QUALITY CONTROL METHODS FOR HERBAL MEDICINE: A MULTIFACETED APPROACH

James Ronald Hennell

A thesis presented to the University of Western Sydney in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

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0.4. 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:

James Ronald Hennell

B.Sc. (Honours)

July 2012
0.5. Dedication

I would like to dedicate this work to my parents, who have given their fullest love, support and motivation throughout my life.
0.6. Acknowledgements

I am grateful and indebted to my supervisor Dr Cheang Soo Khoo (The Compassionate One) for all the gems of knowledge and pearls of wisdom that he has provided over the course of my project. Your extensive experience, knowledge, humour, kindness and patients made my PhD extremely enjoyable and its greatly appreciated.

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<tr>
<td>(-)-ESI</td>
<td>Negative mode ESI</td>
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<tr>
<td>[M-H]</td>
<td>Molecular ion</td>
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<tr>
<td>AAPH</td>
<td>2,2'-Azobis(2-amidinopropane) hydrochloride</td>
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<tr>
<td>ARGCM</td>
<td>Australian Guidelines for Complementary Medicines</td>
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<td>ARTG</td>
<td>Australian Register of Therapeutic Goods</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<td>BP</td>
<td>British Pharmacopoeia</td>
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<tr>
<td>CBoL</td>
<td>Consortium for the Barcode of Life</td>
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<tr>
<td>cGMP</td>
<td>Code of Good Manufacturing Practice</td>
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<td>CM</td>
<td>Complementary Medicine</td>
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<td>DPPH</td>
<td>2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl</td>
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<td>EI</td>
<td>Electron Impact</td>
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<td>ESI</td>
<td>Electrospray Ionization</td>
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<td>ET</td>
<td>Electron Transfer</td>
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<tr>
<td>FID</td>
<td>Flame Ionization Detector</td>
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<td>GC</td>
<td>Gas Chromatography</td>
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<td>HAT</td>
<td>Hydrogen Atom Transfer</td>
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<td>HCA</td>
<td>Hierarchical Cluster Analysis</td>
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<td>HM</td>
<td>Herbal medicine</td>
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<td>KNN</td>
<td>k-Nearest Neighbor Analysis</td>
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<td>LC</td>
<td>High-Performance Liquid Chromatography</td>
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<td>matK</td>
<td>Maturase K</td>
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<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<td>NPPEG</td>
<td>Natural Products (diphenylboric acid 2-aminoethyl ester) and Polyethylene Glycol 4000 (PEG) reagent</td>
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<tr>
<td>OCM</td>
<td>Office of Complementary Medicines</td>
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<td>ORAC</td>
<td>Oxygen Radical Absorbance Capacity</td>
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<td>P-PRC</td>
<td>Pharmacopoeia of the People's Republic of China</td>
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<td>PCA</td>
<td>Principal Component Analysis</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PVDF</td>
<td>Polyvinylidene Difluoride</td>
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<td>Q1 / Q3</td>
<td>Quadrupole 1 / Quadrupole 3</td>
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<td>QA</td>
<td>Quality Assurance</td>
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<td>QC</td>
<td>Quality Control</td>
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<tr>
<td>rbcL</td>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit</td>
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<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
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<td>SD</td>
<td>Standard Deviation</td>
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<td>SIM</td>
<td>Selective Ion Monitoring</td>
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<td>TCM</td>
<td>Traditional Chinese Medicine</td>
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<td>Therapeutic Goods Administration</td>
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<td>TGO</td>
<td>Therapeutic Goods Orders</td>
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<td>TLC</td>
<td>High-Performance Thin Layer Chromatography</td>
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<td>US$</td>
<td>US Dollar</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>$\lambda_{\text{det}}$</td>
<td>Wavelength of detection</td>
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<td>$\lambda_{\text{max}}$</td>
<td>Wavelength of maximum absorption</td>
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0.8. Abstract

The past decade has seen an unprecedented growth in the popularity of complementary medicines in Western countries. As the popularity of complementary medicines continues to grow, serious concerns have been raised about their quality and safety.

Herbal medicine quality control (QC) and assurance (QA) poses a great challenge, as the large assortment of complex chemicals and the compositional variation found within herbal mixtures makes analysis especially difficult. Here, the two commonly used paradigms for herbal quality assessment; the compound- and pattern-based approaches are examined using model systems.

The compound-based paradigm focuses on the quantitative analysis of one or more chemical constituents of an herb or formulation in order to ensure quality and hence product consistency. The complex nature of the 13-herb Endoherb™ formulation made it an exemplar model for assessing this approach. The methodology used in this study demonstrates how the systematic QC and QA of a complex herbal mixture can be carried out using a logical and systematic method for the selection of the analytes, and then developing a method for their sensitive, specific and accurate analysis. Several methods of analysis including high-performance liquid chromatography (LC) with photodiode array (PDA) and mass spectrometric (MS) detection, as well as gas chromatography (GC) with MS and flame ionisation detection (FID) were employed for the specific analysis of the selected analytes based on factors such as their polarity, volatility and presence of a chromophore. Sample preparation is rapid and simple, utilising sonication as the extraction method. The results show that increasing the
organic modifier in the extraction solvent, even for predominantly polar analytes can increase the analyte extraction efficiency.

