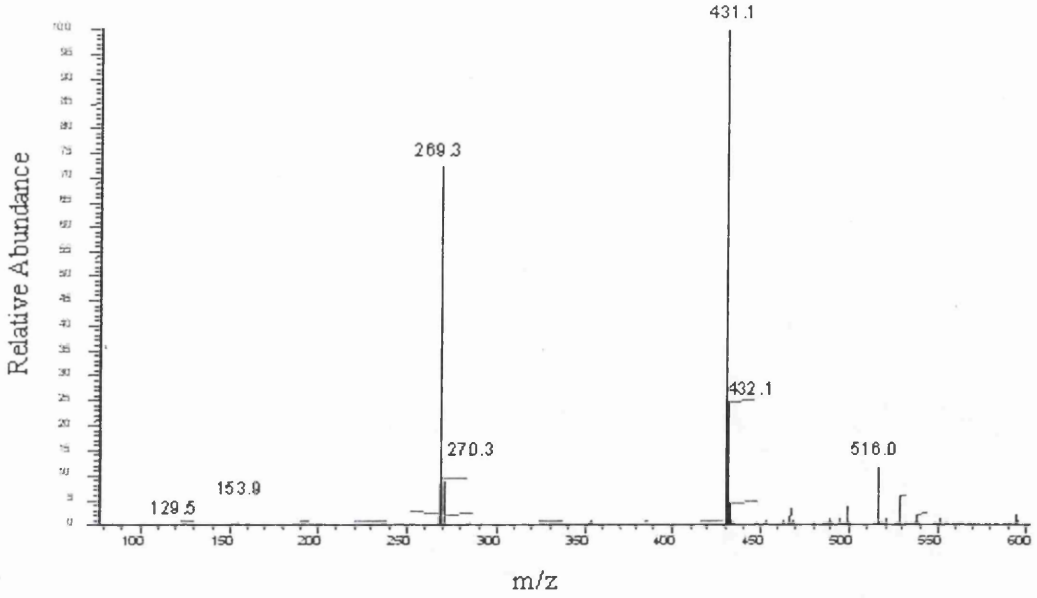


Figure 2.27 *Negative ESI/MS mass spectrum of component 2 from sample 1*

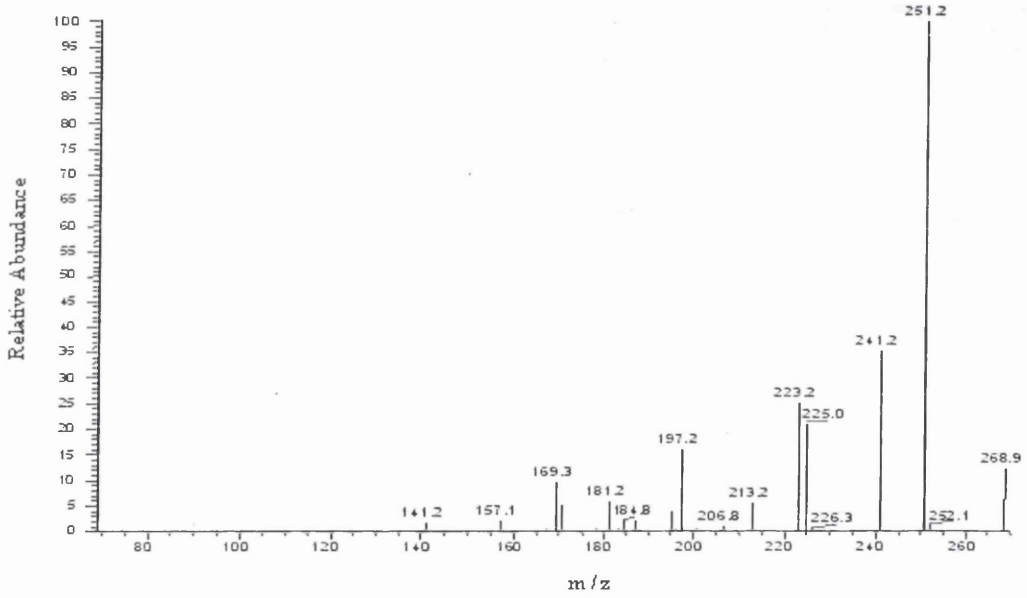


Figure 2.28 *The mass spectrum of negative ESI/MS^2 of m/z 269 (CID=40 %) of component 2*

When negative ESI was used for the component 2 in sample 1, the negative ESI/MS mass spectrum gave an intense flavonoid glycoside ion of m/z 431 of $[M-H]^-$ and its aglycone fragment ion of m/z 269 of [M-H-glucoside]$^-$ [Figure 2.27], the latter ion again corresponds to the $[M-H]^-$ ion of the standard baicalein. The aglycone m/z 269 of $[M-H]^-$ was more intense than that of m/z 271 of $[M+H]^+$ in positive ESI. Fragments of m/z 169 and m/z 251 were also observed. When the molecular ion of m/z 431 was selected as parent ion and the product ions were recorded in different collision induced dissociation (CID) [see Figures 2.30 and 2.31], the negative ESI/MS/MS mass spectrum showed the characteristics of [M-H-90]$^-$ and [M-H-120]$^-$ ions, due to

the cleavage of the O-glycoside moieties [32]. The presences of [M-H-90]$^-$ and [M-H-120]$^-$ ions indicate that a hexose glucoside is linked to the flavonoid. In view of the common occurrence of particular sugar moieties in flavonoids and in accordance to a previous report[33], the molecular mass of the component 2 was 432 and the component 2 is probably baicalein-7-O-glucoside. Figure 2.29 shows the structure of the component 2 from sample 1 and scheme 2.2 shows the proposed mechanism of fragmentation of component 2 from the sample 1 with extract method 1.

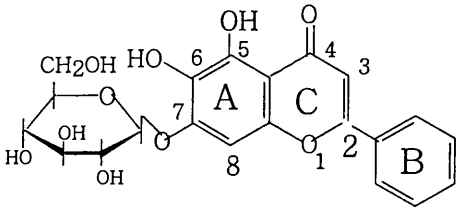
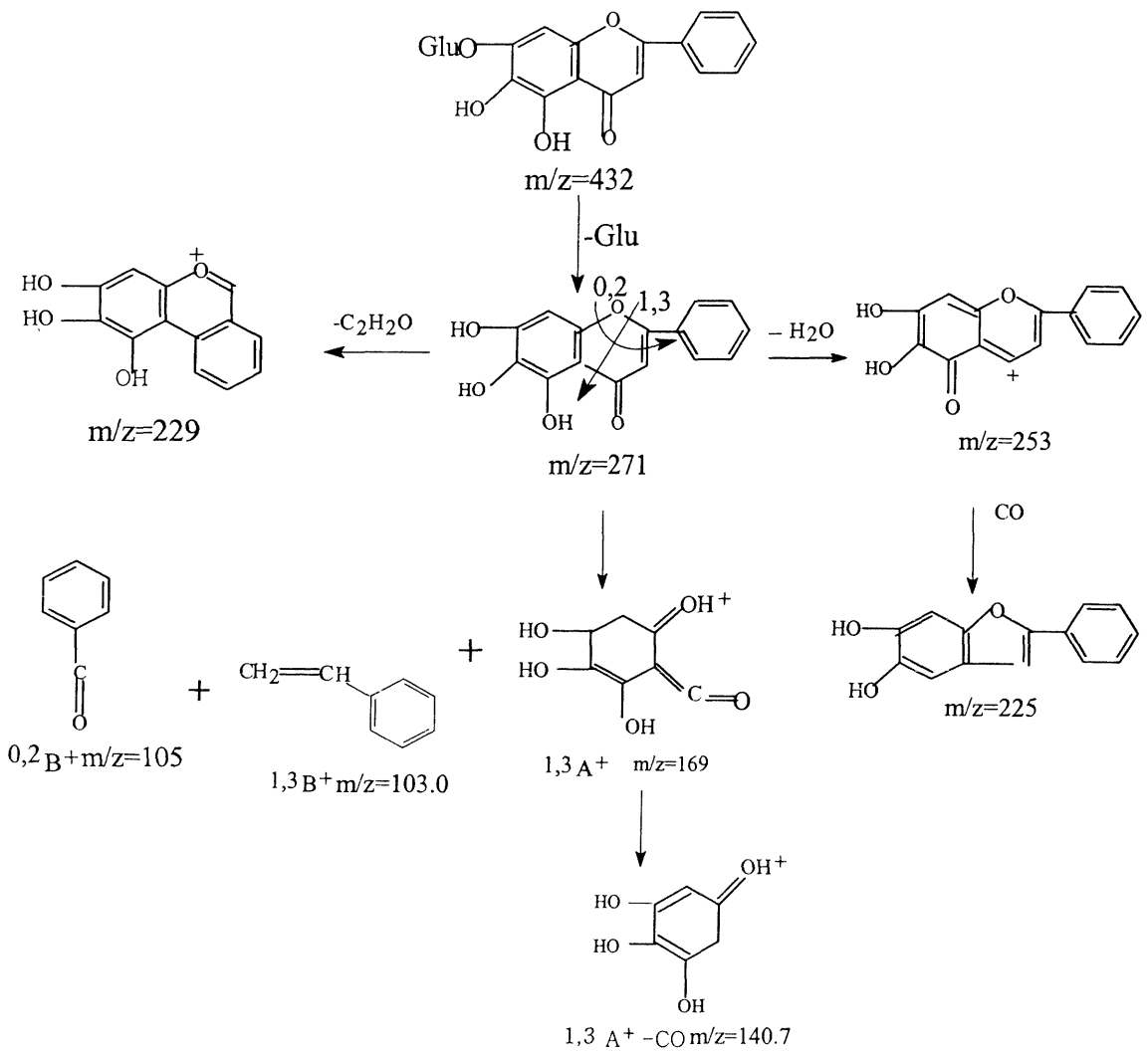


Figure 2.29 *The structure of the component 2 from sample 1*



Scheme 2.2 *Main fragmentations of protonated m/z=271 by positive ESI/MS*^{2}

For free flavonoid aglycone [13], the symbols ^{i,j}A and ^{i,j}B are used to designate primary fragment ions containing A- and B-rings, respectively. The superscripts i and j refer to the bonds of the C-ring that have been broken. These ions can lose small neutral fragments, such as H_2O and CO. These product ions are represented combining $^{i,j}A^+$ or ^{i,j}B with the lost fragments.

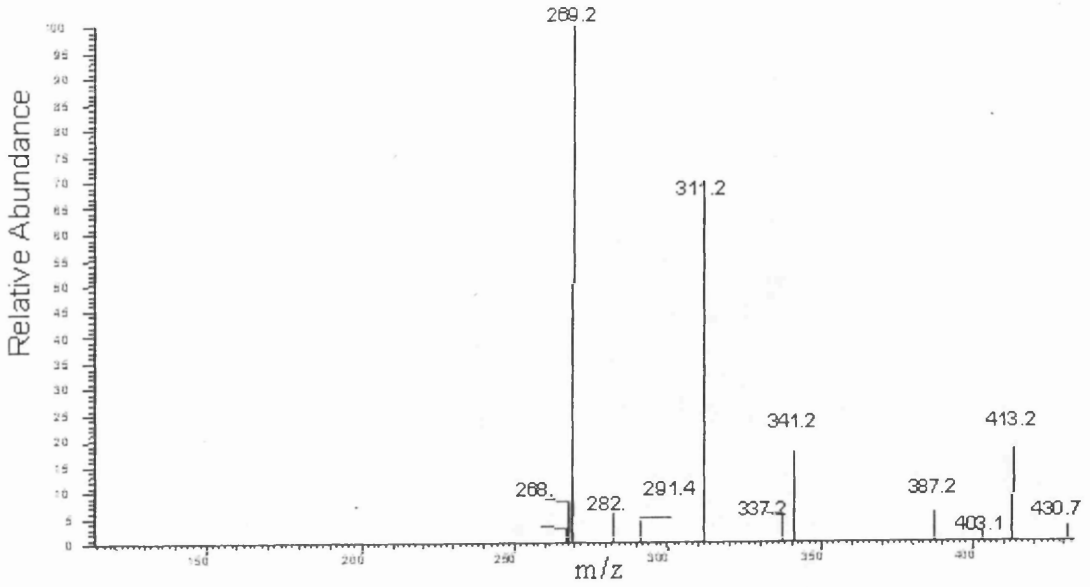


Figure 2.30 *Negative ESI/MS/MS mass spectrum of m/z 431 (CID=35 %) of component 2*

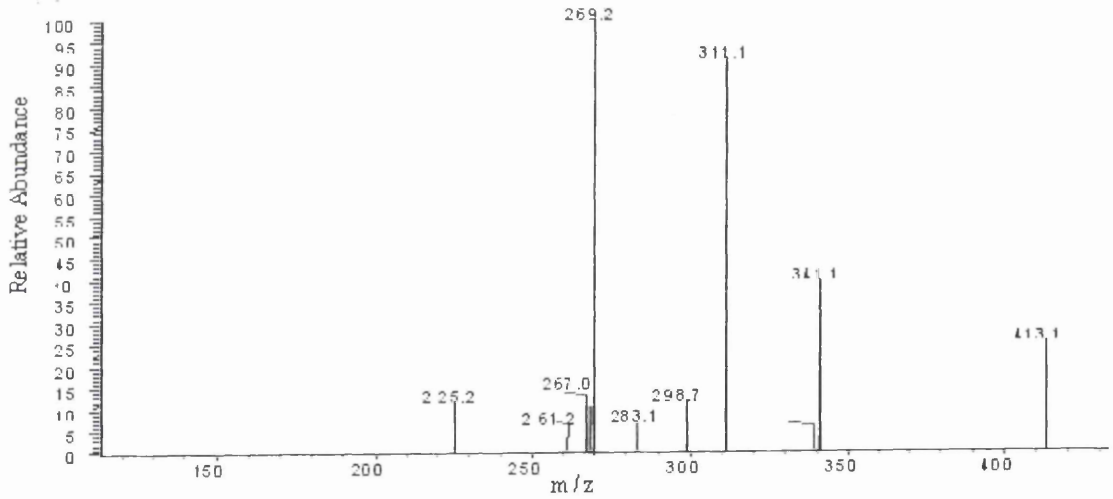
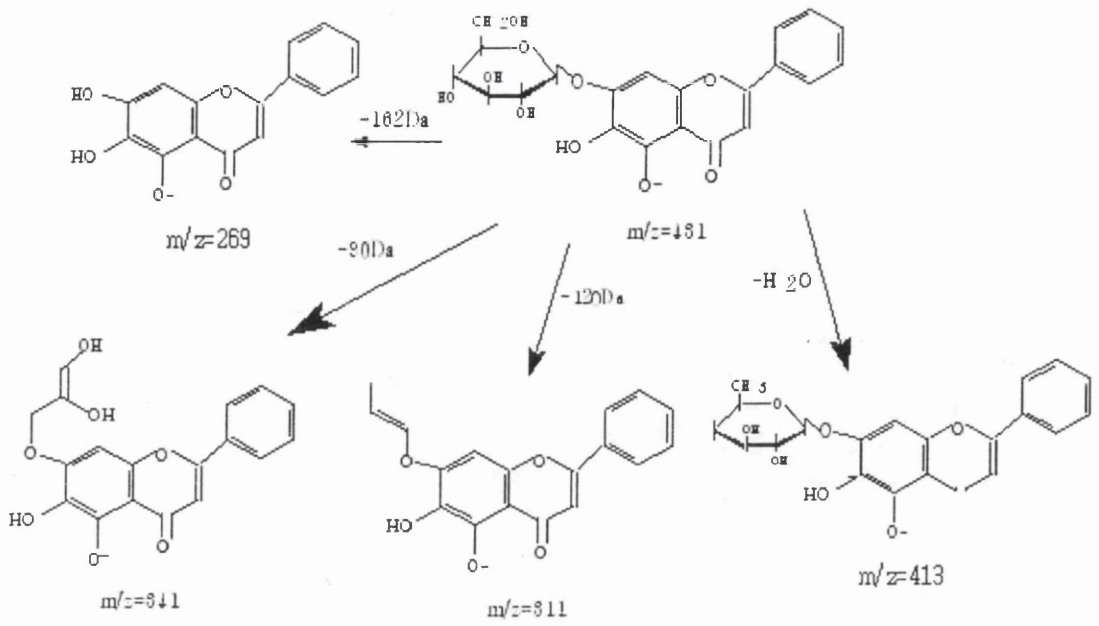


Figure 2.31 *Negative ESI/MS/MS mass spectrum of m/z 431 (CID=40 %) of component 2*



Scheme 2.3 *Main fragmentations of deprotonated m/z 431 by negative ESI/MS/MS*

In conclusion, component 2 of sample 1 from extract method 1 by HSCCC was positively identified as genistein-7-O-glucoside. Due to unavailability of a standard genistein-7-O-glucoside, UV and NMR were needed to further its identification.

2.11.3.1.2 Identification of component 3 from sample 1

Component 3 of sample 1 after HSCCC separation provided 98 % purity. A portion of component 3 was evaporated by dryness and redissolved into methanol and subjected to on line LC/MS analyses. The result is shown in Figure 2.32.

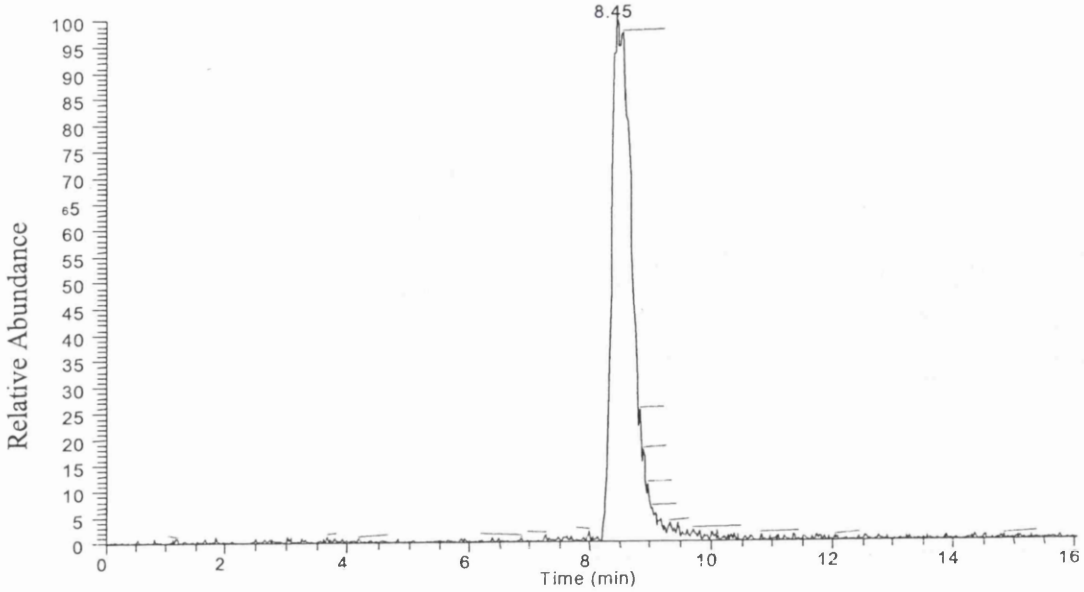


Figure 2.32 *TIC chromatogram of component 3 from sample 1 after HSCCC separation*

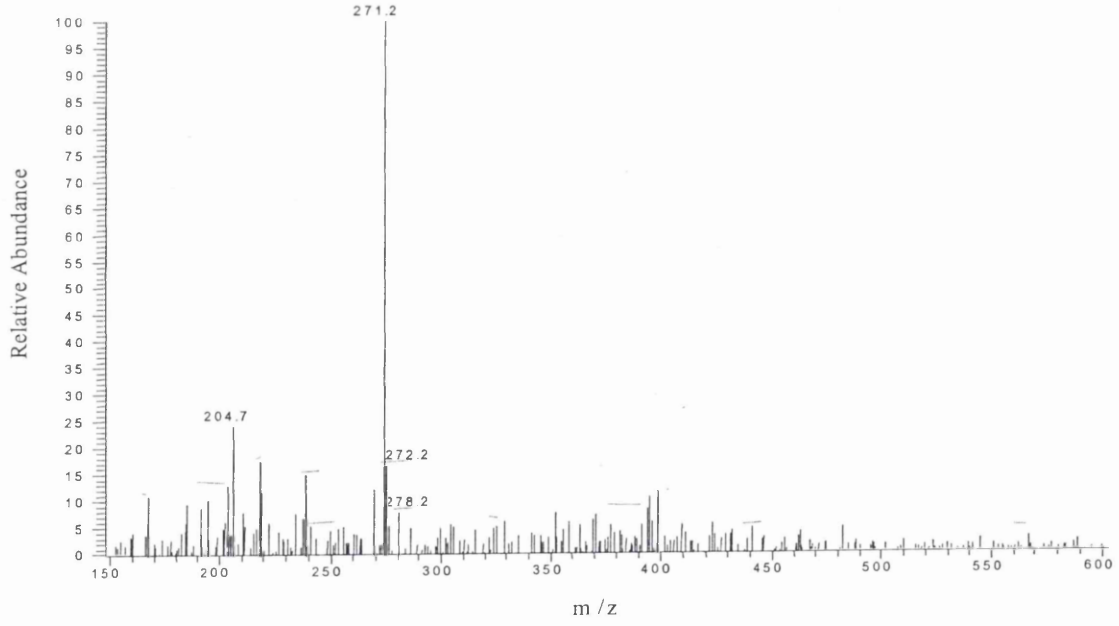


Figure 2.33 *Positive ESI /MS mass spectrum of component 3 from the sample 1 by HSCCC*

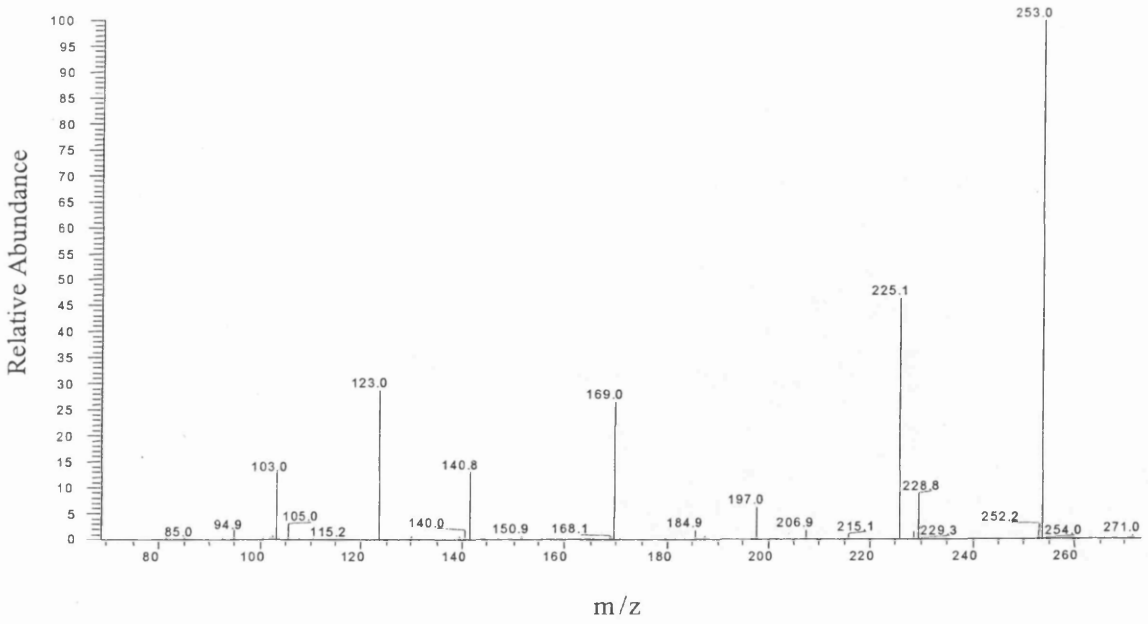


Figure 2.34 *Positive ESI/MS/MS spectrum at m/z 271 (CID=35 %) of component 3 of sample 1*

For component 3, the positive ESI /MS/MS spectrum from m/z = 271 has the same ions as those of component 2. The MS/MS spectrum in Figure 2.34 displayed exactly the same fragmentation patterns as the standard baicalein. HPLC retention time of component 3 also confirmed its identity as baicalein.

2.11.3.1.3 Identification of component 4 of sample 1 from the extraction method 1

Component 4 of sample 1 by HSCCC separation provided 96 % purity. Component 4 collected was evaporated by dryness and redissolved into methanol and subjected to on-line LC/MS analyses. The result is shown in Figure 2.35.

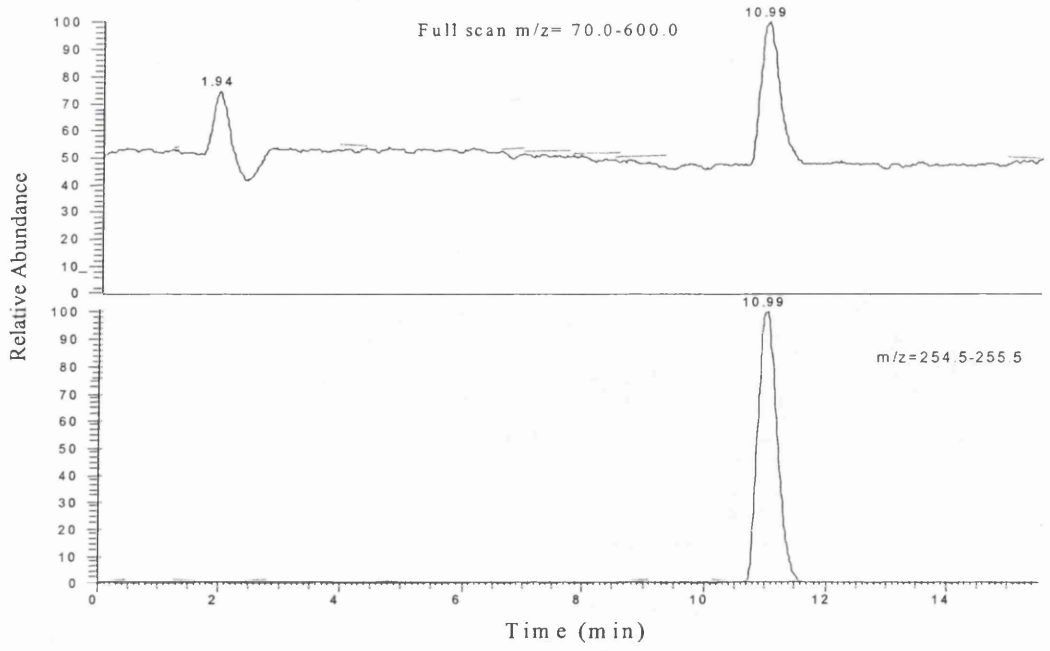


Figure 2.35 *TIC chromatogram of component 4 from sample 1 after HSCCC separation*

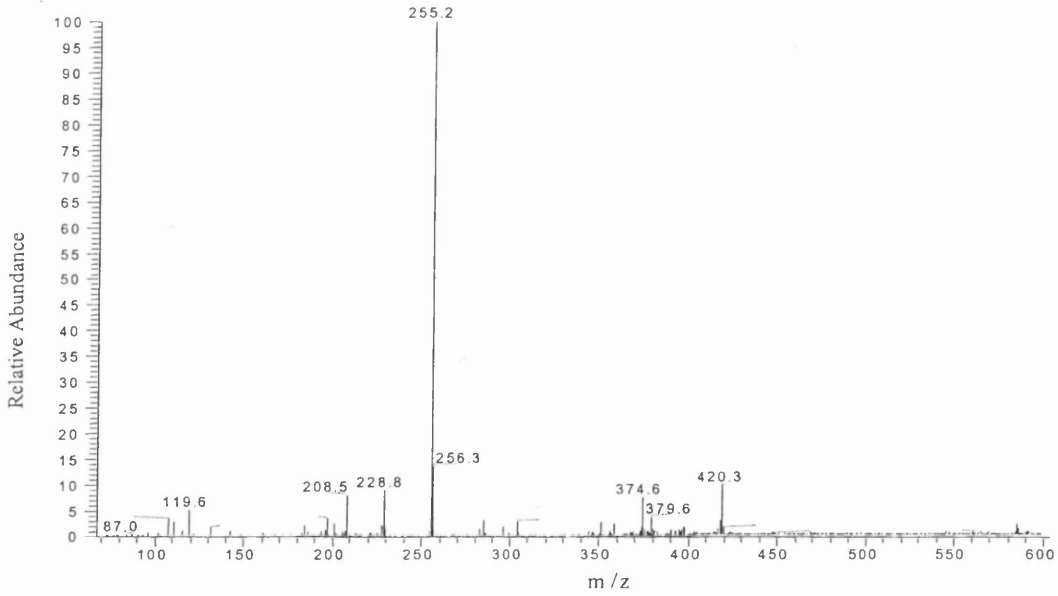


Figure 2.36 *Positive ESI/ MS spectrum of component 4 from the sample 1 by HSCCC*

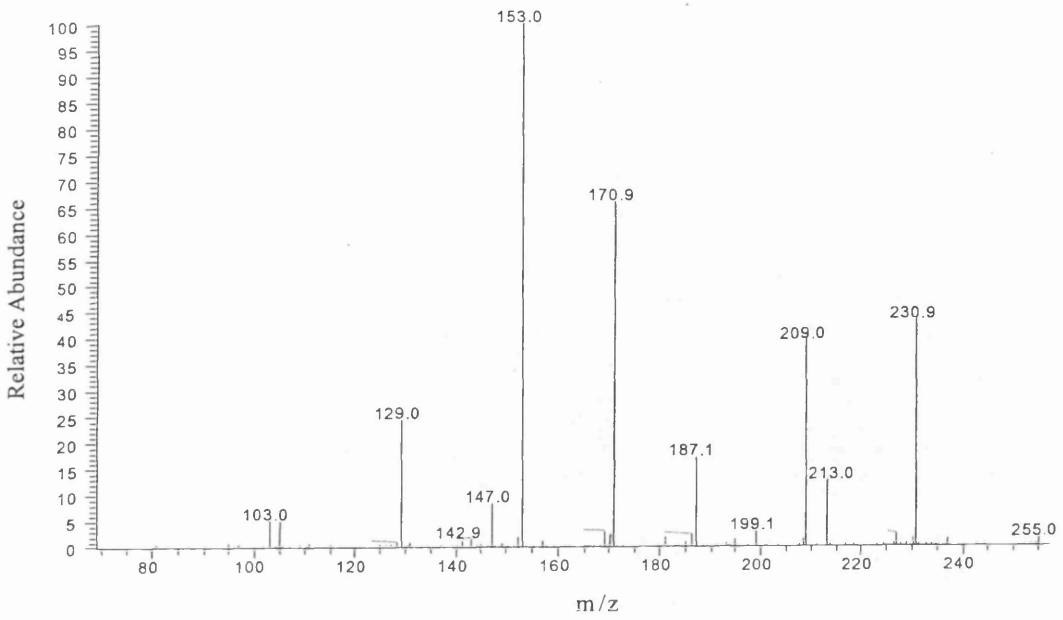
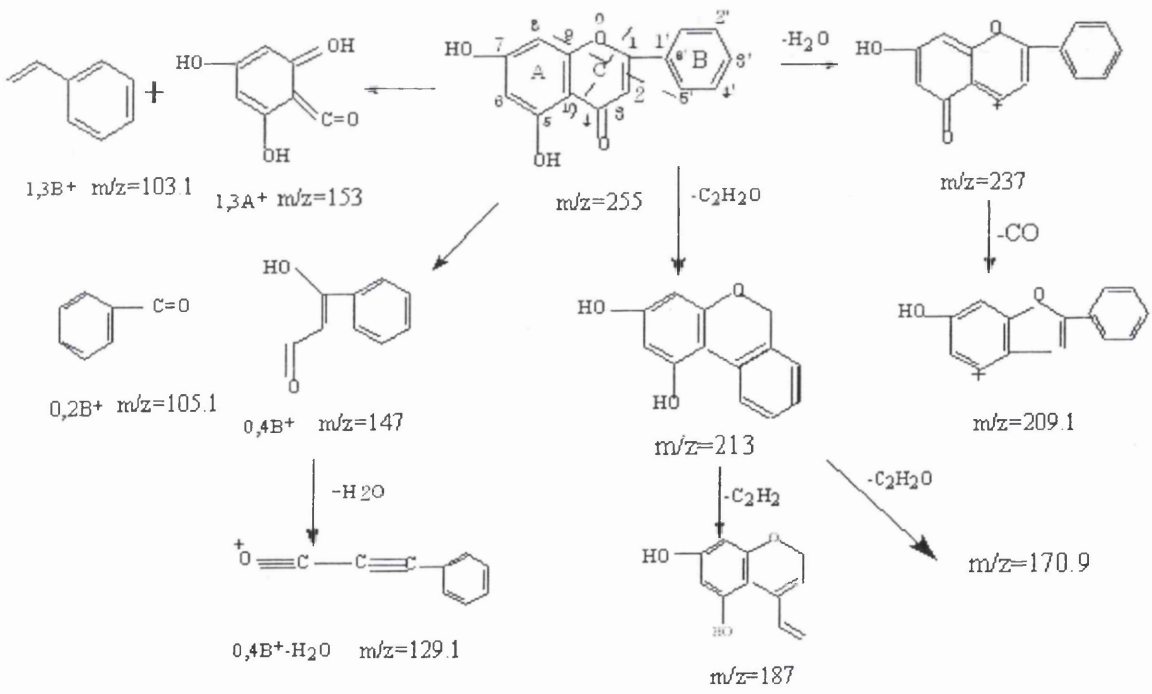


Figure 2.37 *Positive ESI/MS/MS spectrum at m/z 255 of component 4 from sample 1*



Scheme 2.4 *Proposed pathway of component 4 at m/z=255 by ESI/MS²*

Component 4 on electrospray mass spectrometry indicated an M+1 ion at m/z 255 and its proposed MS/MS fragmentation mechanism is shown in Scheme 2.4.

Comparison of component 4 with standard chrysin showed that component 4 gave exactly the same retention time and mass spectrum as those of chrysin. Hence, component 4 of sample 1 was confirmed as chrysin.

2.11.4 Identification of components of sample 2 from extraction method 2

In this sample 2, three fractions were eluted with chloroform-methanol-water=8: 10: 5 by HSCCC. These fractions were identified by HPLC/MS and HPLC/MS/MS.

2.11.4.1 Identification of fraction II in sample 2 with extraction method 2

Fraction II of sample 2 by HSCCC separation contained some impurity compounds and its purity was only 85 %. Figure 2.38 shows the HPLC/MS analysis.

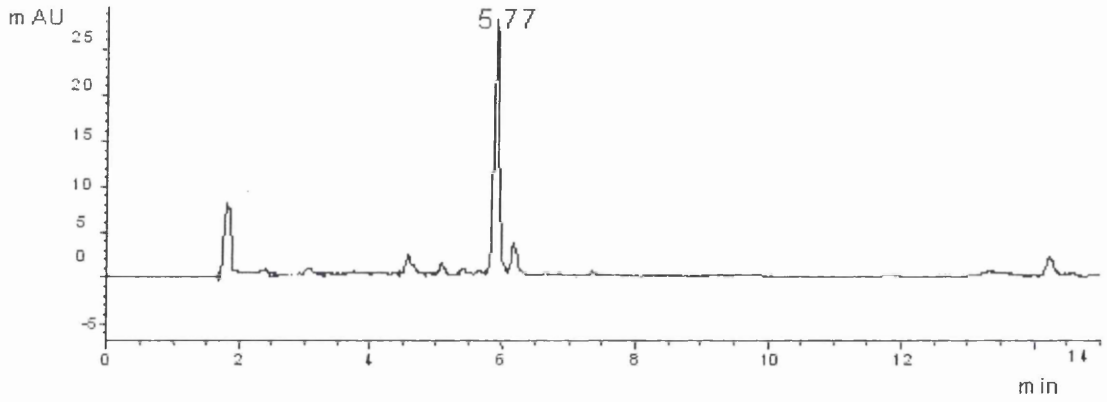


Figure 2.38 *HPLC analysis of fraction II from sample 2 after HSCCC separations*

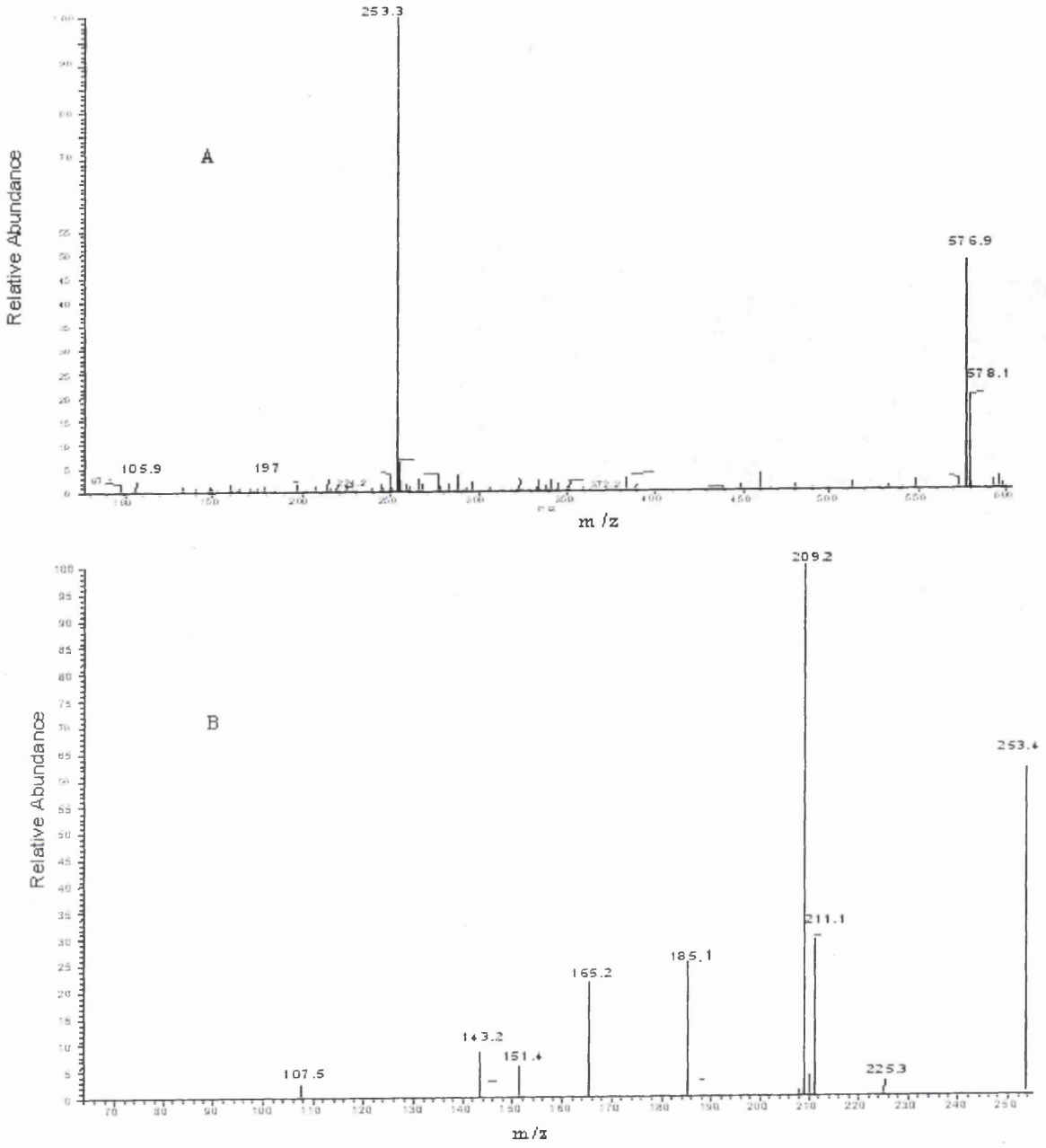


Figure 2.39 *Negative ESI/MS (A) and ESI/MS^2 (B) mass spectra of fraction II from the sample 2*

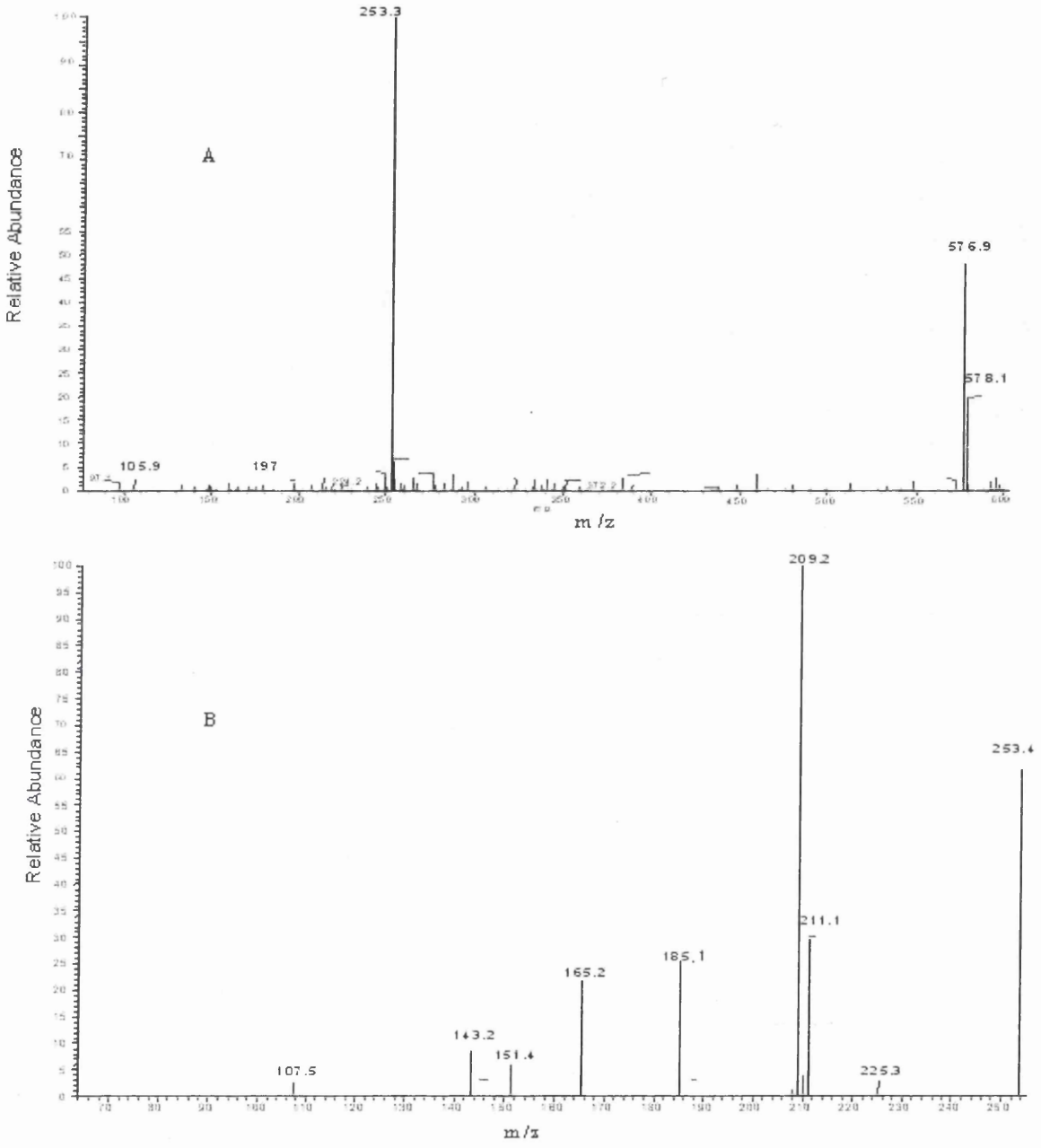


Figure 2.39 *Negative ESI/MS (A) and ESI/MS^2 (B) mass spectra of fraction II from the sample 2*

For fraction II, the $[M-H]^-$ molecular ion at m/z = 577 was very intense and the molecular weight of fraction II is 578. The ESI/MS spectrum of fraction II shows an intense aglycone ion $[A-H]^-$ at m/z =253 due to loss of a neutral fragment of mass 324 amu .The loss of 324 amu between these

peaks confirmed the presence of two hexose residues. The negative MS/MS mass spectrum of product ion of m/z=253 gave the same pattern as those of standard chrysin. According to the report of T. Tomimori [15], the major component of fraction II is possibly chrysin-7-O-diglucoside. However, due to the limited amount and impurity of component, this major component of fraction II has not been completely confirmed.

2.11.4.2 Identification of fraction I in sample 2 with extraction method 2

For fraction I in sample 2, the $[M-H]^-$ molecular ion at m/z 593 can be clearly observed, and the molecular mass of the major component of fraction I is 594. Its negative ESI/MS spectrum showed an intense aglycone ion $[A-H]^-$ at m/z 269 by loss of two neutral fragments of mass 324 amu. The loss of 324 amu between these peaks confirmed the presence of two hexose residues. The product MS/MS spectrum of the ion at m/z=269 exhibits five main diagnostic fragment ions m/z=251, 223, 197, 169 and 143. The fragment at m/z=169 shows the substitution of the A ring by 3 OH groups. In order to confirm this conclusion, fraction I was refluxed for 2 hours at 90°C in a 1.2 mol / ml HCl (methanol) solution and hydrolysed in order to give the corresponding aglycones. After evaporation to dryness using a rotary evaporator and a 35°C water bath, the residue was dissolved in methanol and then subjected to LC/MS/MS analysis. The hydrolysed fraction I gave the same retention time and fragment ions as those of standard baicalein. In view of the common occurrence of particular sugar moieties in flavonoids and the previous report [13], the major component of fraction I is baicalein-7-O-diglucoside. The UV spectrum of fraction I in methanol solution resembled that of standard baicalin and the use of diagnostic shifts suggested the absence of a free hydroxyl at the 7-position. In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of hydrolysed fraction I, the signals appeared at almost the same position as those of baicalein, indicating that the major component of fraction I is baicalein-7-O-diglucoside (Oroxylin B). After HSCCC separation, 92 % purity of baicalein-7-O-diglucoside was obtained. Figure 2.40 shows the LC/MS chromatogram of fraction I in sample 2 after HSCCC separation.

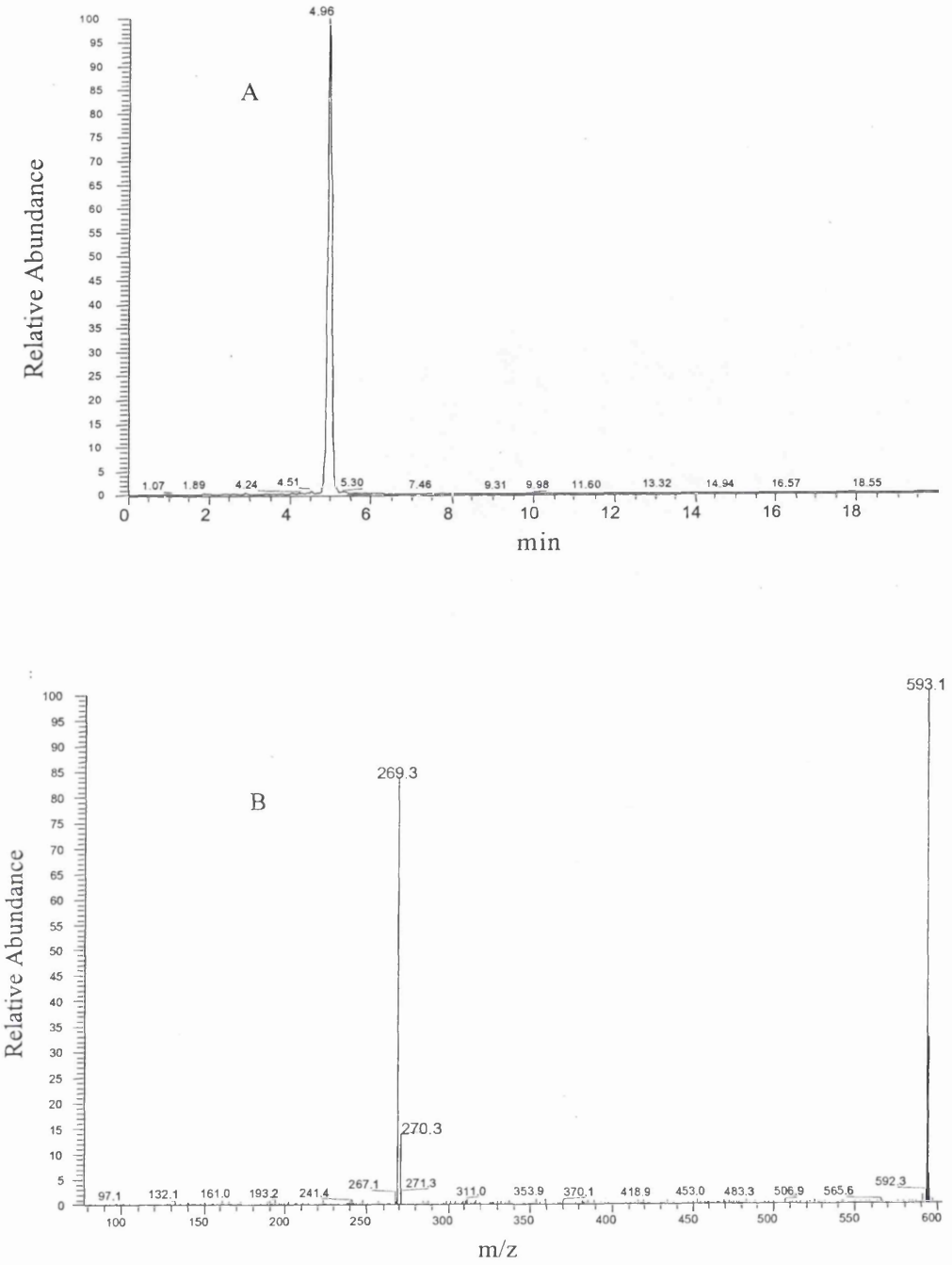


Figure 2.40 *TIC chromatogram (A) and -ESI mass spectrum (B) of fraction I of sample 2*

2.11.4.3 Identification of fraction III in sample 2 with extraction method 2

In fraction III of sample 2, the purity of major component of fraction III was 95 %. It shared the same retention time and mass spectrum as that of component 2 of sample 1. Hence, fraction III of sample 2 was confirmed as genistein-7-O-glucoside.

In conclusion: for sample 2 from extract method 2, fraction I was confirmed as baicalein-7-O-diglucoside and fraction II was confirmed as chrysin-7-O-diglucoside. Fraction III was the same substance as component 2 of sample 1 and was confirmed as baicalein-7-O-glucoside.

2.11.5 Identification of five fractions by HSCCC from the sample 3 with extraction method 3

Five fractions could be resolved from the sample 3 with extraction method 3. These fractions were labelled components A, B, C, D, E. HPLC analysis indicated that five fractions of sample 3 from extraction method 3 shared the same retention times as those of sample 1 and sample 2. These results show that 2-butanol is a strong extraction solvent and all flavonoid aglycone and glycosides are extracted from the seeds of *O indicum.*

Component A from sample 3 gave the same retention time and mass spectra as that of fraction III from sample 2 and it was identified as baicalein-7-O-glucoside. Component B gave the same MS and MS/MS mass spectra as that of fraction I from sample 2, hence, component B was identified as baicalein-7-O-diglucoside. Component C from sample 3 gave the same retention time and mass spectra as component 3 from sample 1. It was identified as baicalein by comparing it with standard baicalein. Component D was identified as chrysin by comparing it with standard chrysin and component E was possibly identified as chrysin-7-O-diglucoside.

Table 2.6 *Negative ESI-MS^n spectra (m/z value) of five components of sample 3*

	Components	Mr	t_R	$[M-H]^-$	MS^n data of compounds
					593 MS^2 269 MS^3
Sample	Component A	594	4.786	593	251,241,225,197,169,141
	Component E	578	5.861	577	577,253MS^2 235, 151,145,127,105
					431$MS^2$311$MS^3$269MS^4
	Component B	432	6.114	431	251,241,225,197,169,141
	Component C	270	9.420	269	269$MS^2$251, 241,225,197,169,141
	Component D	254	11.950	253	253MS^2 235, 151,145,127,105
Standard	chrysin	254	11.948	253	253$MS^2$235, 151,145,127,105
	Baicalein	270	9.413	269	269$MS^2$251, 241,225,197,169,141
					445,269 MS^2 269 MS^3 251,
	Baicalin	446	6.231	445	241,225,197,169,141

Experimental conditions: Spray voltage 3.5 kV, Capillary temperature 200°C; the isolation width for MS^n 1.0 Da: and the collision energy (%) was as follows: for MS^2 40 %; MS^3: 45 %.

Five components were identified from sample 1, sample 2 and sample 3. All these components were successfully separated and purified by HSCC. Our study showed that HSCCC is a strong separation technology especially in the isolation and purification bioactive components from natural plants.

2.12 IDENTIFICATION OF FOUR COMPONENTS BY ^{1}H NMR AND ^{13}C NMR

Component A ($C_{27}H_{30}O_{15}$) is yellow needles. It gave baicalein on acid hydrolysis. In the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum component A, the signals caused by the aglycone moiety appeared at almost the same position as those of baicalin (baicalein-7-O-glucuronide), indicating that component A is baicalein-7-O-diglucoside (Oroxylin B).

Component B ($C_{21}H_{20}O_{10}$) is a yellow powder. Component B was hydrolyzed with 10 % HCl-MeOH under reflux on a hot bath for 3 hr. The mixture was cooled, and the deposited aglycone was collected and recrystallized from MeOH to give yellow powder, which found to be identical with baicalein by direct comparison of their retention times and mass spectra. ^{1}H-NMR (DMSO-d6): δ ppm: 8.49 (1 H, s, H-6), 7.9 -8.0 (2 H, d, J=8.0 Hz, H-2', 6'), 7.4-7.5(3 H, m, H-3', 4', 5'), 6.95 (1 H, s, H-8), 6.91(1 H, s, H-3), 12.46 (1 H, s, H-5). ^{13}C- NMR (DMSO-d6): 182.9 (C-4), 163.8 (C-2), 151.9 (C-9), 149.6 (C-7), 146.8 (C-5), 132.4 (C-4'), 131.2 (C-6), 130.9 (C-1'), 129.5 (C-3', 5'), 126.7 (C-2', 6'), 106.4 (C-10), 105.1 (C-3), 101.2 (C-1"), 94.6 (C-8), 77.7 (C-3"), 76.2 (C-5"), 73.5 (C-2"), 70.0 (C-4"), 60.98 (C-6"). Hence, component B was identified as baicalein-7-O-glucoside.

Component C ($C_{15}H_{10}O_5$) is a pale yellow powder. ^{1}H- NMR (CD_3OD, TMS) δ ppm: 7.8 (2 H, H-2', H-6'); 7.3(3 H, H-3', H-4', H-5'); 6.5 (1 H, H-3) 6.4 (1 H, H-8); ^{13}C-NMR (DMSO-d_6) δ ppm: 95.45 (C-8), 105.75 (C-10), 106.24 (C-3), 127.77 (C-2', C-6') 130.61 (C-3', C-5'), 131.15 (C-6,) 133.32 (C-4'), 133.13 (C-1'), 148.30 (C-9), 152.56 (C-5), 155.55 (C-2), 165.98 (C-7), 184.64 (C-4). Component C was identified as baicalein by direct comparison their retention times and mass spectra with reference standard.

For component D, its ^{1}H NMR (CD_3CD, TMS) δ ppm: 7.9 (2 H, H-2', H-6'); 7.5(3 H, H-3', H-4', H-5'); 6.4 (1 H, H-3) 6.5 (1 H, H-8); 6.1 (1H, H-6) 13C-NMR(CD_3OD) δ ppm: 95.45 (C-8), 99.27 (C-6) 104.31 (C-10), 104.90 (C-3), 126.30 (C-2', C-6') 129.10 (C-3', C-5'), 131.43 (C-1',) 131.93 (C-4'), 158.44 (C-9), 162.16 (C-5), 164.50 (C-2), 165.57 (C-7), 182.74 (C-4). Its ^{13}C-NMR and ^{1}H-NMR signals were completely the same as those of standard chrysin.

Table 2.7 The signals of ^{13}C- NMR spectra of compound A-D and standard flavonoids

^{13}C NMR signals	Component A	Component B	Component C	Component D	chrysin	baicalein
C-2	163.9	163.8	155.5	164.8	164.5	155.5
C-3	104.8	105.1	106.2	104.9	104.9	106.2
C-4	183	182.9	184.6	182.7	182.7	184.6
C-5	146.7	146.8	152.6	162.1	162.1	152.6
C-6	131.1	131.1	131.2	99.27	99.27	131.2
C-7	149.6	149.8	165.9	165.6	165.6	165.9
C-8	94.7	94.6	95.48	95.45	95.45	95.48
C-9	151.8	151.9	148.3	158.4	158.4	148.3
C-10	106.4	106.4	106	104.3	104.3	106
C-1'	130.8	130.8	133.1	131.4	131.4	133.1
C-2',6'	126.7	126.7	127.7	126.3	126.3	127.7
C-3',5	129.5	129.5	130.6	129.1	129.1	130.6
C-4'	132.2	132.4	133.3	131.9	131.9	133.3
C-1"	162.7	101.2				
C-2"	73.4	73.5				
C-3"	77.1	77.7				
C-4"	70.5	70.6				
C-5"	75.9	76.2				
C-6"	61.4	60.98				
C-1"'	103.8					
C-2"'	73.7					
C-3"'	75.9					
C-4"'	69.8					
C-5"'	77.3					
C-6"'	61.4					

2.13 CONCLUSION

Our study demonstrates that high speed CCC is a valuable method for separating, purifying and identifying bioactive components from natural products. Using HSCCC, five flavonoids, chrysin-7-O-diglucoside, baicalein-7-O-diglucoside, baicalein-7-O-glucoside, baicalein and chrysin were separated and purified from the O, indicum seeds with two two-phase solvent systems comprising of chloroform-methanol-water=8:10:5 and hexane-ethyl acetate-methanol-water. Compared to H-

E-M-W, chloroform-methanol-water solvent system is more suitable for the isolation and purification of polar flavonoids from natural plant material. The author recommends H-E-M-W for the isolation and purification of flavonoids because of environment considerations. From 200 mg crude extract, 50 mg baicalein-7-O-glucoside, 10.5 mg baicalein-7-O-diglucoside, 4.5 mg chrysin-7-O-diglucoside, 25 mg baicalein and 45 mg chrysin were be obtained in a run repeated ten times.

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CHAPTER 3

HSCCC/MS study of Flavonoids and Isoflavones in Extracts from Plant Materials

3.1 THE BACKGROUND TO HSCCC/MS

Over the past 20 years, countercurrent chromatography (CCC), which totally eliminates the use of a solid support, has been developing. Countercurrent chromatography technology has advanced in various directions including preparative and trace analysis. During the preceding decade, CCC has been used mostly for preparative scale separation of natural products. However, there are few papers reported on HSCCC/MS. HSCCC coupling to a mass spectrometer was not widely used due to following reasons: 1) HSCCC/MS will result in a large amount of stationary phase being lost and a high baseline noise because high back pressure will arise after interfacing the two instruments; 2) The solvent system usually used in HSCCC would not give good sensitivity if no modified solution was added to help ionization of target components; 3) Most CCC instruments were of preparative scale and therefore unsuitable for lower flow rates required by a mass spectrometer. However, the benefits offered by coupling a mass spectrometer with many analytical instruments play a very important role in the identification and determination of analytes in a complex sample matrix. Various methods coupled with mass spectrometry such as thin-layer chromatography-MS [1], high–performance liquid chromatography/MS [2,3], supercritical fluid chromatography/MS [4] and capillary electrophoresis/MS [5] have been developed and they are useful tools in the study of natural products and in many other aspects of analytical chemistry. However, all of them use solid supports as a stationary phase except capillary electrophoresis-MS, so they do not eliminate various complications arising from the use of solid supports.

HSCCC is a unique liquid-liquid partition technique that does not require the use of a solid support. All components can remain in the separation process and this technique separates components within a mixture according to their different partition coefficients. Normally, on line detection in HSCCC has been almost entirely performed with a UV-VIS absorbance monitor [6]. On many occasions, this kind of monitor results in too much noise because of the carryover of the droplets of the stationary phase during the elution procedure. In addition, a slight change in temperature may cause noise signals from the effluent. Various other detection methods have been reported that include post column reaction [1], laser light scattering detection [2, 3], and HSCCC/FTIR [4, 5]. Most of these detection methods are much less sensitive to the carryover of the stationary phase and, therefore, will improve the quality of tracing the elution curves in HSCCC.

Mass spectrometry is a rapid detection method that lends itself well to many types of chromatography because of its high sensitivity and small amount of sample used. It is important to interface HSCCC with mass spectrometry because it combines the advantage of HSCCC with the low detection limit and identification capability of MS. Recently, Considerable effort has recently been made to HSCCC analytical instruments in order to decrease the flow rate and coil volume for the purpose of coupling HSCCC/MS. CCC/TSP/MS has been successfully applied to the separation of natural products including the analyses of alkaloids, triterponic acids and lignans [7, 8, 9]. Ito developed a HSCCC interface with Frit CI/MS for analyses of indole auxins [10]. This HSCCC column I. D. was 0.3 mm and the column capacity was 6 ml. A new analytical HSCCC has been designed by the Brunel Institute for Bioengineering, which is called Milli CCC. This Milli-CCC J-type apparatus has gears enclosed in a lubricated case to minimize noise. Its volume with one coil mounted in a cantilever style is 4.6 ml with 2.5 m of 0.76 mm bore tubing. It can rotate at a maximum speed of 2100 rpm. Coupling this machine to a mass spectrometer is straightforward [11] and gives separation times ranging from 2.5 to 20 min for flows ranging from 2 ml / min to 0.25 ml / min. In this study, HSCCC/MS has been explored for the separation and identification of flavonoids and isoflavone mixtures by two HSCCC instruments.

3.2 CHARACTERISTICS OF HSCCC/MS

The desired performance of a HSCCC/MS interface is as follows:

1). High enrichment of sample in ion sources.

2). High yield of sample reaching the mass spectrometer.

3). It provides good sample resolution

4). It has a low backpressure to preserve the PTFE flying leads

5). No peak broadening

6). Applicability to non-volatile samples.

When HSCCC is directly interfaced with mass spectrometry, the HSCCC column often breaks due to the high backpressure generated by the interfacing between CCC and the mass spectrometer. Previous work on CCC/MS systems has mostly focussed on the reduced pressure ionization techniques such as frit electron ionization (EI), chemical ionization and fast-atom bombardment (FAB). Some ionization techniques like TSP have reduced this constraint somewhat,

but backpressure can often cause rupturing of the coil itself as it is commonly made from PTFE tubing. With the development of the CCC technique to the analytical scale, the development of stainless steel, high-pressure coils provides an answer to this problem and allows the use of ESI and APCI/MS [16]. Hence, the CCC/ESI/MS and CCC/APCI/MS provide a convenient interface method at atmospheric pressure, suitable for analytical scale separations. In this study, two HSCCC instruments interfaced directly with ESI and APCI mass spectrometry were explored for the separation of mixtures of four standard flavonoids mixture, six standard isoflavones and were also successfully used for the isolation and identification the flavonoids in an ethyl acetate extract from the seeds of *O indicum.*

3.3 EXPERIMENTAL

3.3.1 Solvent and reagents

HPLC grade solvents used in this study included hexane, ethyl acetate, methanol and water. All these solvents were purchased from Fisher Chemicals (Loughborough, UK). AnalR grade formic acid and acetic acid were also used from the same supplier. All solvents were filtered using a vacuum filtration system (Millipore) through a 0.45 μm nylon membrane filter (Alltech Associates Inc, Lancashire, UK), and degassed prior to use. Gases used included oxygen free nitrogen (OFN), helium and air, which were purchased from BOC Ltd. (Surrey, UK).

Standard baicalein-7-O-glucoside was isolated by our lab and checked for purity by UV and NMR, baicalein and chrysin were purchased from SIGMA Company. Standard daidzin, genistin, glycitein, daidzein, genistein, formonetin and biochaninA were purchased from Indofine (Somerville, NJ, USA).

