Study of the Marketplace Variation in the Chemical Profile of Qi Ju Di Huang Wan (Lycium, Chrysanthemum and Rehmannia Formula)

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Table of Contents

**Study of the Marketplace Variation in the Chemical Profile of Qi Ju Di Huang Wan
(Lycium, Chrysanthemum and Rehmannia Formula)**

1 List of Figures

2 List of Tables

3 List of Publications

These first two papers, whilst not directly related to the thesis project, were part of doctoral training.

Su C, Zhang D, Truong J, Jiang C, Lee S, Jarouche M, et al. Effects of a novel herbal formulation JSK on acute spinal cord injury in rats. Restorative Neurology and Neuroscience. 2013;31(5):597-617.DOI: 10.3233/RNN-120303.

Lee S, Khoo CS, Hennell JR, Pearson JL, Jarouche M, Halstead CW, et al. LC determination of albiflorin and paeoniflorin in Bai Shao (Paeonia lactiflora) as a raw herb and dried aqueous extract. J AOAC Int. 2009;92(4):1027-1034.

Submitted article

This paper is largely drawn from Chapter 6

Jarouche M, Khoo CS, Bensoussan A, Hijazi L, Hennell JR. *Lycium barbarum* and *Lycium chinese:* Analysis of the divergence in chemical composition of twelve samples from the medicinal and food market. J Food Comp Anal. 2013.

Articles in final preparation

This paper is largely drawn from Chapter 6

Jarouche M, Khoo CS, Bensoussan A. Method validation samples of L. *barbarum* and L. *chinese* by UPLC-ESI-MS. 2014.

This paper is largely drawn from Chapter 5

Jarouche M, Khoo CS, Bensoussan A. A targeted approach to quality assurance of herbal medicine using Qi Ju Di Huang Wan as an exemplar. 2014

Conference poster

Jarouche M. A study of the variation in chemical composition of twelve *Lycium* samples from the market place (Poster). 8th Annual Congress of the International Society for Complementary Medicine Research (ISCMR); London, United Kingdom 2013.

4 Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in whole or in part, for a degree at this or any other institution.

Candidate's signature:

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B.Med.Sc.

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5 Dedication

I would like to dedicate this work to my children, who have given their endless love and support, and motivation throughout my academic years.

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7 List of Abbreviations

Abbreviation	Definition
(-)-ESI	Negative mode ESI
[M-H]-	Molecular ion
ARGCM	Australian Guidelines for Complementary Medicines
ARTG	Australian Register of Therapeutic Goods
cGMP	Code of Good Manufacturing Practice
CM	Complementary Medicine
DPPH	2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl
EI	Electron Impact
ESI	Electrospray Ionization
ET	Electron Transfer
GC	Gas Chromatography
HCA	Hierarchical Cluster Analysis
HM	Herbal medicine
LC	Liquid chromatography
MS	Mass spectrometry
MRM	Multiple Reaction Monitoring
P-PRC	Pharmacopoeia of the People's Republic of China
PCA	Principal Component Analysis
PVDF	Polyvinylidene Difluoride
SRM	Selective reaction monitoring
Q1 / Q3	Quadrupole 1 / Quadrupole 3
QA	Quality assurance
QC	Quality Control
QJDHW	Qi Ju Di Huang Wan

RSD	Relative Standard Deviation
SD	Standard Deviation
SRM	Selective Reaction Monitoring
TCM	Traditional Chinese Medicine
TGA	Therapeutic Goods Administration
TGO	Therapeutic Goods Orders
US$	US Dollar
UV	Ultraviolet
WHO	World Health Organization
λdet	Wavelength of detection
λmax	Wavelength of maximum absorption

Abstract

Current quality control (QC) of herbal medicines mainly focus on safety concerns– there is mandatory testing for herbal identity, pesticides, heavy metals, bacterial contamination and toxic components if an herb has been known to contain it. There is no requirement to quantify putative active components unless the supplier makes a label claim. Therefore while there is no concern about safety, the consumer is mostly uninformed about herbal quality regarding the amount of putative active components. Quantifying the putative actives is a twostep process – firstly the analytes that reflect herbal quality for the consumer have to be selected and secondly the analytical method has to be developed and validated. The complexity in quantifying the putative actives in a multi herb formulation increases with the number of herbs used. This study demonstrates how to estimate the quality of an eight herb formulation; Qi Ju Di Huang Wan (QJDHW) (Lycium, Chrysanthemum and Rehmannia Formula) described in the Chinese Pharmacopeia for the treatment of liver and kidney disorders.

Nine analytes were systematically selected for monitoring by liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis. The MS/MS detector is particularly suitable for complex matrices because it is highly selective and the chances of peak misidentification is minimised. The developed analytical method was then applied to analyse a total of eleven samples of QJDHW to estimate variability of the medication in the marketplace. There was significant concentration variation of the analytes studied which ranged from 4.4 to 28.9 fold (average 12.6 fold). Due to the large analyte concentration variation observed, it was decided to study the concentration of putative actives of one herb, namely *Lycium barbarum*. This herb is also of interest because two species, L.

barbarum and L. *chinense* are readily available in the marketplace and it is also consumed as a so called 'super food'. The two *Lycium* berries are similar physically and the question arose as to whether the two species have distinguishing chemical profiles. Seven analytes were quantified in the two Lycium species and the fold variation in concentration ranged from 1.8 to 7.8 (average 4.9) across twelve samples obtained from the marketplace. The extracts were also analysed by LC with photodiode array detection and gas chromatography with mass spectrometric detection. Our results indicate that the chemical compositions of the two *Lycium* species are not significantly different (eight L. *barbarum* and four L. *chinense* samples examined) and that the two should be interchangeable.

In addition to targeted analysis where the selected analytes were quantified, untargeted analysis (where all detectable constituents are considered) was also carried out where the chromatographic profile of the test samples (QJDHW and *Lycium*) were compared and statistically analysed by hierarchical cluster analysis (HCA) and principal component analysis (PCA). The conclusions reached by statistical analysis are similar to those reached by targeted analysis – that is, there is significant compositional variation in the QJDHW formulation with no obvious clustering of samples into similarity groups. With L. *barbarum* and L. *chinense* there is also no species clustering indicating that there is no compositional difference between the two species.

Chapter 1

General Introduction

1.1 Background and context of study

There has been a worldwide increase in the popularity and expenditure on complementary medicines in recent years .The extent of evidence around individual product efficacy, safety, mechanisms of action, potential as novel therapeutic agents, and cost-effectiveness is very variable. This is reflected in the two tiered regulatory approach to complementary medicines throughout the world (1). In the first tier requires a lower level of scientific proof of efficacy of medicines, including complementary medicines, which are generally regarded as safe because of their ubiquitous presence in nature or extensive prior exposure to humans. In the second tier evidence requirements are more stringently applied to relatively new pharmaceutical ingredients without any history of human exposure and often making high level therapeutic claims. Increasingly however, complementary medicines are also being applied to address serious disease and in these cases careful analysis of the herbal mixture is required.

There exists a plethora of chemicals in complex herbal mixtures posing a significant challenge to the quality assurance (QA) and quality control (QC) of herbal medicine. Pharmaceutical compounds usually have well-defined compositions and are manufactured to a consistent quality. Of course, the clinical impact on any individual may still be variable. Botanical formulations on the other hand can exhibit significant variation in the concentration of bio-active components and this may also in turn affect clinical outcomes – both safety and efficacy. In our research we sought to develop a pragmatic method for the quality assessment of an herbal formulation based on a targeted approach using model systems. We developed a suitable test of product quality and consistency evaluation through an analysis of eleven of the same herbal medicine samples obtained from several

sources in the Australian marketplace. Identification and quantification of pre-determined bioactive constituents was used to investigate chemical compositional uniformity and batch to batch consistency.

We selected Qi Ju Di Huang Wan (QJDHW) (Lycium, Chrysanthemum and Rehmannia Formula) as an exemplar model for this quality control study. QJDHW is an eight herb formulation described in the Chinese Pharmacopeia for the treatment of many types of ailments with signs of 'yin insufficiency' in the liver and kidneys marked with symptoms of dizziness and vertigo (2).

Biochemically, QJDHW has shown a substantial effect in decreasing the concentrations of angiotensin in plasma and myocardium, reducing the endothelin (ET) content and improving kidney blood stream in rats with essential hypertension (3-5). Recent studies have shown that QJDHW could help to control blood pressure (6, 7). A 2010 systematic review of randomized controlled trials on the effectiveness and safety of QJDHW for the treatment of essential hypertension (10 randomized trials involving 1024 patients were included) suggests that QJDHW combined with antihypertensive drugs is more effective in lowering blood pressure and improving the treatment of essential hypertension than antihypertensive drugs alone. No trials reported severe adverse events related to QJDHW use (8).

A validated method of analysis was developed for the quantitation of nine selected chemical constituents in one sample of the QJDHW formulation. This method was then applied to the analysis of eleven other QJDHW samples. The method validation parameters determined in this study closely follow those described by the European Union Commission decision of August 2002 implementing Council Directive 96/23/EC (9). The variation in concentration of

the analytes for all the samples analysed was evaluated using multivariate statistical analysis techniques.

1.2 Overview of traditional medicine

A tremendous resurgence in the popularity and impetus of traditional medicine has been observed worldwide. Traditional medicine has been defined as the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illnesses (10).

With an ancient cultural foundation traditional medicine has experienced an extensive upsurge in use and has subsequently augmented in economic significance. In many developing countries it is a major source of primary care. In developed countries traditional medicine is an alternative to, or complementary with, western medicine (11).

Annual revenues from traditional medicines in Western Europe reached US$ 5 billion in 2003-2004. In 2010, the sales of these products in the U.S. reached > US$ 5.2 billion dollars (12). In China, product sales totalled US$ 14 billion in 2005. Herbal medicine revenue in Brazil was US$ 160 million in 2007 (13). Herbal medicine has been integrated into preventative care in countries with organised primary health care systems (14).

The World Health Organisation (WHO) reports that there are a growing number of countries adopting national policies for preserving and promoting traditional medicine and developing specific regulatory capacity (13). However, these policies fall short of adequately addressing a number of concerns such as safety, efficacy, quality, rational use, availability and preservation and development of such health care (15, 16). WHO documents that there is a

lack of sound scientific evidence relating to the quantity and quality of the safety and efficacy of traditional medicine to support its use worldwide (15).

1.3 Theories and concepts of Traditional Chinese Medicine

A form of primary health care in China for centuries, TCM has been gaining increased acceptance as an alternative medical system in the developed world. TCM is diverse in its practice embracing an array of techniques including acupuncture and Chinese herbal medicine as well as other modalities. The conceptual phenomenon of health and illness in TCM differs considerably from that in western medicine. It is an organisation of medical practice with its own philosophy, treatment systems and pharmacology thus making it somehow alien to western medical practice. Moreover, as a unique holistic healthcare system aimed at restoring and maintaining the dynamic balance of a human body, it provides a basis for clinical practice that achieves harmony between the person and the social, physical and natural environment.

Therapy in TCM is aimed at specific responses that reflect change at the multi-system and multi-organ levels of the human body. In contrast, western medicine drugs are developed to counteract or neutralize pathological targets or to eliminate pathogenic factors (17).Prescription of medicinal herbs forms to an extent the foundation of TCM where herbal medicines are believed to work in a synergistic manner in treating an ailment. According to theory of TCM, medicinal plants sometimes need to undergo different types of processing such as heating, steaming or soaking to enhance the efficacy or reduce the toxicity of their components before the herbs can be used in the prescription of traditional medicines (18). TCM has an established history in Australia and has developed rapidly in recent years (19), Chinese medicines now account for 3.2% of the total use of complementary medicines (20).

The *Chinese Medicine Registration Act* was approved by the Victorian Parliament in 2000, to regulate the qualifications of traditional Chinese medicine practitioners and dispensers to encourage the safe use of TCM in Victoria. In parallel with the growth of Chinese medicine, serious concerns have been raised about its quality and safety in western countries. In the past, some Chinese medicines have been contaminated with toxic heavy metals and adulterated with prescription drugs (21).

1.4 Plant metabolites

A vast heritage of knowledge in diverse cultures and civilisations has been prompted through vigilant scrutiny by means of exploring the medicinal properties of plants. Medicinal plants were initially employed in an unmodified form then as concentrated extracts thus improving their intensity and uniformity of action (8).

In addition to genetic differences and environmental conditions, nutritional variation and the presence of microorganisms or fungi have a direct impact on the cellular processes in the plants and their responses to stimuli (22, 23). As a result, different levels of small endogenous molecules collectively known as metabolites are produced. (24) Two categories exist, namely primary and secondary metabolites. Primary metabolites, such as amino acids, organic acids and carbohydrates are essential for life and exist in all plants. Secondary metabolites are not directly involved in the normal life cycle but help the plant adjust to its surroundings (25-27).

Higher plants produce a vast number of secondary metabolites, via complex pathways, which are regulated in a highly sophisticated manner (28) . Many of them show strong biological activities, for example the inhibition of DNA and protein synthesis, inhibition of

the nerve system, cardiac activity, and modulation of microtuble structure. Secondary metabolites have therefore been utilized as natural medicines and often such plants containing those compounds have been used as medicinal plants and prescribed in many recipes as forms of crude drugs (29, 30).

Based on the biosynthetic origin of the plant chemical, three basic chemical families exist; phenolic and polyphenolic compounds such as flavonoids, terpenoids such as camphor, and nitrogen-containing alkaloids such as ephedrine. Secondary metabolites have been extensively analysed as part of herbal regulation as some plants or species have unique secondary metabolites that can be monitored as a diagnostic of herbal identity and/or quality.

Nevertheless, natural toxins (usually metabolites produced by plants to defend themselves against various threats such as bacteria, fungi, insects) may be present in plants and subsequently some plant secondary metabolites may pose a significant risk to human health (31-33).

1.5 Phytomedicine – potential safety concerns

The consumption of herbal medicines (HM) is not always safe and free of health risks. The quality and safety of phytomedicines may pose serious concerns. Like conventional drugs, HM can contribute to adverse reactions directly in response to toxic compounds in the plant. Considerable variations in the contents of biologically active constituents have also been reported. In some cases the concentrations of putative active ingredients are lower than expected but occasionally a component could be too high thus the user may consume

more than the maximum recommended dosage making it difficult to ascertain treatment outcomes and safety for patients.

Good QC of HM is important to avoid adverse reactions arising from contamination (such as pesticides, heavy metals and microbes), adulteration, misidentification, improper processing and preparation (34-36). Some of these contaminants have been identified by the Committee for Proprietary Medicinal Products (CPMP) of the European Community (EC) for use in controlling the purity of herbal medications in the European Union (EU) (37, 38).

There are numerous factors that potentially influence the quality, safety and efficacy of herbal medicines.

1.5.1 Species and origin of the raw herb

The biological diversity of a plant may differ from one species to another, so careful distinction between similar species and genetic origins in essential.

1.5.2 Place of origin, collection and growing environment

Differences in growing conditions and geographical origin may impact the chemical profile of the plant so that the same species from different locations may have diverse therapeutic outcomes and toxicity (39). Additionally the growing environment may contribute to heavy metal and pesticide contamination.

1.5.3 Processing and storage of raw herb or extract

The modification of the properties of the medicinal herbs, to reduce toxicity and/or increase therapeutic activity, can be achieved by using different processing methods (40-42). At the same time standardized protocols for storage are essential to avoid deterioration, reduction in essential oils content, bacterial, fungal or chemical contamination.

1.5.4 Manufacturing

Good practice and standardized manufacturing (for example, preparation of the herbal extract and finished product) are vital to the quality and safety of TCM products. Stringent adherence to the criteria for the identification of the medicinal herbs with meticulous QC should be performed according to "*WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants*" (43).

1.5.5 Adulterated and counterfeit drugs

Inadvertent substitution of an intended botanical ingredient with another plant species or an intentional adulteration with cheaper, more readily available plant material may result in contamination of the final product with various natural plant toxins, potentially leading to health problems for consumers. Numerous cases of poisonings by plant toxins in herbal dietary supplements have been reported (44). Examples of toxicity resulting in renal failure and development of urothelial cancer, is the substitution of *Stephania tetranda* with *Aristolochia fangchi* (containing the nephrotoxin aristolochic acid) in a medicine for weight loss (45). Another reported cases is poisoning caused by the neurotoxin anisatin which has been linked to herbal tea supplements labelled as containing Chinese star anise (*Illicium*

verum) (46). The intended component was mistaken with the morphologically similar but toxic Japanese star anise (*Illicium anisatum*).

1.6 Phytomedicine regulation in Australia

There exists an exceptional regulatory system for complementary medicine including phytomedicine in Australia, administered under the guidance of the Australian Therapeutic Goods Administration (TGA).

1.6.1 The Therapeutic Goods Administration

A division of the Commonwealth Department of Health and Aging, Therapeutic Goods Administration (TGA) is responsible for the protection of public health and safety through administering the provisions of the *Therapeutic Goods Act 1989* (47), the (Act) which came into effect in 1991. The Act is supported by the Therapeutics Goods Regulations 1990 and various Therapeutic Goods Orders (TGOs) and determinations, providing a uniform national framework for the import, export, manufacture and supply of therapeutic goods. The object of the Act is to provide for a system of controls relating to the quality, safety, efficacy and timely availability of therapeutic goods.

1.6.2 Listed and Registered medicines

Therapeutic goods such as medicines must generally be entered as either 'registered' or 'listed' medicines on the Australian Register of Therapeutic Goods (ARTG) (48) before they may be supplied in or exported from Australia. Lower risk medicines can be 'listed' on the ARTG, whereas higher risk medicines must be 'registered' (49).

Herbal formulations deemed by the TGA to contain low risk ingredients in acceptable amounts, manufactured in accordance with the code of Good Manufacturing Practice (cGMP) (50), making indications (for therapeutic use) for health maintenance and health enhancement are permitted for use under 'listed' medicines by the TGA. Medicines listed on the ARTG are assigned a unique AUST L number, which must be displayed on the medicine label. There are approximately 4500 plant-based products currently listed (51). "Listed" medicines are assessed by the TGA for quality and safety but not efficacy. Most complementary medicines (for example, herbal, vitamin and mineral products) are "listed" products.

Registered complementary medicines are regarded as posing a relatively higher risk than 'listed' medicines, based on their constituents (that is, if they contain herbs that are restricted by the Australian Standards for the Uniform Scheduling of Medicines and Poisons No 4 *(SUSMP 4)*, *Poisons Standard 2013* (52) or the indications made for the medicine, for example, if the substance is a designated active ingredient that has an established identity and traditional use. These are fully evaluated by the TGA for quality, safety and efficacy prior to being accepted on the ARTG and marketed. Substances can only be registered when appropriate documentation outlining clinical trial evidence is submitted to the Office of Complementary Medicines (OCM) that advises the TGA. Medicines registered on the ARTG are assigned a unique AUST R number, which must be displayed on the medicine label.

1.7 Pharmacovigilance

This is defined as the science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other drug-related problem (53). Although not a legislative requirement, sponsors of registered and listed medicines in

Australia are encouraged to have an effective system of pharmacovigilance in place in order to assure responsibility and liability for their products in the market and to ensure that appropriate action can be taken, when necessary (54).

1.8 Improving quality assessment of herbal formulations

Current regulatory standards predominantly focus on a targeted approach paradigm, where one or two markers or pharmacologically active components in the herb or herbal mixture are monitored for evaluating product quality and consistency. The extent to which these assumptions are valid will likely depend on the type and number of analytes selected for monitoring. The Australian Regulatory Guidelines for Complementary Medicines (ARGCM) guidelines recommends the analysis of at least one unique analyte from each constituent herb in an herbal formulation (50) . This is to detect adulteration of the herbal formulation with different herb parts or substitution with a different herb altogether. The analytes selected should ideally contribute to the therapeutic effect of the medication. This ideal is often not achieved because relatively few plants have chemicals that are absolutely unique to them, but it may be sufficient for the chemical just to be unique in the context of the formulation. There is currently no regulatory criteria for the selection of analytes in complex herbal medicines for QC and QA purposes, except that the analyte, where possible, should contribute to the effect of the formulation (50).

While these measures are enough to ensure the basic safety of the herbal product, it does not necessarily provide information about its quality since the putative bio-actives are not determined unless a label claim regarding their concentrations is shown on the product packaging. Moreover, several methods for the evaluation of herbal products were considered in a comprehensive review on the quality control of herbal medicine suggesting

that the combination of chromatographic fingerprints of HM and the chemometric evaluation might be a powerful tool for their quality control. (55)

1.8.1 Analytical methods for herbal analysis

The concentration of some constituents in botanical products is generally low, requiring highly sensitive and robust analytical hyphenated systems for their analysis to be performed as stringent as possible. Liquid chromatographic (LC) methods are almost universally employed for herbal analysis, usually with photodiode array detection (PDA) but more increasingly with electrospray ionisation tandem mass spectrometry (ESI-MS/MS). The MS/MS detector is preferred due to its greater selectivity, hence reducing the probability of peak mis-identification which may occur in complex matrices. Furthermore, the MS/MS detector can provide peak identity confirmation and is suitable for detecting chemical species which do not show UV absorbance. Ultra performance liquid chromatography (UPLC) rather than high performance liquid chromatography (HPLC) is favoured at present; this is partly attributed to the application of lower flow rates rendering it more amenable for the requirements of the ESI-MS/MS detector, where the solvent is more readily removed because there is less of it. Other advantages of the UPLC include shorter separation times and hence lower solvent (mobile phase) consumption.

Selected reaction monitoring (SRM, also referred to as multiple reaction monitoring, MRM) is performed on a triple quadrupole mass spectrometer. In SRM the first and third quadrupoles are used to select predefined pairs of *m/z* values (also called transitions) corresponding to the precursor ion and a specific fragment ion, which is generated in the second quadrupole which acts as a collision cell. This technique gives precise measurements over a wide dynamic range and with good sensitivity.

1.8.2 Herbal composition of Qi Ju Di Huang Wan

QJDHW is a standard eight herb formula described in the Chinese Pharmacopeia to assist in the management of liver and kidney disease. The constituent herbs and their percentage composition in the formulation are presented in Table 1.1. For consistency, the herbs used in this formulation are referred to by their pinyin name.

Table 1.1 The herbal nomenclature and percentage composition of Qi Ju Di Huang Wan[1]

Pinyin name	Chinese name	Latin pharmaceutical name	Latin botanical name	%w/w
Fuling	茯苓	Poria	Poria cocos (Schw.) Wolf	10.4
Gou Qi Zi	枸杞子	*Lycium* barbaum	*Lycium barbarum* L.	6.8
Ju Hua	菊花	Chrysanthemum	Chrysanthemum x morifolium Ramat	6.8
Mu Dan Pi	牡丹皮	Cortex moutan	Paeonia suffruticosa Andr.	10.4
Shan Yao	山藥	Dioscorea opposita	Dioscorea opposita Thunb.	13.8
Shu Di Huang	熟地黃	Radix rehmanniae praeparata	Rehmannia glutinosa Libosch.	27.6
Shan Zhu Yu	山茱萸	Cornus	Cornus officinalis Siebold &Zucc.	13.8
Ze Xie	澤瀉	Alisma	Alisma plantago-aquatica subsp. orientale (Sam.)	10.4

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[1]Pharmaceutical and botanical names are based on The Plant List (2013). Version 1.1. Published on the Internet; http://www.theplantlist.org/ (accessed 1st January).

1.9 Selection of analytes for QC monitoring

A systematic and justifiable approach is crucial in the selection process of analytes to be monitored for the QC of an herbal formulation. Devising a procedure for reducing the number of analytes to a manageable quantity according to a logical selection scale is essential. The aim is to analyse a small number of the total number of compounds present and for the compounds that are determined to reflect the efficacy of the product for the consumer. The selection criteria considers factors such as the compound's pharmacological activity (particularly in relation to the disease the medication is targeted at), concentration, regulatory compliance, uniqueness in the context of the formulation and availability of the purified compound.

1.9.1 Weighting of selection criteria

The analytes for QC monitoring are ranked in importance and selected according to the following criteria as set out by the National Institute for Complementary Medicine (NICM) (56).

• Concentration of the analyte in the herb

• Uniqueness of the analyte to the herb

• Physical and chemical properties of the analyte (including its stability)

• Availability of the reference analytes

• Bioavailability of the analytes

• Amount of research to support the action of the analyte(s)

• Toxicity of the analyte and the safety limits applied to its use

• Quality of research conducted on the analyte

• Traditional use of the herb

• The part of the plant used in the formulation, such as the seed, root or rhizome

A numerical ranking system from 0 – 5 as presented in Table 1.2 was established using the criteria described. The higher numerical value is indicative of greater significance in monitoring that analyte. A rank of 'x' means the analyte is unsuitable.

The ranking scale was previously developed at the National Institute of Complementary Medicine in an iterative, discursive consensus process based on current scientific literature and laboratory and clinical experience. At each stage of iteration the literature was used to test the model on a common complex herbal formula. After three iterations and application tests, a systematic method was agreed by consensus for the selection, identification and quantification of key chemical markers of importance for the purposes of QA and routine QC of a multi-herb mixture (57).

Table 1.2 The ranking system used to select analytes for the targeted approach analysis of QJDHW

Selection scale	Details of analyte
X	No bioactivity studies
0	Activity not related to disease of interest OR not commercially available
1	Activity indirectly related to disease symptom OR activity not supported by traditional and therapeutic use of herb OR Present at very low concentrations
2	Has bioactivity related to a symptom (major or minor) of the disease, however there is only one study to support this action And Traditional/other use of the plant may be related to the action of the compound And Present in a low concentration ($< 1 \ \mu g \ mL^{-1}$)
3	Has bioactivity related to a symptom (major or minor) of the disease, however there is only one study to support its action And Traditional/other use of the herb and its therapeutic action supports the activity of the compound And Present in a relatively high concentration ($> 50 \ \mu g \ mL^{-1}$)
4	Has high bioactivity related to major symptom(s) of the disease with sufficient evidence to support its activity (≥ 2 reported studies) And Traditional/other use of the herb and its therapeutic action supports the activity of the compound And Present in a relatively high concentration ($> 50 \ \mu g \ mL^{-1}$)
5	Has highest bioactivity related to major symptoms of the disease with sufficient evidence to support its activity (≥ 2 reported studies) And Traditional/other use of the herb and its therapeutic action supports the activity of the compound And Compound and/or its metabolites are known to be bioavailable Or May be toxic and needs to be screened to comply with safety limits And Present in relatively high concentration ($> 50 \ \mu g \ mL^{-1}$)

It is desirable to restrict the number of analytes to 1-2 per herb since this is an eight herb formulation otherwise the analysis may be impractical for routine application in a typical commercial QC laboratory. A total of 9 analytes were chosen for monitoring namely, alisol B, pachymic acid, alisol C, rutin, luteolin, cornuside, paeoniflorin, catalpol and diosgenin. These analytes were selected based on the selection criteria described in Table 1.2 and the application of the criteria for each herb is shown in Tables 1.3 - 1.10. Compromises had to be made when the analytical reference standard was very costly or if there is a long wait for delivery. This was the case with batatasin I in Shan Yao and morroniside in Shan Zhu Yu. Paeoniflorin, rutin, cornuside, luteolin and diosgenin were selected primarily due to their reported biological activity as vasodilators (58) and a systematic review (7) on the effectiveness and safety of QJDHW for the treatment of essential hypertension. Despite the presence of rutin in many herbs, its concentration in *Lycium barbarum* is high compared to the other compounds. It was therefore selected as the analyte for L.*barbarum* in this study. Alisol B and C were shown to have high biological activity with respect to the symptoms of the disease and have been reported as reference markers in the herb Ze Xie (59-61). The references for the claimed biological activities as summarised in Tables 1.3 - 1.10 are presented in the Appendices 1 & 2.

Table 1.3 Compounds in Mu Dan Pi and their bioactive relative ranking based on potential activity relevant to QJDHW

Compounds	Action	Ranking	Standard availability
Paeoniflorin	Analgesic, anti-aggregant, anti-allergic, anti-bacterial, anti-coagulant, anti-inflammatory, anti-mutagenic anti-oxytocic, anti-spasmodic, central nervous system-depressant, hypotensive, immunostimulant, musculotropic, myorelaxant, vasodilator	5	Y
Benzoic acid	Allergenic, antiotitic, antiseptic, choleretic, phytoalexin, tyrosinase-inhibitor, vulnerary	0	Y
Paeonol	Analgesic, anti-aggregant, anti-atherosclerotic, anti-bacterial, anti-inflammatory, anti-mutagenic, anti-pyretic, anti-stress, anti-vasculitic, diuretic, sedative, immunomodulator	3	Y
Pentagalloyl-glucose	Anti-fibrinolytic, anti-inflammatory, anti-mutagenic, antiviral, dehydrogenase-inhibiton	0	N
Oxypaeoniflorin	No bioactivity recorded	X	Y
Bezoylpaeoniflorin	No bioactivity recorded	X	N
3-Hydroxy-4-acetophenone	No bioactivity recorded	X	Y
Paeonolide	Anti-aggregant	0	Y
Paeonoside	Anti-aggregant	0	Y
Campesterol	Anti-oxidant, hypocholesterolemic, anti-inflammatory, anti-tumor, hypolipemic	0	Y

Table 1.4 Compounds in Fu Ling and their bioactive relative ranking based on potential activity relevant to QJDHW

Compounds	Action	Ranking	Standard availability
Pachymic acid	Anti-inflammatory, anti-hyperglycemic, stimulate triglyceride accumulation and reduced glycerol release in 3T3-L1 adipocytes	4	Y
Polyporenic acid C	Anti-inflammatory	2	Y
Dehydrotrametenolic acid	No bioactivity found	X	N
Trametenolic acid	No bioactivity found	X	N
Cerevisterol	No bioactivity found	X	Y
Eburicoic acid	Anti-tumour, anti- inflammatory	0	Y
Poricoic acid A	Anti-tumour	0	N
Poricoic acid H	Anti-tumour	0	N
Poricoic acid	Anti-tumour	0	N
Tumulosic acid	Anti-inflammatory, anti-oxidant	1	Y

Table 1.5 Compounds in Gou Qi Zi and their bioactive relative ranking based on potential activity relevant to QJDHW

Compounds	Action	Ranking	Standard availability
Rutin	Anti-diabetic, anti-inflammatory, anti-obesity, anti-tumour, anti-infective, cytoprotective, neuroprotective	5	Y
p-Coumaric acid	Anti-oxidant, anti-spasmodic, anti-hepatotoxic	2	y
Scopoletin	Analgesic, anti-adrenergic, anti-aflatoxin, anti-asthmatic, anti-hepatotoxic, anti-inflammatory, anti-oxidant, anti-spasmodic	1	Y
Isorhamnetin-3-*o*-rutinoside	Anti-bacterial, hepatoprotective	0	Y
Kaempferol-3-*o*-rutinoside	Neuroprotective, antibacterial, anti-inflammatory, cytoprotective, anti-tumour, cardiovascular	0	Y
Chlorogenic acid	Anti-inflammatory, anti-hepatotoxic, analgesic	1	Y
Vanillic acid	Anti-inflammatory, anti-oxidant, anti-fatigue	0	Y
Caffeic acid	Hepatocarcinogenic, hepatoprotective, hepatotropic, anti-spasmodic, anti-oxidant, anti-inflammatory	1	Y
Quercetin	Anti-inflammatory, anti-obesity, anti-tumour, immunostimulant, immunomodulator	1	Y

Table 1.6 Compounds in Shan Zu Yu and their bioactive relative ranking based on potential activity relevant to QJDHW

Compounds	Action	Ranking	Standard availability
Gallic acid	Topoisomerase-I-inhibitor activity, anti-hepatotoxic, ACE inhibitor, analgesic, anti-allergenic, anti anaphylatic , anti-spasmodic	1	Y
Cornuside	immunomodulatory and anti-inflammatory, antidiabetic, inhibition of CCl_4-induced liver damage	4	y
Morroniside	Neuroprotective, anti-inflammatory	2	Y
Logannin	Neuroprotective	0	Y
Sweroside	Pesticide activity, anti-inflammatory, analgesic	2	Y
Dehydrologannin	No bioactivity recorded	X	N
Ursolic acid	Anti-bacterial, hypolipemic, cytoprotective, anti-inflammatory, analgesic	1	Y
Quercetin	Immunomodulator, analgesic, anti-bacterial, anti-inflammatory, anti-tumour, anti-hypertensive, cyotprotective, immunoprotective	1	y

Table 1.7 Compounds in Ju Hua and their bioactive relative ranking based on potential activity relevant to QJDHW.