The pattern-based method compares the chromatographic profile of the extract of a new batch with that of a target or reference batch. Combined with new genetic and pharmacological methods of analysis, the pattern-based approach can be an important addition to the characterisation of herbal authenticity and quality, especially in regards to the quality of the raw materials. *E. arvense* was used as a model to observe the effect of worldwide cultivation on phytochemical profile, genomic profile and pharmacological activity. LC-MS was shown to provide excellent sensitivity, resolution, and reproducibility for the chemical characterisation of the *E. arvense* extracts. Chromatographic pre-processing of LC-MS profile data using the statistical software ‘R’ was necessary for subsequent statistical analysis as the instrumental contribution to the profiles made the detection of peaks cumbersome and the statistical inferences inaccurate. The novel use of k-nearest neighbour analysis combined with principal component analysis allowed for an objective classification of sample grouping. Chemical measurement of the antioxidant capacity of the *E. arvense* extracts provided a basic and preliminary way to establish quality standards of pharmacological equivalence. Importantly, the results indicate that the implied biological effect of the extracts is not reflected in their chemical profile. Finally, DNA barcoding using the loci *rbcL* and *matK* was successfully used to authenticate the raw materials.
CHAPTER 1.

GENERAL INTRODUCTION
1.1. Background to traditional Chinese medicine

Traditional Chinese medicine (TCM) is an empirical medical system based on traditional theory, pathology, holistic diagnosis and treatment, which differs substantially from the principles of orthodox Western-style medicine. The foundation of TCM is largely based on the prescription of medicinal herbs, known as herbal medicine (HM). The herbs prescribed are believed to work in concert together to treat the diagnosed ailment. In HM, the herbs are mixed, decocted and generally consumed as a tea.

The past decade has seen the HM industry grow from a modest base to a global industry grossing over US$15.6 billion annually. In parts of Asia and Africa, over 80% of the population depend on traditional medicine for health care. It is currently estimated that 20 – 70% of the population from Australia and Europe use some form of complementary or alternative medicine in personal health care, in part due to the increased cost and decreased supply of novel Western pharmaceutical drugs. In countries with organised primary health care systems, HM has been integrated into preventative care.

The World Health Organisation (WHO) has identified that education, training and research to support the safe use of complementary medicines is currently lacking, due in part to the disproportionate increase in usage. Current research has focused on ‘translating’ the traditional theory of HM to a chemical basis by analysing plant secondary metabolites.
1.2. Plant secondary metabolites: a chemical basis for HM

Plants synthesise a structurally diverse assortment of chemicals, known as phytochemicals. These may be classified as primary or secondary metabolites, depending on their role in the plant.\textsuperscript{8} Primary metabolites play a critical role in the normal growth and development of the plant, such as in photosynthesis and reproduction, and includes chemicals such as amino acids and lipids.\textsuperscript{9} The remaining chemicals are known as secondary metabolites and are thought to perform roles in signalling and plant defence, such as protecting plants from herbivores, microbial infection, other plants and UV damage.\textsuperscript{9} Three basic chemical families exist, which are based on the biosynthetic origin of the chemical.\textsuperscript{9,10}

1. Phenolic and polyphenolic compounds such as flavonoids
2. Terpenoids such as menthol
3. Nitrogen-containing alkaloids such as morphine; and sulphur-containing compounds such as glucosinolate derivatives

Secondary metabolites are extensively analysed as part of herbal regulation and in the published literature as a diagnostic in chemotaxonomic studies.\textsuperscript{9} This is because each genus of plant, and even individual species of plant can produce unique secondary metabolites that can be used as a diagnostic of herb identity.\textsuperscript{11}

Plant secondary metabolites have been used throughout history, including in Western culture, for a diverse range of tasks such dyes, glues, oils, waxes, flavours and perfumes.\textsuperscript{9} In the past 50 years research has increased significantly into the uses of secondary metabolites as protective dietary supplements and as a potential source of novel drugs. Secondary metabolites have been reported in the literature to have a
plethora of biological functions in humans, such as antioxidant, antiviral, antibacterial and anticancer effects.\textsuperscript{12,13} However, plant secondary metabolites can present a significant risk to humans.