3.3.2 Instruments

Two HSCCC instruments were studied: one was a prototype J-type coil planet centrifuge HSCCC and the other was J-type Milli CCC apparatus. The first was assembled with two identical bobbins; each with 0.8 mm bore tubing connected in series to provide a total coil volume of 25 ml with a β value of 0.88. This machine is designed to rotate at speed up to 1400 rpm. In this study, the speed up to 1100 rpm was used. The J-type Milli-CCC apparatus has gears enclosed in a lubricated case

to minimize noise and its volume with one coil mounted in a cantilever style is 4.6 ml with 2.5 m of 0.76 mm bore tubing. Both instruments were provided by Brunel Institute for Bioengineering, Brunel University.

Mass spectrometer: A Finnigan AQA mass spectrometer (Thermoquest) was used and this instrument can provide ESI and APCI spectra.

3.3.3 Sample preparation

Standard mixtures of flavonoids and isoflavones were dissolved in methanol (90 %). 50 g of seeds of O. indicum were refluxed for 5 hours in 90 % methanol, the extracts were then filtered and evaporated .The residue was redissolved in 200 ml H_2O and extracted three times with ethyl acetate. Final evaporation yielded 6.5 g of a yellow powder.

3.3.4 HSCCC method for HSCCC instrument

Biphasic mixtures of hexane-ethyl acetate- methanol- water were prepared in the different ratios and purged for 30 minutes with nitrogen to remove any dissolved gases. First, the coil was filled with the upper phase (organic layer) of the biphasic mixture. The coils were rotated in a forward direction at a speed of 1100 rpm and the lower phase (aqueous layer) was pumped into the coil from head to tail at a flow rate of 1.0 ml/minute. When the two layers were observed, the equilibration point was determined when no more stationary phase was eluted (hydrodynamic equilibration). The displaced volume (V_{dis}) could then be calculated by subtracting the volume of the stationary phase retained in the coil at the end of the equilibration process from the coil volume and the extra volume. The extra volume was calculated according to the Wood plot [12] and is described in Chapter 2. When the extra volume was determined, the active mobile phase volume in the coil (V_m) and retained volume of stationary phase (Vs) can be calculated.

3.3.4.1 Measurement of partition coefficient

The different ratios of hexane-ethyl acetate-methanol-water were prepared in 50 ml tubes. After

equilibration through shaking for 5 minutes, approx 1 mg of the test sample was weighted in a 5 ml test tube to which 2 ml each of the equilibrated two-phase solvent system was added. The test tube was stoppered and shaken vigorously for 1 min to thoroughly equilibrate the sample into two phases. Then, accurate equal volumes (100 μl) of the upper and lower phases were pipetted out with a micropipette into 1 ml of appendix. After evaporating the solvent with a rotary evaporator to dryness, the residues were diluted with methanol to 1 ml and analysed by HPLC/MS. The partition coefficients were calculated by the peak area of the upper phase divided by the peak area of the lower phase of unknown components. Table 3.1 shows the partition coefficients of these compounds.

Table 3.1 *Partition coefficients in H-E-M-W solvent system with varying ratio of ethyl acetate*

Solvent system H-E-M-W	Baicalein-7-o-glucoside	Baicalein	Chrysin
1:0.4:1:1	0.041	0.023	1.181
1:0.8:1:1	0.065	0.04	2.028
1:1.2:1:1	0.121	0.06	3.068
1:1.6:1:1	0.184	0.114	4.786
1:2:1:1	0.285	0.078	3.459

3.3.4.2 HSCCC interfacing with a mass spectrometer

Analytical HSCCC can be interfaced directly with ESI/MS or APCI/MS without additional pumps between the mass spectrometer and the HSCCC. When ESI/MS is interfaced with HSCCC, a split of flow rate is required for ESI ionization. Figure 3.1 shows the HSCCC/MS interface.

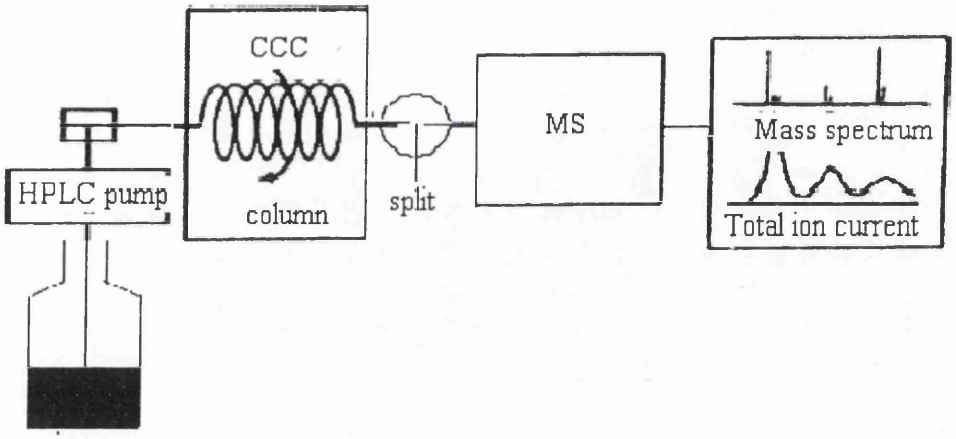

Figure 3.1 *The HSCCC interface with mass spectrometry*

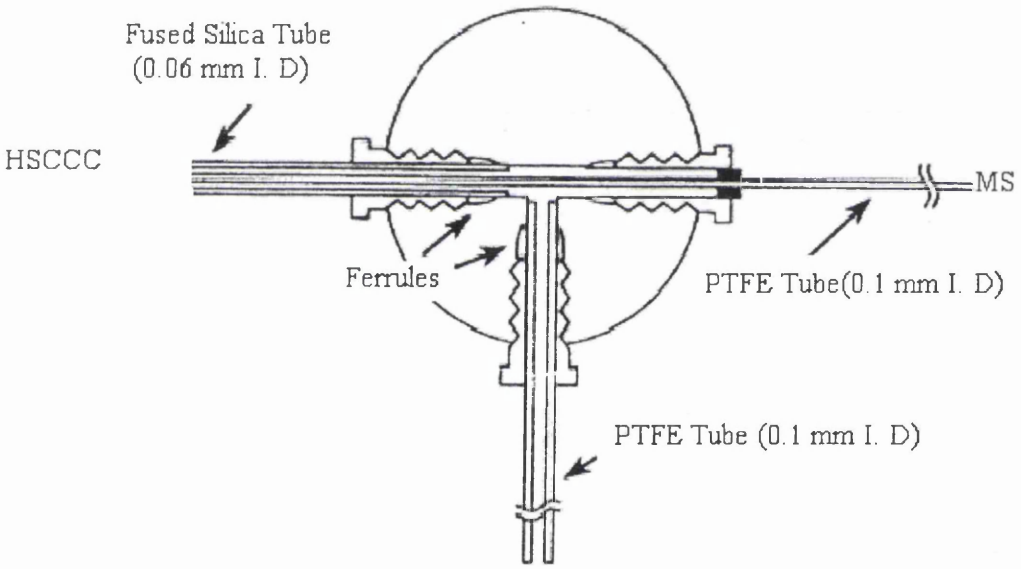

Figure 3.2 *A valco split-tee used in HSCCC interfacing mass spectrometry*

A Finnigan AQA quadrupole mass spectrometer has soft ionization methods and it contains sources for electrospray and atmospheric pressure chemical ionization. Instrument is tuned with a standard calibration solution of polyethylene glycol (PEG) and ammonium acetate. All data acquisition was performed in full scan mode and the scan range for sample was from 100 to 800-amu. The interface between the HSCCC and the mass spectrometer was a Valco split-tee (Supelco, Bellefonte, PA, USA), with a 1:10 ratio, to introduce the eluant from the HSCCC to the ESI source of the mass spectrometer. When HSCCC was interfaced with APCI/MS, no split–tee

was used since the APCI source allows high flow rates up to 2.0 ml / min, so the HSCCC can be directly connected with APCI. Standard solutions baicalein, baicalein-7-o-glucoside and chrysin were tuned by singly infusing each standard solution with the concentration of 10 μg/ml. The optimum conditions for the separation were: Capillary temperature 170 °C; The AQA (source voltage) 20 kV; Probe voltage 3.3 KV; RF lens 0.3 V. These conditions were found to be the optimum for the separations.

3.4 RESULTS AND DISCUSSION

3.4.1 Positive HSCCC/ESI/MS for the separation of a standard mixture of baicalein, chrysin, baicalein-7-O-glucoside and flavone

When a HSCCC interfaces a mass spectrometer to the separation of a standard mixture of baicalein, chrysin, baicalein-7-O-glucoside and flavone, baicalein, chrysin and baicalein-7-O-glucoside were very well resolved in 32 minutes. Another standard flavone was retained in the stationary phase due to its high hydrophobic characteristics. However, baicalein and chrysin gave weak signals, which indicated their low ionization efficiency. Figure 3.3 shows the separation of a standard flavonoid mixture. When the ratio of methanol was increased to effect the separation of flavone and also to improve the ionization efficiency, the flavone was eluted in 32.73 minutes. Figure 3.5 shows the separation of a standard flavonoid mixture when the concentration of methanol was increased.

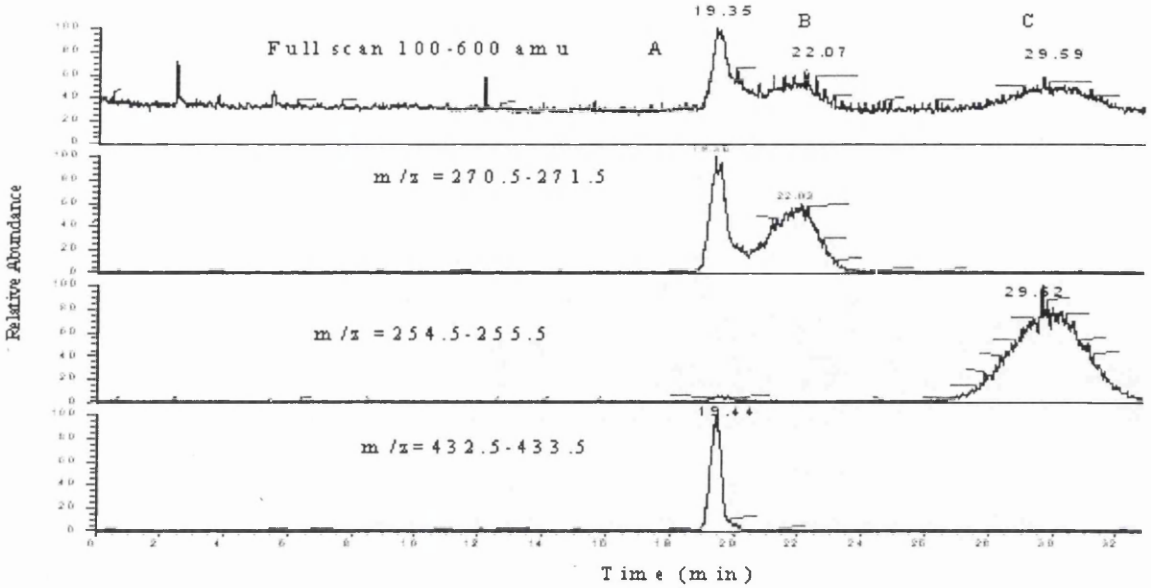

Figure 3.3 *RIC chromatogram of a standard flavonoid* mixture by HSCCC/ESI/MS

*A) : Baicalein-7-O-glucoside; B): Baicalein; C): Chrysin. Sample concentration: 25 µg/ml. HSCCC conditions: P*rototype J-type coil planet centrifuge HSCCC; *Solvent system: H-E-M-W=1:1.2:1:1; Upper phase: Stationary phase; Lower phase: Mobile phase; Stationary retention (%)* S_F*= 44.56; Speed: 1100 rpm; Flow rate: 1.0 ml/min. a split of 1:10 was used between the HSCCC and the mass spectrometer.*

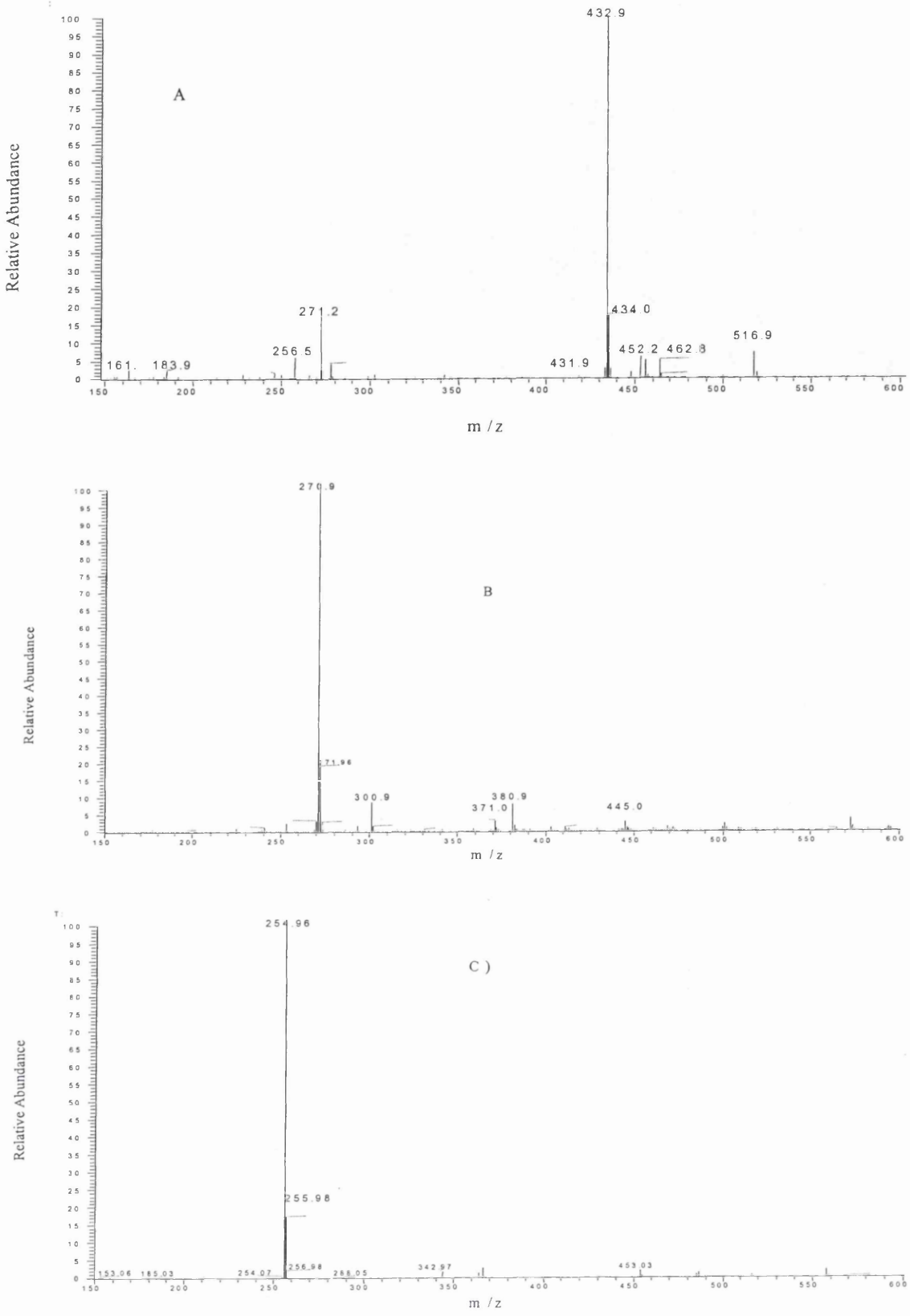

Figure 3.4 *+ESI mass spectra of fractions A, B and C in full scan mode*

In this solvent system, the elution time was 32 minutes and the three components were very well resolved. Another standard flavone was retained in the stationary phase. However, the signals of baicalein and chrysin were very weak. When the ratio of methanol was increased to effect the separation of flavone and also to improve the ionization efficiency, the flavone was eluted. Figure 3.5 shows the separation of a standard flavonoid mixture when the concentration of methanol was increased.

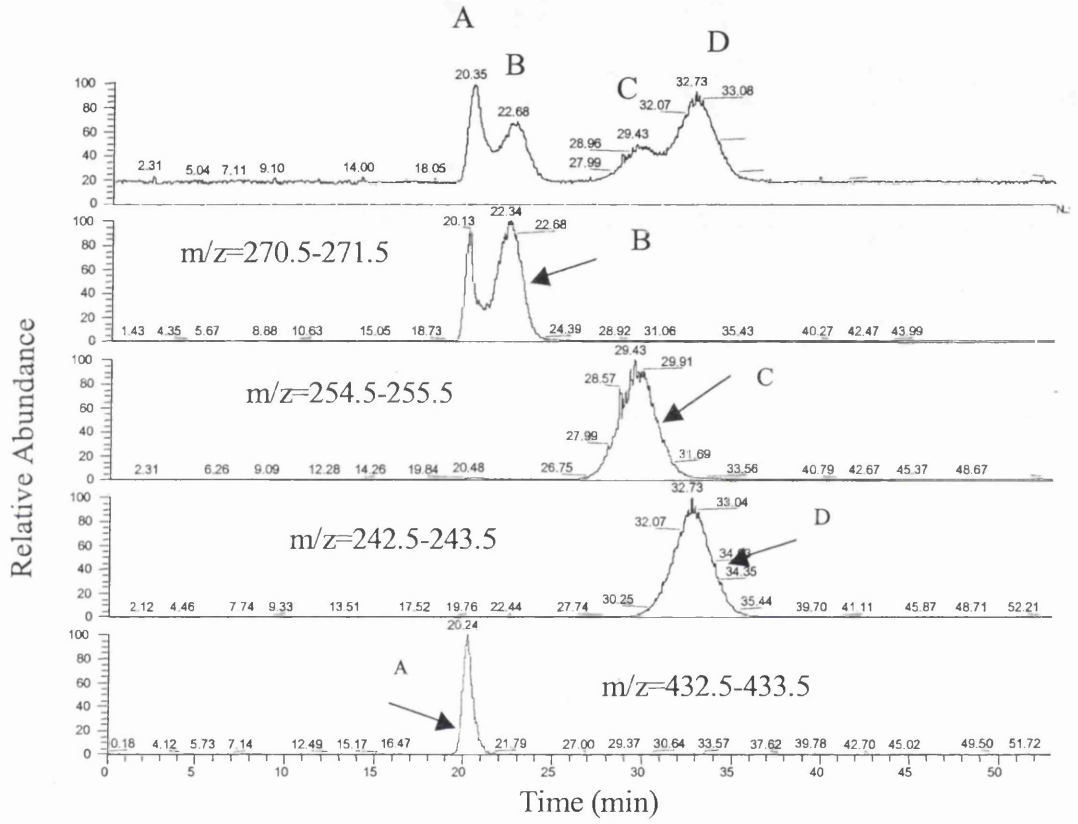

Figure 3.5 *RIC chromatogram of a standard flavonoid mixture by HSCCC/ESI/MS A) Baicalein-7-O-glucoside, B) Baicalein, C) Chrysin, D) flavone. Solvent system: H-E-M-W=1:1.2:1.5:1. For HSCCC conditions see Figure 3.3*

Figure 3.5 shows that when the ratio of methanol was increased from 1 to 1.5 in solvent system of H-E-M-W, the flavone was eluted in 33 minutes, but chrysin and flavone overlapped.

3.4.2 Negative HSCCC/ESI/MS for the separation of standard flavonoid mixture of baicalein, chrysin and baicalein-7-O-glucoside

The results by tuning standard flavonoids showed that -ESI ionization is better than +ESI. A 0.05 % ammonium formate buffer solution of pH=4, which is commonly used in HPLC/MS, was also added between the HSCCC and the mass spectrometer in order to improve the ionization efficiency. Figure 3.6 shows the negative HSCCC/ESI/MS separation results.

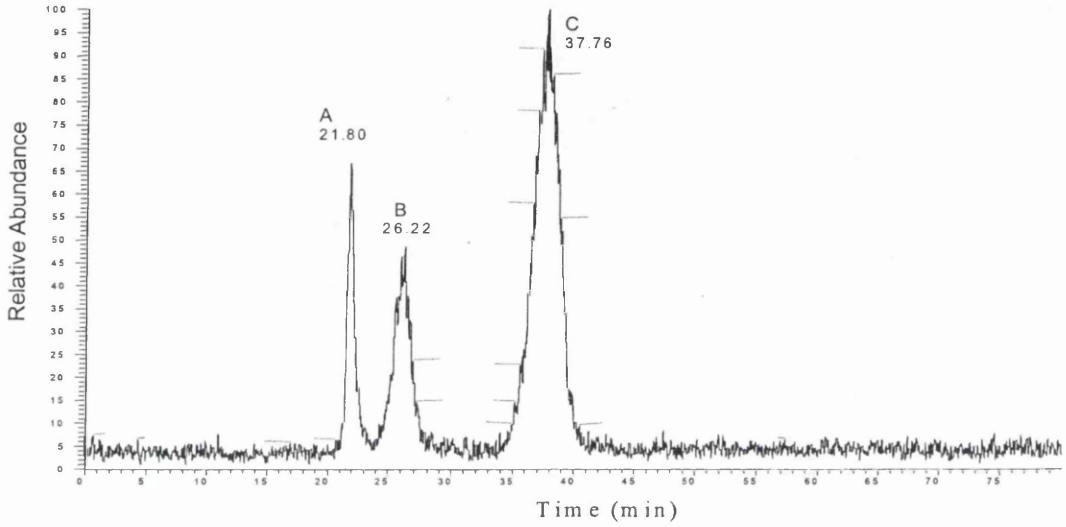

Figure 3.6 *TIC chromatogram of a standard flavonoid mixture by negative HSCCC/ESI/MS A: Baicalein-7-O-glucoside; B: Baicalein; C: Chrysin. Sample concentration was 25 μg /ml. HSCCC conditions: Solvent system: H-E-M-W=1:1.2:1:1; Upper phase: Stationary phase; Lower phase: Mobile phase; Stationary retention (%) S_F= 44.50; Mass spectrometry conditions: Capillary temperature is 170℃; the AQA is 25 kV.*

HSCCC/ESI/MS could effect the separation of flavonoids with good detection limits in the negative mode. However, a split of flow rate has to be used to provide the lower flow rates required for ESI and accurate flow into the mass spectrometer cannot be guaranteed. Sometimes, this disadvantage could greatly change the retention time. APCI can tolerate high flow rate to 2.0 ml/min, so HSCCC/APCI/MS was used to the separation of flavonoids without use of a split.

3.4.3 HSCCC/APCI/MS for the separation of a standard mixture of flavonoids

Baicalein-7-O-glucoside, baicalein, chrysin and biochaninA belong to the intermediate flavonoids; so, APCI is also a suitable ionization mode. When HSCCC/APCI/MS was employed in the

separation of standard flavonoid mixture in the negative mode, baicalein-7-O-glucoside, baicalein, chrysin and biochaninA were resolved very well. Figure 3.7 shows the separation results by HSCCC/APCI/MS.

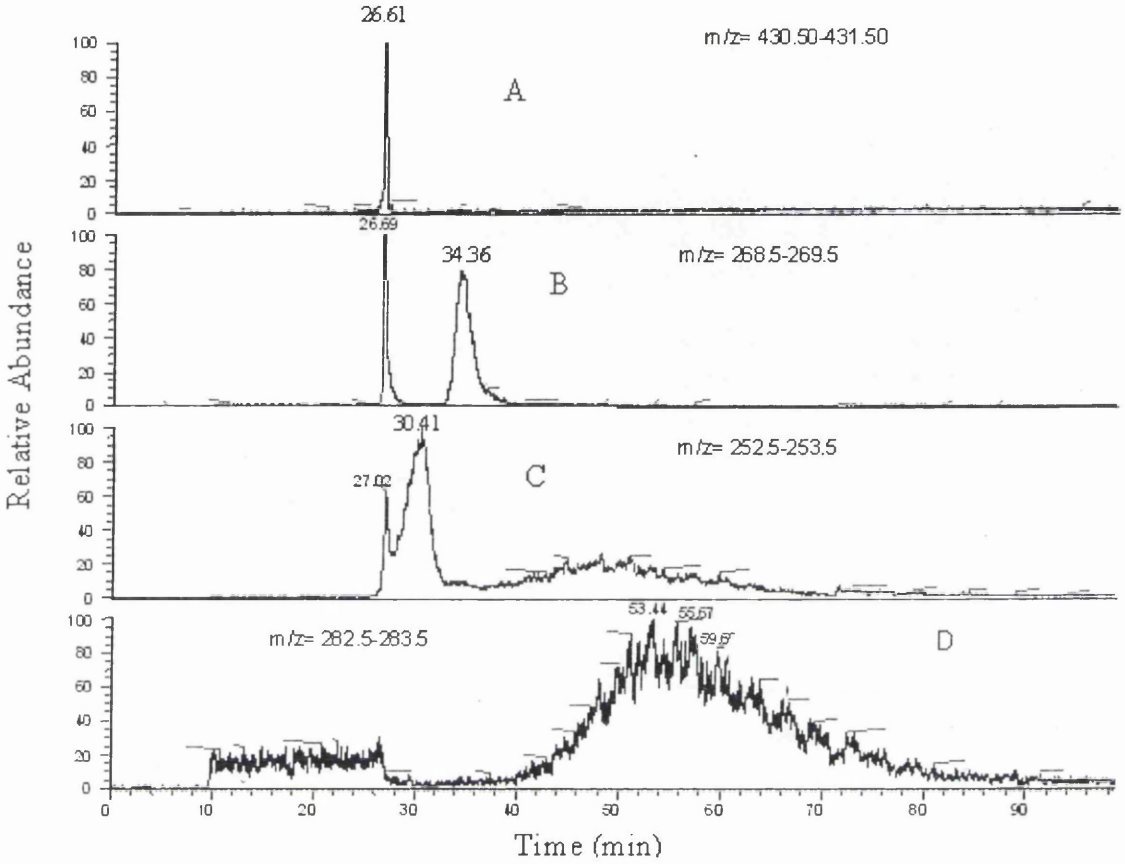

Figure 3.7 *RIC chromatograms of a standard flavonoid mixture*

A: Baicalein-7-o-glucoside; B: Baicalein; C: Chrysin. Sample concentration was 25 μg/ml. HSCCC condition: Solvent system: hexane-ethyl acetate-methanol-water=1: 4: 1: 1; Upper phase: Stationary phase; Lower phase: Mobile phase; Stationary retention (%)S_F = 57.08; Speed 1100 rpm; Flow rate: 1.0 ml/min.

Mass spectrometer conditions: Capillary temperature 250°C; AQA 20 V; Corona 3.00 V.

Our studies of HSCCC/MS indicate that negative APCI mode could also effect the ionization of flavonoids. HSCCC/APCI/MS is better than HSCCC/ESI/MS because no split was used in the separation process. This result can be used for the separation and characterization of flavonoids in the seeds of *O indicum*.

3.4.4 Application of HSCCC/APCI/MS for the separation of flavonoids from the ethyl acetate extract of the seeds of *O indicum*.

O indicum is a small to medium sized trees found in China and India. Its seeds are known as the crude drug 'Mu Hu Die' in China and it has been used as an analgesic and anti-inflammatory agent for the treatment of cough, bronchitis and other diseases. Its importance has already been described in chapter 2. HSCCC/APCI/MS can provide an important guide for the selection of solvent systems for preparative CCC. Figure 3.8 shows the separation of the ethyl acetate extract from the seeds by HSCCC/APCI/MS.

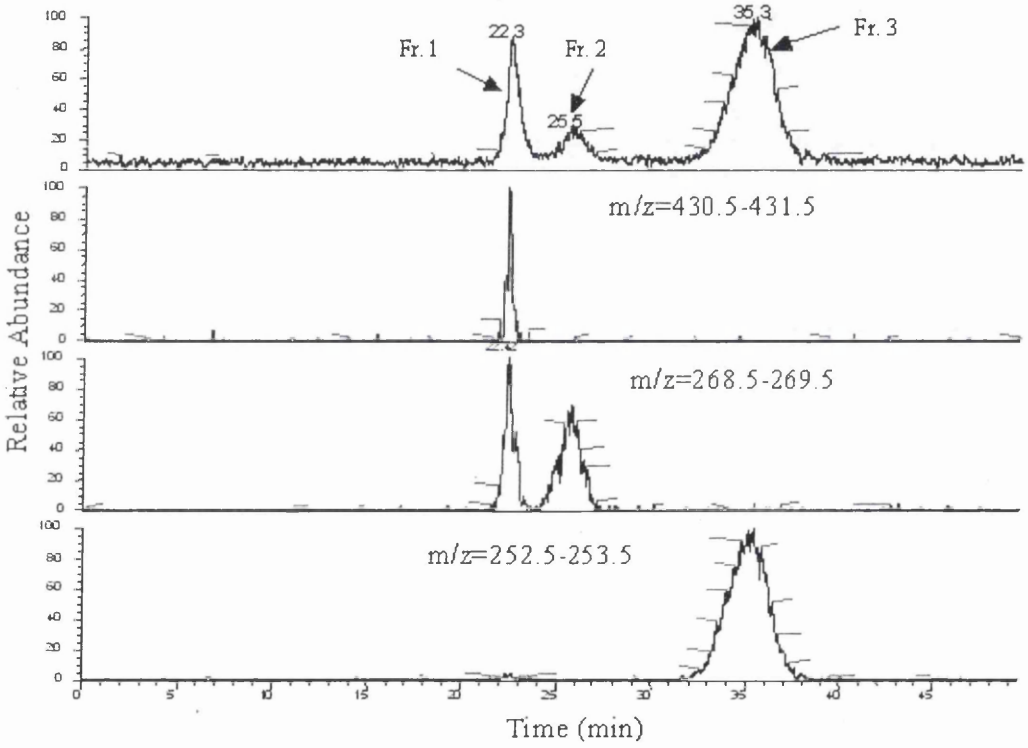

Figure 3.8 *TIC and RIC chromatograms of the ethyl acetate extract of the seeds of O, indicum by HSCCC/APCI/MS separation*

Fr1: baicalein-7-O-glucoside; Fr 2: baicalein; Fr3: chrysin. HSCCC and mass spectrometry conditions see Figure 3.6.

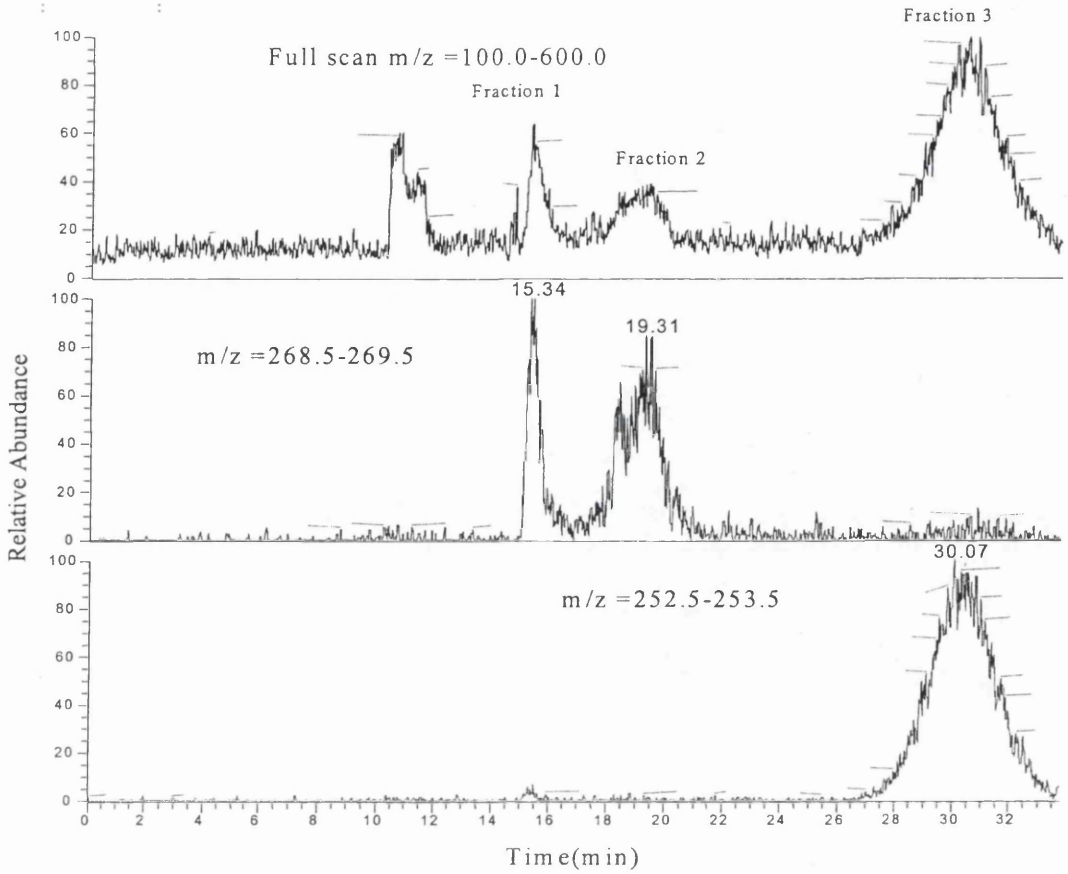

Figure 3.9 *TIC and RIC chromatograms of flavonoids of ethyl acetate extract from the seeds of O, indicum in H-E-M-W=1:1.2:1.5:1 solvent system.*
Other HSCCC and mass spectrometry conditions see Figure 3.6

As can be seen from Figure 3.9, when the ratio of methanol was increased, the elution time of flavonoids was shortened and all flavonoids could be resolved very well. Figures 3.10, 3.11 and 3.12 show mass spectra of fractions 1, 2 and 3

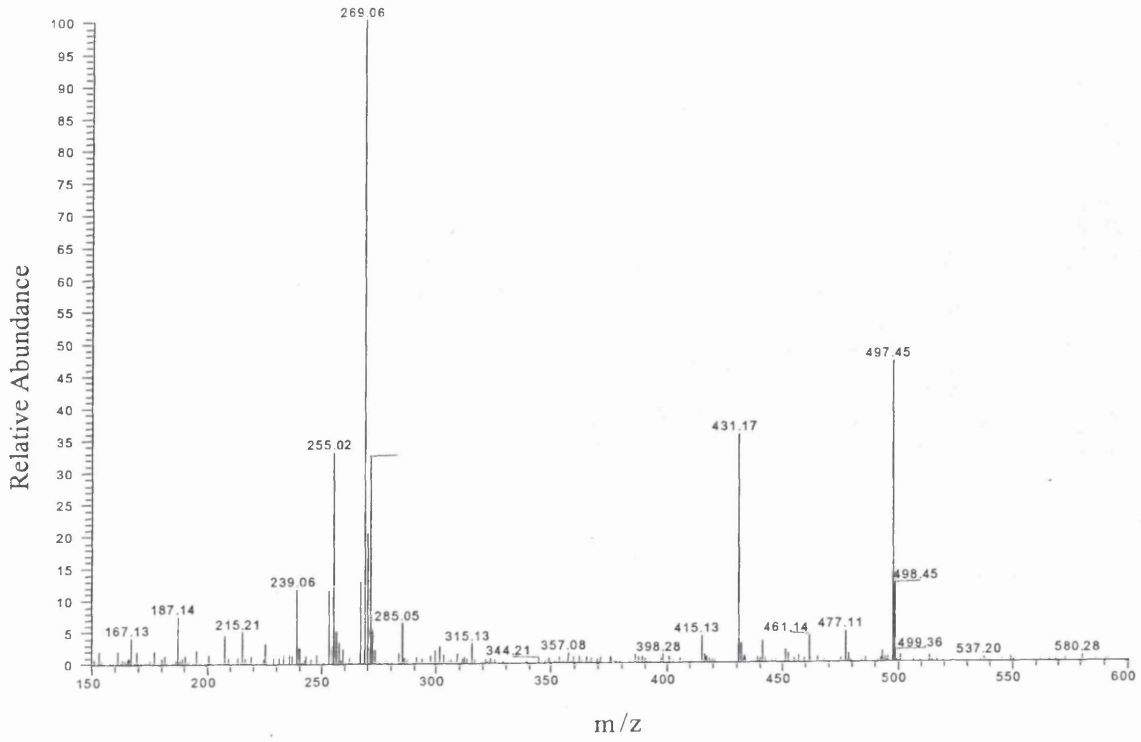

Figure 3.10 *Negative APCI mass spectrum of fraction 1 in full scan mode*

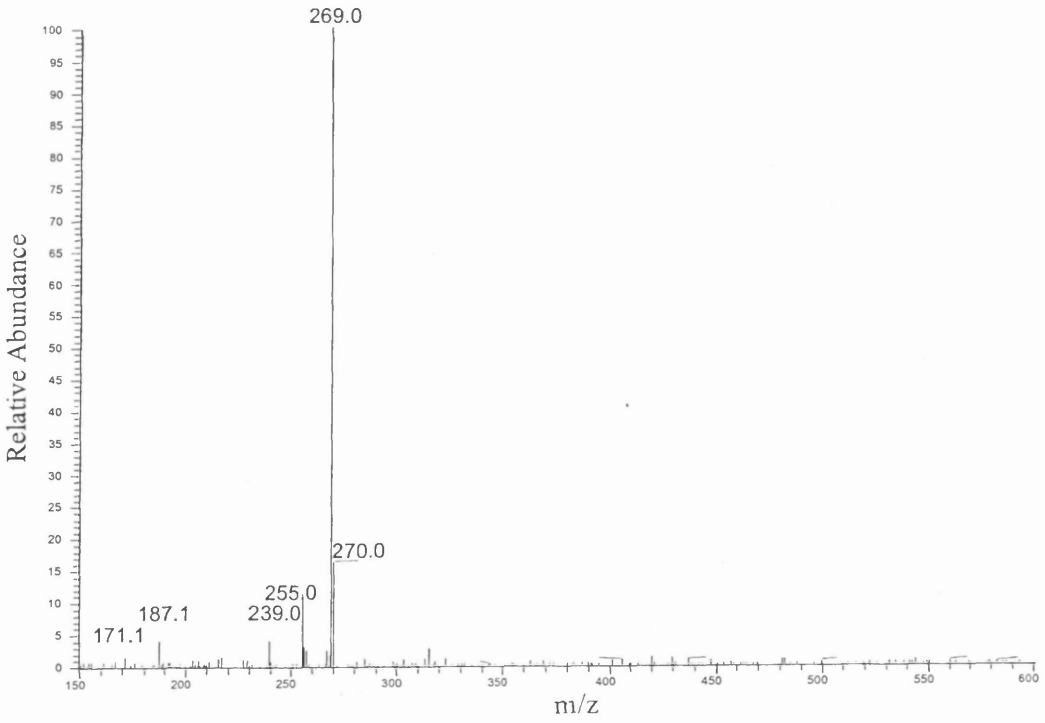

Figure 3.11 *Negative APCI mass spectrum of fraction 2 in full scan mode*

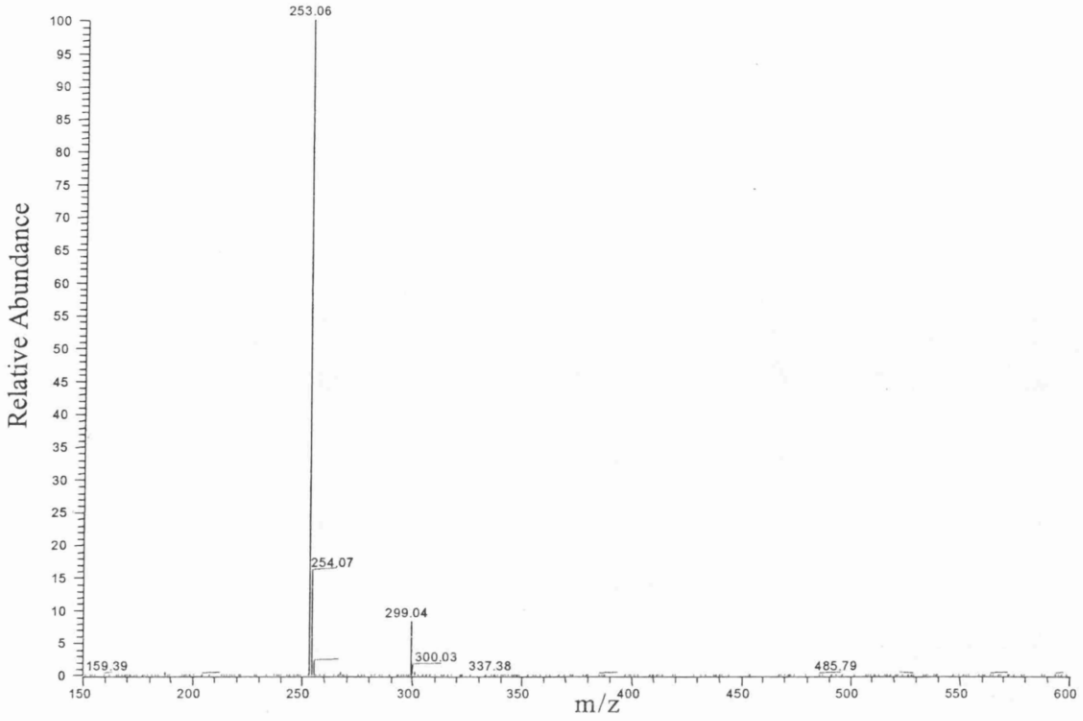

Figure 3.12 *Negative APCI mass spectrum of fraction 3 in full scan mode.*

Conclusion: HSCCC/APCI/MS was successfully used for the separation of flavonoid components in the ethyl acetate extract from the seeds of O, indicum. Three components were obtained with high purities and were identified as baicalein-7-O-glucoside, baicalein and chrysin. This result indicated that hexane-ethyl acetate-methanol-water=1:1.2:1:1 is a suitable solvent system for the separation of flavonoids and can be used for preparative CCC.

3.4.5 HSCCC/APCI/MS for the separation of a standard mixture of isoflavones

Daidzein, genistein, glycitein, genistin, daidzein and glycitin are common isoflavones, which exist in soy food and in some plants such as red clover. In recent years, an increasing interest in the isoflavones has focused on their separation and their identifications in soy food and plant due to their benefits on human health. Q. Du [13] used chloroform-methanol-butanol-water to separate a crude soybean extract and genistin and glycitin were separated with high purities. However, chloroform is a problem for environmental considerations. In our study, HSCCC/APCI/MS was used for the first time for the separation and identification of six isoflavones using a hexane-ethyl acetate-methanol-water solvent system.

3.4.5.1 HSCCC/APCI/MS for the separation of six isoflavones with isocratic elution

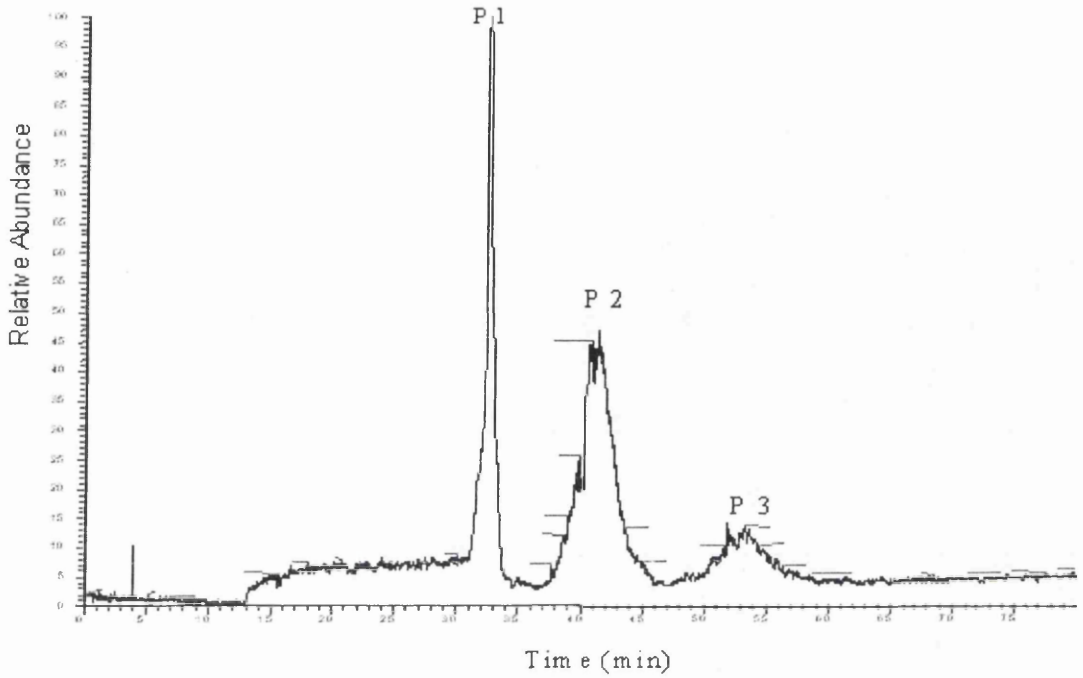

Figure 3.13 *TIC chromatogram of a mixture of six isoflavones by HSCCC/APCI/MS*

Sample: A standard mixture of genistin, daidzin, genistein, daidzein, glycitein and formonetin. P1: genistin+daidzin; P2: daidzein; P3: genistein; BiochainA and formonetin were l retained in the coil; Solvent system: H-E-M-0.2 % formic acid = 1:4:1.6:4; (%) S_F = 56.04. Other HSCCC and mass spectrometry conditions see Figure 3.6.

As we can see from Figure 3.13, the solvent system of H-E-M-H =1:4:1.6:4 could only resolve daidzein and genistein, and genistin and daidzin co-eluted. BiochaninA and formonetin were retained in stationary phase. When the ratio of methanol was increased to 2.5, a short elution time was obtained and biochaninA was eluted as well. Figure 3.14 shows the results obtained with this system.

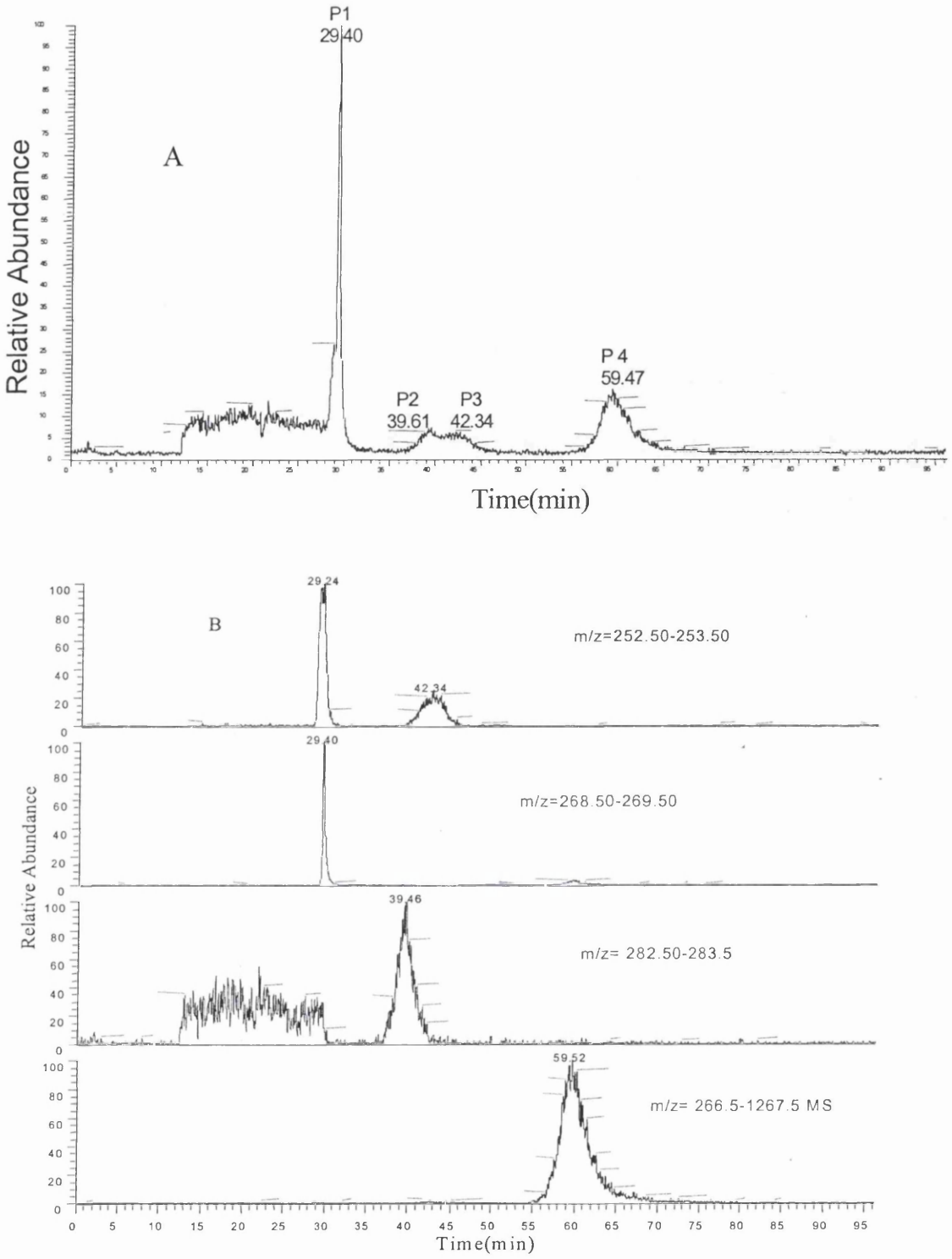

Figure 3.14 *TIC (A) and Reconstructed (B) chromatograms of HSCCC/APCI/MS for separation of a standard mixture of 6 isoflavones*

P1: genistin+daidzin; P2: glycitein; P3: daidzein; P4: formonetin. Biochanin A still retained in the coil; Solvent system: H-E-M-0.2 % formic acid=1:4:2.5:4; (%) S_F=44.68

Figure 3.14 shows that genistin and daidzin could not be resolved easily. This is because that genistin and daidzin share very similar structures and polarity. The two components were also difficult to separate by RP HPLC (Chapter 4 shows the results of HPLC). In this case, we obtained resolution by decreasing the proportions of ethyl acetate and methanol.

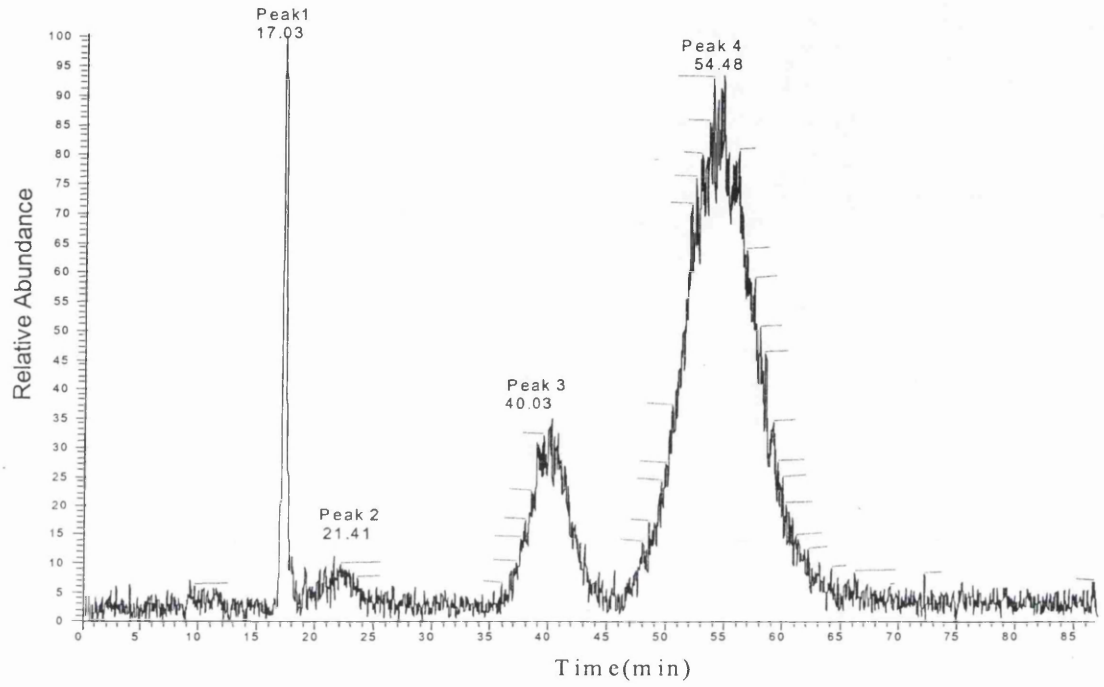

Figure 3.15 *TIC chromatograms of HSCCC/APCI/MS for the separation of a mixture of six isoflavones*

Sample: 50 μg / ml standard isoflavone mixture. Peak 1: Genistin; Peak 2: Daidzin +glycitein; Peak 3: Genistein; Peak 4: Formonetin. HSCCC conditions: Solvent system: H-E-M-W=1:1.2:0.8:1; Upper phase: Stationary phase; Lower phase: Mobile phase; Stationary retention (%) S_F= 47.08; Speed 1100 rpm; Flow rate: 1.0 ml/min. Mass spectrometer conditions see Figure 3.6.

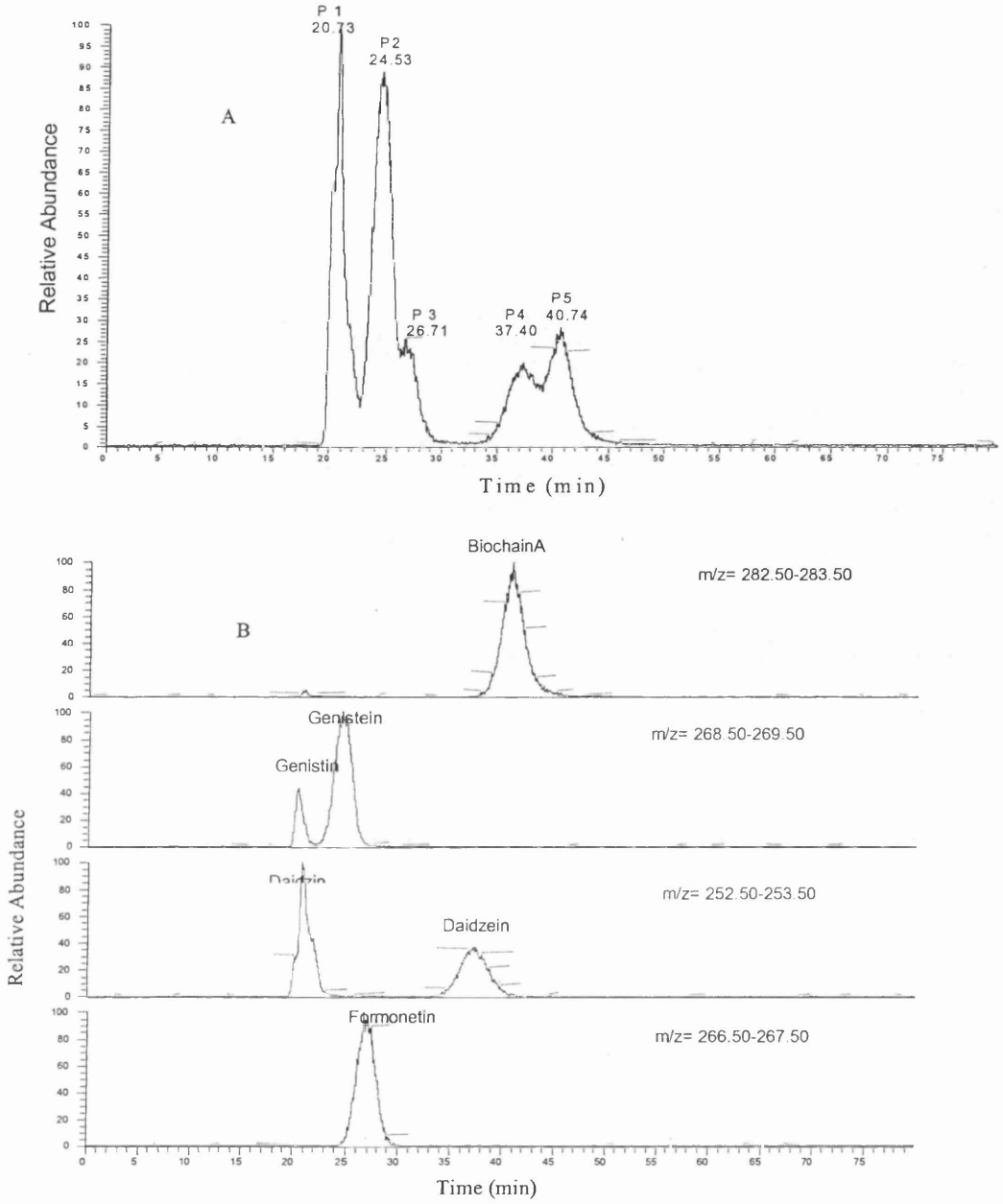

Figure 3.16 *TIC (A) and reconstructed (B) chromatograms for the separation of a mixture of 6 standard isoflavones*

Solvent system: H-E-M-W=1:1.2:1:1. For other HSCCC and mass spectrometer conditions see Figure 3.6

Conclusion: In the isocratic elution mode by HSCCC/APCI/MS, it is difficult to resolve the six isoflavones in a single run due to their similar polarity and structures. Gradient elution in HPLC has the advantage over isocratic elution in terms of separation large range of polar components. So, gradient elution was also explored for the separation in HSCCC/APCI/MS.

3.4.5.2 HSCCC/APCI/MS for the separation of six isoflavones with gradient elution

3.4.5.2.1 Separation of six isoflavones with methanol gradient elution of H-E-M-W=0.5:1:0.3:1 to 0.5:1:1.5:1

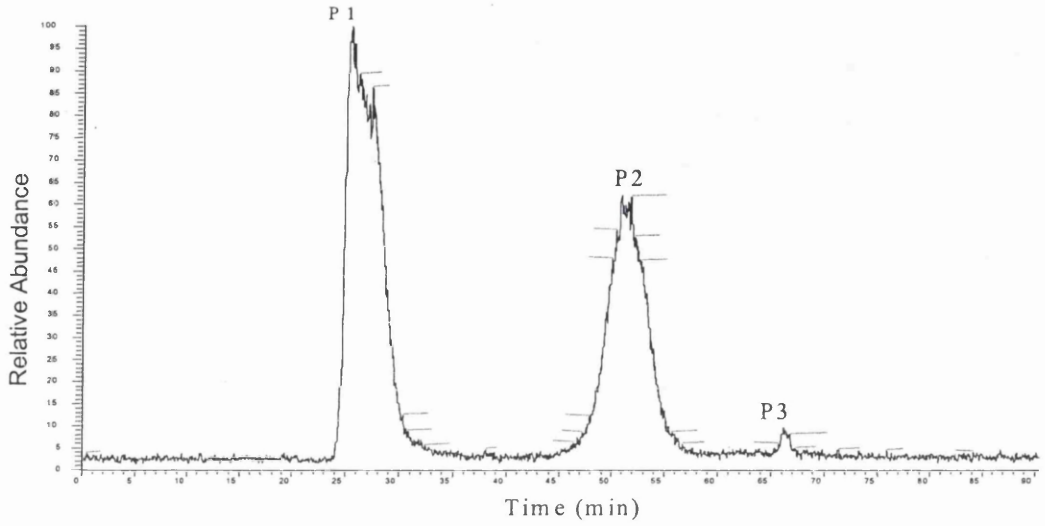

Figure 3.17 *TIC of HSCCC/APCI/MS for the separation of a 6 standard isoflavone mixture H-E-M-W=0.5:1:0.3:1 to 0.5:1:1.5:1. A): Lower phase of biphasic solvent H-E-M-W=0.5:1:0.3:1, B): Lower phase of biphasic solvent H-E-M-W=0.5:1:1.5:1; Rotor speed: 1100 rpm; Gradient elution programme: 0-15 min, 100 % A, 15-25 min, 100 % A to 50 % A, 25-45min, 50 % B-100 % B, 45-100 min, 100 % B. Mass spectrometer conditions see Figure 3.6. P1: genistin+daidzin; P2: glycitein+genistein+daidzein; P3: biochaninA*

3.4.5.2.2 Separation of a mixture of isoflavones with methanol gradient elution of H-E-M-W=0.5:1:0.3:1 to 0.5:1:1.5:1 with a different elution programme.

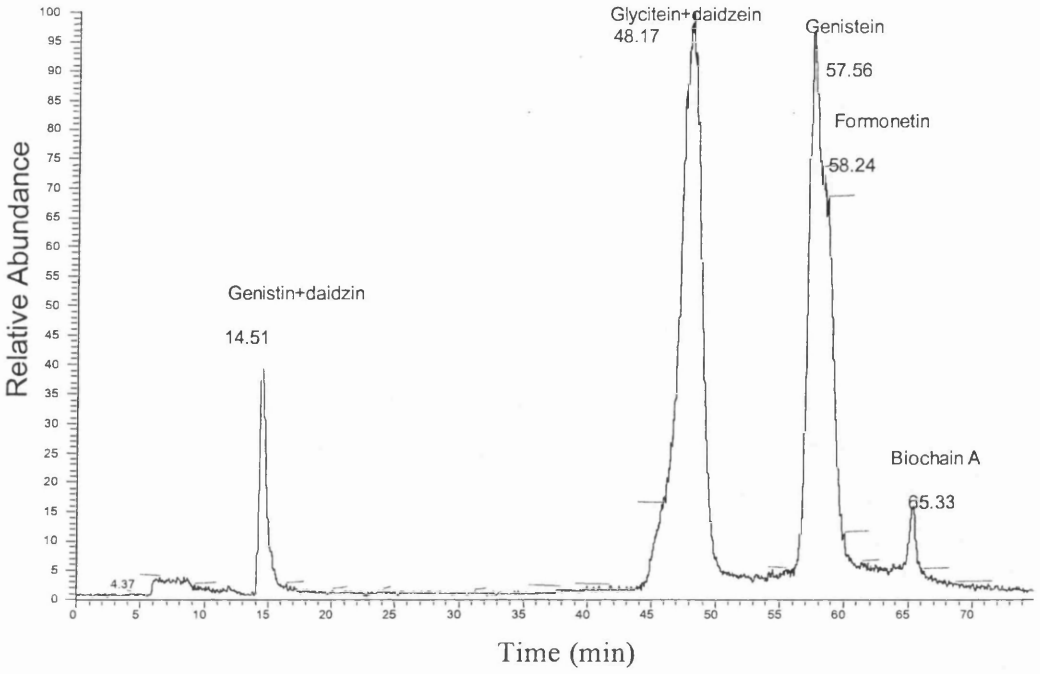

Figure 3.18 *TIC of HSCCC/APCI/MS for the separation of a mixture of 6 isoflavones* A): Lower phase of biphasic solvent H-E-M-W=0.5:1:0.3:1, B): Lower phase of biphasic solvent H-E-M-W=0.5:1:1.5:1; Rotor speed: 1100 rpm; Gradient elution programme: 0-20 min, 100 % A, 20-35 min, 100 % A to 50 % A, 35-100 min, 50 % B-100 % B. Mass spectrometer conditions see Figure 3.6.

After varying the gradient elution programme, biochaninA was eluted in 65 minute and resolved from genistein. However, genistin coeluted with daidzin and glycitein coeluted with daidzein as well. When increasing the ratio of hexane in the gradient elution programme, a better resolution was obtained. Figure 3.19 shows the result of this gradient elution.