Compound	Action	Ranking	Standard availability
Acacetin-7-*O*-β-D-glucoside	Aldose-reductase inhibitor	0	Y
Apigenin-7-*O*-β-D-glucoside	Anti- inflammatory	1	Y
Luteolin	Anti-oxidant, hepatoprotective effects and inhibitory effect on aortic vascular smooth muscle cell proliferation, anti- inflammatory, anti-tumour	4	Y
Chlorogenic acid	Anti-diabetic, anti -oxidant, anti-inflammatory	2	Y
Kaempferol	Antioxidant, antispasmodic, anti-inflammatory, antiaggregant, neuroprotective	1	Y
Myricetin	5-Lipoxygenase-inhibitor, cyclooxygenase-inhibitor	0	Y
Quercitrin	Anti-oxidant, anti-carcinogenic activities via inhibition of neoplastic transformation by blocking activation of the MAPK pathway and stimulation of cellular protection signalling	0	Y
Diosmetin 7-*O*-glucoside	Aldose-reductase inhibitor, anti-alzheimeran, anti-dementia, anti-mutagenic	X	Y
Chrysanthenone	Pesticide, dentifrice	X	Y
Chrysanthemol	Anti-inflammatory	1	Y
Cumambrin-A	Herbicide, pesticide	X	Y

Table 1.8 Compounds in Ze Xie and their bioactive relative ranking based on potential activity relevant to QJDHW

Compound	Action	Ranking	Standard availability
Alisol A	Anti-hypercholesterolemic, natriuretic	0	Y
Alisol B	Natriuretic, anti-hypercholesterolemic	0	Y
Alisol C	Hepatoprotective	2	Y
Epialisol-A	No recorded activity	X	N
Alisol F	Hepatoprotective	2	Y
Alisol-C-monoacetate	Anti-hypercholesterolemic, diuretic, hepatoprotective, hypokalemic	4	Y
Alisol-B-monoacetate	Anti-hypercholesterolemic, hepatoprotective	4	Y
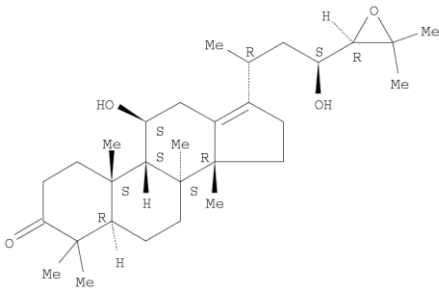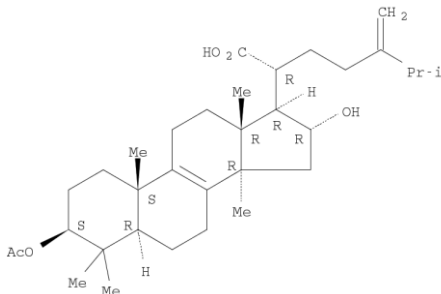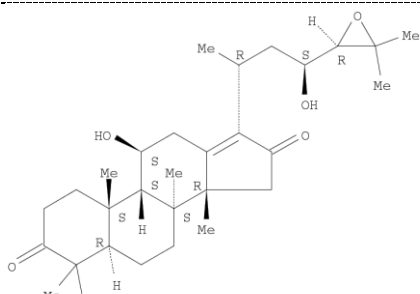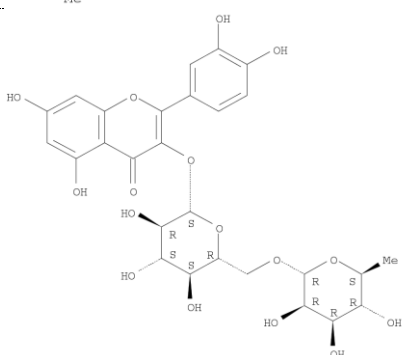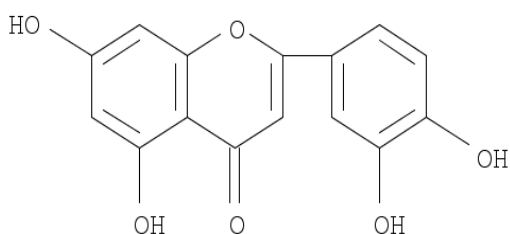
Table 1.9 Compounds in Shu Di Huang and their bioactive relative ranking based on potential activity relevant to QJDHW

Compound	Action	Ranking	Standard availability
Catalpol	Hepatoprotective, neuroprotective, anti-diabetic, anti-ischemic	5	Y
Aucubin	Anti-inflammatory, anti-leukemic, anti-oxidant, anti-prolactin, hepatoprotective	3	Y
Glutamic acid	Antiepileptic, antihyperammonemic, antilithic, anti-prostatitic, anti-retardation, anxiolytic; neurotoxic	0	Y
5-Hydroxymethyl furfural	Improving blood circulation, potent anti-sickling effects	1	Y
Melittoside	No reported activity	X	N
Acteoside	Anti-inflammatory, anti-tumour	0	Y
Leonuride	Hepatoprotective	X	N
Rehmannioside D	Hepatoprotective	2	Y
Rehmannioside A	No reported activity	X	N
Rehmannioside B	No reported activity	X	N

Table 1.10 Compounds in Shan Yao and their bioactive relative ranking based on potential activity relevant to QJDHW

Compound	Action	Ranking	Standard availability
Diosgenin	Anti-fatigue, anti-inflammatory , anti-stress , estrogenic, hepatoprotective, hypocholesterolemic	4	Y
Dioscin	Anti-tumour	0	y
Progenin II	No reported activity	X	Y
Batatasin III	Allelopathic, fungicide, pesticide	0	Y
Tristin	No reported activity	X	N
Batatasin-I	Anti- inflammatory	2	Y

Table 1.11 Chemical structures of determined compounds

Compound	Chemical structure
Alisol B	
Pachymic acid	
Alisol C	
Rutin	
Luteolin	

Cornuside
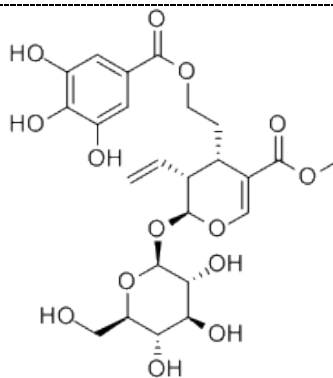
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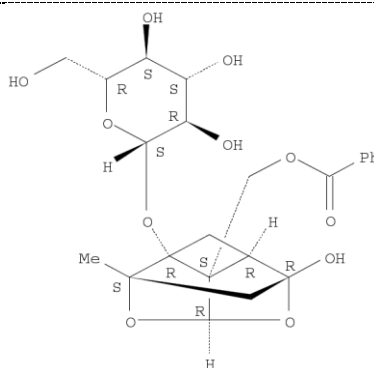
Catalpol
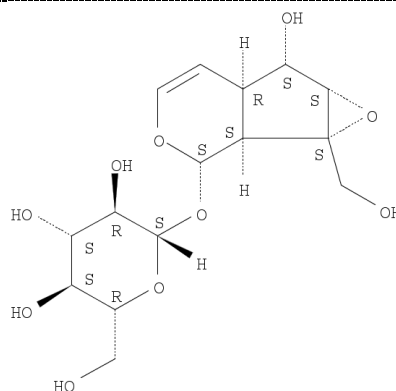
Diosgenin
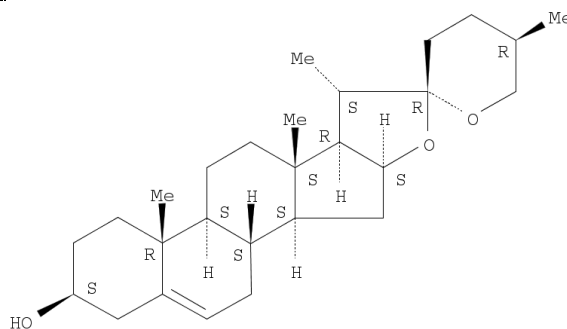
1.10 Analytical method development

1.10.1 Significance of analytical method validation

Analytical method validation is a process employed to establish that the proposed method is fit for its claimed analytical purposes (62). This may be accomplished by adopting a minimum series of validation experiments and obtaining analytical results with an acceptable uncertainty level (or RSD). The key analytical method validation parameters are described in section 1.10.2. Analytical method development is performed for one source of the herb or herbal formula, and applied to the other herbal sources accordingly.

1.10.2 Analytical method validation parameters

1.10.2.1 Analyte identity confirmation by LC-MS/MS

Analyte peak identity confirmation with MS/MS detection is achieved by comparing the *m/z* values and their relative intensities obtained for at least two product (or daughter) ions arising from MRM of the selected precursor ion. Then intensities of the ions should match within specified tolerances as described by the European Commission Directorate for Agriculture on MS analysis (9).

1.10.2.2 Accuracy

An analytical method needs to be assessed for accuracy or trueness in the agreement between the results of the proposed method and the results of an established reference method, or with the expected results from a standard reference. In this study it is determined from fortification (or spiking) recoveries carried out at the 50, 100 and 200 %

levels (above the unfortified sample). Fortification is carried out using a mixed fortification standard solution, where the ratios of the concentration of the standards correspond to their relative concentrations in the unfortified sample. A volume of fortification solution is added to the sample such that the analyte peak height (or area) will increase by approximately 50, 100 and 200 %. After adding the fortification solution the solvent is allowed to evaporate before starting the analysis. The unfortified and the three fortification levels are each analysed with 7 replicates to give a total of 28 samples which are extracted and analysed.

1.10.2.3 Precision

The replicates performed for the analysis enable a reasonable estimate of the method precision. The analytical method is assessed for repeatability and reproducibility. Repeatability is the intra-day precision and reproducibility is the inter-day precision (analysis carried out on the same equipment). The measure of precision is usually expressed in terms of standard deviation (SD) or relative standard deviation (RSD) of n = 7 replicate sample extractions and analyses. The contributing uncertainties from sample preparation and instrumental analysis are reflected in the observed method SD. The instrumental contribution to the method SD may be estimated by injecting one solution multiple (usually n = 7) times and the SD from the sample extraction and preparation steps is then estimated by difference. Most studies observe that the instrumental contribution to method SD is less than the contribution from the sample preparation step(s) unless the analyte concentrations are very low and the instrument is operating at close to its detection limit.

1.10.2.4 Linearity

The linearity of an analytical method is its ability to produce test results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of analyte(s). The linearity of a calibration curve is determined by a series of injections of standard solutions which contain the analyte present in the herbal extract. Method linearity is tested from extraction to instrumental analysis over a range in which the analyte concentration can reasonably be expected to lie. In this study the method linearity is tested from approximately the analyte in the unfortified sample to the 200% fortification concentration.

1.10.2.5 Detection limit

The limit of detection (LOD) is the lowest concentration of the analyte that may be detected in a sample after applying the appropriate analytical methodology. Usually the detection limit is taken as 3 times the SD of a set of replicates.

The limit of quantitation (LOQ) is the lowest measured amount of analyte in a sample that can be quantified within a specified degree of accuracy and reproducibility. The method LOQ is generally defined as 10 times the SD of a set of replicates.

1.10.2.6 Stability

The term stability refers to the analyte decomposition represented by a decrease in concentration of the standard and sample under specified storage conditions (>2% decrease in analyte peak area) as a function of time. Standard and sample solution stabilities are both determined with most investigators observing that analytes are less stable in the sample solution compared to the standard solution (which is usually prepared in methanol). Storage conditions are typically at 4 ^{o}C in the fridge, usually wrapped in Aluminium foil to minimise photodecomposition.

1.11 Multivariate analysis of phytochemical variations in Qi Ju Di Huang Wan

Multivariate statistical analysis provides a means of analysing data and extracting differentiating features from complex chromatographic data (63). The first step of the data analysis is to explore the structure of the data and find any relevant groupings. At this stage, the data analysis is unsupervised, that is, there are no assumptions about sample

association. The technique allows for an unbiased view at the data and gives useful information about the variability of a data set. The technique used in this study is principal component analysis (PCA).

1.11.1 Principal component analysis (PCA)

The statistical technique of principal component analysis (PCA) is commonly used in chemometrics as a tool to reduce the complexity of chromatographic profiles to a more user friendly form, such as a 2-dimensional plot (64). This technique exposes the variance in a data set to careful inspection without in-built biases (65).

The variance is expressed through factors called principal components (PC). There are an infinite number of possible PC's but in plant metabolomics only 2 or 3 are needed to explain nearly all the variance within a dataset. Principal component 1 or PC-1 can be described as the scaling transformer that describes the most of the variance in a dataset, while principal component 2 or PC-2 is the next and so on. This is greatly advantageous since the variation in the chromatographic and spectral data is explained by a few PC's compared to the original, large number of variables. It allows discovery of trends, groupings and outliers within a dataset.

The raw data is pre-processed by removing any unwanted signals such as solvent peak. Data handling techniques require the same variable to contain equivalent information for every chromatogram. For this, MS data provide useful information on the underlying compounds. Commonly applied pre-processing techniques include offset correction to reduce baseline drift which is largely contributed by gradient changes in the mobile phase. Beer's Law assumes that the absorption of light at a given wavelength is due entirely to the absorptivity

of the constituents in the sample. Therefore, any variations in the chromatogram due to spectrometer and sampling error should be eliminated prior to data analysis. This is followed by normalization and binning. Normalizing divides each point in the spectrum or chromatogram by all the points in the spectra or chromatogram. This is very useful since it reduces the effects of dilution in a sample. Binning collapses groups of frequencies into single frequency values and this helps correct the peak drifts of narrow peaks.

After the pre-processing, PCA is performed on the dataset. The two main PCA methods are classical and robust. Classical PCA weights all samples in a data set equally, while robust PCA down weights more extreme samples. In general classical PCA is very useful in analysing the variability of a dataset while robust PCA is better at picking outliers. Robust PCA is better at picking outliers because it detects using orthogonal distance rather than using good leverage samples far away from the core of the dataset. Since performing both techniques would be time-consuming, the robust ellipse is projected on a classical PCA score plot.

Classical PCA can be either noscaled, autoscaled or Pareto scaled. When the data set is noscaled, the bigger peaks have bigger loadings, while autoscaled data equally loads all peaks. Pareto scaling is a compromise between the two scaling techniques and is preferred option in plant analysis (66, 67).

Chapter 2

Introduction to *Lycium*

2.1 Use of untargeted analysis for assessing herbal quality

The objective of untargeted chromatographic profiling analysis is to describe a characteristic chromatographic profile or a fingerprint pattern in a given herb/herbal material by determining all detectable constituents without necessarily identifying nor quantifying a specific compound in a single analysis (68, 69). In contrast targeted analysis is more exclusive as it focuses on a specific group of chemicals with identification and quantification of the selected constituents within the group (70). Targeted analyses are important for assessing the characteristics of a specific group of compounds in the sample under determined conditions.

In untargeted analysis the chromatogram is analysed as a chromatographic profile from a selected start to finish time point. Here all the observed peaks (their position and magnitude) within that time window are considered. In targeted analysis only the peaks with retention times matched to known standards (and thereby quantified) are considered.

2.1.1 Estimation of herbal quality through untargeted analysis

The chemical components in an herb comprise of compounds with a wide range of polarities and belong to a variety of chemical classes including sugars, flavonoids, terpenoids, and phenolics. This, and their wide concentration range, can make their simultaneous analysis challenging. This potential problem is side stepped by untargeted analysis where the chromatographic profile of the test herb is compared to that of an herb sample known to be of good quality. This approach to quality estimation may also be applied to herbal extracts or formulations, where the characteristics of the chromatographic profile of a new batch of herbs are compared to a reference batch. There is, however, some subjectivity in judging

the degree of similarity or difference between the profiles through statistical analysis. Of the various profiling techniques, untargeted analysis using LC-MS is a promising tool for investigating the diversity of phytochemicals (71, 72).

2.2 Description, reported bioactivity and uses of the *Lycium berry*

Goji is a relatively new name given to the fruit of *Lycium barbarum* and *Lycium chinense,* which is marketed as a food and dietary supplement in many countries. It is generally accepted that the *chinense* species is grown in southern China whereas *barbarum* is grown in the north and the plant is somewhat taller. There is no report to indicate if the fruit of these species are different. The berries are consumed as an ingredient in Chinese cuisine, as a concentrated juice or infused in liquor and as the dried fruit.

The *Lycium* genus comprises of approximately 75 species and is one of the largest and most widely distributed genera in Solanaceae native to arid and subarid regions of south America, southern Africa, Eurasia and Australia (73) . It grows naturally in Asia, primarily in northwest China and Tibet (16). The fruit is dried in the shade till the skin shrinks and then sun dried till the outer skin becomes dry and hard but the pulp is still soft. China is the main supplier of *L. barbarum* berry products, with exports totalling US$120 million in 2004. This production was derived from 82,000 ha farmed nationwide, yielding 95,000 tons of the berries (74).

The Chinese Pharmacopoeia (CP) reports that Goji berries are used to enhance liver and kidney function. The earliest known Chinese medicinal monograph documenting its therapeutic use dates back to approximately 2300 years. The fruit is an ingredient in many herbal formulations, especially those used for replenishing 'vital essence', enriching kidney 'yin', tonifying the kidney and liver, 'moistening' the lungs, improving vision and promoting

longevity. According to the CP, only the fruit of *L. barbarum* has medicinal use (2) though the *chinense* is also available in the marketplace.

Lycium berries are a rich source of antioxidants (75) and they reportedly contain constituents capable of acting as free-radical scavengers, peroxide decomposers and synergists. Compounds present include alkaloids, amides, peptides, flavonoids, coumarins, lignans, terpenoids, sterols, and steroids, organic acids and their derivatives, carotenoids, nutrients, and essential oils (76, 77). L. *barbarum* polysaccharides (LBP) are estimated to comprise 5–8% of the dried fruit (78). Recent studies reveal that LBP possess a range of biological activities, including anti-aging, neuroprotection (79, 80), endurance enhancing, increasing metabolism, glucose control in diabetics, glaucoma protecting (81, 82), anti-oxidant properties, immunomodulation (83), anti-tumor and cytoprotection properties. *Lycium* also contains phenolics such as flavonoids which have free radical scavenging activity.

A study to evaluate the protective effects of extracts from the fruits of *Lycium barbarum* as pre-treatment in an experimental stroke model, showed neuroprotective function in the retina when given prophylactically. Decreasing immune-reactivity for aquaporin-4 and glial fibrillary acidic protein in LBP treated brains and improving neurological deficits as well as decreased infarct size, hemispheric swelling and water content was noted (84). The flavonoids of *L. barbarum* completely inhibited the heat output from phorbol myristate acetate-stimulated polymorphonuclear leukocyte and could also inhibit the heat output from L1210 cells (85).

The phytochemical profile of botanical products may be affected by factors such as species type, growing conditions (soil, climate, rainfall), age of plant, time of harvest, post-harvest

treatment and storage conditions (86). Variations in chemical profile may alter the therapeutic effectiveness of the herb. Carrying out quality control (QC) ensures a more consistent product and hence some predictable outcome for the consumer. The most commonly used QC approach is by the identification and quantitation of key analytes or putative active components. This may be complemented by pharmacological and biological testing to give an estimation of herbal quality. The type and number of tests carried out is largely determined by cost factors and the practicality of the task in a routine commercial laboratory.

2.2.1 Studying the chemical composition of Lycium barbarum and Lycium chinense

As a significant component in Qi Ju Di Huang Wan (QJDHW), the *Lycium* berry is designated as a 'health food' and is readily available on the marketplace. We were able to obtain eleven different samples of QJDHW – five of the samples specified the *Lycium* species used and the remainder did not. The Chinese Pharmacopoeia (CP) specifies the *barbarum* species. In light of this ambiguity and the relatively widespread use of the *Lycium* herb, it was decided to investigate if there is a difference in the chemical composition of these two species of *Lycium*. To achieve this, the initial step in determining if the two *Lycium* species are different is to develop a validated analytical method for a set of selected analytes; targeted analysis, then to apply the method to the twelve samples (eight *L. barbarum* and four *L. chinense*). The selection of marker compounds in *Lycium* follow the same criterion listed in section 1.9 chapter 1.

The chromatograms of the twelve samples of the herb were also subjected to chemometric analysis by unsupervised pattern recognition methods, namely principal component analysis (PCA), and hierarchical cluster analysis (HCA); namely untargeted analysis.

2.2.2 DNA and spectroscopic studies of Lycium barbarum and Lycium chinense

There has been a study reporting the application of sequence characterized amplified region analysis to authenticate *L. barbarum* and its adulterants (87). The most common species studied were *L. barbarum* cv. '*Tianjinense*' and *L. chinense* var. *Potaninii.* Random amplification of polymorphic DNA profiles generated from the *Lycium* samples allowed for the detection of two characteristic bands that were isolated and sequenced. The study concluded that two primer sets based on the sequences, could amplify a single specific band in samples of *L. Barbarum*, whereas no bands were detected in samples of *L. chinense* var. *potaninii.* In another study eight species of *Lycium* fruits were differentiated by Fourier-transform infrared spectroscopy (IR), second derivative IR spectra and two-dimensional correlation IR. Their results indicated that the chemical constituents in these species of herbs were not distinctively different; however vary in terms of peak intensity (88).

2.3 Chemometric analysis and anti-oxidant testing of *Lycium*

2.3.1 Chemometric analysis

In recent years, the use of multivariate statistical techniques (or chemometrics) on chemical data has gained increasing attention as a tool to assess the herbal raw material quality, and to describe similar and discriminating chemical characteristics. Chemometrics uses a combination of mathematical, statistical, and other logic-based methods to efficiently manage and interpret chemically-derived data (89). Principal component analysis (PCA) and hierarchical cluster analysis (HCA) are multivariate statistical techniques commonly used in chemometric studies to identify important components or factors that explain most of the variances in a chromatogram. They are designed to reduce the number of variables such as

peak height measurements to a small number of uncorrelated variables (principal components) while attempting to preserve the relationships present in the original data. PCA is aimed at finding and interpreting similarities and differences between features in a data set (90) .The key idea of PCA is a quantification of the significance of variables that explain the observed grouping and patterns of the inherent properties of the individual objects. Plotting the samples on the PCs can yield a 1-, 2- or 3- dimensional plot depending on the number of PCs chosen. Similar samples often group together in the same area of the scores plot. Plotting the variables on the loadings plot can identify the variables responsible for the grouping observed in the scores plot.

HCA is a group of multivariate techniques whose primary purpose is to assemble objects based on the characteristics they possess. Its objective is to sort cases (monitoring points) into groups, or clusters, so that the degree of association is strong between members of the same cluster and weak between members of different clusters. The resulting clusters of objects should then exhibit high internal (within-cluster) homogeneity and high external (between clusters) heterogeneity. The Euclidean distance usually gives the similarity between two samples and a distance can be represented by the difference between analytical values from the samples (91).

The R language for statistical computing (known as 'R') is a powerful tool used for chemometric analysis (92). One of R's major advantages is that it is a free program, with many users around the world donating add-on packages that can perform a wide array of functions. One such package is 'msProcess,' which is used in this study to pre-process chromatographic data. 'msProcess' can remove instrumental noise, baseline drift, peak retention time variations, identify peaks, and quantify peak height (93). The 'Stats' package

included in R contains many of the commonly used statistical techniques such as PCA and HCA.

2.3.2 Antioxidant testing of Lycium

Oxidative stress, mediated by reactive oxygen species (ROS), has been implicated as a major cause of cellular injuries in a variety of clinical abnormalities (94). The formation of hydroxyl radicals and other ROS initiates lipid peroxidation and causes DNA damage and protein residual modification. Crude extracts or fractions from edible antioxidant sources may help prevent or alleviate many ROS-related diseases (95).

A preliminary measure of pharmacological activity is the investigation of the radical scavenging capacity. Natural antioxidants such as phenolic constituents, which play an important role in the oxidation process by being preferentially oxidized by the attacking radical, are abundant in *Lycium* berries.

It is relevant to assess the antioxidant activity of a complementary medicine as they may be characterised as chemically equivalent, though they may contain different pharmacologically active chemicals not detectable by the chemical analysis used. Similarly, extracts with different chemical profiles may exhibit the same biological activity if the phytochemicals responsible for the difference in the profile are biologically inert (86). The pharmacological tests measures some bulk or overall property of the herbal extract whereas chemical testing measures the concentration of a small number of chemicals.

The two main methods by which a compound can function as an antioxidant are hydrogen atom transfer and electron transfer (ET). In this study the antioxidant activities of the *Lycium* extracts are evaluated using ET mechanisms. ET reactions such as those using 2, 2-di (4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) involve a redox reaction between the DPPH and the

antioxidant compound being measured. DPPH is a stable synthetic free-radical and has been widely used for measuring free-radical scavenging activity (96).

The DPPH method use gallic acid as a reference for antioxidant capacity. That is, these assays measure how much better (or worse) the *Lycium* extracts are at being antioxidants than gallic acid.

2.4 Analytical method development

A validated analytical method was developed for the quantitation of selected analytes in a representative sample of *Lycium barbarum*, subsequently the method was then applied to all other *Lycium* samples obtained.

2.4.1 Analytes selected for QC monitoring

As described in Chapter 1, analytes selected for monitoring were chosen following the criteria developed within the National Institute for Complementary Medicine (NICM). The selected analytes are given in Table 2.1.

Table 2.1 Analyte selection ranking in *Lycium*

Selected analyte	Action[1]	Ranking
Rutin	Anti-hepatotoxic, antioxidant, cAMP-phosphodiesterase-inhibitor, vasopressor, vasodilator, anti-inflammatory, cytoprotective	5
Isorhamnetin-3-*o*-rutinoside	Anti-inflammatory, antioxidant, hepatoprotective, pro-oxidant	4
Kaempferol-3-*o*-rutinoside	Anti-hepatotoxic, antioxidant, iNOS-Inhibitor,cAMP-phosphodiesterase-inhibitorTNF-alpha-inhibitor,neuroprotective	4
Coumaric acid	Anti-hepatotoxic, antioxidant	3
Scopoletin	Anti-hepatotoxic, antioxidant	3
Caffeic acid	Antioxidant, anti-inflammatory, anti-hepatotoxic, hepatotropic	2
Chlorogenic acid	Hepatotropic, anti-inflammatory	2

---

[1] References to reported activity may be found in appendix 1.

2.5 Chromatographic data analysis

Liquid chromatography with photodiode array detection (LC–PDA) and/or tandem mass spectrometry (LC–MS/MS), and gas chromatography–mass spectrometry (GC–MS) can provide accurate quantitation data for the selected analytes (97). This targeted analysis can be combined with chemometric profiling (untargeted analysis) which considers all the chromatographic peaks, to give another interpretation of the chromatographic data. Results obtained by analysis of the targeted and untargeted data are compared to observe if they yield similar conclusions.

2.6 Aim and objectives

The aim is to investigate if *Lycium barbarum* (eight samples) and *L. chinense* (four samples) have significantly different chemical or chromatographic profiles.

The objectives of the study are to:

1. Employ a systematic method for the selection of analytes for the quantitative analysis of *Lycium*.

2. Develop and validate an analytical method for the quantitation of the selected analytes in the dried raw herb sample.

3. Apply the analytical method to quantify the selected analytes in the *Lycium barbarum* and *chinense* samples sourced from the Sydney marketplace.

4. Statistically analyse the chromatographic profiles of *Lycium barbarum* and *chinense* obtained by LC-PDA, -MS/MS and GC-MS to evaluate their similarities and differences.

5. Use a chemical antioxidant assay to assess the anti-oxidant activity and to observe if it correlates with the herbal quality determined by chemical analysis.

Chapter 3

Analysis of Qi Ju Di Huang Wan

This section describes the methodology, equipment and reagents used in the experimental analysis of Qi Ju Di Huang Wan.

3.1 Equipment and reagents

3.1.1.UPLC-MS instrumentation

A Waters ACQUITY UPLC system (Waters Corporation, Milford, USA) coupled to a Waters Xevo TQ MS triple quadrupole mass spectrometer fitted with a Z-Spray™ source and electrospray ionisation (ESI) probe. Argon was the collision gas. Chromatographic separation was achieved on an Acquity BEH C18 (1.7 μm, 2.1mm X 50mm) column. The instrument was controlled using the Waters MassLynx™ version 4.1 program. The injection volume was set at 3 μL and column heater at 28 ^{o}C.

3.1.2 Miscellaneous equipment

An Adam AFA-210LC analytical balance (Adam Equipment Co., Australia) and a Sartorius SE-2 micro analytical balance (Sartorius Australia, Australia) were used to weigh the samples and standards. A Powersonic 420 ultrasonic bath (Thermoline Scientific, Australia) was used to help dissolve the sample. A Beckmann GP centrifuge from Beckmann Coulter (NSW, Australia) was also used. Solutions were passed through a Millipore 0.22 μm centrifuge filter with microporous membrane purchased from EMD Millipore (Millipore, Billerica, MA, USA).

3.2 Reagents, chemicals and samples

LC grade acetonitrile (Mallinckrodt Chemicals Ltd., UK), LR grade ethanol (95%), methanol and formic acid (90%) (Bio lab, Australia) were used. The gases used were ultra-high purity grade air, argon, helium, hydrogen and nitrogen (Coregas, Australia). Purified water (> 18 MΩ cm) was obtained from an Elga Purelab Prima and Purelab Ultra high purity water

system (Biolab, Australia). Alisol C (98.6%), alisol B (96%) catalpol, (98%),rutin (98%), luteolin (98%), diosgenin (97%) were of primary grade (Sigma-Aldrich, Australia). Paeoniflorin (98.7%), pachymic acid (97.9%), and cornuside (98%) were of secondary grade (Phytomarker Ltd., Tianjin, China). The primary grade standards have purity and spectroscopic characterisation while the secondary grade standards have purity by LC-PDA only. The calibration curves were prepared with standard purity adjustment.

Eleven samples of the 'Qi Ju Di Huang Wan' herbal formula were obtained from suppliers in the Australian marketplace. There were six suppliers who provided the sample and four of whom provided multiple batches. Most of these samples were donated and the donors requested to remain anonymous. Sample A-III was used for the method validation.

3.3 Preparation of LC mobile phase

Mobile phase A (0.1% formic acid in water) was prepared by adding 900 mL water to a 1000 mL volumetric flask followed by 1.1 mL formic acid before making up to volume with water. Mobile phase B was acetonitrile. Mobile phases were degassed by sonication for 5 min and filtered through a 0.45 µm polyvinylidene difluoride (PVDF) membrane filter before use.

3.4 Sample preparation and extraction

Each dried aqueous extract of the herbal formulation was ground to ≤ 200 µm. Approximately 0.5 g of each sample was weighed into a 10 mL conical flask and 10 mL 70% aqueous methanol was added and the mixture sonicated for 1 h with occasional stirring and then centrifuged at 4000 *rpm* for 10 min to pellet out the insoluble excipient. The supernatant was passed through a 0.2 µm PVDF membrane filter into 2 mL auto-sampler vials with glass insert for LC/MS analysis. The resultant liquid was stored at 4°C if not used on the same day.

3.5 Preparation of individual standards and mixed standard stock solution

This section describes the preparation of the individual standard solutions, mixed standard stock solution and working calibration solutions. Two mixed stock standard solutions were prepared. The first mixed standard solution contained alisol B, pachymic acid, alisol C, rutin, catalpol, luteolin, diosgenin and the second contained cornuside and paeoniflorin. This was done because the concentrations of cornuside and paeoniflorin are about higher than the other analytes and they show better solubility in ethanol than in methanol.

3.5.1 Preparation of individual standards of alisol B, pachymic acid, alisol C, rutin, catalpol, luteolin, diosgenin, and the mixed standard stock and working solutions

Individual solutions of 1000 µg mL^{-1} alisol B, pachymic acid, alisol C, rutin, catalpol, luteolin and diosgenin were prepared by weighing 5.0 mg of the standard into a 5 mL volumetric flask and adding approximately 3 mL of methanol before sonicating for 5 min or until the solid has dissolved. The solution was allowed to cool before making up to volume with methanol.

A mixed standard stock solution containing 40 µg mL^{-1} alisol B, 40 µg mL^{-1} pachymic acid, 25 µg mL^{-1} alisol C, 150 µg mL^{-1} rutin, 20 µg mL^{-1} catalpol, 70 µg mL^{-1} luteolin and 20 µg mL^{-1} diosgenin was prepared by adding 1.50, 0.70, 0.40, 0.40, 0.25, 0.20 and 0.20, mL of the respective individual standards into a 5 mL volumetric flask and making up to volume with methanol. This mixed stock solution was used as the fortification solution for the analyte recovery studies and for preparing the working calibration solutions.

The mixed standard stock solution was diluted 20-fold to give an intermediate mixed standard solution. This was prepared by diluting 50 µL of the mixed stock solution to 1000 µL with methanol. This intermediate mixed standard was diluted 1, 1/5, 1/10, 1/50 and

1/100 to give the mixed standard working solutions. The volume of mixed standard working solution used was 1.00 mL.

3.5.2 Preparation of cornuside and paeoniflorin mixed standard stock and working standard solutions

A mixed standard stock solution containing 25000 µg mL^{-1} cornuside and 25000 µg mL^{-1} paeoniflorin was prepared by weighing 25 mg of the respective standards into a 5 mL volumetric flask and adding approximately 3 mL ethanol before sonicating for 5 min. The solution was allowed to cool before making up to volume with ethanol. This mixed stock solution was used as the fortification solution for the analyte recovery studies and for preparing the working calibration solutions.

The cornuside and paeoniflorin mixed standard stock solution was diluted 40-fold to give an intermediate mixed standard calibration solution. This was prepared by diluting 25 µL of the mixed standard stock solution to 1000 µL with 95 % methanol. This intermediate mixed standard was diluted as shown in Table 3.1 to give the mixed working standard solutions.

Table 3.1 Dilution volumes for working standard solutions

Fold dilution	Volume of standard (µL)	Volume of methanol (µL)
1/100	100	900
1/5	200	800
1/2.5	400	600
1/1.7	600	400
1/1.25	800	200
1	1000	0

3.6 LC-MS conditions

All analytes were detected in a single analytical run which took 5 min using the LC and MS conditions described.

3.6.1 Optimisation of MS/MS conditions for the detection of each analyte

The MS/MS parameters for each analyte was developed automatically with the Waters MassLynx v 4.1 software and IntelliStart program. This was achieved by direct infusion of the individual standard solutions at a concentration of 100 μg mL^{-1} in methanol and a flow rate of 0.1 mL min^{-1}. The molecular ion mass was entered into IntelliStart and the software automatically determined the optimum cone voltage for the selected precursor ion to produce at least two product ions and the corresponding collision energies required to achieve this. A report was automatically generated for each compound, specifying the optimized settings for the multiple reaction monitoring (MRM) method. The report also plots the daughter ion spectrum along with cone voltage and collision energies used. The MS conditions were selected so as to give at least two product ions to achieve analyte identity confirmation and quantification which meets the standards set by the European Commission Directorate for Agriculture guidelines. The most prominent product ions were used for quantification.

3.6.2 LC conditions

The column was set at 28 °C and mobile phase flow rate at 0.3 mL min^{-1}. Mobile phase A consisted of 0. 1% formic acid in water and mobile phase B consisted of acetonitrile. The gradient elution program is summarised in Table 3.2.

Table 3.2 Mobile phase gradient program for the LC-MS/MS method

Time (min)	% Water (with 0.1% *v/v* formic acid)	% Acetonitrile
Initial	90	10
1	75	25
1.5	55	45
2.5	35	65
3	25	75
3.5	20	80
4	0	100
4.5	90	10
5	90	10

*Flow rate = 0.3 mL min^{-1}

The injection mode was set at partial loop; with the full loop size being 10 μL. The injection volume was 5 μL. The MS ESI source was operated in both (-) and (+) modes.

3.6.3 MS conditions

Source conditions were set as follows: Nitrogen was the desolvation gas (800 L/h heated to 350 °C) and argon as the collision induced dissociation gas (0.15 mL min^{-1}) and this gives rise to a collision cell pressure of 4.3×10^{-6} Bar. The scan time was 0.005 s. The extractor cone was 2 V and cone gas flow 20 L/h. The source temperature was 150 °C, the capillary voltage in the (+) ESI mode was –3.2 kV and 2.00 kV in the (-) ESI mode.

Two MRM transitions (or product *m/z*'s) were chosen for each target analyte, with the most abundant used as the quantifier and the other the qualifier.

3.6.4 MS conditions for analysis of the analytes

The ESI polarity, precursor and product ions monitored, and the argon collision voltages required to achieve those transitions and the dwell times used are summarised in Table 3.3. Q1 and Q3 are both operated with a peak width of 3 AMU and a scan time of 2 s.

Table 3.3 UPLC-MS-MS monitoring of analytes in the herbal formula

Analyte	ESI polarity	Precursor m/z	Product m/z	Respective voltages (V)	Dwell time (s)
alisol B	+	$[M+H]^+ = 447$	89, 121	26, 18	0.039
pachymic acid	+	$[M+H]^+ = 529$	295, 452	24, 18	0.039
alisol C	+	$[M+H]^+ = 530$	451, 469	20, 16	0.062
rutin	-	$[M-H]^- = 609$	255, 271, 300	40, 62, 50	0.028
catalpol	+	$[M+H]^+ = 380$	165, 183	12, 14	0.028
luteolin	-	$[M-H]^- = 285$	133, 151	34, 26	0.028
diosgenin	+	$[M+H]^+ = 415$	253, 271	26, 18	0.039
cornuside	-	$[M-H]^- = 479$	125, 169	20, 8	0.028
paeoniflorin	-	$[M-H]^- = 541$	121, 449	54, 32	0.028

3.7 Chemometric analysis

The study of the variation of targeted analytes in the herbal formulation obtained from various suppliers was achieved by chemometric analysis. The software used was the '**R** Project for Statistical Computing' (v.2.14.2) for data processing and statistical analysis. Specific packages used with **R** are detailed as follows. The package 'msProcess' was used to modify the chromatograms by removing instrumental noise, baseline drift, identifying peaks, removing peak retention time variations between samples and to quantify peak height. This minimises sample variation due to instrumental factors. The representative chromatograms for all the samples needed to be prepared before PCA could be performed on a data set. The raw data were converted into a comma-separated value file (CSV).

Chapter 4

Experimental procedure for *Lycium barbarum and Lycium chinense*

This section describes the metholodgy, equipment and reagents used in the experimental analysis of *Lycium babarum* and *Lycium chinense*.

4.1 Equipment and reagents

4.1.1 GC-MS instrumentation

An Agilent 7890A GC with a 5975C inert XL EI/CI mass selective detector (MS) and CombiPal autosampler (Agilent, Australia) was used. The column was a HP-5MS (30 m × 0.25 mm ID, 0.25 µm film thickness; J&W Scientific, USA).

4.1.2 HPLC-PDA instrumentation

Analysis was carried out on a Varian (California, USA) Prostar solvent delivery system comprising of 2 × 210 single pumps equipped with a 335 photodiode array detector (PDA) , a '9×0 mm' analytic flow cell (Varian Inc., Australia), column valve module 500 and 430 auto sampler injector. Solvents were degassed using a model AF DG2 in-line degasser (Waters, USA). The system was controlled using Varian Star Workstation version 6.20. A Phenomenex Luna C18 column (150 x 4.6 mm, 5 µm) (California, USA), and a security C18 guard column (3 × 4.6 mm, 5 µm) was the analytical column.

4.1.3 UPLC-MS/MS instrumentation

A Waters ACQUITY UPLC system (Milford, MA, USA) consisting of a binary sample manager, sample manager including the column heater, detector and sample organiser coupled to a Xevo TQ tandem-quadrupole mass spectrometer equipped with a Z-spray electrospray

interface was used. The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient mixed under high pressure. There is a built-in solvent degassing as well as solvent select valves to choose from up to four solvents. The ACQUITY system is capable of pumping mobile phase at pressures up to 15,000 psi. Negative electrospray ionization (ESI) was performed in the multiple reaction monitoring (MRM) mode. The loop size 10 µL. Separation was carried out on an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 µm).

4.1.4 Miscellaneous equipment

The herb grinder was a M 20 Universal mill IKA® (Werke Staufen, Germany). The ultrasonic bath was a Branson 1510 from Branson Ultrasonics (NSW, Australia), and the centrifuge as a Beckmann GP from Beckmann Coulter (NSW, Australia). An Adam AFA-210LC analytical balance (Adam Equipment Co., Australia) and a Sartorius SE-2 micro analytical balance (Sartorius Australia, Australia) were used to weigh the samples and standards.