1.3. Risks of HM

Serious concerns have been raised about the quality and safety of HM, particularly in Western countries. In the past, HM have been contaminated with organic and inorganic matter and some have been adulterated with prescription drugs.\textsuperscript{14,15} Furthermore, the quality of the herb with respect to concentration of potentially biologically active components is generally not known. In some cases the concentrations of putative active ingredients are lower than expected but occasionally a component could be too high, so the user may consume more than the maximum recommended dosage.\textsuperscript{3}

Adverse reactions to TCM are classified as either intrinsic or extrinsic. Intrinsic reactions, which are directly related to the active medicine itself, can be either type A, where they have predictable toxicity such as an overdose; or type B where they have an idiosyncratic reaction such as anaphylaxis.\textsuperscript{16} Intrinsic reactions account for approximately 8\% of adverse reactions.\textsuperscript{3} The remaining adverse reactions are extrinsic in nature, and are as a result of some failure of good handling or good manufacturing processes, such as herb misidentification or the presence of contaminants in the herb like heavy metals, pesticides or microbes.\textsuperscript{16} An appropriately designed QC and QA procedure for these herbs should minimise these risks.
1.3.1. Misidentification of raw materials

Herbal nomenclature and a general lack of certified herbal reference materials are contributors to raw material misidentification. HM are currently named in four common ways: the common name, the translated or pinyin name, the pharmaceutical name, and the binomial botanical name.\textsuperscript{17} Often the common and pinyin names can represent more than one species or even different plant parts. The use of authenticated herbs can also lead to ambiguity, considering the term species represents ‘more or less arbitrary and subjective man-made units’ and ‘there are no objective infallible criteria for rank-determination in plant classification.’\textsuperscript{18}

1.3.2. Lack of chemical standardisation and characterisation

A significant portion of QC in the manufacture of herbal formulations is carried out by ‘QC-by-input’ whereby the manufacturer only needs to show that the quantity of each herbal extract in the herbal formulation complies with the label claim when the bulk mixture is prepared. At this time there is no requirement by the Australian Therapeutic Goods Administration (TGA) that putative actives be quantified, though the regulatory environment is steadily moving towards tighter control. A significant number of suppliers now provide standardised extracts, which are extracts standardised to achieve a target concentration of one marker and occasionally two markers. The selected marker is usually a pharmacologically active component and is present in relatively high concentration in the herb. The assumption is made that the other non-standardised components do not contribute significantly to the activity of the herb.

Quantitation of putative active components is desirable because plants are a variable raw material. Factors such as genetic drift; environmental conditions such as soil,
climate, rainfall; the age and plant part used; time of harvest, post-harvest treatment, storage and processing all contribute to this variation.\textsuperscript{19,20} These variations can contribute significantly to batch-to-batch variation of the product and hence alter its effectiveness.

1.3.3. *Substitution and adulteration of the herbs*

Herbal substitution during the preparation of HM may occur somewhat innocuously as the common and pinyin names for herbs can sometimes cover more than one botanical species or for more economic reasons such as a shortage or increased cost of the original ingredient.\textsuperscript{15} Regardless of the reason, the consequence may be a poor quality or even unsafe product.\textsuperscript{21} A well-publicised example is the substitution of *Stephania tetranda* with *Aristolochia fangchi* in a medicine designed to help with weight loss. *A. fangchi* contains the nephrotoxin aristolochic acid that resulted in rapidly progressive interstitial nephritis with terminal renal failure for those taking it.\textsuperscript{15}

HM are sometimes adulterated with Western prescription drugs in order to increase their efficacy.\textsuperscript{15} For example, an epileptic patient fell into a coma after taking an HM in China laced with phenytoin, an anti-epileptic drug. No mention was made of any prescription drugs on the packaging.\textsuperscript{22}

1.4. *Herbal medicine regulation in Australia*

1.4.1. *Therapeutic goods administration*

The TGA is the Australian Government body responsible for the regulation of medicines, including complementary medicines (CM) based on the Therapeutics Goods Act 1989.\textsuperscript{23}
As presented in Figure 1.1, CM is legally a general term for many kinds of medicines, which also covers TCM.24

![Diagram of Complementary medicines]

**Figure 1.1: Different types of medicines classified by the TGA as complementary medicines.**24

The Therapeutics Goods Act 1989i requires therapeutic goods that are imported or manufactured for supply in Australia be included on the Australian Register of Therapeutic Goods (ARTG).25

1.4.2. Listed and registered complementary medicines

HM can be recorded with the TGA as either a listed product or as a registered product.20 Formulations can be listed and assigned an AUST L number if they contain ingredients that the TGA considers to be of low public health concern. Listed products must be manufactured by a TGA-licensed manufacturer based on the code of good manufacturing practice (cGMP).25 Approximately 4500 plant-based products are currently listed.16 Formulations can be registered and assigned an AUST R number if they contain herbs that are either restricted by the Standards for the Uniform Scheduling of Drugs and Poisons, if the substance is a designated active ingredient that has an established identity and tradition of use, or has been identified by the TGA as being of some health concern.16,26 Substances can only be registered when appropriate
documentation outlining clinical trial work is submitted to the Office of Complementary Medicines (OCM) that advises the TGA.26

1.4.3. Pharmacopoeia and official standards

The British Pharmacopoeia (BP) and Therapeutic Goods Orders (TGOs) are the official standards for regulatory purposes in Australia.27 The BP and TGO monographs provide the minimum requirements that must be met for a medicine to comply with the cGMP. The requirements of the monographs must be met except where a justification for not doing so is authorised by the TGA. The TGA can consider the suitability of other pharmacopoeia monographs or standards on a case-by-case basis.