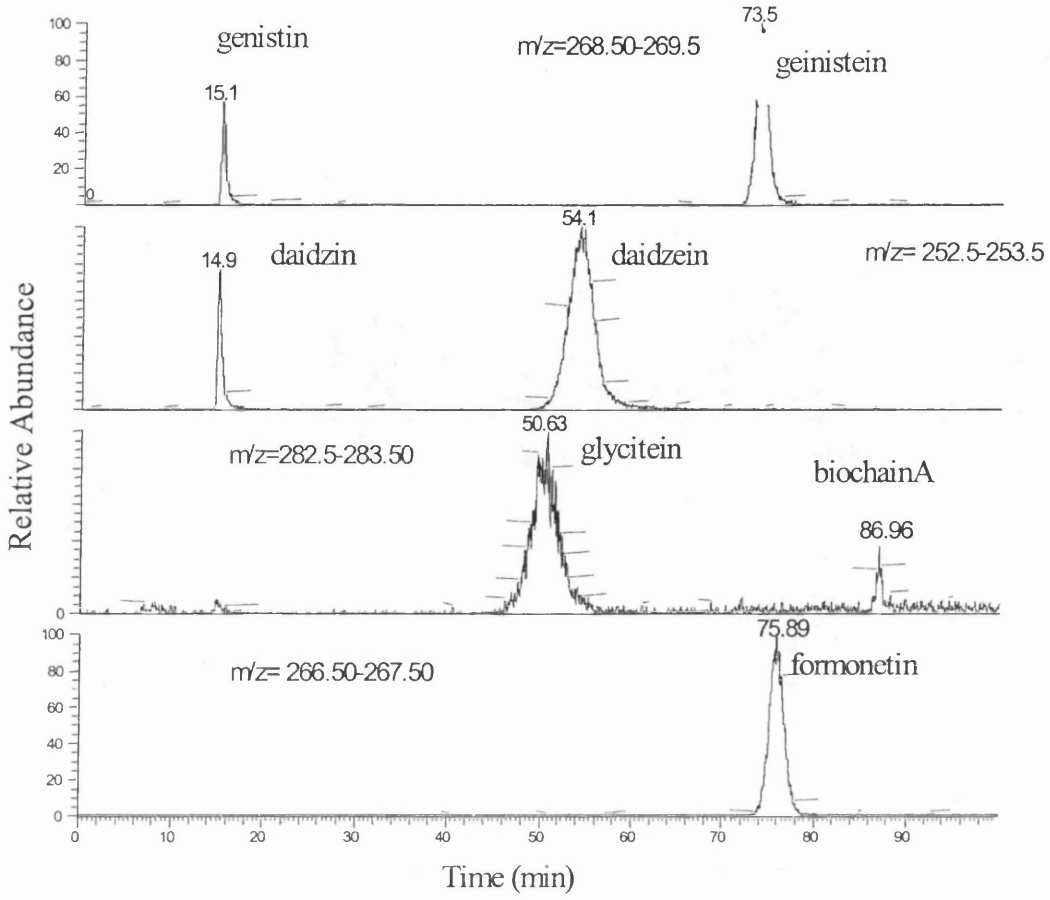

Figure 3.19 *Reconstructed chromatograms of HSCCC/APCI/MS for the separation of a mixture of 6 isoflavones*

A): Lower phase of biphasic solvent H-E-M-0.2 % formic acid =1:1:0.3:1.5, B): Lower phase of biphasic solvent H-E-M-0.2 % formic acid=1:1:1.5:1.5; Rotor speed: 1100 rpm; Gradient elution programme: 0-20 min, 100 % A; 20-60 min, 0 % B to 100 % B; 60 –100 min, 100 % B. Mass spectrometer conditions see Figure3.6. After finished the elution, stationary phase was retained in coil 2.5 ml.

Gradient elution in HSCCC/APCI/MS gives better resolution than the isocratic elution. Genistin and daidzin were still difficult to separate by the gradient elution due to their similar structures and polarity. This result is not unexpected, since it is also difficult to separate them by HPLC. The HSCCC/APCI/MS cannot compete with HPLC/MS in separation efficiency.

3.4.6 Milli HSCCC/ESI/MS for the separation of flavonoids from the ethyl acetate extract of the seeds of *O indicum* in positive mode.

Milli CCC is a good analytical instrument. Not only can separations in an analytical timescale be conducted, the system can be connected to a mass spectrometry detector for on-line CCC/MS separation. This machine also reduces the noise level by allowing the design to feature an enclosed gearbox. Figures 3.20 and 3.21 show the CCC/MS results for the separation of flavonoids of the ethyl acetate extract from the seeds of *O, indicum* with different flow rates.

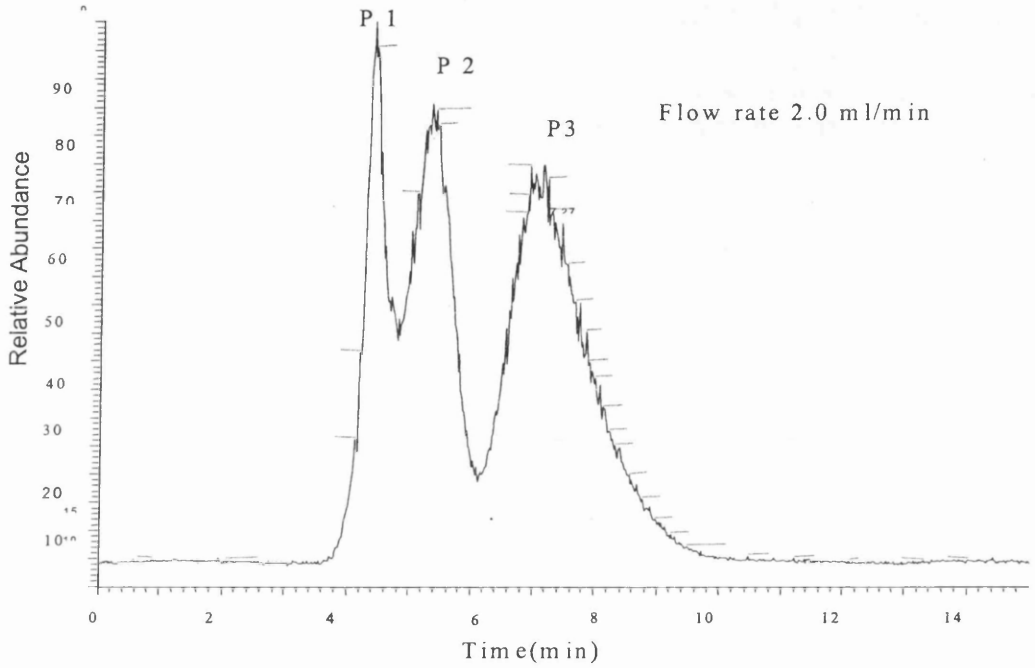

Figure 3.20 *SIM chromatogram of HSCCC/ESI/MS in positive mode for the separation of ethyl acetate extract from the seeds of O, indicum*

HSCCC conditions: Milli CCC, H-E-M-W=1: 1.2:1:1; Upper phase: stationary phase; Lower phase: Mobile phase; Rotation speed: 1800 rpm; Flow rate: 2.0 ml/min. P1: baicalein-7-O-glucoside; P2: baicalein; P3: chrysin. A split of 1:20 was used between the HSCCC and the mass spectrometer.

Mass spectrometer conditions: Probe temperature: 450°C; Ionization mode: +ESI; Capillary voltage (kV): 4.50; Source voltage (V): 40; RF Lens (V):0. 3.

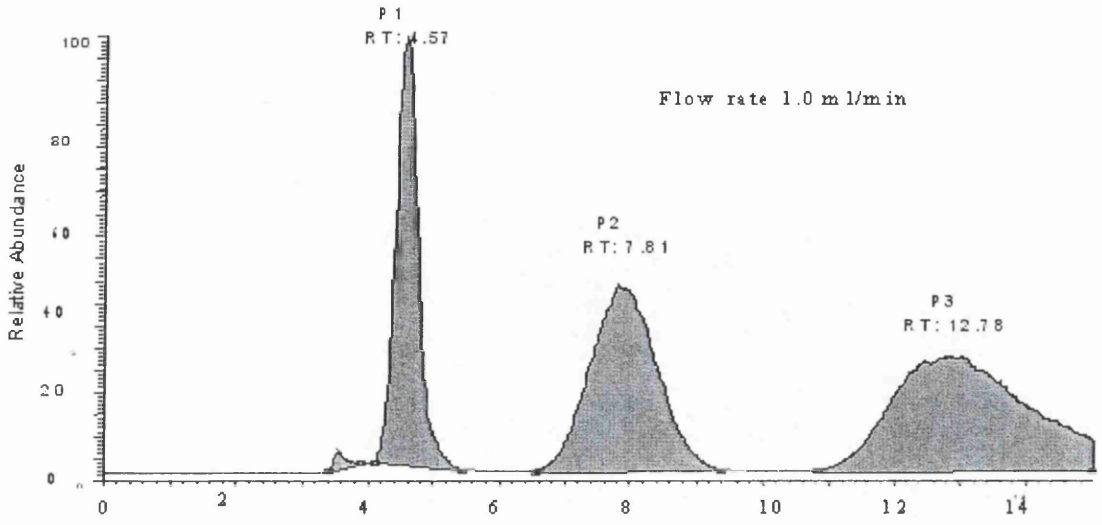

Figure 3.21 *SIM chromatogram of HSCCC/ESI/MS in positive mode for the separation of ethyl acetate extract from the seeds of O, indicum*

Figure 3.21 show that the whole separation can be finished in 15 minutes for the analysis of flavonoids from the natural product and good resolutions were obtained. Milli CCC is an excellent instrument for interfacing with the mass spectrometer. Compared with previous analytical HSCCC, it operates in less noise level and can maintain high resolution with low stationary retention (S_F<25 %) due to high rotation speed. This machine is portable and economical. It can be a complementary instrument in the modern analytical laboratory and encourage more analytical HSCCC application for coupling with the mass spectrometer.

3.5 CONCLUSION AND DISCUSSION

Our study for standard isoflavones showed that six standard isoflavones could be resolved by HSCCC/APCI/MS if gradient elution was used. However, genistin and daidzin were difficult to be resolved by the H-E-M-W solvent system. When the isocratic elution mode was used, six isoflavones could not be resolved in a single run by this solvent system. This result can direct our preparative CCC for the separation of isoflavones from soybean. More work will be done in the separation of isoflavones from soybean by HSCCC/MS in future. A good resolution of three

flavonoids from the seeds of *O indicum* was also obtained by HSCCC/MS with the two HSCCC instruments.

At present, HSCCC/MS could not compete with HPLC/MS in efficiency and resolution due to its low separation efficiency. Many disadvantages such as back pressure; stationary bleeding and low reproducibility limit its development. However, its support-free, low cost, 100 % recovery of target compounds make it very attractive for the screening and identification of bioactive components and for sample clean-up procedures. The analytical HSCCC can also be used for the selection of solvent systems due to low consumption of solvent and short separation times. Furthermore, HSCCC/MS can be a complementary analytical instrument to solve some separation and identification problem, which is difficult to be solved by HPLC/MS.

3.6 REFERENCES

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CHAPTER 4

Comparison of Different HSCCC Instruments in the Separation of the Extract of the Seeds of *O indicum*

4.1 INTRODUCTION

Analytical and preparative instruments are available for most separation methods such as thin layer chromatography (TLC) and high performance liquid chromatography. This has not been the case in CCC, where until very recently, only apparatus for preparative separation have been widely available. In preparative CCC, selection of a suitable solvent system had to be carried out by alternative methods. Normally, shaking test, TLC and high performance liquid chromatography are used to choose suitable solvent systems. The best approach, although time consuming, was to partition the sample between the two phases of a solvent system, followed by quantitative analysis of the two layers. To a certain extent, TLC provided useful information for the solvent selection process [1, 2], but sometimes TLC gave false information. This disadvantage of CCC has now been to some extent put aside by the introduction of analytical HSCCC. Several papers have already shown that analytical HSCCC is very useful for methods development. Zhang et al [3, 4] studied the separation of flavonoids from Hippophae Rhamnoides by analytical and preparative HSCCC using 43- and 280- ml coils, respectively. The whole elution could be finished in 15 minutes for the separation of five compounds using analytical HSCCC. This example clearly demonstrates the advantage of analytical instrumentation for methods development in preparative HSCCC. In other applications, W.D. Conway [5] used analytical HSCCC to evaluate 1-butanol -water and heptane-1-butanol/water solvent systems for the separation of nucleosides and of drug metabolites. The analytical separation was achieved in 25 minutes. Further progress in the development of solvent systems can be expected from the use of fast and solvent-saving analytical instrumentation.

In general, analytical HSCCC is defined as having coils of less than 50-ml volume and having tubing of internal diameters of 1 mm or less [6]. Most separations have been carried out with coils made of 0.85-mm I.D. tubing. Presently, there are two trends in the development of analytical instrumentation: (1) building instruments with small bore tubing and design for high rotational speeds, and (2) building instruments that can be used for analytical and preparative separations. The second goal can be achieved by connecting several coils in series or by building combined coils that contain analytical and preparative coils on the same holder. A good reason for this instrument is that we could use analytical coils for rapid solvent system selection and save on solvent consumption. Compared with HPLC and other analytical methods, efficiencies and resolution reported in analytical HSCCC clearly indicate that this technique will not compete with

for high efficiency. From this point, analytical HSCCC may become a useful method for the following applications: 1) method development, (2) microscale separations, and (3) measurements of partition coefficients for preparative HSCCC. Hence, analytical instrumentation with its speed and low solvent consumption represents a practical way to evaluate solvent systems for preparative HSCCC.

Another application of analytical HSCCC is in microscale isolation where the advantages of analytical HSCCC are recognized. T. -Y. Zhang [7] reported the microscale separation of isoquinolines from Rheum palmatum. L. Lee [8] separated the Vinca alkaloaids vincamine and vincine in hexane-ethanol-water (6: 5: 5) and they compared the separation with that achieved by reversed-phase HPLC. In HSCCC, crude extracts and other complex samples can be separated without filtration prior to injection. Without solid support, risks of losing compounds will not occur through irreversible adsorption onto stationary phase material. This finding makes the method very attractive for the screening of bioactive components and for sample clean-up procedures. Rapid measurement of partition coefficients is the third field where analytical HSCCC is applied

4.2 PREPARATIVE HSCCC

In countercurrent chromatography history, preparative CCC remained a very important tool for the separation and purification of natural products. The isolation of compounds of interest from natural sources, such as plant extracts, microbial fermentation, or animal tissues, presents a number of difficulties. Some of the compounds of interest are present as minor components of extremely complex mixtures. Even though adsorption chromatography, high performance liquid chromatography (HPLC), for example, has sufficient resolution power to separate such mixtures at an analytical scale, the scale-up of these methods into a preparative scale still has practical difficulties. The irreversible adsorption of minor components of denaturation on a solid phase is a common problem encountered in solid phase chromatography. Furthermore, the costs involved in the scale-up of adsorption chromatography from analytical scale to preparative can be extremely expensive. Recently, high-speed countercurrent has widely been used for rapid enrichment of compounds of interest for final purification.

In this chapter, we compared different column volumes from a preparative scale to an analytical

instrument for the separation of flavonoids. There were two kinds of instruments used in our research. Both were supplied by Brunel Institute for Bioengineering, Brunel University. One was a J-type planet centrifuge analytical and preparative HSCCC. This machine was equipped with a HP1100 pump, a UV spectrophotometer detector and a sample collector. A manual sample injection valve was used to introduce the samples into the column. The CCC has four coils that are wound tightly one two separate bobbins on one rotor; each bobbin containing two concentrically wound coils of PTFE tubing with a total volume of 495 ml. In our studies, three columns were chosen and their volumes are shown in Table 4.1. The other instrument was Milli CCC analytical HSCCC and its volume of coil is only 4.9 ml.

Table 4.1 *Comparison of different HSCCC instruments*

	Column volume (ml)	β-value	Maximum speed (rpm)
J-type Quattro CCC	227.2	0.7-0.83	1000
	169.9	0.7-0.80	1000
	94.9	0.8-0.86	1000
	49.9	0.83-0.86	1000
Milli CCC	4.90	0.68-0.79	2100

4.3 EXPERIMENTAL

4.3.1 Solvent and reagents

Analytical grade solvents used in this study included hexane, ethyl acetate, methanol and water. All these solvents were purchased from Fisher Chemicals (Loughborough, UK).

4.3.2 Sample preparation

Samples 1 and 2 were prepared by extraction method 1 and extraction method 2 (see section 2.6.5 of Chapter 2). For sample 1 three major components and one minor component were obtained and identified as baicalein-7-O-diglucoside, baicalein-7-O-glucoside, baicalein and chrysin. They are labelled components 1, 2, 3 and 4. Figure 4.1 shows the HPLC analysis of the sample 1

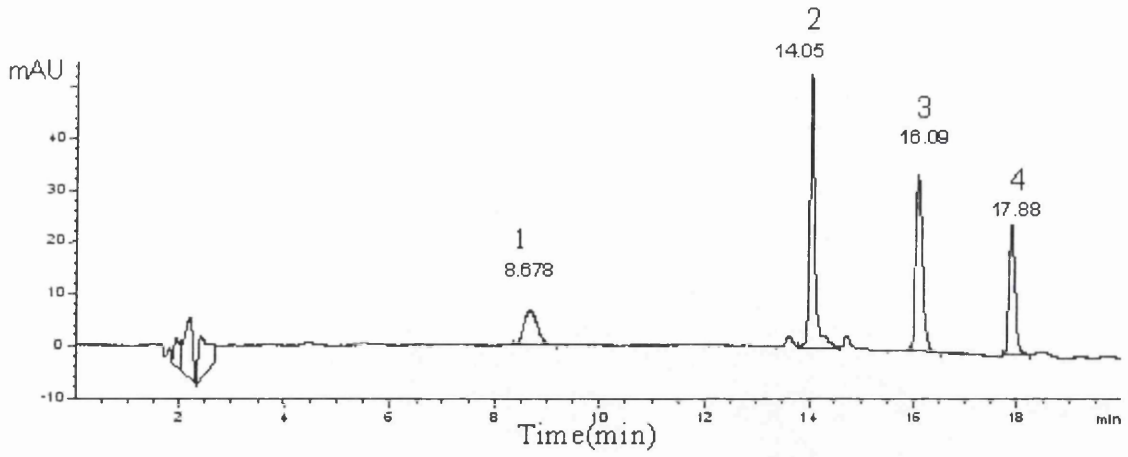

Figure 4.1 *HPLC chromatogram of sample 1 from extract method 1*

Experimental conditions: Cosmosil C_{18} RP column (150 × 4.6 mm I.D Phenomenex); Temperature: 30℃; Flow rate: 1.0 ml / min; Wavelength of 275.5 nm.1: baicalein-7-O-diglucoside; 2: baicalein-7-O-glucoside; 3: baicalein; 4: chrysin. A gradient elution of formic acid 0.2 % (A) and 100 % acetonitrile (B) was employed: 0 -10 min 80 % A and 20 % B; 10-12 min 55 % A, 45 % B; 12-20 min 20 % A, 80 % B.

Sample 2 from extraction method 2 contains two major components and one minor component (see chapter 2 for detail). They are labelled A, B and C, and identified as baicalein-7-O-diglucoside, baicalein-7-O-glucoside and chrysin-7-O-diglucoside. Figure 4.2 shows the HPLC analysis of sample 2.

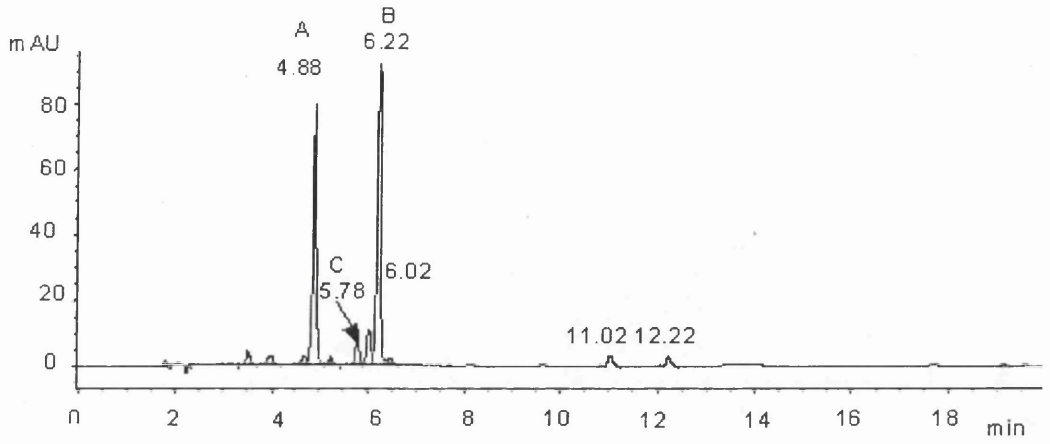

Figure 4.2 *HPLC chromatogram of sample 2 from extract method 2*

(Experimental conditions see Figure 4.1)

A: Baicalein-7-O-diglucoside; B: Baicalein-7-O-glucoside; C: Chrysin-7-O-diglucoside.

4.3.3 HSCCC method

Biphasic mixtures of hexane-ethyl acetate-methanol- water (H-E-M-W) were prepared in the different ratios and purged for 10 minutes with nitrogen to remove any dissolved gases. First, the coil was filled with the upper phase (organic layer) of the biphasic mixture. The coils were rotated in a forward direction at a speed of 1100 rpm and the lower phase (aqueous layer) was pumped into the coil from head to tail at a constant flow rate. When the two layers were observed, the equilibration point was determined when no more stationary phase was eluted (hydrodynamic equilibration). The retention volume of the system could then be calculated by subtracting the volume of the stationary phase eluted at the end of the equilibration process and extra volume from the total volume (for the calculation equation see section 2.6.3 of Chapter 2).

4.4 RESULTS AND DISCUSSION

4.4.1 Separation of flavonoids of sample 2 in column volume of 169.9 ml

Sample 2 contained two major compounds and some small components. Our previous studies (see

Chapter 2) already identified that the two components are baicalein-7-O-diglucoside and baicalein-7-O-glucoside. According to previous studies, their polarities are greater than aglycone flavonoids, therefore, a lower ratio of methanol should be used to the separation of the flavonoid glycosides by a H-E-M-W solvent system. A ratio of H-E-M-W = 0.2: 1.6: 0.2: 1.6 was used for the separation of sample 2.

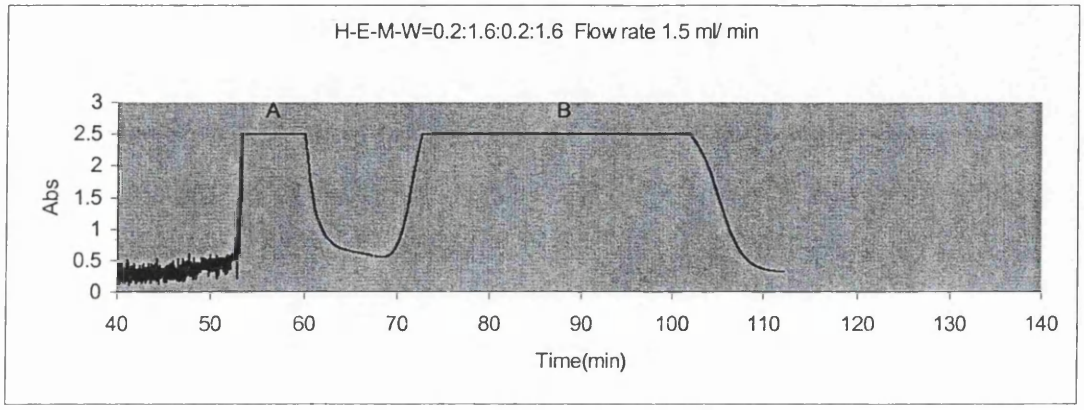

Figure 4.3 *HSCCC chromatograms of the separation of sample 2*

A: Baicalein-7-O-diglucoside; B: Baicalein-7-O-glucoside.

Experimental conditions: Coil volume: 169.9 ml; Stationary phase: Organic phase; Mobile phase: Aqueous phase; Rotation speed: 800 rpm; Flow rate: 1.5 ml/min; Sample volume: 4.0 ml; Sample concentration: 20 μg /ml; Direction of motor: Reverse; S_F *(%) = 67.6.*

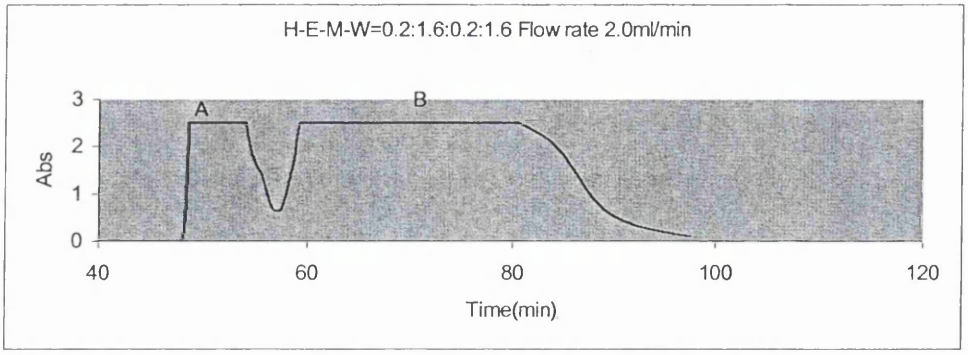

Figure 4.4 *HSCCC chromatogram of the separation sample 2*
(HSCCC experimental conditions see Figure 4.3)

Figures 4.3 and 4.4 show that components A and B are resolved but component C is co eluted with component B. When the flow rate was increased, A and B also overlapped.

4.4.2 Separation of flavonoids of sample 2 in column volume of 94.9 ml

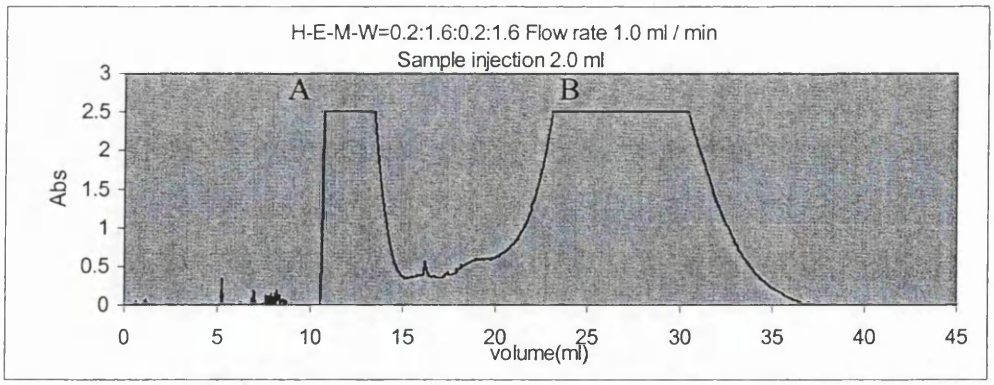

Figure 4.5 *HSCCC Chromatogram of the separation of sample 2*

HSCCC experimental conditions: Stationary phase: Organic phase; Mobile phase: Aqueous phase; Rotation speed: 800 rpm; Flow rate: 1.0 ml/min; Sample volume: 4.0 ml; Sample concentration: 20 μg /ml; Direction of motor: Reverse; S_F *(%) = 73.5.*

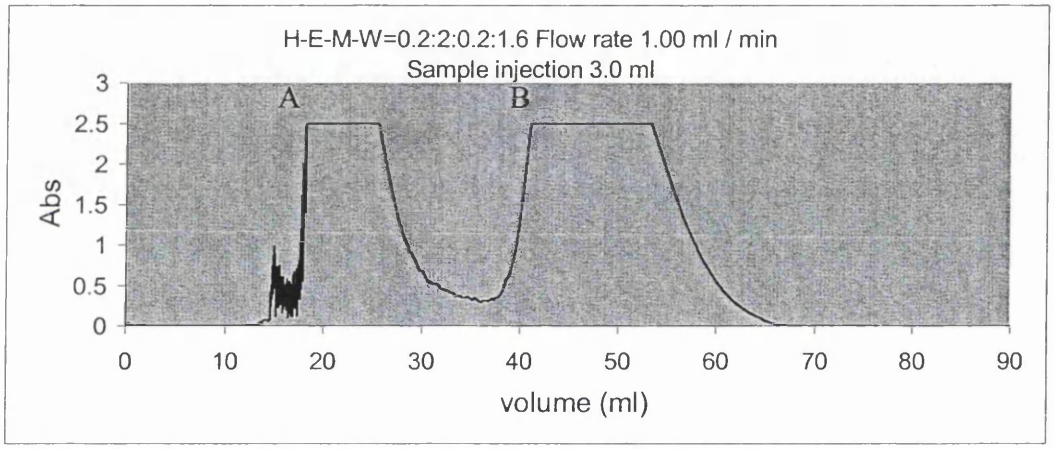

Figure 4.6 *HSCCC chromatogram of the separation of sample 2*

HSCCC conditions see Figure 4.5

When sample 2 was separated in a 94.9 ml coil volume, A and B are also resolved but component C still coeluted with A. When increasing the volume of sample injection, the resolution was not affected. These results are shown in Figures 4.5 and 4.6.

4.4.3 Separation of flavonoids of sample 2 in column volume of 49.9 ml

When the flow rate is 0.5 ml /min, the third component C is resolved from B. When increasing the flow rate, component C coeluted with component B. These results indicate that lower flow rate can effect the separation of component C. Figures 4.7 a) to d) show the effect of flow rates on the

separation.

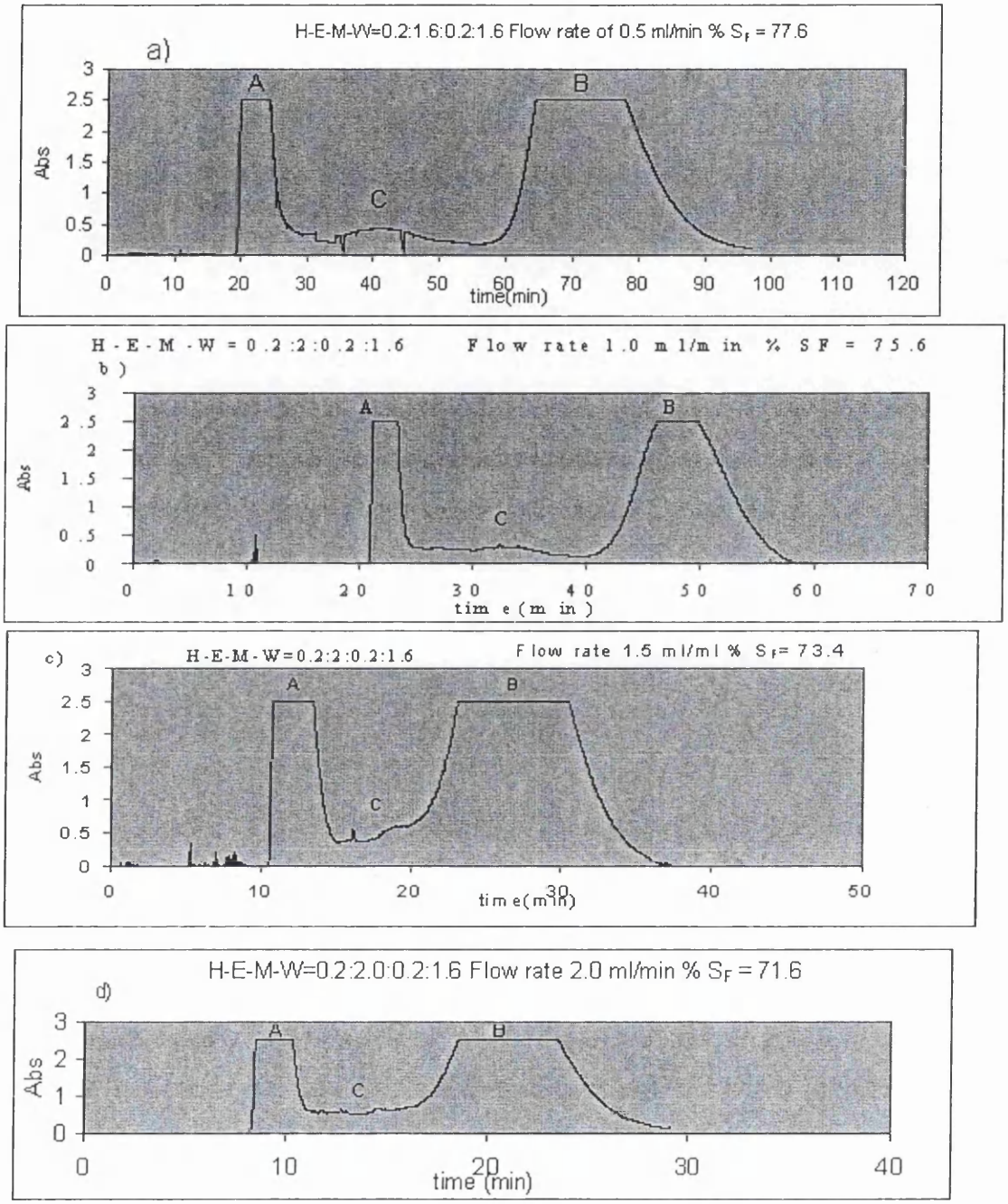

Figure 4.7 *HSCCC chromatogram of the separation of sample 2 in different flow rates*

HSCCC experimental conditions: Coil volume: 49.9 ml; Stationary phase: Organic phase; Mobile phase: Aqueous phase; Rotation speed: 800 rpm; Flow rate: 1.0 ml/min; Sample volume: 4.0 ml; Sample concentration: 20 µg /ml; Direction of motor: Reverse.

4.4.4 Separation of the flavonoids of sample 2 by milli CCC

In this Milli CCC, the whole separation can be finished in 8 minutes with good resolution of A and B (see Figure 4.8). The analytical time can compete with HPLC and very little solvent is consumed in the process. It is very useful to the selection of solvent system for preparative HSCCC.

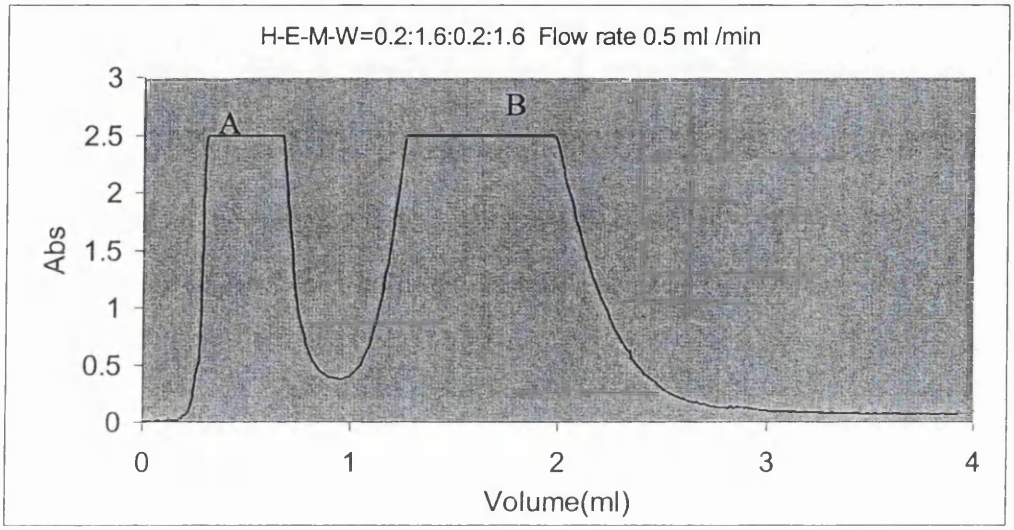

Figure 4.8 *HSCCC chromatogram of the separation of sample 2*

HSCCC conditions: Coil volume: 4.9 ml; Stationary phase: Organic phase; Mobile phase: Aqueous phase; Rotation speed: 1800 rpm; Flow rate: 0.5 ml/min; Sample volume: 200 µl; Sample concentration: 20 µg /ml; Direction of motor: Reverse; % S_F = 44.9.

4.4.5 Separation of the flavonoids of sample 1

4.4.5.1 Separation of the flavonoids of sample 1 in column volume of 49.9 ml

In this coil, the whole analytical time is 100 minutes when the flow rate is 1.0 ml / min and all components 2, 3 and 4 can be resolved very well as shown in Figure 4.9.

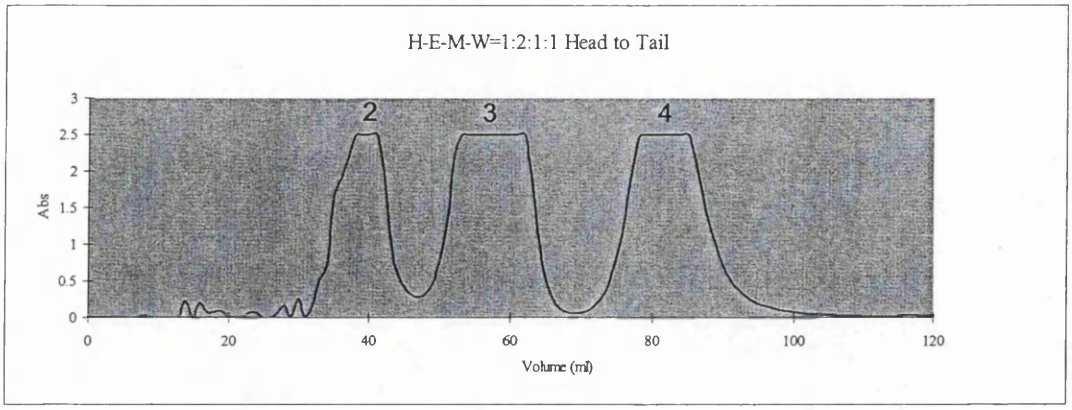

Figure 4.9 *HSCCC chromatogram of the separation of sample 1*

HSCCC experimental Conditions: Coil volume: 49.9 ml; Stationary phase: Organic phase; Mobile phase: aqueous phase; Rotation speed: 800 rpm; Flow rate: 1.0 ml/min; Sample volume: 0.5 ml; Sample concentration: 20 µg/ml; Direction of motor: Reverse; % S_F = 67.6.

4.4.5.2 Separation of flavonoids of sample 1 in column volume of 4.9 ml

Figure 4.10 shows that milli CCC separates three components in 25 min at a flow rate of 0.5 ml /min and gives very good resolution for the separation of sample 1. When increasing the flow rate to 2.0 ml /min, three components can be separated in only 6 minutes with good resolution. The whole consumption of solvent is only 30 ml. The separation time and peak symmetry can compete with that of HPLC analysis. This shows that analytical HSCCC is a powerful tool in the analytical field and more attention should be paid to its utility in research studies. When the flow rate was increased to 2.5 ml, the components 2 and 3 could not be resolved as shown in Figure 4.11.

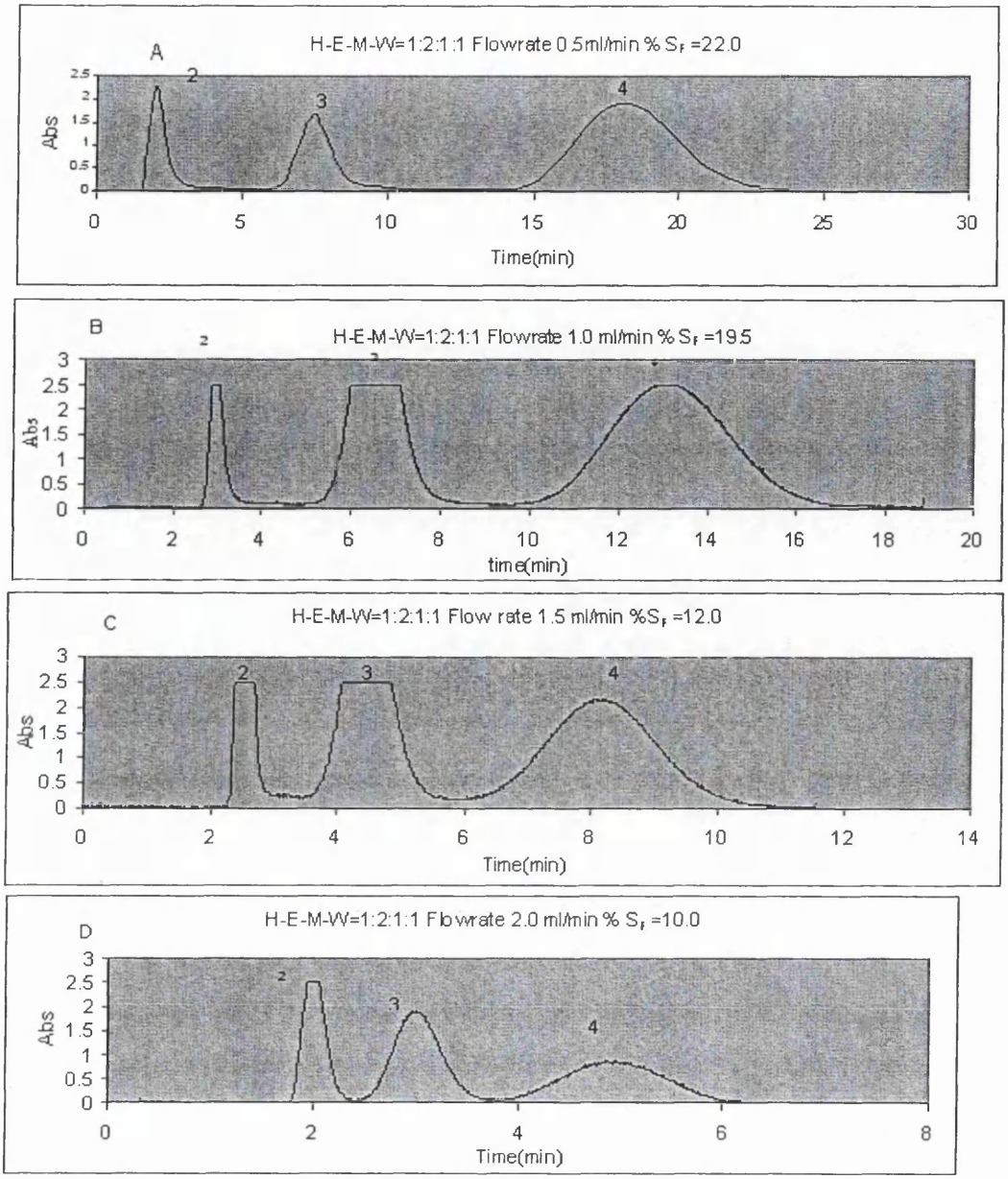

Figure 4.10 *HSCCC chromatogram of the separation of sample 1 at different flow rates HSCCC conditions see figure 4.8*

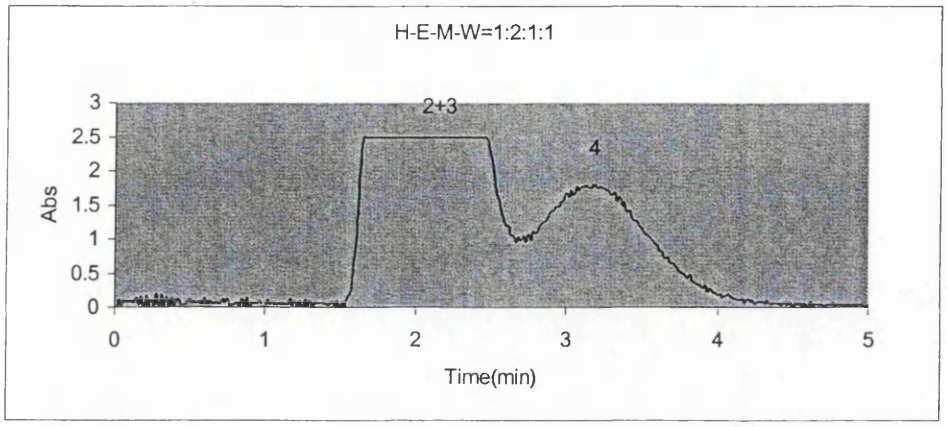

Figure 4.11 *HSCCC chromatogram of the separation of sample 1 at flow rate of 2.5 ml/min. HSCCC conditions see Figure 4.8*

4.5 COMPARISON OF PARTITION COEFFICIENCY

Our studies of different CCC from semi-preparative CCC (coil volume 169.9 ml) to analytical CCC (4.9 ml) for the separation of sample 1 and sample 2 showed that preparative and analytical CCC can gave very similar resolution. Their partition coefficients were calculated in Tables 4.2 and 4.3.

Table 4.2 *Comparison of partition coefficients K_A and K_B*

	Column volume (ml)	K_A	K_B	K_A/K_B
J-type Brunel CCC	169.9	2.09	1.31	1.79
	94.9	5.71	2.13	2.21
	49.9	2.41	0.67	3.68
Milli CCC	4.9	14.7	3.19	4.61

Table 4.3 *Comparison of partition coefficients with different column volumes*

	Column volume (ml)	K_2	K_3	K_4	$K_{2,3}$	$K_{3,4}$
J-type Brunel CCC	49.9 ml Flow rate 1.0 ml/min	1.28	0.85	0.58	1.02	1.04
Milli CCC	Flow rate 0.5 ml/min	5.88	1.32	0.53	2.44	1.83
	Flow rate 1.0 ml / min	1.68	0.72	0.36	2.18	1.45
	Flow rate 1.5 ml/min	1.24	0.68	0.39	1.68	1.21
	Flow rate 2.0 ml/min	1.21	0.80	0.48	1.30	1.26

Tables 4.2 and 4.3 show that all K values of components from sample 1 and sample 2 are the range of 0.5 – 6. The solvent system of hexane-ethyl acetate-methanol-water is a good choice for separation of the flavonoids from natural products by HSCCC.

4.6 CONCLUSION

We compared different column volumes from preparative scale to analytical instrument for the separation of the flavonoids from the seeds of Oroxylum indicum. Analytical CCC gave the same resolution as preparative CCC. Hence, analytical CCC is the best way for rapid selection of solvent system for preparative CCC. In the milli CCC, the whole consumption of solvent in run to run is only 30 minutes. This separation time can compete with that of HPLC. So, analytical CCC is a valuable analytical tool in analytical laboratories.

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CHAPTER 5

Quantitative Determination of Isoflavones in Nutrition Supplements by LC/MS and LC/MS/MS

5.1 INTRODUCTION TO PHYTOESTROGENS

5.1.1 The sources of phytoestrogens

Phytoestrogens are members of classes of polyphenolic compounds synthesized by plants. They include isoflavones and other flavonoids, lignans, coumestanes and zearalenones[1]. Figure 5.1 shows their basic structures. Phytoestrogens are found in plants and in many food supplements such as red clover, oilseeds, nuts and soybean. In contrast to the wide-ranging presence of other flavonoids in higher plants, they are primary confined to one group of plants, the sub-family of the Leguminosae [2]. Recently, isoflavones have also been found in beer and bourbon [3], indicating that their distribution in the plant kingdom may be wider than previously thought. Although phytoestrogens are found in a variety of plants, soy food including soybean, soy flour, tofu and soy drink appear to be the most abundant source among commonly consumed foods. Soy foods, in particular, are rich in a class of phytoestrogens known as isoflavones. The isoflavones are distinctive in that they possess notable biology activity. Unlike other flavonoids, most of which are harmless substances, the isoflavones have insecticidal, pesticidal and anti-fungal properties. In recent years, there has been a significant increase in the number of papers published annually on phytoestrogens. The reason for this rapidly rising interest has been the association of many phytoestrogens and other members of the polyphenol family with specific diseases or toxicity-related issues.

5.1.2 The relationship between phytoestrogen consumption and cancer prevention

Phytoestrogens, which share similar structures with steroidal oestrogens, have an ability to activate the oestrogen receptor and have been shown to exert estrogenic effects on the genital tract of female animals [4]. In the United States and other western countries, their typical diets are high in saturated fat, low in dietary fibre, and low in soy foods, whilst in the Asian nations, their diet is very high in soy foods, low in saturated fat and high in dietary fibre [5]. Epidemiological studies have shown a relationship between the consumption of soy foods and low rates of certain diseases, including coronary heart disease, hormone-dependent cancers such as breast, prostate, and colon cancer and osteoporosis [6]. Increasing evidence points out that the consumption of soy supplements might protect against heart disease and bladder cancer [7]. The social awareness of a

healthy dietary consumption has in recent years become much more apparent, since extensive nutrition research has indicated that diet plays an important factor in the genesis of disease. Soybeans and soy foods have become increasingly recognized because of the health benefits seen in the traditional Asian diet. Due to these facts, in the western countries, the range of different dietary supplements available through pharmacies, health-food shops and supermarkets, has been growing over the last decade. One group of these supplements is soy based health supplements containing phytoestrogens, which called isoflavones or soy protein. Recently, a number of studies explored the concentration of isoflavones in soy-based supplements and their metabolites in biological fluid after consumption of soy food [8, 9,10]. Many nutritional companies prefer to add some isoflavones extracted from soybean into their products.

5.1.3 Isoflavones in soybean

Soybean contains three types of isoflavone aglycones, daidzein, genistein, and glycitein, in four chemical forms [11]: aglycones themselves (daidzein, genistein, and glycitein); β-glucoside conjugates (daidzin, glycitin, and glycitin); 6"-O-acetyl-β-glucoside conjugates (6"-O-acetyldaidzin, 6"-O-acetylglycitin, and 6"-O-acetylgenistin); and 6"-O-malonyl-β-glucoside conjugates (6"-O-malonylgenistin, 6"-O-malonylglycitin, and 6"-O-malonyldaidzin). Figure 5.2 shows their structures. In soybeans, the predominant glycosidic conjugate is the 6"-O-malonyl-β-glucoside at the 7-position of isoflavones. This conjugate undergoes decarboxylation to 6"-O-acetyl-β-glucoside or hydroxylation to the β-glucoside during preparation of soy foods. The common isoflavones genistein, glycitein and daidzein and their β-glucoside conjugates are present in soybeans. Therefore, most research focussed on the determination of genistein, glycitein, daidzein and their β-glucoside conjugates.

After consumption of soy food, it is thought that the aglycone of the isoflavones can be absorbed directly from the gastrointestinal tract, where the glucoside conjugates require cleavage by intestinal bacteria to the aglycones. In addition to conjugation, genistein and daidzein, the major isoflavones in soybeans, are metabolised to dihydrogenistein and dihydrodaidzein, respectively. Generally, the major forms of these isoflavones and their metabolites in human urine are the glucuronide, sulfate and sulfoglucuronide conjugates [12]. The aglycone forms are important as they possess biological activity and the sulfate conjugates of the aglycones may also be as important as aglycones because they may be deconjugated to the aglycone form in a manner

similar to that of the oestrogen sulfates.

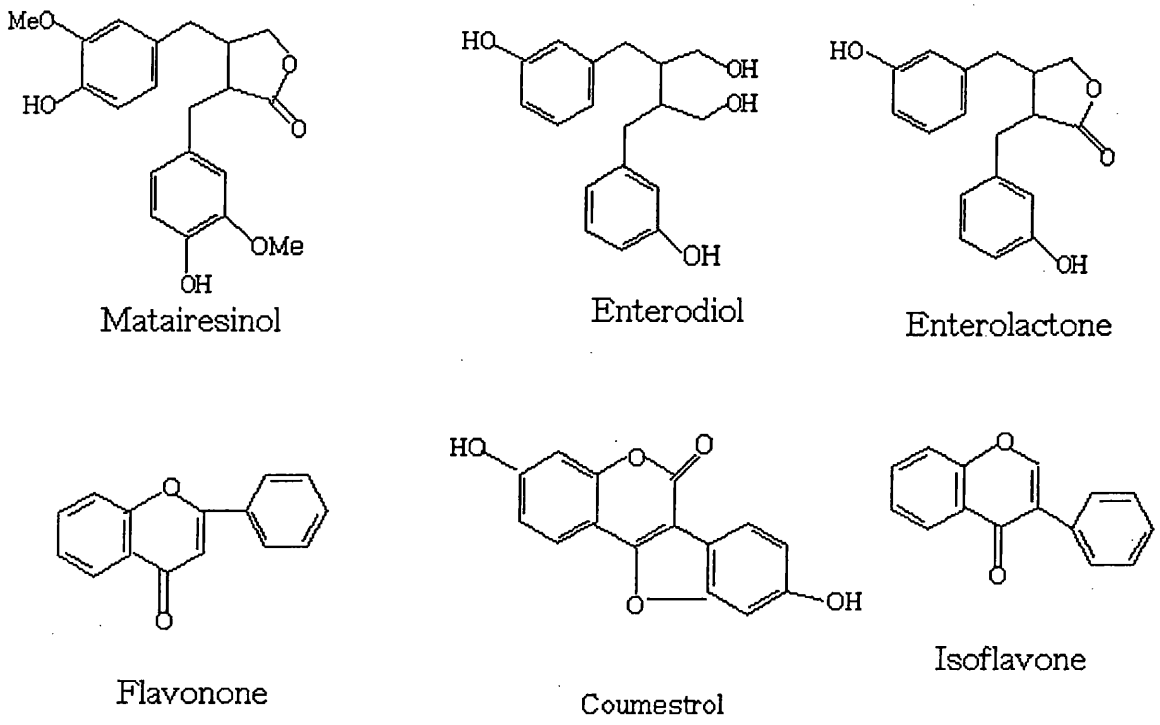


Figure 5.1 *Basic structures of Ligan, Coumestrol, Flavone and Isoflavone*

5.2 QUANTITATIVE METHODS OF THE ISOFLAVONES IN FOOD MATRICES AND BIOLOGICAL FLUIDS

There are a lot of analytical methods that can be used for the determination of phytoestrogens. The older methodology for the assay of isoflavones utilized spectrophotometry, fluorometry, or other techniques such as paper chromatography and thin layer chromatography [13]. However, since isoflavones predominately occur as glucuronide and sulphate conjugates in mammals, hydrolysis of biological samples prior to analysis have been introduced in order to reduce the complexity of matrix. In 1997, the principal method for the analysis of phytoestrogens (and other polyphenols) was gas chromatography (GC/MS) [14]. This approach required the use of a mass spectrometer coupled to the GC instrument (GC/MS) as well as chemical derivatization of the phytoestrogens. Although the technique is sensitive and accurate, the method requires time-

consuming purification, hydrolysis and the preparation of volatile derivatives, since the conjugated forms cannot be directly analysed by GC/MS. High-performance liquid chromatography (HPLC) [15, 16] has a widespread application in phytoestrogens research, and has proved to be a highly effective analytical tool. CE is a relatively new separation technique compared to other chromatographic methods. Basically, separation by CE is a result of difference in electrophoretic mobilities of charged species in an electric field in small diameter capillaries. This method can give advantages of rapid, high-resolution separation (up to 10^6 theoretical plates) with sample volumes in the nanoliter range, resulting in excellent mass detection limits. Aramendia and co-workers [17] explored the use of on-line CE/MS for separation and characterization of selected isoflavones. This method can provide fast separation of genistein, daidzein, biochaninA and isoliquiritigenin. However, pseudobatigenin, formononetin and biochaninA co-migrated in this system and although they could be resolved using CE/MS, it is still difficult for quantitation. In addition, sensitivity of this system relied on many factors and optimum analytical signal for this system was not stable which resulted in poor reproducibility and accuracy. Other non-chromatographic methods such as immunoassay techniques [18, 19] can also be used for the determination of isoflavones; however, these approaches could not resolve all isoflavones. HPLC [20-22] has a widespread application in phytoestrogens research, and has proved to be a highly effective analytical tool. HPLC combined with ultraviolet absorbance (UV) has been extensively used for the analysis of the isoflavones of coumestrol, daidzein, genistein, formonetin, and biochaninA in legumes [23, 24]. The weakness of these detection methods is their non-specificity leading to the possibility of sample matrix interference. Franke and co-workers have contributed significantly to phytoestrogens analysis using reverse phase HPLC [25-27]. This group developed good methods for the efficient extraction and quantitative analysis.

Mass spectrometry is currently the most sensitive and selective analytical method for the rapid qualitative and quantitative analysis of known compounds as well as the identification of unknown compounds from crude and partially purified samples of natural supplements [28] and has been applied to soy isoflavones [29] .Its unique ability to filter and isolate molecular ions with specific mass-to-charge (m/z) ratios from a complex mixture makes MS invaluable for analysis. In this chapter, an integrated approach consisting of HPLC, LC/MS/MS has been used for the quantification and identification of isoflavones in nutritional supplements.

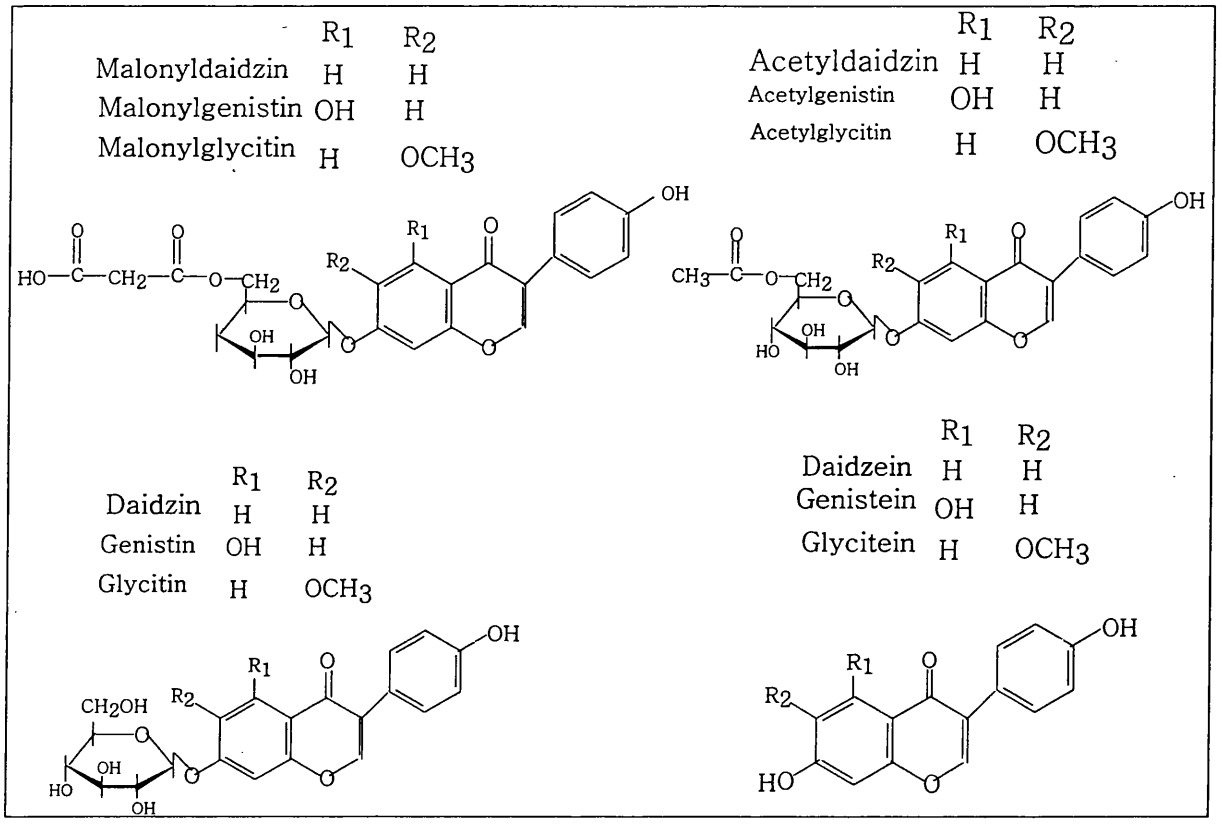


Figure 5.2 *The structures of the isoflavones in soy food*

5.3 AIMS

Most studies have quantified the isoflavone content of foods, in terms of the genistein, glycitein and daidzein content, following an acid or enzymatic hydrolysis step. More rapid and accurate quantitative methods are clearly needed regarding the isoflavone glycoside conjugates and aglycone content of both high and low soy foods and also regarding their rates of absorption, metabolism, excretion, and overall bioavailability. The content of isoflavones varies with the source of soy food and processing. For example, Japanese soy can contain a 10-fold higher proportion of the malonylglucosides to glycosides than do US soy supplements. Acetylglucosides are generally found at low levels in the intact minimally processed soybean. It is accepted that malonyl isoflavones are stable in foods but unstable in solution at high temperatures, where they will undergo decarboxylation to the corresponding acetyl ester, and that this degradation is the source of most measured acetylglucosides in soy food [30]. At room temperature the rate of degradation slows to an acceptable level, whereas heat processing, enzymatic hydrolysis, and

fermentation all alter the distribution of isoflavone forms.

The principal objective of this research was to develop a routine qualitative and quantitative method to determine daidzein, genistein, glycitein, daidzin, glycitin, genistin, 6"-O-acetyldaidzin, 6"-O-acetylglycitin and 6"-O-acetylgenistin content in selected high and low isoflavones in nutrition supplements. A successful method for the quantitative determination of 9 isoflavones was developed by on line LC/APCI/MS. The extraction and hydrolysis method for the isolation of the isoflavones from three nutrition supplements was also studied.

5.4 EXPERIMENTAL

A Hewlett Packard 1100 HPLC system was interfaced to a LCQ Ion Trap Mass Spectrometer (Finnigan MAT, San Jose, CA.USA).

5.4.1 Chemicals and standards

HPLC grade solvents of methanol, water, ethyl acetate, formic acid, acetic acid and acetonitrile were purchased from Fisher Chemicals (Loughborough, UK). Genistein, daidzein, biochaninA were obtained from Aldrich. Genistin, glycitin and daidzin were obtained from Indofine (Somerville, NJ, USA) and acetyldaidzin, acetylglycitin and acetylgenistin were purchased from Plantech (Reading, UK). All solvents were filtered using a vacuum filtration system (Millipore) through a 0.45 μm nylon membrane filter (Altech Associates Inc., Lancashire, UK), and degassed prior to use. Gases used included oxygen free nitrogen (OFN), helium and air, which were purchased from BOC Ltd. (Surrey, UK).

A stock standard solution was prepared by accurately weighing, on a five-place analytical balance to the nearest 10.00-mg. 10.00 mg daidzin, 10.00 mg glycitin, 10.00 mg genistin, 10.00 mg daidzein, 10.00 mg genistein and 10.00 mg glycitein were separately dissolved in 50 ml methanol to give a final concentration of 200 μg/ml, respectively. The solution was stable for at least 2 months at room temperature. This solution was used in recovery experiments with soy sample and to prepare working standards. Acetylglucoside isoflavones were dissolved freshly into DMSO prior to use. All standards were kept in the ultrasonic bath for 10 min to confirm complete solution. The quantitation standard was appropriately diluted with methanol and stored at 0°C and

biochanin A was used as internal standard. Acetyl isoflavones were available in limited quantities and not stable. Calibration standards of acetyl forms were always prepared just prior to use and were diluted in a single step to final concentration.

5.4.2 HPLC separation conditions and quantitative parameters

5.4.2.1 Optimisation conditions of the isoflavones in soybean nutrition supplements

Generally, sodium acetate buffer and triethylamine acetate buffer can be used for separation in HPLC. However, these buffer solutions are not suitable for HPLC/MS on line separation. In this case, formic acid or acetic acid can be used in the mobile phase. HPLC was performed with a Hewlett-Packard autosampler, gradient pump and an HP1100 photodiode-array detection system (Hewlett-Packard, Wilmington, DE, USA). UV detection at 275 nm was applied.

Separation was achieved with two RP analytical columns; one was a Luna C_{18} RP column (100 × 4.6 mm I.D, 3 μm stainless steel Phenomena) using gradient elution with temperature of 30°C, flow rate of 0.2 ml/min, the other was a Luna C_{18} RP column (150 × 4.6 mm, 5 μm, Phenomenex). Gradient elution was also used.

5.4.2.2 Extraction procedures

Extraction of isoflavones from soy foods is a difficult challenge. Extraction of the isoflavone from soy supplements has commonly been achieved with a hot methanol and water mixture with different ratios of volume. Eldridge [35] reported complete extraction by stirring soy samples in aqueous methanol for 4 h at 60°C. After removal of the solid residue by filtration, the filtrate was directly assayed for isoflavones by HPLC without further sample treatment. But this method results in complete conversion of 6"-O-malonyl and 6"-O-acetyl-isoflavone-β- glycosides to β -glucoside and aglycone forms. Wang and Murphy [32] utilized acidified acetonitrile at room temperature for extraction of isoflavones from foods, but follow this step with rotary evaporation at less than 30°C to remove the acidified acetonitrile followed by dissolution in 80 % (v/v) methanol. This laborious procedure makes extraction and analysis of large numbers of samples difficult. In this study, a lot of different solvents were studied to extract the samples and an

optimised extraction was obtained.