4.1.5 Chemicals and reagents

The analytical reference standards rutin (94%), caffeic acid (99%) *p*-coumaric acid (98%), chlorogenic acid (95%), and scopoletin (99%) were purchased from Sigma-Aldrich (St Louis, MO); kaempferol-3-*o*-rutinoside, isorhamnetin-3-*o*-rutinoside were of second grade from Phytomarker Ltd. (Tianjin, China).

LC grade methanol and AR grade ethyl acetate were obtained from Biolab (VIC, Australia). AR grade formic acid was obtained from Univar (NSW, Australia). Phosphorus pentoxide (used as desiccant) was from Sigma-Aldrich (St Louis, MO).

DPPH (or 2,2-diphenyl-1-picrylhydrazyl) and gallic acid was from Sigma–Aldrich (NSW, Australia). Air, argon, helium, hydrogen and nitrogen were of ultra-high purity grade from Coregas (NSW, Australia). Purified water (>18 MΩ cm) was obtained from an Elga Purelab Prima and Purelab Ultra high purity water system (Biolab, NSW, Australia).

4.2 *Lycium* samples

4.2.1 Plant material

Twelve samples of the dried raw herb (8 labelled as the *barbarum* and 4 as the *chinense* species) were obtained from markets (local supermarkets, herbal stores and herbal companies) in Sydney. The authenticity of the raw material was established by chromatographic profile comparison against a certified reference sample of *L. barbarum* (batch number AAT15209CRB) purchased from Alkemists Pharmaceuticals (CA, USA). A certified reference sample of *L. chinense* was not commercially available.

4.2.2 Sample preparation

The raw samples were dried over phosphorus pentoxide in a desiccator for one week, then ground to pass through a 250 μm sieve before storing in the desiccator under vacuum.

4.3 Preparation of individual standards and mixed standard stock solution

This section describes the preparation of the individual standard solutions, mixed standard stock solution and working calibration solutions.

4.3.1 Preparation of individual standards of rutin, isorhamnetin-3-o-rutinoside, kaempferol-3-o-rutinoside, coumaric acid, scopoletin, caffeic acid, chlorogenic acid; and the mixed standard stock and working mixed standard solutions

Individual solutions of 5000 µg mL^{-1} of rutin, chlorogenic acid, coumaric acid were prepared by weighing 50.0 mg of the standard (on a microbalance) into a 10 mL volumetric flask and adding approximately 7 mL of methanol before sonicating for 5 min or until the solid is dissolved. The solution was allowed to cool before making up to volume with methanol.

In a similar way, individual solutions containing 500 µg mL^{-1} isorhamnetin-3-*o*-rutinoside, kaempferol-3-*o*-rutinoside, scopoletin, caffeic acid were prepared from 5.0 mg of the standard. The solutions were stored at -4 °C and discarded if it was not used within 2 weeks.

A mixed standard stock solution containing 1140 µg mL^{-1} rutin, 39.6 µg mL^{-1} chlorogenic acid, 89.4 µg mL^{-1} coumaric acid, 60 µg mL^{-1} isorhamnetin-3-*o*-rutinoside, 11.6 µg mL^{-1} kaempferol-3-*o*-rutinoside, 17.4 µg mL^{-1} scopoletin and 1.64 µg mL^{-1} caffeic acid was prepared by adding 5.7, 1.7, 3.7, 2.5, 0.50, 0.80 and 0.10, mL of the respective individual standards into a 25 mL volumetric flask and making up to volume with methanol. This mixed standard stock solution was used as the fortification solution for the analyte recovery studies and for preparing the working calibration solutions.

The working calibration solution was prepared by diluting 50, 150, 250, 500, 1000, and 1200 µL of the mixed standard stock solution to 1000 µL with methanol.

4.4 Extraction of the dried raw herb

Various aqueous solutions were investigated; the solvent that resulted in the largest overall peak area was subsequently selected for further analysis. The concentration of the analyte in the unfortified samples was determined by weighing a known amount of the ground raw

herb approx. (1.0 g) into a 10 mL volumetric flask and extracted by sonication in 7 mL 50% aqueous methanol for 2 x 30 min, (with a 15 min cooling interval between sonications). After cooling the flask was made up to volume with the extraction solvent and mixed by vortexing. The supernatant was transferred to a centrifuge tube and centrifuged at 4000 rpm for 10 min. An aliquot of the supernatant was filtered through a 0.22 μm PVDF membrane filter into a 2 mL auto sampler vial for analysis. The filtrate was stored at 4 °C if not analysed on the same day. Each sample was analysed in seven replicates (extraction and analysis).

4.4.1 Recovery studies for the raw herb

To determine the analyte extraction efficiency of the method, an accurately weighed amount of ~1.0 g of the ground herbal sample was transferred into 10 mL volumetric flasks, and 0.5 mL of the fortified stock solution was added to the 100 % fortification level. For the 50 and 200% fortification levels, the amounts were adjusted proportionately. The concentration of the mixed fortified solution was arranged such that for the 100% fortification level the resultant peak area should be double that of the unfortified sample. Seven replicates were carried out for each fortification level to give a total of twenty one samples for the three fortification levels. The solvent was allowed to evaporate overnight in a fume hood.

4.5 Methods

4.5.1. Chromatographic conditions

4.5.1.1 GC-MS

The injection volume programmed into the autosampler was 1 µL, pre-cleaning the syringe with ethanol once, then the sample five times before each injection. The syringe was rinsed with ethanol five times after each injection. The injector was set at 200 °C using a split ratio of 10: 1. The column pressure was programmed to maintain a constant flow of 1.5 mL min^{-1}. The initial oven temperature is held at 50 °C for 1 min, then increased at a rate of 10°C/ min to 300°C and held at 300°C for 3 min. The oven temperature was then increased to 350 °C for 2 min after the run to clean the column. The MS transfer line was maintained at 250 °C, the EI source at 230 °C and the quadrupole at 150 °C; the helium carrier gas was set to flow at 1 mL min^{-1}; The MS scan range was 40-500 *m/z*.

4.5.1.2 HPLC-PDA

The injection volume was set at 10 µL and column temperature at 30°C. The PDA was set to acquire data from 200 - 400 nm. The mobile phase was acetonitrile (A) and water containing 0.1% formic acid (B) and a flow rate of 1 mL min^{-1}. The elution program is summarised in Table 4.1.

Table 4.1 Mobile phase gradient program for the HPLC-PDA analysis

Time (min)	% Water (with 0.1% *v/v* formic acid)	% Acetonitrile
Initial	95	5
5.0	95	5
55.0	90	10
80.0	80	20
81.0	5	95
90.0	5	95
91.0	95	5
100.0	95	5

4.5.1.3 UPLC

The column temperature was 28°C and sample manager 5 °C. The sample loop size was 10

µL and the injection volume 3 µL. A gradient elution program consisting of 0.1% aqueous

formic acid (A) and methanol (B), as summarised in Table 4.2 was used. The flow rate was

0.20 mL min^{-1}.

Table 4.2 Mobile phase gradient program for the UPLC-MS/MS analysis

Time (min)	% Water (with 0.1% *v/v* formic acid)	% Methanol
Initial	80	20
1.0	75	20
6.0	55	40
11.0	35	70
13.0	25	100
15.0	80	20
15.5	80	20

4.5.1.4 Mass spectrometer

The ESI source conditions were set as follows; nitrogen desolvation gas at 800 L/h heated to

350 °C, and argon as the collision induced dissociation gas at 0.15 mL min^{-1}. This resulted in

a collision gas pressure maintained at 4.3×10^{-6} bar in the collision cell.

The scan time was 0.005 s. The extractor cone was 3 V and cone gas flow 20 L/h. The source

block temperature was 150 °C, the capillary voltage in the (-) ESI mode was 2.40 kV. Manual

tuning for optimal cone voltage and collision energy was performed using the instrument's

built-in fluidics system. The dwells times for the MRM channels were automatically

calculated by the software, and a desired number of 20 data points was selected for peak

determination. The inter-channel delay time was set to 3 ms and the inter-scan delay time

was 3 ms at all times. System operation and data acquisition were controlled using the

Waters Mass Lynx 4.1 software. Two MRM product (or transition *m/z*'s) were chosen for each target analyte, with the most abundant used as the quantifier and the other the qualifier. Two product ions with matching intensities between the standard and sample peaks meet the analyte identity confirmation standard set by the European Commission Directorate for Agriculture guidelines (9).

4.5.1.5 MS conditions for analysis of the analytes

The ESI polarity, precursor and product ions monitored, and the argon collision voltages required to achieve those transitions and the dwell times used are summarised in Table 4.3.

Table 4.3 UPLC-MS/MS monitoring parameters for analytes in the raw herb

Analyte	ESI polarity	Precursor *m/z* [M-H]⁻	Product *m/z*	Respective collision voltages (V)	Dwell time (s)
rutin	-	609	255, 271, 300	50,62,40	0.016
isorhamnetin-3-o-rutinoside	-	623	271, 299, 315	66, 48, 30	0.017
kaempferol-3-o-rutinoside	-	593	227, 255, 284	62, 54, 34	0.017
coumaric acid	-	164	93, 120	12, 26	0.016
scopoletin	-	191	103, 147, 176	24, 20, 16	0.016
caffeic acid[1]	-	179	135	15	0.195
chlorogenic acid	-	353	93, 85	50, 50	0.095

[1] Only one product ion was observed for caffeic acid.

4.6 Chemometric analysis

The chromatographic profiles of *Lycium* were subject to the chemometric techniques of principal component analysis (PCA) and hierarchical cluster analysis (HCA) which can help identify components that explain most of the variances in a system.

Data processing and statistical analysis was performed using the "R" statistical computing package (98) and the add-on package "msProcess" (99). This enables chromatogram modification by removal of instrumental noise, peak retention time variation between samples, baseline drift, and identification of peaks thereby minimising sample variation due to instrumental factors. Extract chromatograms were pre-processed according to the stated method. Peaks were identified in each chromatogram and then aligned to remove small variations in peak retention times between chromatograms. Whenever a peak was detected in any chromatogram the amplitude at that particular retention time was measured across all chromatograms in order to build a matrix of peak amplitudes.

4.7 DPPH anti-oxidant assay

4.7.1 The anti-oxidant assay carried out was the DPPH (or 2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay.

The method adapted from Blois et al (1958) and Molyneux et al (2004). The method estimates DPPH activity relative to a gallic acid standard (100) (101). All reagents were prepared in 80% aqueous methanol. The gallic acid standard curve was constructed by measuring the absorbance of a gallic acid stock (6 mM) diluted to give 0.3, 0.6, 1.5, and 3 mM working standards. The samples were prepared by dissolving 100 mg of the extract in

10 mL (final concentration: 0.1 g L^{-1}) solvent. Serial dilutions (1 in 10 and 1 in 100) of the stock sample solution were also prepared. The solvent, 80% aqueous methanol solution, was used as the reagent blank. 180 µL of the DPPH reagent (250 µM) was applied to each well of a 96 well plate. In triplicate, 20 µL of each working standard, sample or blank was added to the DPPH reagent to make a total volume of 200 µL in each well. To correct for sample blank absorbance (i.e. absorbance not due to the DPPH), sample blanks were made in triplicate by adding 180 µL of 80% aqueous methanol to the well and adding 20 µL of sample. The plate was shaken at 700 rpm for 30 min in the dark prior to measuring the absorbance at 515 nm (POLARstar OPTIMA; BMG). The sample antioxidant scavenging capacity is reported as the gallic acid equivalent.

Chapter 5

Results and Discussion of Analysis of

Qi Ju Di Huang Wan

5.1 Extract production

The herbal tablet obtained from various commercial sources was powdered and sieved to ensure uniformity in particle distribution and homogeneity for sampling. The powdered sample was extracted by sonication in 70% aqueous methanol to produce an extract for chromatographic analysis. This extraction procedure was tested first as it has been successfully used in our labs for similar work.

5.2 Analytical method development

Table 3.3, chapter 3 summarises the ESI polarities, collision voltages for MRM and the precursor ion and product ions for each analyte. Representative chromatograms for analysis of the selected analytes in the sample monitored in SRM mode are presented in Figure 5.1.

The LC-ESI-MS/MS analysis was divided into time segments such that the different optimum MS/MS experimental conditions could be applied for each of the chemical compounds. Therefore the chromatogram obtained could be regarded as a segmental SRM profile, with each segment selectively optimised for a particular representative chemical analyte in the herb extract.

The narrowing down of the quadrupole data acquisition window facilitated the convenient analysis of the analytes both in the herbal sample extract and in the mixed standard. Chromatograms obtained for the precursor and the product ions monitored in SRM mode for each analyte in the sample and the standard are shown in Figures 5.2 – 5.15. A summary of the results for the method validation parameters determined is presented in Table 5.1.
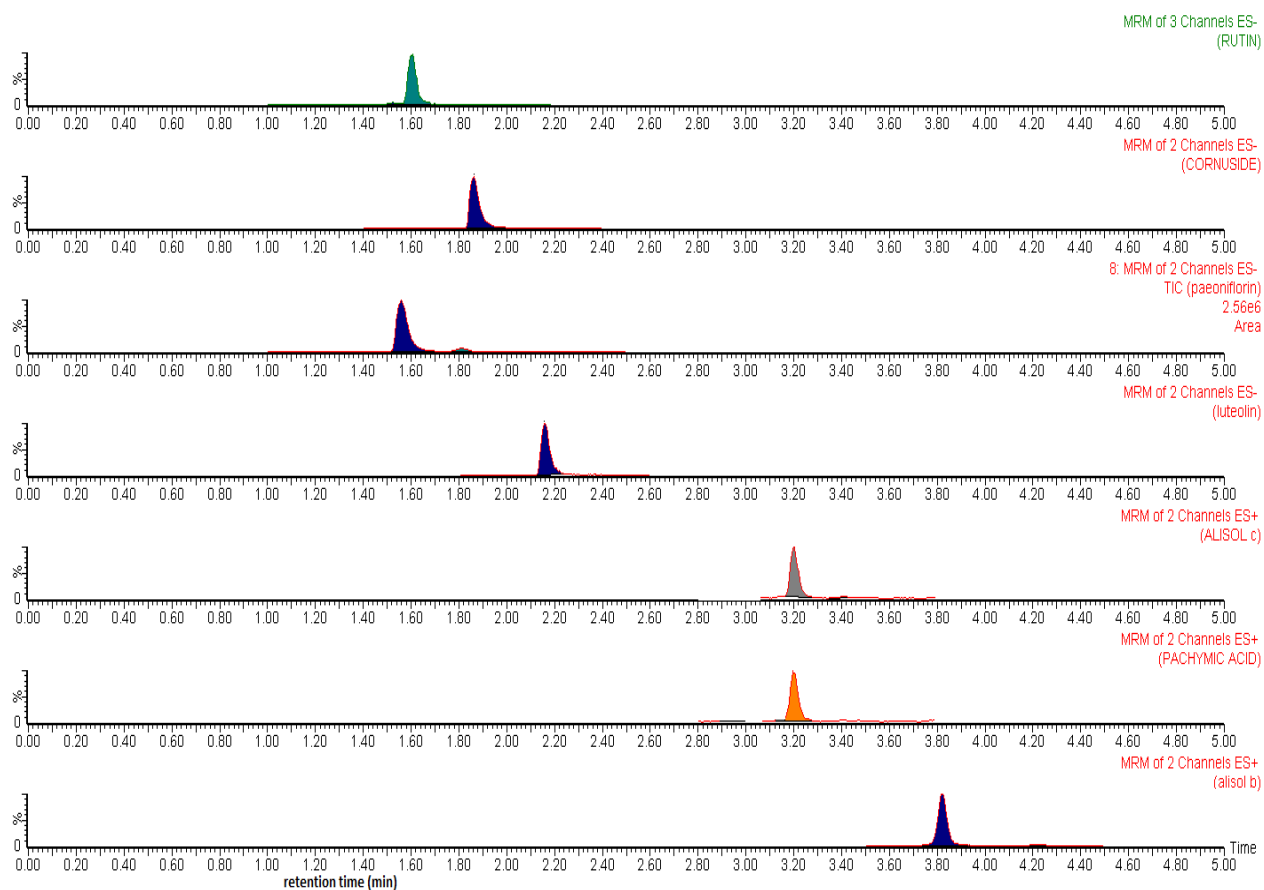
Figure 5.1   SRM of alisol B, pachymic acid, alisol C, rutin, luteolin, cornuside and paeoniflorin present in a representative sample of Qi Ju Di Huang Wan.

5.2.1 Analyte identity confirmation by comparison to reference standard
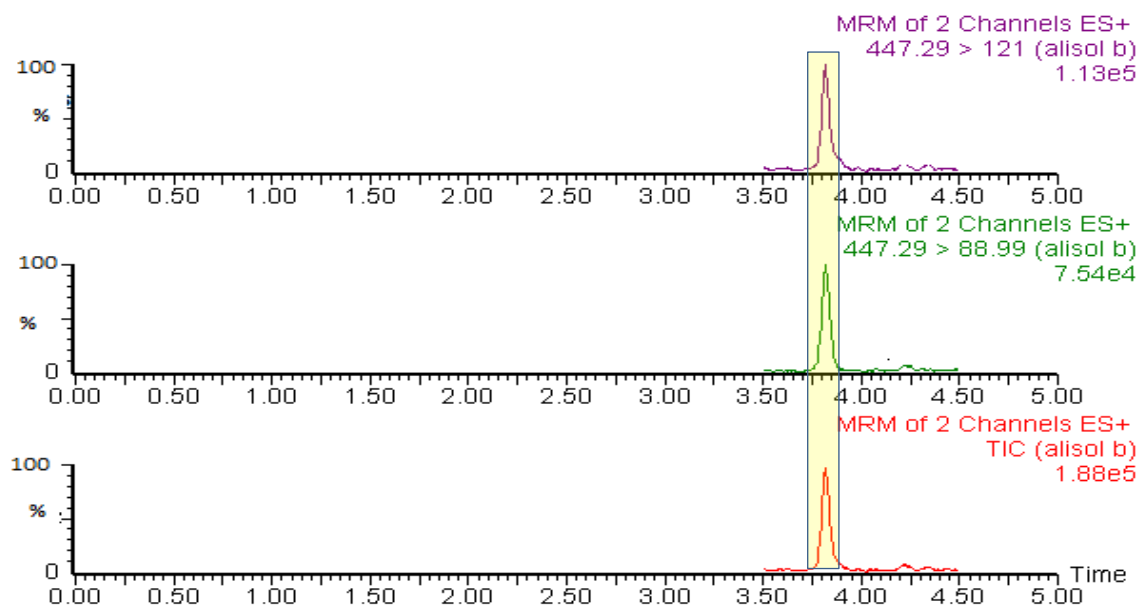
Figure 5.2 Representative LC-MS chromatogram of the precursor and product ions of alisol B in the sample monitored by SRM
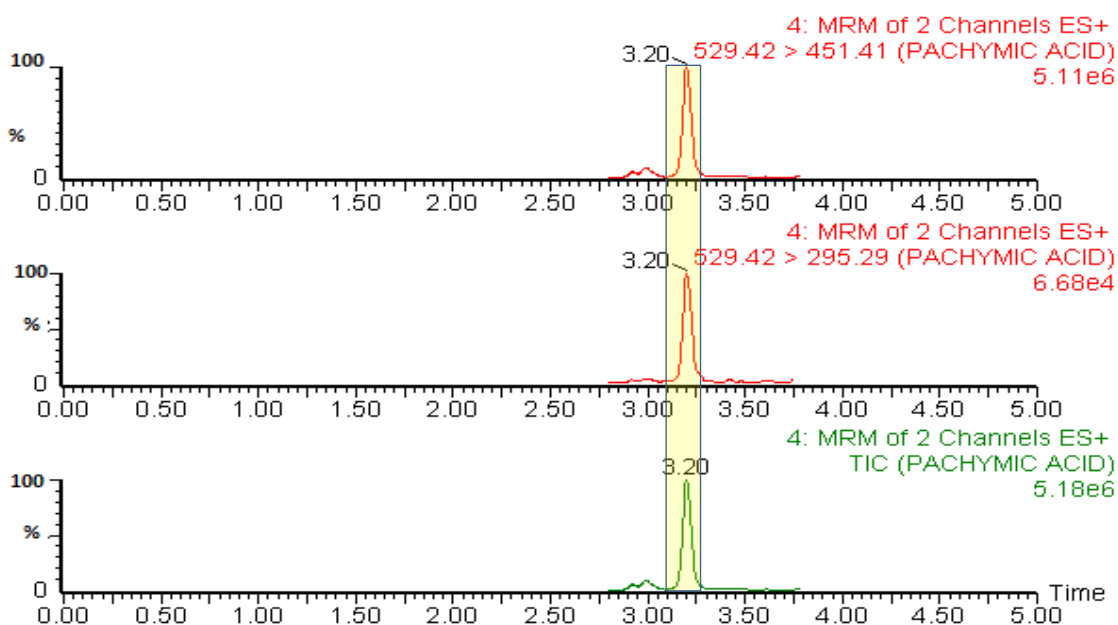
Figure 5.3 Representative LC-MS chromatogram of the precursor and product ions of pachymic acid in the sample monitored by SRM
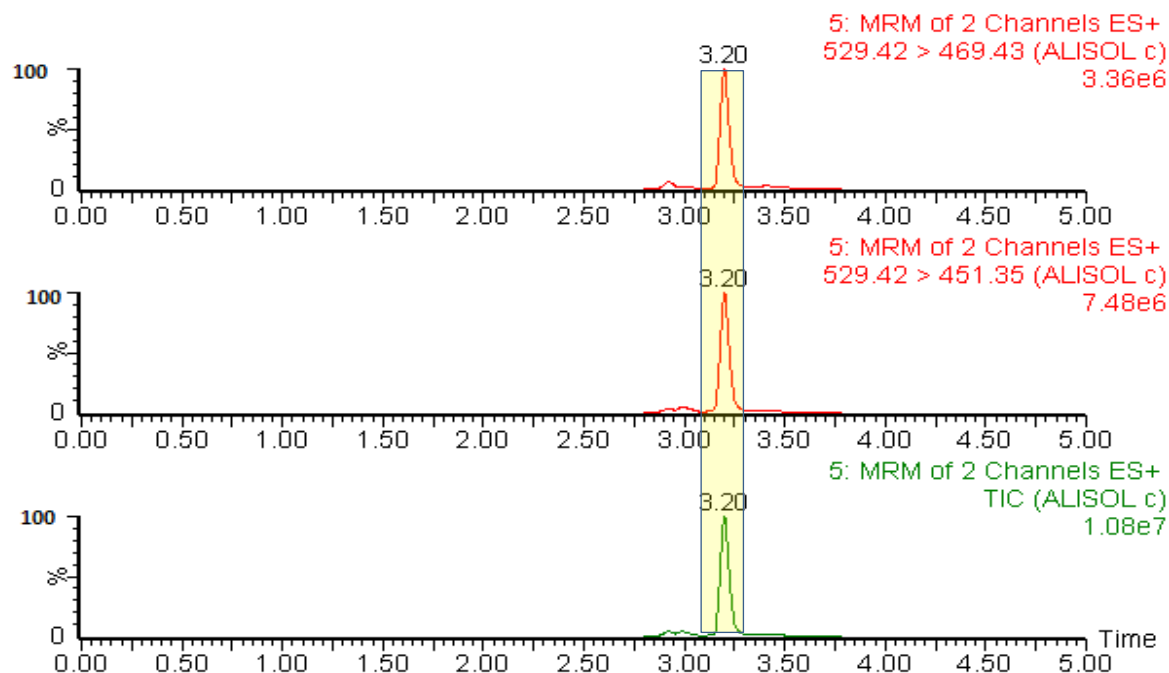
Figure 5.4 Representative LC-MS chromatogram of the precursor and product ions of alisol C in the sample monitored by SRM
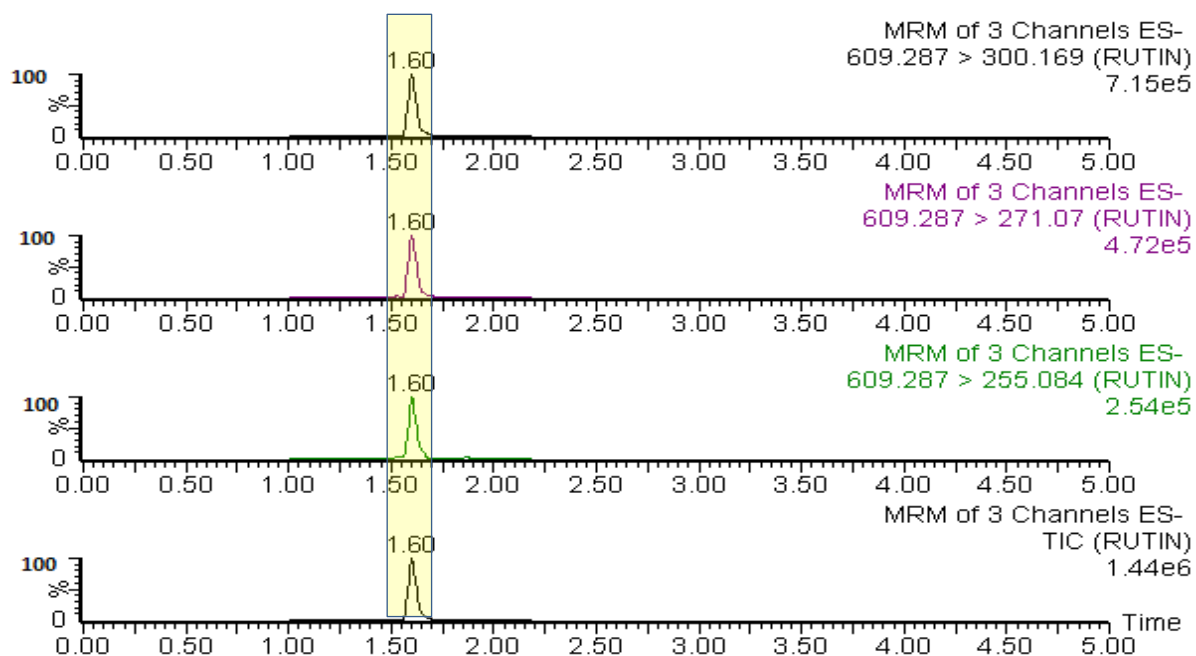
Figure 5.5 Representative LC-MS chromatogram of the precursor and product ions of rutin in the sample monitored by SRM
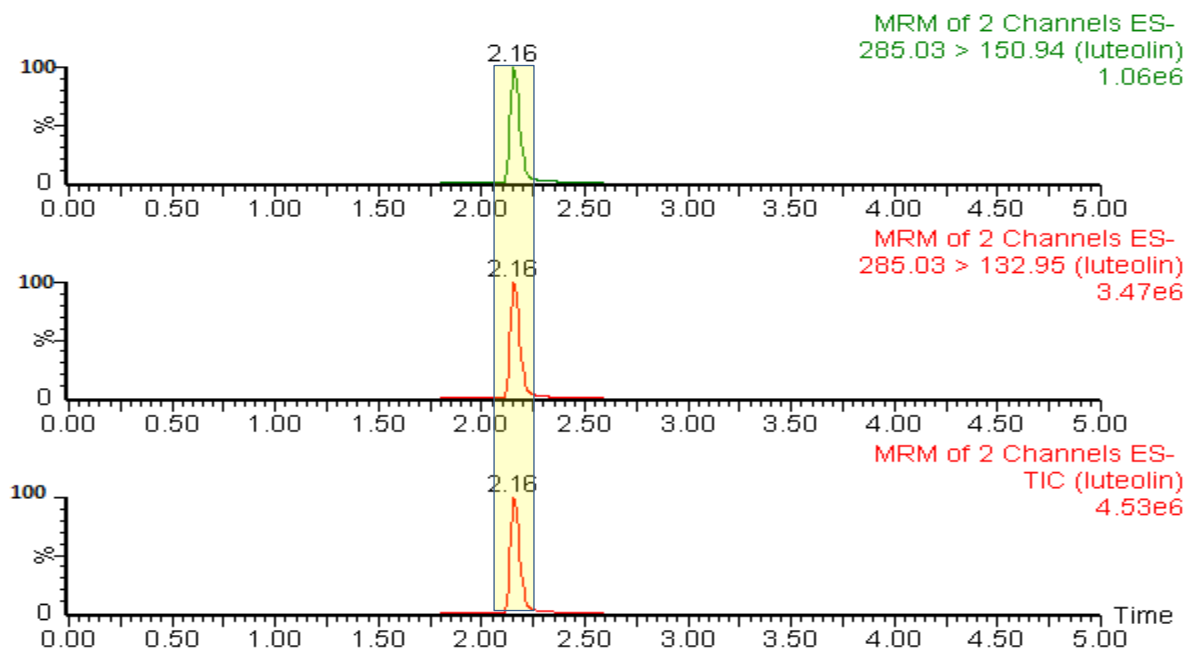
Figure 5.6 Representative LC-MS chromatogram of the precursor and product ions of luteolin in the sample monitored by SRM
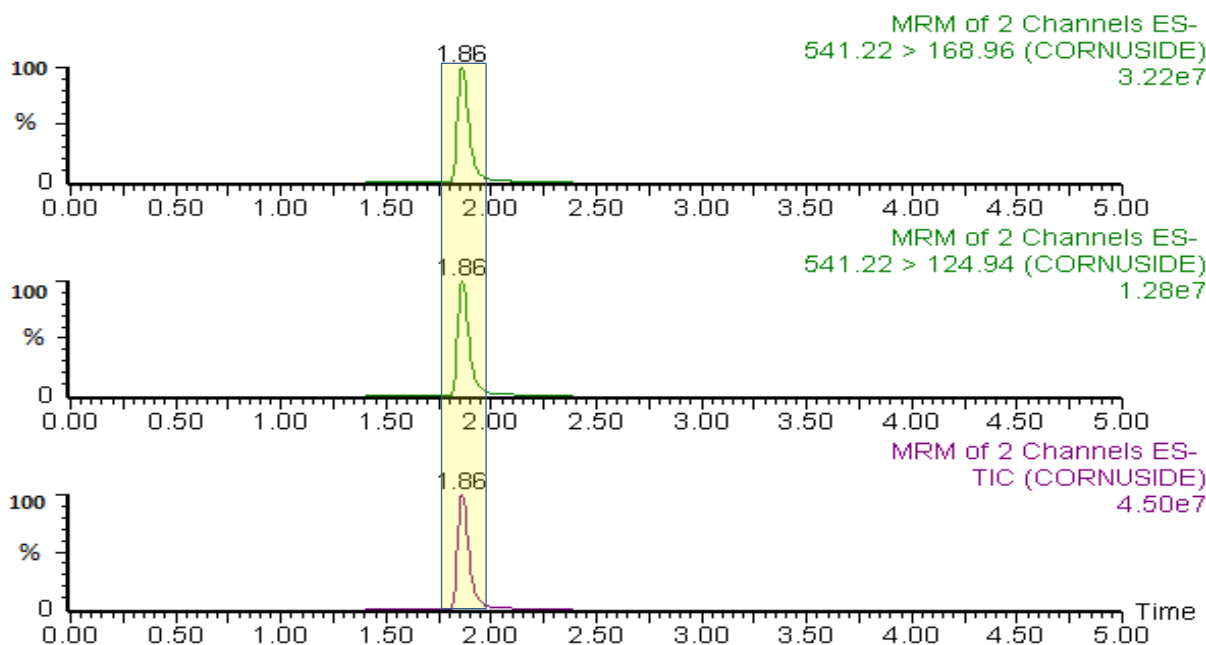
Figure 5.7 Representative LC-MS chromatogram of the precursor and product ions of cornuside in the sample monitored by SRM
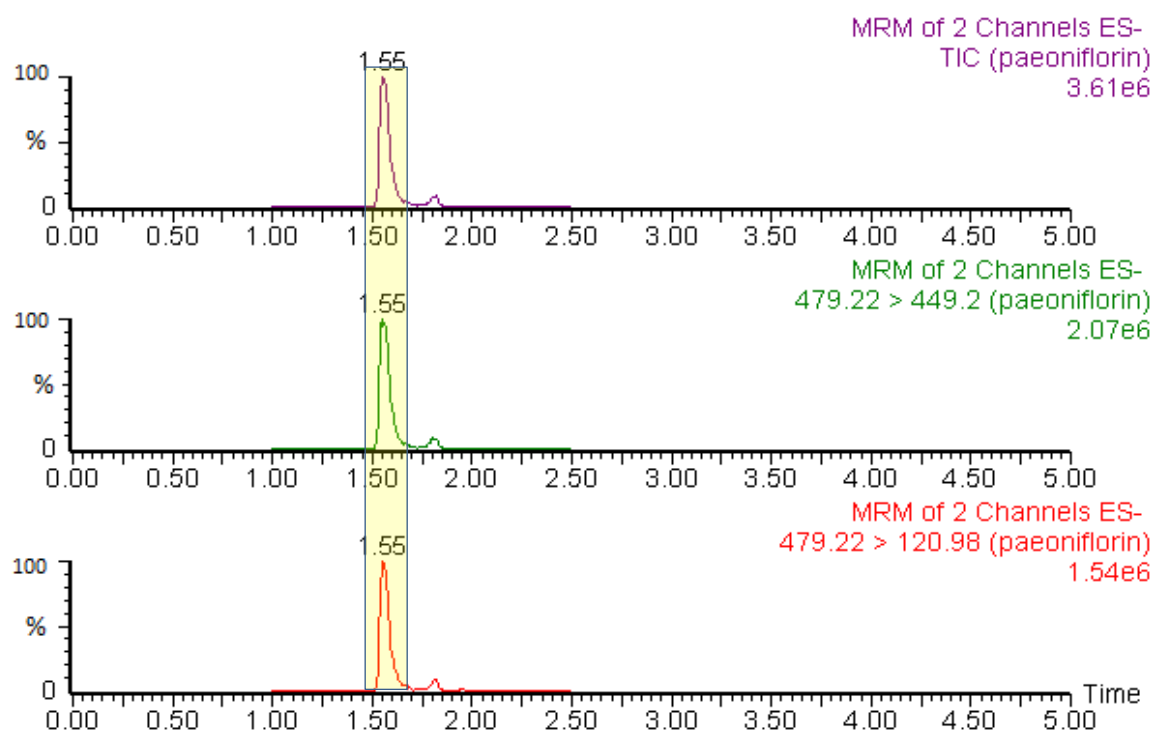
Figure 5.8 Representative LC-MS chromatogram of the precursor and product ions of paeoniflorin in the sample monitored by SRM
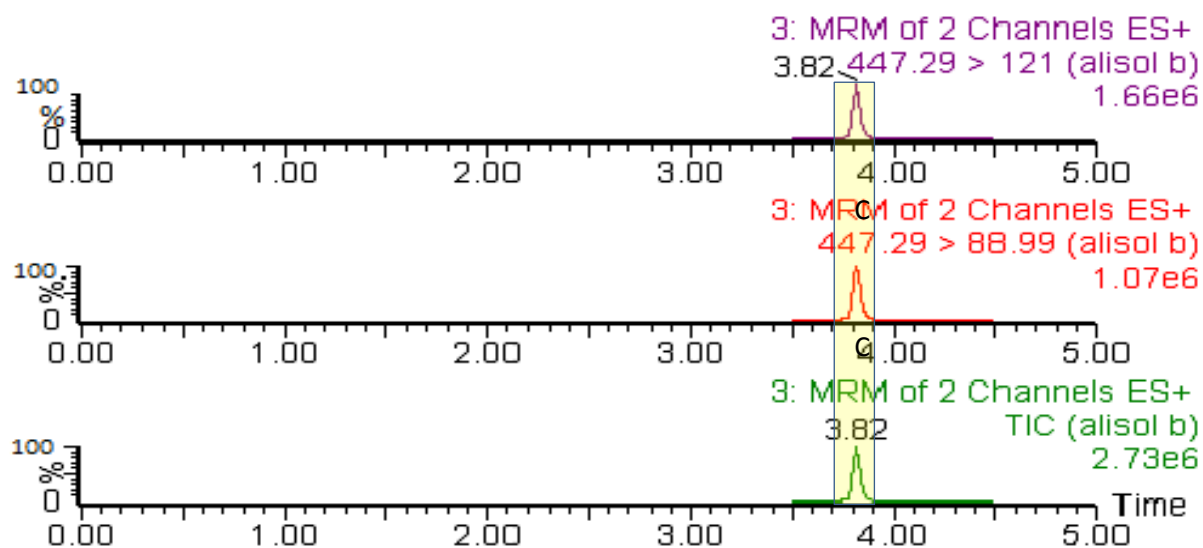
Figure 5.9 Representative LC-MS chromatogram of the precursor and product ions of alisol B reference standard monitored by SRM
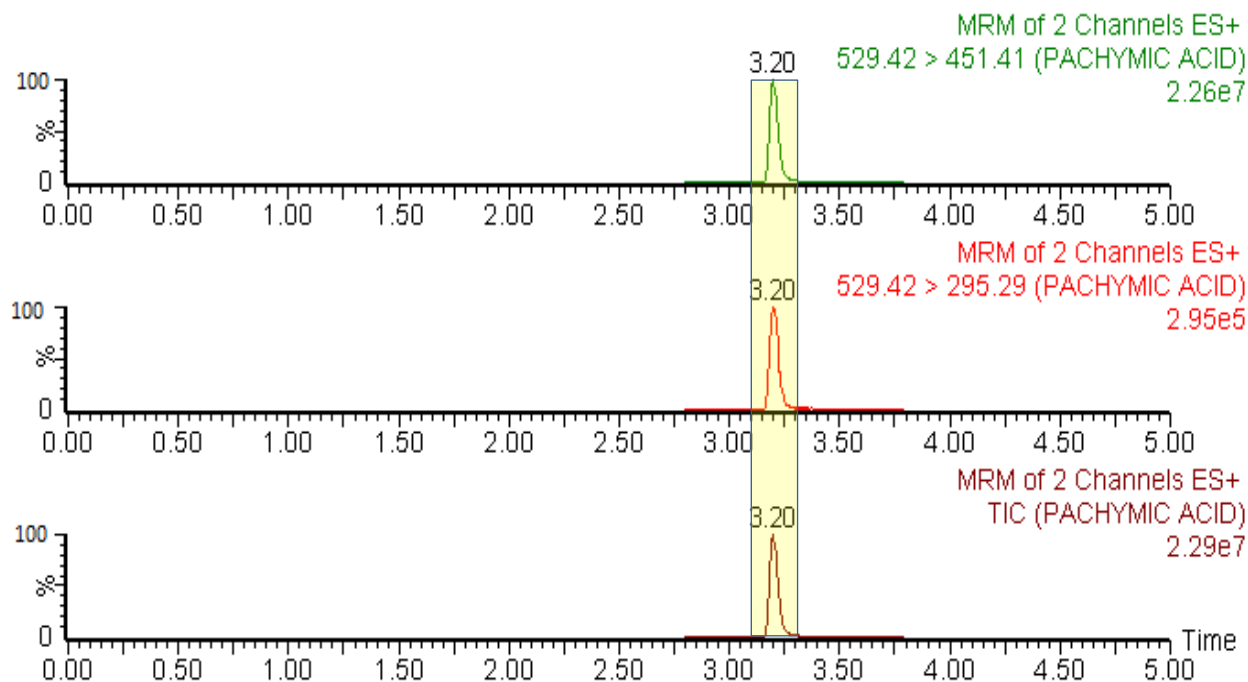
Figure 5.10 Representative LC-MS chromatogram of the precursor and product ions of pachymic acid reference standard monitored by SRM
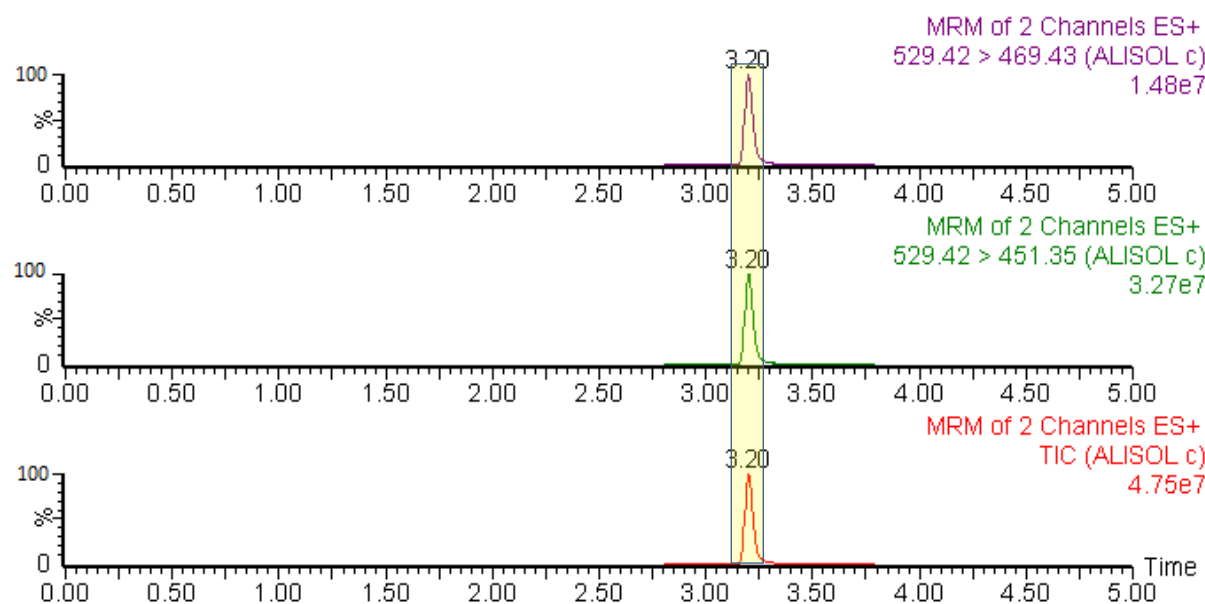
Figure 5.11 Representative LC-MS chromatogram of the precursor and product ions of alisol C reference standard monitored by SRM
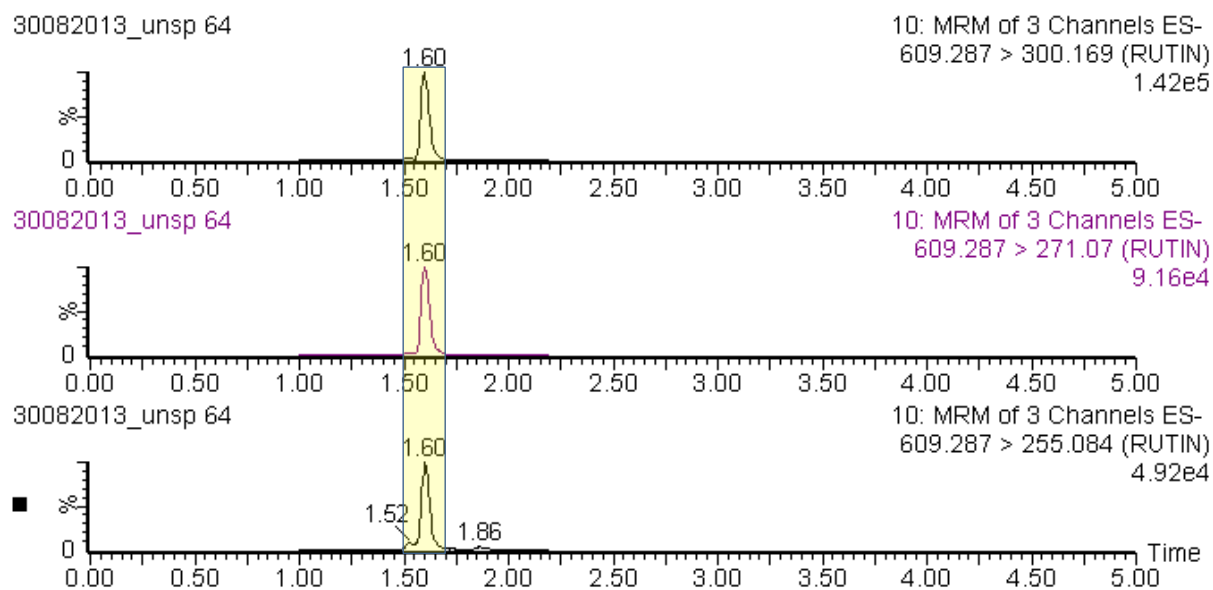
Figure 5.12 Representative LC-MS chromatogram of the precursor and product ions of rutin reference standard monitored by SRM
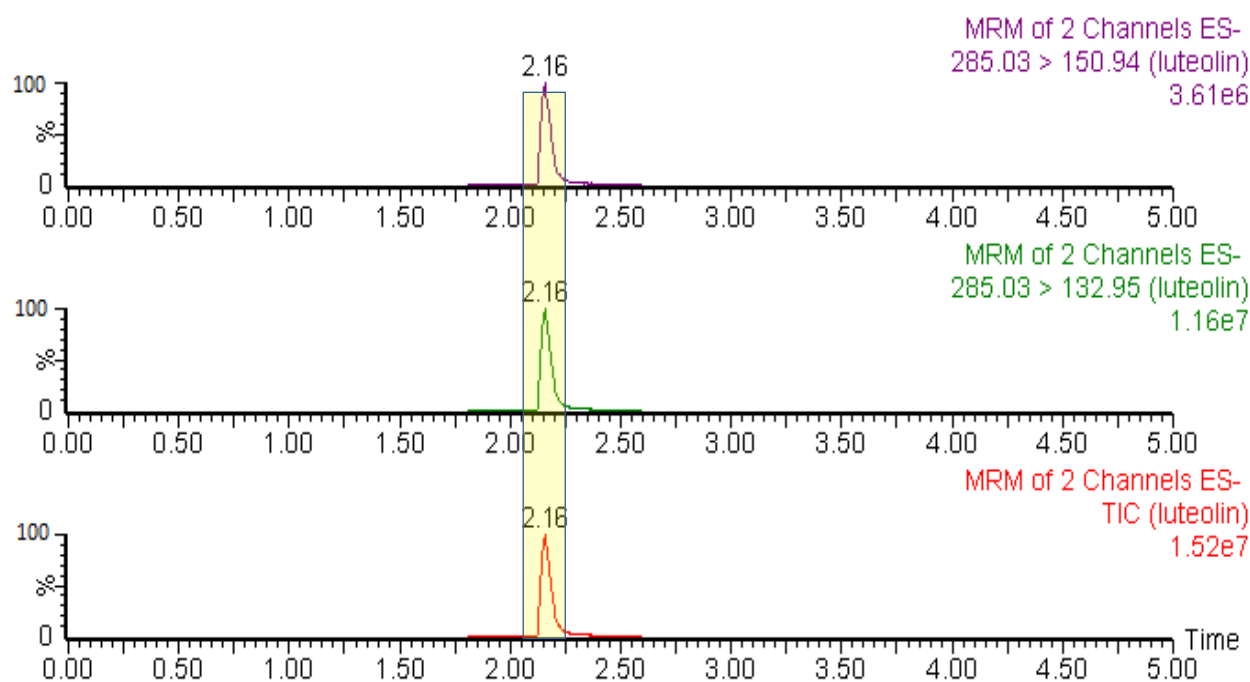
Figure 5.13 Representative LC-MS chromatogram of the precursor and product ions of luteolin reference standard monitored by SRM
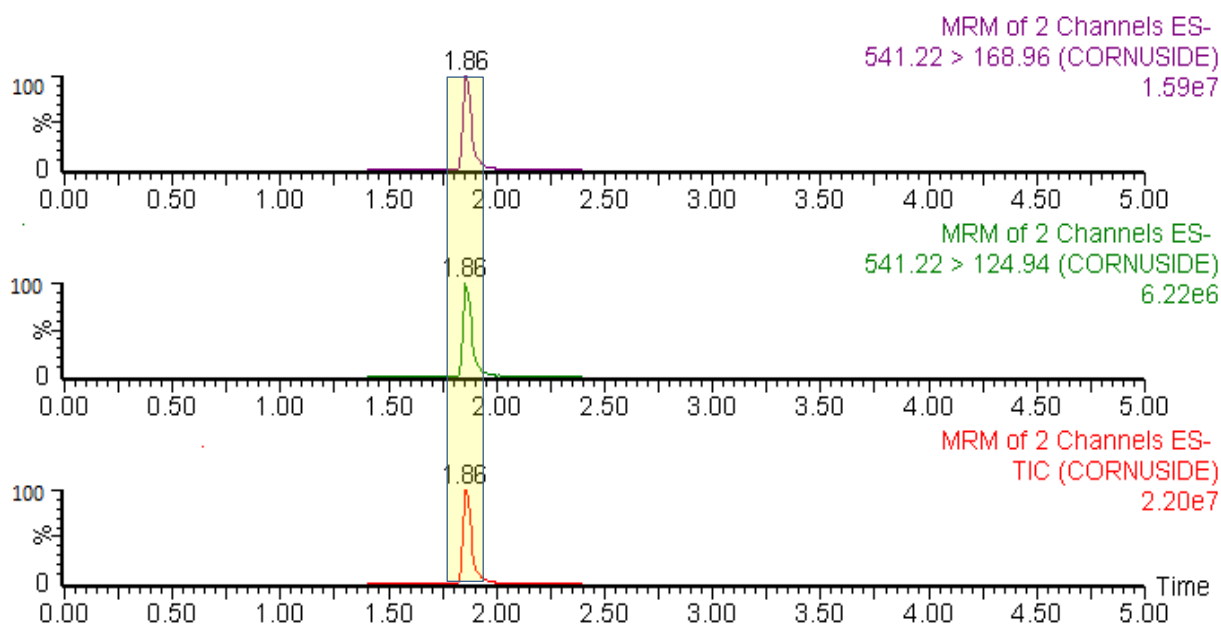
Figure 5.14 Representative LC-MS chromatogram of the precursor and product ions of cornuside reference standard monitored by SRM
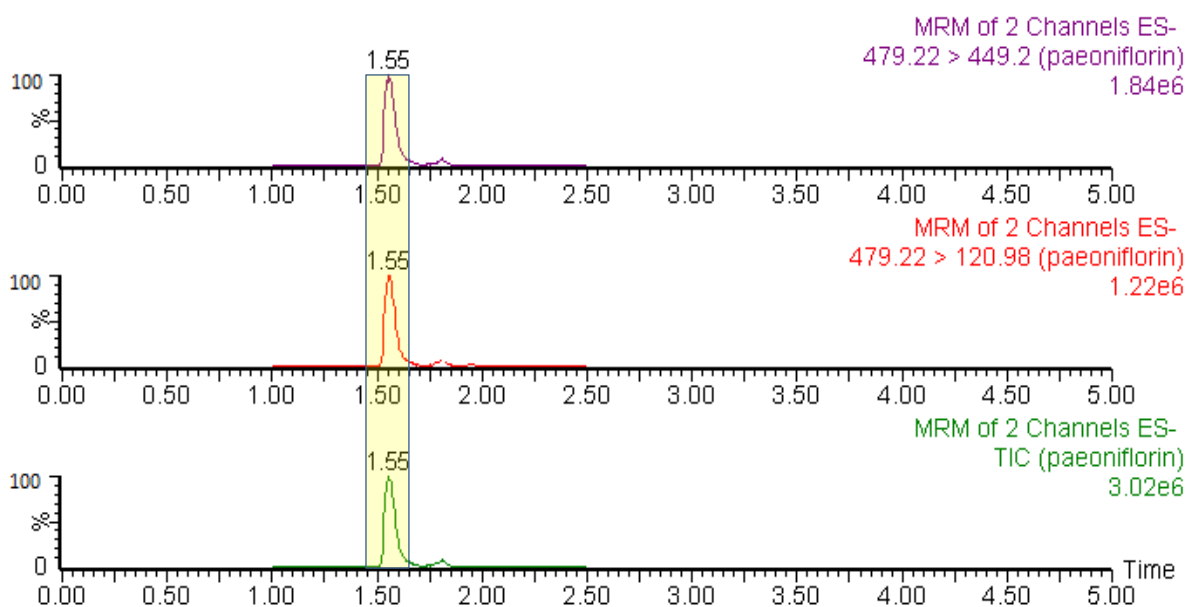
Figure 5.15 Representative LC-MS chromatogram of the precursor and product ions of paeoniflorin reference standard monitored by SRM