Approximately 0.2000 g of soy nutrition supplement was accurately weighed into a screw cap 20 ml test tube. 4 ml of water was added to the sample and swirled to suspend, then 16- ml volume of acetonitrile was added and shaken briefly to mix. The sample tube was extracted for 40 minutes on a sonic bath at 25°C. After 40 minutes, 1 ml of samples were centrifuged for 10 min at 2000 g to pellet insoluble matter and eliminate foam. A portion of the supernatant was removed with a syringe, filtered through a 0.45 μm PVDF filter into a sample vial and diluted with 80 % acetonitrile to 1 ml and analysed by HPLC. This extraction procedure presented here minimized handling of the samples during preparation. The sample was extracted directly by 80 % acetonitrile and transferred to the HPLC system without an evaporation procedure.

5.4.3 LC/MS method

5.4.3.1 LC/ESI/MS method

Gradient elution was generally used and mixtures of acetonitrile-acetic acid or acetonitrile-formic acid were used for the mobile phase. In our study, a rapid LC/ESI/MS method utilized a Luna RP C_{18} column, 100 × 2.0 mm with 3 µm packing. Injection volume was 5 μl. Solvent A was 0.1 % (v/v) acetic acid and solvent B was acetonitrile. The flow rate was 0.2 ml/min in order to transfer eluant to the mass spectrometer in the ESI scan mode. Column temperature was held constant at 35 °C and the gradient was started immediately upon injection of the sample. LC/ESI/MS analysis was done on an LCQ ion trap mass spectrometer. The LCQ was used in the negative ion mode with electrospray ionisation (ESI). The heated capillary temperature was 190°C and the electrospray voltage was 4.5 kV. The system was calibrated according to manufacturer instructions and was tuned using the calibration solution.

5.4.3.2 LC/APCI/MS method

A Luna RP C_{18} column, 150 × 4.6 mm with 5 μm packing was used with injection volume of 10 µl. Solvent A was 0.1 % (v/v) acetic acid, Solvent B was acetonitrile and the flow rate was 0.8-ml/min. The entire column flow was directed into the MS system and the column temperature was maintained at 35°C. The column was held constant at 12 % B for 10 minutes of the run, followed by a two-step linear gradient, to 30 % B over 30 min, then to 90 % B over 2 min. The column was

washed at 90 % B for 6 min and equilibrated 10 min. Total sample to sample run time was 58 minutes. Detection was by UV absorbance at 260 nm and LC/APCI/MS analysis was performed on an LCQ ion trap mass spectrometer in the negative ion mode. The vaporizer temperature was 500°C, sheath gas flow rate was 90 arbitrary, the discharge current was 5.0 V, Capillary temperature was 170°C, the capillary voltage was –30 kV and the electrospray voltage was 4.5 kV.

5.4.3.3 Recovery experiment

Three samples of the soy nutritional supplements, each spiked with known quantities of reference standards in low, middle and high concentrations were extracted according to the same extraction method. The isoflavones in these six samples were determined by LC/MS. The ratio of the found to the expected isoflavone contents in the spiked samples was the recovery efficiency of the extraction.

5.5 RESULTS AND DISCUSSION

5.5.1 Chromatographic conditions and quantitation

Reversed-phase high performance liquid chromatography (HPLC) with UV detection has been the method of choice for isoflavone analysis. Liquid chromatography/mass spectrometry (LC/MS) [33, 34] has also been widely used, especially for clinical studies of isoflavone metabolites in animals and humans. However, there are few reports on simultaneous quantitative analysis of all isoflavones in soy based nutritional supplements by LC/MS. Simultaneous separation of genistein (GE), genistin (G), daidzin (D), daidzein (DE), glycitin (GL) and glycitein (GLE) by HPLC has been achieved with gradient elution [35-37] and isocratic elution [38], but the separation time was long. In order for simplicity and rapidity of separation, optimisation of the extraction and separation method was investigated. Figure 5.3 shows the HPLC separation from a mixed gradient elution programme.

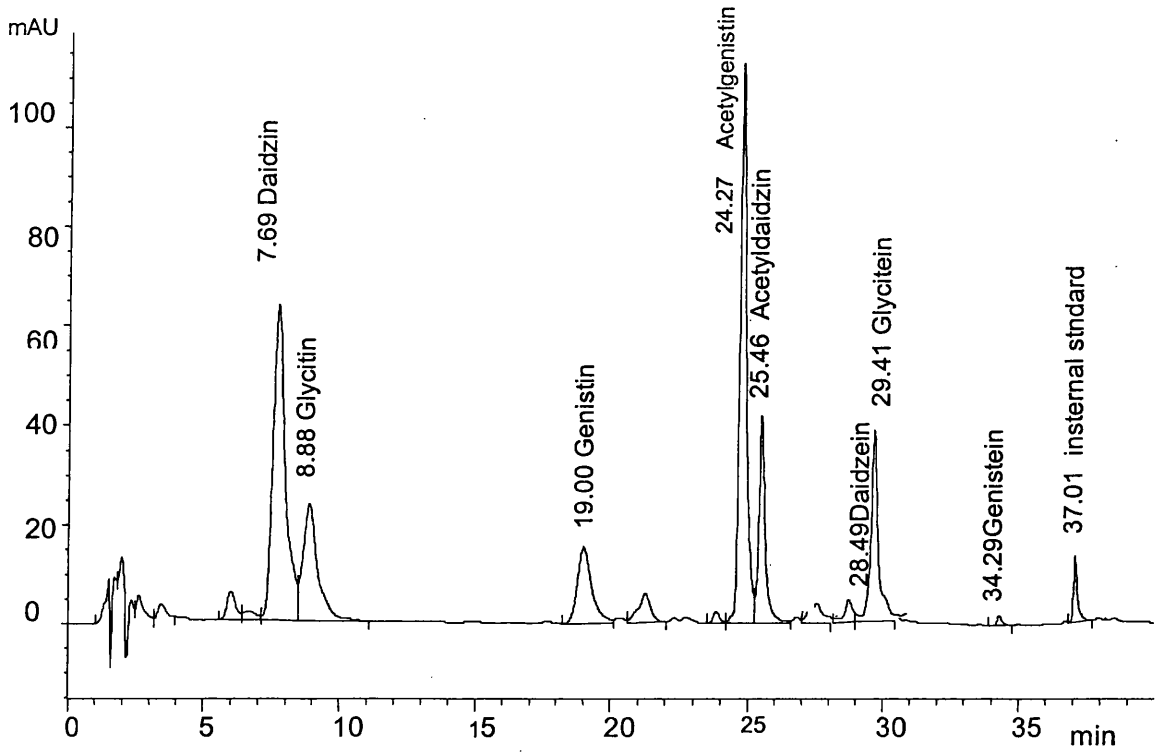


Figure 5.3 *LC Chromatogram of a soy nutritional supplement (BiochaninA as internal standard)*

Experimental conditions: Reverse column; Luna C_{18} (3 μm), 100 × 4.6 mm).

Table 5.1 *Gradient elution programmes used in Figure 5.3*

Time (min)	A %	B %	Flow rate (ml/min)
0	85	15	0.2
10	85	15	0.2
28	65	35	0.2
32	20	80	0.2
40	20	80	0.2

A: 1 % acetic acid. B: Acetonitrile

Figure 5.3 shows that this gradient elution programme could resolve all the isoflavones present in soy supplement, but daidzin and glycitin, acetyldaidzin and acetylglycitin partially overlapped and made it inaccurate for quantitative determination. Another gradient elution programme was also explored for separation and quantitation of isoflavones by LC/APCI/MS. Figure 5.4 shows the separation results obtained by this method.

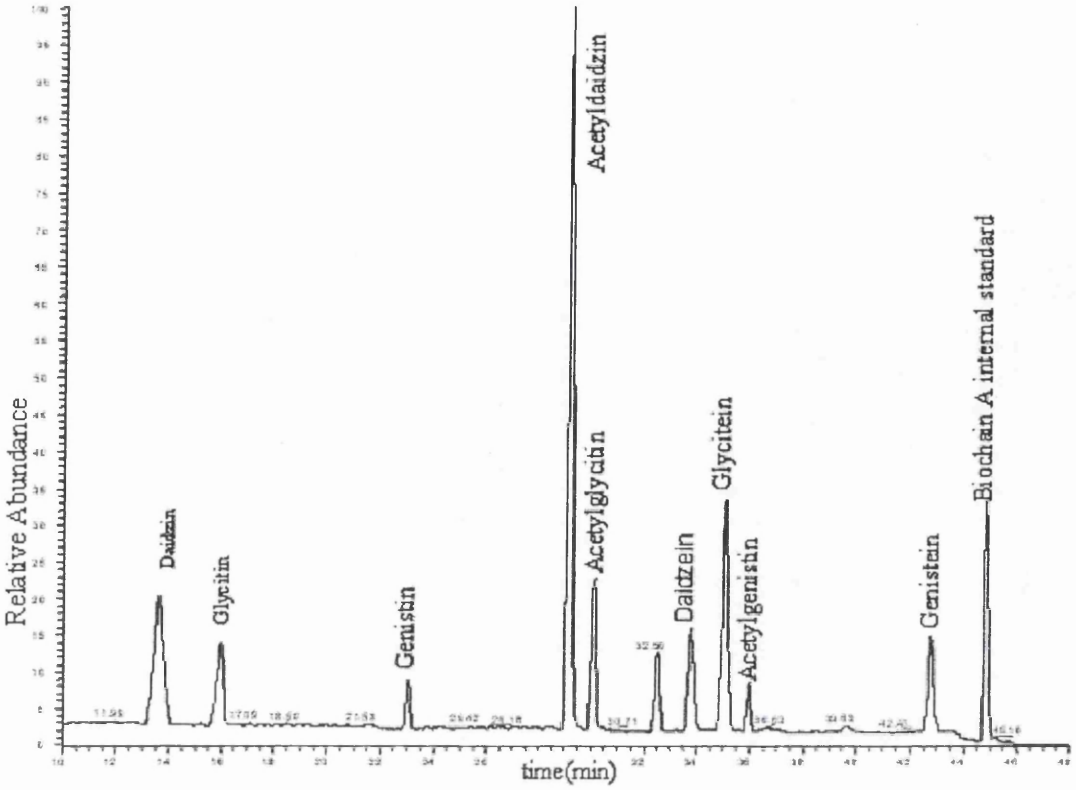


Figure 5.4 *LC -APCI-MS Chromatograms of 9 standard isoflavones*

Mass spectrometry conditions: Vaporizer temperature: 500°C; Sheath gas flow rate: 90 arbitrary; Discharge current: 5.0 V; Capillary temperature: 170°C, Capillary voltage: –30 kV; the electrospray voltage: 4.5 kV. HPLC experimental conditions: Reverse column; Luna C_{18} (5 μ), 150 × 4.6 mm.

Table 5.2 *gradient elution programmes in LC/APCI/MS*

Time (min)	A %	B %	Flow rate (ml/min)
0	88	12	0.8
10	88	12	0.8
40	65	35	0.8
42	10	90	0.8
48	10	90	0.8

A : 0.1 % acetic acid (A) and B: acetonitrile

Figure 5.4 shows that negative LC/APCI/MS can provide good resolution of all the isoflavones. On the other hand, the entire column flow was directly into the mass spectrometer without a split of flow rate and a stable flow rate was guaranteed in the analysis process. In order to get accurate

quantitative results, negative LC/APCI/MS was employed to our studies. Acetonitrile and acetic acid were usually chosen to be the mobile phase for the isoflavone separation. Isocratic and gradient elution were explored in our study. Isocratic elution did not resolve all 9 isoflavones due to their large range of polarities. So, in our study, gradient elution was used for the separation in the nutritional supplements.

5.5.2 Improved extraction procedure

5.5.2.1 Comparison of different extraction methods

Acetonitrile, ethanol and methanol were evaluated for their efficiency in the extraction of isoflavones from soy nutrition food. Extractions were carried out with and without the addition of hydrochloric acid. 0.2 g of supplement 1 was extracted with the same method as 5.4.2.2. 80 % acidified ACN means that 4 ml 0.1 mol/L acetonitrile was used instead of 4 ml water in 5.4.4.4. 80 % acidified MeOH or EtOH mean that 4 ml 0.1 mol/L methanol or ethanol was used instead of 4 ml water in 5.4.2.2. Table 5.3 shows the results from different solvents for the extractions of isoflavones from nutrition supplement sample 1.

Table 5.3 Comparison of different solvent for extraction of isoflavones *

(%, V/V)	D	GLE	G	AD	AGL	DE	GLE	AG	GE
80 %ACNMean (μg/ml)	19.0	9.7	11.0	39.2	13.8	1.2	1.9	13.5	0.3
Acidified (80%)ACN (Mean) μg/ml	17.2	8.7	11.6	38.3	13.4	1.3	1.9	13.1	0.3
Acidified(80%)MeOH Mean(μg/ml)	16.3	7.9	10.6	37.4	13.3	1.2	1.9	12.6	0.3
80%MeOH (Mean) μg/ml	17.6	8.8	11.9	39.9	14.0	1.2	1.8	13.5	0.3
Acidified(80%)EtOH (Mean) μg/ml	17.3	8.6	10.7	38.5	13.3	1.2	1.8	13.2	0.3
80 % EtOH (Mean) μg/ml	17.4	8.7	10.8	38.6	13.5	1.2	1.8	13.4	0.3

* *Single analysed of soy nutrition supplement extracted at the indicated concentration and D: daidzin, AD: acetyldaidzin, DE: daidzein, GL: glycitin, AGL acetyglycitin, GLE: glycitein, G: genistin, AG: acetylgenistin, GE:genistein*

Table 5.3 indicates that acetonitrile as an extraction solvent can obtain high concentration of the isoflavones, so acetonitrile was chosen to be the extraction solvent. An advantage was that the sample could be directly subjected to LC/MS without evaporation process because the acetonitrile was chosen as the mobile phase.

5.5.2.2 The effect of ultrasonic extraction time on the extraction efficiencies

Our studies indicated that the ultrasonic bath extraction of the sample can greatly shorten the time of extraction but the temperature was controlled under 25°C in order to avoid deesterification to β-glucoside from acetyl glucoside form. Table 5.4 shows the effect of ultrasonic time on the extraction of isoflavones. The amounts of sample and extraction method see 5.4.4.4

Table 5.4 *Effect of ultrasonic time on efficiency of extraction of isoflavones**

Ultrasonic time (min)	Daidzein	Glycitin	Genistin	Acetyl daidzin	Acetyl glycitin	Daidzein	Glycitein	Acetyl genistin	Genistein
10	11.97[a]	7.110	3.257	38.3	13.4	0.350	0.257	13.1	0.043
20	12.17	7.386	3.327	37.3	13.3	0.362	0.258	12.9	0.045
40	12.44	7.413	3.676	37.2	13.4	0.358	0.251	12.8	0.044
60	15.77	8.606	4.312	28.9	10.2	0.531	0.300	9.5	0.06
120	15.70	8.654	4.157	25.4	9.2	0.541	0.340	8.7	0.065
240	16.62	8.870	4.478	19.6	7.1	0.593	0.405	7	0.107
Overnight	21.68	13.09	5.202	0	0	1.314	0.844	0	0.220

* Single analysed of soy nutrition supplement extracted at the indicated ultrasonic time

[a] The mean concentration of isoflavone (μg/ml) at the indicated ultrasonic time in triplicate times

Table 5.4 indicates that the concentrations of isoflavones are maintained constant in 20-40 minutes of ultrasonic time. Too long a ultrasonic time will result in the deesterification of acetyl isoflavones due to the increase of temperature during the sonic time. In order to avoid the hydrolysis of acetyl isoflavones, the sample should be maintained at room temperature in throughout the period.

Table 5.5 *Effect of ACN concentrations on efficiency of extraction of isoflavones* *

ACN (%, v/v)	Daidzein	Glycitin	Genistin	Acetyl daidzin	Acetyl glycitin	Daidzein	Glycitein	Acetyl genistin	Genistein
40	13.5	5.4	8.3	32.1	10.8	1.1	1.9	11.3	0.3
50	14.3	7	9.4	33.3	11.7	1	0.8	11.2	0.3
60	16.1	7.8	10.6	37.4	13.3	1.2	1.9	12.6	0.3
70	16.7	7.6	11.2	37.9	13.2	1.2	1.8	12.9	0.4
80	17.6	8.8	11.9	39.9	14	1.2	1.8	13.5	0.4
90	15.7	7.9	10.4	37.2	13.1	1.2	1.8	12.8	0.3

* *Single analysis of soy supplement extracted at the indicated acetonitrile concentration. Results are concentrations of isoflavone at individual retention times*

5.5.2.3 The effect of refluxing time on the extraction efficiencies

Most papers reported the extraction of isoflavones by refluxing sample in acidified methanol. All acetyl and glucoside isoflavones were converted into aglycone isoflavones. In our study, hydrolysis of isoflavones was explored and genistein were found to be partly decomposed if extraction time was more than 60 minutes. Table 5.6 showed the effect of refluxing time on the concentration of isoflavones.

Table 5.6 *The effect of refluxing time on the concentration of isoflavones**

Refluxing time (min)	Daidzin	Glycitin	Genistin	Acetyl daidzin	Acetyl glycitin	Daidzein	Glycitein	Acetyl genistin	Genistein
10	19.34	331.5	312.3	17.63	1.670	8.680	0.605	1.727	0.085
20	19.36	332.5	322.2	17.14	1.638	0.874	0.604	1.735	0.091
40	17.43	265.3	301.9	5.511	0.507	1.600	0.929	0.386	0.132
60	17.62	224.7	294	1.678	0	3.557	1.654	0	0.265
120	9.081	59.3	151.8	0	0	11.03	3.133	0	0.622
180	1.656	0	12.5	0	0	15.72	3.602	0	0.523
240	0.120	0	0	0	0	17.71	3.696	0	0.325

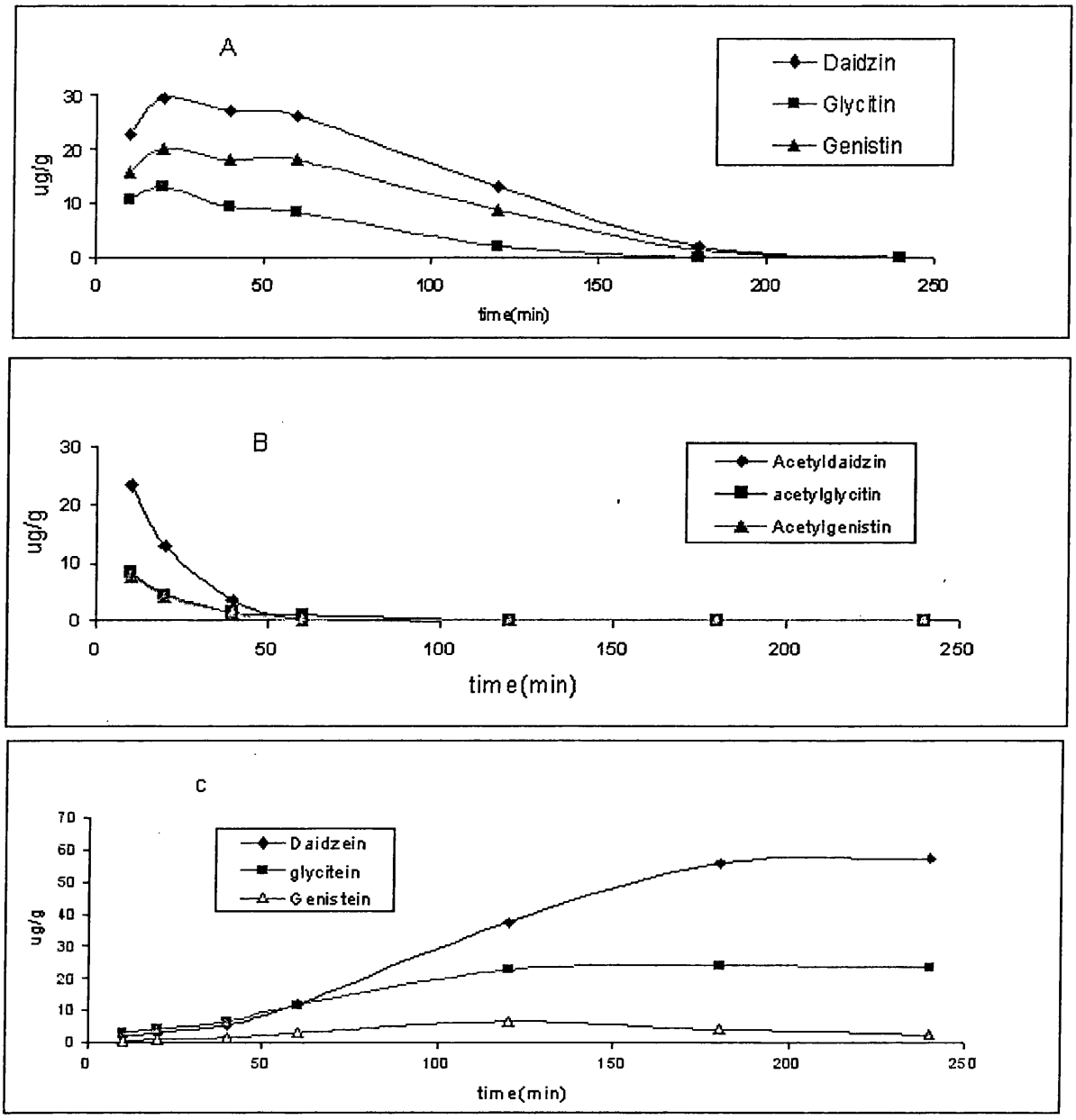


Figure 5.5 *The concentration of isoflavones against the refluxing time*

Figure 5.5 clearly shows that the concentrations of isoflavones in soy supplement attained the largest values after refluxing for 10 min, and that the concentrations of acetyl isoflavones decreased with increased refluxing time. At the same time, the concentrations of glucoside isoflavones decreased slowly. These results show that the acetyl isoflavones converted to their glycosides and aglycones simultaneous. After refluxing 60 min, all the acetyl isoflavones convert into their glycosides and aglycones and increasing the refluxing time resulted in the conversion of glycosides into aglycones. About refluxing 4 hrs, all glycoside isoflavones converted to aglycones. One important phenomenon is that the aglycone became unstable when the refluxing time was increased. We can see from Figure 5.5 B that the concentration of genistein decreased when

refluxing time was 4 hours. Hence, the hydrolysis time should be considered carefully.

5.5.3 Recoveries determination

The recoveries of daidzin, glycitin, genistin, daidzein, glycitein and genistein were measured by the addition of the 6 standard isoflavones into the soy based nutrition supplement covering a range of concentrations of 0.5 to 6 μg/ml. The spiked samples were then processed through the whole extraction procedure. Their concentrations were determined by their individual standard curve. Table 5.7 shows the results of the recoveries.

The method showed good recoveries that were close to 100 % and the mean recoveries of the individual standards ranged from 102.7 to 111.0 for daidzein, 95.27 to 108.1 for glycitein. 97.27 to 104.0 for genistin, 93.50 to 100.4 for daidzin, 92.36 to 101.7 for glycitin and 94.67 to 101.9 for genistein. The higher recoveries (>100 %) were found for lower amounts of spiked standards. This was probably due to some impurities co-eluting in the nutrition food that were significant at the lower spiked levels.

Table 5.7 *Recoveries of 6 isoflavones*

Recoveries(%)	Daidzein	Glycitein	Genistin	Daidzin	Glycitin	Genistein
2	109.0	99.98	97.27	100	101.7	98.65
2	111.0	101.4	99.95	100.4	92.36	94.67
2	101.4	104.2	101.6	102.5	94.63	102.4
2	99.85	102.5	98.45	97.67	96.84	99.87
2	104.5	107.8	97.43	98.45	102.4	100.4
Mean(n=5)	105.15	103.176	98.94	99.804	97.586	99.20
SD	4.79	3.01	1.83	1.87	4.38	2.87
RSD(%)	4.55	2.92	1.85	1.88	4.49	2.89
1	102.7	95.27	102.7	93.64	93.27	101.0
1	105.0	105.2	104	93.5	98.08	99.69
1	106.4	105.3	100.9	99.36	98.51	99.25
1	106.7	108.1	100.4	99.84	97.51	101.9
1	110.0	106.3	103.5	103.5	102.7	103.5
Mean(n=5)	106.2	104.0	102.3	97.97	98.01	101.1
SD	2.67	5.04	1.59	4.32	3.35	1.72
RSD(%)	2.51	4.84	1.55	4.41	3.42	1.70
3	102.3	98.45	97.86	96.33	94.85	96.42
3	105.8	105.8	100.6	98.74	96.65	97.32
3	106.8	103.1	100.2	98.99	100.8	100.9
3	102.8	100.4	98.42	97.46	98.33	95.43
3	104.3	102.8	96.33	101.3	95.33	99.45
Mean(n=5)	104.4	102.1	98.68	98.56	97.19	97.90
SD	1.92	2.80	1.75	1.86	2.43	2.24
RSD (%)	1.84	2.74	1.77	1.89	2.50	2.29

1: Low concentration added; 2: Middle concentration added; 3: High concentrations

5.5.4 Reproducibility

The repeatability and reproducibility of the method was evaluated by carrying out six replicate determinations on the same day and six on three different days. Inter-assay relative standard deviation (RSD) and intra-assay relative standard deviation are shown in Table 5.8.

Table 5.8 *Reproducibility of HPLC analysis of the soy nutrition supplement*

Isoflavone	Concentration (mg/g)					
	Within-day			Between-days		
	Mean*	SD	RSD	Mean	SD	RSD
Daidzin	20.79	0.458	1.87	21.33	0.70	4.19
Glycitin	14.78	0.565	3.82	14.92	0.32	9.09
Genistin	6.73	0.086	1.27	7.10	0.26	7.35
Acetyldaidzin	58.46	0.394	0.67	59.16	1.70	2.87
Acetylglycitin	5.37	0.064	1.19	5.37	0.14	2.67
Daidzein	0.827	0.086	10.39	0.62	0.072	11.6
Glycitein	0.369	0.014	3.79	2.35	0.07	2.83
Acetylgenistin	6.98	0.090	1.29	6.95	0.19	2.74
Genistein	0.154	0.008	5.19	2.37	0.05	1.93

* Mean value of n=6

Table 5.8 shows that repeatability and reproducibility are obtained by our method. All isoflavones gave good repeatability and reproducibility but daidzein. Daidzein is the most hydrophobic isoflavone in all isoflavones with low concentration. So, the quantitative determination should be done in three days after sample treatment in order to obtain good reproducibility. Hence, this method can be used for quantitative determination.

5.5.5 LC/APCI/MS studies of the nutrition supplements

Both LC/APCI/MS and LC/ESI/MS were studied for the separation of the isoflavones using a Finnigan MAT LCQ ion trap, where the sources were principally operated in the negative ion mode. For completeness, positive ion modes were also explored, but the negative ion ionisation methods were deemed superior. Negative LC/ESI/MS gave lower sensitivities only for the acetyl isoflavones compared to negative LC/APCI/MS but the split had to be used to lower the flow rate

into the mass spectrometer and the accurate flow rate cannot be guaranteed. In this study, LC/APCI/MS in the negative ion mode was used for qualitative and quantitative determination of the isoflavones. Prior to LC/APCI/MS analysis, parameters such as the source voltage, the sheath and auxiliary gas pressures, the heated capillary temperature, the vaporizer temperature, discharge current, electrospray voltage, and the tube lens offset voltage, were optimised by loop injection of a standard solution of isoflavones at 0.8 ml/min into the APCI source.

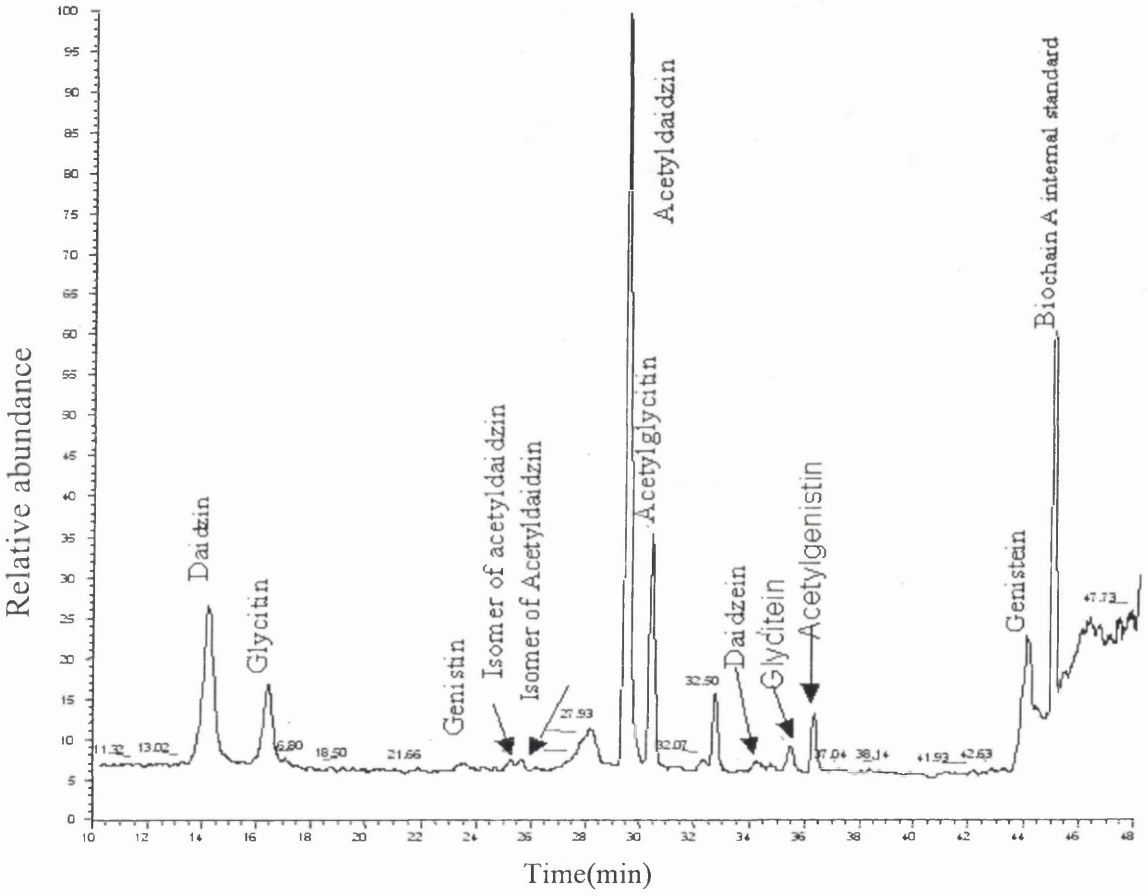


Figure 5.6 *TIC chromatogram of one soy supplement by negative LC/APCI/MS*

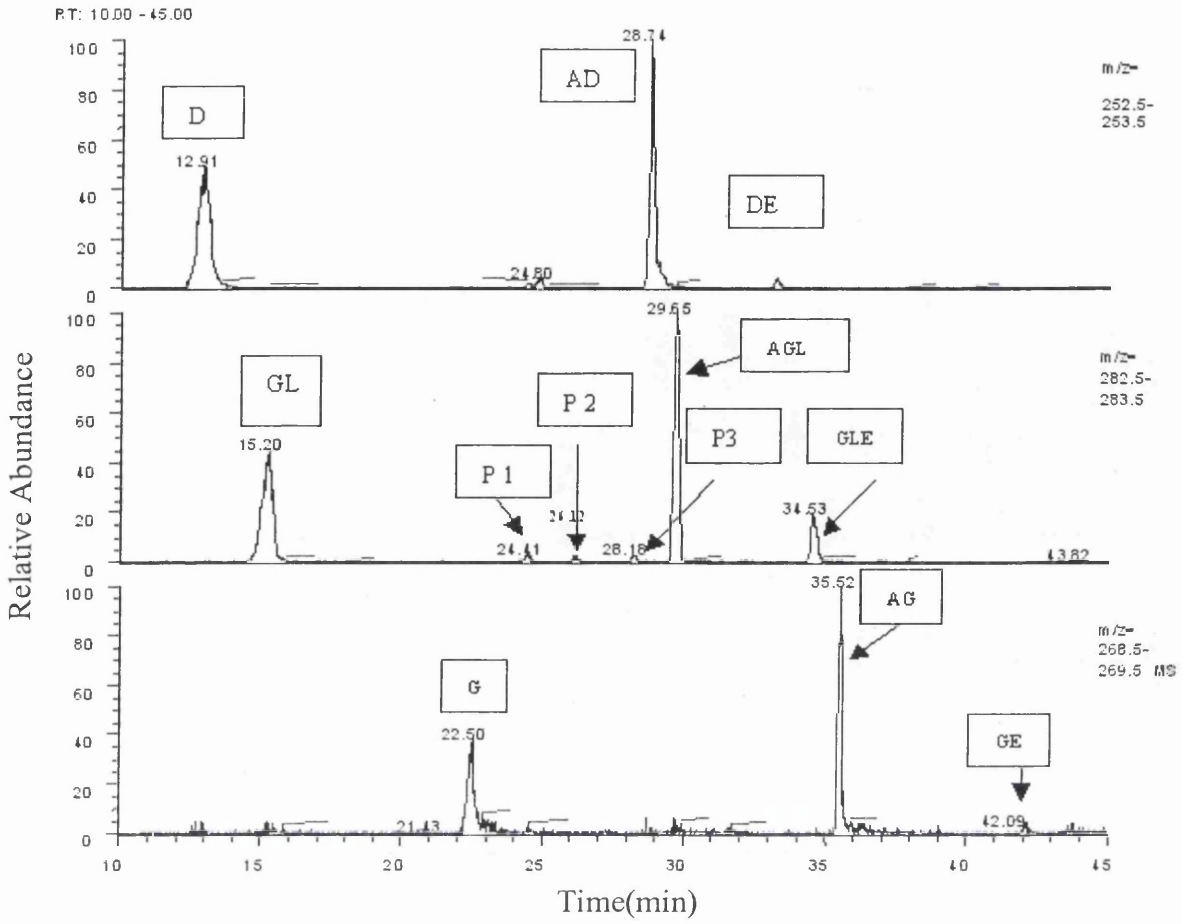


Figure 5.7 RIC *chromatogram for all the isoflavones of one nutrition supplement*

D: daidzin, AD: Acetyldaidzithn, DE: daidzein, GL: glycitin, AGL: acetyglycitin, GLE: glycitein, G: genistin, AG: acetylgenistin, GE: genistein, P1, P2, P3: unknown components. Mass spectrometer conditions see Figure 5.4

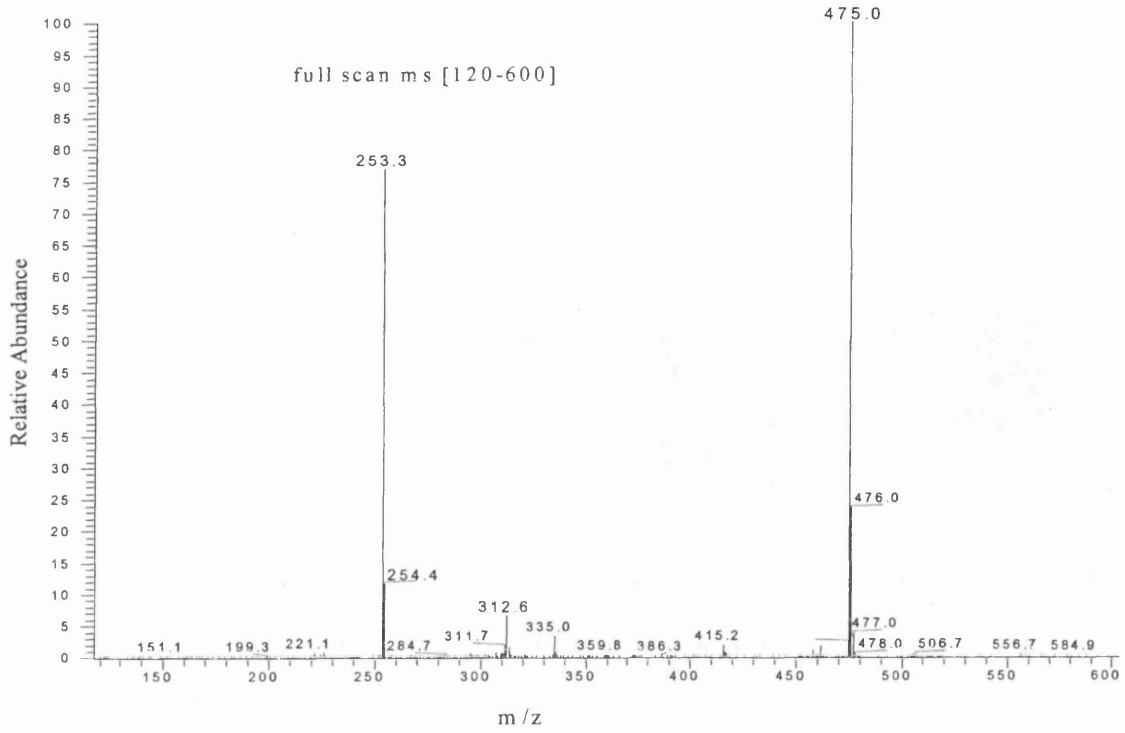


Figure 5.8 *Negative mode LC/APCI/MS mass spectrum of D (daidzin)*

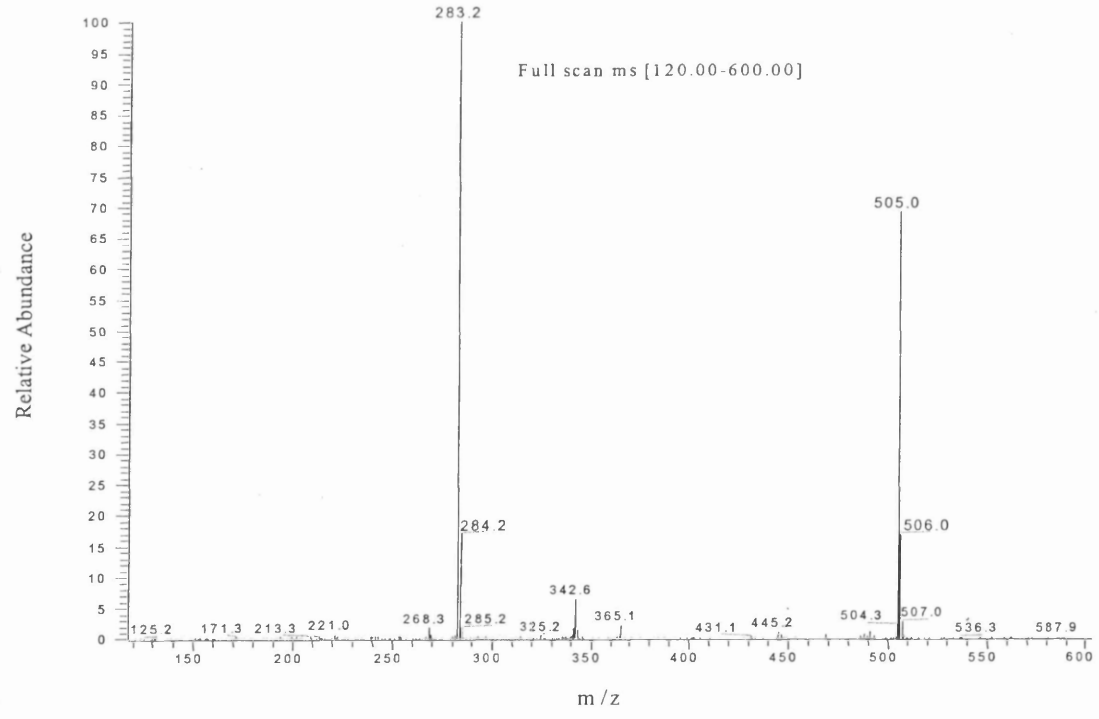


Figure 5.9 *Negative mode LC/APCI/MS mass spectrum of GL (glycitin)*

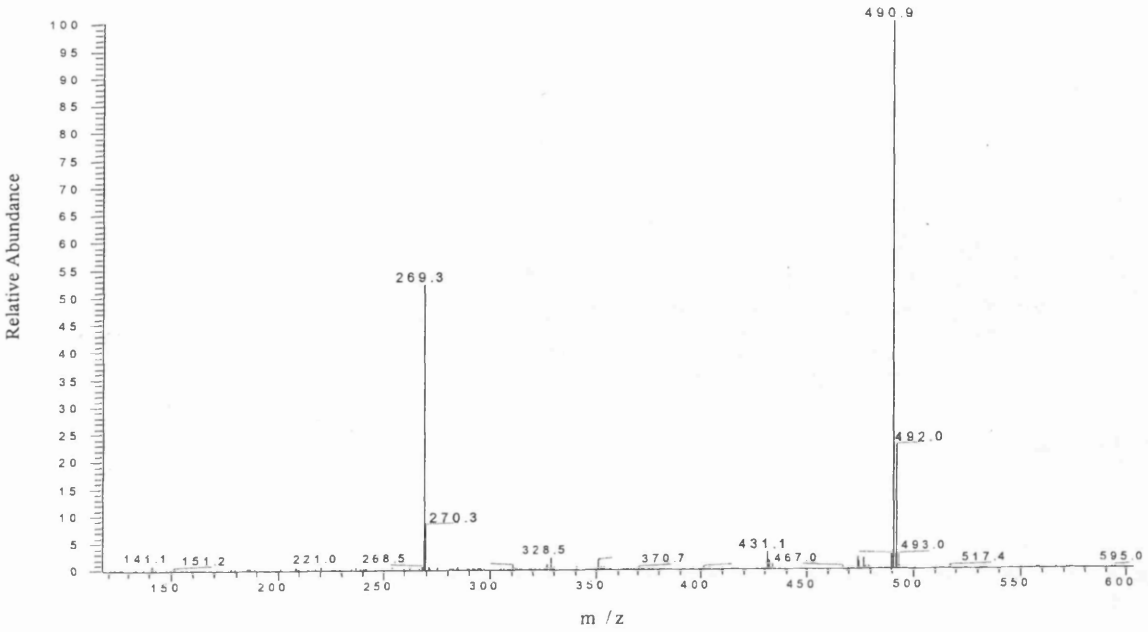


Figure 5.10 *Negative LC/APCI/MS mass spectrum of G (genistin)*

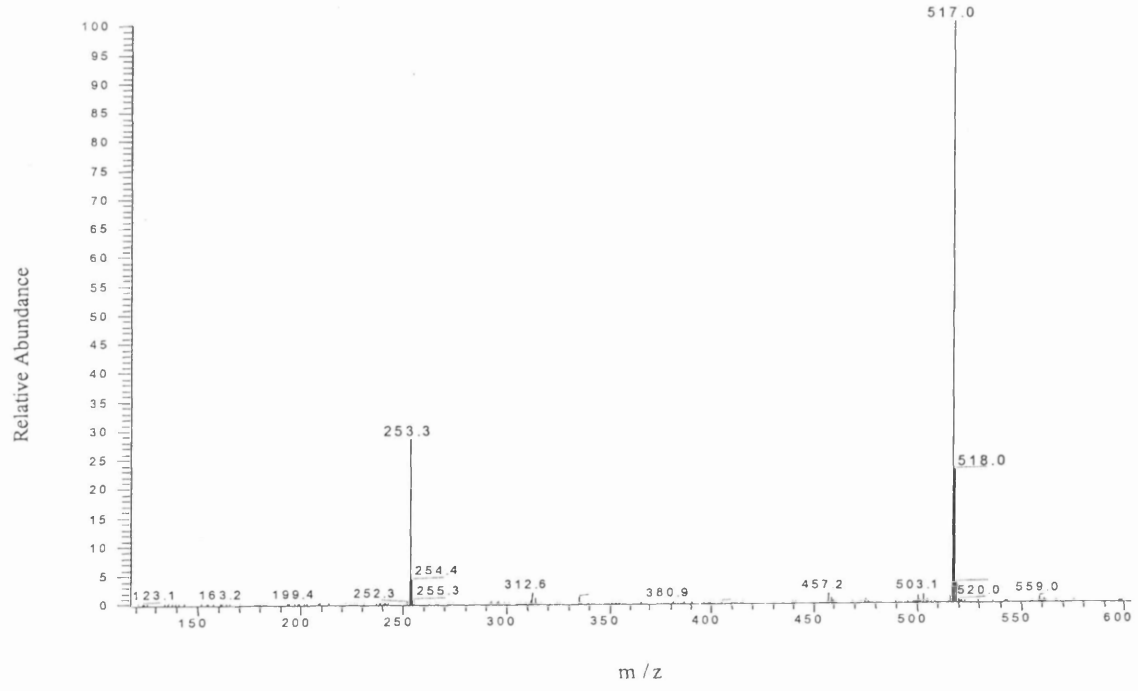


Figure 5.11 *Negative LC/APCI/MS mass spectrum of AD (acetyldaidzin)*

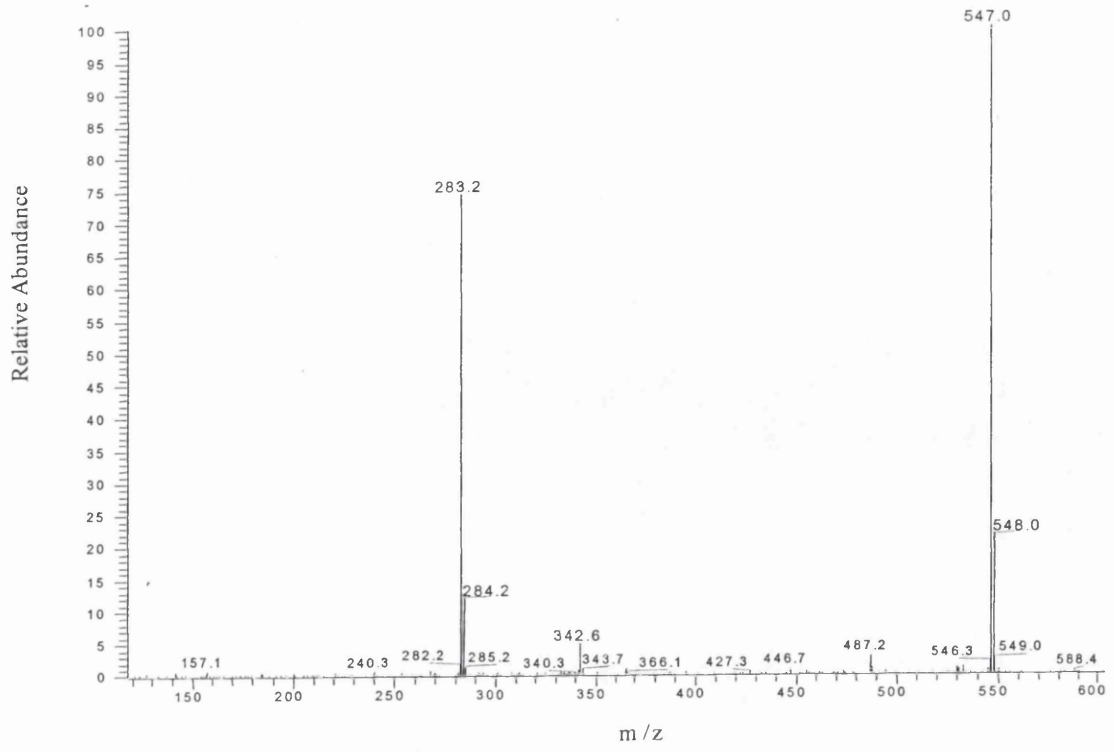


Figure 5.12 *Negative LC/APCI/MS mass spectrum of AGL (acetylglycitin)*

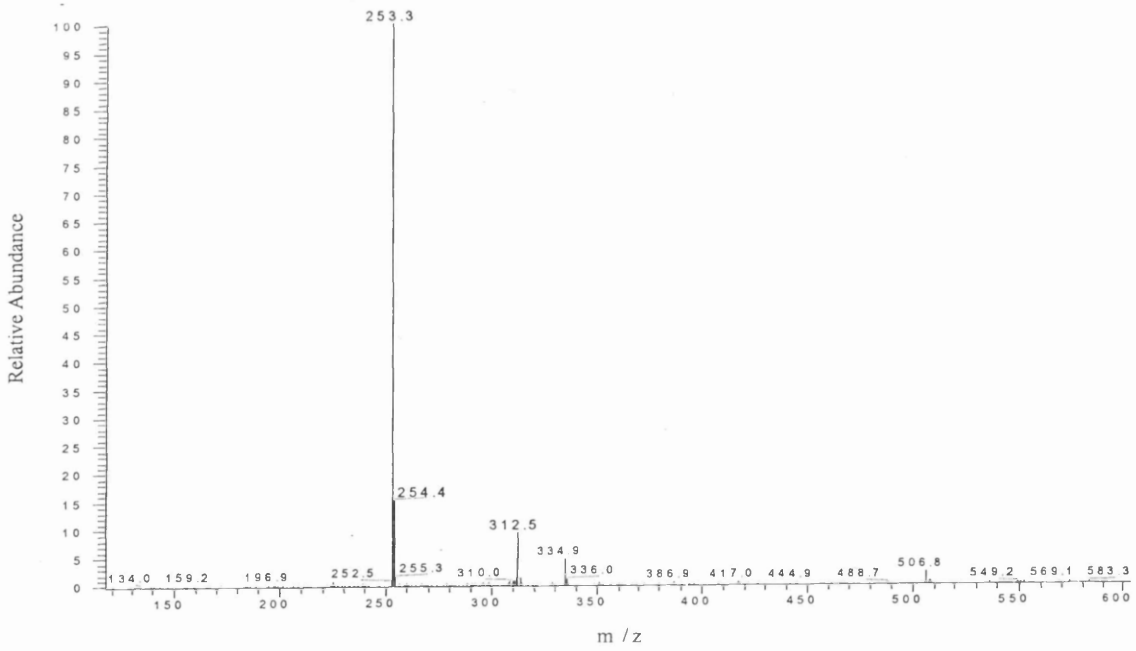


Figure 5.13 *Negative LC/APCI/MS mass spectrum of D (daidzein)*

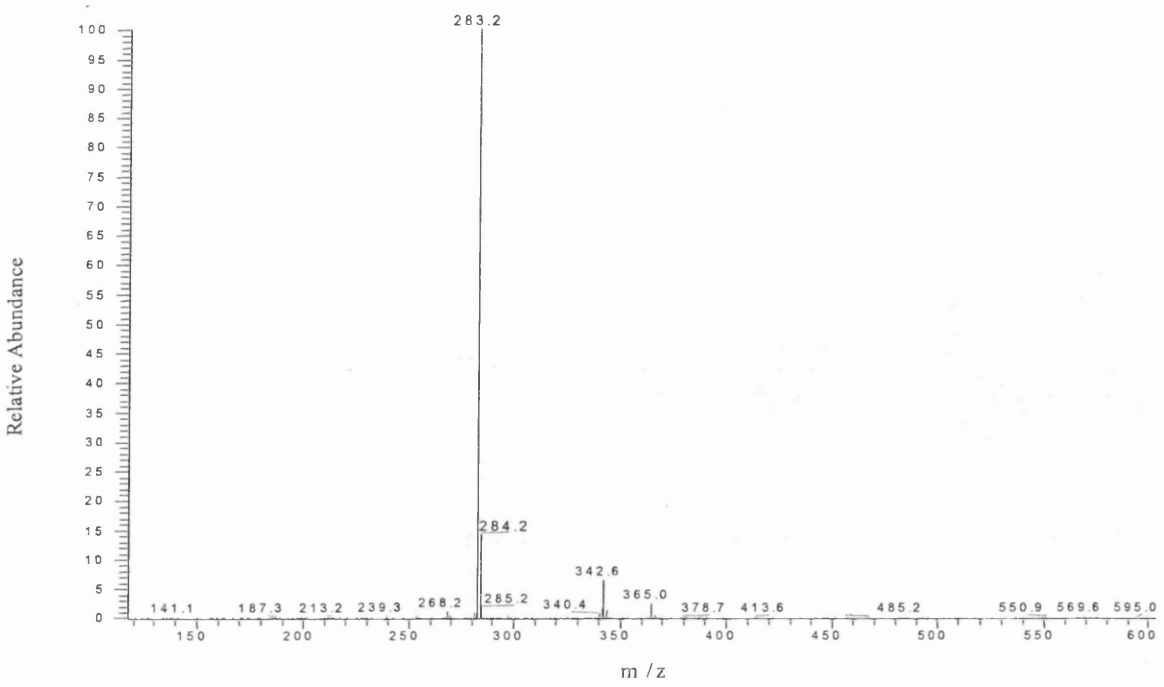


Figure 5.14 *Negative LC/APCI/MS mass spectrum of GLE (glycitein)*

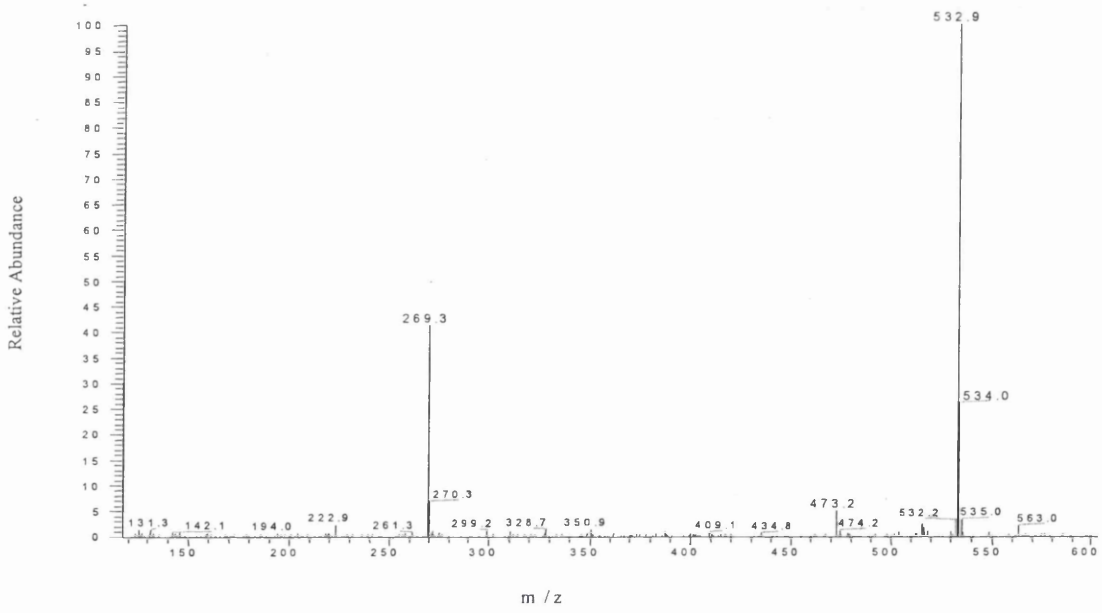


Figure 5.15 *Negative LC/APCI/MS mass spectrum of AG (acetylgenistin)*

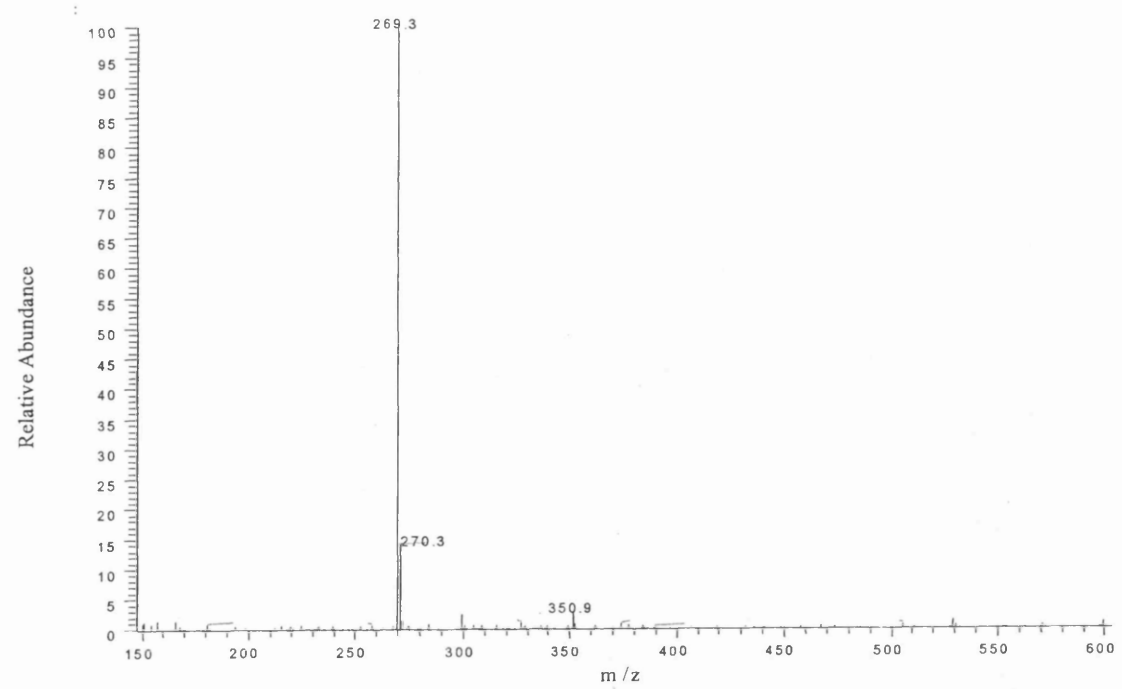


Figure 5.16 *Negative LC/APCI/MS mass spectrum of GE (genistein)*

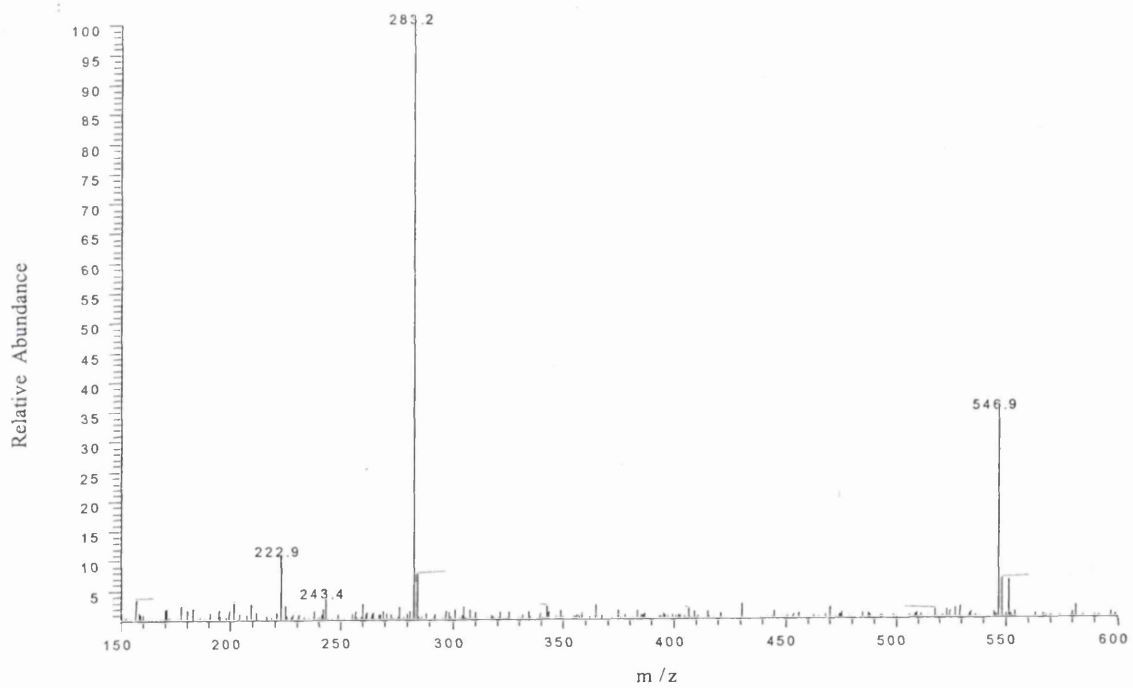


Figure 5.17 *Negative LC/APCI/MS mass spectrum of unknown peak 1*

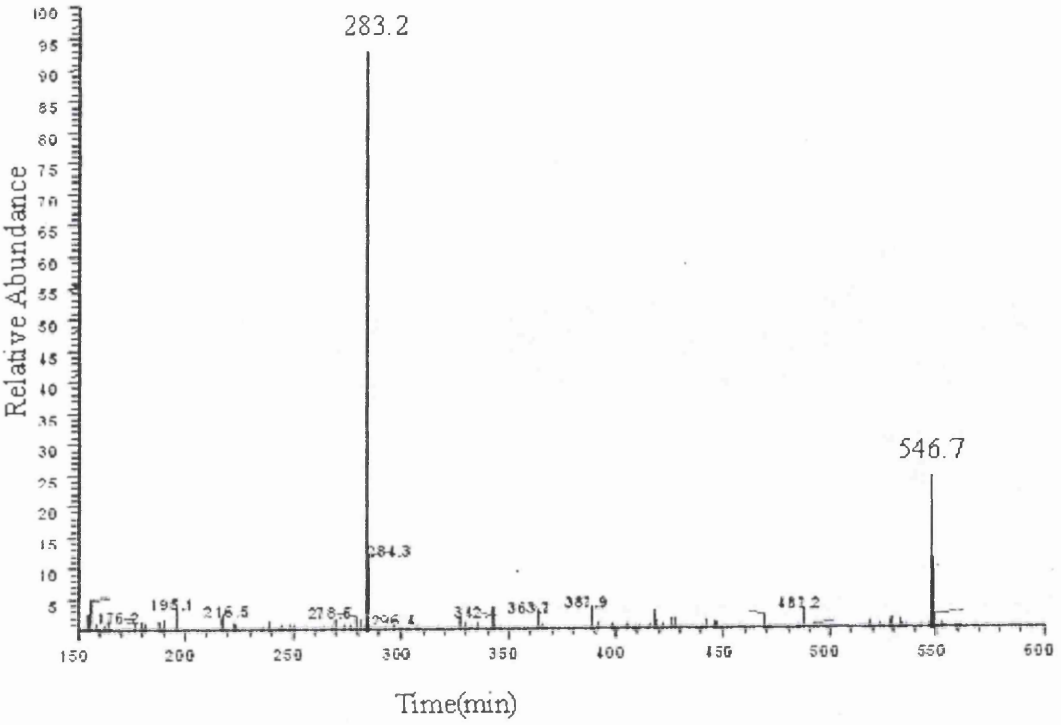


Figure 5.18 *Negative LC/APCI/MS mass spectrum of unknown peak 2*

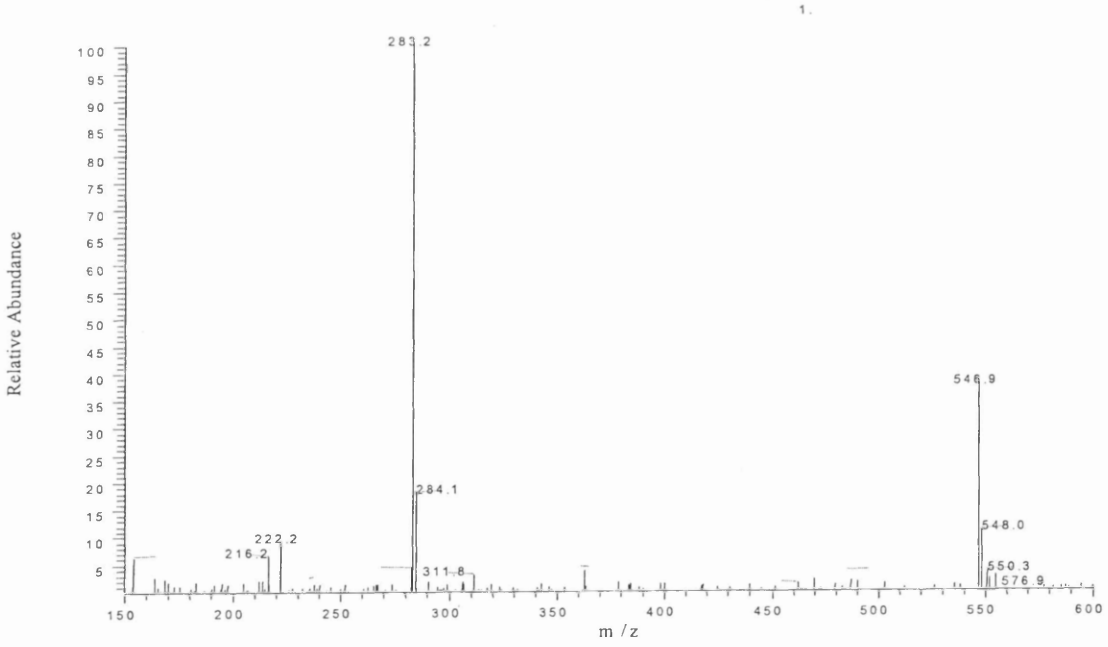


Figure 5.19 *Negative LC/APCI/MS mass spectrum of unknown peak 3*

In the analyses of isoflavones of nutrition supplements by full scan negative LC/APCI/MS, Their molecular ion plus acetate acid of $[M-H+60]^-$ ions of acetyl and glycoside isoflavones gave

intense negative ion spectra at m/z=532.9 for acetylgenistin, m/z=546.9 for acetylglycitin, m/z=517 for acetyldaidzin, m/z=491 for genistin, m/z=475 for daidzin and m/z=505 for glycitin because acetate acid was used in the mobile phase. For the acetyl isoflavones and glucoside isoflavones, O-glycoside moieties in the 7-position increase their hydrophilic characteristics due to exist of hydroxyl groups in the O-glycosides, which make them easily attached by acetate acid group. So, all their molecular ion plus acetate acid $[M-H+60]^-$ ions of acetyl and glycoside isoflavones gave intense negative ion spectra in negative LC/APCI/MS. For all the isoflavones a free aglycone ion constituted the base peaks at m/z=269 for acetylgenistin, genistin and glycitin, at m/z=253 for acetyldaidzin, daidzin and daidzein and at m/z=283 for acetylglycitin, glycitin and glycitein. 9 isoflavones were detected in the sample and malonyl isoflavones were not detected in the three nutrition supplement samples. Three unknown components marked P1, P2, P3, which have the same mass spectrum as acetylglycitin, were detected. In order to have enough information and identifications of the components under analyses, LC/APCI/MS^n was used to analyse all the isoflavones.