Identity confirmation for the analytes is achieved by comparing the relative intensities of the *m/z* of the two product ions obtained for the sample and standard. The intensities should agree to within the pre-set limits set by the EU standards for maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques (9). At least two product ions are required to meet the criteria set for analyte peak identity confirmation however selecting more than two predefined transitions measured during one acquisition cycle (typically 2 s to maintain proper definition of analyte elution profiles) is limited in order to maintain a practical dwell time.

5.3 Method validation parameters

5.3.1 Analytical identity confirmation

Identity confirmation of the analyte peak by LC-ESI-MS/MS is achieved by comparing the relative abundances of the *m/z* product ions obtained for the sample and standard. The SRM collision voltage (collision gas) is selected so as to produce two product ions for identity confirmation which meet the standards set by the European Commission Directorate for Agriculture guidelines (9). The agreement of the relative intensities for the product ions in the standard and samples should fall within the specified tolerances (102). The MS analyte identity confirmation results are presented in Table 5.1.

Table 5.1 Identity confirmation results of analytes monitored by selective reaction monitoring

Analyte	*m/z*	Relative intensity		Relative difference (%)*	Permitted tolerance (%)**	Pass/fail
		Standard	Sample			
alisol B	121	100	100	-		
	89	65	66	1.5	± 15	pass
pachymic acid	451	100	100	-		
	295	77	83	7.8	± 15	pass
alisol B	469	100	100	-		
	451	45	46	2.2	± 15	pass
rutin	300	100	100	-		
	271	65	66	1.5	± 15	pass
	255	34	35	2.8	± 15	pass
luteolin	151	100	100	-		
	132	33	29	12	± 15	pass
cornuside	169	100	100	-		
	125	41	40	2.4	± 15	pass
paeoniflorin	449	100	100	-		
	121	75	72	4.0	± 15	pass

*Relative difference = [Intensity of sample –intensity of standard)/ (intensity of standard)] x 100

**Maximum permitted tolerance of the European Union guidelines is ±15 (9)

5.3.2 Accuracy

The analyte recovery results across all the fortification levels (50, 100 and 200% of the unfortified concentration) for Qi Ju Di Huang Wan are presented in Table 5.2.The average recovery was 95% (range 85 to 115 %) with an average RSD of 3.4% (range 1.9 to 4.5%).

Table 5.2 Fortification recovery of analytes

Analyte	Fortification level[2]							
	50%		100%		200%		average[3]	
	%recovery	%RSD	%recovery	%RSD	%recovery	%RSD	%recovery	%RSD
pachymic acid	89	3.3	92	3.4	88	5.7	90	4.1
alisol C	85	2.2	83	1.7	87	5.9	85	3.3
luteolin	110	2.9	116	3.1	119	3.6	115	3.2
paeoniflorin	84	3.4	92	2.9	83	5.4	86	3.9
cornuside	116	2.3	108	3.2	101	3.2	108	2.9
rutin	84	1.8	97	1.6	91	2.2	91	1.9
alisol B	87	5.2	90	4.2	92	4.1	90	4.5
catalpol	> LOD[4]							
diosgenin	> LOD[5]							

[2] % Recovery ± %RSD calculated from seven replicate extractions and analyses
[3] Average recovery of all three fortification levels ± % RSD
[4] LOD is 0.005 mg g^{-1}
[5] LOD is 0.003 mg g^{-1}

Most recoveries show <100% recovery which is the common observation with most recovery studies due to the inevitable losses that occur in the various steps of the extraction process. Recoveries of >100% is often attributed to co-eluting components but the selectivity of the MS/MS technique makes this unlikely unless the co-eluting substance have the same product *m/z*. In this study this occurs only once, namely with alisol C and pachymic acid have one same product ion with *m/z* = 451 and the same retention time (3.22 min). Recoveries of >100% may be due to ionisation suppression by matrix components in the sample which are absent in the standard solutions. The peak intensity of an LC-MS/MS signal is related to the ionization efficiency of the individual analyte while passing from the solution phase to the gas phase. Some compounds will co-elute from the column and go through the ionization process simultaneously. Some co-eluting analytes in the solution may compete in the ionization process and change the ionization efficiency of the analyte of interest. As an example take alisol C and pachymic acid they have the same product ion and the same retention time as presented in Table 5.1.

In this study the recoveries obtained (average 90 to 115%) and precision of recoveries (1.9 to 4.5% RSD) were satisfactory for the concentrations studied. More importantly, the good recovery precision means that the result can be corrected for recovery.

Cross-talk interference by residual molecules, which is common to all mass spectrometers that employ high pressure collision cells, occur when successive product ions are monitored in the SRM mode and the time used to clear previous ions in the collision cell is not sufficient. However the recovery results allow this interference to be detected corrected for if desired.

5.3.3 Precision of quantitation

Table 5.3 Linearity of calibration curve, quantitation results, precision of quantitation and precision of retention time (RT)

| Analyte | Linearity (r^2) | Precision[a] | | LOD[b] | LOQ[c] |
		Amount (mg g^{-1}) ± %RSD	RT (min) ± %RSD	(mg g^{-1})	(mg g^{-1})
alisol B	> 0.99	2.35 ± 4.2	3.81 ± 0.2	0.31	1.04
pachymic acid	> 0.99	3.99 ± 1.5	3.2 ± 0.009	0.18	0.62
alisol C	> 0.99	5.24 ± 0.76	3.2 ± 0.15	0.09	0.31
rutin	> 0.99	0.62 ± 4.8	1.6 ± 0.25	0.12	0.41
luteolin	> 0.99	0.59 ± 1.7	2.15 ± 0.23	0.04	0.14
cornuside	> 0.99	2.9 ± 1.64	1.86 ± 0.16	0.29	0.98
paeoniflorin	> 0.99	1.27 ± 3.15	1.5 ± 0.13	0.12	0.41

[a] Average and RSD calculated from n = 7 replicates.
[b] Limit of detection (LOD) is three times the standard deviation (SD) for each analyte in AIII
[c] Limit of detection (LOQ) is ten times the standard deviation (SD) for each analyte in AIII

5.3.4 Stability

For accurate quantitation, standards and samples were stored at -4 $^{\circ}$C freezer; with stability

for up to 72h the peak area of the analyte did not decrease by >2%

5.4 Concentration of target analytes in eleven samples of Qi Ju Di Huang Wan herbal formulation

Table 5.4 Concentration of the selected analytes in the eleven samples studied

Analyte	Concentration[a] (mg g^{-1}) ± %RSD											Fold variation[b]
	AI	AII	AIII[c]	BI	BII	BIII	CI	CII	DI	DII	EI	
alisol B	4.02 ±3.2	2.88 ±3.2	2.50 ±4.3	2.50 ±4.1	1.48 ±5.1	2.88 ±4.1	2.75 ±3.3	2.88 ±3.4	0.59 ±3.6	2.37 ±4.2	2.24 ±3.3	6.8
pachymic acid	3.85 ±2.1	0.21 ±7.8	4.05 ±1.5	<LOD	1.16 ±3.8	< LOD	0.38 ±4.8	<LOD	<LOD	0.25 ±6.5	0.70 ±5.2	16.2
alisol C	4.93 ±3.5	0.68 ±6.8	5.26 ±1.2	<LOD	1.99 ±3.2	< LOD	0.72 ±4.7	0.25 ±6.6	<LOD	0.42 ±6.7	0.52 ±6.1	12.5
rutin	0.71 ±7.1	0.98 ±4.8	0.66 ±4.1	0.7 ±5.1	1.16 ±4.1	< LOD	2.90 ±3.1	1.82 ±3.9	<LOD	0.66 ±5.1	<LOD	4.4
luteolin	0.53 ±6.6	0.75 ±5.8	0.58 ±4.3	0.19 ±5.8	0.27 ±6.1	< LOD	1.22 ±4.2	0.61 ±5.3	< LOD	4.09 ±5.2	0.88 ±4.2	21.5
cornuside	3.47 ±1.3	1.23 ±4.6	2.94 ±2.4	0.52 ±6.2	0.12 ±7.2	<LOD	0.31 ±5.8	0.90 ±6.3	<LOD	0.24 ±5.8	1.17 ±4.8	28.9
paeoniflorin	1.50 ±2.9	1.10 ±3.9	1.30 ±3.9	0.26 ±7.7	0.60 ±6.2	0.70 ±6.1	0.95 ±4.6	0.74 ±6.8	<LOD	0.95 ±5.8	0.60 ±6.2	5.7

[a] Average calculated from seven replicates ±% RSD

[b] Fold variation = (highest concentration)/ (lowest concentration). (<LOD values omitted from this calculation)

[c] Analytical method validation performed on this sample

[d] Samples labelled with the same alphabet come from the same supplier

Sample AIII was used for the method validation studies and the developed method was subsequently applied for the analysis of the other samples. Samples with the same alphabet are the same brand so their observed differences may be indicative of inter-batch variation. The most noticeable fold variation is observed for cornuside (28.9 fold) followed by luteolin (21.5 fold), pachymic acid (16.2 fold) and alisol C (12.5 fold). The fold variation for other constituents is much lower by comparison, namely alisol B (6.8 fold), paeoniflorin (5.7 fold) and rutin (4.4 fold) nevertheless the variations remain substantial.

There is also a significant analyte concentration variation in the different batches of the same brand. If these bioactive constituents play a pivotal role in the proposed putative actions such as physiological potency of the medicine, then there may be a reasonable expectation that the effectiveness of the later, and hence its physiological outcome for the consumer, would also vary noticeably.

5.5 Chemometric analysis

The LC-MS-SRM datasets are represented as complex matrices. This complexity can be untangled by the application of chemometric methods that are used to reduce the dimensions of the LC-MS-SRM data for visualization purposes and to identify inherent patterns among sets of spectral measurements.

The chromatograms of Qi Ju Di Huang Wan samples were prepared prior to commencement of any chemometric analysis. Raw chromatographic data were converted into a comma-separated value file (CSV). The data files for each sample were accessed using R (v.2.14.2). The R language for statistical computing (known simply as 'R') is a powerful tool for chemometric analysis. One of R's major advantages is that it is free, with many users around

the world donating add-on packages that can perform a wide array of functions. Programming code based on the —chemometrics package written by Varmuza and Filzmoser was developed for the PCA analysis (63). A graphics wrapper for the displays was provided by the —ChemoSpec package written by Hanson (102).

The raw data is pre-processed by removing any unwanted signals such as sugar moieties and solvent peaks. The data is then organised into a set and drifting baselines are corrected. Improving data point stability in the chromatogram was achieved by noise removal resulting in a significant reduction in the number of data points required for plotting (200-250 data points). The data set for each analyte was transformed into a workable R data object and a relevant stack plot obtained. Baseline data was statistically corrected and the data then normalized and binned. Normalizing divides each point in the spectrum or chromatogram by all the points in the spectra or chromatogram. This is very useful since it reduces the effects of dilution in a sample. Binning collapses groups of frequencies into single frequency values and this helps correct the peak drifts of narrow peaks. However, it is important to note that baseline data correction did not make any significant changes in the chromatogram or spectrum as the analysis was performed in single reaction monitoring mode, and specifically as a segmental SRM profile, with each segment selectively optimised for a particular representative chemical analyte in the herbal extract.

5.6 Principal component (PCA) analysis

PCA was implemented to screen for any projected variability that is predominantly due to quantitative changes in analyte concentration. The unsupervised segregation was checked by principal component analysis (PCA) using Pareto-scaled data. The initial step in data analysis is exploring data structure and any relevant groupings; there are no assumptions

about sample association at this stage (unsupervised segregation). In Pareto scaling the variable mean is subtracted from each variable (column of the data) and then each variable is divided by the square root of the standard deviation. Pareto scaling falls in between no scaling at all and auto-scaling and gives the variable a variance equal to its standard deviation instead of unit variance.

The first objective in the data analysis process is to reduce the dimensionality of the complex data set to enable easy visualization of any component clustering of the different groups of samples. Thus, the loading plot gives an indication of the components that most strongly influence the patterns in the score plot.

5.6.1 Alisol B

Baseline data was statistically corrected, the peak maximums, minimums and mean were checked for Gaussian distribution and minimise retention time shift due to solvent conditions.
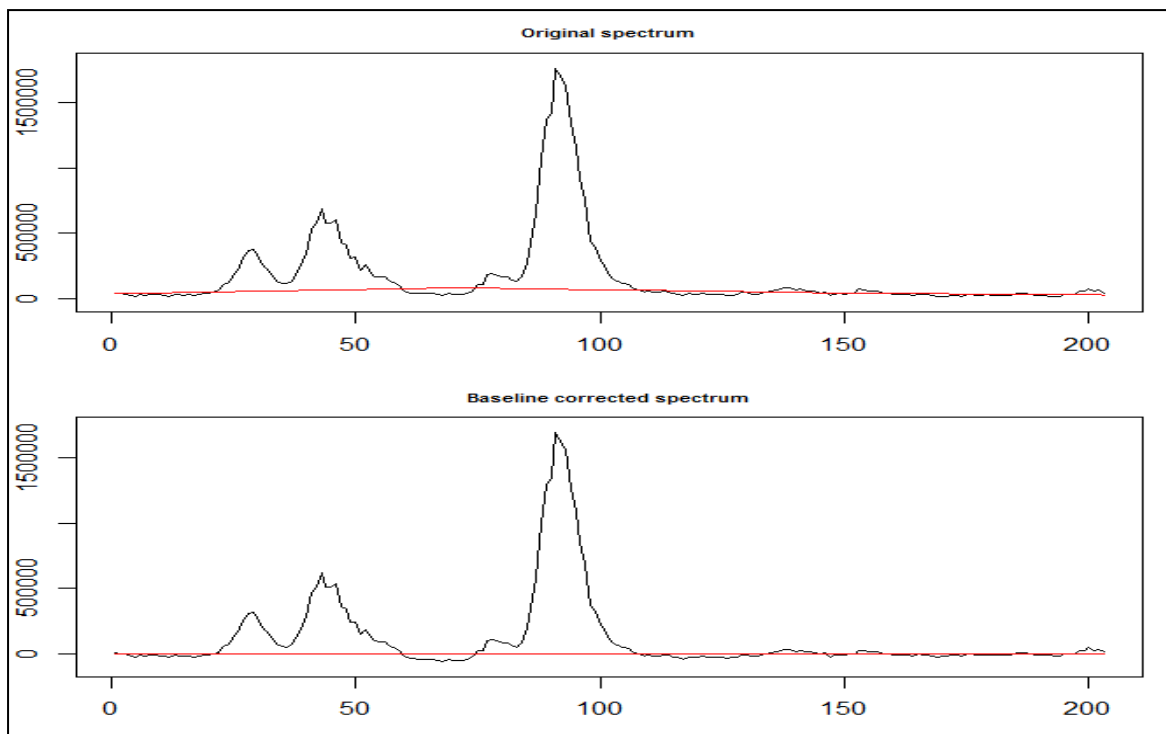
Figure 5.16 Representative baseline correction of alisol B

In the figure above, the x-axis represents the number of data points in alisol B spectrum and the y-axis is the counts. Alisol B data files from all eleven samples were meshed into a —R.data file and a stack plot was developed for the preliminary inspection of the region of interest.
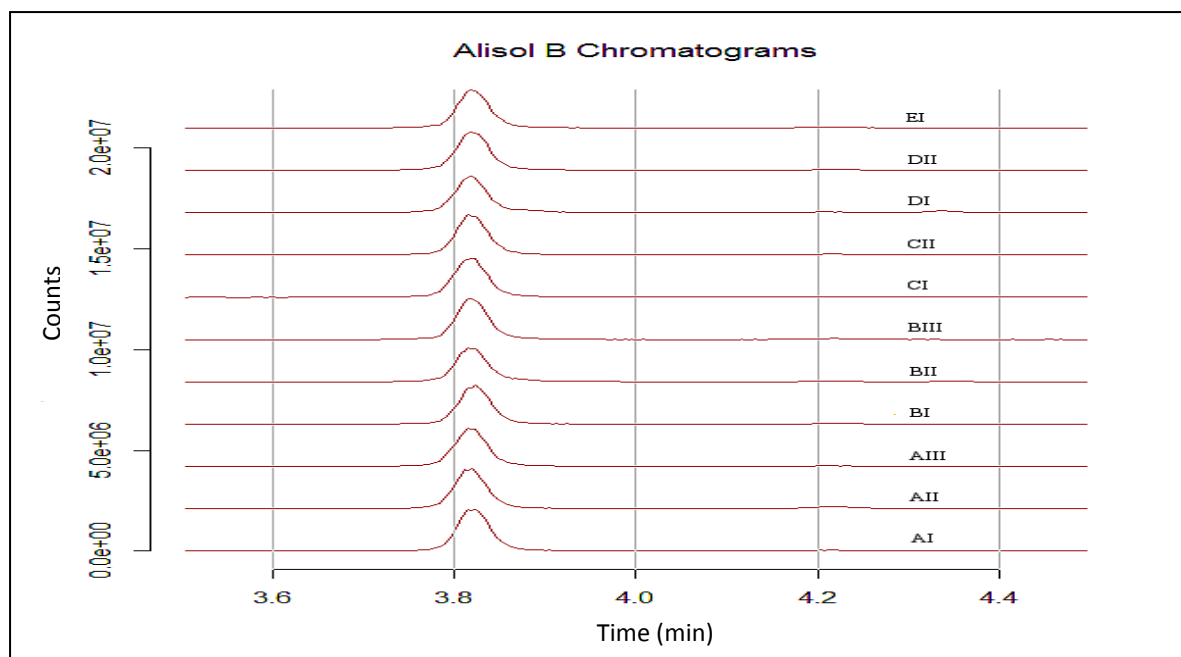
Figure 5.17 Mass spectra overlay of alisol B detected by selective reaction monitoring in eleven samples of Qi Ju Di Huang Wan

The PCA options that explained the greatest amount of the variance in the dataset was selected. The Pareto scaling option was chosen as it is a compromise between weighting all the peaks equally and weighting the biggest peaks the most. The loadings plot of the scaling technique was checked before PCA was performed.
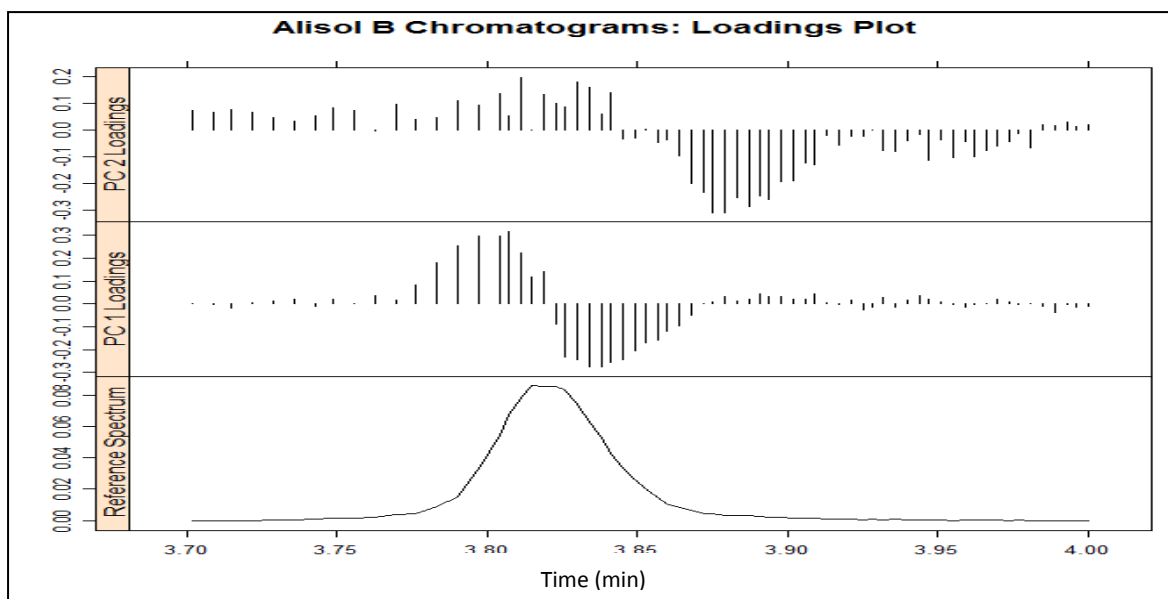
Figure 5.18 Representative loadings plot for alisol B used for PCA analysis

In the loadings plot, each line is a "data point" that has been loaded into the Pareto scaling

matrix .The loadings are varied across PC-1 and PC-2.
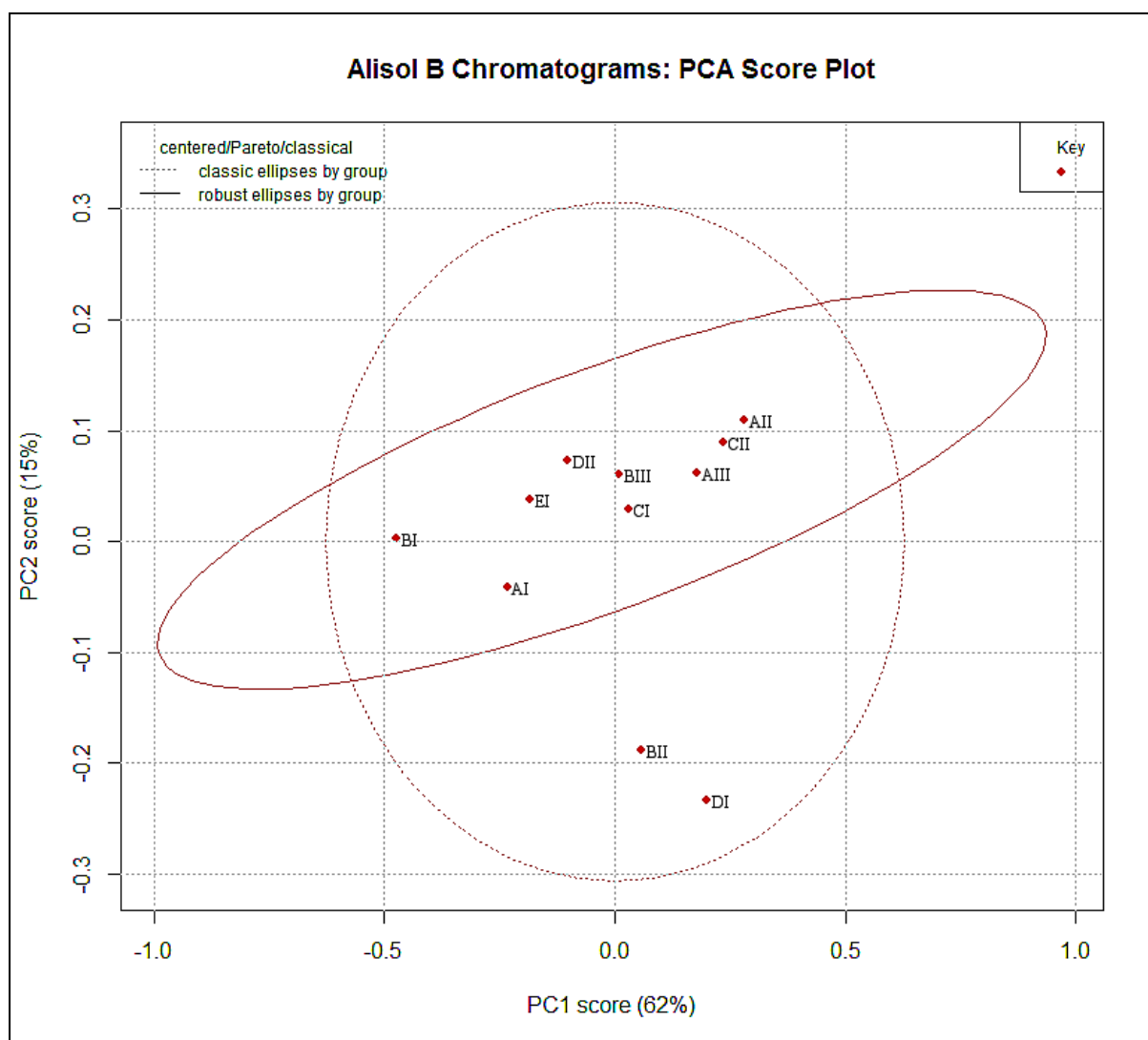
Figure 5.19 Scores plot of a two-component PCA model of LC-MS chromatograms showing clustering pattern of eleven samples of Qi Ju Di Huang Wan according to variation in concentration [1]
(Note: Data for plot obtained from Table 5.4)

The cumulative score for PC-1 and PC-2 is 77%, which means that these PC's explain most of

the variability within the data set with very good confidence. The PCA scores plot provides a

rationale regarding the variability in concentration of the analyte of interest in Qi Ju Di

Huang Wan samples. The robust ellipse envelops the core of the dataset, showing us that

the possible outliers are samples BII, DI. One possible explanation of this clustering is

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[1] The robust ellipse envelops the core of the dataset, and may be used for the detection of outliers. Samples falling outside the ellipse may be indicative that the species are different possibly due to adulteration.

evident as shown in Table 5.4 where both samples are shown to constitute low concentrations of the targeted analytes in comparison to the remainder.