5.5.6 LC/APCI/MS^2 Analysis of the isoflavones

MS^n has been used to study flavonoids existing as their glycosylated conjugates and their aglycone flavonoid structures [39, 40]. M. A. Aramendia[17] determined the structure of isoflavones by using HPLC/APCI/MS^n .Cunniff et al[41] determined the structures of various flavonoids using an LCQ ion trap mass spectrometer, and the utility of the instrument in the field of natural supplement research was clearly demonstrated. The LCQ can perform MS^n operations in a step-wise manner, and precursor ions are isolated prior to a subsequent MS^n experiment. Hence, the isolation step ensures that the fragmentation spectra at each stage less complex than traditional MS-MS data. Cunniff results have shown the powerful ability of MS^n in exposing core flavonoids. In our study, LC/APCI/MS/MS was conducted using a Finnigan ion trap (the operating conditions applied were the same as for Figure 5.4. Collision energies are shown in Table 5.9).

Table 5.9 *Major supplement ions with relative high intensities for isoflavones in sample, acquired by negative mode LC/APCI/MS/*MS

Isoflavones	Precursor	Supplement Ions (relative intensities)	Collision energy
Daidzin	415	142.3(18), 143.5(9), 236.6(21) 252.3(100), 253.4(97), 295(10). 319.2(28), 415.2(19)	35
Glycitin	445.4	124.8(2), 268.3(2), 282.2(19), 283.2(100), 325.2(9), 355.2(3), 430.2(5), 445.4(12)	30
Genistin	431.2	170(1), 268.3(100), 269.3(67), 293.3(1), 311.1(16), 323.4(3), 341.1(5), 381.5(1), 431.2(11)	33
Acetyldaidzin	457.1	252.3(100), 253.4(73), 267.3(4), 295.2(27), 325.2(4), 397.2(5), 446. (4), 457.1(5)	35
Acetylglycitin	487.3	268.2(7), 282.3(100), 283.2(61), 325.2(25), 355.2(5), 427.3(4), 472.2(8), 487.3(9)	
Daidzein	253.2	152.3(14), 183.3(21), 197.3(64), 209.4(25), 223.4(29), 224.3(100), 225.3(69), 235.4(14)	45
Glycitein	283.2	240.3(0.1), 266.2(0.1), 267.5(0.6), 268.2(100), 283.5(0.3)	32
Acetylgenistin	473	267.3(1), 268.3(100), 269.3(20), 311.2(9), 323.3(1), 341.3(6), 413.2(3), 473.3(6)	35
Genistein	269	169.2(26), 181.2(27), 197.2(39), 201.2(50), 213.2(28), 224.3(43), 225.3(100), 241.2(47)	45
P1	283.2	82.8(0.6), 140.1(0.5), 158.6(0.5), 167(0.5), 255.4(1.3), 267.5(0.6), 268.2(100),	32
P2	283.2	126.5(0.4), 137.9(0.8), 159.7(0.4), 162.3(0.5), 214.8(0.4), 267.5(1), 268.2(100), 283.2(0.3)	32
P3	283.4	124.6(0.4), 207.2(0.2), 219.3(0.3), 267.3(2.1), 268.2(100), 272.9(0.4), 283.4(0.2)	32

5.5.6.1 MS^2 Analysis of Acetyl isoflavones

The nomenclature used to define the various fragment ions has been proposed by Claeys and co-workers [42]. For free aglycones, the symbols $^{i,j}A^+$ and $^{i,j}B^+$ are used to designate primary fragment ions containing A- and B-ring, respectively. The superscripts i and j refer to the bonds of the C-ring that have been broken. These ions can lose small neutral fragments, such as H_2O and

CO. These supplement ions are represented combining $^{i,j}A^+$ or $^{i,j}B^+$ with the lost fragments. In order to obtain accurate product ion fragments from the precursor ion, molecular ion of $[M-H]^-$ was chosen as precursor ion even though the $[M-H-CH_3COOH]^-$ were detected instead of ions of $[M-H]^-$ due to the use of acetic acid in the composition of the mobile phase. For acetyl isoflavones, the APCI/MS^2 spectra exhibited their fragments $[M-H-120]^-$ and $[M-H-90]^-$ characteristic of the cleavage of the O-glycoside moieties when the ions of $[M-H]^-$ are parent ions. Figures 5.20-5.22 show their APCI/MS/MS when their molecular ions were selected as precursor ions and the product ions were recorded. Scheme 1 shows the proposed pathway of their fragmentations.

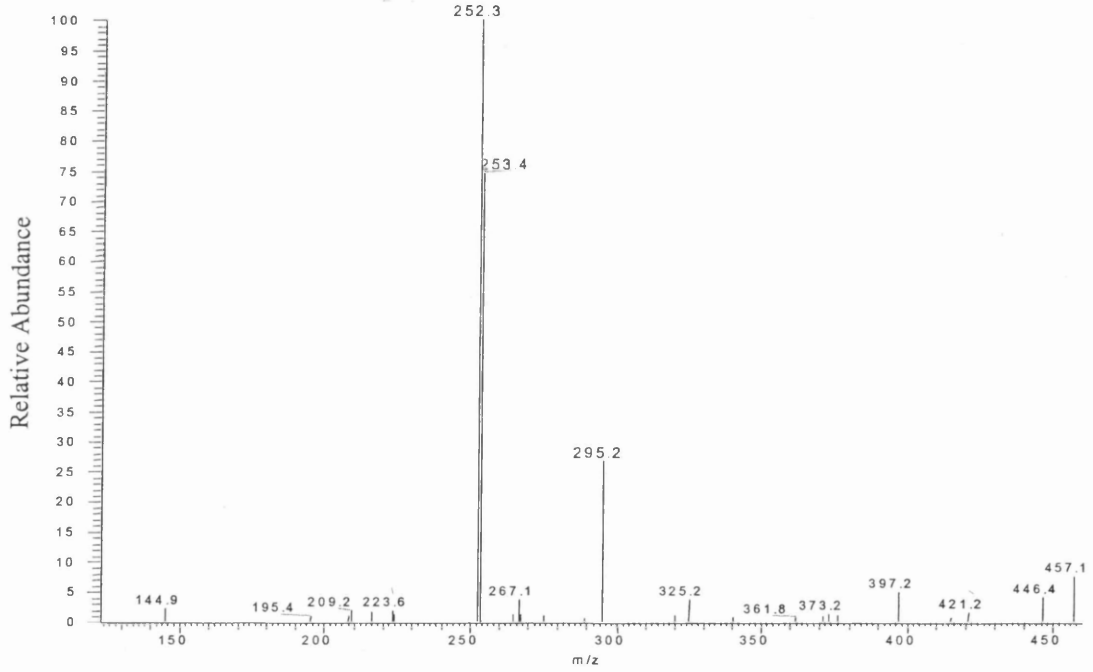


Figure 5.20 *Negative LC/APCI/MS^2 mass spectrum of acetyldaidzin*

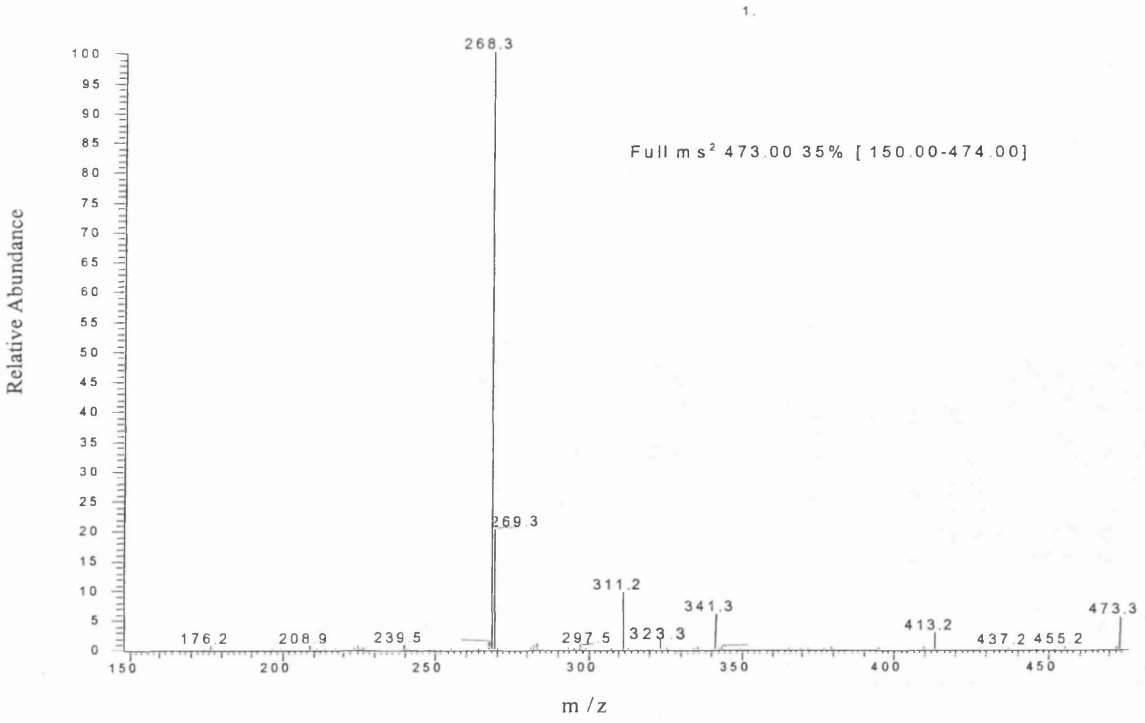


Figure 5.21 *Negative LC/APCI/MS^2 mass spectrum of acetylgenistin*

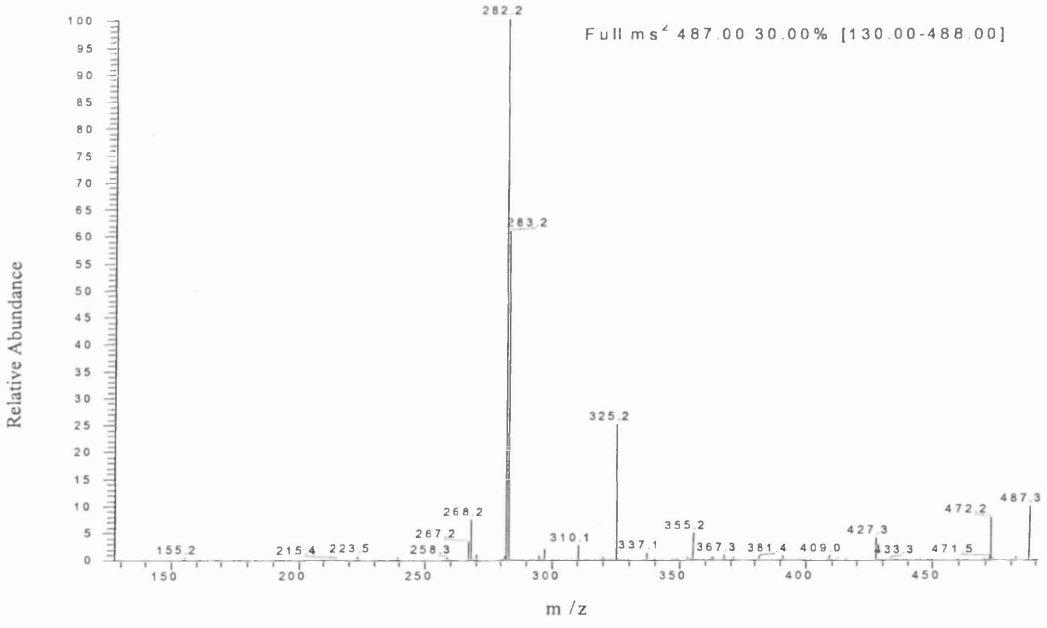
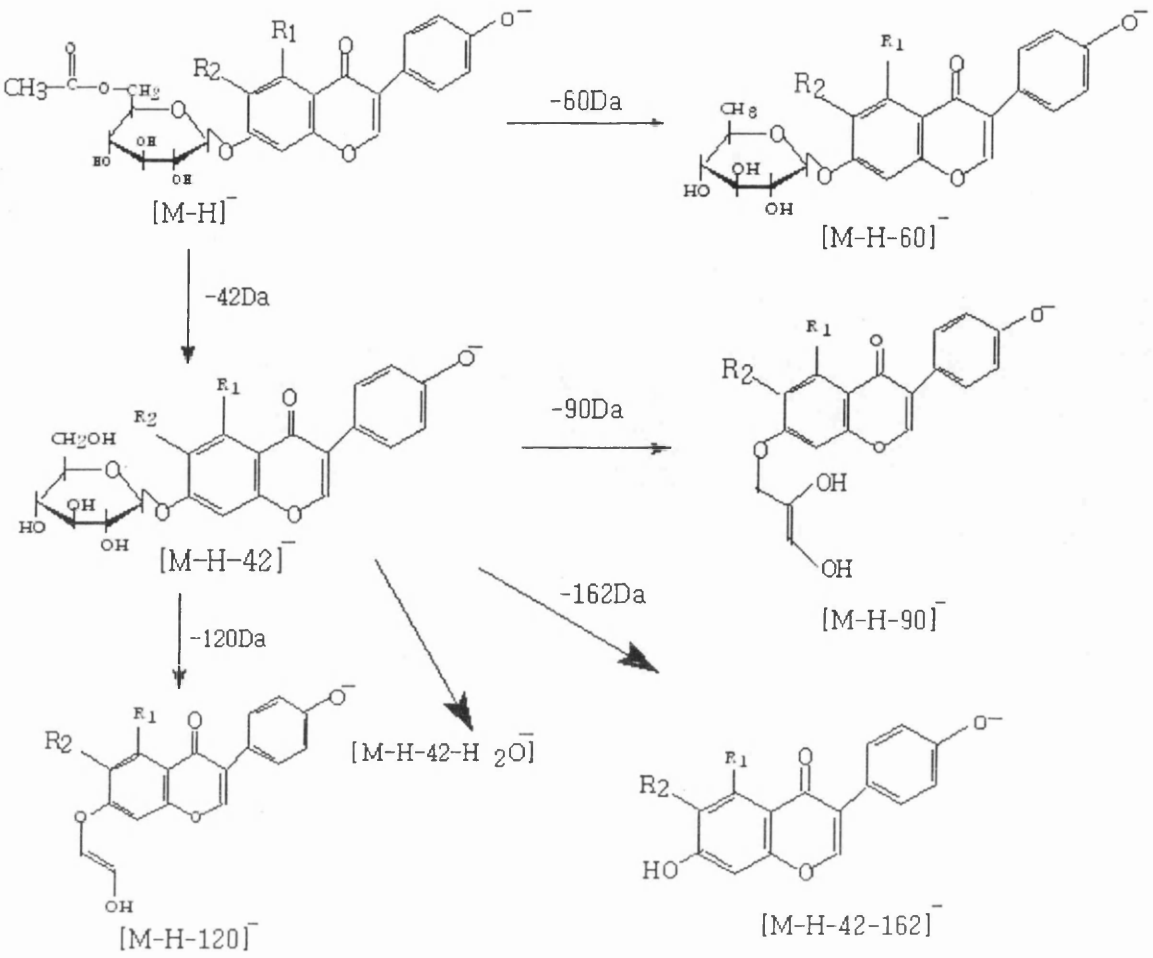


Figure 5.22 *Negative LC/APCI/MS^2 mass spectrum of acetylglycitin*



	R_1	R_2	m/z
Acetyldaidzin	H	H	457
Acetylgenistin	OH	H	473
Acetylglycitin	H	OCH_3	487

Scheme 5.1 *Proposed pathway fragmentations of acetyl isoflavones*

5.5.6.2 MS^2 Analysis of glycoside isoflavones

Glycoside isoflavones gave very similar fragments to the acetyl isoflavones when $[M-H]^-$ were selected as their precursor ions and product ions were recorded. Figures 5.23-5.25 show their negative APCI/MS^2 spectra and scheme 2 gives the proposed pathway of fragmentations.

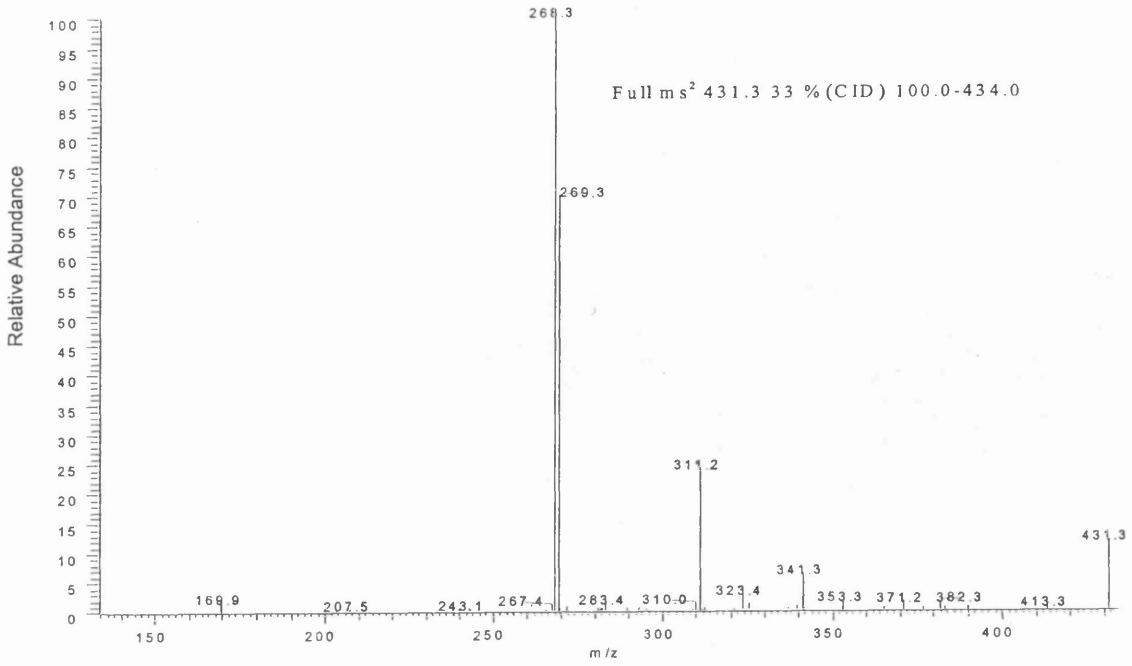


Figure 5.23 *Negative LC/APCI/MS² mass spectrum of genistin*

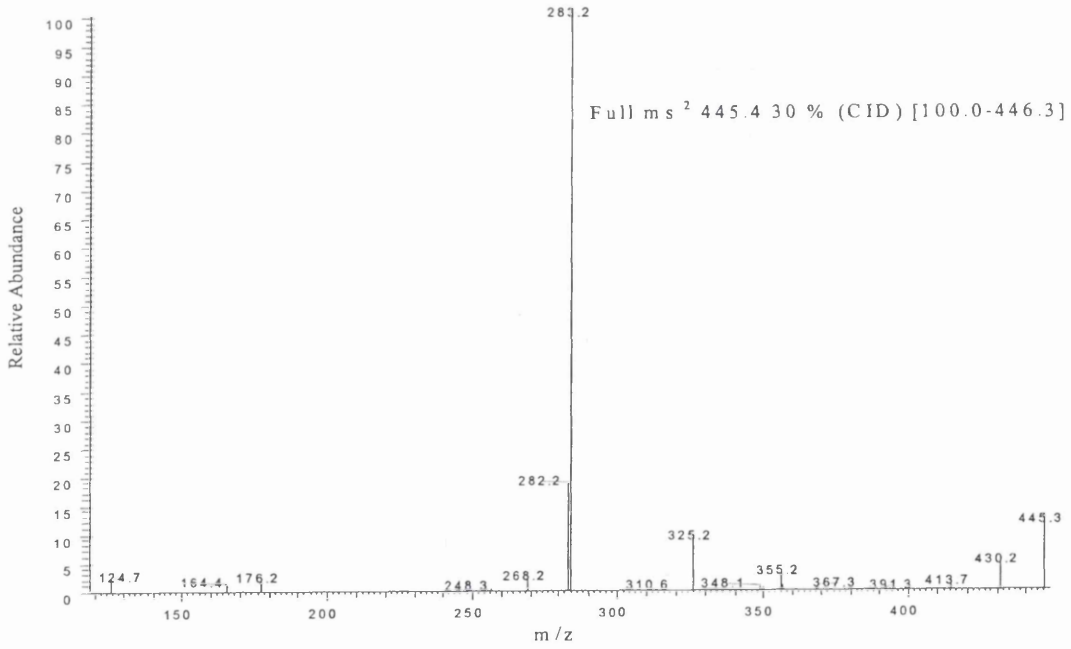


Figure 5.24 *Negative LC/APCI/MS² mass spectrum of glycitin*

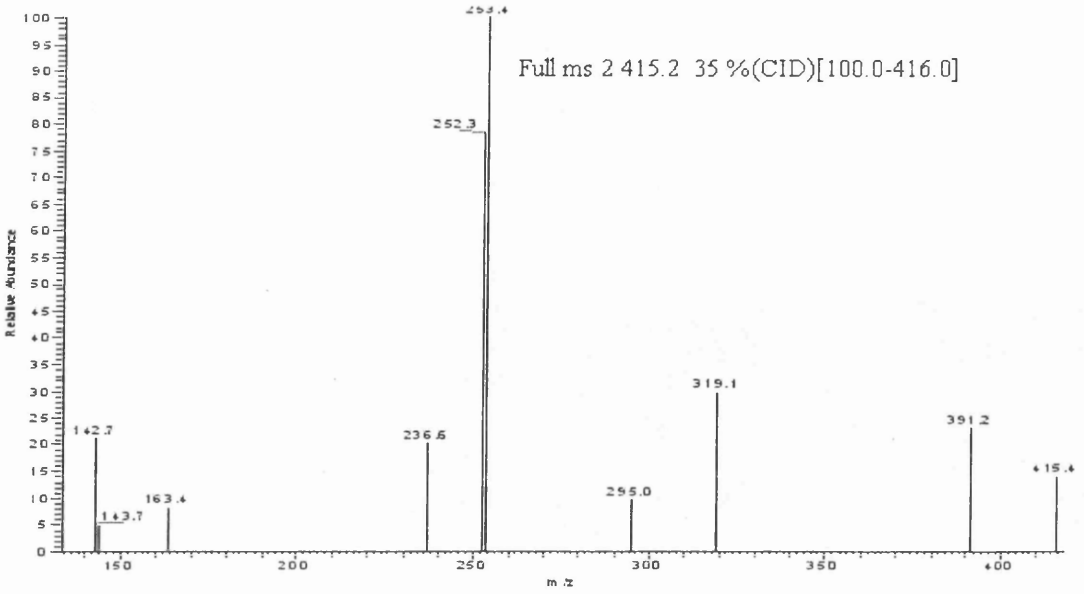
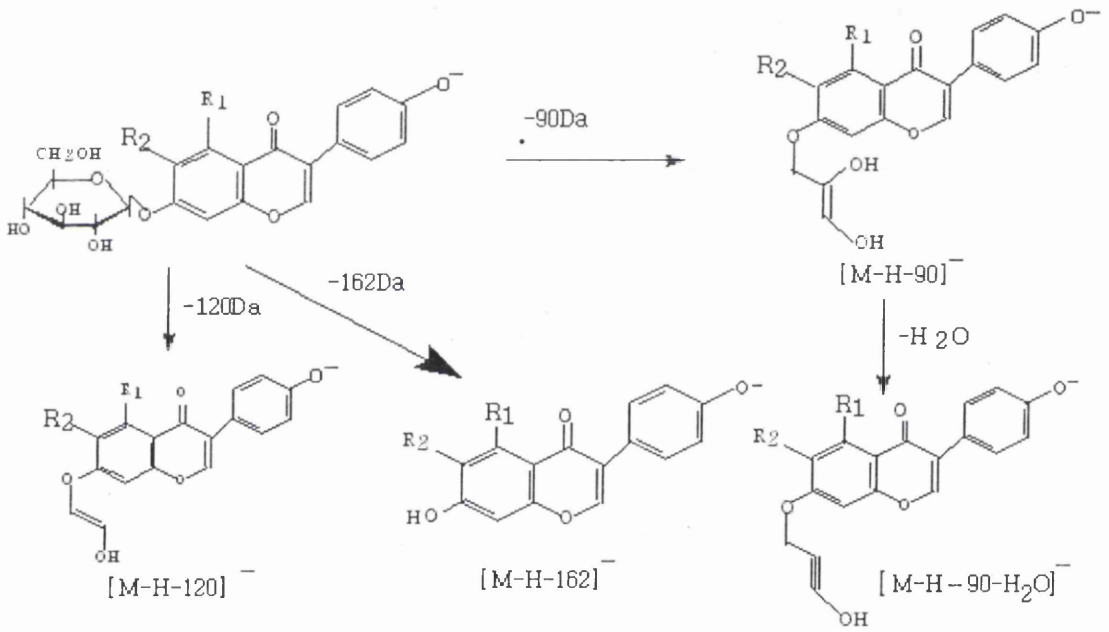


Figure 5.25 *Negative LC/APCI/MS^2 mass spectrum of daidzin*



	R1	R2	m/z
Daidzin	H	H	415
Genistin	OH	H	431
Glycitin	H	OCH_3	445

Scheme 5.2 *Proposed pathway fragmentations of glycoside isoflavones*

5.5.6.3 MS^2 Analysis of aglycone isoflavones

5.5.6.3.1 MS^2 Analysis of daidzein

APCI/MS^2 studies of daidzein, genistein and glycitein can provide more information for their structural elucidation and identification of unknown compounds. The results of extensive studies give scope as a molecular fragmentation fingerprint with which to index and identify each target component within mixtures of unknown compounds. For daidzein, glycitein and genistein, different collision energies were required to provide a satisfactory fragmentation pattern, whilst retaining the presence of the $[M-H]^-$ precursor ions. Figures 5.26-5.28 give their negative APCI/MS^2 spectra when the ions of the $[M-H]^-$ were selected as precursor ions and supplement ions were recorded.

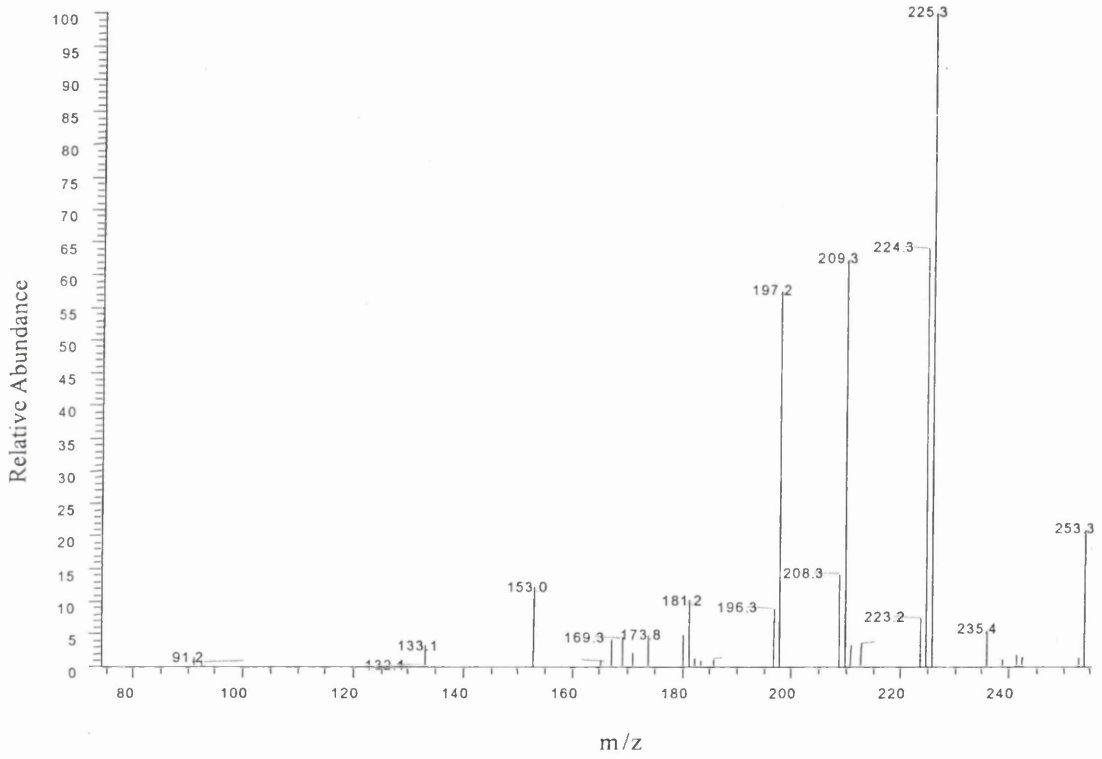
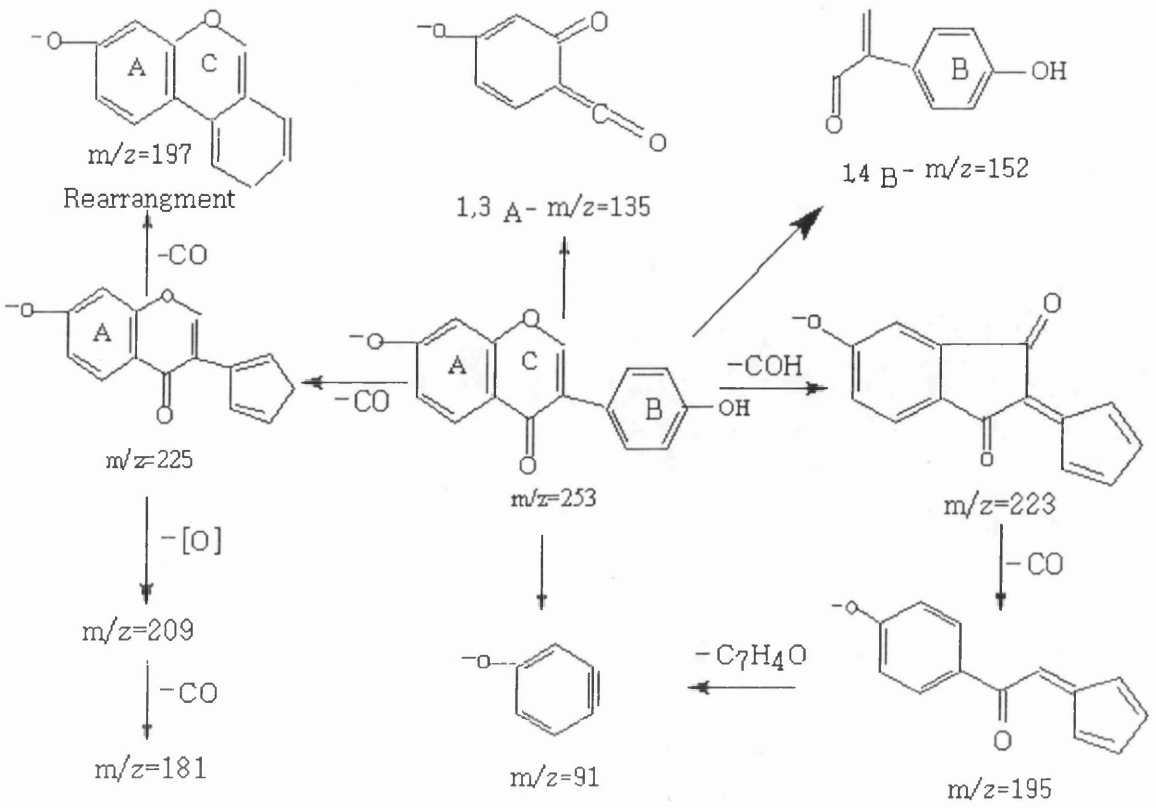


Figure 5.26 *Negative LC/APCI/MS^2 spectrum of daidzein at 253.3[M-H]$^-$ (CID 45%)*



Scheme 5.3 *Proposed pathway fragments of daidzein*

For daidzein, m/z=224 and 225 appear as major fragments in the negative APCI/MS^2 spectrum, implying losses of CHO, and CO, respectively. This type of fragmentation is indicative of phenol-containing compounds such as baicalein, daidzein, genistein and chrysin. The appearance of m/z=196 may suggest a further loss of CO or CHO from the other phenolic group. A notable ion at m/z=197 may imply a loss of CO from m/z=225 and rearrangement.

Aramendia et al [17] have suggested a fragmentation pathway for genistein and daidzein relating to ions at m/z 135 and 91 by negative APCI/MS/MS, but in our case, the ion of m/z=133 was detected instead of m/z=135. The ion of m/z=91 is a very important fragment of the structure elucidation of flavonoids and isoflavones. This fragment results from the ion of the B-ring, which indicated an OH group in the B ring of daidzein. Unfortunately, the signal of ion at m/z=91 is very weak for daidzein in our case and the signal of ion at m/z=106.8 from genistein is weak as well. The ion of m/z=181 is possibly formed by the loss of O from m/z=197 and the ion of m/z=153 is further loss of CO from m/z=197. According to our elucidation, the ion of m/z=197 may imply one OH group in the A-ring from daidzein. According to our elucidation, the ion of m/z=213 should be observed from the mass spectrum of genistein in its APCI-MS^2 due to two OH groups

in the ring B.

5.5.6.3.2 MS^2 Analysis of genistein

The MS^2 fragmentation mass spectrum (see Figure 5.27) of genistein shows similarities to that observed for daidzein and the ions of m/z=225 and 197 are observed as well. This is to be expected, since the two compounds are very similar. However, the ion of m/z=106.8 is observed with a very weak signals instead of m/z 91, indicating two OH groups in the B-ring of genistein. From the mass spectrum of genistein, the fragments at m/z=225 and 224 indicate respective losses of m/z=44 and 45, which could be the losses of CO_2 or CH_2CHOH, and CO_2H or CH_3CHOH. The fragment of m/z 213 may result from the loss of CO from m/z=241 and imply two OH groups in the ring A. So, it is possible to differentiate the daidzein and genistein from the m/z=213 ion. Scheme 5.4 also gives the proposed pathway fragments of genistein. Further inspection of the daidzein and genistein standard solutions were acquired by performing MS^3 and MS^4 analysis on the base peak product from the MS^2 study. Indeed, we can obtain significant value in ascertaining differences or similarities between identical m/z results of the two different compounds by using MS^3 to MS^4

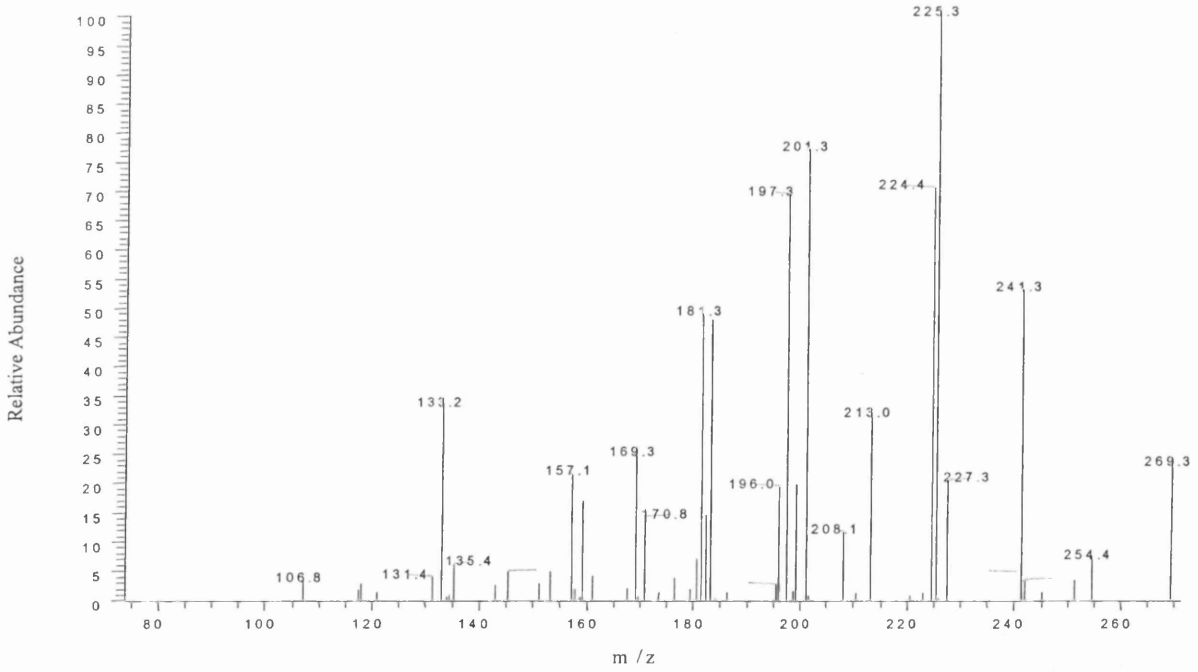
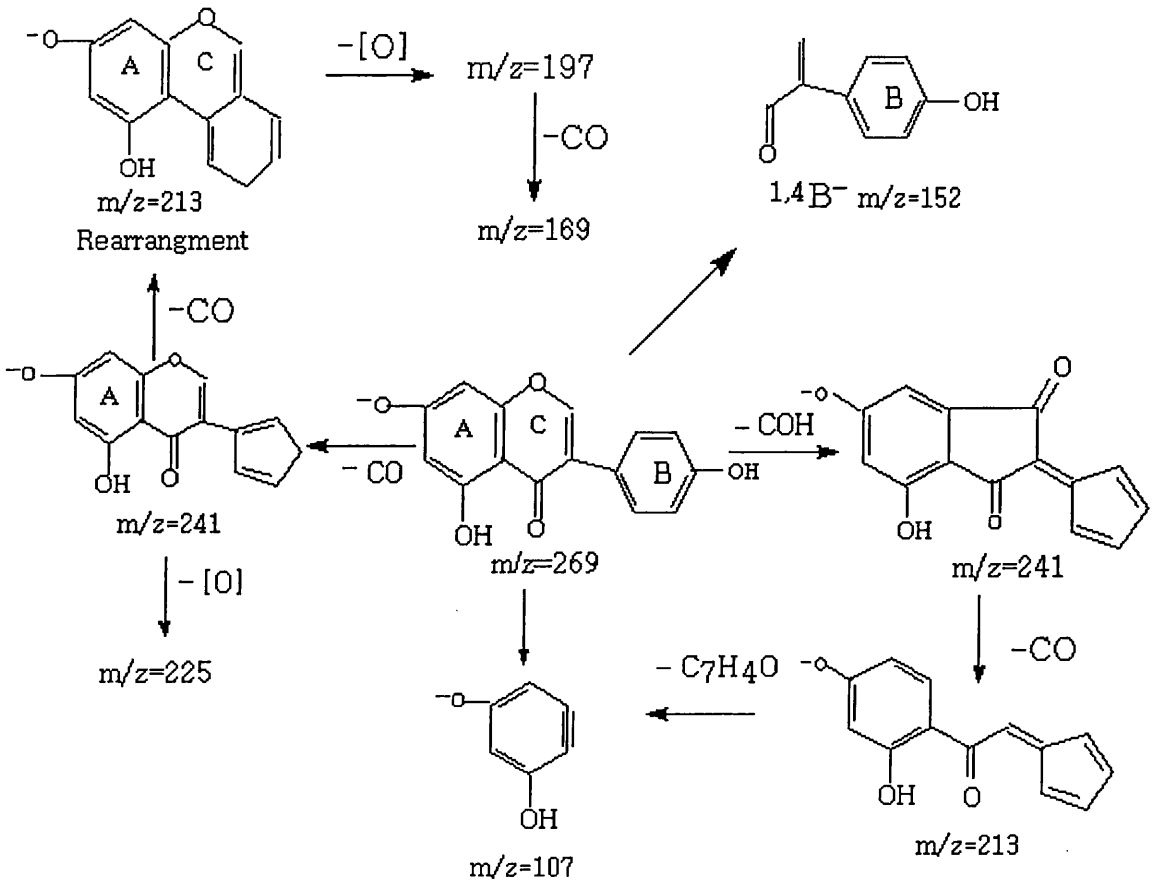


Figure 5.27 *Negative LC/APCI/MS^2 spectrum of genistein at 269.3 $[M-H]^-$ (CID 45 %)*



Scheme 5.4 *The proposed pathway fragments of genistein*

5.5.6.3.3 MS^2 Analysis of glycitein

The negative LC/APCI/MS^2 mass spectrum of glycitein only gave a strong ion at m/z=268 $[M-H-CH_3]^-$, even increasing the collision induced energy (CID) to 45 %. This result was also observed in the unknown peaks 1, 2 and 3 when the same conditions of APCI-MS^2 were used. As a rule, isoflavones with a methoxy group (biochaninA, glycitein and glycitin) exhibit one fragment ion at m/z=$[M-H-CH_3]^-$ when a high extraction cone voltage is used. In order to further investigate the structure information especially for the unknown compounds, further MS^3 studies of the glycitein and unknown peaks 1, 2 and 3 were performed.

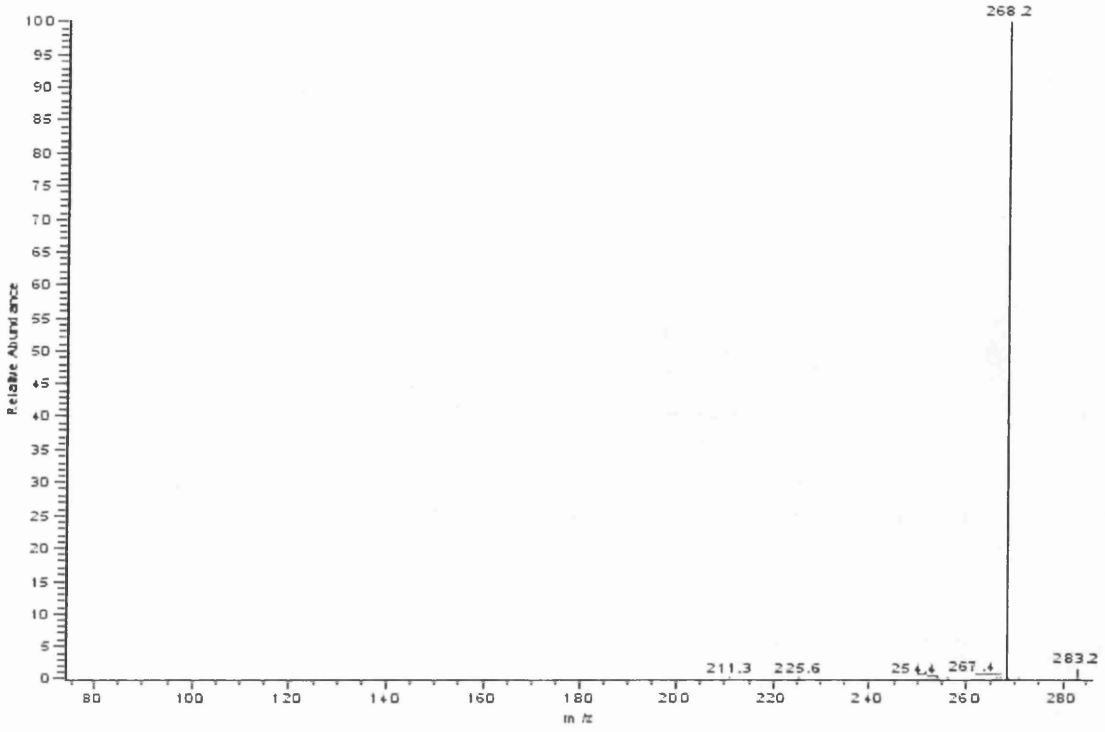


Figure 5.28 *Negative LC/APCI/MS^2 spectrum of glycitein 283.2 [M-H]$^-$(CID 30 %)*

5.5.6.4 MS^3 Analysis of aglycone isoflavones

For the glycitein, as we expected, the negative APCI/MS^2 mass spectrum only gave the base peak of m/z=268 and Peaks 1. 2 and 3 exhibit the same mass spectra as that of the glycitein in negative APCI/MS^2. These fragments are not enough to differentiate them. Further inspection of the daidzein, genistein, and glycitein peaks 1, 2 and 3 were acquired by performing MS^3 on the base product ions resulting from the MS^2.

In our study, peaks 1, 2 and 3 gave the same precursor [M-H]$^-$ ion and product ion at m/z=283. We can conclude that compounds 1, 2 and 3 all are isomers of acetylglycitin. However, the differences of their structure can be obtained by MS^3.

For peak 1, the mass spectrum of negative APCI/MS^3 gives a very different pattern from that of glycitein, indicating their different structures. We cannot elucidate the structure of peak 1 due to low concentration. We can still conclude that peak 1 belongs to the 6"-O-acetyl glycoside

isoflavonoid due to the precursor presence of fragment of $[M-H-CH_3CO-162]^-$ and peak 1 is the isomer of acetylglycitin. Figure 5.29 shows the MS^3 spectrum of peak 1

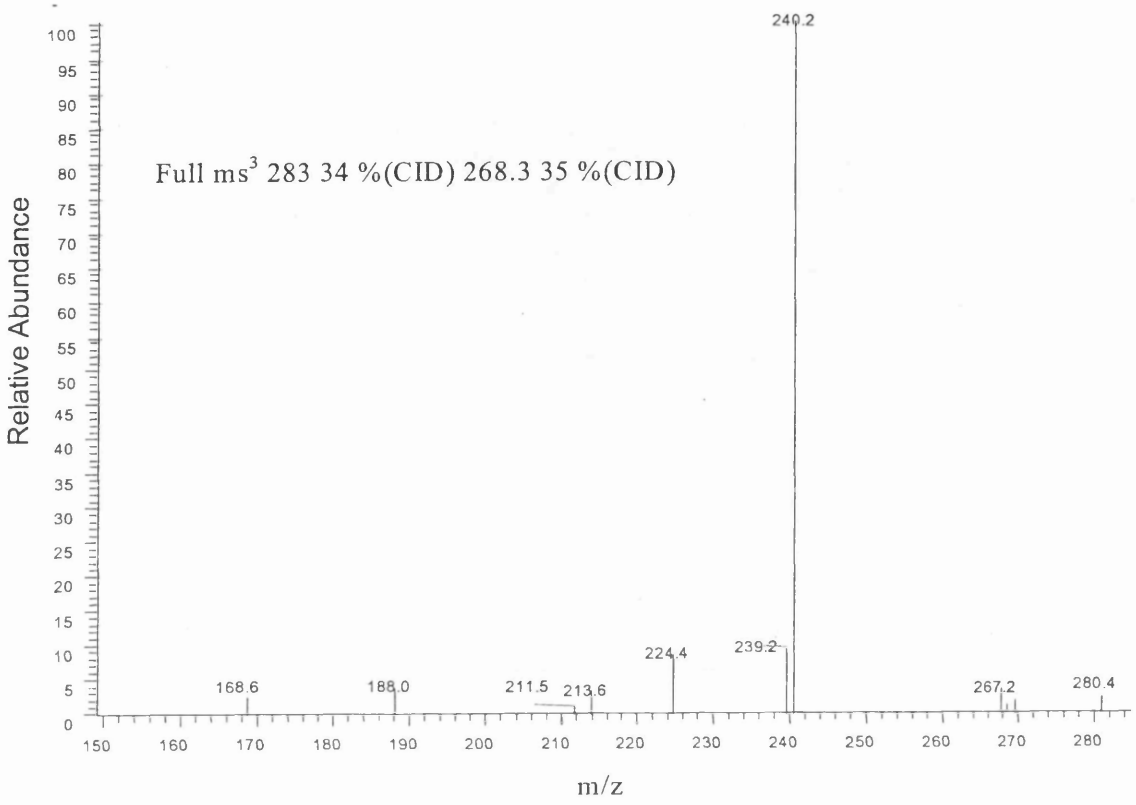


Figure 5.29 *Negative LC/APCI/MS^3 mass spectrum of peak 1*

The MS^3 experiments of the ion at m/z=283 of peaks 1-3 displayed product ions due to the losses of 15(CH_3), 29(CHO), and 28 (CO), representing a methylated flavonoid. Attempts were made to elucidate the structure of the ion at m/z=283 on the basis of its products. For this purpose, we compared the MS^3 data of the standard methylated isoflavones glycitein; $[M-H]^-$ 283Da) with those of the ion at m/z=283 of peaks 2 and 3. We found that the MS^3 mass spectrum of peak 3 is very similar to that of glycitein. On the basis of these pieces of evidence, peak 2 is proposed to be 5-methoxy-acetyldaidzin. We could not elucidate their structures merely based on MS^2 and MS^3 data. We could conclude that peaks 2 and 3 are all isomer of acetylglycitin. Although the elucidation of unknown compounds is still difficult without the aid of reference materials, we can still get some useful structure information of unknown compounds. Figures 5.30-5.32 show their negative APCI/MS^3 spectra of peaks 2, 3 and glycitein. Table 5.10 shows their major ions with relative high intensities acquired by negative LC/APCI/MS^3.

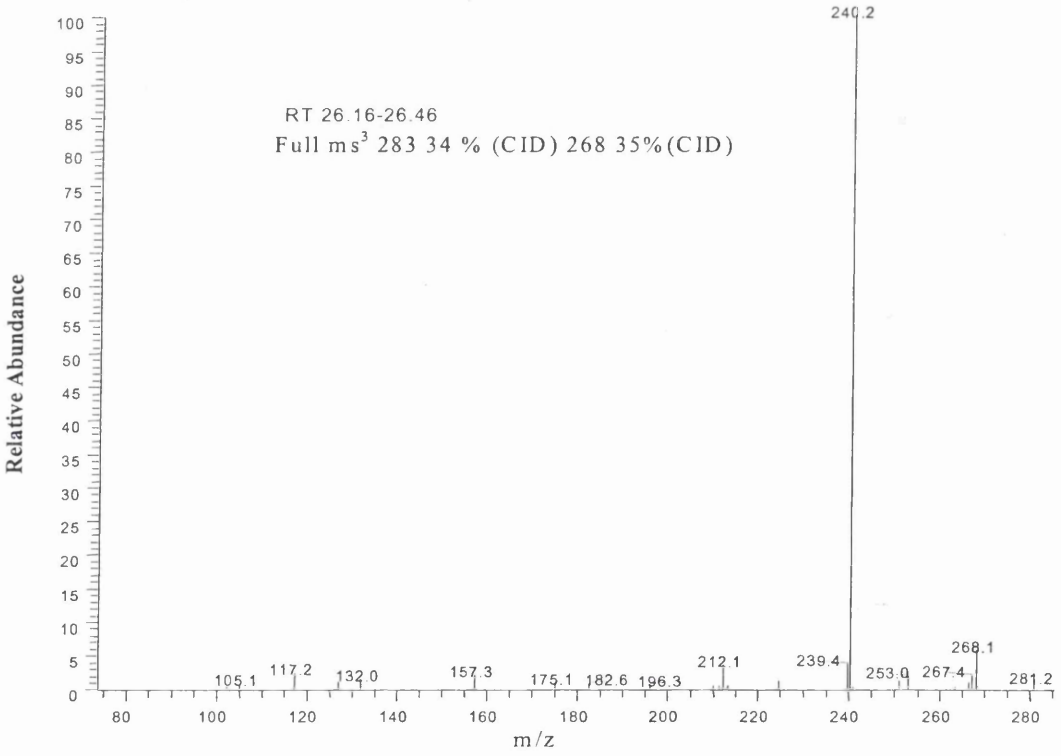


Figure 5.30 *Negative mode LC/APCI/MS^3 mass spectrum of peak 2*

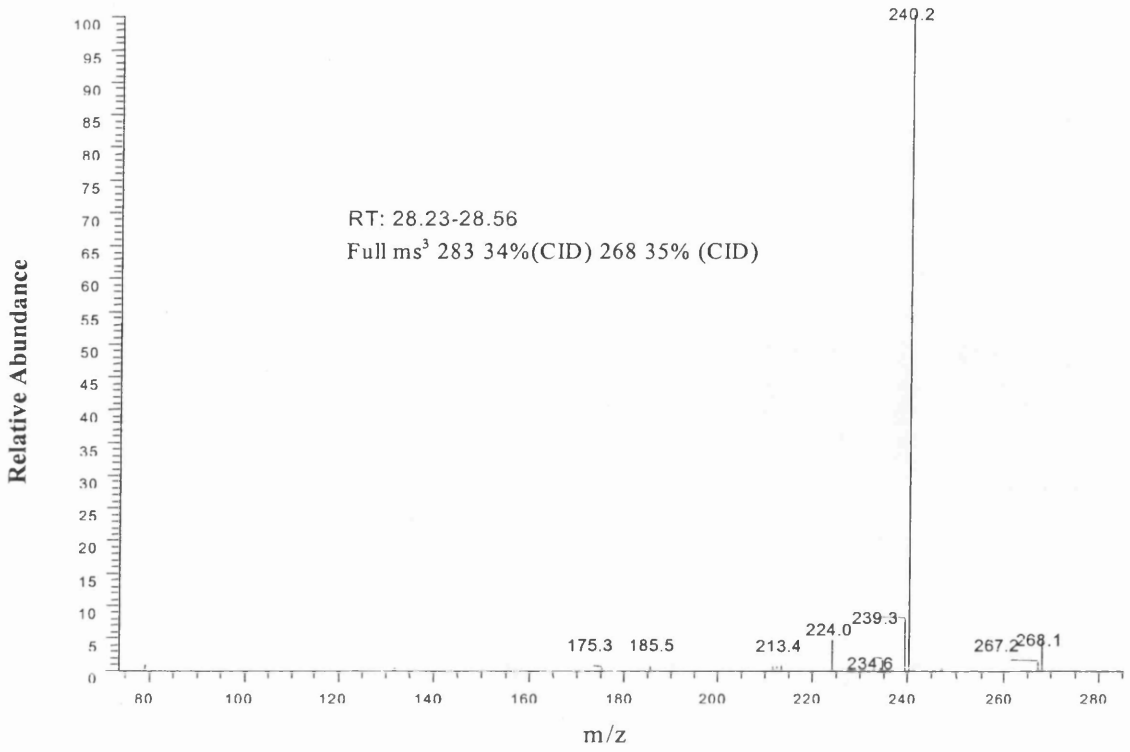


Figure 5.31 *Negative mode LC/APCI/MS^3 mass spectrum of peak 3*

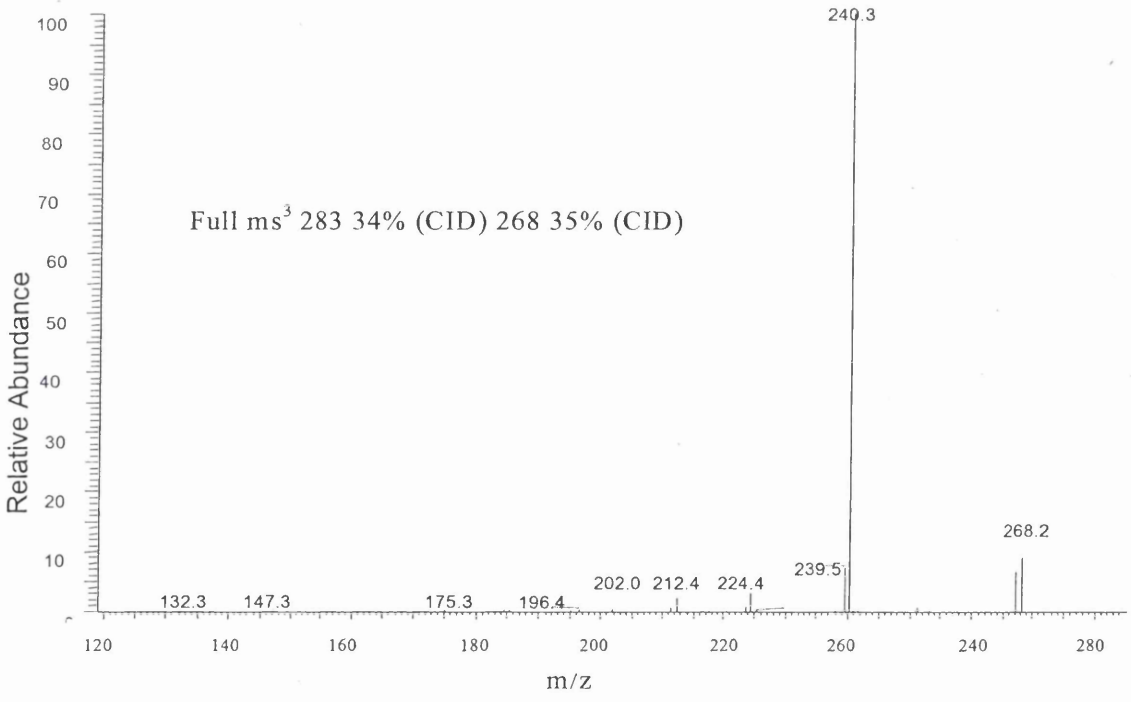
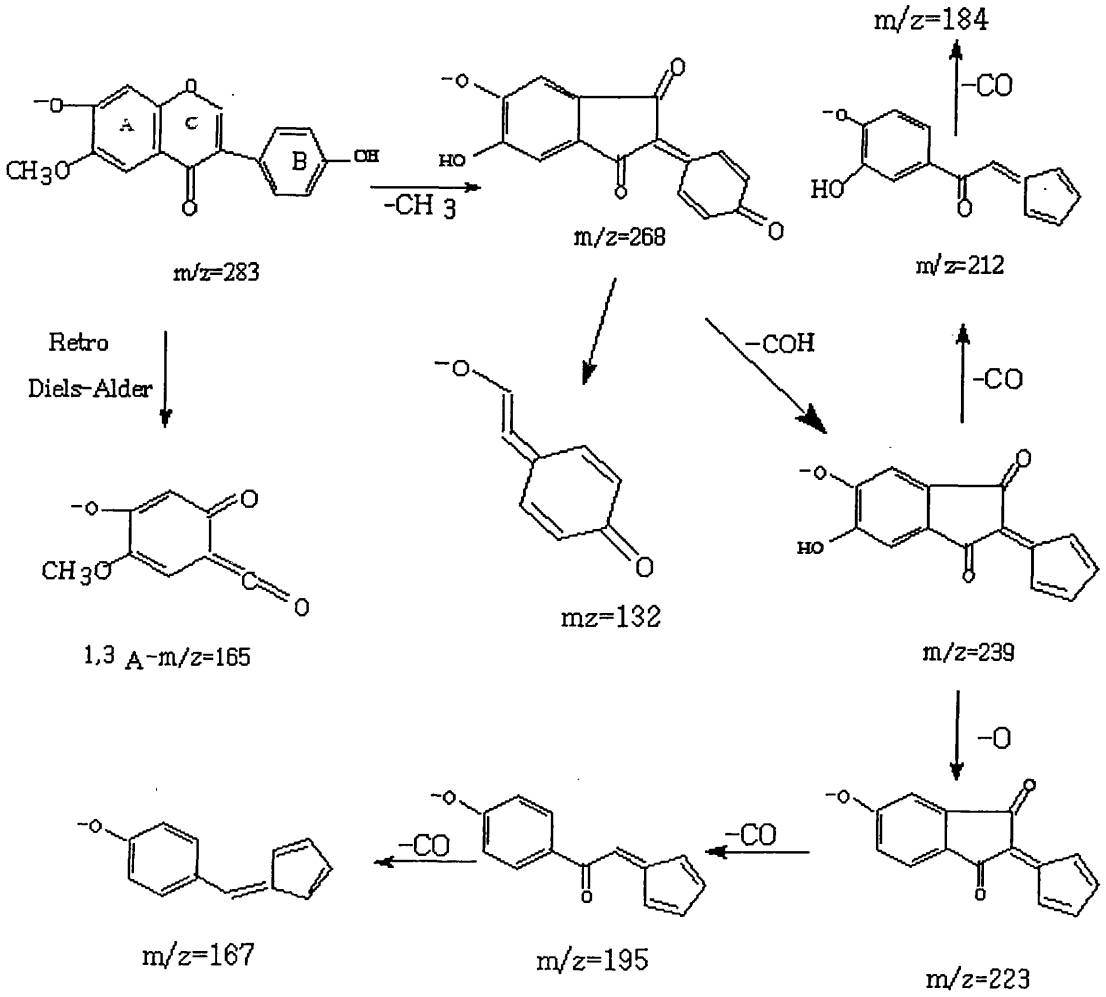


Figure 5.32 *Negative LC/APCI/MS^3 mass spectrum of glycitein*



Scheme 5.5 *The proposed pathway fragments of glycitein*

Table 5.10 *Major ions with relative high intensities acquired by negative LC/APCI/MS*^{3}

Component	First-stage Precursor Ion and CID (%)	Second-stage Precursor Ion and CID (%)	Third-stage Precursor Ion and intensity (%)
Daidzein	253(44)	225(40)	95.1(71), 115.1(16), 169.3(100), 197.2(54), 200.4(13), 215.2(8)
Genistein	269(42)	225(40)	180.3(61), 181.7(59), 195.3(100), 197.1(65), 211.4(38)
Glycitein	283(35)	268.3(35)	224.4(3), 239.5(7), 240.3(100), 267.3(7), 268.2(9)
Peak 1	283(34)	268(35)	187.9(2), 203(3), 213.6(2), 224.3(7), 239.1(4), 240.2(100), 267.3(4), 268.0(3)
Peak 2	283(34)	268(35)	157.3(2), 212.3(3), 239.4(3), 240.3(100), 253(2), 267.4(2), 268.1(6)
Peak 3	283(34)	268(35)	224(5), 239.3(9), 240.2(100), 267.2(1), 268.1(5)

5.5.7 Linear Response Curve for LC/APCI/MS Analysis

Standards of the six readily available isoflavones were used for routine calibration and recovery experiments. The solution was stable for at least 2 months at room temperature. These solutions were used in recovery experiments with soy sample and to prepare working standards. malonyl and acetyl isoflavones were not stable and available. Most quantitative determination of isoflavones in food or nutrition food was measured in aglycones after the hydrolysis and only six standard materials were needed instead of 12. However, our study of optimisation of extraction of isoflavones indicates that some unknown compounds will hydrolyse to aglycone isoflavones and inaccurate results could be obtained. In the report of T. Nurmi[43], quantitative results of the isoflavones in aglycones measured after the hydrolysis were far lower than the values given by the

producer. In my opinion, the hydrolysis method is a possible reason. Hence, if standard malonyl and acetyl isoflavones are made available, more accurate determination of isoflavones will be obtained. Our aim of the study was to develop an HPLC/MS method to analyse all isoflavones in the nutrition products (provided by Cultech company, Swansea, UK). Acetyl isoflavones and Malonyl isoflavones were purchased from Plantech (Reading, UK). Their purities were checked by HPLC and dissolved in DMSO solution. All acetyl and Malonyl isoflavones were prepared freshly for the calibration. We also found that the malonyl and acetyl isoflavones are relatively stable when they are dissolved in dimethylsulfoxide (DMSO). All the isoflavones of nutrition products were quantitatively determined using HPLC. For experimental conditions see Figure 5.4. The calibration range of acetyl isoflavones are 0-25 μg/ml, glycoside isoflavones 0-10 μg/ml and aglycone isoflavones 0-10 μg/ml. Figures 5.33, 5.34 and 5.35 show their calibration range and linear relative coefficient r values, respectively.