5.6.2 Pachymic acid
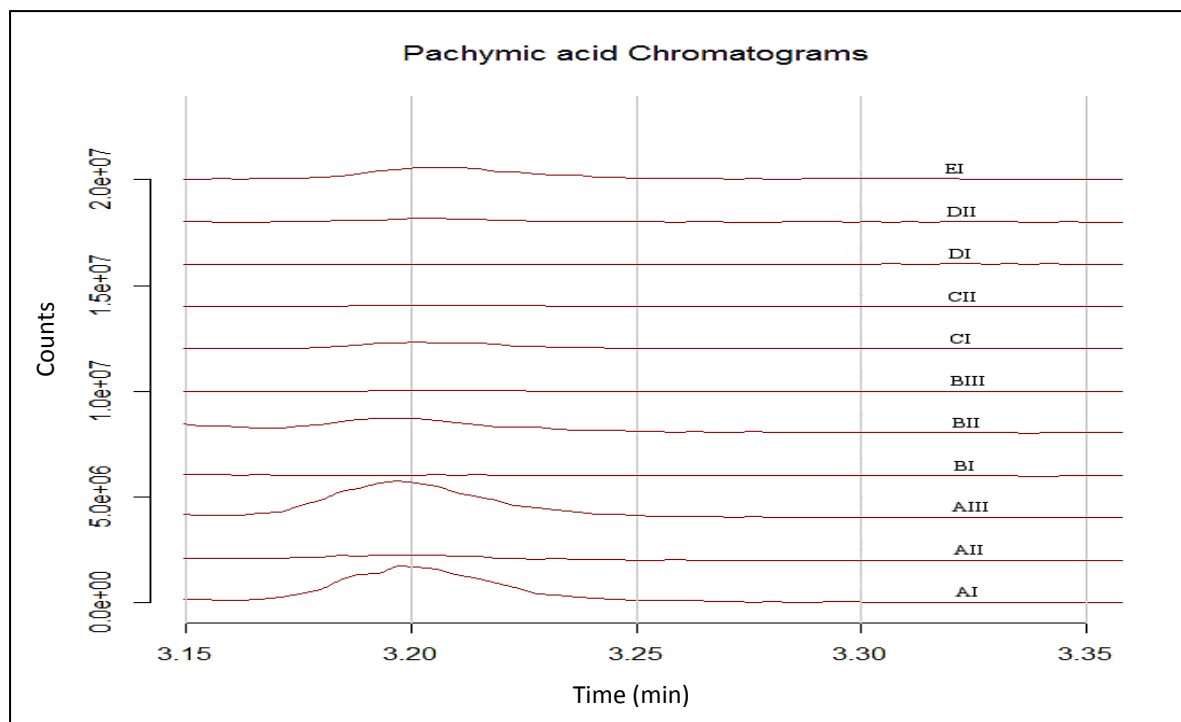
Figure 5.20 Mass spectra overlay of pachymic acid detected by selective reaction monitoring in eleven samples of Qi Ju Di Huang Wan
(Note: all the figures start with a baseline at '0.0 e+00' but shown at different starting points for clarity only).

Pachymic acid was shown to be below the limit of detection in most samples, this finding does not contribute to any significance in the variations observed in the PCA analysis.

Figure 5.21 Scores plot of a two-component PCA model of LC-MS chromatogram showing clustering according to variation in concentration

The cumulative score for PC-1 and PC-2 is 75%, which means that these PC's explain most of the variability within the data set. It is important to note that the results shown in the PCA scores plot of pachymic acid are in agreement with the findings evident in Table 5.4 where the outliers samples DI, BI, BIII and CII all fall below the limit of detection.

5.6.3 Alisol C
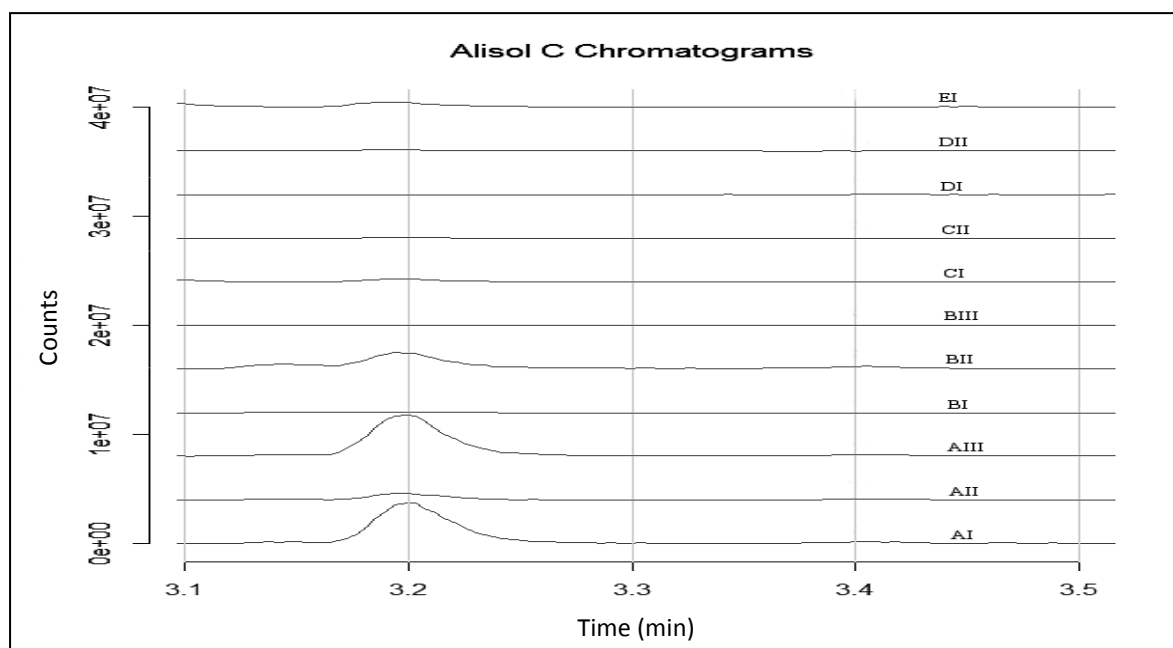
Figure 5.22 Mass spectra overlay of alisol C detected by selective reaction monitoring in eleven samples of Qi Ju Di Huang Wan
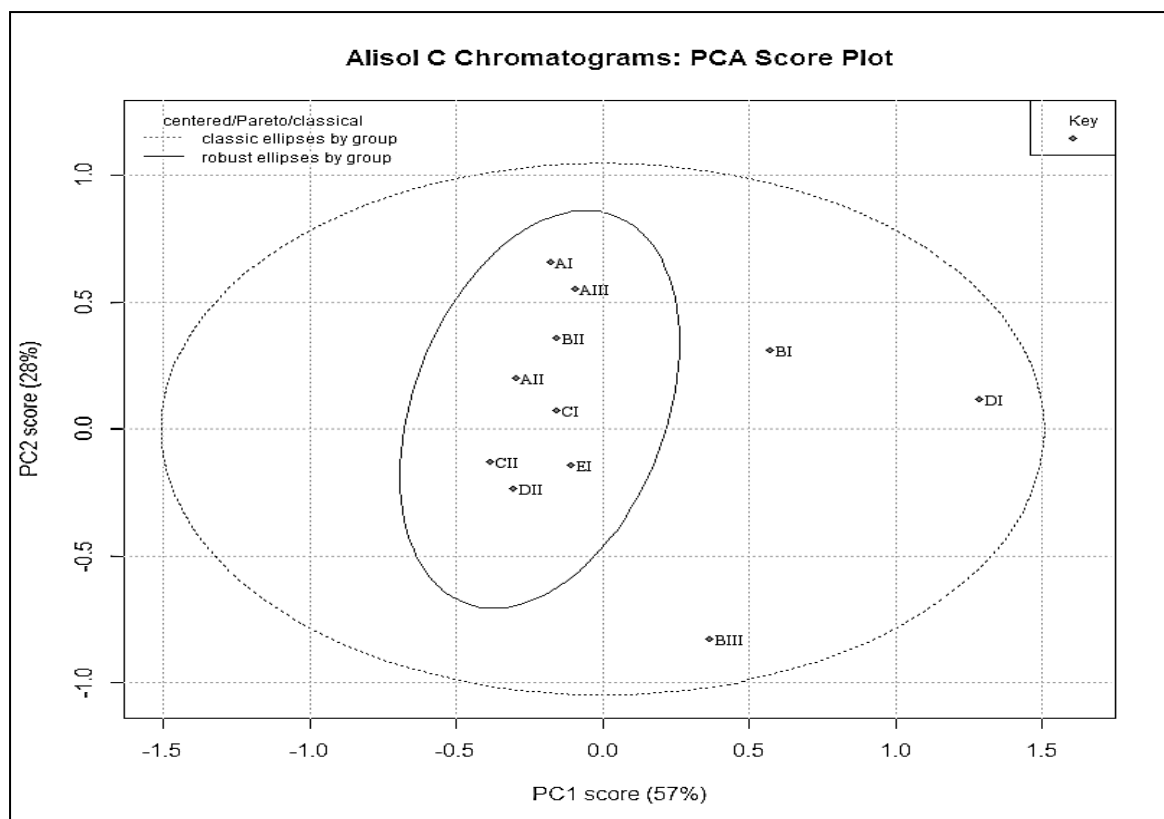
Figure 5.23 Scores plot of a two-component PCA model of LC-MS chromatograms showing clustering according to variation in concentration

The cumulative score for PC-1 and PC-2 is 85%, which means that these PC's explain most of the variability within the data set. The PCA scores plot of alisol C in the samples confirms the findings presented in Table 5.4 where the outliers DI, BI and BIII all fall below the limit of detection.

5.6.4 Rutin
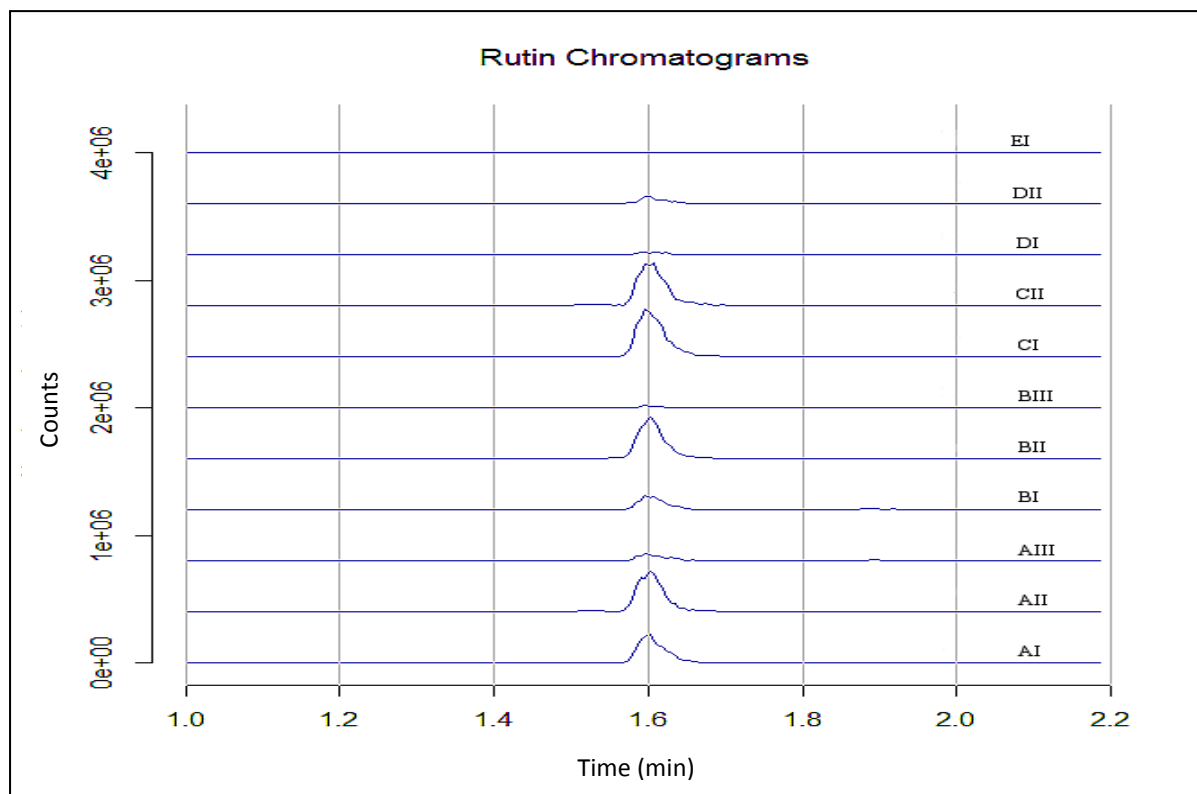
Figure 5.24 Mass spectra overlay of rutin detected by selective reaction monitoring in eleven samples of Qi Ju Di Huang Wan
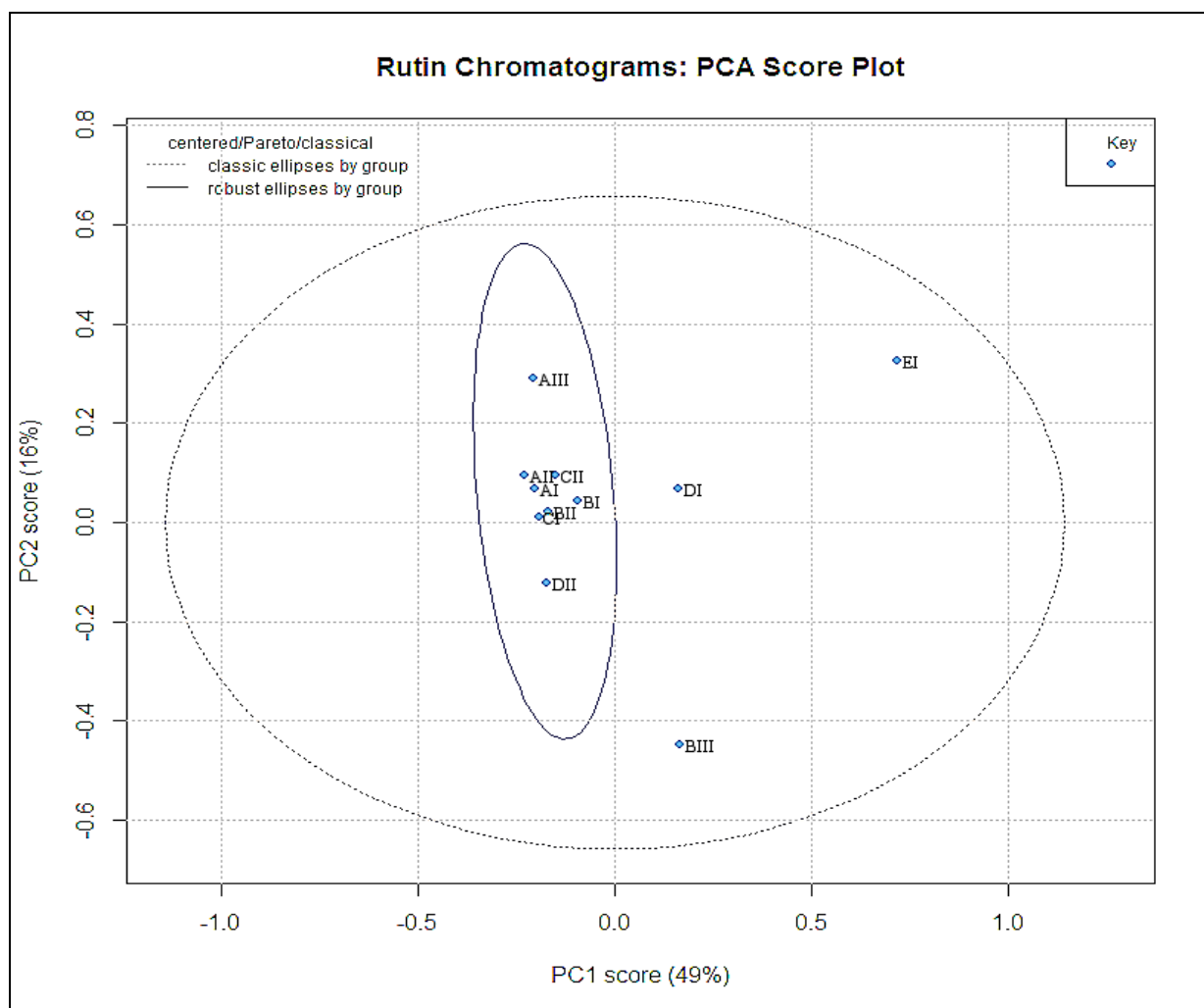
Figure 5.25 Scores plot of a two-component PCA model of LC-MS chromatograms showing clustering according to variation in concentration

The cumulative score for PC-1 and PC-2 is 65%, which means that these PC's explain most of the variability within the data set with very good confidence. The PCA scores plot of rutin samples confirms the findings presented Table 5.4. The outliers DI, EI and BIII fall below the limit of detection.

5.6.5 Luteolin
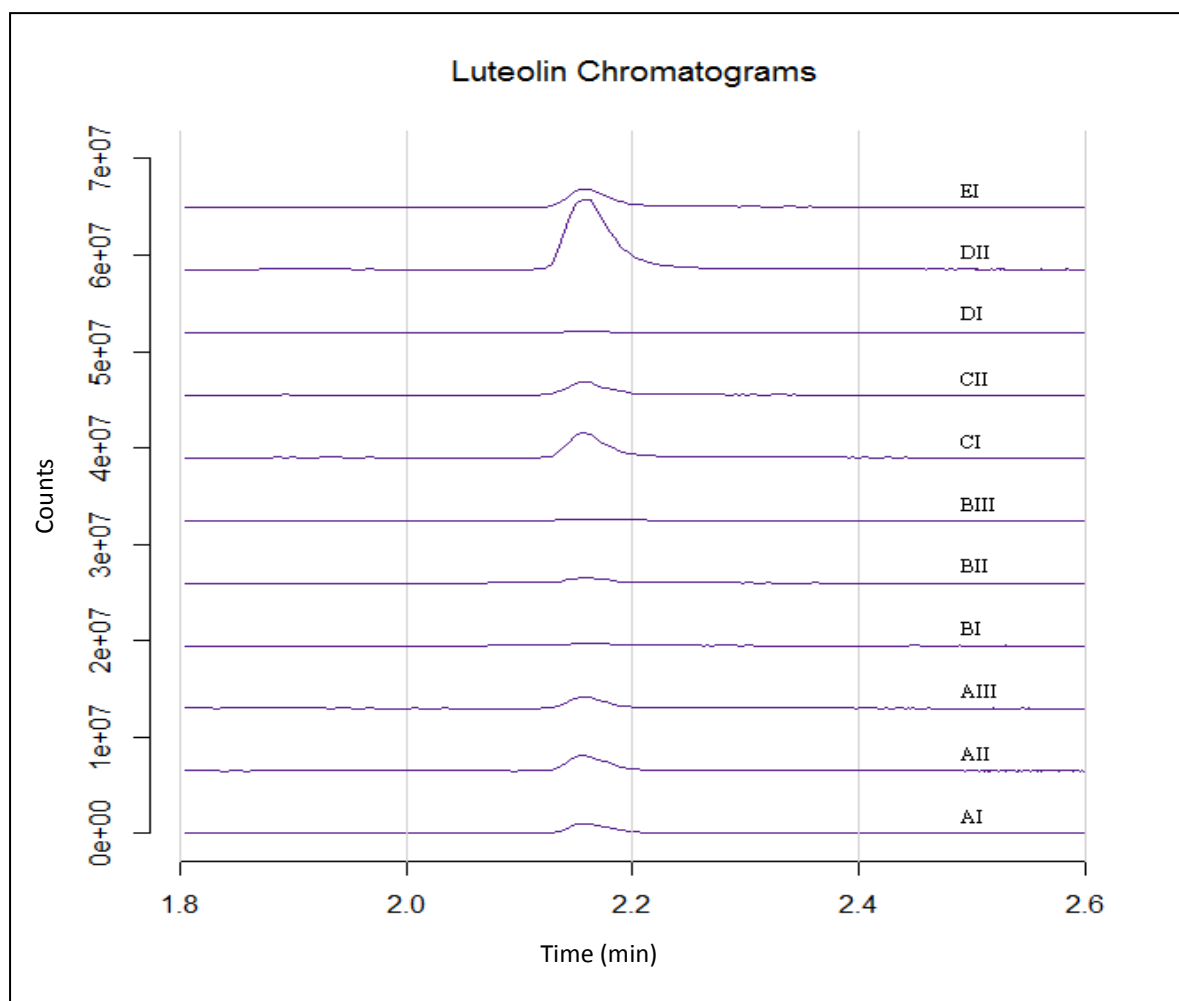
Figure 5.26 Mass spectra overlay of luteolin detected by selective reaction monitoring in eleven samples of Qi Ju Di Huang Wan
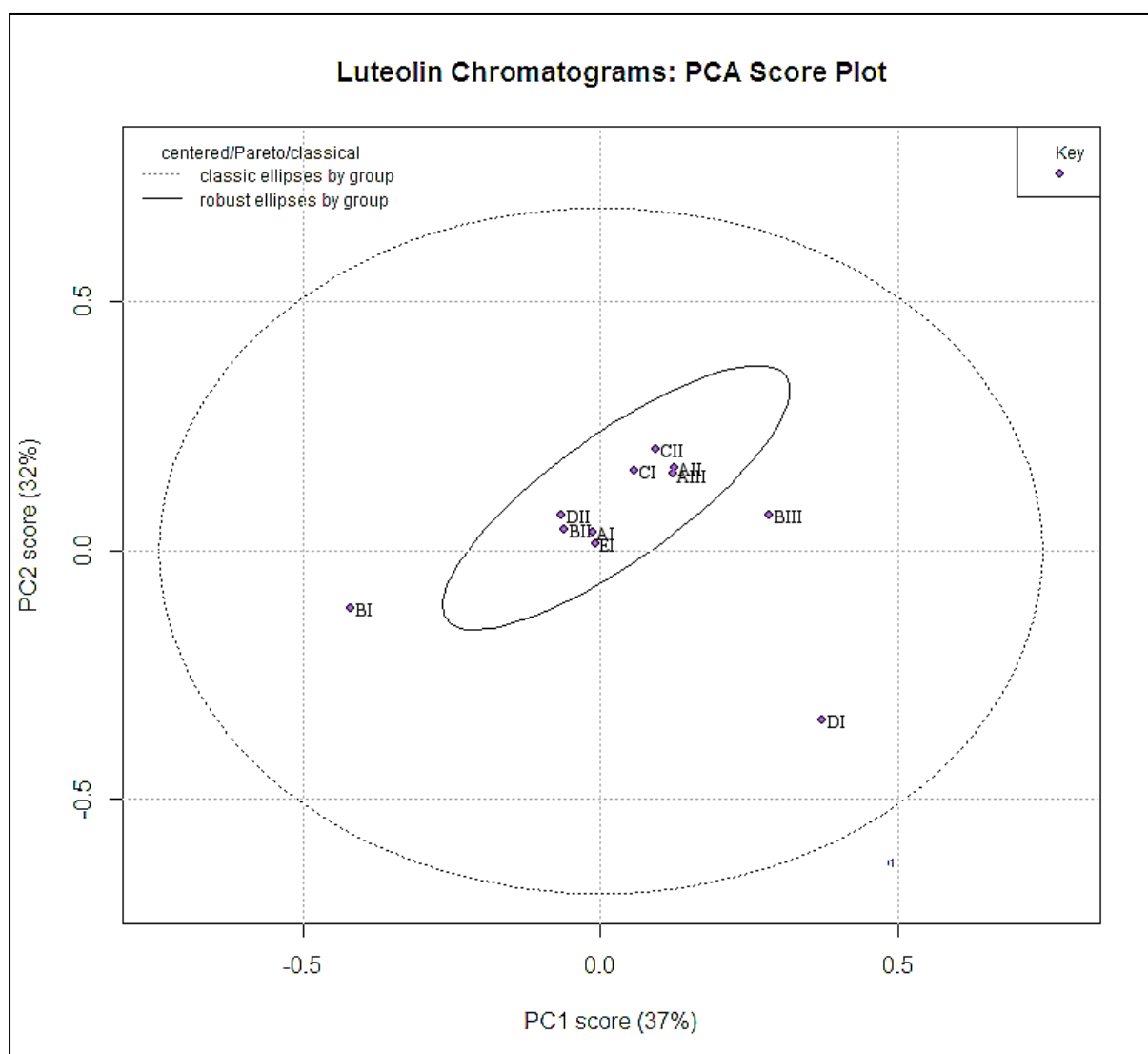
Figure 5.27   Scores plot of a two-component PCA model of LC-MS chromatogram showing clustering according to variation in concentration of luteolin

The cumulative score for PC-1 and PC-2 is 69%, which means that these PC's explain most of the variability within the data set with very good confidence. The PCA scores plot of luteolin samples confirms the findings presented in Table 5.4 where the outliers BI and BIII fall below the limit of detection. In sample DI, luteolin is detected at a very low concentration which is likely to be the justification for the observed clustering patterns.

5.6.6 Cornuside
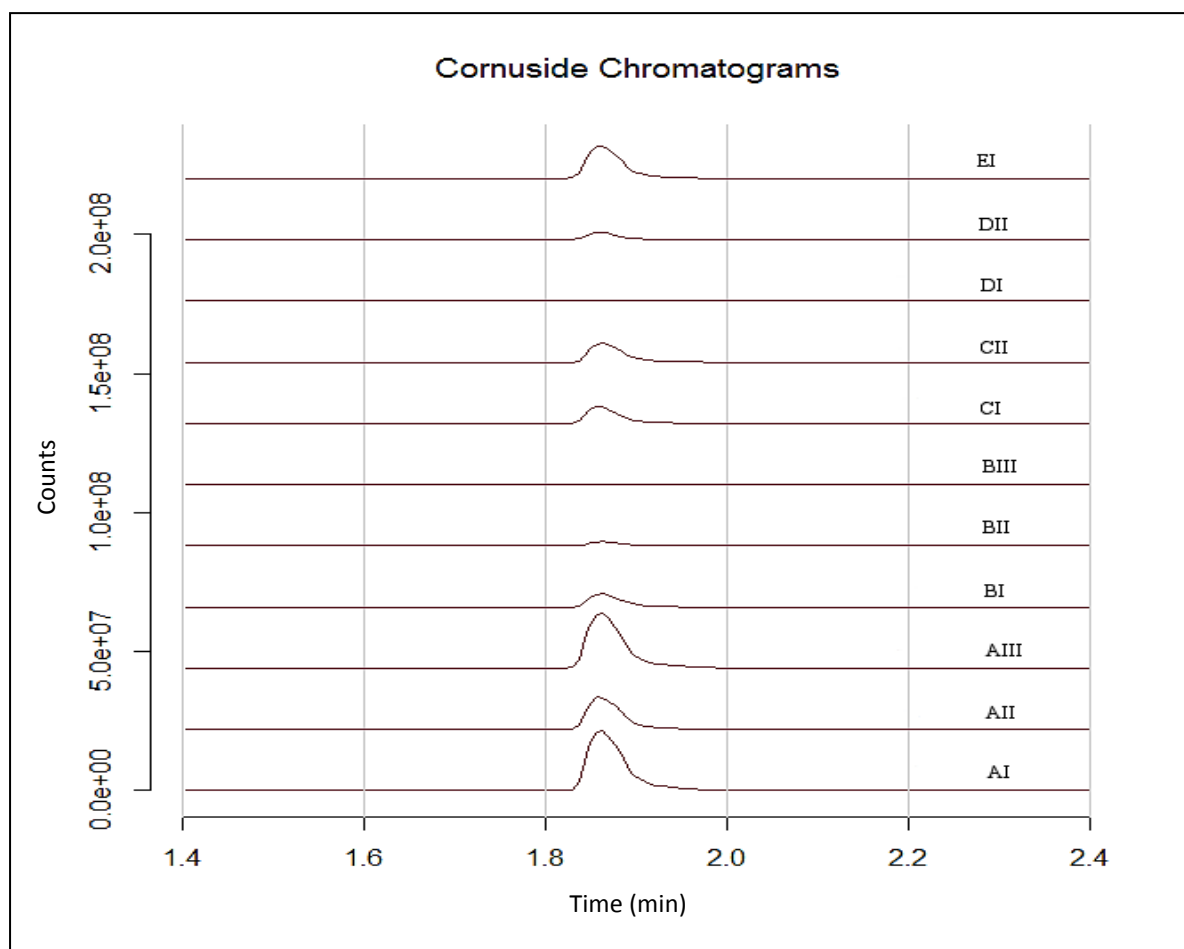
Figure 5.28 Mass spectra overlay of cornuside detected by selective reaction monitoring in eleven samples of Qi Ju Di Huang Wan
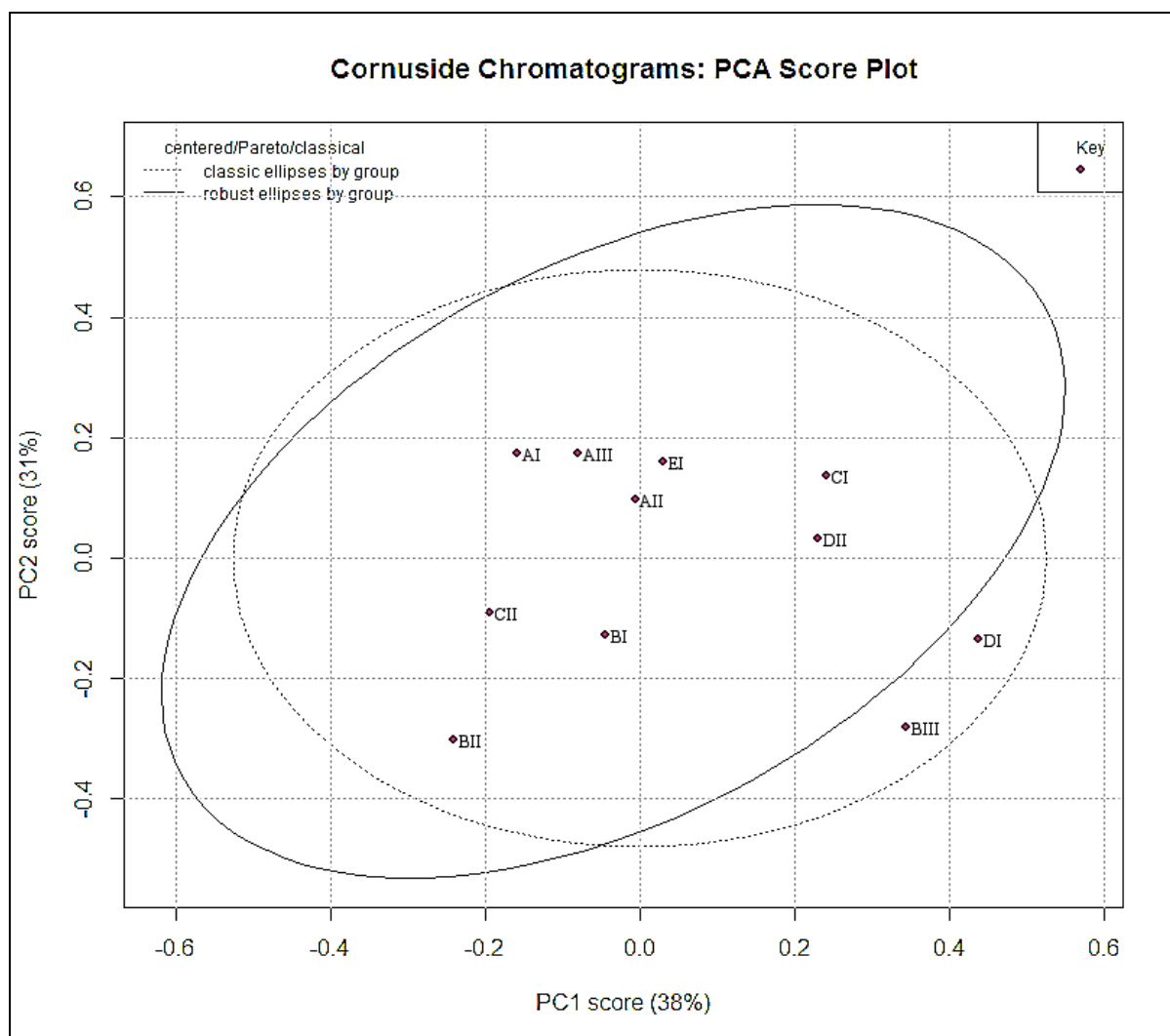
Figure 5.29 Scores plot of a two-component PCA model of LC-MS chromatogram showing clustering according to variation in concentration of cornuside

The cumulative score for PC-1 and PC-2 is 69%, which means that these PC's explain most of

the variability within the data set with very good confidence. The robust ellipse envelops the

core of the dataset; two outliers can be observed BIII and DI which is in agreement with

data found in Table 5.4 where both samples fall < LOD.