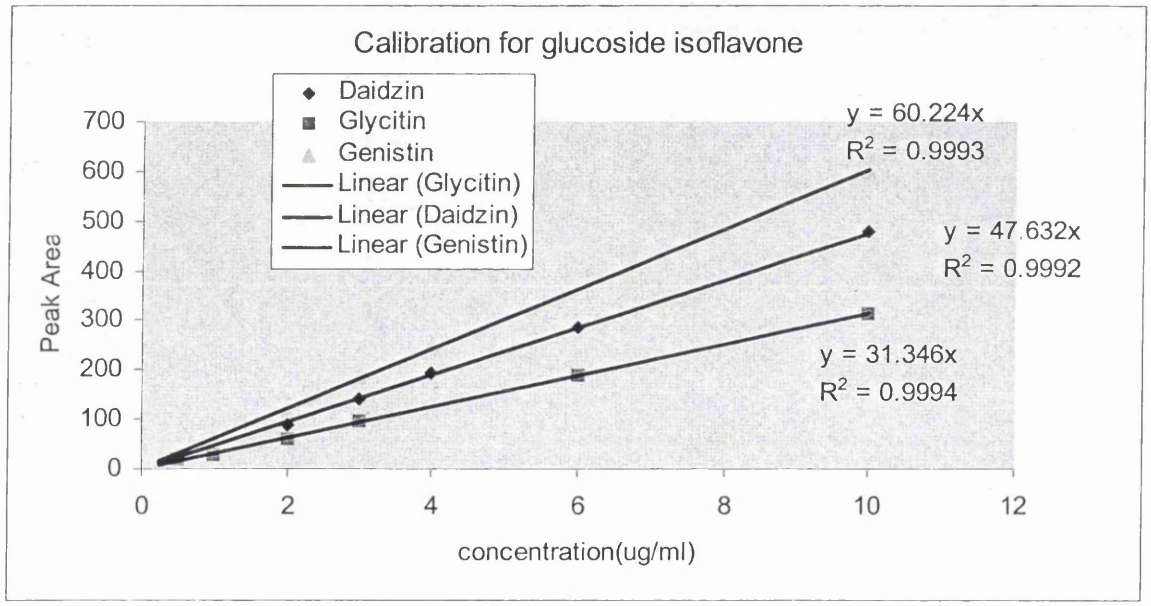


Figure 5.33 *Calibration curve for the analyses of the daidzein, glycitein and genistein*

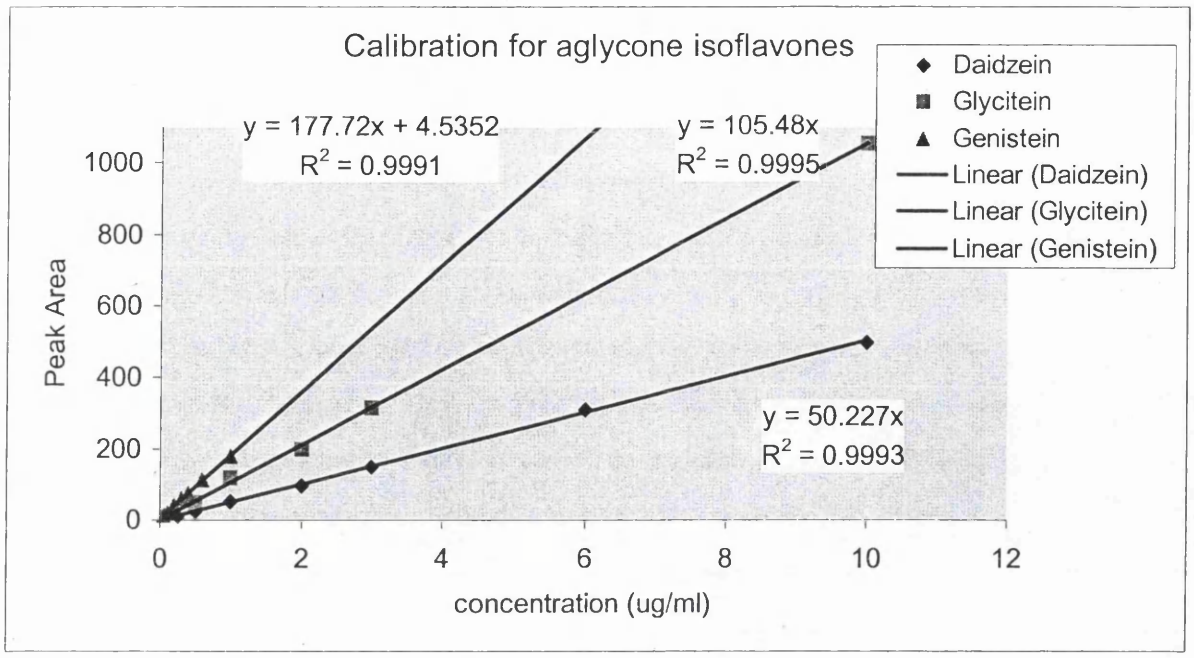


Figure 5.34 *Calibration curve for the analyses of the daidzin, glycitin and genistin*

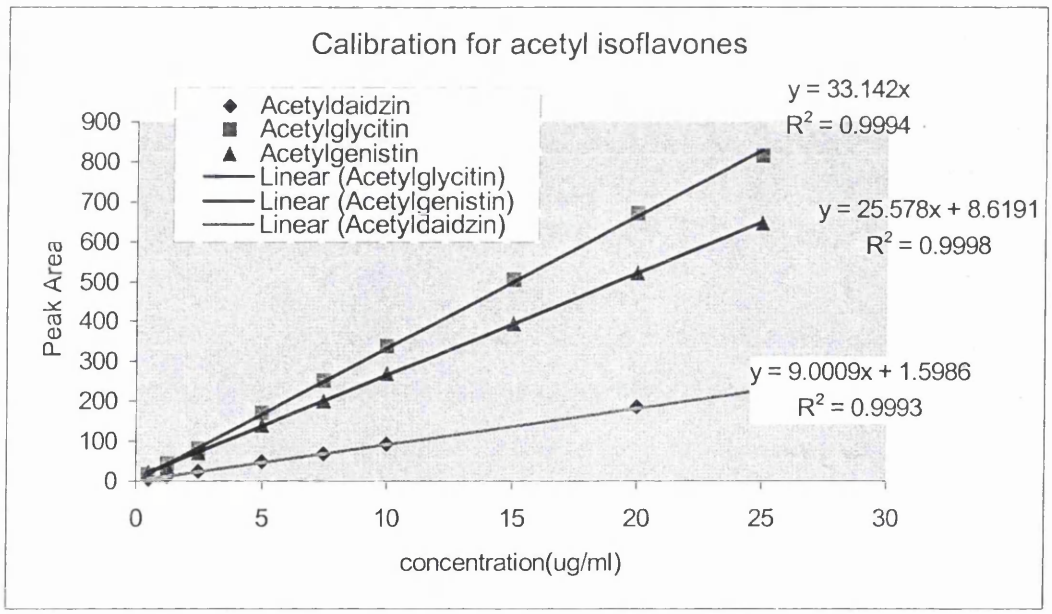


Figure 5.35 *Calibration curve for the analyses of the acetyl isoflavones*

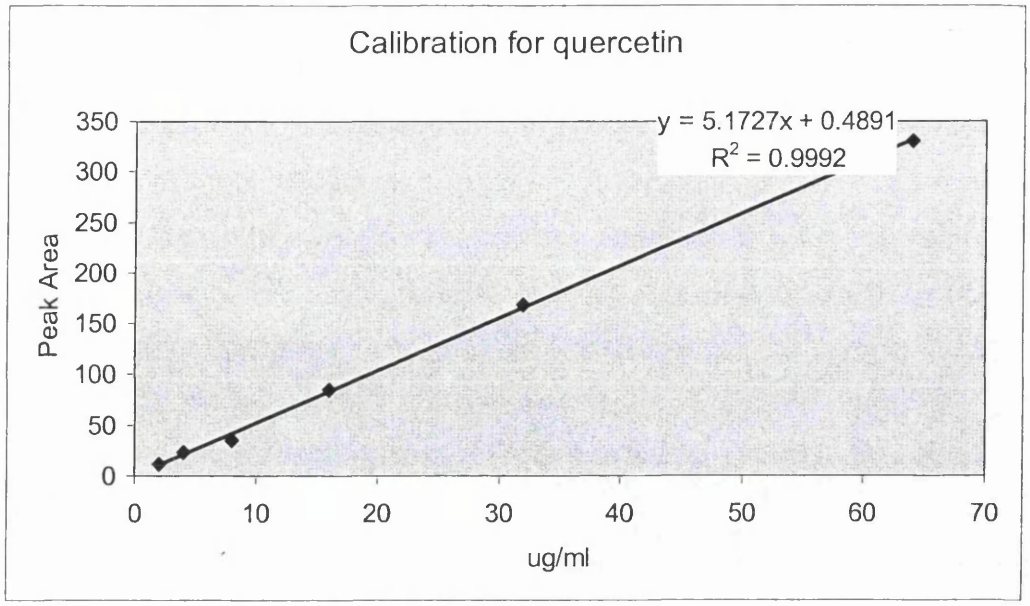


Figure 5.36 *Calibration curve for the analysis of quercetin*

5.5.8 Isoflavone concentration of nutrition supplements

Three nutrition supplements were quantified by our methods. They represent high isoflavones, middle isoflavones and low isoflavones.

5.5.8.1 Isoflavones in high content nutrition supplement (10 % total content of isoflavones)

In a sample of 10 % soy extract, acetyl isoflavones were its prominent compounds and malonyl isoflavones were not detected. It indicates that the content and the isoflavone forms can vary due to different fermentation process. Glycoside and aglycone isoflavones only represented a small portion of the total isoflavones. Figure 5.37 shows its analysis result by negative LC/APCI/MS.

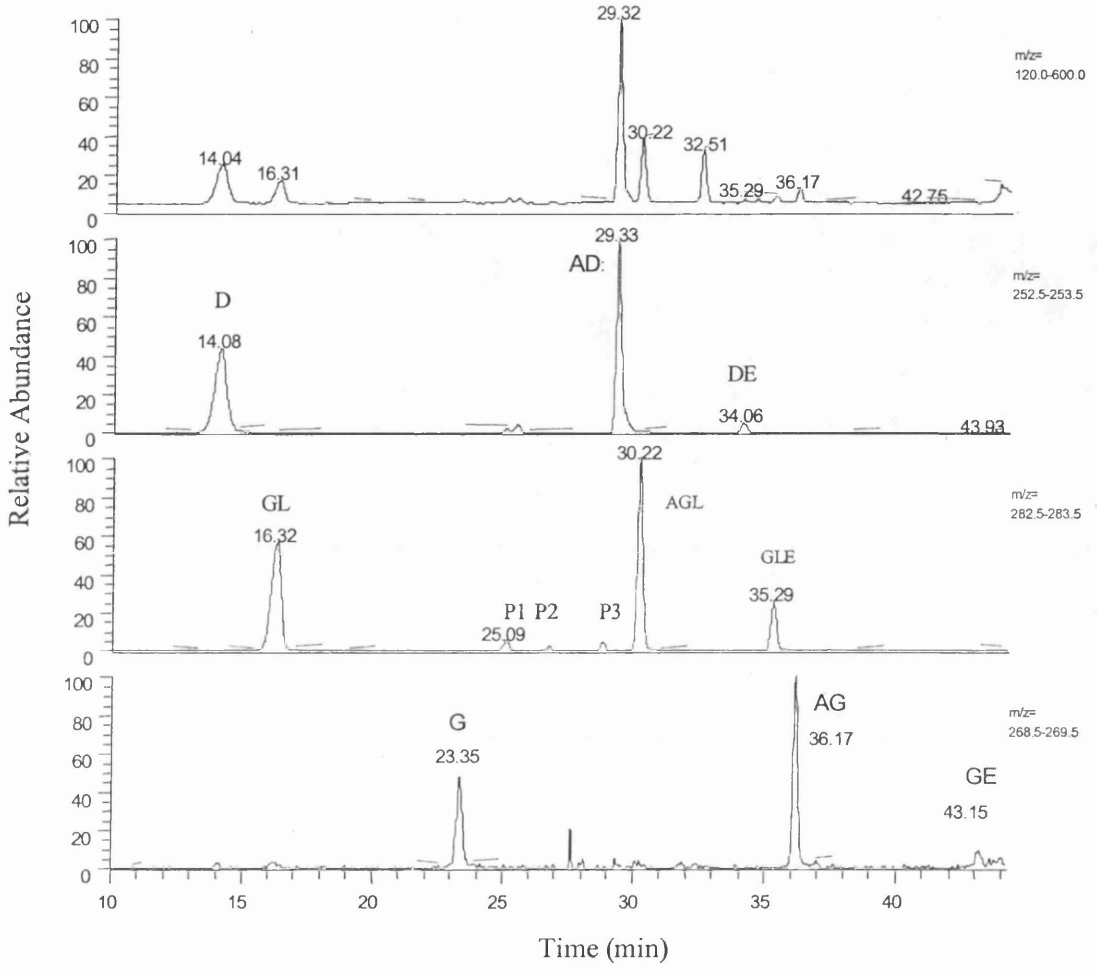


Figure 5.37 *LC/APCI/MS Chromatograms of 10 % soy extract*

(Experimental conditions see Figure 5.4)

D: daidzin, AD: Acetyldaidzin; DE: daidzein; GL: glycitin; AGL: acetyglycitin; GLE: glycitein; G: genistin; AG: acetylgenistin; GE: genistein; P1, P2, P3: unknown components. Mass spectrometer conditions see Figure 5.4

Nutrition supplement 2 was a capsule containing some other filling material and another extract from the fruit. It is marked as an antioxidant health supplement. Quercetin was its prominent additive for the purpose of antioxidant activity and the content of all isoflavones were marked 15 mg/capsule. Our result for isoflavones was 10.20-mg/capsule samples. In the supplement, all isoflavones were prominent in the glycoside form and amounts of acetyl and malony isoflavones were very low. This result showed that a different fermentation process was used by producers for

this supplement. The amount of glycone isoflavones was very low but all isoflavones can be detected by our method.

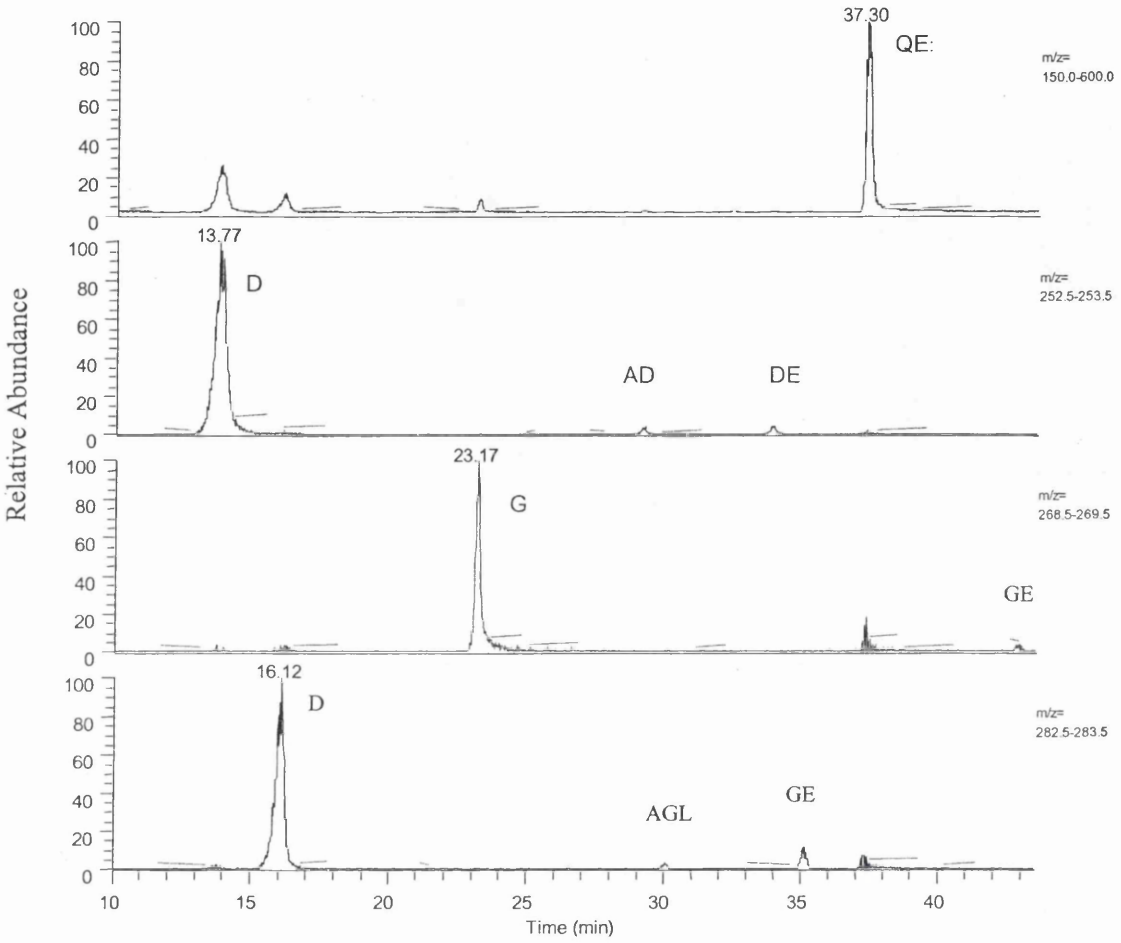


Figure 5.38 *LC/APCI/MS Chromatograms of soy antioxidant*

D: daidzin; AD: Acetyldaidzin; DE: daidzein; GL: glycitin; AGL: acetyglycitin;GLE: glycitein; G: genistin; AG: acetylgenistin; GE: genistein; QE: quercetin; P1, P2, P3: unknown components. Mass spectrometer conditions: see Figure 5.4

In nutrition supplement 3, the amount of soy concentrate was determined of 20 mg/g of the total content. On the supplement label a value of 2 % isoflavones was given. The distribution of isoflavones was a little different from supplement 1. The malonyl isoflavones were not detected and daidzin and acetyldaidzin were prominent. On the other hand, all the aglycone isoflavones gave a relatively high concentration. Table 5.11 shows our quantitative results of the three

nutrition supplements. Generally, the manufacture only labelled the content of total isoflavone. In fact, the content and forms of isoflavone varied with different fermentation process. If some health effects are expected after the soy supplement consumption, it is very necessary to measure their distribution and content.

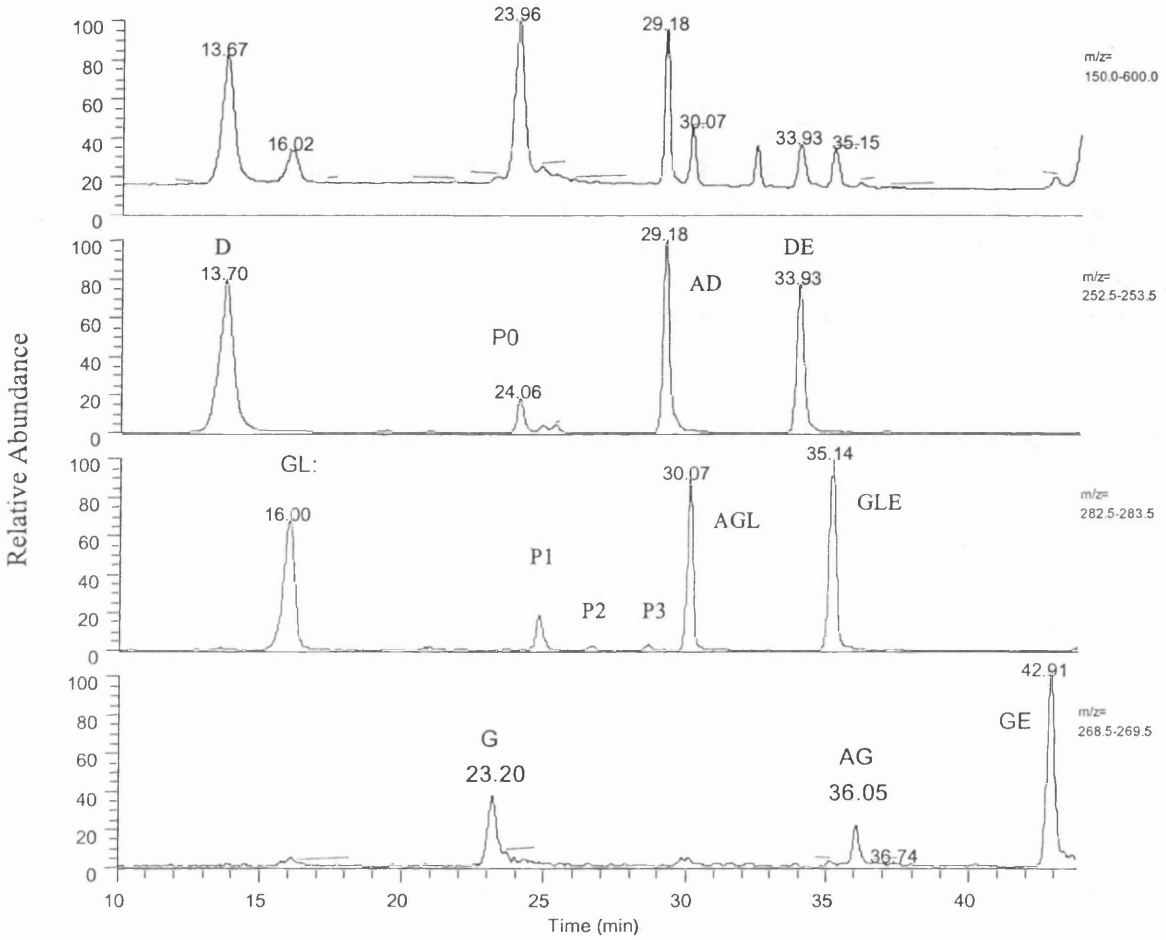


Figure 5.39 *LC/APCI/MS Chromatograms of sample complex*

D: daidzin; AD: Acetyldaidzin; DE: daidzein; GL: glycitin; AGL: acetyglycitin; GLE: glycitein; G: genistin; AG: acetylgenistin; GE: genistein; P1, P2, P3: unknown components. Mass spectrometer conditions see Figure 5.4

Table 5.11 *The isoflavone concentration of the soy supplements in ground samples*

Supplement	Supplement1 (Powder) mg/g[b]	Supplement 2[b] (Capsule 0.7286[a]) mg/capsul[e]	Supplement[b] (Powder) mg/g[b]
Daidzin	20.79	5.084	4.15

Glycitin	14.78	2.582	2.598
Genistin	6.73	1.542	0.858
Acetyldaidzin	58.46	0.605	12.98
Acetylglycitin	5.37	0.302	1.738
Daidzein	0.827	0.059	0.844
Glycitein	0.369	0.016	0.216
Acetylgenistin	6.98	0	1.03
Genistein	0.154	0.014	0.414
Total isoflavone	111.5	Total isoflavone: 10.20 mg Quercetin: 49.80 mg	24.83
The values labelled	100	Total isoflavone: 15 mg Quercetin: 50 mg	20

[a] Weight of the capsule itself in not included. [b] Mean value of the triplicate

5.6 CONCLUSION

In the present study a negative HPLC/APCI/MS method to analyse isoflavones was developed and 3 soy based health supplements were analysed to determine whether the isoflavone content data given by the producers corresponds with the measured value. 9 forms of isoflavones were quantified with high recoveries and accuracies. This method described for the extraction and HPLC analysis of isoflavones provides a means to speed extraction and analysis of isoflavones using the preferred solvent, acetonitrile. This solvent is also as mobile phase for HPLC analysis, hence, the elimination of the need to evaporate the acetonitrile greatly reduce the time and effort in sample preparation. Good reproducibility and recoveries were also obtained by this method. This method also gave a lot of useful information about unexpected compounds eluted together with isoflavones, and will allow rapid analysis of isoflavones, and may be particularly useful for analysis of purified commercial supplements, especially for the analysis of nutrition supplements.

If some health effects are expected after the soy supplementation, they are exclusively related to the active part of the isoflavone molecule. Our studies indicated that different processes of soybean could vary the forms of isoflavones. In nutrition supplement 1, the weight of the acetyl isoflavones is approximately 71 % of the total isoflavones weight and aglycone isoflavones are minor parts of the total isoflavones (8 %). In nutrition supplement 2, the glucoside unit is major forms of the total isoflavones with composition of 80 %. Aglycone isoflavones existed in all nutrition supplements with low percentage. Researchers, health practitioners and consumers are not always aware of these aspects. Our studies can provide accurate distribution of the form of isoflavones and more samples need to be done further.

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CHAPTER 6

Quantitative Determination of Isoflavones and Their Metabolites in Human Urine by LC/APCI/MS and LC/APCI/MS/MS

6.1 BACKGORUND TO ISOFLAVONES

Diphenolic phytoestrogens are plant substances that show some structural similarity to estradiol-17 β and to the estradiol receptor producing estrogenic effects. There are three main groups of nonsteroidal dietary phytoestrogens of flavones, isoflavones and coumestans. The main known phytoestrogens are the isoflavones genistein, daidzein. In recent years, soybeans and soy foods have become increasingly recognized because of the health benefit seen in the traditional Asian diet, which is very high in soy foods, low in saturated fat, and high in dietary fibre [1, 2]. For example, women in China, where soy consumption is high, have a lower risk for breast cancer and 36 % lower plasma estrogen levels when compared to women in Britain where soy consumption is low [3].

Epidemiological studies have shown a correlation between the consumption of soy foods and low rates of certain diseases, including coronary heart disease, hormone-dependent cancers such as breast, prostate, and colon cancer, osteoporosis, and problems associated with menopause and menstrual irregularities. In the United States and other western countries where the risk of these diseases is high, the typical diet is high in saturated fat, low in dietary fibre, and low in soy foods when compared to Asian nations [4].

6.2 BASIC METABOLITES OF ISOFLAVONES

In soy foods, phytoestrogens are found primarily as glycoside conjugates with aglycones comprising a relatively small percentage of the total isoflavones [5]. In the body, the isoflavone aglycones are metabolised to isoflavone glucuronides and sulfates or undergo further metabolism to products such as equol, dihydrogenistein and dihydrodaidzein [6, 7]. It is thought that the aglycones are absorbed directly from the gastrointestinal tract, whereas the glucoside conjugates require cleavage by intestinal bacteria to the aglycones prior to absorption and are then transformed by intestinal bacteria to hormone-like compounds. The mechanisms, through which the phytoestrogens may influence sex hormone production, metabolism and biological activity and exert anticancer, cancer protective, antiatherogenic, bone-maintaining effects, seem to depend, at least in part, on their mixed oestrogen agonist-antagonist properties. Furthermore, these weakly oestrogenic molecules were demonstrated to effect intracellular enzymes, protein synthesis, growth factor action, malignant cell proliferation, cell differentiation, angiogenesis, and apoptosis.

In addition to conjugation, genistein and daidzein, the major isoflavones in soybeans, are metabolised to dihydrogenistein and dihydrodaidzein, respectively [8]. The aglycone forms are important as they possess biological activity. The sulfate conjugates of the aglycones may also be important as carriers of aglycones to target tissues, since they may be deconjugated to the aglycone form in target tissues in a manner similar to that of the estrogen sulfates. Zhu BT et al [9] reported that the sulfate conjugates themselves might be biologically active as daidzein-7, 4'-di-O-sulfate has been reported to competitively inhibit sterol sulfatase in hamster liver microsomal fractions. The glucuronide conjugates could also be biologically active, as deglucuronidation of estradiol- and estrone-3β-D-glucuronides has been demonstrated in Syrian hamster kidney and liver lysosomes and microsomes.

6.3 AIMS

The classes of compounds to be detected and identified in human body fluids can be classified into two groups, lignans and isoflavonoids. Both of them are of plant origin with molecular weights and structures similar to those of steroids. Accurate methods to detect and quantitate isoflavone aglycones and their active metabolites are necessary to conduct complete pharmacological studies in these compounds because they could be important modulators of the human hormonal system and hormone action. The following isoflavonoid phytoestrogens have been identified or detected in human urine: formonetin, methylequol, daidzein, dihydrodaidzein, O-desmethylangolensin, genistein, and 3' 7-dihydroxyisoflavan and some other metabolites. Several methods have been reported in the literature to measure isoflavones and their metabolites in body fluids as well as foods. All of these methods require digestion or hydrolysis of sulfate and glucuronide conjugates prior to analysis. A comprehensive analysis of isoflavones and metabolites was obtained using a GC-MS method to separate and detect isoflavones and metabolites in urine [10]. Using this method, the aglycone and all metabolites can be quantitated. However, the method is very time-consuming, costly, requires multiple extractions and is not practical for large studies. HPLC methods have been developed to quantitate aglycones and total isoflavones [10, 11] but these methods require a 10-20-fold concentration of urine sample prior to analysis, and also do not quantitate levels of specific metabolites. Recently, an RIA has been reported to measure genistein and daidzein aglycone levels in serum [13, 14] although the radioimmunoassays are valuable methods, they are not yet commercially available, do not quantitate metabolites, and may lack specificity.

Recently, application of the ion trap detector to the LC/MS analysis of phytoestrogens has been reported [15, 16] providing a LC/MS method to estimate urinary concentration of genistein and daidzein, and their sulfate and glucuronide conjugate in urine samples. In this study, 52 ± 4 % and 26 ± 4 % of genistein was found in rat as aglycone and sulfate conjugate, respectively. Valentin-Blasini et al [17] used APCI/MS/MS for the measurement of seven phytoestrogens in human serum and urine. This method uses enzymatic deconjugation of the phytoestrogen metabolites followed by SPE and reverse-phase HPLC. The method allows detection of isoflavones and lignans with a limit of detection in the low parts per billion range (ng/ml). In this paper, an improved LC/MS method to detect isoflavones and their metabolites in urine was studied. When coupled with enzymatic digestion prior to analysis, this method quantified isoflavone aglycones and the sulfate and glucuronide conjugates. This method permitted the measurement of phytoestrogens with high sensitivity and specificity and simplified the sample pre-treatment procedure. Only 0.5-1 ml of urine sample was needed and is suitable for use in pharmacological studies.

6.4 EXPERIMENTAL

6.4.1 Reagents and Chemicals

Genistein, daidzein and glycitein were purchased from Indofine (Somerville, NJ, USA). Dihydrogenistein, 3'-hydrodaidzein, 8-hydrodaidzein, equol, O-desmethylangolensin and dihydrodaidzein were purchased from Plantech (Reading, UK). Formic acid (96 %, ACS reagent grade), ammonium formate (97 %), ammonium acetate, and dimethylformamice (99.8 %), ACS reagent grade), were obtained from Aldrich. Acetic acid (glacial, ACS certified). Ascorbic acid, 12-mol/ml hydrochloric acid and HPLC grade solvents (methanol, acetonitrile, methyl tert-butyl ether, dichloromethane, dimethylsulfoxide) were purchased from Fisher Scientific. β-Glucuronidase/sulfatase (product number G-0876) was purchased from Sigma. Because synthetic conjugates of glucuronidase or sulfate isoflavones are commercially unavailable, our assays built on the measurements of free isoflavones of genistein, daidzein and glycitein after treatment sample with enzymes [18, 19]. Hence, the total isoflavone (total genistein, total daidzein and total glycitein) is defined as the total amount of the isoflavone that is in the free form after treatment of the sample with β-Glucuronidase/sulfatase enzymes from Helix pomatia (G-0876). The free isoflavones (free genistein, daidzein and glycitein) were defined as the free isoflavones of sample

without treatment of sample by enzyme.

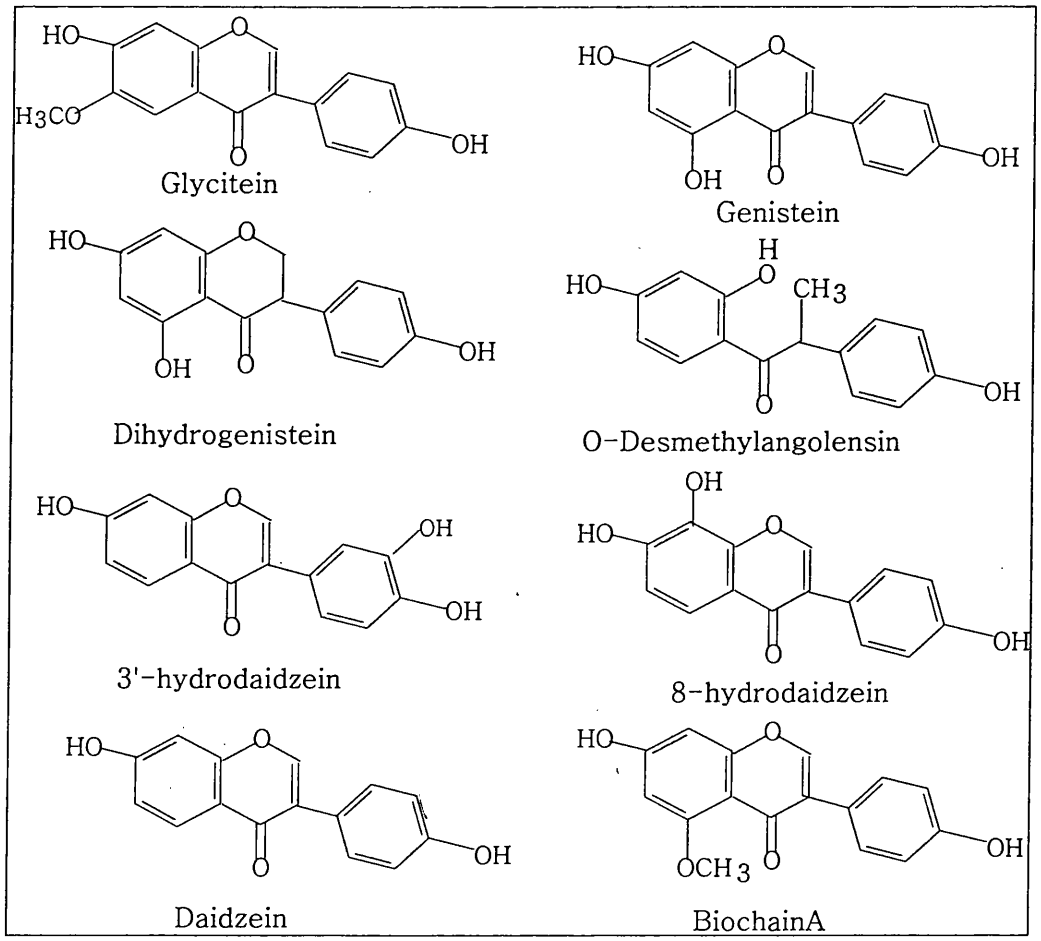


Figure 6.1 *Structures of isoflavones and their major* metabolites

6.4.2 Methods

6.4.2.1 LC/MS separation of isoflavones

The separation of isoflavones by a reverse phase column was studied. Two LC methods were developed to separate isoflavones and their metabolites. A Luna C_{18} HPLC column (100 × 4.6 mm, 3 μm) was used for separation of free isoflavones and a Luna C_{18} HPLC (150 × 4.6 mm, 5 μm column was used for separation of total isoflavones and sulfate isoflavones. Mobile phase was composed of solvent A (0.05 mol/L ammonium formate, pH = 4) and solvent B acetonitrile. Linear gradient programs were used to elute the isoflavones. Tables 6.1 and 6.2 show the gradient programme. Isoflavones were detected using a Finnigan LCQ mass spectrometer by negative

APCI full scan mode.

Table 6.1 *A gradient elution programme for method A)*

Time (min)	Solvent A	Solvent B	Flow rate (ml/min)
0	90	10	0.4
24	65	35	0.4
26	20	80	0.4
32	20	80	0.4

Table 6.2 A *gradient elution programme for method B)*

Time (min)	Solvent A	Solvent B	Flow rate (ml/min)
0	88	12	0.8
16	80	20	0.8
24	65	35	0.8
26	55	45	0.8
32	30	70	0.8
40	15	85	0.8

Standard stock solutions of genistein, daidzein and glycitein were prepared in dimethylsulfoxide(DMSO). Standard stock solutions of dihydrodaidzein, 3'-hydrodaidzein, 8-hydrodaidzein, O-desmethylangolensin, and dihydrogenistein were dissolved in methanol. Calibration standards were made by adding appropriate amounts of these stock solutions to a blank matrix (urine presumed to be free of the isoflavone analytes). Peak area response ratios were plotted against the ratios of analyte to internal standard (biochanin A at 100 ng/ml).

6.4.2.2 Analysis of isoflavones in urine

6.4.2.2.1. Free isoflavones in urine

100 μg/ml internal standard of biochainA was added to 1 ml of urine in a 30 ml glass centrifuge

tube and 6 × 3ml of methyl tert butyl ether (MTBE) was added to extract free isoflavones from urine. An end-over-end rotating mixer was used for approximately 30 min to facilitate the extraction. The samples were centrifuged at ca 2000 g for 10 min. The methyl tert butyl ether layer was then transferred to a 20 ml glass tube and concentrated to dryness under N_2 at 40 °C. The residue was dissolved in 2 ml of methanol: 0.05 M ammonium formate, pH 4.0 (50:50), using a vortex mixer as needed for reconstitution. 40 μl of the reconstituted material was then transferred to an auto sampler vial.

6.4.2.2.2 Total isoflavones in urine

Aliquots (500 μl) of urine were transferred to a 10-ml glass disposable centrifuge tube and treated with 0.5 ml of a mixture of β-glucuronidase/sulfatase from Helix pomatia to hydrolyze glucuronide and sulfate conjugates of genistein, daidzein and glycitein. The mixture of enzyme should be made up freshly and contained 0.15 g ascorbic acid in 10 ml of 0.2 M acetate buffer, pH 4.0, and 500 μl of β-glucuronidase/sulfatase.

6.4.2.2.3 Free plus sulfate conjugates of isoflavones in urine

Aliquots (1ml) of urine in a 20 ml glass disposable centrifuge tube were frozen and concentrated to dryness by lyophilization. To the residue were added (in the order listed), 600-μl dimethylformamide, 10 μl 6 M HCl, and 3 ml dichloromethane. The tubes were tightly capped and heated overnight (15-18 h) at ca. 37°C in order to effect solvolysis. After the incubation, the samples were dried under N_2 for 40 minutes to remove the dichloromethane.

Free Aglycone	Urine + 1 ml pH = 6 NH_4AC, extracted with MTBE three times
Glucuronide	Urine + 1 ml pH = 6 NH_4AC, enzyme mixture incubation 8 hrs Extracted with MTBE three times
Sulfates	Urine (frozen and lyophilization + 600 μl dimethylformamide +3 ml dichoromethane + 20 μl 6M HCl incubation 8 hrs and extracted with MTBE three times

Figure 6.2 *The experimental procedure of isoflavones*

6.4.2.3 Optimization of the mass spectrometric analysis of the isoflavones and their metabolites

Optimization of the mass spectrometric analysis of the standard isoflavones was performed by setting up "tune files" for various isoflavones and their metabolites in which the variable parameters of the mass spectrometer were optimized for the analysis of the protonated or deprotonated molecule of the isoflvones and their metabolites being tuned for. Both negative ESI and APCI were used for tuning all standard isoflavones by loop injection into the mass spectrometer. The negative APCI ionization mode was found to be much better than that the negative ESI.

A Finnigan LCQ mass spectrometer was used to optimize the detection and analysis of isoflavones and negative APCI was chosen to analyze for the isoflavones. The parameters of vaporizer temperature, sheath gas, aux gas, capillary temperature, capillary voltage and tube lens offset were optimized for the maximum abundance of $[genistein\text{-}H]^-$ and $[daidzein\text{-}H]^-$. The sheath nitrogen gas flow and auxiliary nitrogen gas flow was set at 90 and 5 arbitrary units, respectively. Capillary temperature was held at 180 °C. Capillary voltage was set at –40 V and tube lense offset was set at –20 V. The divert valve was employed to allow flow into the mass spectrometer only during analytes elution time.

6.4.2.4 Soy feeding study

8 Healthy volunteers (three of them are Asian people and five of them are British), aged 30-55 years were recruited for a 5-week study. All subjects were given a food list, which they were asked to avoid one week before study. A baseline level of phytoestrogens in urine was determined by analyzing a 24-h urine sample collected the day before the study started. During seven days, the subjects consumed genistein, daidzein and glycitein soy nutrition food every day; all urine was collected in the first three days (presumed the isoflavones were ejected totally after 72 hours). In the first three days of the second week, the urine was collected again. In the first three days of the third week and fourth week, urine samples were collected. All urine containers had ascorbic acid and sodium azide added as preservatives (0.1 % concentration of each). Samples were stored at –20 °C.

6.5 RESULTS AND DISCUSSION

6.5.1 Definitions of free isoflavones and total isoflavones

In our study, the following definitions are used. Unconjugated isoflavones recovered in urine after extraction (no enzymatic digestion) are referred to as " free aglycones". Isoflavones recovered from β-glucuronidase/sulfatase-treated urine are referred to as " total aglycones"(free aglycones plus aglycones released from sulfated and glucuronide conjugates). Isoflavones recovered from treatment with acid are defined as free plus sulfate isoflavones.

6.5.2 Sample extraction Procedure

Urine contains a complex mixture of compounds such as urea, amino acid, creatinine, catecholamines and ascorbic acid, amongst this, the modified isoflavones are minor components and hence considerable purification is required prior to HPLC analysis.

6.5.2.1 Selection of buffer solution

A lot of solvents and buffer solutions were chosen to extract the isoflavones and their metabolites. In general, diethyl ether, ethyl acetate and MTBE solvents were usually chosen to extract isoflavones and their metabolites. The isoflavones were efficiently recovered from urine upon direct extraction with these solvents. In our study, MTBE was considered to be a good extract solvent because it afforded markedly cleaner chromatograms [20]. Different buffer solutions of ammonium formate, ammonium acetate with different pH values of 4, 6 and 10 were optimized for the extraction of urine samples. The MTBE with buffer of pH=6 was found to efficiently recovery the free isoflavones from urine sample. Hence, MTBE with buffer of pH=6 ammonium formate solution was chosen as extraction solvent.

6.5.2.2 Enzymatic digestion

There are three main approaches to obtaining information on conjugates. The first is to conduct selective enzymatic hydrolysis to provide data on total sulfates, total glucuronides, and free aglycones, making all actual measurements as concentrations of the aglycones. The second approach involves separation of the classes of conjugates by a multistep chromatographic

processes, hydrolysis of these separate fractions, and measurement of the related aglycones, usually by GC/MS. GC methods for analyzing phytoestrogen have historically been designed around the limited ability of GC columns to elute polyphenolics. All GC-based methods for phytoestrogens analysis involve derivatization steps in order to reduce molecular polarity and increase volatility. These constraints result in a labor-intensive method involving many solid-phase extraction steps and separate GC/MS injection per sample determination. The extensive preparative works make this method unsuitable for routine use. The third possible approach is the direct measurement of all conjugates simultaneously and aglycones without any of the sample processing steps.

The only analytical technique currently capable of meeting this requirement for quantification of intact conjugates is LC/MS/MS [19]. The applicability of mass spectrometry to this area has been demonstrated by the measurement of isoflavone glucuronides in rat urine by LC/ESI/MS and ion trap LC/MS/MS, isoflavone glucuronides, and sulfates from human blood and the detection of sulfated genistein metabolites by LC/ESI/MS. The limiting factor has been the lack of suitable standard conjugates for identification and quantitation.

6.5.3 LC/APCI/MS negative ionization mode for the separation of isoflavones and their metabolites

6.5.3.1 LC/APCI/MS analysis of isoflavones and their metabolites

Reversed-phase HPLC has been utilized extensively for the quantitative and qualitative analysis of isoflavones in crude plant material and food products. However, the matrix is complex in urine and the concentrations of free isoflavones are too low for HPLC analysis with UV detection. In our study, LC/APCI/MS and LC/ESI/MS with negative mode were used to analyse the isoflavones and their possible metabolites, and LC/APCI/MS can give better detection limits. We developed two LC methods A and B (Tables 6.1 and 6.2) to separate the free isoflavones and total isoflavones, respectively. Figures 6.3 and 6.4 show LC/APCI/MS results with negative ion full scan mode for the separation of isoflavones by methods A and B, respectively.

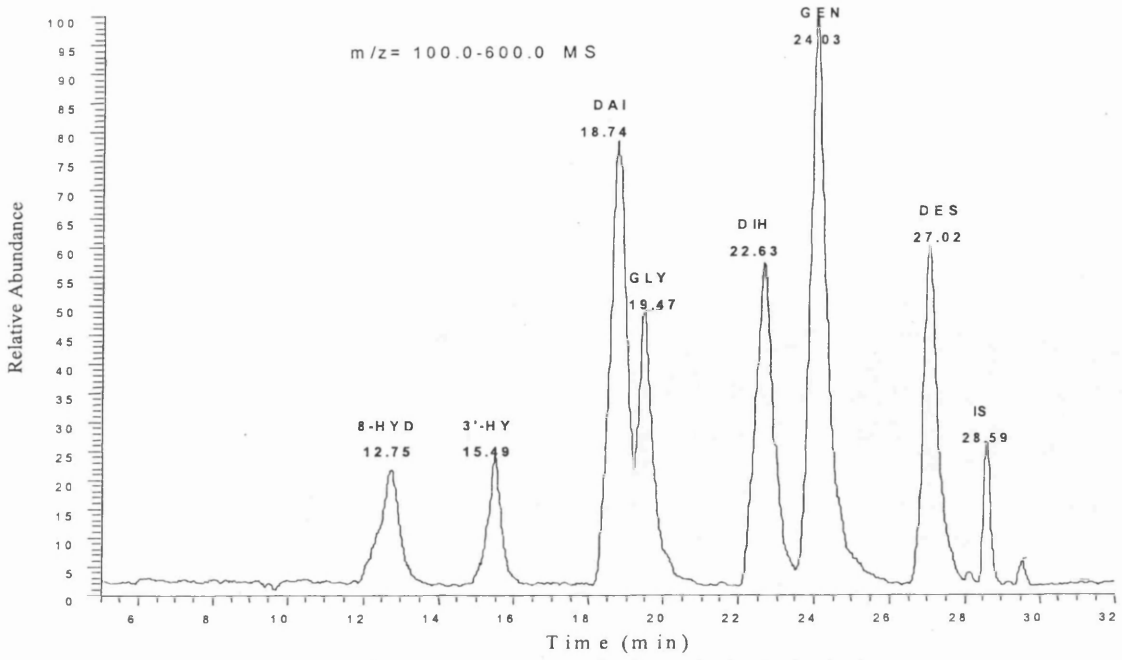


Figure 6.3 *TIC full scan chromatogram of [M-H]⁻ ions of isoflavones and their metabolites standards by LC/APCI/MS*

8-HYD=8-hydrodaidzein, 3'-HYD=3'-hydroxydaidzein, DAI=daidzein, GLY=glycitein, GEN=genistein, DIGE=dihydrogenistein, DID=dihydrodaidzein, DES=desmethylangolensin. IS=internal standard (BiochainA as internal standard).

Negative APCI and source conditions: Vaporizer Temperature (°C): 550; Sheath Gas flow (arbitrary units): 85; Auxillary gas flow (arbitrary units): 5; Discharge current (μA): 5; Heated capillary temperature (°C): 180; Capillary voltage (kV): -40; Tube Lens Offset (V): -20.

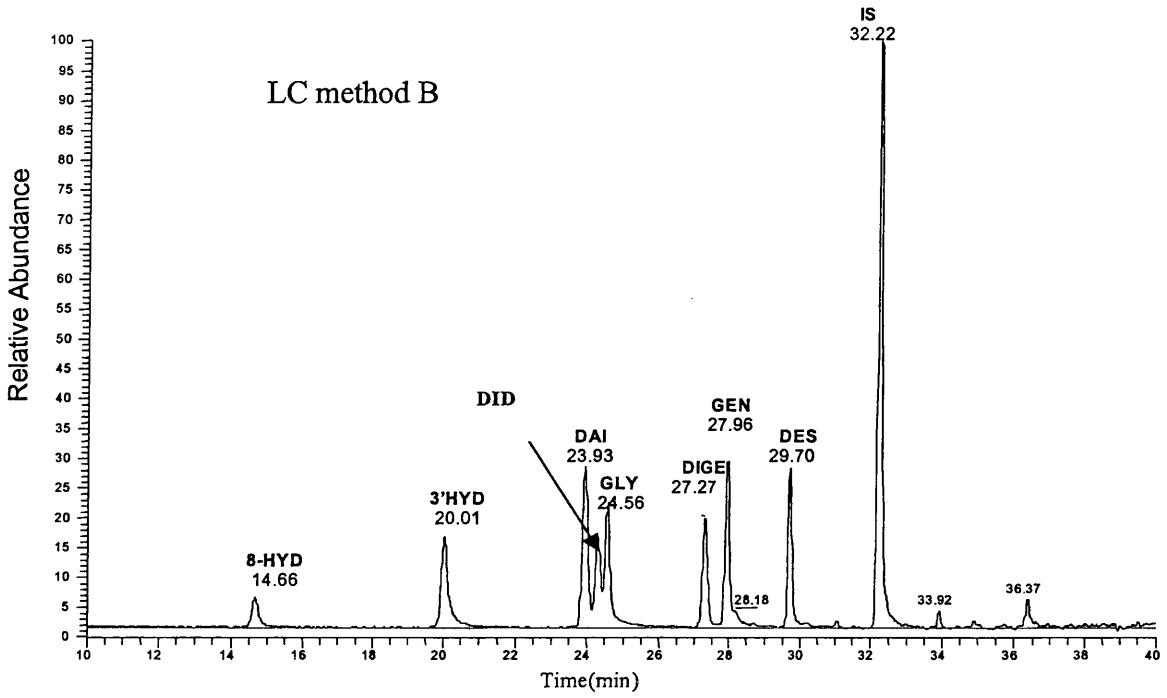


Figure 6.4 *TIC full scan chromatogram of isoflavone and metabolite standards by LC/APCI/MS 8-HYD: 8-hydrodaidzein; 3'-HYD: 3'-hydroxydaidzein; DAI: daidzein; GLY: glycitein; GEN: genistein; DIGE: dihydrogenistein; DID: dihydrodaidzein; DES: desmethylangolensin; IS=internal standard (BiochainA as internal standard). Mass spectrometry conditions see Figure 6.3.*

In method A, the separation can be finished in 32 min and all isoflavones and some metabolites can be resolved, but this method cannot resolve dihydrodaidzein and glycitein. In the free isoflavone, dihydrodaidzein cannot be detected, so, method A was used to determine the concentration of free isoflavones. In method B, all components gave good resolution and all isoflavones and their metabolites can be resolved. This method was used to quantitate and identify unknown components in subjects especially for good metabolism subjects.

6.5.3.2 Sensitivity, Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Sensitivity in mass spectrometry is defined as the ratio of the ionic current change to the sample change in the source. Limit of detection is defined that the signal and noise became statistically indistinguishable. It is the smallest sample quantity that yields a signal that can be distinguished from the background noise (generally a signal equal to ten times the background noise).

The limit of detection depends considerably on the abundance of the ionic species that is measured. The more abundant the measured ionic species with respect to all of the ions derived from the analyzed molecule, the higher is the limit of detection. The goal is thus to produce a signal that is as intense as possible. Several methods allow this goal to be reached, such as modification of the ionization conditions, ionization mode or derivatization of the sample, in order to increase the number of ions produced in the source or to reduce their fragmentation.

Another factor influencing the sensitivity corresponds to the time length of the signal integration. The longer that time, the more intense is the signal. Three acquisition modes exist: full scan mode, selected-ion monitoring (SIM) mode and selected-reaction monitoring (SRM) mode. These three modes are cited in ascending order of their effect on the sensitivity. Table 6.3 shows the results of limit detections of isoflavones and their metabolites.

Table 6.3 *Limit of detections of isoflavones and their metabolites*

Compound	LOD (full scan) pmol	LOD (SIM) pmol	
dihydrodaidzein	74.1	37.0	(255)
3'-hydrodaidzein	62.7	23.5	(269)
daidzein	28.0	12.2	(253)
glycitein	30.0	15.4	(283)
dihydrogenistein	55.4	14.8	(271)
genistein	45.4	2.20	(269)
desmethylangolensin	116.3	0.31	(257)

6.5.3.3 Fragmentation of standard isoflavones

MS/MS and MS^n have been widely applied to the structure elucidation of compounds from a wide variety of sources as it offers a large amount of information regarding the structural composition

of unknown compounds. In chapter 4, we have shown this approach to be effective in structure elucidation in the case of isoflavones when MS/MS fragmentation was used. It has been noted that fragmentation experiments assist in structural elucidation in two ways. First they enable the comparison of the fragmentation pathways of known isoflavones with naturally occurring compounds identified as those same isoflavones. Secondly knowledge of the fragmentation processes assists in the interpretation of the fragmentations of unknown, potentially novel compounds. Interpretation of MS/MS spectra can be used in the identification of phytoestrogens. Kulling et al [21] claimed that the location of the hydroxyl groups of the metabolites can be obtained from LC/MS with positive APCI/MS/MS using the base peak $[M+H]^+$ as well as fragment ions derived from the molecular ions by a retro Diels-Alder reaction. These ions can be used to determine the number of OH groups in the A-ring of the molecules. Fragment ions due to the loss of H_2O and CO were also observed in the MS^2 spectrum indicative of substituent positioning. S. E. Silcocks [22] also reported that the retro Diels-Alder decomposition product, a predominant ion in the CID spectra of all flavonoids obtained using quadrupole ion trap mass spectrometry, could be used to determine the number of substituents. In our study, MS/MS and MS^n were also used to interpret isoflavones and their metabolites by fragmentation and proposed pathway was also suggested.

6.5.3.3.1 Fragmentation of dihydrogenistein

Dihydrogenistein is metabolite of genistein. Its bimolecular ion of m/z 542.8 $[2M-H]^-$ is also detected in the full scan mode. In addition, ions of m/z 338.9 $[M-H+HCOONa]^-$ and m/z 316.5 $[M-H+HCOO-]^-$ are observed, respectively. The ion of m/z 211.2 $[M-H]^-$ is the base peak of dihydrogenistein.

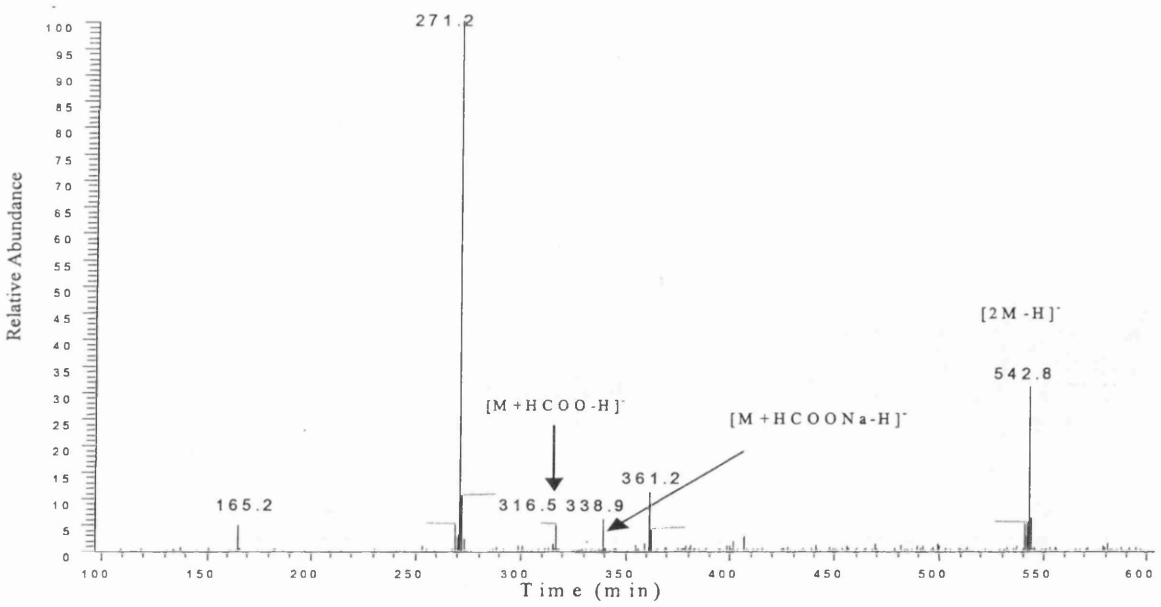


Figure 6.5 *MS full scan spectrum of the dihydrogenistein*

There are few papers reporting on the fragmentation of dihydrogenistein. In our studies, the fragmentation of dihydrogenistein was explored by negative APCI/MS^2 and APCI/MS^3. Using negative APCI/MS^2 and APCI/MS^3, the product ions m/z 137.1 and 165.2, which were postulated to contain the A-ring of the isoflavonoid molecule [23, 24] were observed. Since the difference between dihydrogenistein and dihydrodaidzein is the additional 5-hydroxyl group in the former compound, the A-ring-containing product ions of the respective metabolite are expected to differ by a mass of 16. This difference was actually observed with the postulated A-ring containing product ions (m/z 109.2 versus m/z 93.1). Figures 6.6 and 6.7 show the results analysed by negative APCI/MS^2 and APCI/MS^3.

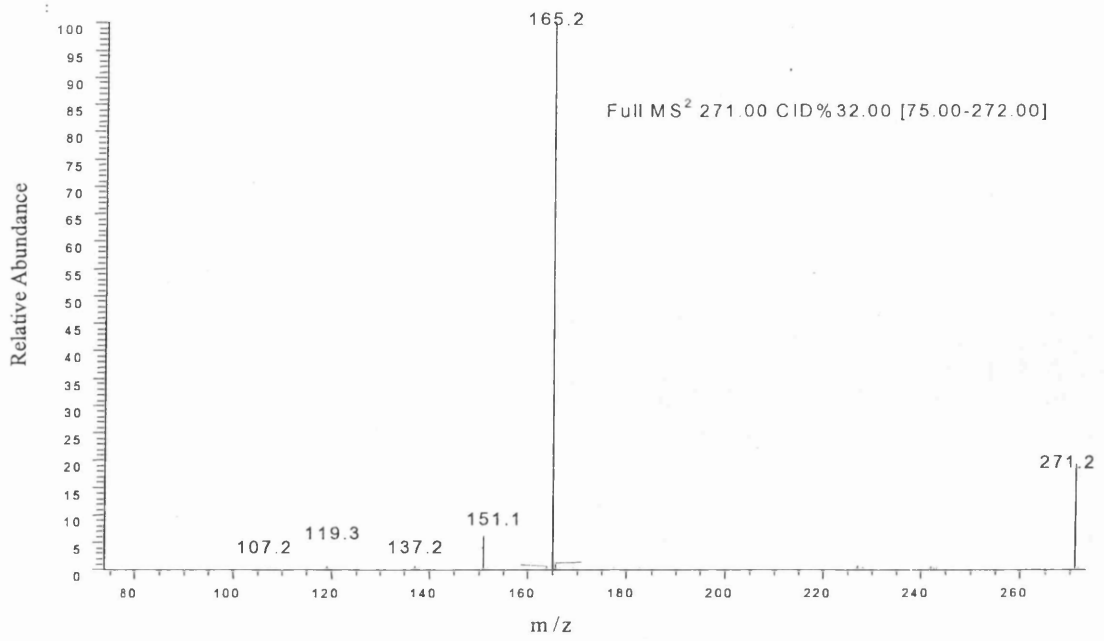


Figure 6.6 *MS/MS spectrum of the deprotonated molecule of dihydrogenistein (m/z=271)*

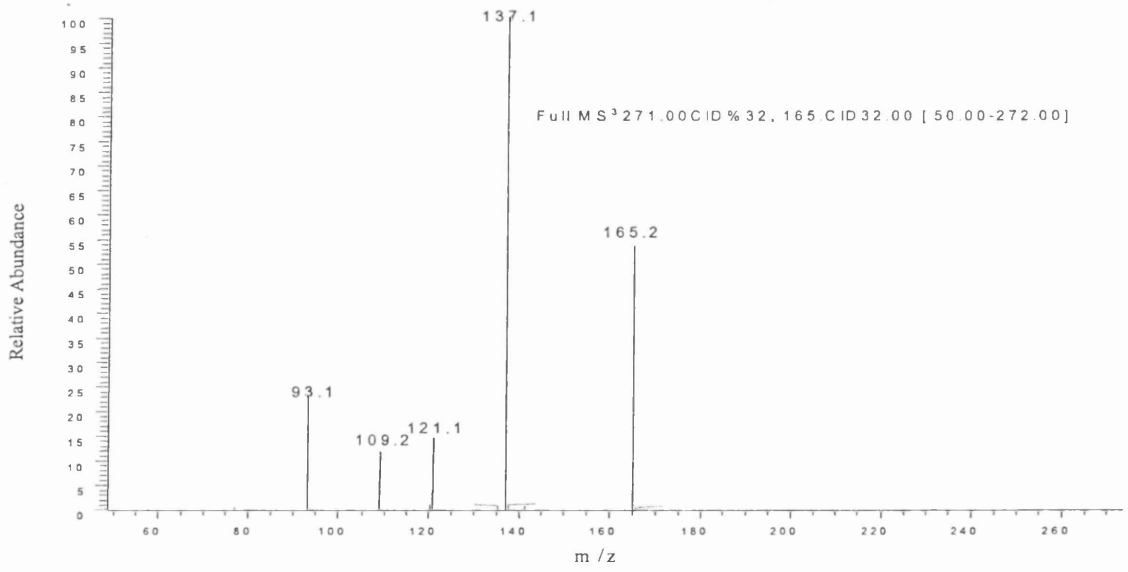


Figure 6.7 *MS^3 spectrum of deprotonated molecule of dihydrogenistein (m/z271→ m/z165)*

6.5.3.3.2 Fragmentation of dihydrodaidzein

Comparison of fragmentations of dihydrogenistein with those of dihydrodaidzein shows that the product ions of m/z= 165 from dihydrogenistein and m/z=149 from dihydrodaidzein are their differential fragment ions. Product ions of m/z= 109.2 from dihydrogenistein and m/z=93.2 from

dihydrodaidzein are also important fragments for their structure differences. Product ions of m/z=165 and 109.2 are fragments from the A-ring of dihydrogenistein which shows two hydroxyl groups in the A-ring. Product ions of m/z=149 and 93.2 are fragments from the A-ring of dihydrodaidzein which shows only one hydroxyl group in the A-ring. Figures 6.8-6.10 show the results analysed by negative APCI/MS^2 and APCI/MS^3. Scheme 6.1 shows the proposed pathway of dihydrogenistein and dihydrodaidzein.

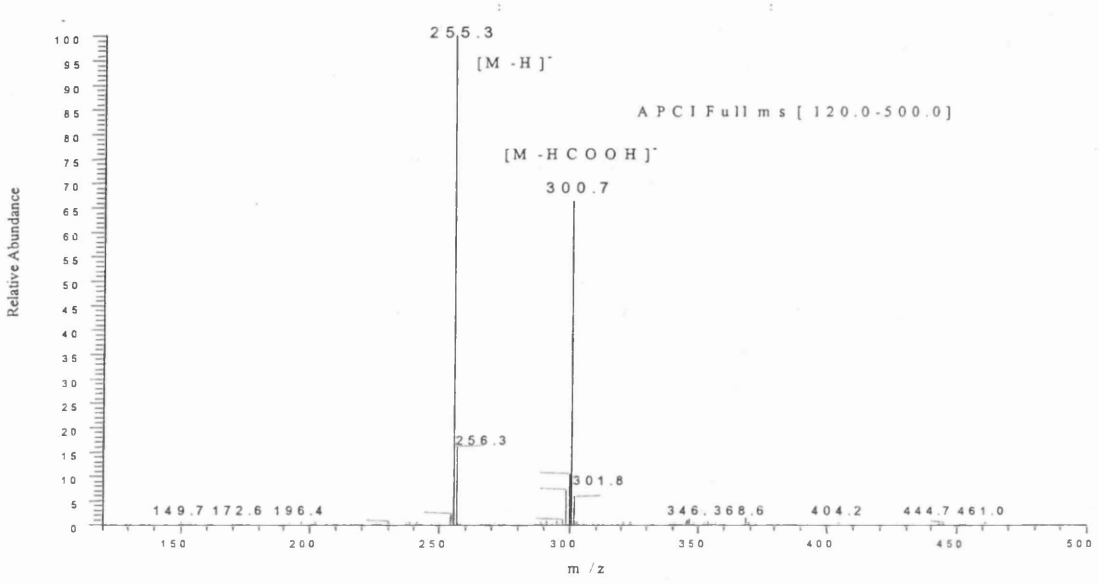
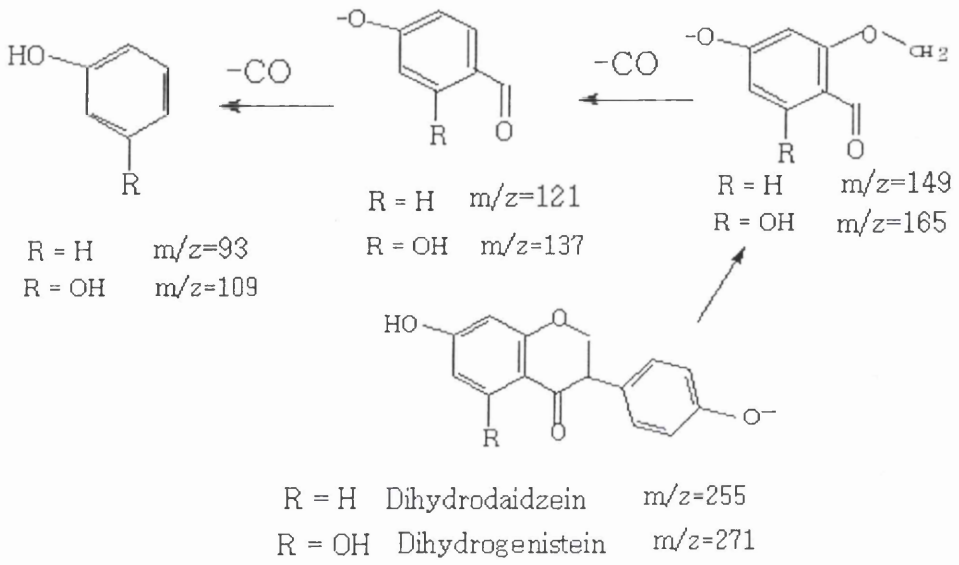


Figure 6.8 *MS full scan spectrum of the dihydrodaidzein*



Scheme 6.1 *Proposed pathway of dihydrogenistein and dihydrodaidzein*

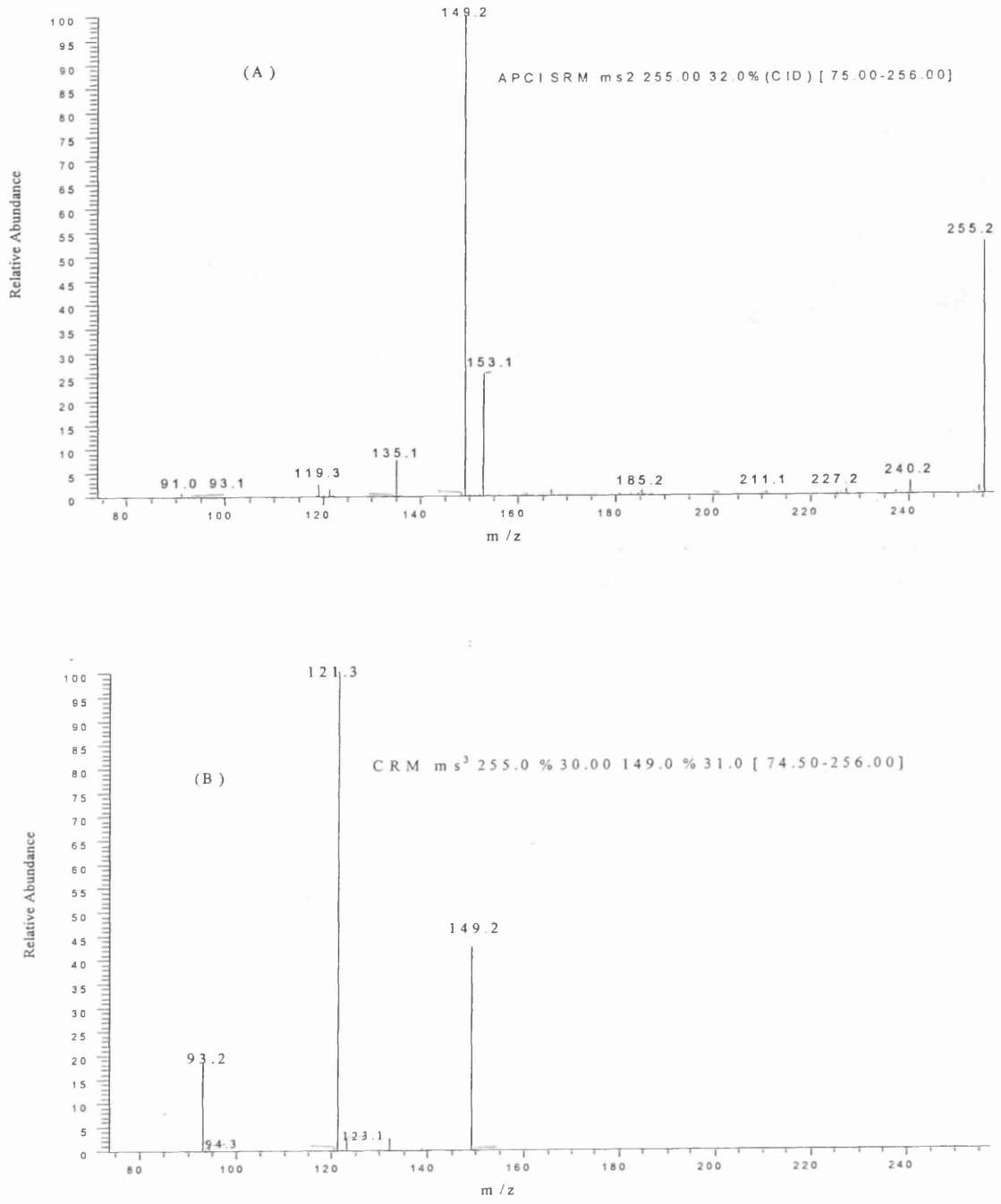
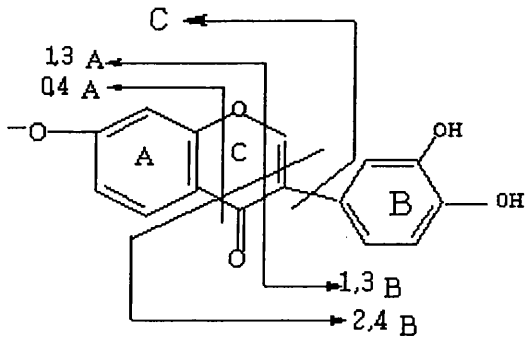


Figure 6.9 *MS/MS (A) and MS^3 spectra (B) of dihydrodaidzein*

6.5.3.3.3 Fragmentation of 3'-hydroxydaidzein

3'-Hydroxydaidzein and 8-hydroxydaidzein both belong to aglycone isoflavones. Characteristic fragments in the mass spectra of isoflavones can be expressed by fission of the deprotonated ion into A- and B- ring derived fragments. These fragmentations usually involve one of the two competing pathway which are similar to the pathways of flavonoids. The major fragments from molecule deprotonated ion are depicted as follow:



The negative ion spectra by APCI/MS^2 gave a lot of useful structural information. The main fragmentations of the M^- ions produced under different CID energies conditions were the structurally informative $^{1,3}A$ (m/z 135), $^{0,4}A$ (m/z 93) and $^{1,3}B$ (m/z 135) ions for 3'-hydroxydaidzein, formed by cleavage of two bonds of C-ring. These ions provide information of the substituents on the A and B rings. Other ions formed by the losses of small molecules were useful for the identification of the presence of specific functional groups. The successive losses of CO-groups directly from the M^- led to the formation of a series of ions at m/z 241, 213, 197 and 167. The fragment of m/z 213 may result from the loss of CO of m/z 241 and imply two OH groups in the B-ring. Figures 6.10-6.13 show the spectra of 3'-hydroxydaidzein analysed by APCI/MS and APCI/MS^n.

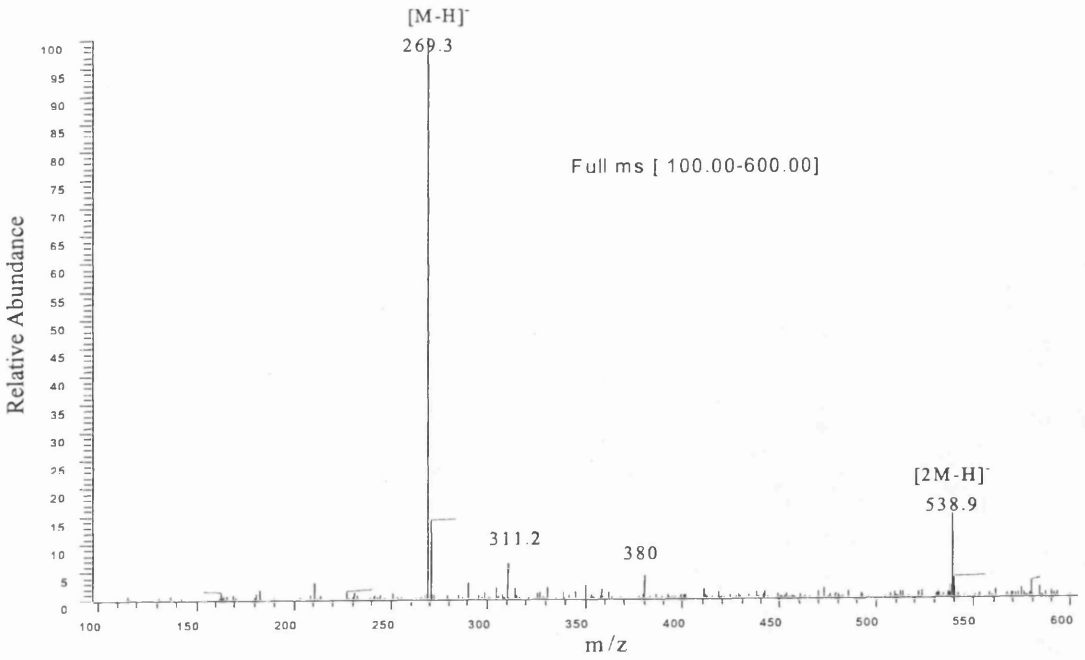


Figure 6.10 *MS full scan spectrum of the 3'-hydroxydaidzein*

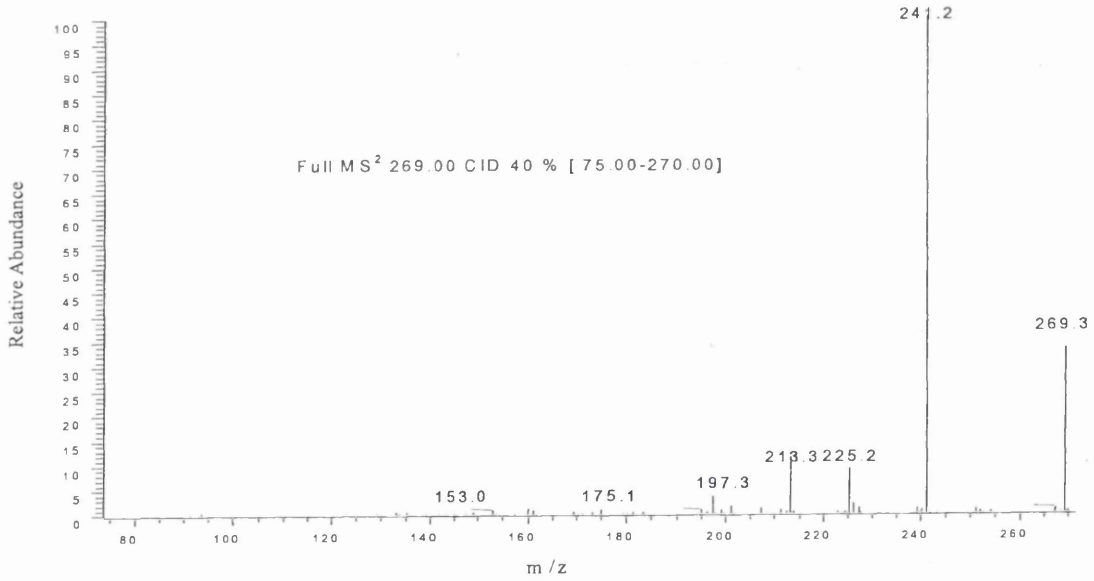


Figure 6.11 *MS/MS spectrum of the deprotonated molecule of 3'-hydroxydaidzein*

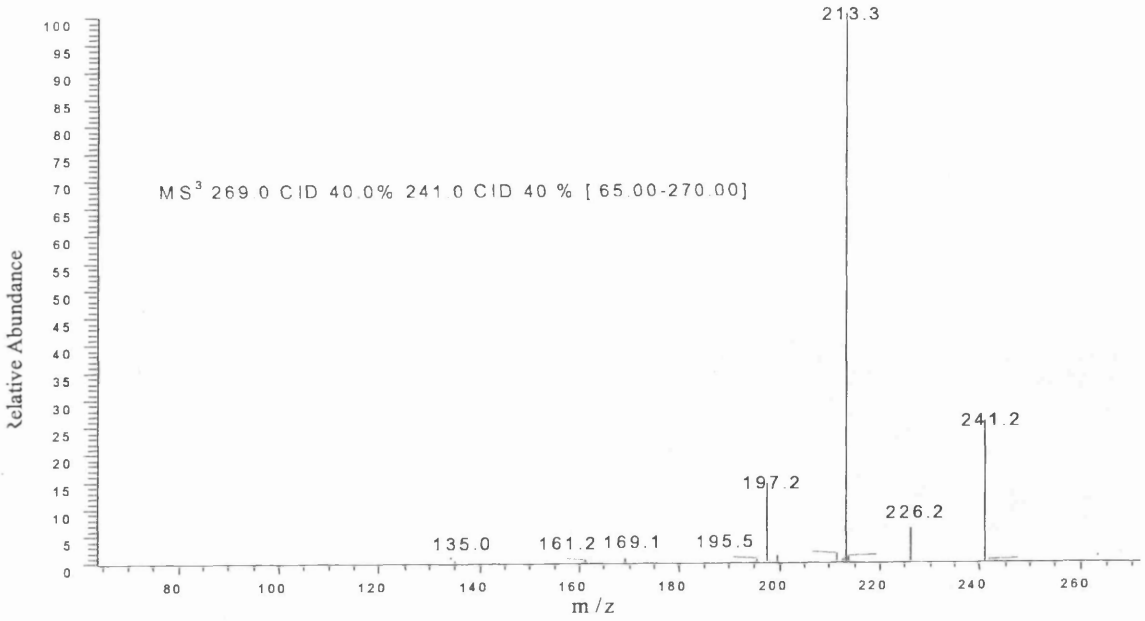


Figure 6.12 *MS^3 spectrum of the deprotonated molecule of 3'-hydroxydaidzein*

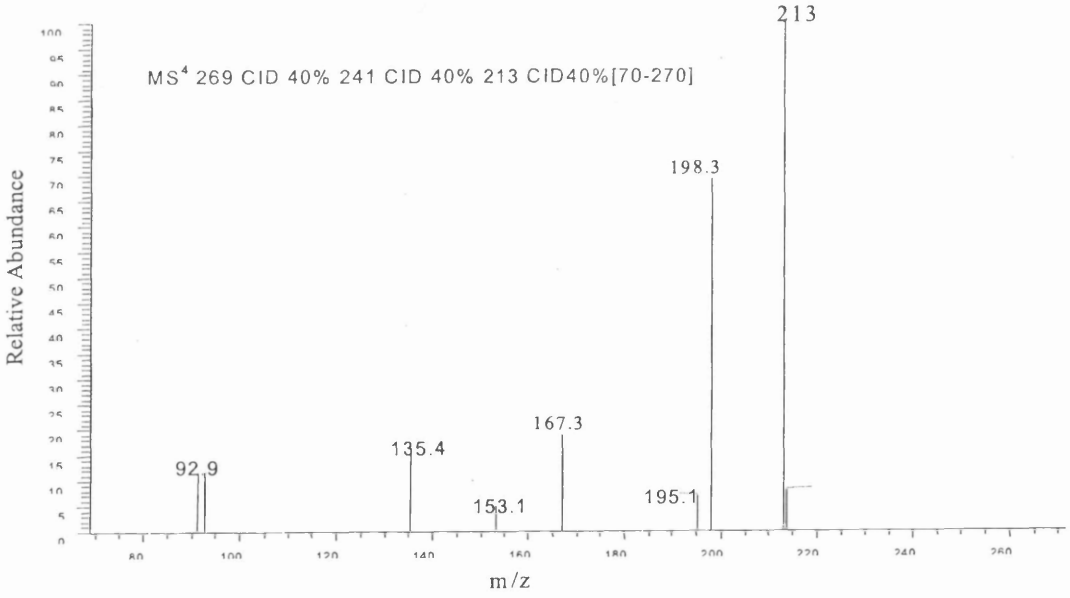


Figure 6.13 *MS^4 spectrum of the deprotonated molecule of 3'-hydroxydaidzein*

6.5.3.3.4 Fragmentation of 8-hydroxydaidzein

The main fragmentations of the M^- ions produced by 8-hydroxydaidzein are similar to those of 3'-hydroxydaidzein due to their similarity of structure. Structurally informative $^{1,3}A$ (m/z 153) ion shows two hydroxyl groups in the A-ring indicating the presence of specific functional groups. The successive losses of CO or O directly from the M^- led to the formation of another series of ions at m/z 241, 213, 197 and 181. Figures 6.14-6.16 show spectra of 8-hydroxydaidzein analysed by APCI/MS and APCI/MS^n.

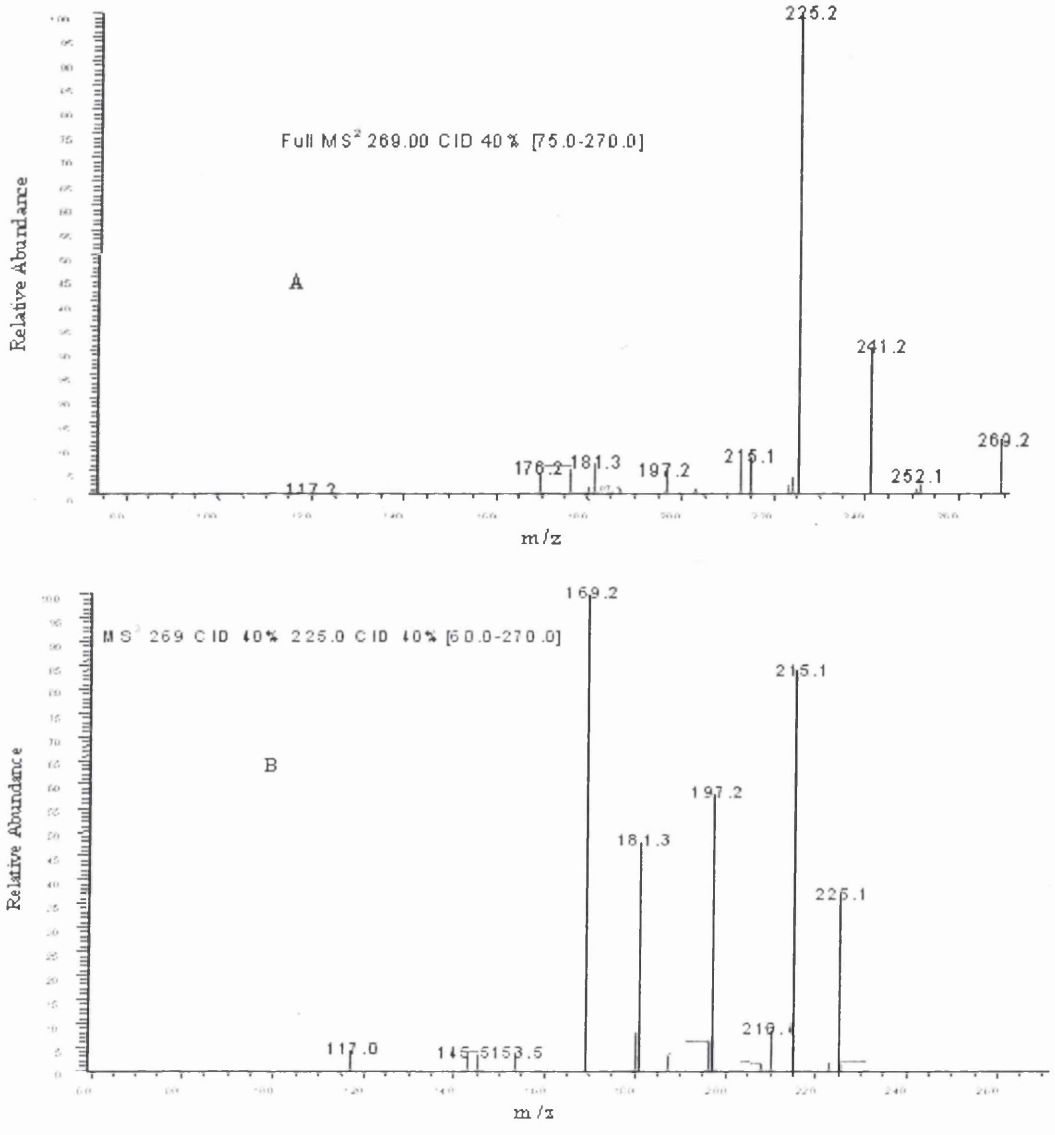


Figure 6.14 *MS/MS (A) and MS^3 spectrum (B) of 8-hydroxydaidzein*

6.5.3.3.5 Fragmentation of *O*-desmethylangolensin

O-desmethylangolensin is already described as a metabolite detected in the human urine and is the metabolite from daidzein [25, 26]. The structural analysis results from a molecule deprotonated ion fragmentation spectrum of m/z 257 $[M-H]^-$. The product ions m/z 119 and 147 were postulated to contain the B-ring of the isoflavonoid molecule. In the APCI/MS/MS spectrum, the fragment m/z 239 exhibits the highest intensity. Its formation can be explained by the loss of H_2O of O-desmethylangolensin. The fragment m/z 147 is the α- cleavage of O-desmethylangolensin. The presence of m/z 163 and m/z 109 ions indicated one hydroxyl group in the A-ring. Figures 6.15-6.17 show the spectra of O-desmethylangolensin analysed by negative APCI/MS and APCI/MS^n. Scheme 2 suggested the proposed fragmentation pathway of the O-desmethylangolensin.

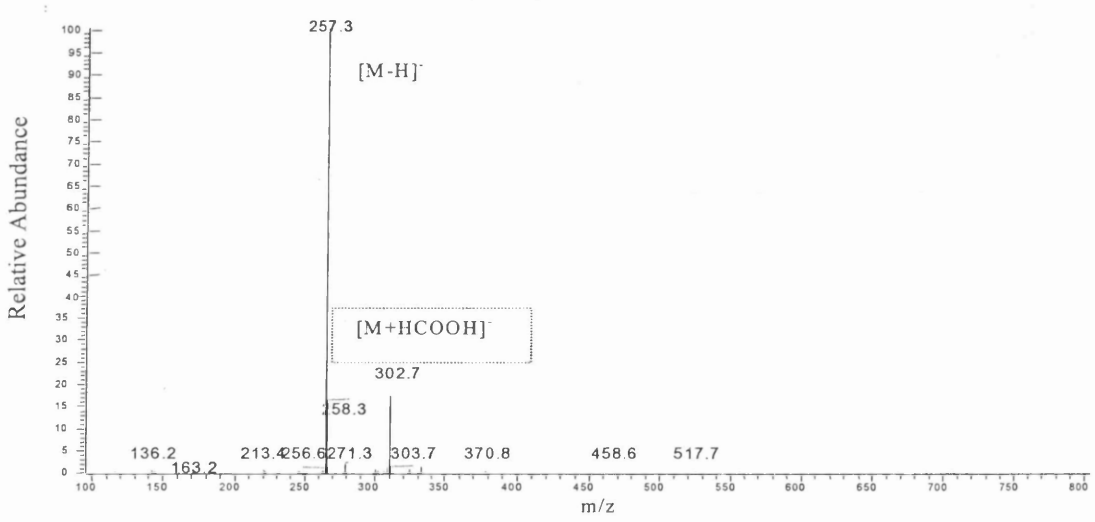


Figure 6.15 *MS full scan spectrum of O-desmethylangolensin*

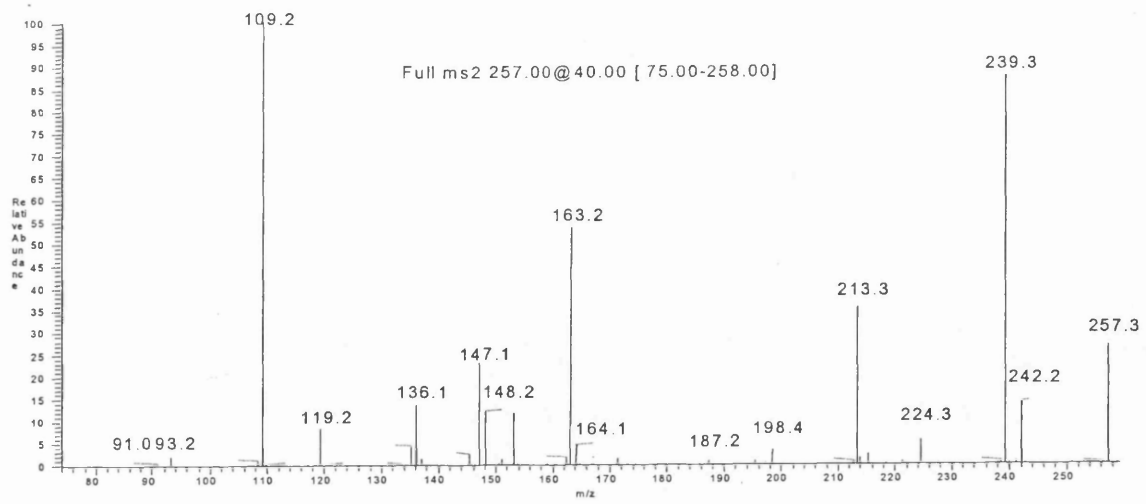


Figure 6.16 *MS^2 spectrum of the deprotonated molecule of O-desmethylangolensin*

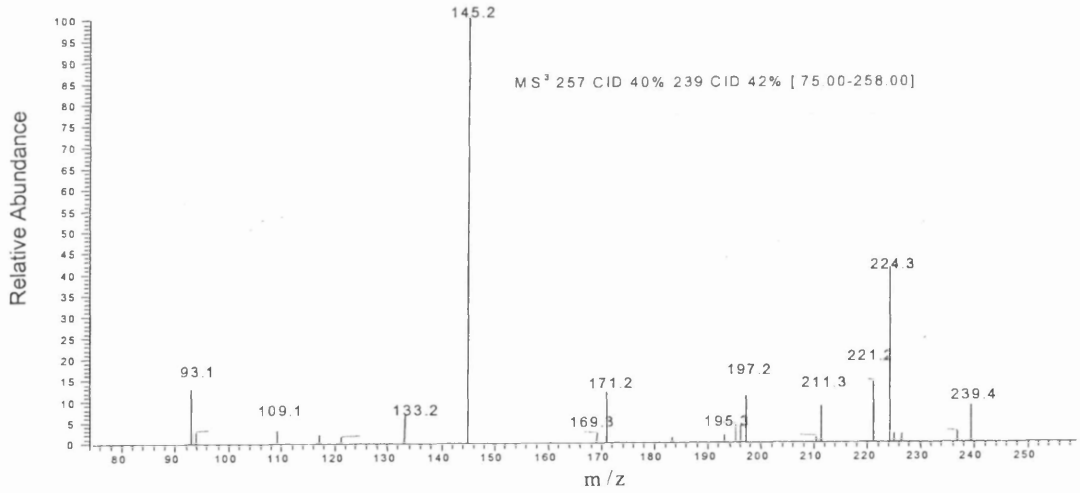
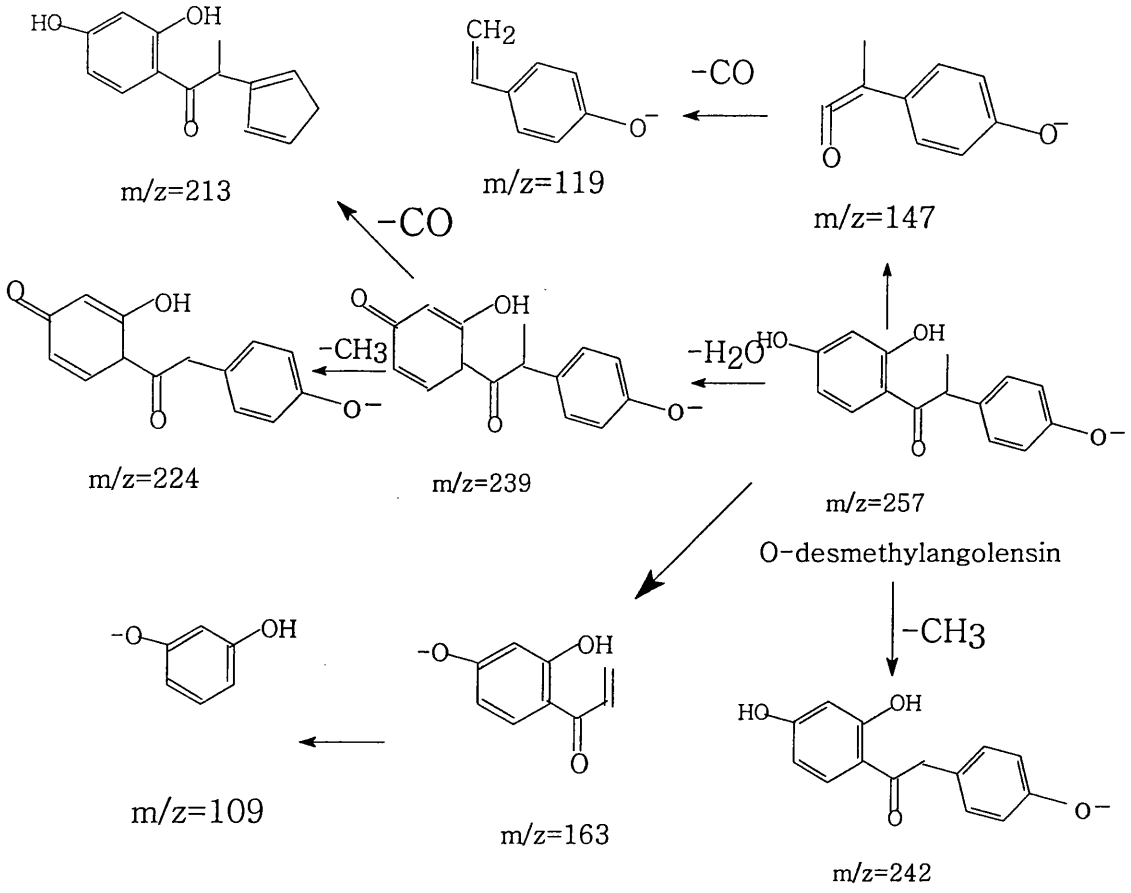


Figure 6.17 *MS^3 spectrum of the deprotonated molecule of O-desmethylangolensin*



Scheme 2 Proposed fragmentation pathways of *O*-desmethylangolensin

6.6 IDENTIFICATION OF ISOFLAVONES AND THEIR METABOLITES IN URINE

120 urine samples from 8 health subjects were purified and analyzed by HPLC/MS and HPLC/MS^n to determine their content of isoflavones and metabolites. In urine samples, the concentrations of free isoflavones from the subjects were very low, but can be detected from most subjects especially Chinese people. Total isoflavones were detected in all subjects but varied with different countries and diet habits.

6.6.1 Free isoflavones

Free isoflavones comprise a relatively low percentage of all isoflavones in soy nutrition food and

most of the isoflavones exist in the form of glycosides. When full scan LC/APCI/MS was used to detect the free isoflavones, only the free isoflavones of daidzein and genistein were detected in Chinese people and vegetarian people in UK due to the low sensitivity of full scan mass spectrometry, but the free isoflavones can be detected in most subjects when LC/MS with Single Ion Monitoring (SIM) was used. Figure 6.18 shows the free isoflavones results from a Chinese man by LC/APCI/MS/SIM.

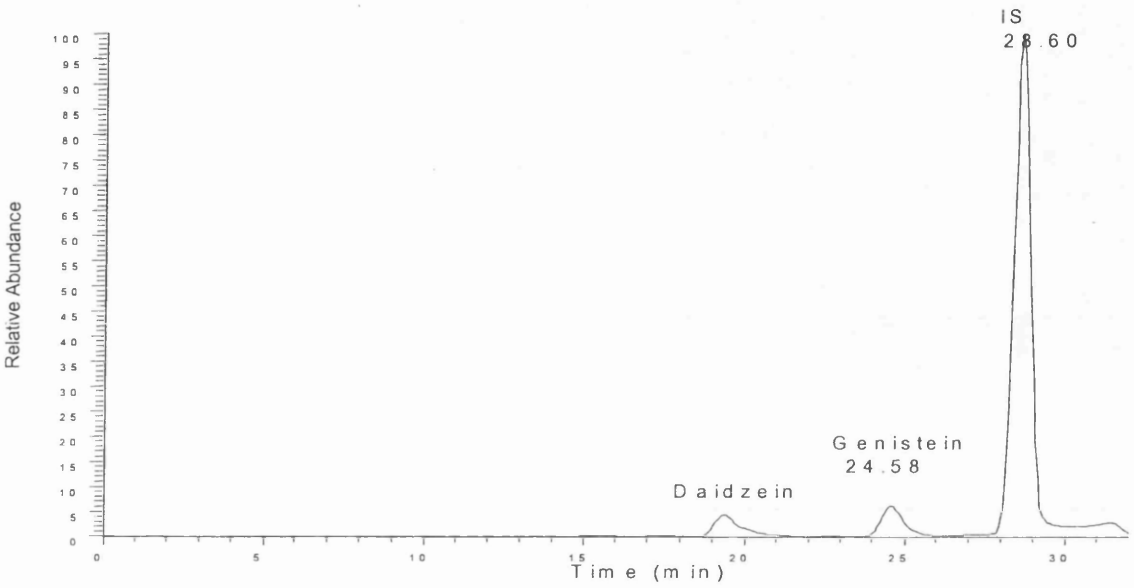


Figure 6.18 *The free isoflavones results from a Chinese man by LC/APCI/MS (SIM). (HPLC analysis methods see Table 5.2)*

6.6.2 Total isoflavones of urine

When soy-base nutrition food is consumed, the free isoflavones can be directly absorbed by the gastrointestinal tract, whereas the glucoside conjugates require cleavage by intestinal bacteria to the aglycones prior to absorption. In addition to conjugation, genistein and daidzein, the major isoflavones in soybeans, are metabolized to dihydrogenistein and dihydrodaidzein, respectively. It is generally accepted that the glucoside forms ingested with foods are hydrolyzed and the free aglycones are then converted to sulfate and glucuronide conjugates by the liver and the other epithelial surfaces. These biological conjugates circulate in the plasma and are excreted in the urine and feces. So, the urine sample needs to be hydrolyzed by enzymes in order to cleavage the sulfate and glucuronide isoflavones to free isoflavones.

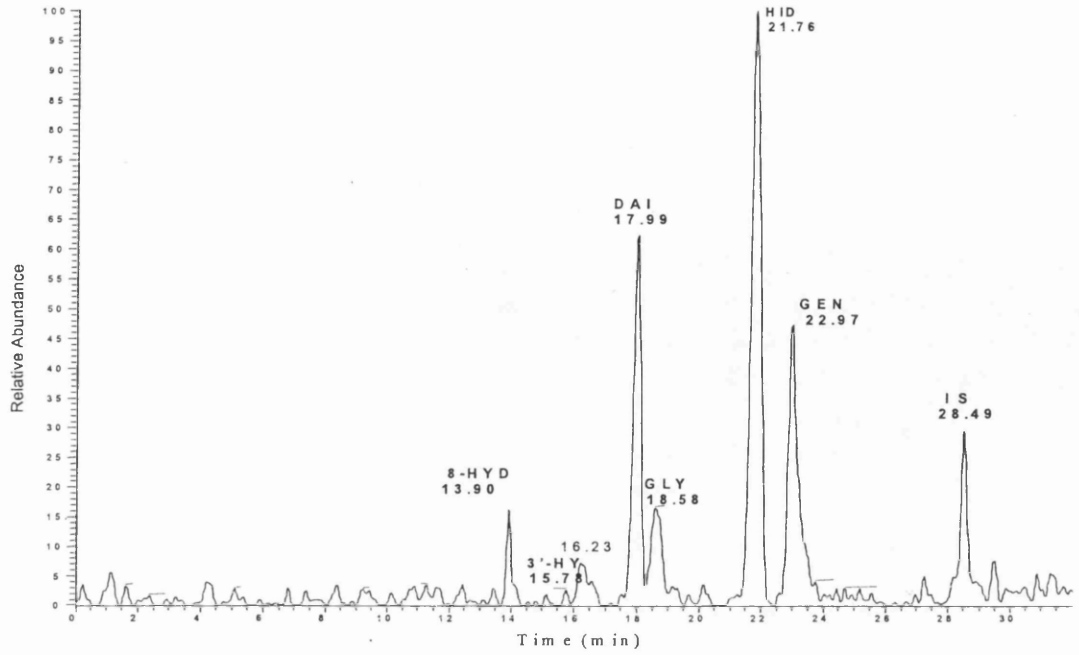


Figure 6.19 *TIC chromatogram of total isoflavones and their metabolites of a urine sample from a Chinese man obtained by LC/APCI/MS (HPLC analytical conditions see Table 6.2)*

Figure 6.19 shows the baseline of a urine sample extract from a Chinese man before taking soy based nutrition supplements. This subject immigrated to the UK ten years ago and he maintains an Asian diet and consumed soy food including soymilk, soybean and tofu very often. After hydrolysis with mixed glucuronidase/sulfatase enzymes, the aglycone content of urine increased markedly, as would be expected. Daidzein, glycitein, genistein were detected and at the same time, 8-hydrogenistein, dihydrogenistein and desmethylangolensin, which are metabolites of genistein and daidzein, were also detected.

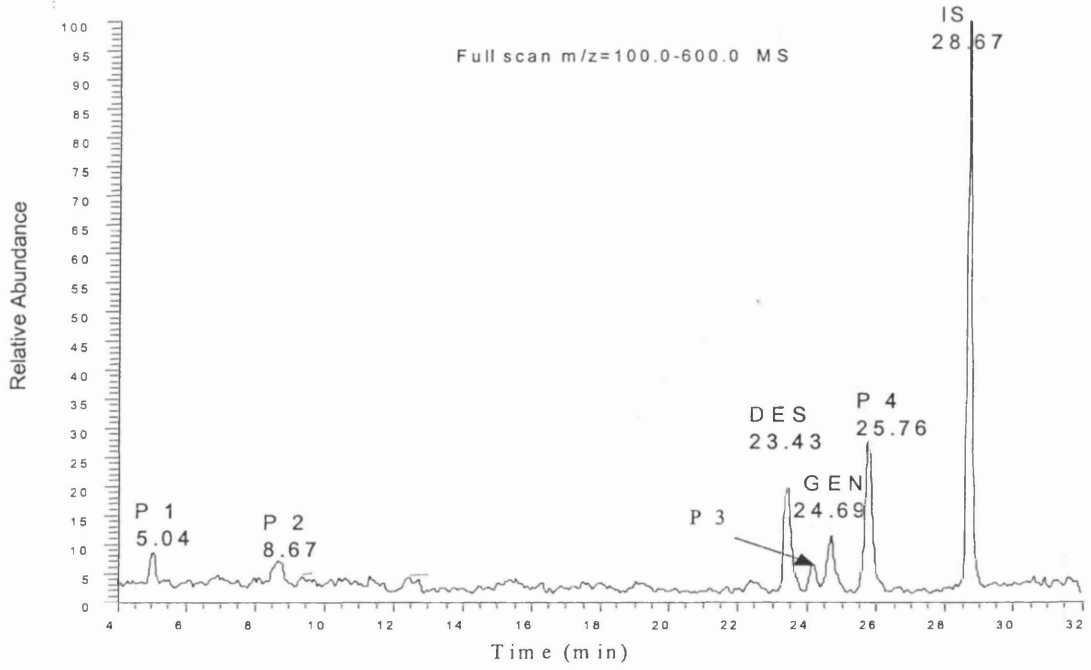


Figure 6.*20* *TIC of total isoflavones and their metabolites of a urine sample from a Chinese woman by LC/APCI/MS (HPLC analytical conditions see table 5.2)*
8-HYD=8-hydrodaidzein, 3'-HY=3'-hydrodaidzein, DAI=daidzein, GLY=glycitein, GEN=genistein, DIH=dihydrogenistein, DES=desmethylangolensin. IS=internal standard

Figure 6.20 shows results of a urine sample from a Chinese woman after taking soy based nutrition supplements for two weeks. This subject immigrated to the UK two years ago and her diet has changed and she does not consume soy food a lot in daily life. Only desmethylangolensin and genistein were detected. New peaks P1, P 2, P3 and P 4 were detected. This result shows that the change of diet can influence metabolism of isoflavones.

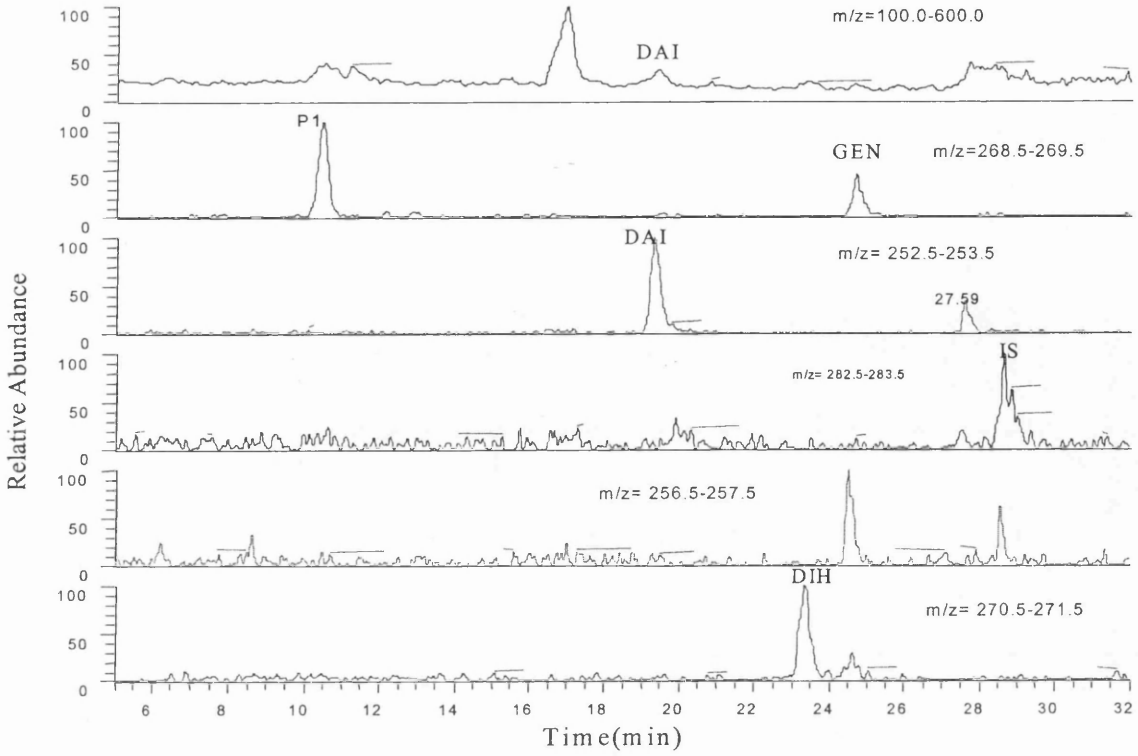


Figure 6.21 *RIC chromatogram of total isoflavones and their metabolites of a urine sample from a British woman by LC/APCI/MS*

Figure 6.21 shows the results of a urine sample from a British woman after taking soy –based nutrition supplements. This subject is a vegetarian and consumed more soy food than normal British people. Daidzein, genistein and dihydrogenistein were detected but glycitein and desmethylangolensin were not detected. Another new peak, which has the same m/z of daidzein, was also detected. This new peak could not identified due to the unavailability of standard material.

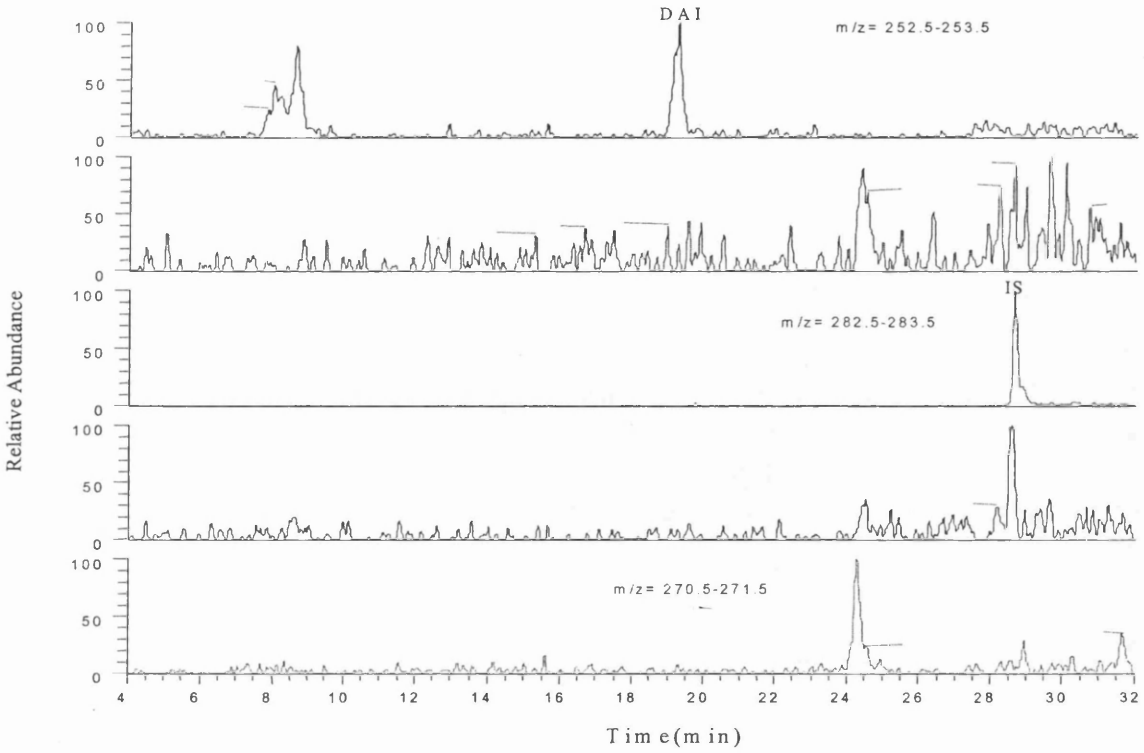


Figure 6.22 *RIC chromatogram of total isoflavones and their metabolites of a urine sample from a British man by LC/APCI/MS*

Figure 6.22 shows the results of urine sample from a British man after taking soy –based nutrition supplements. This subject represents typical British people who consumed low soy food and high fat food. Genistein, glycitein and desmethylangolensin were not detected but dihydrogenistein and daidzein were detected.

6.6.3 Identification of peaks of isoflavones in urine sample

The Chinese people studied all seemed to show good metabolism and many metabolites were detected in their urine. In order to detect all the possible metabolites in urine, HPLC method B was used to quantitate and identify the isoflavones and their metabolites in the urine sample. Figure 6.23 shows the results of a urine sample from a Chinese woman

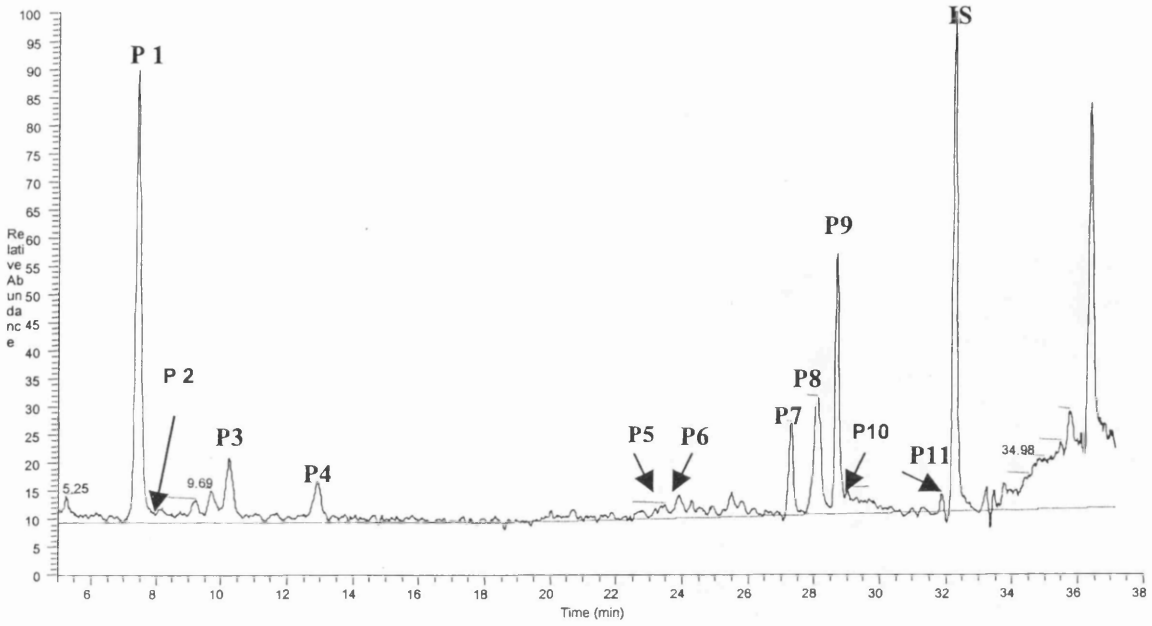


Figure 6.23 *TIC full scan chromatogram of total isoflavones and their metabolites in a urine sample from a Chinese woman by LC/APCI/MS (HPLC method sees Table 6.2)*

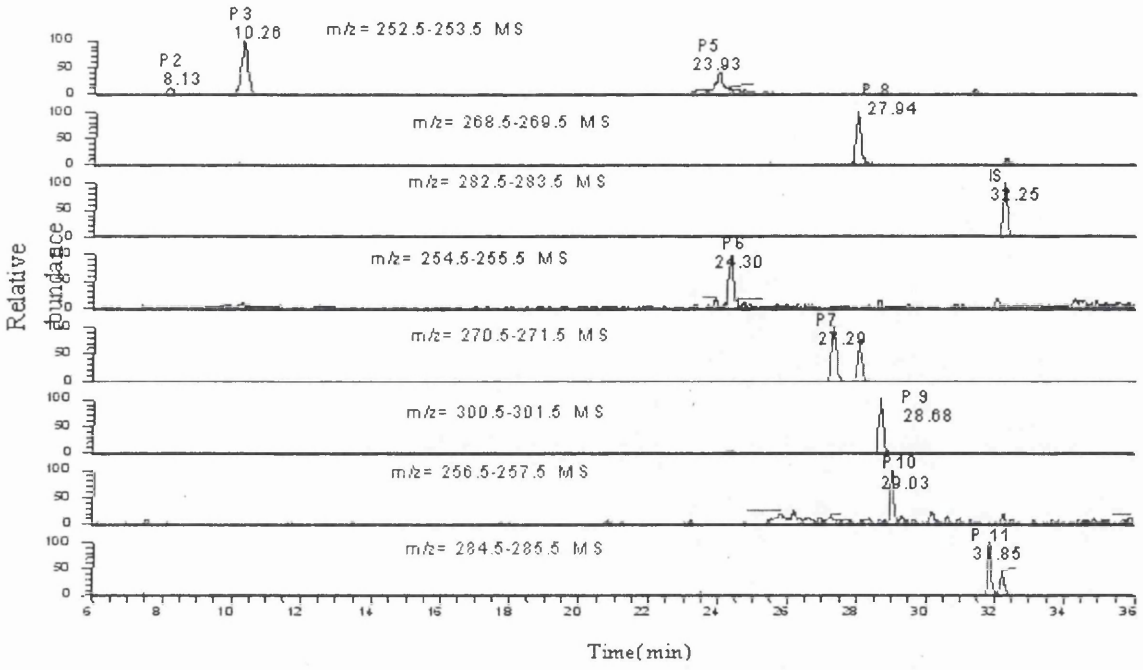


Figure 6.24 *TIC chromatofram of total isoflavones and their metabolites in aurine sample from a Chinese woman by LC/APCI/MS (HPLC method sees Table 6.2*

This subject has a good metabolism and many metabolites were detected in her urine sample with high concentrations. Some unknown components also presented in the subject. Our studies indicated that all Chinese people are good metabolic subjects. In order to identify all components in the urine sample, this subject was chosen to be analysed using LC/APCI/MS and LC/APCI/MS^2.

6.6.3.1 Analysis of peak 1 by LC/APCI/MS and LC/APCI/MS^2 in the urine sample in Figure 6.23

Peak 1 gave the mass of m/z=178 in negative APCI/MS mode and the mass of m/z=180.2 in positive APCI/MS mode (see Figure 6.25). This component appeared in urine sample with high concentration in all subjects. We could not give a conclusion that this component is from isoflavone metabolite or urine itself due to unavailability of standard material.

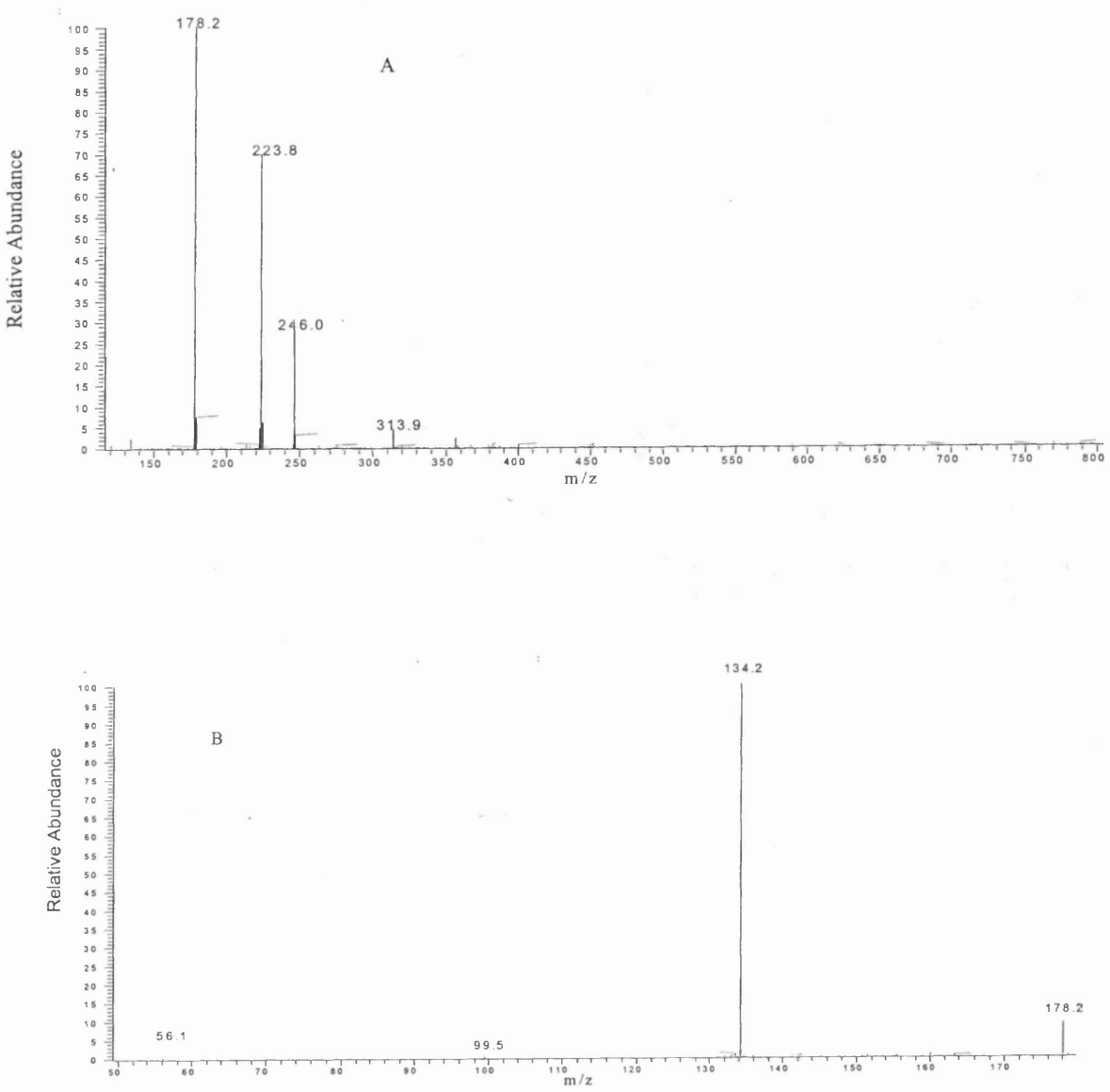


Figure 6.25 *LC/MS (A) and LC/MS^2 (B) spectra of peak 1 at m/z=178(CID=30%)*

6.6.3.2 Analysis of peak 2 by LC/APCI/MS and LC/APCI/MS^2 of peak 2 in the urine sample in Figure 6.23

Peak 2 gave the molecular mass of 252.9(see Figure 6.26). It is an isomer of daidzein and the concentration is very low. Since an authentic reference compound was not available, we could not identify its structure. We can only conclude that this component is an isomer of daidzein.

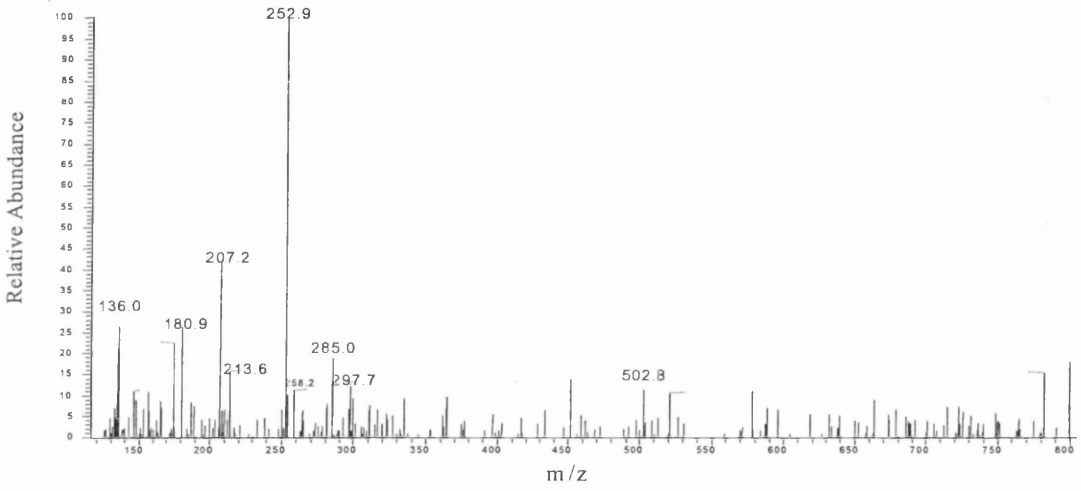


Figure 6.26 *LC/APCI/MS full scan mass spectrum of peak 2 in retention time of 8.13 min*

6.6.3.3 Analysis of peak 3 by LC/APCI/MS and LC/APCI/MS^2 of peak 3 in the urine sample in Figure 6.23

Peak 3 gave the precursor ion m/z 253 in negative LC/APCI/MS (see Figure 6.27) and m/z 255 in positive LC/APCI/MS. Comparison of the MS^2 spectrum with that of daidzein showed that they gave very similar product ions. We can conclude that this compound is the isomer of daidzein, but the structure cannot be identified due to the unavailability of an authentic reference.

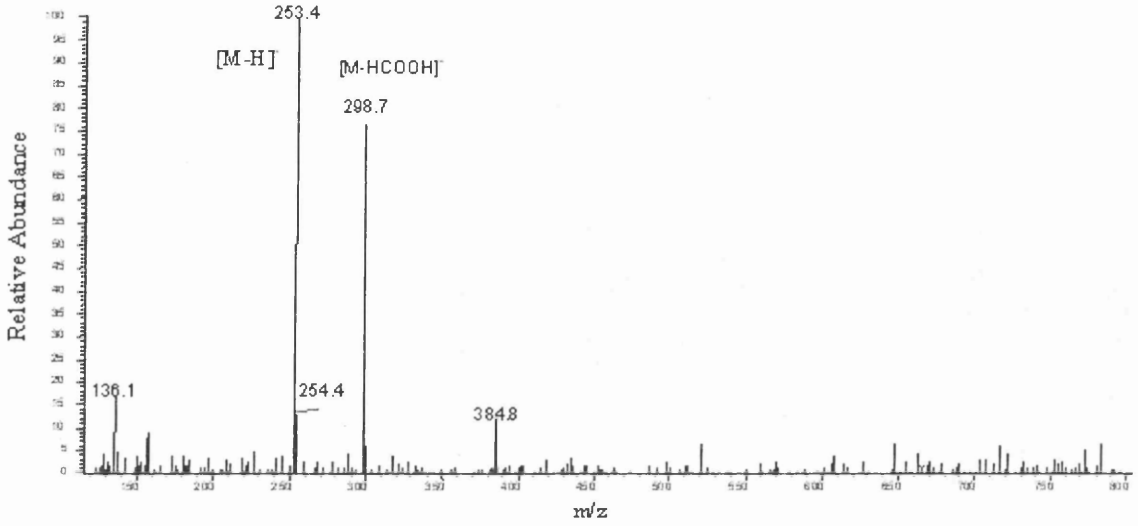


Figure 6.27 *LC/APCI/MS full scan mass spectrum of peak 3 in retention time of 10.26 min*

6.6.3.4 Identification of peak 4 by LC/APCI/MS and LC/APCI/MS^2 of peak 4 in urine sample in Figure 6.23

Peak 4 gave the molecular mass m/z=269. It gave the same retention time as that of standard 3'-hydroxydaidzein and was identified as 3'-hydroxydaidzein by comparing the retention time and mass spectra with a standard solution.