It is interesting to note that although the PCA analysis enabled the detection of outliers

below LOD, however samples BII and DII were also expected to be detected as outliers and

that was not the case. This outcome may be improved with more samples in the data set and using more advanced techniques like ANOVA.
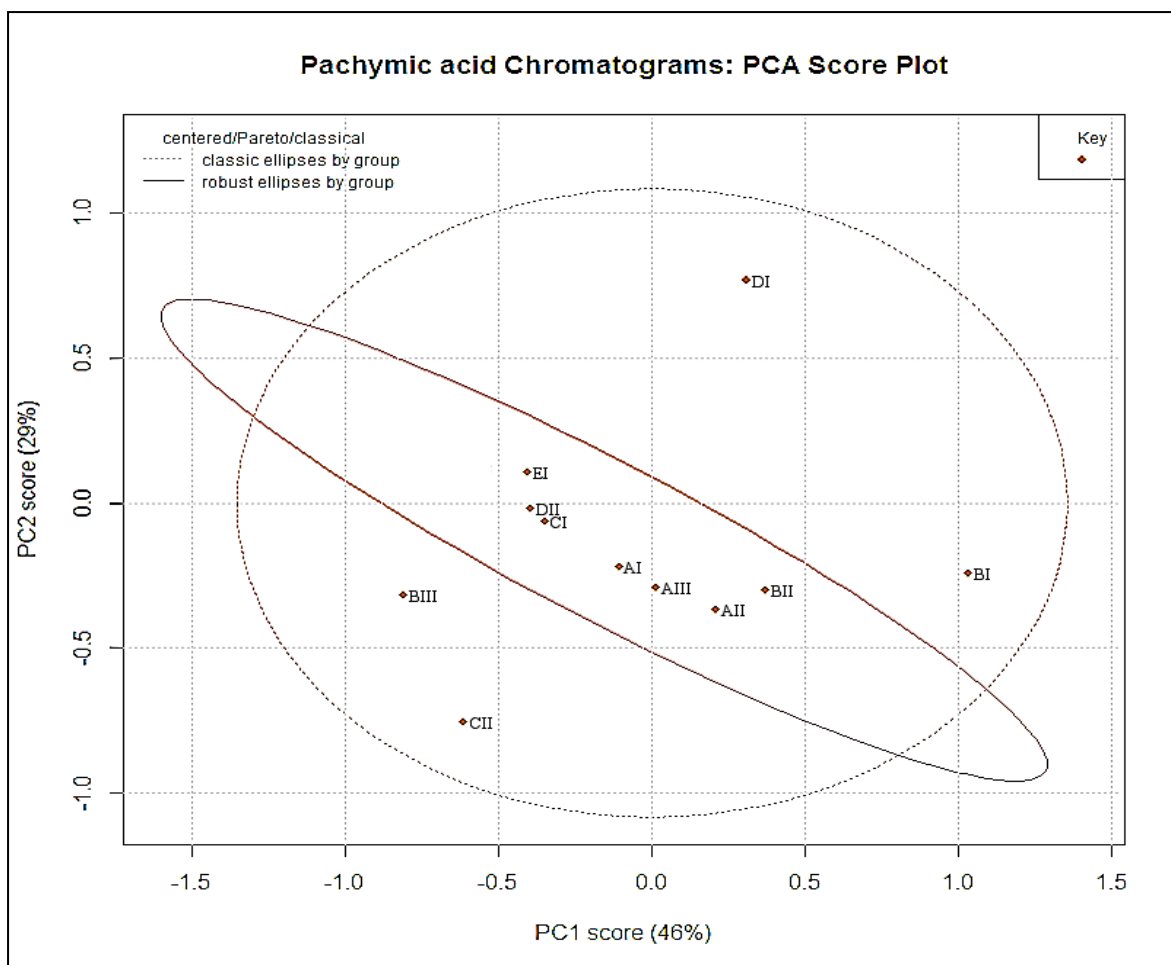
5.6.7 Paeoniflorin
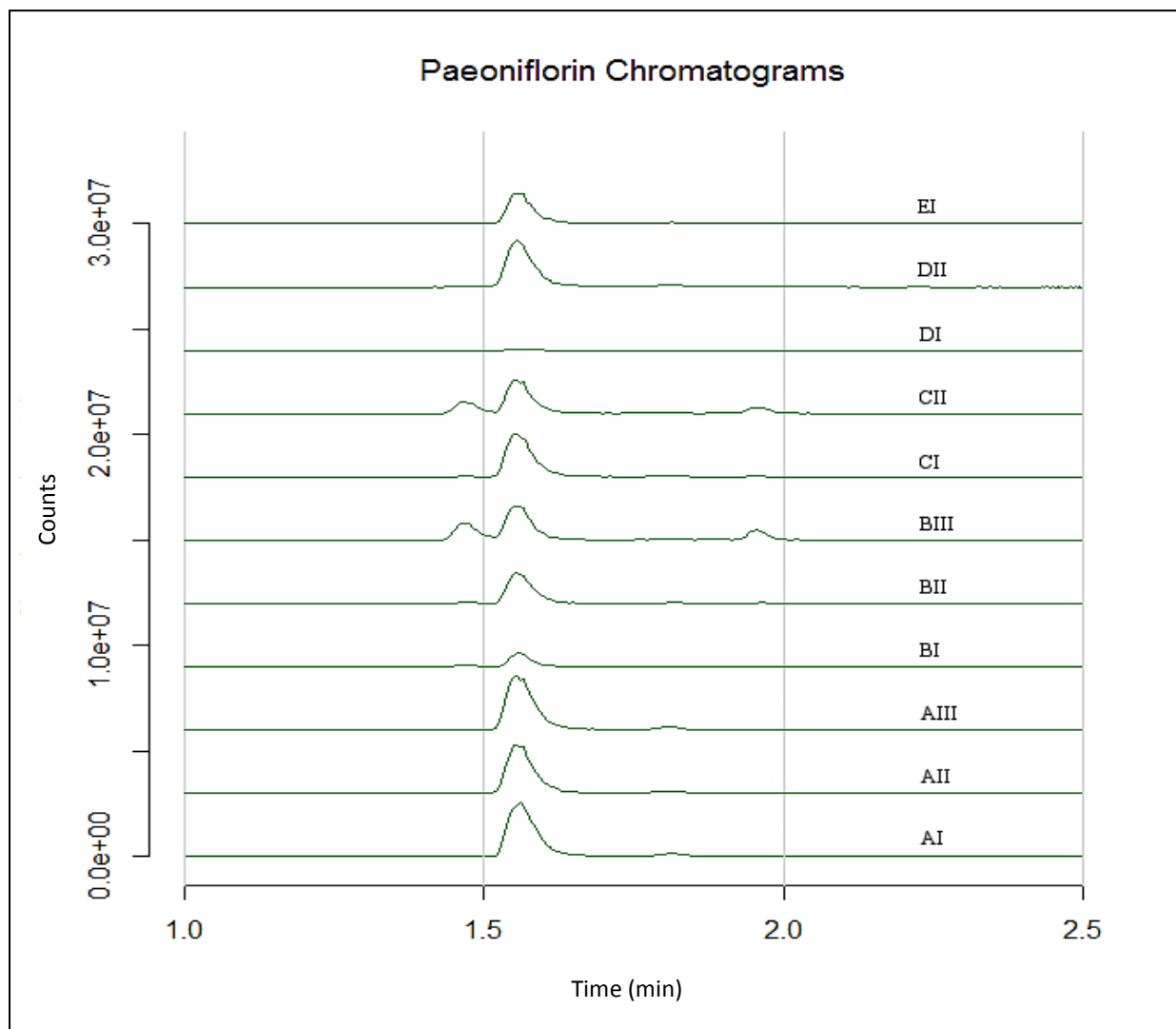
Figure 5.30 Mass spectra overlay of paeoniflorin detected by selective reaction monitoring in eleven samples of Qi Ju Di Huang Wan
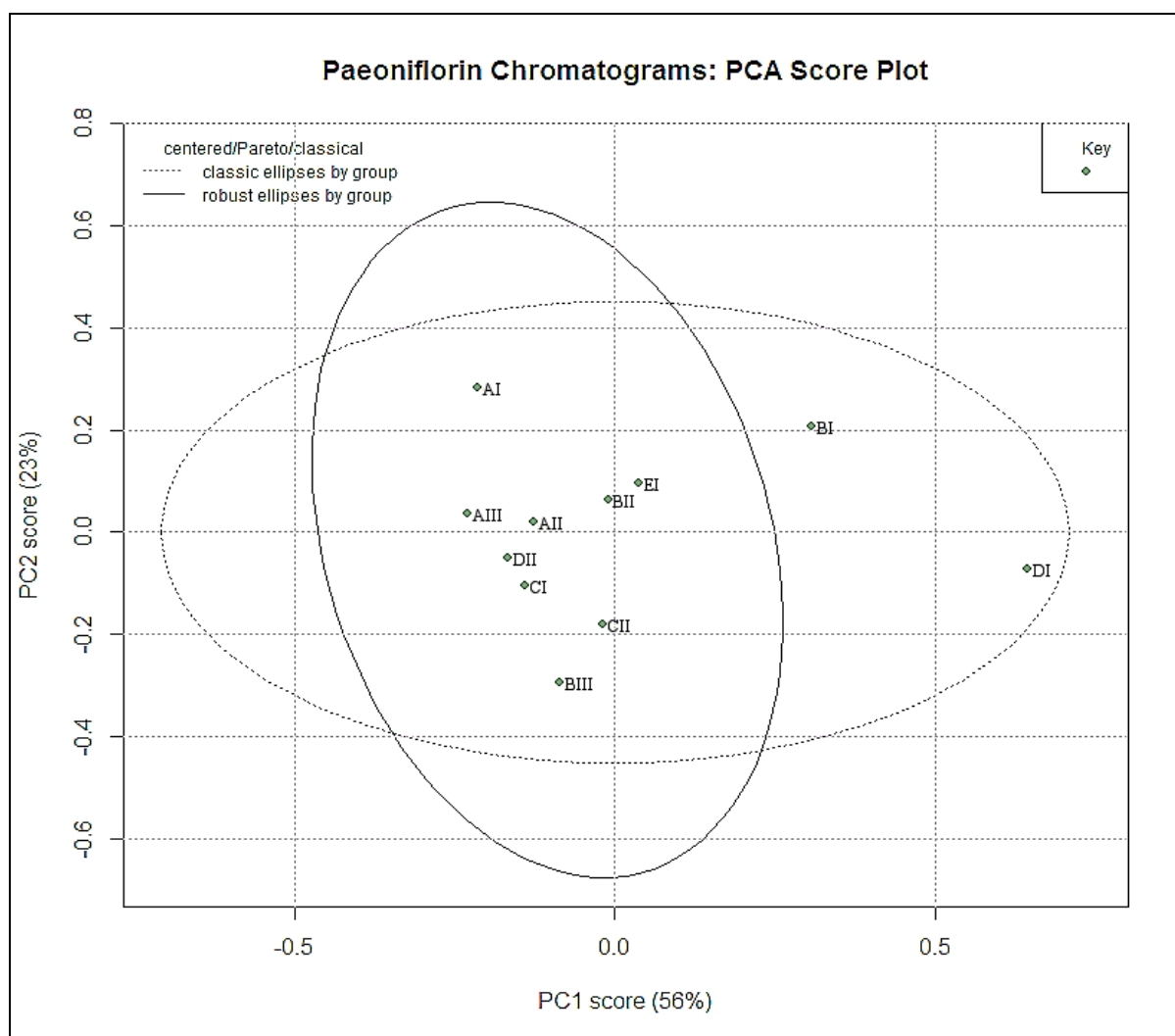
Figure 5.31   Scores plot of a two-component PCA model of LC-MS chromatograms showing clustering according to variation in concentration of paeoniflorin

The cumulative score for PC-1 and PC-2 is 79%, which means that these PC's explain most of the variability within the data set with very high confidence. The robust ellipse envelops the core of the dataset; two possible outliers can be observed BI and DI. The observed clustering maybe pertaining to the fact that paeoniflorin was <LOD in sample DI, and present in very low concentration in sample BI. In this case the results are desirable as the PCA analysis could afford the detection of outliers possibly due to low concentration and below their limit of detection.

5.7 Summary of findings

A targeted LC-ESI-MS/MS method was developed for the quantification of selected analytes in the herbal formulation except for catalpol and diosgenin where the concentrations were too low for reliable detection. Good resolution of the analyte peak and the satisfactory signal-to-noise ratio enabled for the reproducible and accurate quantitation of the analytes of interest.

The analytical method was validated to test the accuracy with average recoveries ranging from 90% to 115% across all spiking levels. The recoveries demonstrate that the effectiveness of the analytical method in testing for the range of concentrations selected for each analyte. Preliminary identity confirmation of the predetermined analytes was achieved by comparing the MS spectra of the analyte peak from the sample and the pure standard. The *m/z* product ion intensities for each analyte were compared between the sample and the pure standard and the results of the relative ion intensity difference was within the guidelines specified by the European Commission Directorate for Agriculture guidelines (2002) (9).

PCA was implemented for all eleven Qi Ju Di Huang Wan samples to ascertain the variability of the commercial samples. PCA is a well-established statistical method for the analysis of complex data. Similar samples tend to form clusters on the scores plot whereas dissimilar samples will be found at greater distances, with the loadings plot indicating the original variables responsible for the similarity or dissimilarity. As with all statistical analysis, the overall reliability of the result is dependent on the sample size. This in turn enables mapping the quantitative variability for each analyte and the qualitative variability for each herb present in any given herbal formulation.

Pertinent to our studies there seems to be an observed clustering pattern of three main groups in terms of the quality of the herbs used in the herbal formulation; this is unequivocally contingent on the monitored analytes. Sample BI, DIII and DI were of the poorest quality in terms of quantitative analysis followed by samples CII, EI, DII and BII being in the mid-range and finally samples AI, AII and AIII being the most prominent.

Chapter 6

Results and Discussion of Analysis of *Lycium*

6.1 Preparation of the *Lycium* raw herb for analysis

The dried raw herb obtained from various commercial sources was prepared as described in section 4.2, chapter 4. The powdered sample was extracted by sonication in 50% aqueous methanol to produce an extract for chromatographic analysis.

6.2 Analytical method development

100 μg mL^{-1} individual solutions of each standard were infused into the MS/MS to optimize the detection parameters for each of the seven analytes and to select two appropriate transitions (product ions) for each compound using the IntelliStart software with the ESI in the positive and negative modes. The (-) ESI mode was selected for subsequent analysis because higher ion counts were observed in this mode. In (-) ESI mode the [M-H]$^-$ ion is generated. Table 4.3 in chapter 4 summarises the precursor ions used, the collision voltages for SRM and the product ions for each analyte.

6.2.1 Representative LC-ESI-MS/MS chromatograms for Lycium

Representative chromatograms for analysis of the selected analytes in the sample monitored in SRM mode are presented in Figure 6.1 as a spectra overlay, with the detector output displayed for the time window (or segments) set for each analyte. At each time window the predetermined optimum MS/MS experimental conditions is applied for each analyte. Chromatograms obtained for the product ions monitored in SRM mode for analytes in the sample and the standard are shown in Figure 6.2.a. – Figure 6.8b.
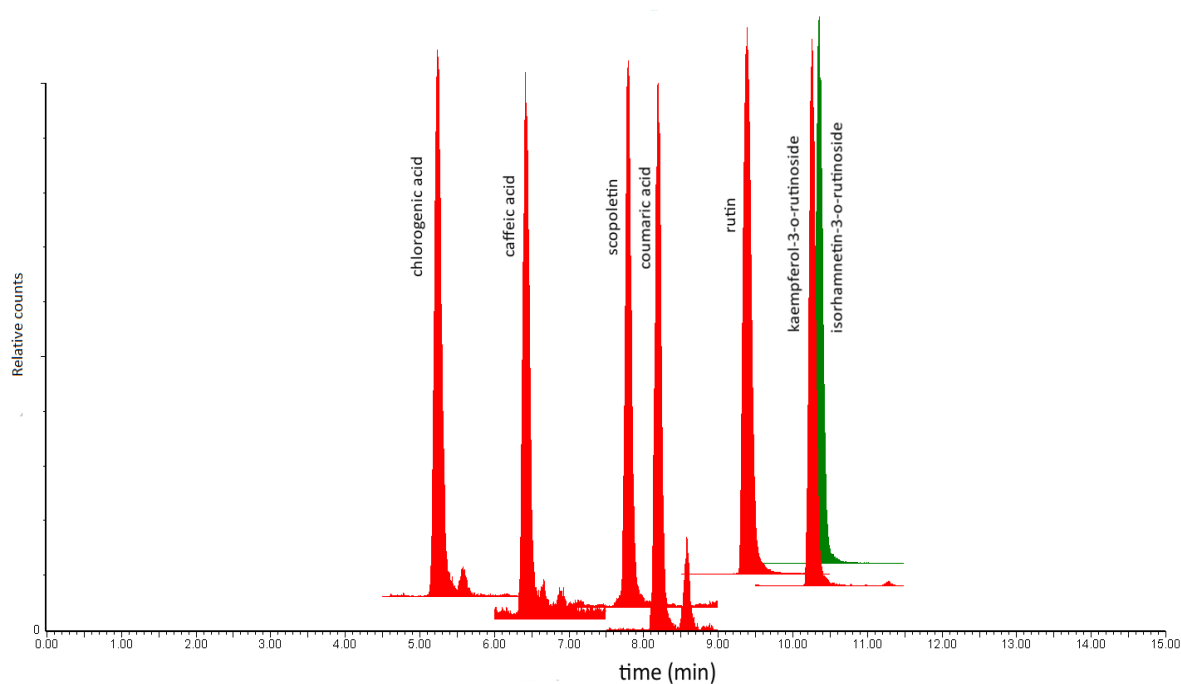
Figure 6.1   Representative spectra overlay of analytes monitored in a sample of *Lycium* analysed by selective reaction monitoring mode (SRM)
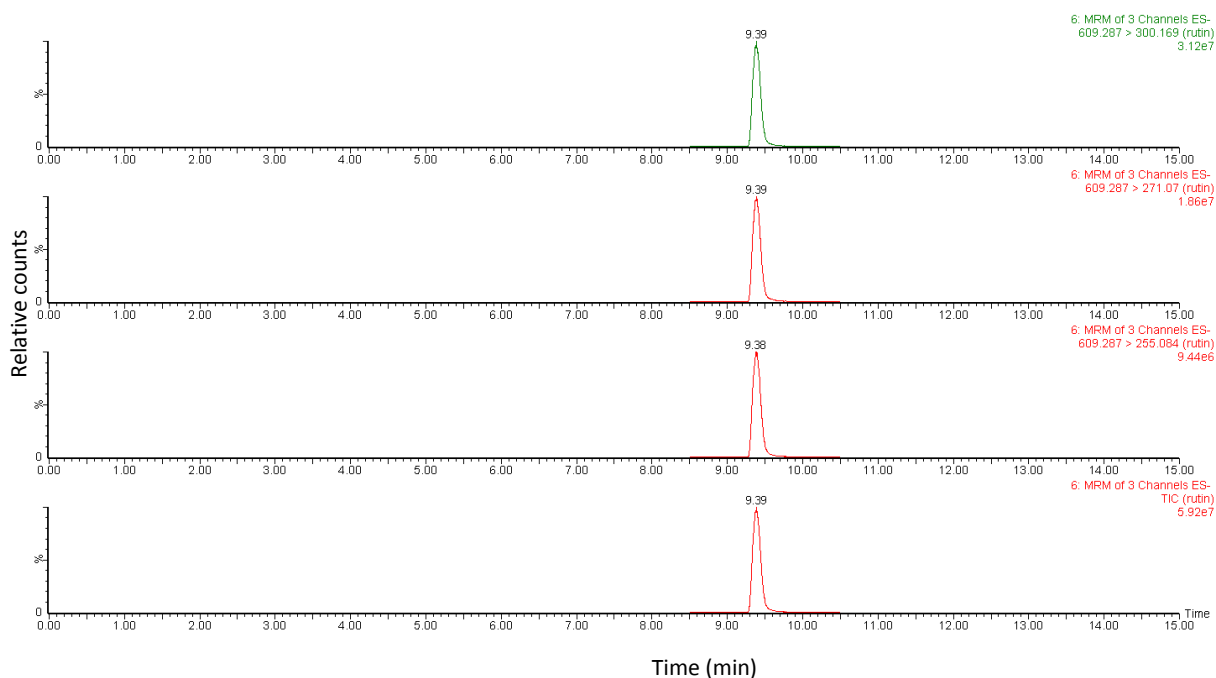
Figure 6.2a   Representative chromatogram of the product ions and total ion chromatogram of rutin monitored in the *Lycium* sample
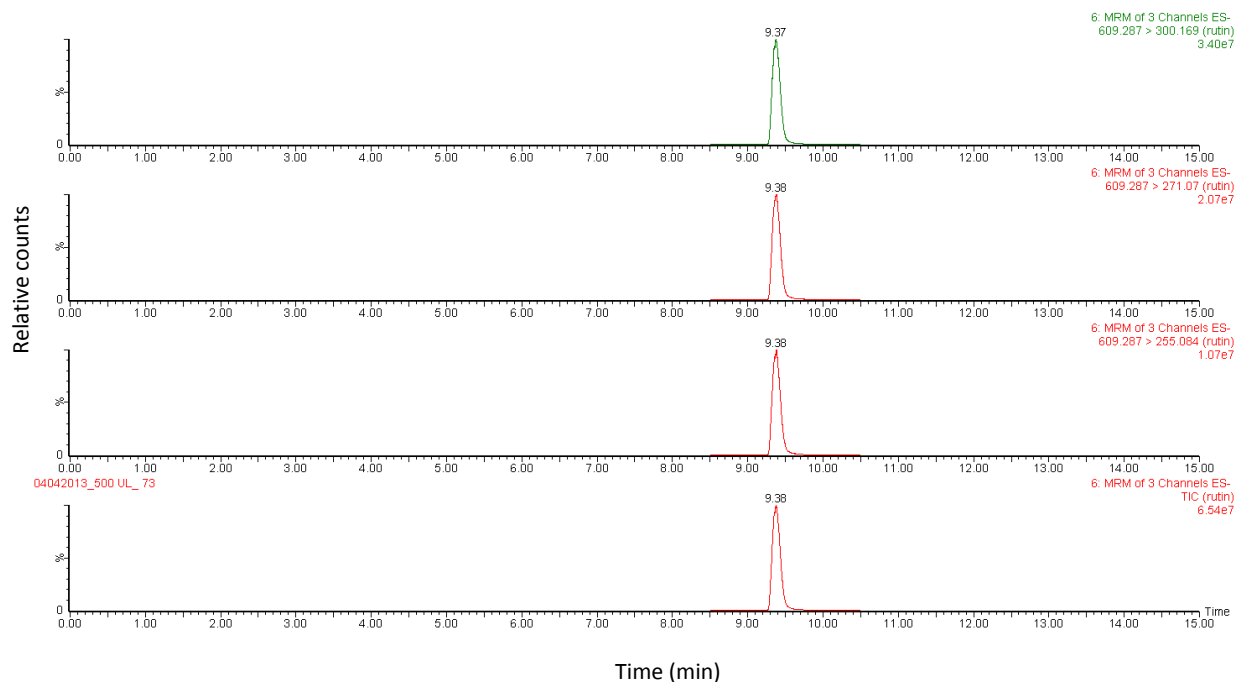
Figure 6.2b   Representative chromatogram of the product ions and total ion chromatogram of rutin monitored in the mixed standard solution
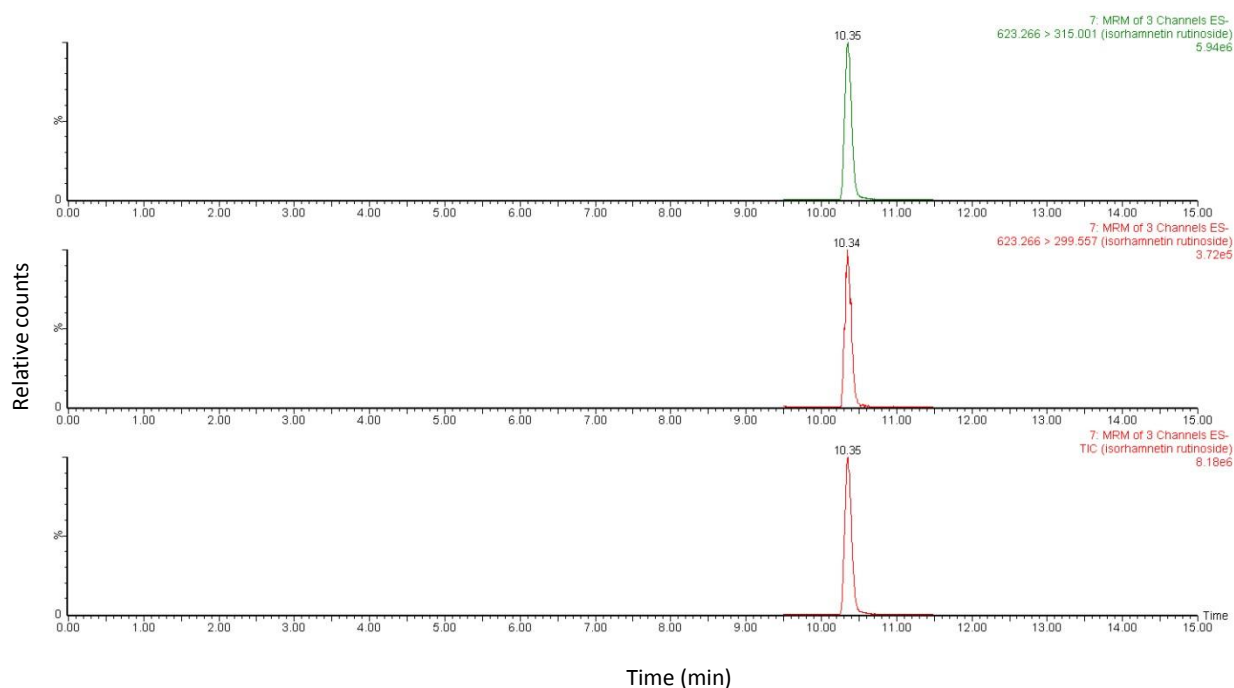
Figure 6.3a   Representative chromatogram of the product ions and total ion chromatogram of isorhamnetin-3-*o*-rutinoside monitored in the *Lycium* sample
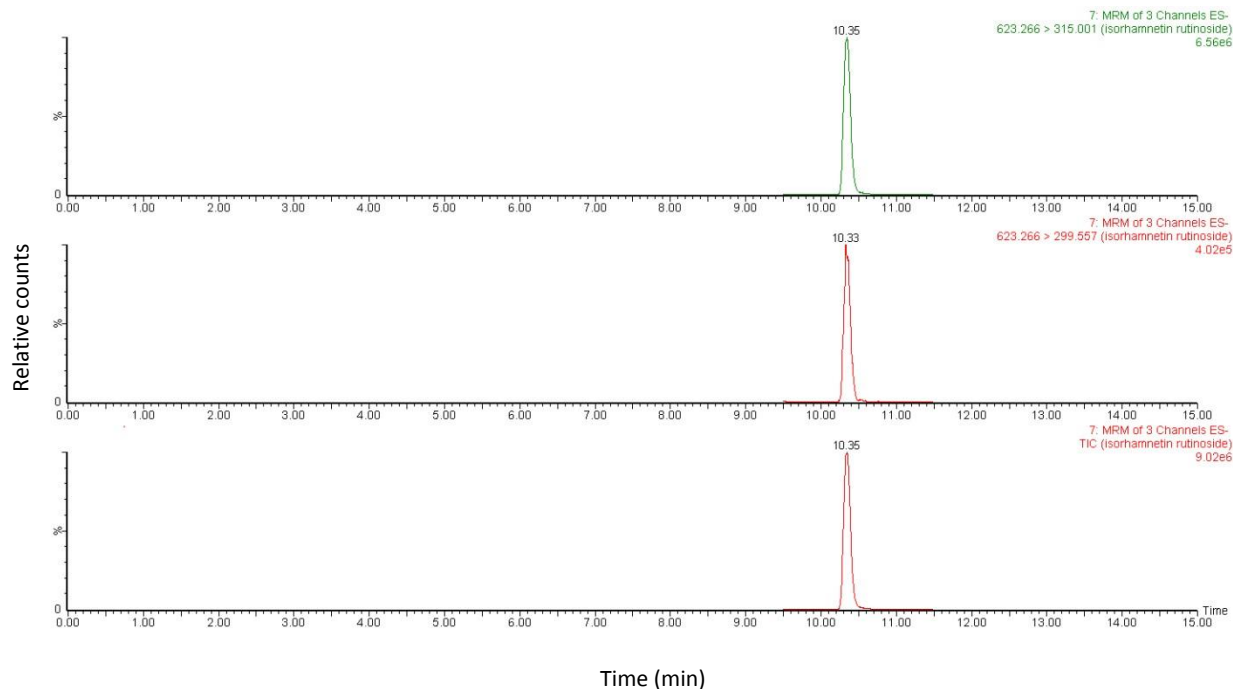
Figure 6.3b   Representative chromatogram of the product ions and total ion chromatogram of isorhamnetin-3-*o*-rutinoside monitored in the mixed standard solution
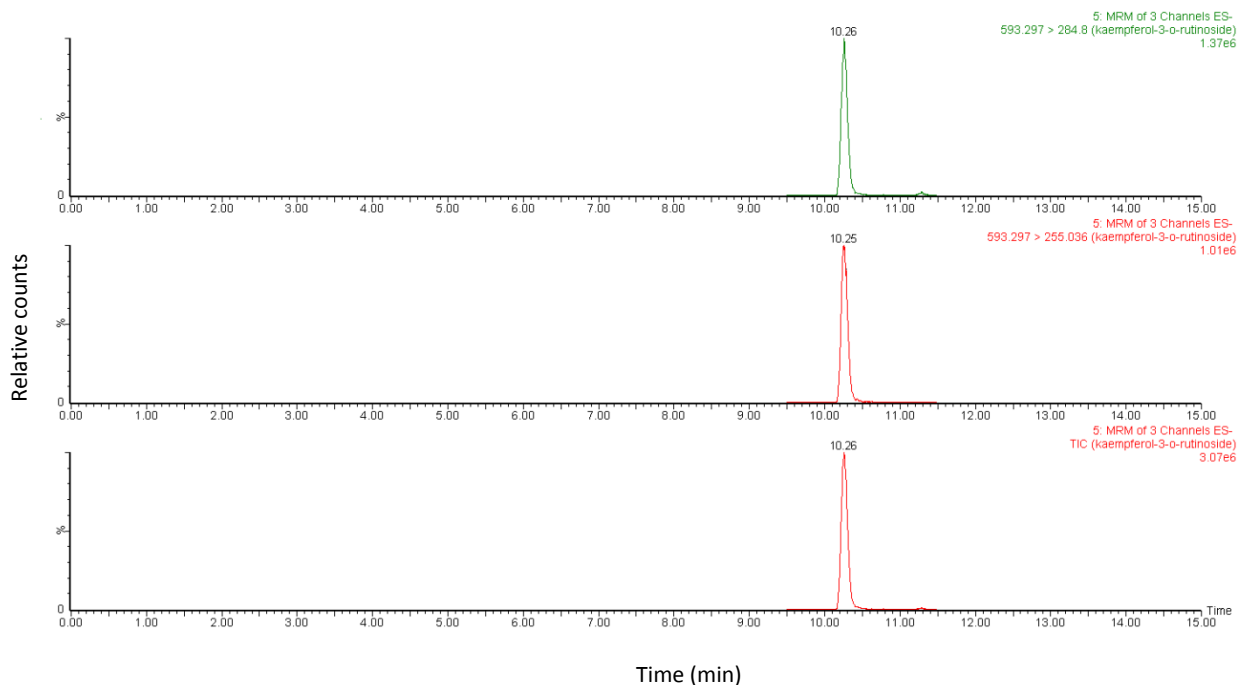
Figure 6.4a   Representative chromatogram of the product ions and total ion chromatogram of kaempferol-3-*o*-rutinoside monitored in the *Lycium* sample
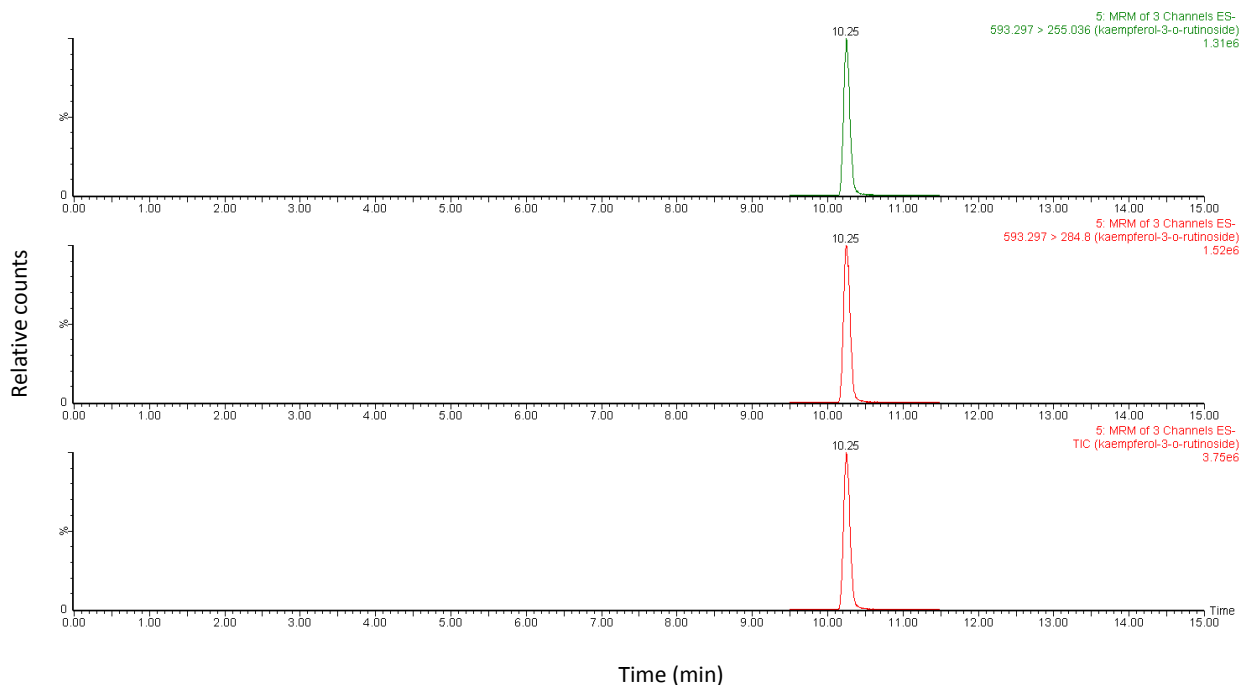
 Figure 6.4b   Representative chromatogram of the product ions and total ion chromatogram of kaempferol-3-*o*-rutinoside monitored in the mixed standard solution
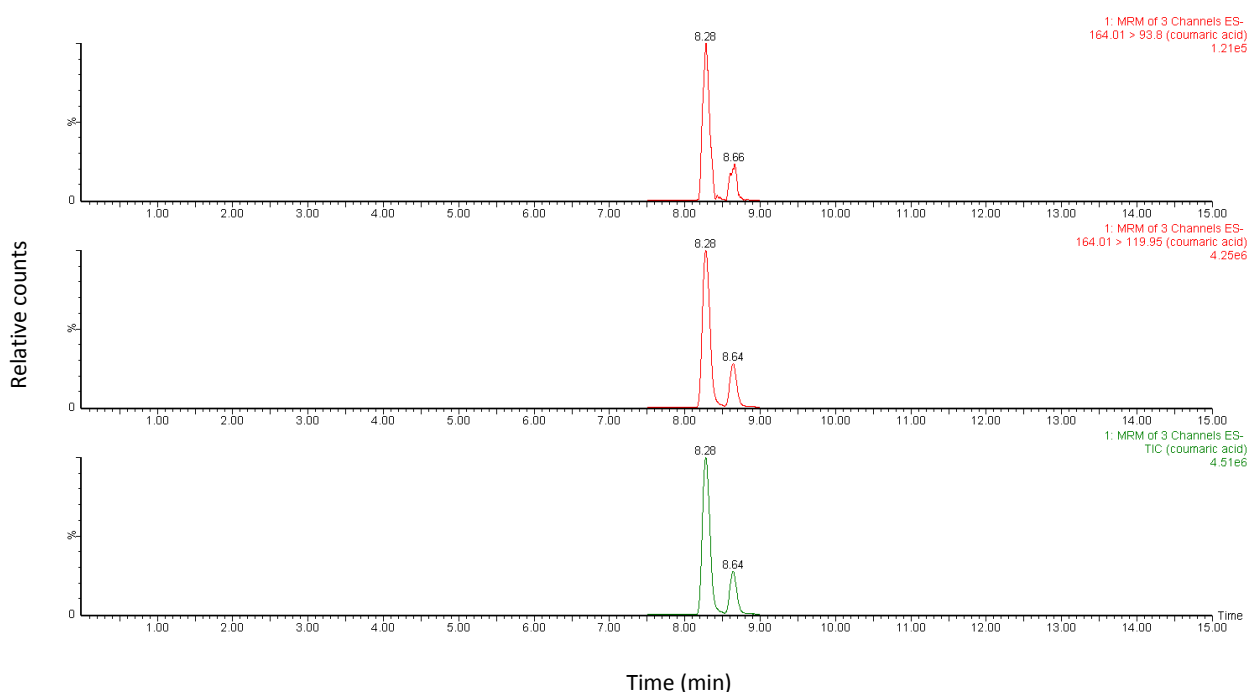
Figure 6.5a   Representative chromatogram of the product ions and total ion chromatogram of coumaric acid monitored in the *Lycium* sample
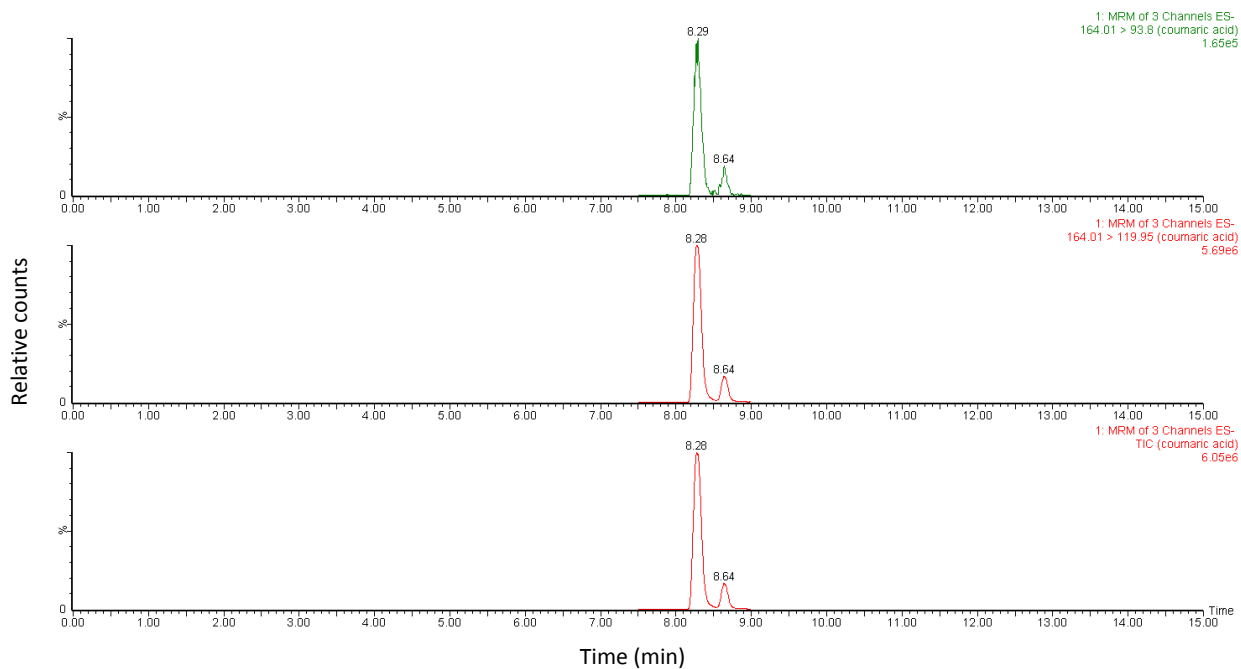
Figure 6.5b Representative chromatogram of the product ions and total ion chromatogram of coumaric acid monitored in the mixed standard solution
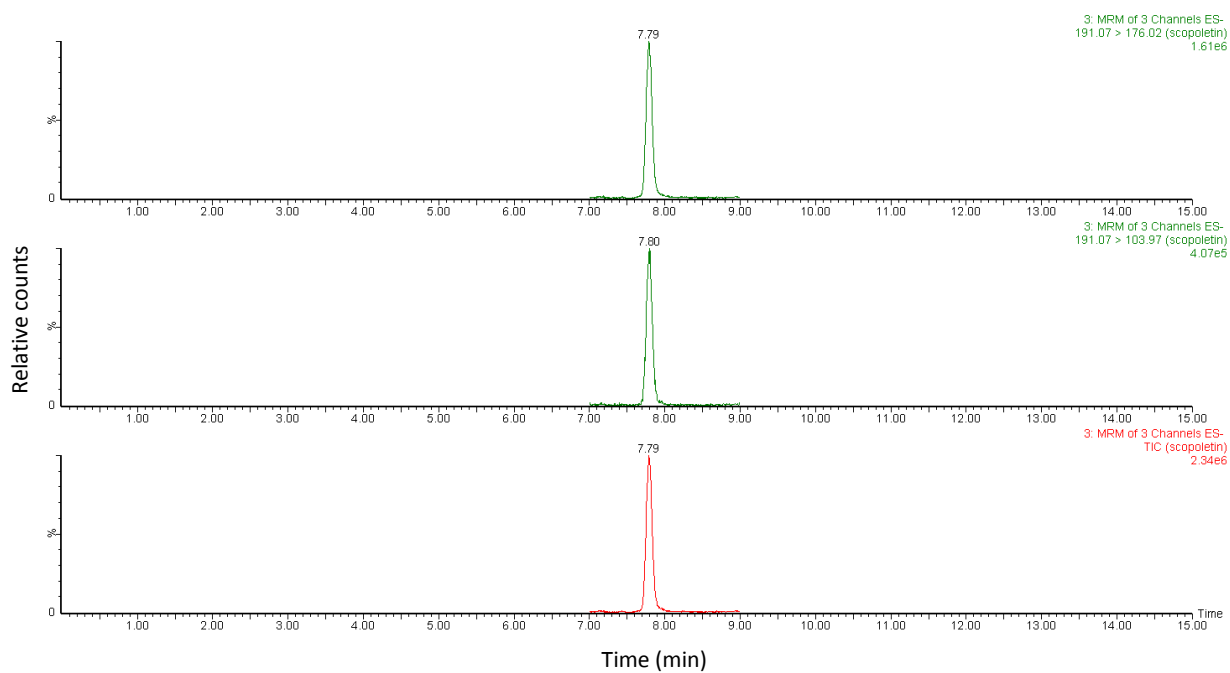
Figure 6.6a   Representative chromatogram of the product ions and total ion chromatogram of scopoletin monitored in the *Lycium* sample
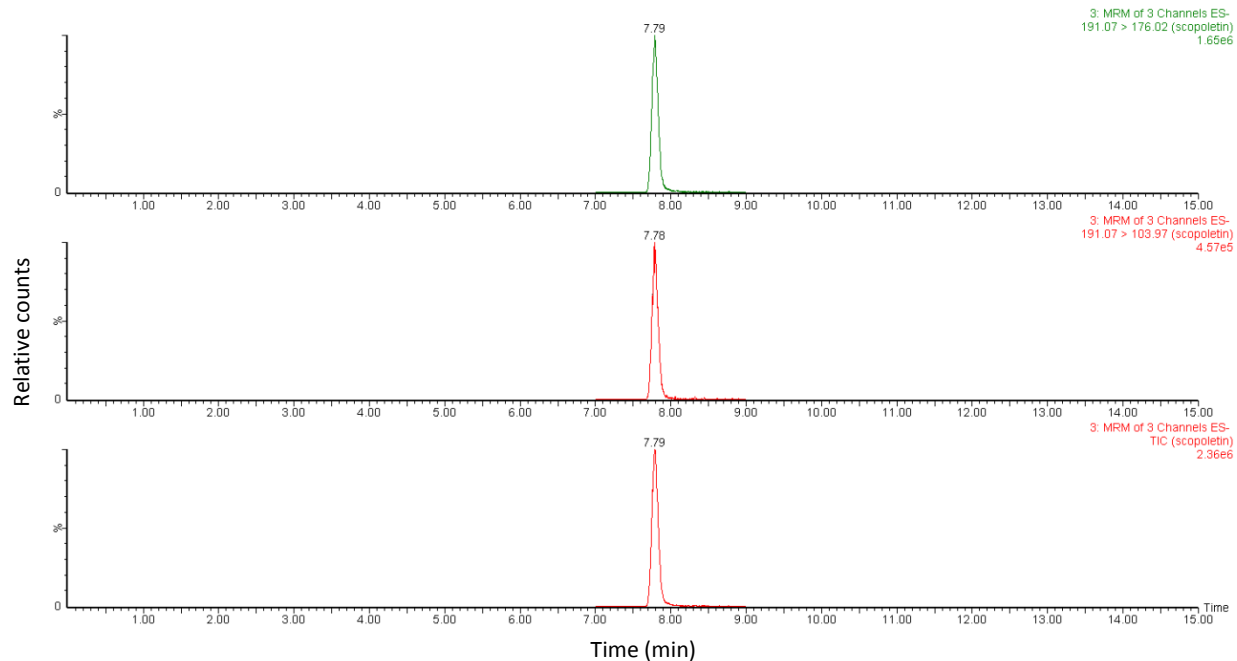
Figure 6.6b   Representative chromatogram of the product ions and total ion chromatogram of scopoletin monitored in the mixed standard solution
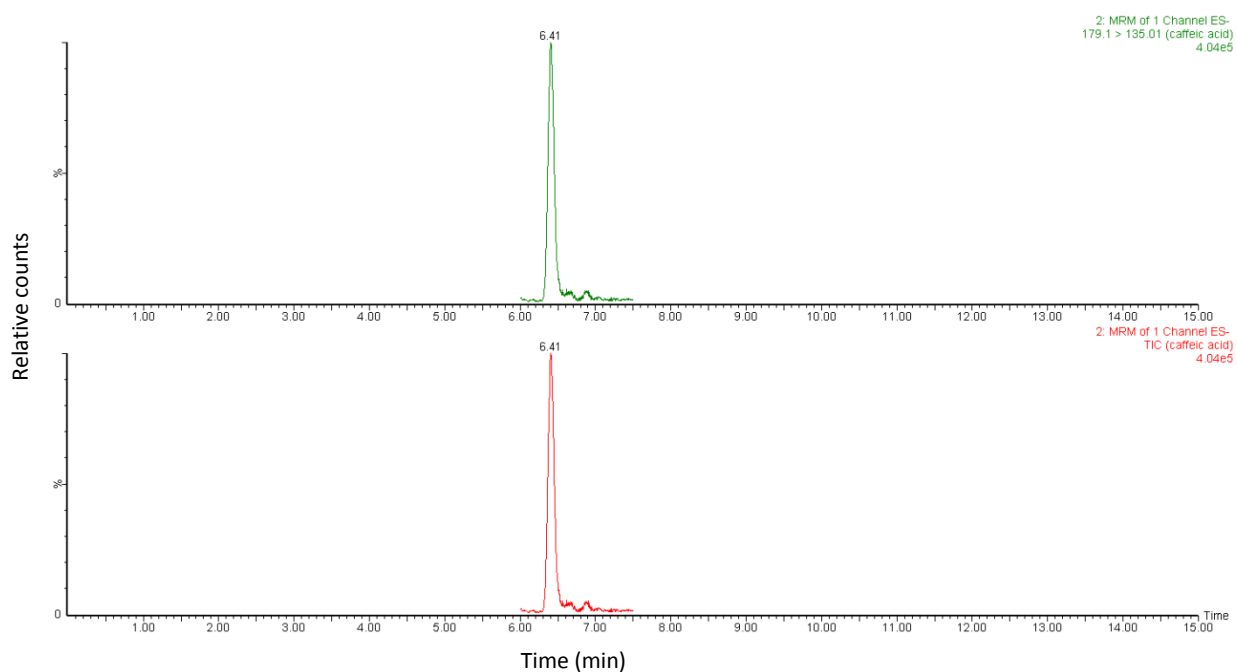
Figure 6.7a   Representative chromatogram of the product ion and total ion chromatogram of caffeic acid monitored in the *Lycium* sample
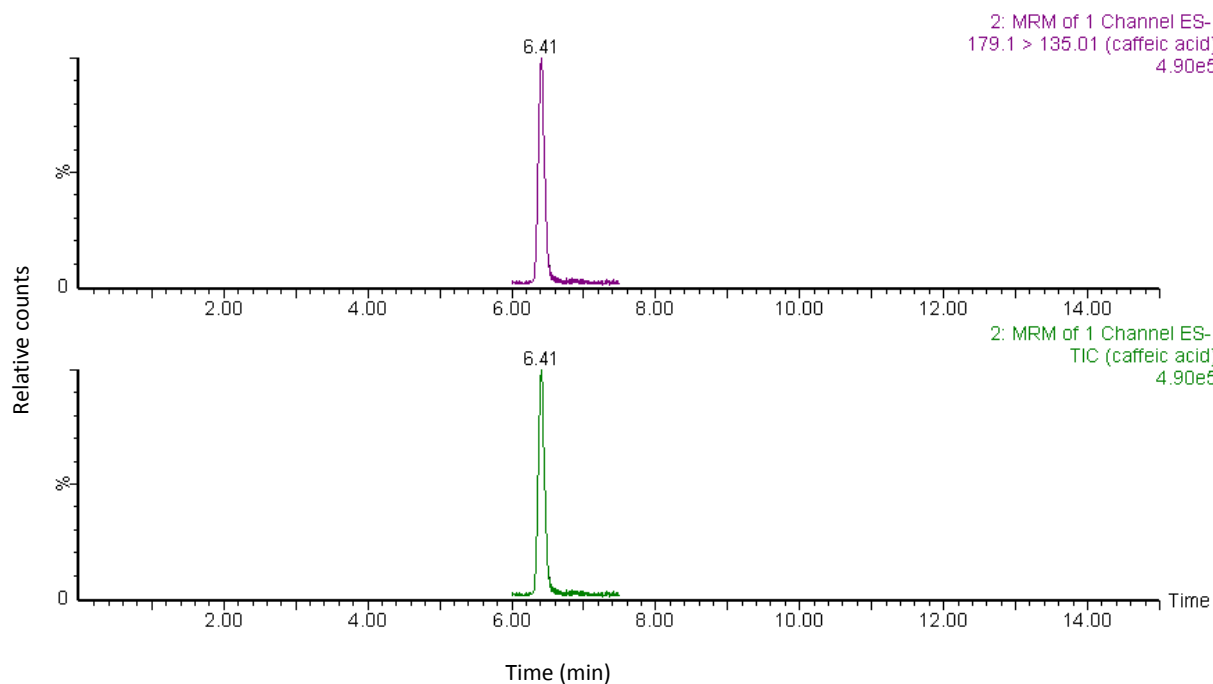
Figure 6.7b   Representative chromatogram of the product ion and total ion chromatogram of caffeic acid monitored in the mixed standard solution
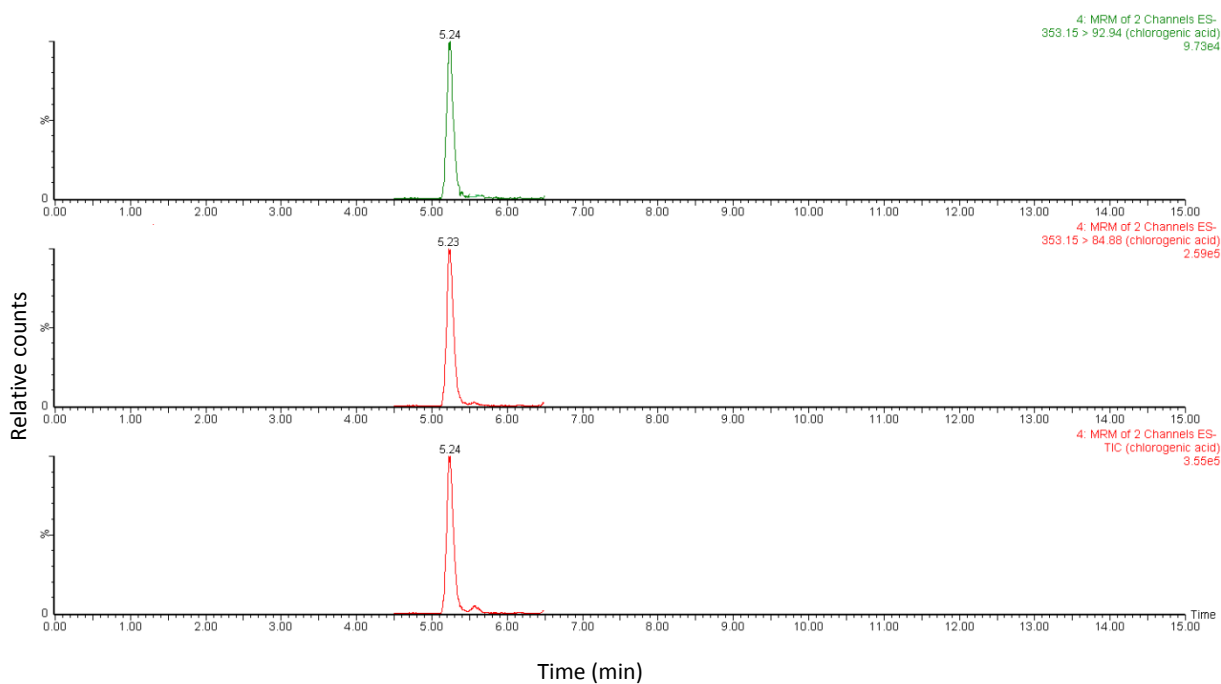
Figure 6.8a   Representative chromatogram of the product ions and total ion chromatogram of chlorogenic acid monitored in the *Lycium* sample
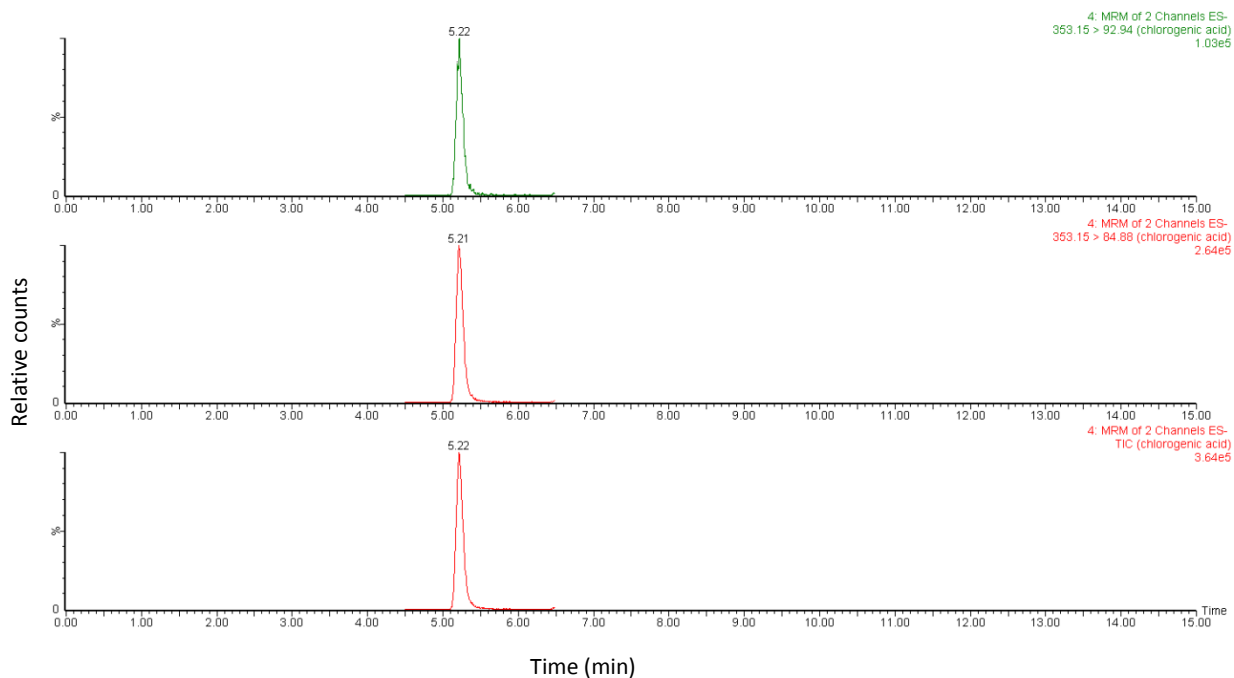
Figure 6.8b   Representative chromatogram of the product ions and total ion chromatogram of chlorogenic acid monitored in the mixed standard solution

The profile of the MRM peaks observed for the standard and sample solutions are essentially the same – their retention times and peak shape match closely. The relative intensities of the product ions for each analyte are also similar and this is summarised in Table 6.1. A close match between the relative intensities of the product ions obtained for the sample and standard solutions is a perquisite for analyte identity confirmation.

6.3 Analytical method validation

This section describes the results obtained for the method validation parameters examined.

6.3.1 Analyte identity confirmation

According to the guidance document set by the European Commission Directorate for Agriculture guidelines for the LC–MS/MS, identification was conducted based on the retention time, on two (at least) selected ion transitions (productions) and on their relative abundance (103). Analyte identity confirmation results are presented in Table 6.1.

Table 6.1 Analyte identity confirmation in *Lycium* by comparison of product ion intensities obtained for the sample and standard chromatograms

Analyte	*m/z*	Relative intensity [1]		relative difference (%) [2]	Permitted tolerance (%) [3]	Pass/fail permitted tolerance
		standard	sample			
	300	100	100	-		pass
rutin	271	61	60	1.6	± 20	pass
	255	31	30	3.2	± 25	pass
isorhamnetin-3-*o*-rutinoside	315	100	100	-		pass
	299	61	53	14	± 20	pass
kaempferol-3-*o*-rutinoside	284	100	100	-		pass
	255	86	73	16	± 25	pass
coumaric acid	120	100	100	-		pass
	93	29	28	3.5	± 25	pass
scopoletin	176	100	100	-		pass
	103	40	36	10	± 25	pass
caffeic acid	135	100	100	-		pass
chlorogenic acid	191	100	100	-		pass
	93	50	37	26	± 25 [4]	fail

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[1] Average calculated from 7replicates
[2] Relative difference = [Intensity of standard –intensity of sample) / (intensity of standard)] x 100
[3] Maximum permitted tolerance of the European Union guidelines is ± 20%
[4] Maximum permitted tolerance of the European Union guidelines is ± 25%

6.3.2 Accuracy

In this study, method accuracy is determined by fortification recoveries carried out at 50, 100 and 200% of the unfortified analyte concentration. The recovery results for *Lycium* are presented in Table 6.2 .The average recovery was 98.5% (range 90.8% to 109.4%) with an average RSD of 4.5% (range 1.7% to 9.2%). These recoveries and RSD's are good for the analyte concentrations measured.

Table 6.2   Recovery results for *Lycium* fortification studies

Analyte	Fortification level[5]							
	50%		100%		200%		Average [6] %recovery	%RSD
	%recovery	%RSD	%recovery	%RSD	%recovery	%RSD		
rutin	93.1	3.7	90.2	7.9	89.1	8.6	90.8	6.7
isorhamnetin-3-*o*-rutinoside	105.6	3.4	101.7	1.7	100.5	2.5	102.6	2.8
kaempferol-3-*o*-rutinoside	92.2	3.8	94.5	2.9	95.2	2.5	93.9	3.1
coumaric acid	98.4	7.5	88.6	9.3	102	3.8	96.3	6.8
scopoletin	89.6	7.0	95.3	5.5	91.2	3.3	92.0	5.3
caffeic acid	89.9	7.1	110.8	9.5	113.7	4.2	104.8	1.9
chlorogenic acid	101	9.2	112.8	8.2	114.3	7.4	109.4	4.5

[5] %Recovery ± %RSD calculated from seven replicate extractions and analyses
[6] Average recovery of all three fortification levels ± %RSD

6.3.3 Precision of quantitation

Linearity of the calibration curve, precision of retention time, precision of quantitation and quantitation results are presented below in Table 6.3.

Table 6.3 Linearity of calibration curve, quantitation results, precision of quantitation and precision of retention time (RT)

Analyte	Linearity (r^2)	Precision [1]		LOD [2] (mg g^{-1})	LOQ [3] (mg g^{-1})
		Amount (mg g^{-1}) ± %RSD	RT [4](min) ± %RSD		
rutin	> 0.99	34.8 ± 4.3	9.37 ± 0.02	0.42	1.4
isorhamnetin-3-*o*-rutinoside	> 0.99	1.6 ± 2.8	10.30 ± 0.01	0.13	0.4
kaempferol-3-*o*-rutinoside	> 0.99	0.8 ± 6.1	10.20 ± 0.01	0.14	0.47
coumaric acid	> 0.99	10.3 ± 6.8	8.30 ± 0.02	0.25	0.84
scopoletin	> 0.99	1.7 ± 4.3	7.75 ± 0.03	0.06	0.20
caffeic acid	> 0.99	0.1 ± 9.3	6.35 ± 0.01	0.04	0.12
chlorogenic acid	> 0.99	4.1 ± 5.9	5.21 ± 0.01	0.74	2.47

[1] Average and RSD calculated from *n* = 7 replicates
[2] LOD = limit of detection determined as 3x SD, measured for sample LB7
[3] LOQ = limit of quantitation determined as 10x SD, measured for sample LB7
[4] RT = retention time

The LOQ and LOD values are sufficiently low to quantify the concentrations of all the analytes of interest in the *Lycium* raw dried herb with confidence as shown in Table 6.3. The calibration curves for each analyte show excellent linearity with R^2 ≥0.999.

6.3.4 Stability

For accurate quantitation the stability of the sample and standard needs to be determined. The standards and samples were stored at -4 ^{o}C freezer and analysed over a 72 h period. The criterion for stability is set at a peak area decrease of ≥2% and over this period the analytes in the sample and standard are stable.

Table 6.4   Observed variations across all *Lycium* samples

Sample[2]	Concentration (mg g^{-1}) ±%RSD [1]						
	Rutin	Isorhamnetin-3-0-rutinoside	Kaempferol-3-0-rutinoside	Coumaric acid	Scopoletin	Caffeic acid	Chlorogenic acid
LB1	16.1 ± 9.3	0.37 ± 3.0	0.37± 8.5	6.8 ± 4.45	0.77 ± 6.7	0.18 ± 3.8	3.7 ± 5.4
LB2	43.1 ± 8.5	0.56 ± 4.8	0.26 ± 1.9	10.3± 7.0	0.33± 9.0	< LOD	< LOD
LB3	19.5 ± 3.3	0.94 ± 7.5	0.43 ± 4.9	10.6± 4.1	1.4 ± 3.8	0.075 ± 9.4	1.4 ± 6.6
LB4	19.1 ± 3.8	1.23 ± 6.8	0.58 ± 7.8	10.2 ± 5.3	1.3 ± 6.9	< LOD	1.1 ± 5.4
LB5	48. 2 ± 6.2	1.46 ± 5.2	0.78 ± 7.8	12.1± 5.7	0.78 ± 6.7	0.15 ± 7.1	3.7 ± 5.3
LB6	23.1± 2.8	0.62 ± 6.5	0.41 ± 5.8	11.2 ± 4.1	1.1 ± 6.0	0.24 ± 7.0	4.1 ± 5.8
LB7[3]	34.8 ± 4.3	1.65 ± 2.8	0.77 ± 5.9	10.3 ± 6.9	1.6 ± 4.3	0.11 ± 9.3	4.1 ± 5.9
LB8	49.2± 3.3	1.43 ± 6.6	0.67 ± 4.6	12.2 ± 5.9	2.1 ± 6.9	0.15 ± 7.0	7.1 ± 9.0
LC1	25.3 ± 2.5	1.23 ± 4.4	0.43 ± 8.0	9.7 ± 5.8	2.5 ± 6.4	0.09 ± 6.1	2.9 ± 8.3
LC2	21.5 ± 3.6	0.98 ± 2.6	0.51 ± 3.7	10.2 ± 5.6	1.5 ± 5.5	0.16 ± 4.3	4.2 ± 7.2
LC3	35.1 ± 6.7	1.11 ± 3.5	0.44 ± 3.6	8.6 ± 7.1	2.3 ± 5.1	0.32 ± 6.4	9.1 ± 7.3
LC4	33.1± 4.0	1.25 ± 3.4	0.63 ± 4.9	10.2 ± 7.0	2.6 ± 4.7	0.18 ± 6.7	5.1 ± 5.3
Fold variation[4]	3.1	3.9	6.0	1.8	7.8	4.3	7.3

[1] Average calculated from 7 replicates ± % RSD
[2] LB = *Lycium barbarum*, LC = *Lycium chinense*
[3] Method validation performed on LB7
[4] Fold variation = (highest concentration) / (lowest concentration); (<LOD values omitted from this calculation)

Sample LB7 was used for analytical method development. All seven analytes were detected in the sample at ≥ 0.1 mg g^{-1}. The developed method was then employed for the analysis of the other *Lycium* samples. The greatest analyte variation was observed for scopoletin (7.8 fold) and chlorogenic acid (7.3 fold), followed by kaempferol-3-0-rutinoside (6.0 fold) and caffeic acid (4.3 fold). The fold variation for other constituents is lower by comparison, namely coumaric acid (1.8 fold), isorhamnetin-3-0-rutinoside (3.9 fold) and rutin (3.1 fold). It is reasonable to believe that these significant variations in the concentration of key analytes in the herb will have an impact on its efficacy for the consumer.

6.4 Phytochemical profiling

Phytochemical profiling offers integral characterization of a given herb using multiple qualitative and quantitative measures, encompassing the wide-ranging properties of a given herb. HPLC and GC coupled to MS are the primary instruments used in such analysis.

No significant qualitative differences were observed in the profiles of *Lycium barbarum* and *Lycium chinense* obtained using LC-PDA and GC-MS analysis as may be seen in Figures 6.9 – 6.10.
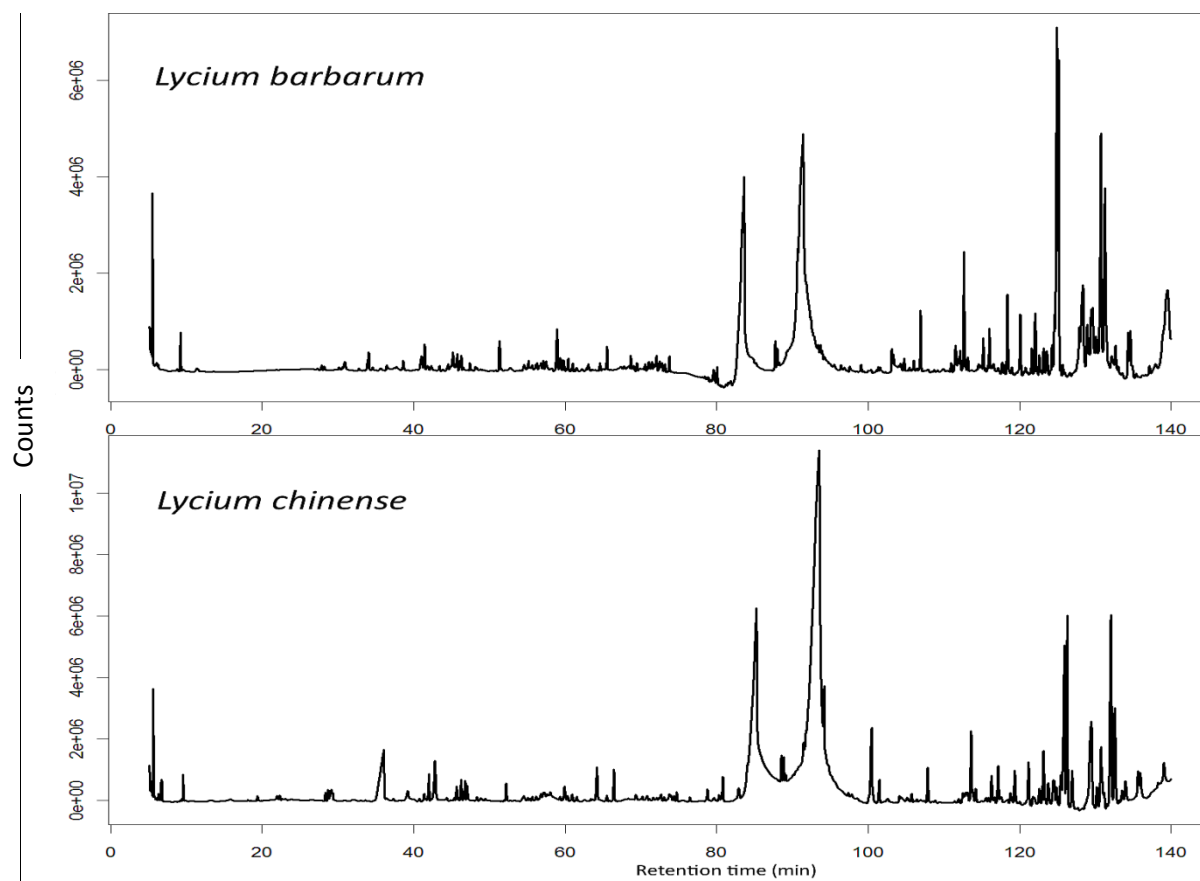
Figure 6.9 Representative GC-MS profile of *Lycium barbarum* and *Lycium chinense*
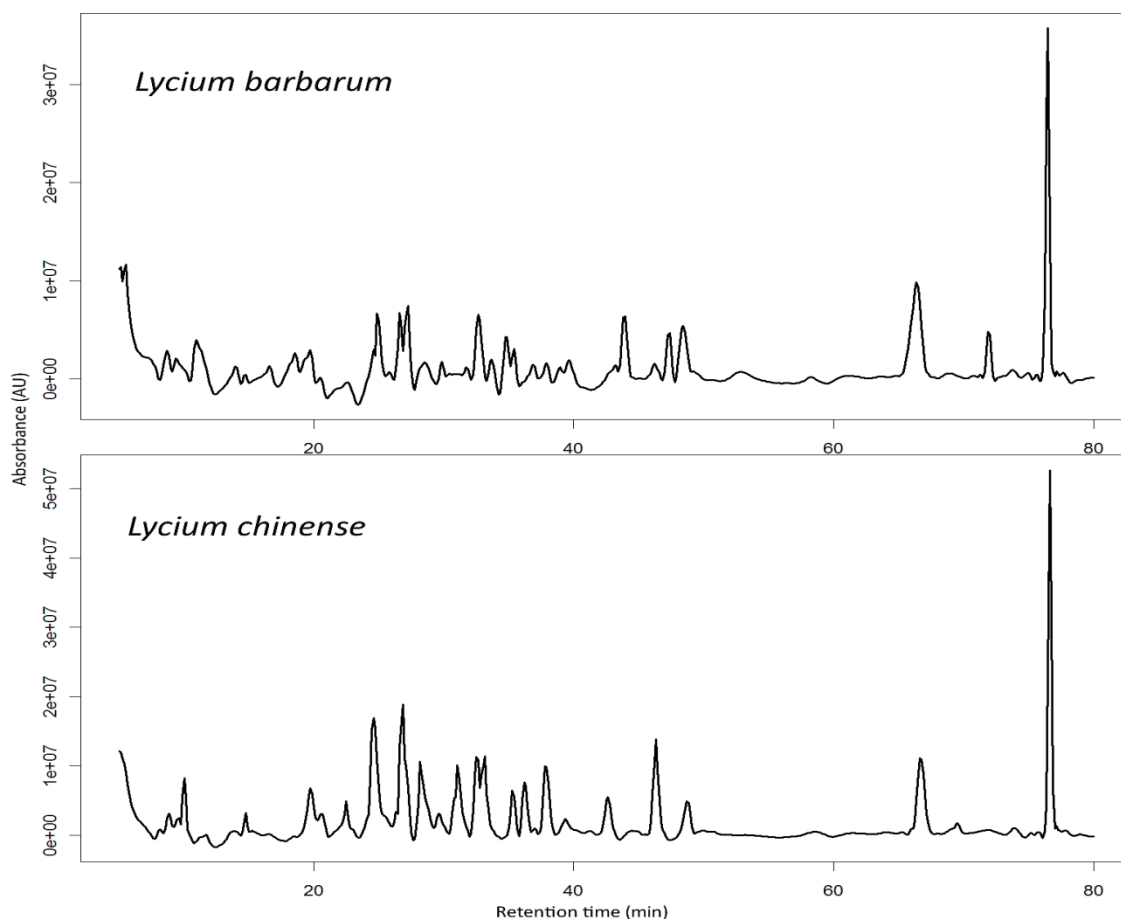
Figure 6.10   Representative LCP-DA profile of *Lycium barbarum* **and** *Lycium chinense*

Chromatographic characterization of the *Lycium* samples using LC-PDA was carried out at 280 nm. The detection of peaks in a chromatogram is important for qualitative and quantitative analysis because the amount of information increases as more peaks are detected. The use of two different chromatographic techniques to compare the chemical profiles of *Lycium* labelled as *barbarum* or *chinense* provides stronger confidence in the conclusions reached. GC-MS gives the profile of the volatile components while LC-PDA gives the profile of moderately polar and non-polar UV-absorbing components.