6.6.3.5 Analysis of peak 5 by LC/APCI/MS and LC/APCI/MS^2 of peak 5 in urine sample in Figure 6.23

Peak 5 gave the molecular mass m/z=253 in negative LC/APCI/MS. It gave the same retention time as that of standard of daidzein and was identified as daidzein by comparing the retention time and mass spectra with a standard solution.

6.6.3.6 Identification of peak 6 in urine sample in Figure 6.23

Peak 6 gave molecular mass m/z=255.2 in negative LC/APCI/MS and m/z=257 in positive LC/APCI/MS. Its MS^2 gave the same mass spectrum as that of standard dihydrodaidzein. So, peak 6 was identified as dihydrodaidzein by comparing their retention times and full scan and MS^2 mass spectra. Figure 6.28 shows its negative APCI/MS and APCI/MS^2 spectra.

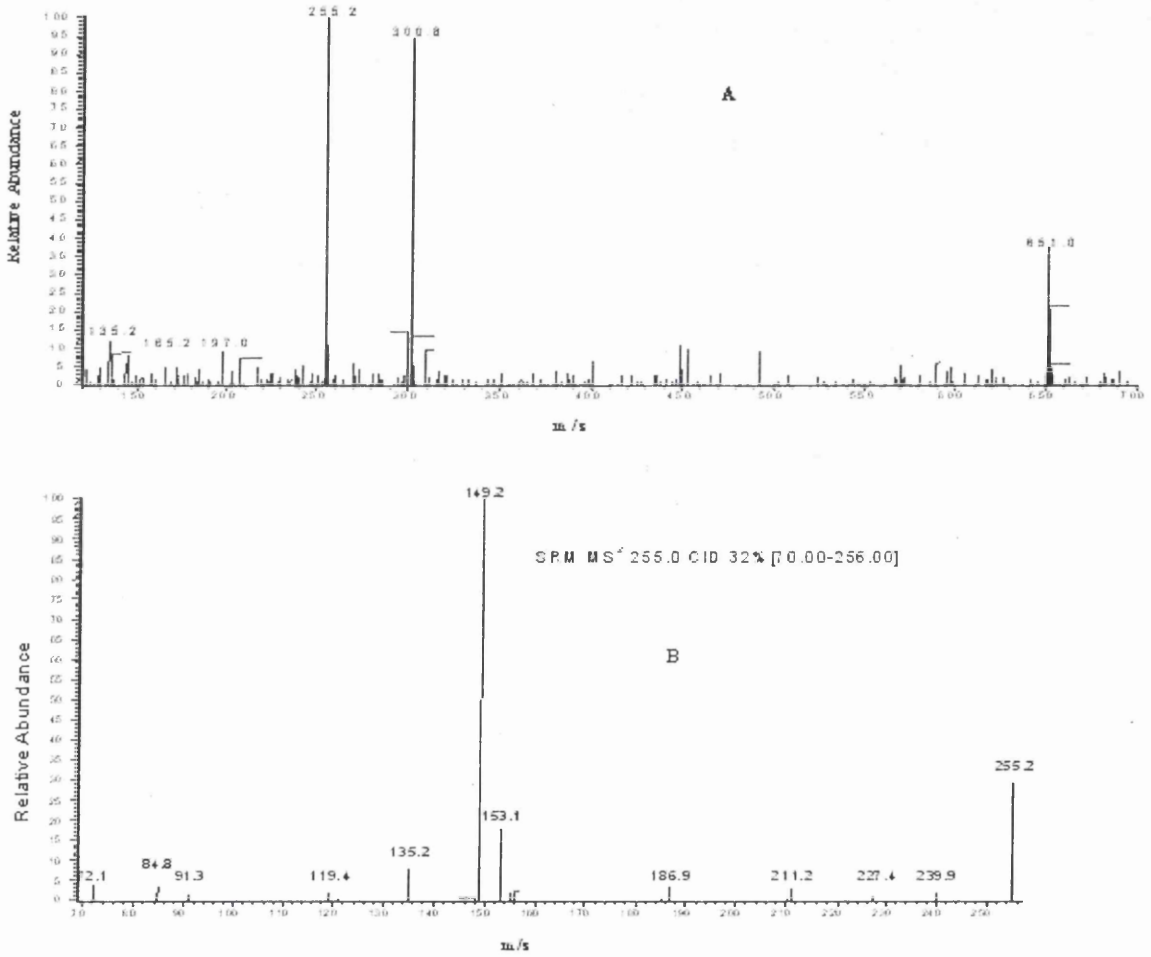


Figure 6.28 *LC/MS (A) and LC/MS^2 (B) spectra of peak 6 in retention time of 24.3 min*

6.6.3.7 Identification of peak 7 in urine sample in Figure 6.23

Peak 7 was identified as dihydrogenistein by comparing their retention times and MS full scan and MS^2 mass spectra (see Figure 6.29).

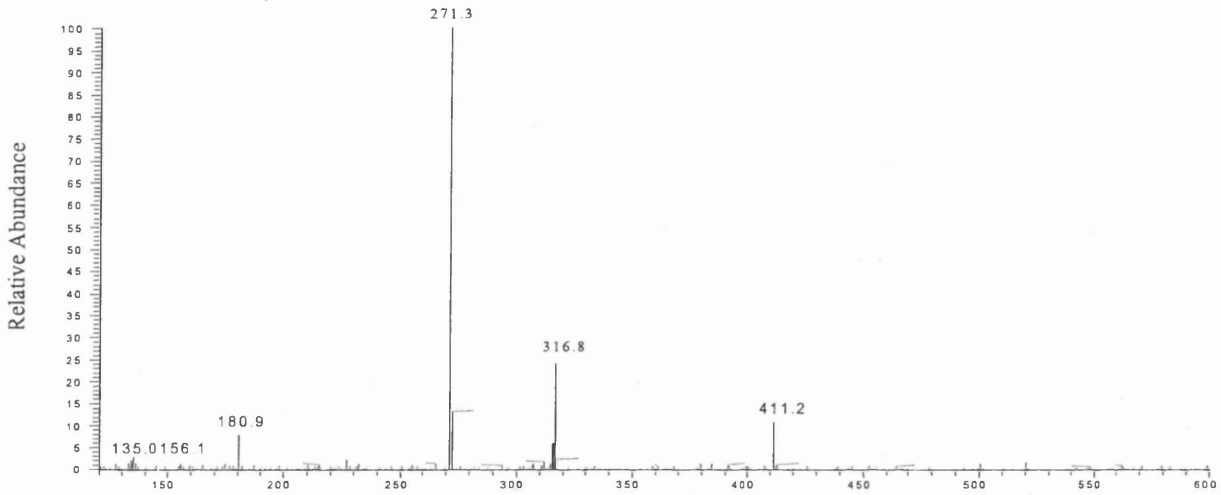


Figure 6.29 *LC/MS full scan spectrum of peak 7 in retention time of 27.29 min*

6.6.3.8 Identification of peak 8 in urine sample in Figure 6.23

Peak 8 was identified as genistein by comparing retention time and MS full scan and MS^2 mass spectra (see Figure 6.30).

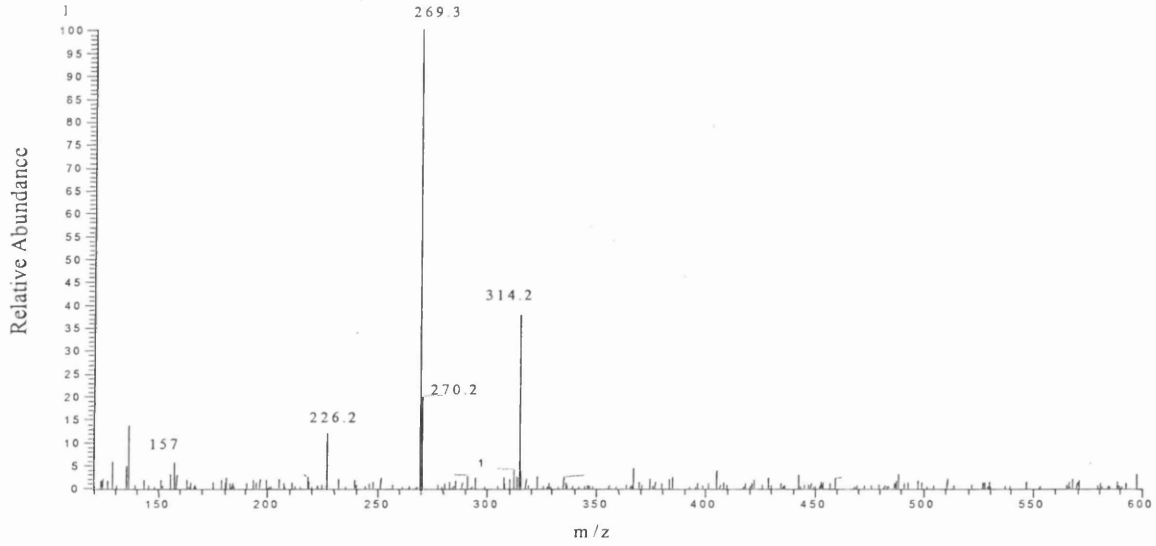


Figure 6.30 *LC/APCI/MS full scan spectrum of peak 8 in retention time of 27.94 min*

6.6.3.9 Identification of peak 9 in the urine sample in Figure 6.23

Peak 9 was identified as enterodiol by comparing the retention time and mass spectrum with the authentic reference compound(see Figure 6.31). This component was not studied further.

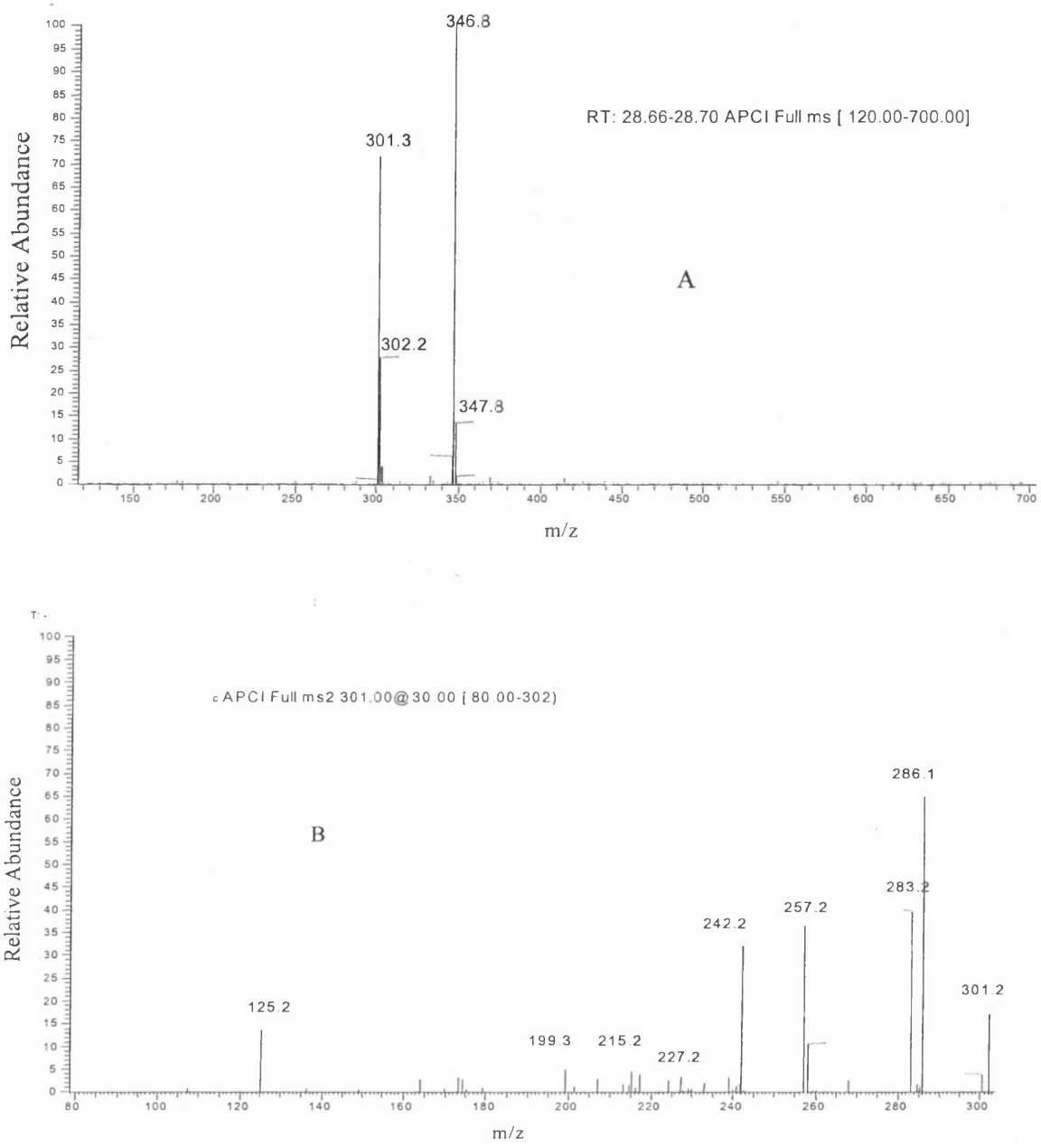


Figure 6.31 *LC/MS (A) and LC/MS^2 (B) spectra of peak 9 in retention time of 28.68 min*

6.6.3.10 Identification of peak 10 in urine sample in Figure 6.23

Peak 10 was identified as *O*-desmethylangolensin by comparing retention times and full scan and MS^2 mass spectra with an authentic reference sample of *O*-desmethylangolensin (see Figure 6.32)

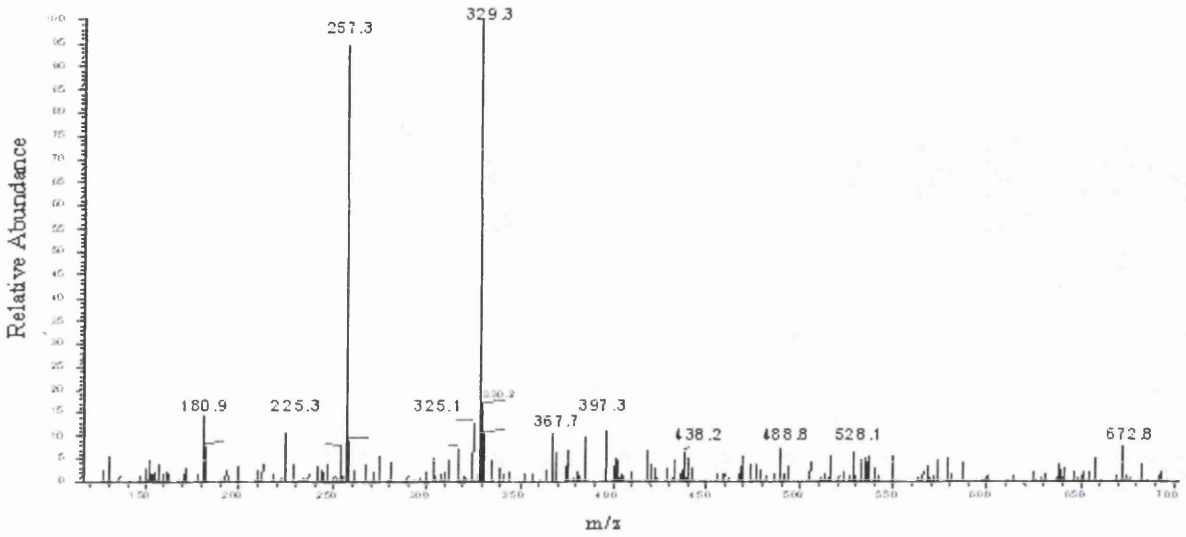


Figure 6.32 *LC/APCI/MS full scan spectrum of peak 10 in retention time of 29.03 min*

6.6.3.11 Identification of peak 11 in urine sample in Figure 6.23

The MS/MS spectrum of peak 11 shows that this component has a methoxyl group in the structure due to the mass loss of 15 from the molecular ion m/z=285.3(see Figure 6.33). The retention time of this component is very close to that of glycitein. The product ion of m/z 243 indicated typical ions of isoflavonoids. According to other reports, the component is possibly dihydroglycitein. Further work needs be done for identification of its structure.

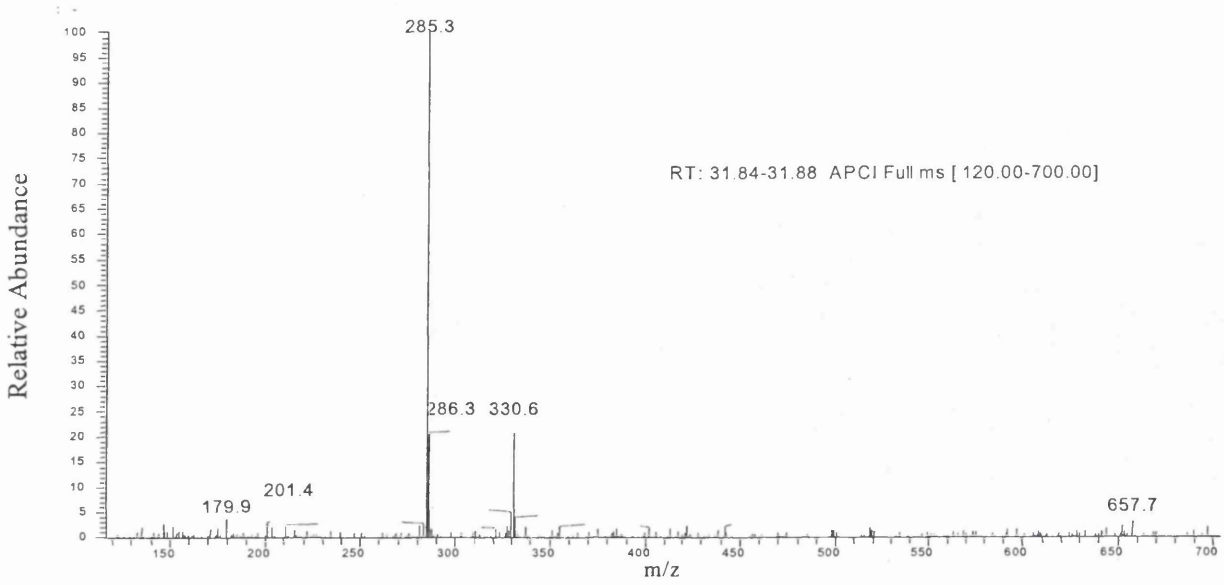


Figure 6.33 *LC/MS full scan spectrum of peak 11 in retention time of 31.86 min*

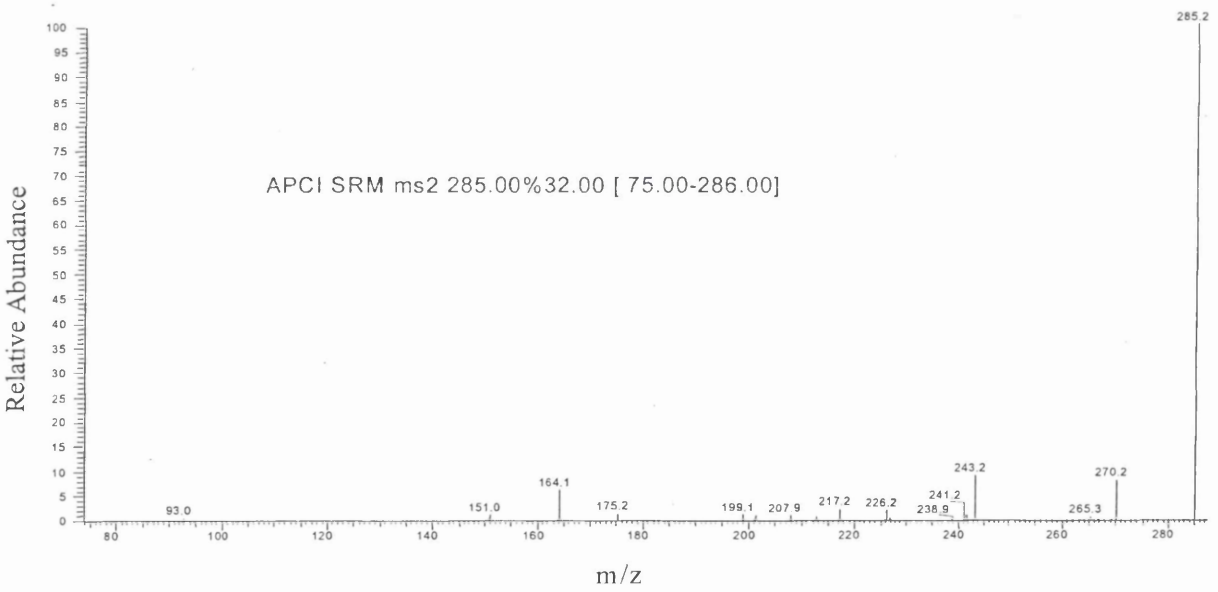
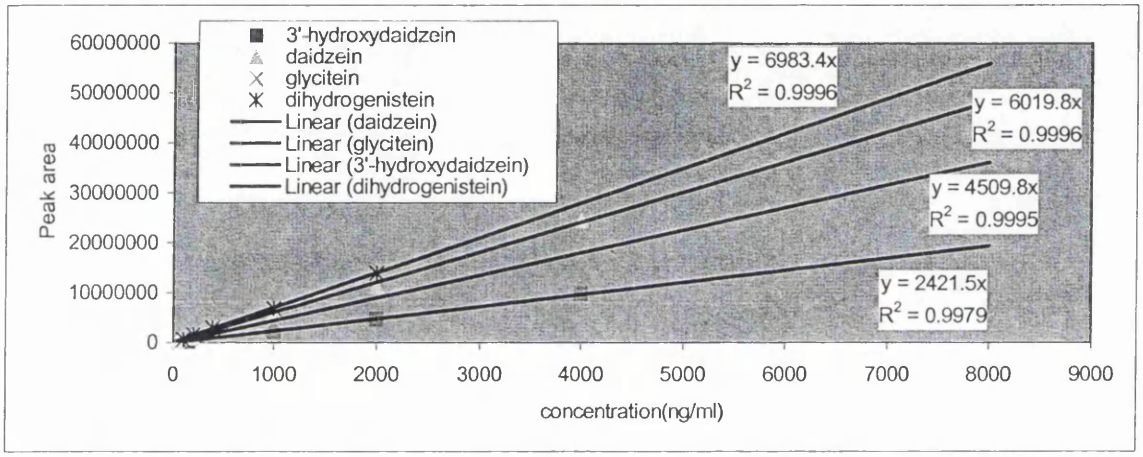


Figure 6.34 *LC/MS/MS full scan spectrum of peak 11 in retention time of 31.86 min*

6.7 VALIDATION PROCEDURES

6.7.1 Full scan calibration of isoflavones and their metabolites

The standard curves exhibited excellent linearity in the range from 0.05 to 8.0 μg/ml in full scan negative LC/APCI/MS. All the correlation coefficients are greater than 0.999, with intercept values that did not deviate significantly from the origin (Figure 6.35). However, this calibration range is too high for the concentration of isoflavones in urine sample and more volume of sample was needed for covering this range. In order to simplify the pre-treatment of samples and increase the sensitivity of detection, single ion monitoring scan mode was used to calibrate the isoflavones and their metabolites. Figure 6.36 shows the TIC chromatogram by negative LC/APCI/MS/SIM.



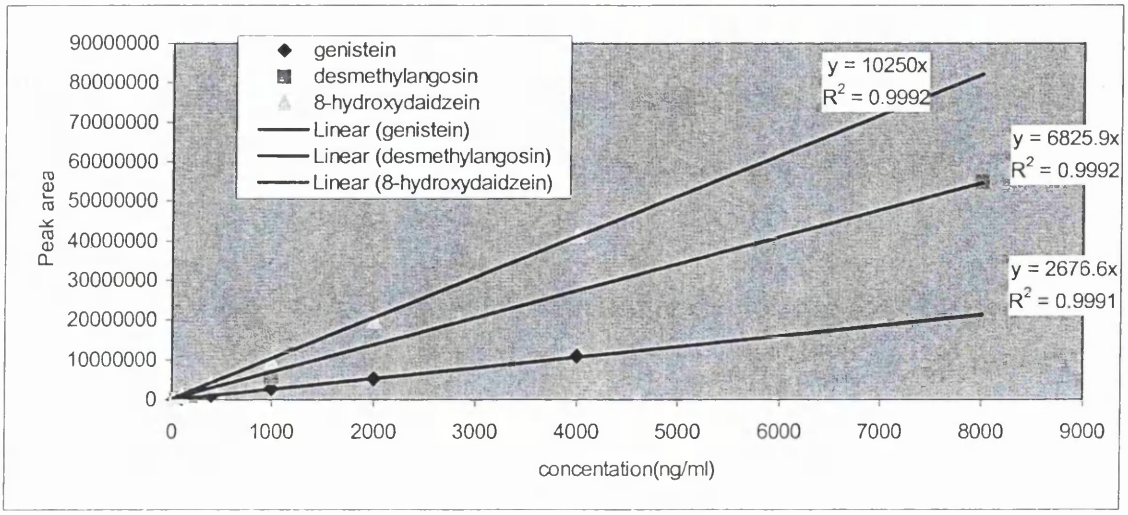


Figure 6.35 *Calibration curves of isoflavones and metabolites by LC/APCI/MS full scan mode*

6.7.2 Single ion monitoring (SIM) calibration of isoflavones and their metabolites

Single ion monitoring was used to determine the standard curve of the isoflavones. In single ion monitoring, only ions of selected mass-to-charge ratio are monitored, hence, generally the selected ion monitoring scan mode provides a higher sensitivity than the full scan mode. In Figures 6.37 and 6.38, SIM of ions corresponding to m/z=269, 253, 283, 271, 257 $[M-H]^-$ of 8-hydrodaidzein, 3'-hydrodaidzein, genistein, daidzein, glycitein, biochanin A, dihydrogenistein and dihydrodaidzein were used to calibrate the isoflavones and their metabolites.

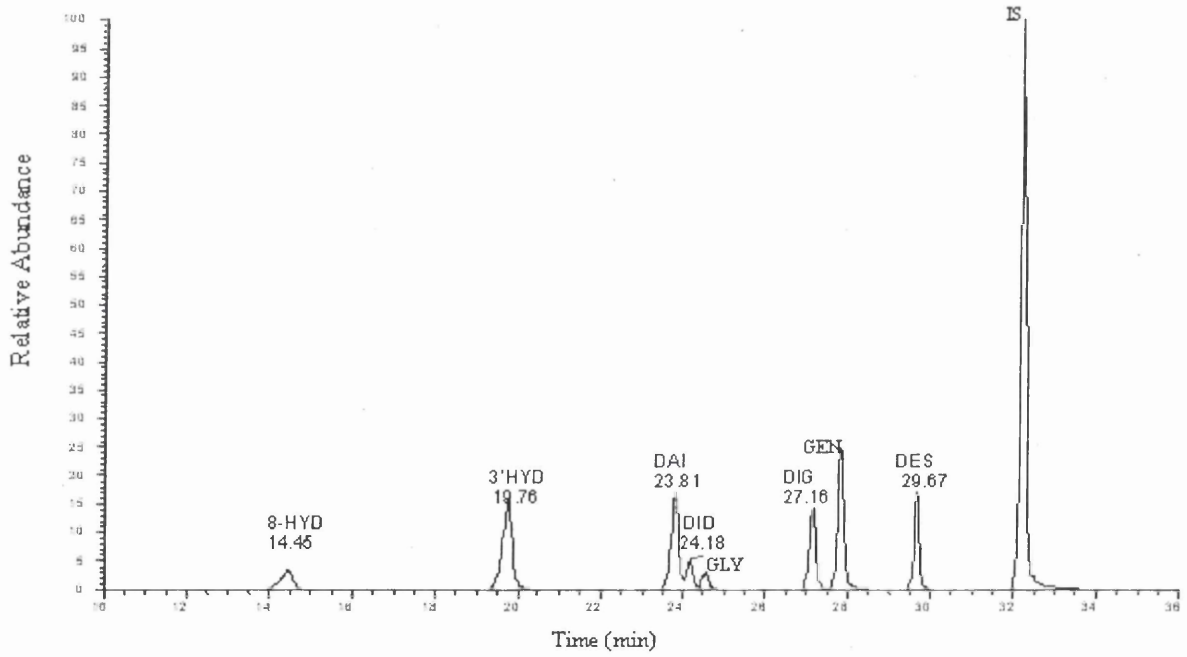
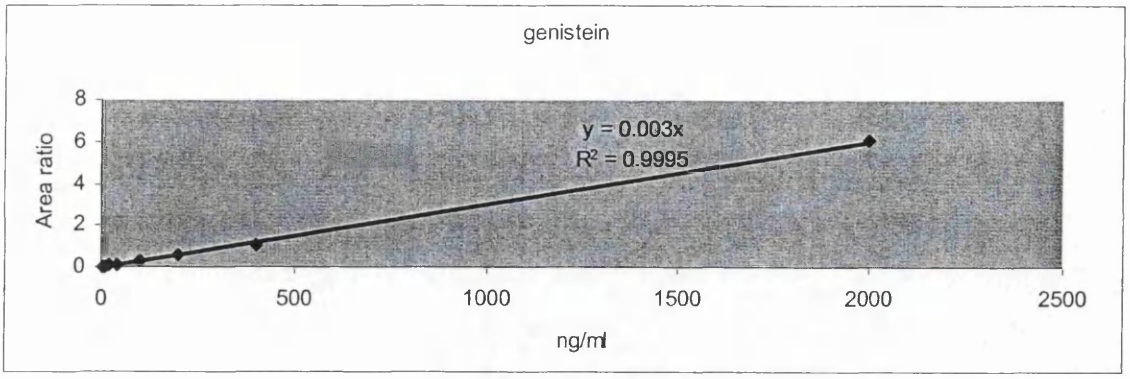
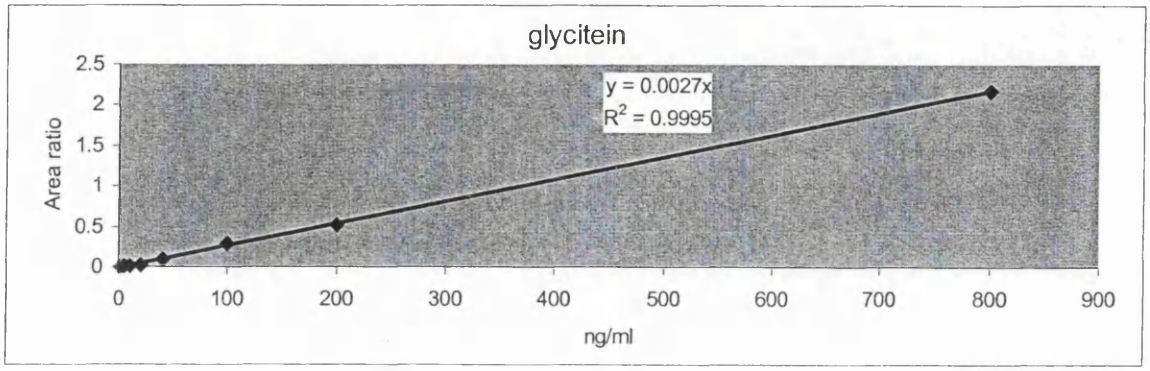


Figure 6.36 *LC/APCI/MS SIM chromatogram of a standard mixture of isoflavones and their metabolites*





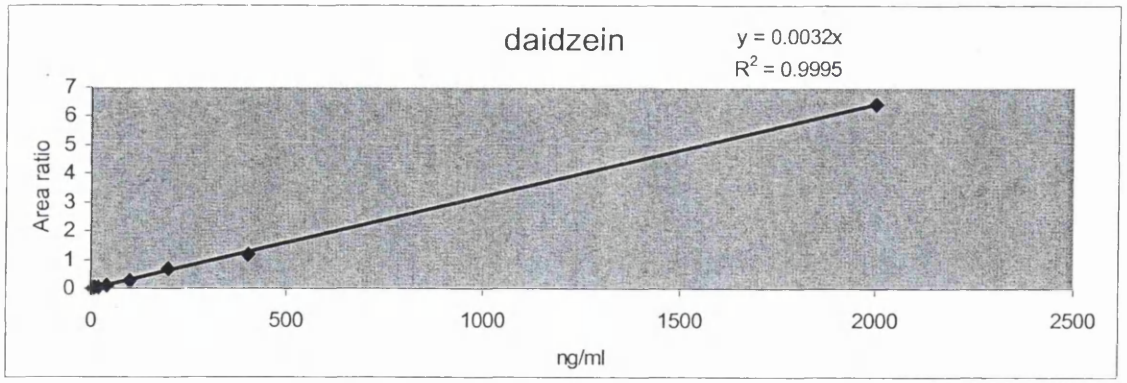


Figure 6.37 *Calibration curves of isoflavones by LC/APCI/MS (SIM)*

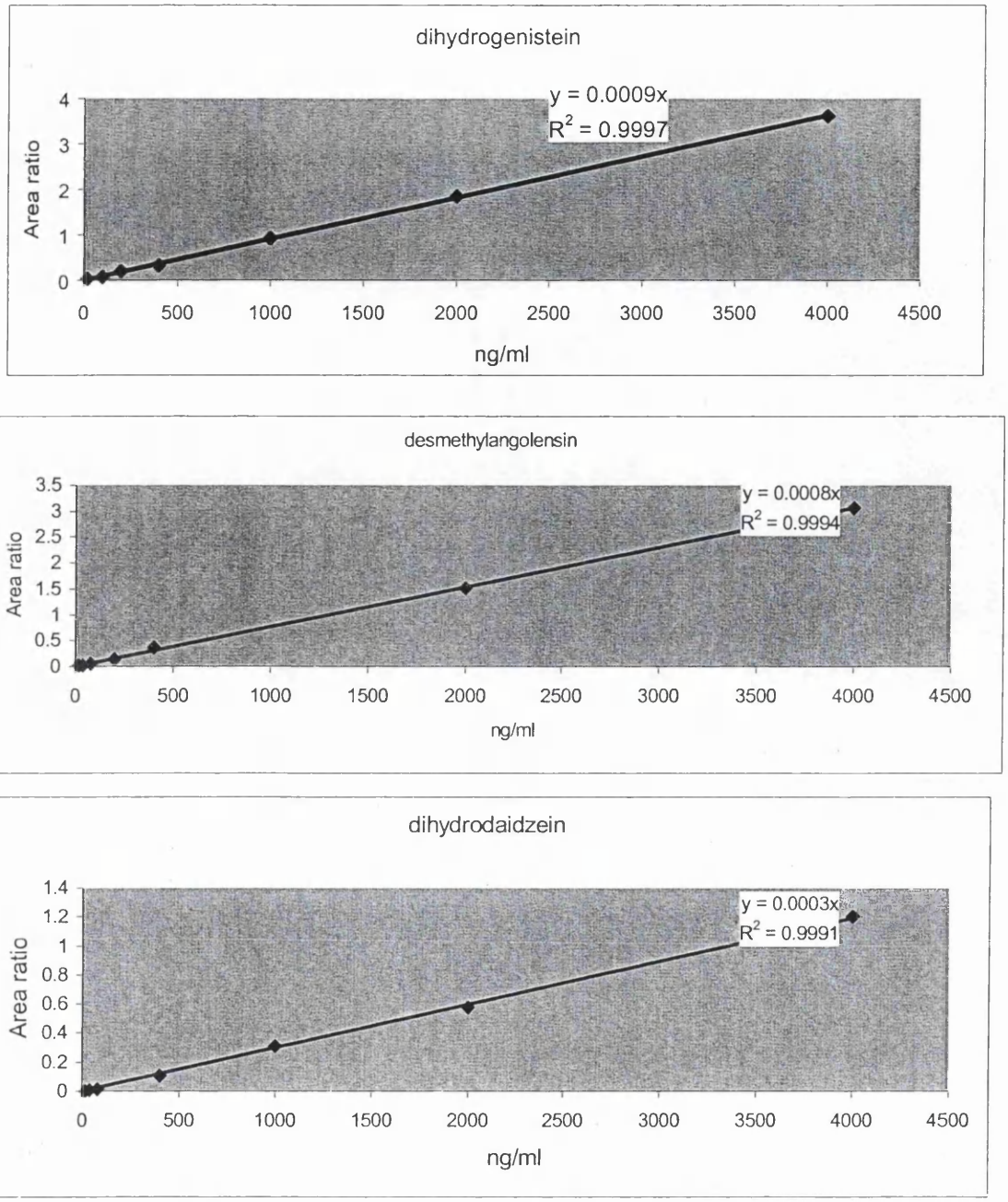


Figure 6.38 *Calibration curves of isoflavone metabolites by LC/APCI/MS (SIM)*

Figures 6.37 and 6.38 show that calibration by SIM can give an excellent linear range from 5-4000 ng/ml with a high sensitivity. Calibration curves for free genistein, daidzein and glycitein were generated by analysis of fortified urine extracts. The standards ranged in concentration from 5-2000 ng/ml for genistein and daidzein (10 standards in duplicate), and from 2-800 ng/ml for glycitein (8 standards in duplicate). All the r^2 values of the calibration curves were 0.9995.

Calibration curves for dihydrogenistein, dihydrodaidzein and *O*-desmethylangolensin ranged in 5-4000 ng/ml with $r^2 > 0.999$. These calibration curves could cover the range of the concentration in urine sample. The mean determined value for each analyte's set of control urine samples was within 20 % of the nominal value. The RSD around this mean value did not exceed 20% and meet the acceptance criteria for both precision and accuracy. Hence, in our study, SIM was used to determine the concentrations of isoflavones and their metabolites.

6.7.3 Extraction recoveries of isoflavones

Extraction recoveries were determined in triplicate aliquots in several assays by adding isoflavones and their metabolite standards to samples prior to extraction or prior to digestion plus extraction. Three concentrations covering low, middle and high calibration ranges, were chosen to evaluate their extraction recoveries. Since the urine samples were dried and reconstituted before injection on the LC/MS, these recoveries include any loss due to multi-step treatment of samples. In order to decrease loss in the treatment of sample, all urine samples were extracted three times by MTBE and all organic phases combined for next step to take them to dryness. The mean extraction recoveries for all aglycones in high and middle concentrations are showed in Tables 6.4 and 6.5.

Table 6.4 *Extraction recoveries of free isoflavones in high concentration*

	High control, % recovery						Mean	SD
8-HYD (2μg/ml)	93.86	100.8	92.73	91.72	99.44	92.89	95.24	3.860
3'-HYD (2μg/ml)	98.71	91.58	94.05	97.69	99.53	96.80	96.39	3.025
DAI (2μg/ml)	92.83	98.48	91.08	94.40	99.48	95.81	95.34	3.241
GLY (2μg/ml)	85.44	105.1	87.88	94.49	103.0	98.31	95.70	7.953
DIH (2μg/ml)	96.80	95.73	86.93	85.09	92.52	84.85	90.38	5.390
GEN (2μg/ml)	99.47	103.2	99.42	96.94	93.14	97.21	98.24	3.378
DES (2μg/ml)	94.32	92.55	88.68	94.19	95.64	92.18	92.93	2.435

Table 6.5 *Extraction recoveries of free isoflavones in urine with middle control*

	Middle control, % recovery						Mean	SD
8-HYD (100ng/ml)	91.33	95.03	89.17	91.87	89.34	92.56	91.84	1.97
3'-HYD (100ng/ml)	102.3	108.5	98.09	97.66	100.3	105.3	101.9	3.88
DAI (100g/ml)	107.7	101.5	102.5	106.6	102.3	101.4	103.7	3.39
GLY (100ng/ml)	89.64	105.1	87.88	94.49	103.0	98.31	92.89	3.27
DIH (100ng/ml)	110.3	108.6	111.4	107.6	102.8	104.5	107.5	3.32
GEN (100ng/ml)	112.5	107.3	113.7	110.5	108.4	109.9	110.4	2.41
DES (100ng/ml)	96.39	102.4	103.2	99.49	95.78	98.32	99.26	3.05

** 8-HYD=8-hydroxydaidzein, 3'-HYD=3'-hydroxydaidzein, DAI=daidzein, GLY=glycitein, DIH=dihydrogenistein, GEN=genistein, DES=O-desmethylabgolensin*

Tables 6.4 and 6.5 show that the extraction recoveries of free isoflavones in urine were quite high. Daidzein and genistein were over 100 % in low concentrations. Daidzein, glycitein, genistein and internal standard appeared to be completely extracted using this procedure. However, for the recoveries of total isoflavones after enzyme hydrolysis, the recoveries were lower than that of free isoflavone without enzyme hydrolysis. Table 6.6 shows the recoveries of isoflavones when standards were added to drug free urine for hydrolysis by enzyme.

Table 6.6 *Extraction recoveries of isoflavones in urine after hydrolysis with enzyme*

	% recovery						Mean	SD (%)
8-HYD (2μg/ml)	93.86	89.33	92.73	91.72	92.44	92.89	92.16	1.551
3'-HYD (2μg/ml)	96.71	91.58	94.05	97.69	85.88	92.80	93.13	4.230
DAI (2μg/ml)	92.43	97.48	89.01	94.67	99.37	94.00	94.49	3.669
GLY (2μg/ml)	84.34	89.65	88.22	94.00	104.0	98.23	93.07	7.189
DIH (2μg/ml)	93.82	90.73	87.03	85.60	90.05	84.03	88.54	3.639
GEN (2μg/ml)	99.50	104.2	92.44	96.04	99.85	96.22	98.04	4.050
DES (2μg/ml)	85.66	90.00	86.88	91.45	90.24	92.00	98.04	2.544

* *8-HYD=8-hydroxydaidzein, 3'-HYD=3'-hydroxydaidzein, DAI=daidzein, GLY=glycitein, DIH=dihydrogenistein, GEN=genistein, DES=O-desmethylabgolensin*

6.7.4 Determination of accuracy, precision and limit of quantitation

6.7.4.1. Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. Typically, accuracy is represented and determined by recovery studies, but there are three ways to determine accuracy: 1) comparison to a reference standard, 2) recovery of the analyte spiked into blank matrix or 3) standard addition of the analyte. Generally, an analyte recovery would be determined from three replicate measurements each of three different concentration preparations. All nine values are averaged and used for final accuracy determination. The results of these measurements then are compared to results obtained by other methods or results reported on a certificate of analysis from an external source.

In our study, quality control solutions were prepared independently from calibration standards using DMSO to dissolve each analyte. The stock solutions were then diluted to various concentrations within the calibration curve range, including one high concentration, one middle concentration, and one low concentration at the desired limit of quantitation. These samples were prepared in quintuplicate for determination of assay accuracy, precision, and limit of quantitation.

Precision can be defined as " the degree of agreement among individual test results when the procedure is applied to multiple samplings of a homogeneous sample [25]. A more comprehensive definition proposed by the International Conference on Harmonization (ICH) [26] divides precision into three types: 1) repeatability, 2) intermediate precision, and 3) reproducibility. Repeatability is the precision of a method under the same operation conditions over a short period time. One aspect is instrumental precision. This is measured by the sequential, repetitive injection of the same sample 10 or more times, followed by the averaging of the measured values and determination of the relative standard deviation (RSD) of all sample. Sometime it is termed as

intra-assay precision.

Intermediate precision is the agreement of complete measurements (including standards) when the same method is applied many time times within the same laboratory. This can include full analysis on different days, instruments, or analysts, but would involve multiple preparations of samples and standards. Reproducibility examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

The precision often is expressed by the standard deviation (SD) or relative standard deviation (RSD) of a data set. If n is referred to the measurement times, the average value obtained from those n measurements is defined as

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n}$$

Where x_i are the individual measurements on the sample. The standard deviation of these data is then

$$SD = \sqrt{\frac{\sum_{i=1}^{n} x_i^2 - \bar{x})^2}{n-1}}$$

and the relative standard deviation (RSD) or coefficient of variation (CV) is

$$RSD\ (\%) = 100\ SD/x$$

6.8 COMPARISON OF CONCENTRATION OF ISOFLAVONES AND THEIR METABOLITES IN ALL FORMS

6.8.1 Determination of free isoflavone

In the urine sample, the free isoflavones of daidzein and genistein were readily observable in most urine sample from one-week soy-based nutrition food consumed. Chinese people had a higher concentration than those from UK. However, some vegetarian subjects had a high concentration of isoflavone aglycones. The concentration of free isoflavones ranged from non-detectable (baseline sample) to 10 % of the total daidzein content and 12 % for total genistein. The concentration of free isoflavone of glycitein was not determined by LC/MS/SIM because of its low concentration. Daidzein and genistein were detected from Chinese people before taking soy-based supplements (baseline). Figure 6.39 shows a summary of free isoflavones levels in all subjects.

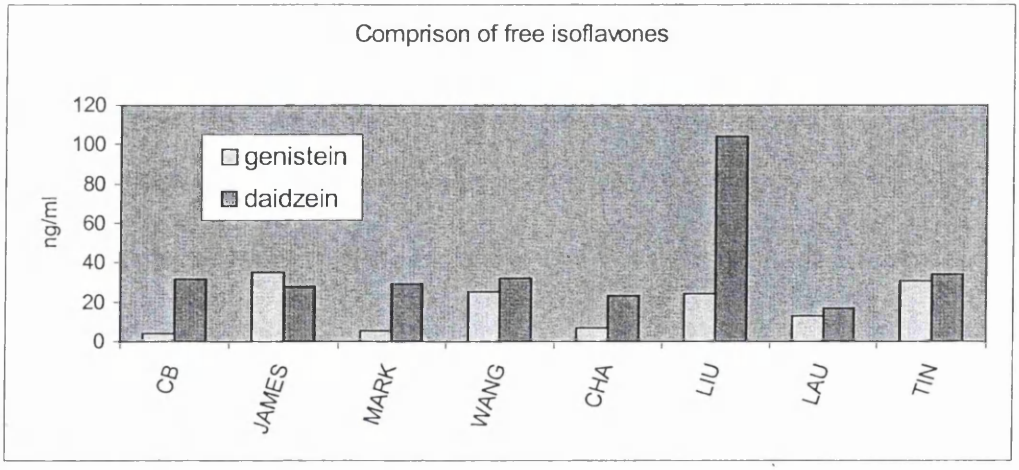


Figure 6.39 *Comparison of concentrations of free isoflavone of all subjects*

Table 6.7 *Mean concentration (μmol/l n=5) of free isoflavone in urine of subjects*

	CB	JAMES	MARK	WANG	CHA	LIU	LAU	TIN
Genistein	0.015	0.131	0.019	0.093	0.026	0.091	0.048	0.114
Daidzein	0.125	0.109	0.116	0.128	0.092	0.411	0.066	0.135

CB, MARK, CHA are typical UK people, LIU, JAMES and WANG are Chinese people, TIN and LAU are vegetarian UK people.

Precision and accuracy of the assay of free isoflavones was determined with five replicate urine control samples that were analyzed at each of three concentration levels of genistein, daidzein and glycitein (at high, middle, and LOQ levels of the calibration curve). The accuracy and precision data for this assay are provided in Tables 6.8 and 6.9

Table 6.8 *Urine assay validation data for free isoflavone (without hydrolysis)*

	Concentration (ng/ml) (middle control)						
	8-HYD	3'-HYD	DAI	GLY	DIH	GEN	O-DES
Mean	45.25	42.5	37.84	44.66	49.23	117.1	37.67
SD	3.10	3.84	2.33	1.18	1.45	4.26	1.46
RSD (%)	6.85	2.69	6.15	2.64	2.95	3.64	3.87
CON	45.25±2.48	42.5±3.1	37.84±1.86	44.66±0.94	49.23±1.16	117.1±3.4	37.67±1.16

Table 6.9 *Urine assay validation data for free isoflavone (without hydrolysis)*

	Concentration (ng/ml) (high control)						
	8-HYD	3'-HYD	DAI	GLY	DIH	GEN	O-DES
Mean	95.47	102.6	161.2	92.68	119.1	184.8	126.1
SD	6.24	3.84	5.32	3.69	5.92	5.79	2.69
RSD (%)	6.54	3.74	3.29	3.98	4.97	3.14	2.13
CON	95.47±4.9	102.6±3.1	161.9±4.3	92.68±2.9	119.1±4.7	184.8±4.6	126.1±2

6.8.2 Determination of total isoflavone

After hydrolysis with mixed glucuronidase/sulfatase enzymes, the aglycone content of urine increased remarkably, as would be expected. Daidzein, genistein were detected with high concentration in subjects. At the same time, 8-hydrogenistein, dihydrogenistein and O-desmethylangolensin, which are metabolites of genistein and daidzein, were also detected in some subjects especially Chinese people.

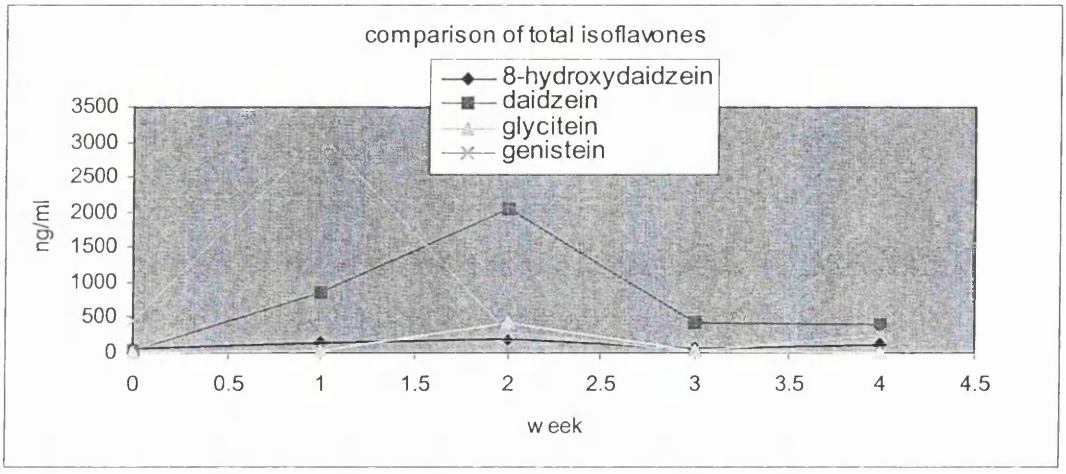


Figure 6.40 *Concentration of total isoflavones and their metabolites of a UK woman*

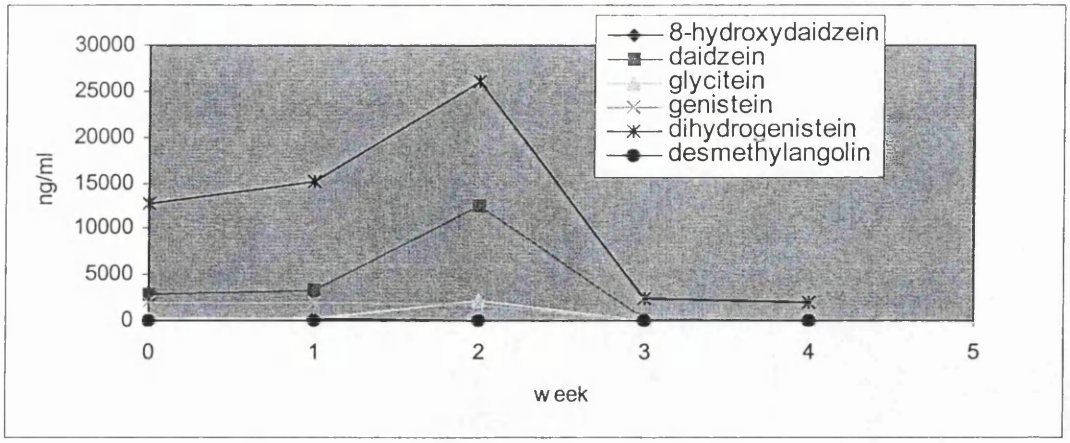


Figure 6.41 *Concentration of total isoflavones and their metabolites of a Chinese woman*

Figures 6.40 and 6.41 indicate that the concentrations of isoflavones and their metabolites in a Chinese woman were much higher (average 10 times) than those in a UK woman. These results are consistent with other reports [32]. Surprisingly, dihydrogenistein and 8-hydrogenistein, which are metabolites of genistein and daidzein appeared in high concentration in our study.

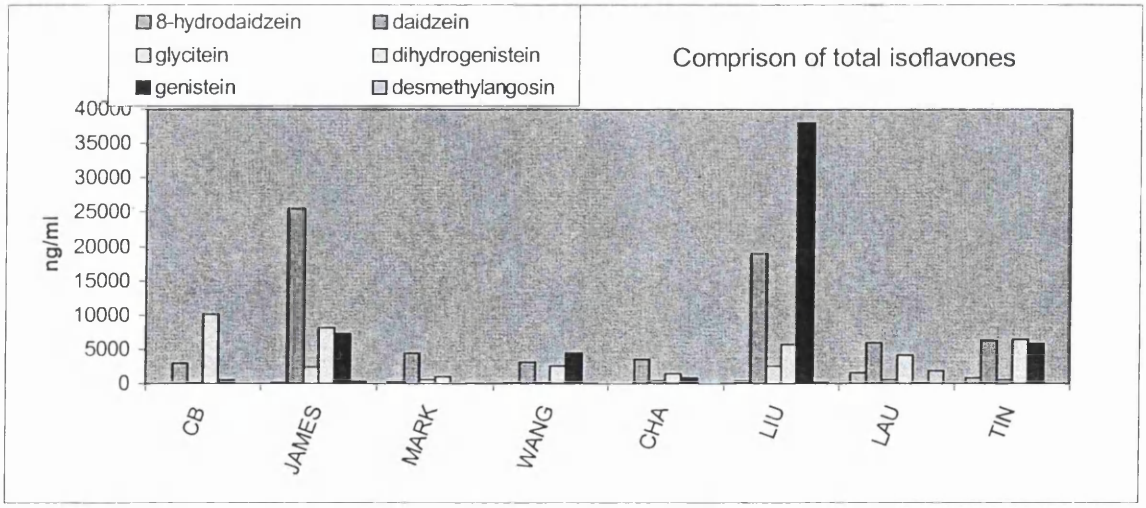


Figure 6.42 *Comparison of concentrations of total isoflavone of all subjects*

Table 6.10 *Urine assay validation data for Total isoflavone (after hydrolysis)*

	Concentration (ng/ml) (high control)						
	8-HYD	3'-HYD	DAI	GLY	DIH	GEN	O-DES
Mean	152.2	318.5	1714	381.1	735.0	1029	249.4
SD	4.25	7.16	53.70	8.27	12.98	50.40	10.49
RSD (%)	2.79	2.25	3.13	1.20	1.76	4.89	4.20
CON	152.2±3.4	318.5±5.7	1714±6.0	381.1±6.6	735.0±10.3	1029±40	249.4±8.4

Table 6.11 *Mean concentration (µmol/l) of total isoflavone in urine of subjects*

	Concentration (µmol/l)					
	8-HYD	DAI	GLY	GEN	DIH	O-DES
LAU	0.960	23.62	2.21	15.21	0.52	6.96
LIU	1.405	74.40	8.89	21.19	26.95	0.37
WAN	0.486	12.51	0.75	9.37	17.05	0.14
CB	0.174	11.53	1.17	37.39	2.16	0.09
TIN	3.220	24.54	1.91	23.61	22.89	0.04
MARK	1.798	17.57	2.20	3.91	0.24	0.78
JAM	0.595	100.5	8.46	30.09	28.47	0.95
CHA	0.082	14.23	1.37	5.14	3.47	0.13

CB, MARK, CHA are typical UK people, LIU, JAMES and WANG are Chinese people, TIN and LAU are vegetarian UK people.

6.8.3 Determination of sulfate isoflavone

Isoflavones combined with sulfate were also detected in urine samples with higher concentration than those of free isoflavones. Figure 6.43 shows the comparison of concentrations of sulfate isoflavones.

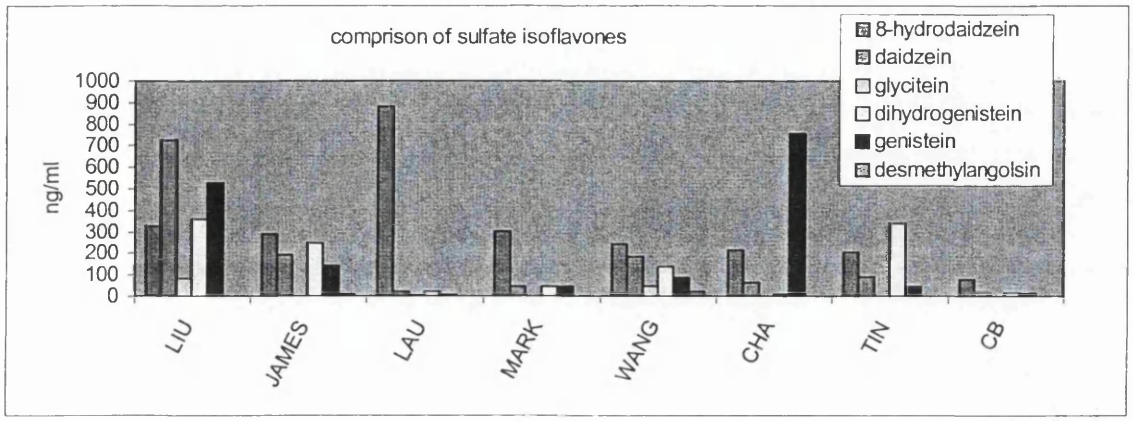


Figure 6.43 *Comparison of concentrations of sulfate isoflavone of all subjects*

Table 6.12 *Mean concentration (μmol/l) of sulfate isoflavone in urine of subjects*

	Concentration (μmol/l)					
	8-HYD	DAI	GLY	DIH	GEN	O-DES
LIU	1.21	2.85	0.288	1.326	1.955	0
JAME	1.07	0.756	0	0.908	0.526	0.041
LAU	3.26	0.092	0.017	0.078	0.026	0
MARK	1.121	0.172	0	0.176	0.166	0
WANG	0.896	0.716	0.162	0.512	0.323	0.081
CHA	0.804	0.253	0	0.027	2.80	0
TIN	0.767	0.355	0	1.243	0.172	0.007
CHB	0.290	0.096	0	0.061	0.048	0

CB, MARK, CHA are typical UK people, LIU, JAMES and WANG are Chinese people, TIN and LAU are vegetarian UK people.

6.9 DISCUSSION AND CONCLUSION

In this study, a rapid, convenient, reproducible, and sensitive assay based on reverse-phase LC/APCI/MS has been developed which permits the sensitive and specific measurement of isoflavones and their main metabolites in urine. Daidzein, genistein glycitein, dihydrogenistein, dihydrodaidzein and *O*-desmethylangolensin were identified and quantitated simultaneously with high recoveries. The relatively high extraction recoveries of the analytes in urine are consistent with the results obtained by other investigators using a similar method [19] and is comparable to that obtained with alternative procedures including chromatography [29, 30]. Our procedures were reproducible and reliable and afforded limits of quantitation of approximately 2 ng/ml for all 6 isoflavones and their metabolites in urine sample. The linearity, accuracy, precision and stability of analytical method were also evaluated and gave satisfactory results.

This assay is ideal for use in determining subject compliance in long-term feeding studies using soy food products, and for the measurement of exposure to isoflavones during uncontrolled feeding by the general population. It also has value in pharmacokinetic experiments involving individual isoflavones or soy foods.

We also found that all isoflavones and their metabolites are quite stable and do not appear to decompose rapidly even when left at room temperature over a short period of time. In order to ensure good quality and to control for losses and degradation of the compounds throughout a multistep method, internal standards should be used for all the compounds measured. An ideal internal standard should be a compound structurally related to the analyte and having a similar polarity but with a retention time that does not overlap with the other peaks in the chromatogram. Only few isoflavone investigators have used internal standards to adjust for analyte loss in extraction and analysis. Flavone, biochaninA and 4-hydroxybenzophenone were chosen to be internal standards. BiochaninA was found to be best due to its high purity and good resolution from analytes. Some people utilize deuterated internal standards for each isoflavonoid by exchanging hydrogen atoms for deuterium atoms [31]. Consequently, the retention times of the non-deuterated compounds and the deuterated standards are very close. The main drawback of

this method is that it is relatively complicated mainly because so many compounds are assayed and because high sensitivity is needed.

The difficulty met in our validation studies with isoflavones and metabolites involves the lack of a readily available supply of glucuronide and sulfate conjugates of the analytes to use as standards, resulting in our having to determine the free isoflavones instead of their conjugates. But the results of the validation data for conjugated materials when free isoflavone is used demonstrate that the analytes of interest are recoverable after treatment with enzyme hydrolysis. Our studies indicated that no endogenous materials are released by the gluronidase/sulfatase enzyme or by the solvolysis procedures that interfere with assay linearity, accuracy, precision or specificity. Other laboratories have attempted to utilize standards synthesized with biological enzyme systems [32] or have purified them from bile.

Unlike GC/MS, the reverse-phase LC/APCI/MS assay method does not require derivatization of the isoflavones, nor is an extensive work up of the sample necessary. Although isoflavones readily form ions in the APCI interface, the addition of ammonium buffer to the elute increases the sensitivity of genistein. The presence of the ammonia would increase the proportion of negatively charged phenolate ions of each of the isoflavones and increase the sensitivity for all isoflavones and their metabolites. For the genistein, there is hydrogen bonding between the 5-hydroxyl group and the 4-carbonyl oxygen [33], which limits the ionization process. By adding ammonium buffer, the hydrogen bonding is minimized and improved sensitivity was observed in daidzein. Equol, which is metabolite of daidzein, was detected with very low sensitivity when a standard solution of equol subjected to HPLC/MS. This metabolite equol was not detected in all objects urine samples.

Specificity in the assay is obtained by collision-induced dissociation (CID) of the parent isoflavone molecular ion and selection of product fragment ions. A very weak peak corresponding to the minor soy isoflavone glycitein was detected in Chinese subjects. Both biochaninA and

glycitein readily lose their methyl group during fragmentation, as previous reported by Aramendia et al [34].

The high recovery of isoflavone conjugates from urine was obtained by hydrolyzing them enzymatically directly in the urine and then extracting the aglycones with organic solvent. This approach was originally introduced by Lundh et al [35] and has been recently used by Xu et al [36]. The isoflavones were more efficiently recovered from a urine sample with MTBE extraction than with solid phase extraction. The high efficiency of recovery of isoflavones from urine by hydrolysis and extraction with MTBE made it unnecessary to use isotope dilution methods to correct for losses.

An unresolved problem is the structure elucidation of some unknown compounds. It is also difficult to propose suitable fragmentation pathways to explain all fragments from isoflavones and their metabolites by LC/MS/MS.

Quantitative data are essential when this knowledge is applied to prevention and management of disease and for the provision of appropriate diets for individuals and populations. A basic requirement for an understanding of the biological role of a diet-derived compound is the determination of their metabolites. Despite immense research efforts, the role of phytoestrogens in the maintenance of health and prevention of disease in humans has not been defined. Epidemiological evidence and experimental data from animal studies are highly suggestive of the beneficial effects of phytoestrogens on human health, but the clinical data supportive effects are not too much, or are awaiting design and execution of appropriate prospective large-scale clinical studies. The three major aims of the study were achieved. The following summary highlights its achievements and new findings, and identifies areas where further research is absolutely necessary.

1. The principal aim of this study was to develop a sensitive and specific LC/MS method for the identification and quantification of the biologically most important phytoestrogens and their metabolites. The method measures daidzein, genistein, glycitein, dihydrogenistein, 8-

hydroxydaidzein, dihydrodaidzein and O-desmethylangolensin. The method is a useful supplement to the LC/MS methods already available for phytoestrogens in human biological fluids.

2. The LC/MS-SIM method has been applied successfully to qualify and quantify daidzein, genistein, glycitein, dihydrogenistein, 8-hydroxydaidzein, dihydrodaidzein and O-desmethylangolensin isoflavones with high recoveries and specificity.

3. Our development of LC/APCI/MS assay for isoflavones and their metabolites in the free, sulfate conjugate and total conjugate forms in urine, provide novel analytical methods with which the pharmacokinetics and pharmacodynamics of the principle active forms of soy isoflavones can be studie . The methods we described here can determine very low levels of detection accurately in human urine. It can be possible used in the quantitative determination in human plasma and feces. Further work on these samples will be done in near future.

6.10 REFERENCES

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