6.5 Multivariate analysis

6.5.1 Processing of chromatograms in preparation for multivariate analysis

Prior to multivariate analysis, shifts in peak retention time between chromatograms due to unavoidable experimental variations, such as column aging and small mobile phase composition changes, have to be corrected for. The package msProcess was employed for pre-processing of chromatographic data to remove variables such as instrumental noise, baseline drift and peak retention time variations.

Once each chromatogram is processed, they are compared to each other to determine a set of representative peaks present in most of the chromatograms.

6.5.2 Statistical analysis

The multivariate statistical techniques of hierarchical cluster analysis (HCA) and principal component analysis (PCA) were used to visualize the differences and similarities between the *Lycium* samples based on peak intensity. A cluster dendrogram is plotted for HCA and a scores plot for PCA. These plots enable us to look for grouping of samples. PCA was also used to determine which chromatographic peaks and therefore phytochemicals are responsible for the observed differentiation (if any).

In this study, an efficient method for the analysis of variance between clusters was applied and Euclidean distance was selected as measurement. The clustering results of an unsupervised hierarchical analysis are illustrated as a dendrogram in Figure 6.11a for the GC-MS data and Figure 6.12a for the LC-PDA data. Figure 6.11b shows the HCA scores plot for the GC-MS data and Figure 6.12b for the LC-PDA data. The dendrograms for

the GC-MS and LC-PDA show a grouping pattern into two main clusters, with no apparent

clustering of *L. barbarum* and *L. chinense* into distinct groups.
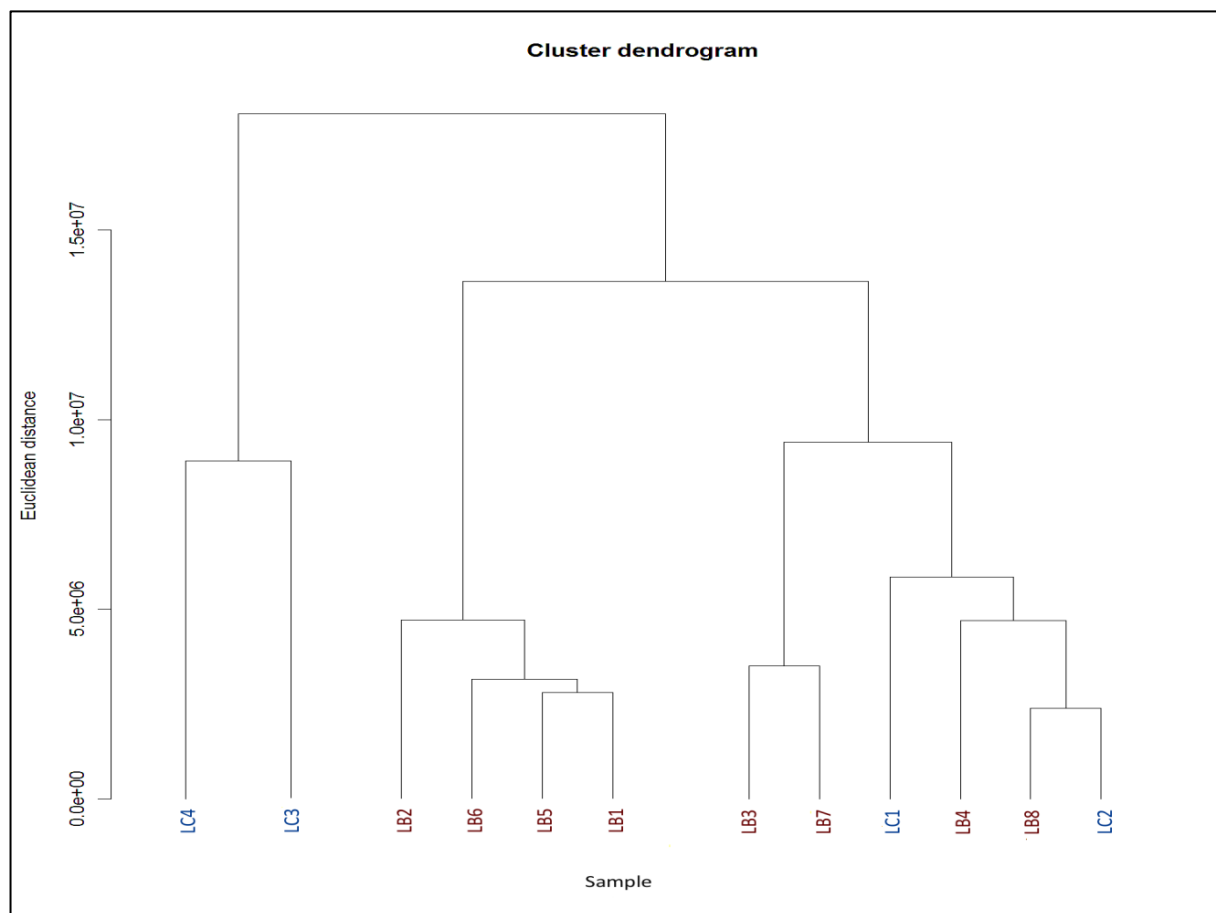
Figure 6.11a Representative GC-MS HCA cluster analysis of *Lycium* samples

The scores plot for the GC-MS analysis (Figure 6.11b) shows a similar result to that obtained for HCA cluster dendrogram analysis, namely the *L. chinense* samples LC3 and LC4 appear in a separate region. However since the *L. chinense* (LC1 and LC2) appear to cluster with the other *L. barbarum* samples a conclusion is made that *L. barbarum* and *L. chinense* are not distinctly different.
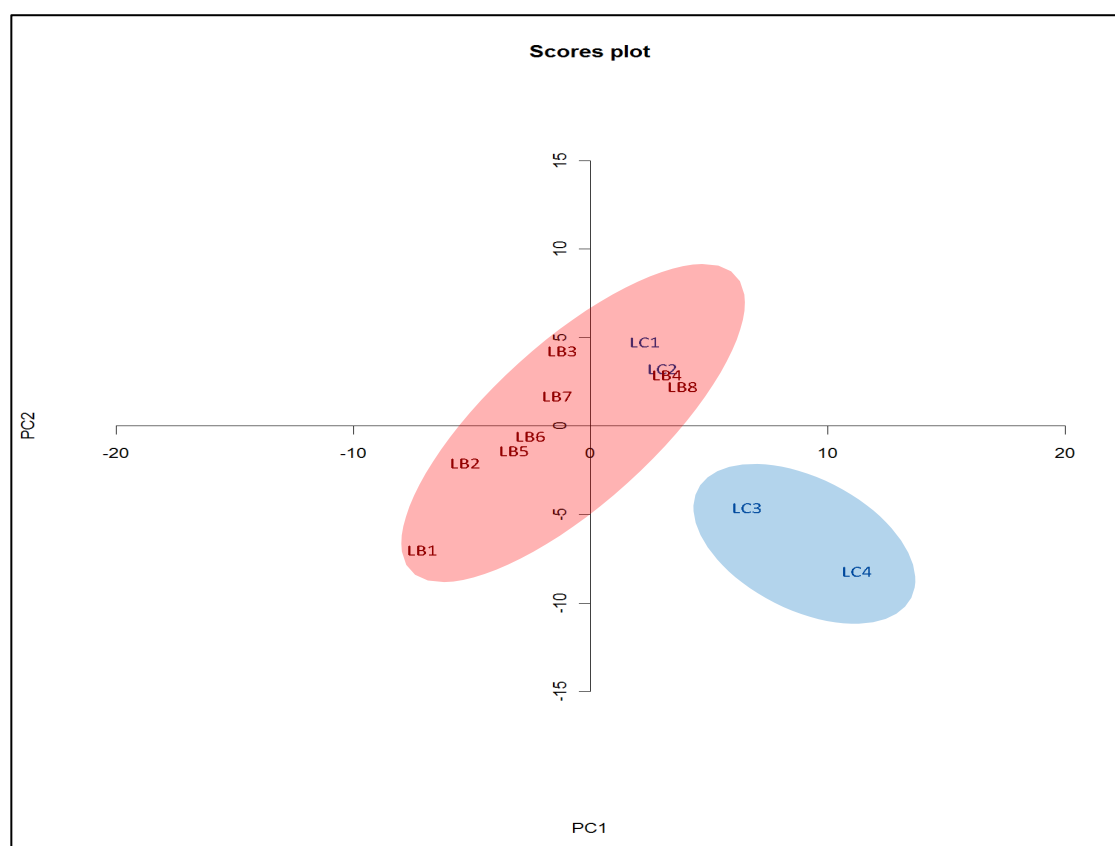
Figure 6.11b GC-MS PCA scores plot of *Lycium* samples
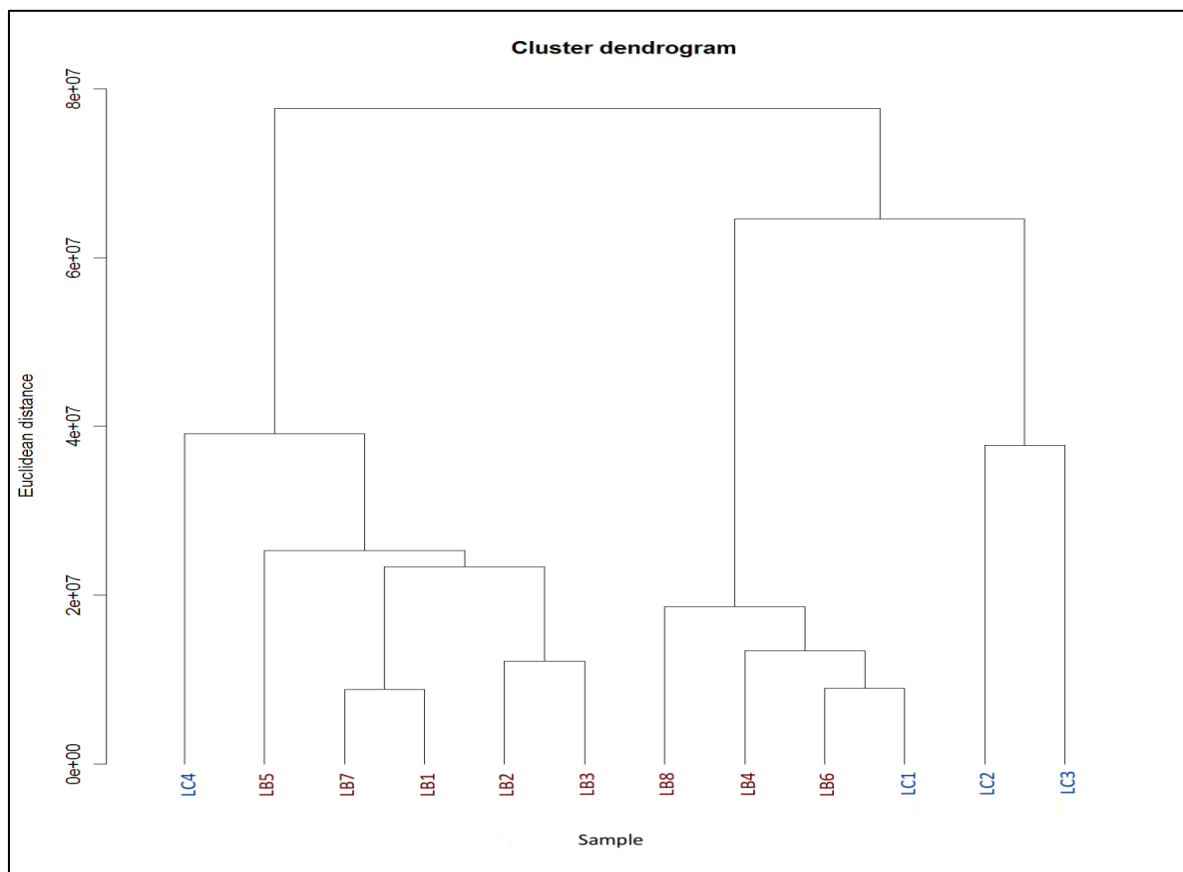
Figure 6.12a Representative LC-PDA HCA cluster dendrogram of *Lycium* samples
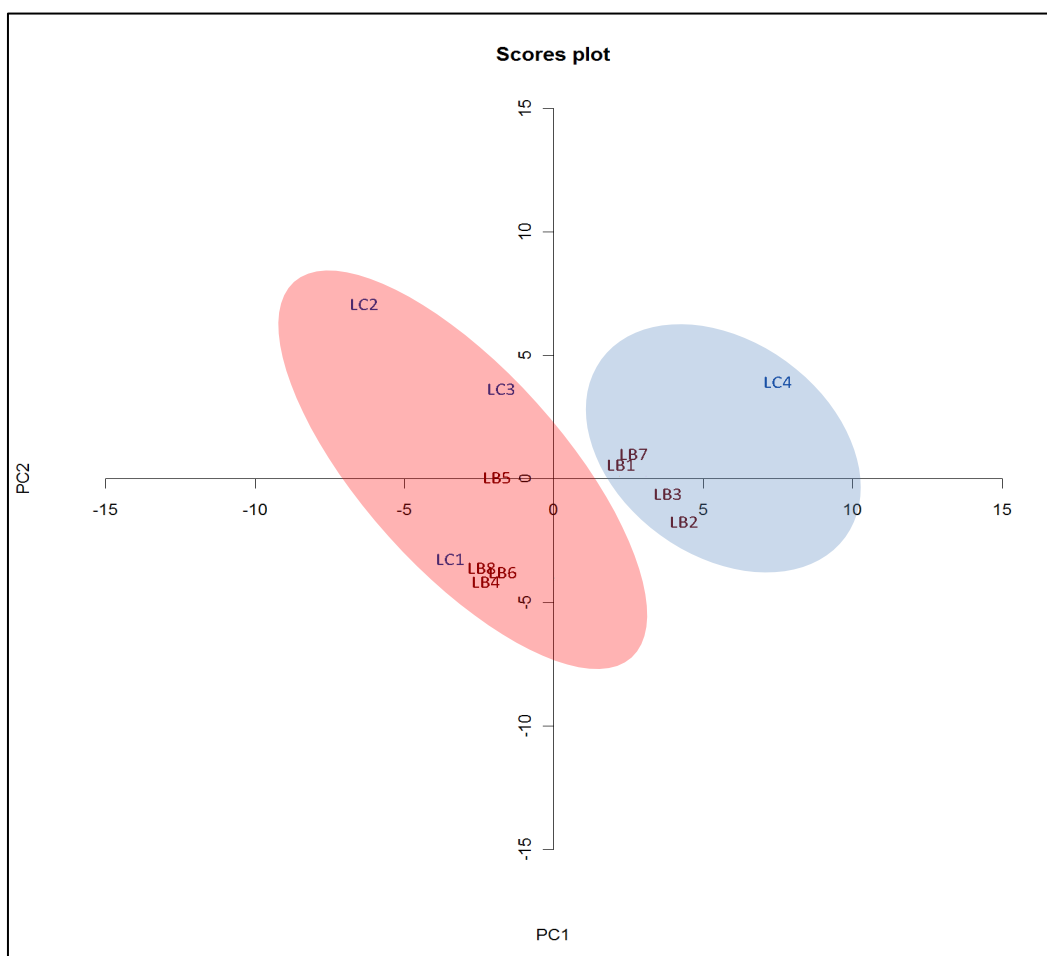
Figure 6.12b LC-PDA scores plot of *Lycium* samples

A HCA and PCA analysis of the GC-MS and LC-PDA chromatograms demonstrate that there is no apparent phytochemical difference between the two *Lycium* species, however definitive conclusions cannot be drawn at this stage as the assumption is made that the *L. barbarum* and *L. chinense* samples have been correctly labelled – we were able to obtain one certified *L. barbarum* sample but an authenticated sample of *L. chinense* was not available. Assuming that the samples are correctly labelled, our findings are in agreement with a reported study where eight species of *Lycium* berries were analysed by Fourier-transform infrared spectroscopy (FTIR), second derivative IR spectra and two-dimensional correlation IR (88). Their results indicated that the chemical constituents in these species

of herbs were not distinctively different and varied only in some IR peak intensities .

Since there is almost no cost difference between the two *Lycium* species, there is unlikely to

be any motivation to mislabel the species.

6.7 DPPH radical scavenging capacity of *Lycium*

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity of *Lycium* samples

was determined. The DPPH assay is an electron transfer based assay involving a redox

reaction between the DPPH and the antioxidant compound being measured, using gallic

acid as a reference for antioxidant capacity. That is, the assay measures how much better

or worse the *Lycium* extract is at being an antioxidant compared to gallic acid. The sample

DPPH antioxidant scavenging capacity reported as the gallic acid equivalent (GAE) is shown

in Table 6.5. Samples LC1 and LC4 samples show the highest antioxidant capacity across all

samples.

Table 6.5 Summary of DPPH assay results for the *Lycium* samples

Sample [1]	Gallic acid equivalent (µg mg^{-1})
LB1	1.41
LB2	1.91
LB3	1.56
LB4	1.69
LB5	1.59
LB6	1.82
LB7	1.63
LB8	1.27
LC1	2.03
LC2	1.91
LC3	1.56
LC4	2.64
Fold variation[2]	1.87

The GAE range from 1.41 to 2.64 (mean = 1.75 µg mg^{-1}) with a fold variation of 1.87. The mean GAE is 1.61 for *L. barbarum* and 2.04 for *L. chinense* but this difference is unlikely to be significant due to small sample size of the latter. The results in Table 6.5 are presented in the form of a chart to obtain an alternate view of the variations observed. This again shows that three out of the four *L. chinense* species cluster at the top for DPPH activity.

[1] LB = *Lycium barbarum*, LC = *Lycium chinense*
[2] Gallic acid fold variation = (highest GAE)/ (lowest GAE)
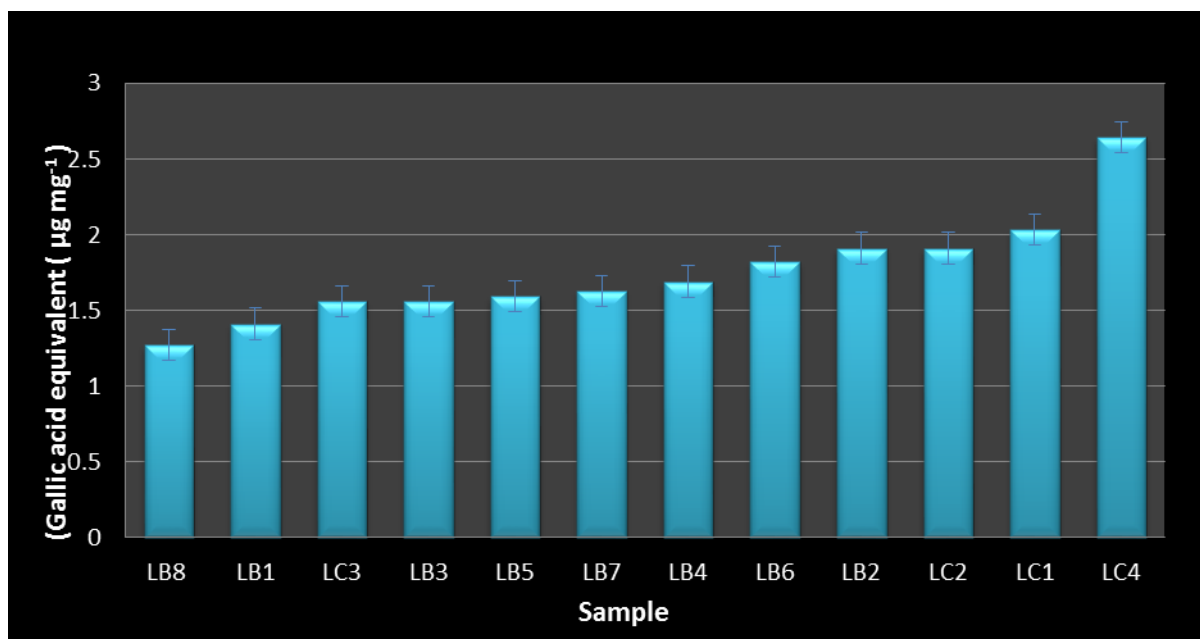
Figure 6.13 *Lycium* **samples arranged in increasing DPPH activity**

A chemical antioxidant assay such as the DPPH-based assay maybe considered albeit fairly basic and preliminary as it can be useful as a rapid and simple method for the evaluation of the pharmacological variability of a given herb. A downside of this assay is that its measurements are yet to be correlated with biological or physiological activity (96). However there are several other types of antioxidant assays that may be considered in the assessment of different antioxidant parameters.

6.8 Correlation between DPPH activity (as GAE) and analyte concentrations

An evaluation of the correlation coefficient was established by linear regression analysis between the individual analytes and total analyte concentration and their related activity in the assay. Values obtained were r^2 = 0.012, 0.101, 0.01, 0.35, 0.07, 0.16 and 0.05 for rutin, isorhamnetin-3-*o*-rutinoside, kaempferol-3-*o*-rutinoside, coumaric acid, scopoletin, caffeic

acid and chlorogenic acid respectively. The correlation coefficient of the total analyte concentration was 0.091. These results indicate that no particular analyte can be identified to make a major contribution to DPPH activity and similarly no correlation with total analyte concentration was demonstrated.

Chapter 7

General Summary and Conclusions

7.1 Summary

Herbal medicine has a long therapeutic history over thousands of years and continues to play an essential role in healthcare. An estimated 80% of the world's inhabitants rely mainly on traditional medicines for their primary healthcare. However, plant based medicines comprise a plethora of phytochemical constituents, which trigger concerns around ensuring consistent quality and safety. As natural products, inherent compositional variations between crops pose a significant challenge in terms of their quality control.

The complexity of applying QC to multi-herb medicines increases with each additional herb in a medicine. Moreover the therapeutic activity of the medicine depends on its phytochemical constituents. The development of analytical methods which can reliably profile the phytochemical composition in a way that accurately reflects its physiological efficacy remains a major challenge.

Current regulatory standards require herbal identity confirmation (only for single herbs), typically by high performance TLC and basic safety testing (heavy metals, pesticides and bacterial contamination). While these measures might be enough to ensure the basic safety of the herbal product, they do not necessarily provide information about its quality since the putative bio-actives are not determined unless there is a label claim regarding the concentration of particular active ingredients.

7.2 Evaluation of the quality of Qi Ju Di Huang Wan herbal mixture through analysis of targeted analytes

The complex composition of Qi Ju Di Huang Wan (QJDHW) and its common availability in the marketplace made it a suitable subject of investigation for the assessment of product quality. The proposed quality assessment methodology is suitable for routine use in a well-equipped commercial QC laboratory.

Eleven samples of QJDHW were obtained from several sources in the Australian marketplace; the analytes were selected based on the selection criteria described and samples were analysed using the developed methodology. An evaluation of the variations observed in the concentration of monitored bioactive constituents indicated significant intra and inter batch variations amongst the samples, with fold variations ranging from 4.4 to 28.9. Should these bioactive constituents play a pivotal role in the putative actions such as physiological potency then there may be a reasonable expectation that the effectiveness of the medicine in terms of its physiological outcome for the consumer, would also vary noticeably and hence be somewhat unpredictable.

Quantitation of the appropriate bioactives would provide the consumer with greater confidence regarding product efficacy. The development of stronger regulations will lead to improved quality of herbal products. This will also ensure that commercially sold herbal extracts and formulations do not have herbal substitutions or prescription drugs added in order to boost the efficacy.

A targeted analytical approach to testing commercial herbal products will allow regulators to monitor and map the variability of herbal products available in the marketplace. It is important to bear in mind that achieving an 'absolute' consistency in chemical profile may not necessarily be possible, considering the natural variations in the starting material.

However, the variants in chemical profile in addition to the fixed (diagnostic) chemical traits should be characterized in order to distinguish the natural variants from adulterant or substituent chemistries.

Despite the fact that we have demonstrated how to chemically characterise an herbal formulation and single herb, we have not performed comprehensive biological testing to determine which sample is the most biologically or physiologically active. The assumption is made is that the sample with the highest analyte concentrations would be the most active. While this appears a reasonable assumption, the link should be established by means of biological testing. If a formulation has been thoroughly tested, for example in human trials, and found to be effective, its chromatographic profile could be established and used as a reference where future batches could be manufactured to match this target profile as closely as possible. To achieve this, the composition of the formulation might be altered – therefore instead of a constant herbal composition, the composition is varied to achieve a 'constant' chromatographic profile or target analyte profile.

7.3 Quality evaluation of *Lycium barbarum* and *Lycium chinense* through targeted and untargeted analysis

7.3.1 Targeted analysis

L. barbarum is a component of the QJDHW formulation. An investigation into the phytochemical composition and variability of *Lycium barbarum* and *Lycium chinense* was performed. For the targeted analysis, analytes of interest were selected by the same method employed for QJDHW and a validated method was developed for their quantitation. The results indicate no significant difference in chemical composition between the two *Lycium* species (eight *L. barbarum* and four *L. chinense* samples). Fold variations for analytes

determined ranged from 1.8 to 7.8 amongst samples. It is rational to postulate that significant variations in the concentration of key analytes in the herb will have an impact on its efficacy and hence on herbal formulations which use it as a major constituent.

7.3.2 Untargeted analysis

Here the chromatographic profiles of the herbs are compared rather than the concentrations of a set of targeted analytes. The GC-MS and LC-PDA chromatographic profiles of the extracts were compared and analysed by the multivariate statistical techniques of PCA and HCA for their phytoequivalence. The results indicate little qualitative differences between the *L. barbarum* and *L. chinense* species. The results from the targeted and untargeted analysis are in harmony – namely there is no significant difference between the chemical profiles of the two *Lycium* species.

7.4 Future work

This research established that it is viable to statistically analyse the chromatographic profiles of the herbal extracts to check for phytoequivalence. It would be interesting to investigate if a statistical analysis of an NMR spectrum of the herbal extracts would also place the samples in similar positions in the cluster dendrogram and scores plot. If so, the NMR technique might represent an alternative method to assess phytoequivalence which is relatively fast to perform.

Other spectral characterisation techniques such as NMR and IR could also be included in product characterisation. Further investigations may be the use of time-of-flight LC-MS for the characterisation of large molecules such as polysaccharides.

This research only carried out one pharmacological test, namely DPPH testing and found that it did not correlate with the analytes tested. This is not surprising as the constituents are not strong antioxidants and they are not present in high concentrations. Potential future research could include the evaluation of the herbal samples for a range of pharmacological and biological activities (in-vitro and in-vivo analysis) relevant to the formulation to observe if the chemical ranking correlates with the biological testing results.

Finally, the methods adopted here need to be communicated to industry and the scientific community to ensure harmonisation of herbal QC methods.

References

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APPENDICES

Appendix 1

This section describes selected reported literature for the bioactivity of analytes monitored in *Lycium*.

<u>**Rutin**</u>

Anti-hepatotoxic: Guardia T, Rotelli AE, Juarez AO, Pelzer LE. Anti-inflammatory properties of plant flavonoids - effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. Il Farmaco. 2001; 56(9):683-687. DOI: 10.1016/S0014-827X(01)01111-9

Anti-oxidant: Qian J-Y, Liu D, Huang AG. The efficiency of flavonoids in polar extracts of *Lycium chinense* Mill fruits as free radical scavenger. Food Chem. 2004; 87(2):283-8. DOI: 10.1016/j.foodchem.2003.11.008

Anti-inflammatory: Wen CC, Chen HM, Yang NS. Developing phytocompounds from medicinal plants as immunomodulators. In: Shyur LF, Lau ASY, editors. Advances in botanical research: Recent trends in medicinal plant research. 2012;62:197-272. DOI: 10.1016/B978-0-12-394591-4.00004-0

cAMP-phosphodiesterase-inhibitor: Marles RJ, Farnsworth NR. Antidiabetic plants and their active constituents. Phytomedicine. 1995; 2(2):137-189. DOI: 10.1016/S0944-7113(11)80059-0

Vasodilator: Prince PSM, Priya S. Preventive effects of rutin on lysosomal enzymes in isoproterenol induced cardio toxic rats: Biochemical, histological and in vitro evidences. Eur J Pharmacol. 2010; 649(1–3):229-35. DOI: 10.1016/j.ejphar.2010.08.054

Cytoprotective: Amagase H, Farnsworth NR. A review of botanical characteristics, phytochemistry, clinical relevance in efficacy and safety of *Lycium barbarum* fruit (Goji). Food Res Int. 2011; 44(7):1702-1717. DOI: 10.1016/j.foodres.2011.03.027

<u>**Isorhamnetin-3-0-rutinoside**</u>

Anti-inflammatory: Kim J, Lee KW, Lee HJ. Polyphenols suppress and modulate inflammation: Possible roles in health and disease. In: Watson RR, Preedy VR, Zibadi S, editors. Polyphenols in human health and disease. San Diego: Academic Press; 2014. 393-408.

Antioxidant: Amagase H, Sun B, Borek C. *Lycium barbarum* (goji) juice improves in vivo antioxidant biomarkers in serum of healthy adults. Nutr Res. 2009; 29(1):19-25. DOI: 10.1016/j.nutres.2008.11.005

Hepatoprotective: Zhang R, Ah Kang K, Piao MJ, Kim KC, Kim AD, Chae S, et al.

Cytoprotective effect of the fruits of *Lycium chinense* Miller against oxidative stress-induced hepatotoxicity. J Ethnopharmacol. 2010; 130(2):299-306. DOI: 10.1016/j.jep.2010.05.007

Pro-oxidant: Matkowski A, Jamiolkowska-Kozlowska W, Nawrot I. Chinese medicinal herbs as source of antioxidant compounds - where tradition meets the future. Curr Med Chem. 2013; 20(8):984-1004. DOI: 10.2174/092986713805288888

Kaempferol-3-0-rutinoside

Anti-hepatotoxic: Kim SY, Kim HP, Huh H, Kim YC. Antihepatotoxic zeaxanthins from the fruits of *Lycium chinense*. Arch Pharm Res. 1997; 20(6):529-532. DOI: 10.1007/BF02975206

Anti-oxidant: Le K, Chiu F, Ng K. Identification and quantification of antioxidants in Fructus lycii. Food Chem. 2007; 105(1):353-63. DOI: 10.1016/j.foodchem.2006.11.063

iNOS-inhibitor: Oh YC, Cho WK, Im GY, Jeong YH, Hwang YH, Liang C, et al. Anti-inflammatory effect of *Lycium* Fruit water extract in lipopolysaccharide-stimulated RAW 264.7 macrophage cells. Int Immunopharmacol. 2012; 13(2):181-189. DOI: 10.1016/j.intimp.2012.03.020

TNF-alpha-inhibitor: Li H, Liu X, Yang H, Zhu L. Effects of *Lycium barbarum* on the behavior, body weight and TNF-alpha level of rat treated with binding. Wei sheng yan jiu - Journal of Hygiene Research. 2007; 36(6):743-745.

Neuroprotective: Ho YS, Yu MS, Lai CSW, So KF, Yuen WH, Chang RCC. Characterizing the neuroprotective effects of alkaline extract of *Lycium barbarum* on β-amyloid peptide neurotoxicity. Brain Res. 2007; 1158:123-134. DOI: 10.1016/j.brainres.2007.04.075

Coumaric acid

Anti-hepatotoxic : Kiso Y, Suzuki Y, Watanabe N, Oshima Y, Hikino H. Antihepatotoxic principles of Curcuma longa rhizomes. Planta Med. 1983; 49(11):185-187. DOI: 10.1055/s-2007-969845

Anti-oxidant: Zhong Y, Shahidi F, Naczk M. Phytochemicals and health benefits of Goji berries. Dried Fruits: Blackwell Publishing Ltd.; 2013: 133-144.

Scopoletin

Immunomodulatory : Tang WM, Chan E, Kwok CY, Lee YK, Wu JH, Wan CW, et al. A review of the anticancer and immunomodulatory effects of *Lycium barbarum* fruit. Inflammopharmacol. 2012; 20(6):307-314. DOI: 10.1007/s10787-011-0107-3

Anti-hepatotoxic: Kumar S, Pandey AK. Chemistry and biological activities of flavonoids: An

overview. The Scientific World Journal. 2013;2013:16. DOI: 10.1155/2013/162750

Anti-oxidant: Wu WB, Hung DK, Chang FW, Ong ET, Chen BH. Anti-inflammatory and anti-angiogenic effects of flavonoids isolated from *Lycium barbarum* Linnaeus on human umbilical vein endothelial cells. Food Funct. 2012;3:1068-1081. DOI: 10.1039/C2FO30051F

Caffeic acid

Antioxidant: Okutan H, Ozcelik N, Yilmaz HR, Uz E. Effects of caffeic acid phenethyl ester on lipid peroxidation and antioxidant enzymes in diabetic rat heart. Clin Biochem. 2005;38:191–196. DOI: 10.1016/j.clinbiochem.2004.10.003

Anti-inflammatory: Luo Q, Cai Y, Yan J, Sun M, Corke H. Hypoglycemic and hypolipidemic effects and antioxidant activity of fruit extracts from *Lycium barbarum*. Life Sci. 2004; 76(2):137-149. DOI: 10.1016/j.lfs.2004.04.056

Anti-hepatotoxic: Yao X, Peng Y, Xu LJ, Li L, Wu QL, Xiao PG. Phytochemical and biological studies of *Lycium* medicinal plants. Chem Biodivers. 2011; 8(6):976-1010. DOI: 10.1002/cbdv.201000018

Chlorogenic acid

Hepatotropic: Marques V, Farah A. Chlorogenic acids and related compounds in medicinal plants and infusions. Food Chem. 2009; 113(4):1370-1376. DOI: 10.1016/j.foodchem.2008.08.086

Anti-inflammatory: Chang RCC, So KF. Use of anti-aging herbal medicine, *Lycium barbarum*, against aging-associated diseases. What do we know so far? Cell Mol Neurobiol. 2008; 28(5):643-652. DOI: 10.1007/s10571-007-9181-x

Appendix 2

This section describes selected reported literature for the bioactivity of analytes monitored in Qi Ju Di Huang Wan.

<u>Paeoniflorin</u>

Analgesic : Ding HY, Lin HC, Teng CM, Wu YC. Phytochemical and pharmacological studies on Chinese Paeonia species. J Chin Chem Soc (Taipei). 2000;47: 381-388.
DOI: 10.1002/jccs.200000051

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