1.5. Overview of HM quality control strategies

The QC and QA of HM is major a challenge because herbal products have a complex chemical composition and are subject composition variation.

Besides ensuring safety, the aim of a QC program is to ensure a consistent product and hence some predictable outcome for the consumer. The aim of ensuring product safety is well defined, such as having limits on heavy metals, pesticides, microbial contamination, and confirmation of herbal identity, which are relatively straightforward to achieve. In a very small number of cases, a particular chemical might have to be monitored because it is toxic or may be toxic at high concentrations. Achieving product consistency is a more complex proposition with three broad possible approaches:19

1. Chemical testing
2. Biochemical testing
3. Biological response testing
Ideally chemical, biochemical and biological testing should all be performed but this ideal is unlikely to be achieved in a real-world manufacturing environment due to time and cost constraints.

1.5.1. Chemical testing

Chemical characterisation is universally used as it relies on technology and expertise typically found in most chemistry QC laboratories as well as being comparatively fast to carry out, though the analytical method development process itself may be quite time consuming.

1.5.1.1. Analytical equipment

Herbal medicines contain potentially hundreds of analytes, usually in low concentration. Methods of analysis must therefore focus on being able to separate these analytes and determine them specifically and accurately. Chromatographic techniques such as high-performance thin layer chromatography (TLC), high-performance liquid chromatography (LC) and gas chromatography (GC) are extensively used for the quality assessment of HM.

TLC is commonly used for the rapid identification of herbal extracts. TLC has been successfully used to differentiate and identify several herbal species.²⁸ Sample application, plate development and visualisation can easily be optimised.²⁹ Unfortunately a number of key steps rely heavily on operator skill, giving rise to poor reproducibility and its semi-quantitative nature.¹⁴

LC is by far the most popular chromatographic technique for analysing herbal products, due to the generally non-volatile nature of large secondary metabolites produced by
One of the major advantages of LC is the ability to hyphenate with various detectors, the main ones being the photodiode array (PDA) and mass spectrometer (MS) detectors.

GC is a sensitive method of analysing volatile components or non-volatile components that can be made volatile by chemical derivatisation. GC is routinely hyphenated to detectors such as the flame ionisation detector (FID) and MS.

1.5.1.2. Analytical paradigms

Current regulatory standards predominantly focus on a compound-based paradigm, where quantitative analysis of one or more chemical constituents of an herb or formulation are used to quantify quality and hence product consistency. This approach assumes that those constituents not quantified do not make a significant contribution to the therapeutic effect of the medication. Consequently, this method does not give a complete picture of an herbal product, as many components may be responsible for the purported therapeutic effect and may work synergistically or antagonistically with each other. The extent to which these assumptions are valid will likely depend on how competently the type and number of analytes have been selected for monitoring. Obviously the more analytes the better, but this has to be balanced against the cost, complexity and hence practicality of the analytical task in an industry QC laboratory.

HM have been referred to as a ‘black box’ due to the plethora of unknown chemicals contained within them. The pattern-based method compares the chromatographic profile of an extract of a new batch with that of a target or reference batch, without necessarily identifying or quantifying the detected chemicals. Identifying chemicals is however desirable if they have been shown to have some pharmacological effect.
Traditionally, comparison between batches is performed using TLC and LC, however the LC profile is more reproducible as the result is less dependent on operator skill. Statistics are often utilised to determine whether the variation between the observed chemical profiles are within acceptable limits.

1.5.2. **Biochemical testing: DNA barcoding**

Characterisation of plants at the genome level has been touted as the method by which unequivocal herb identification can be achieved. DNA barcoding is a technique where polymerase chain reaction (PCR) is used for the amplification of a small *locus* of genomic DNA, usually chloroplast DNA of the plant. DNA sequences are then identified by comparison to sequences deposited in public repositories such as GenBank. A limitation of DNA barcoding is that it is generally only applicable to the raw herbal starting material and not to the prepared extracts. This can be problematic since most commercial herbal formulations are prepared by mixing single herb extracts rather than producing an extract from the raw herb mixture. Thus the application of DNA barcoding must be introduced at the raw material supplier to identify the raw herb prior to manufacture.

1.5.3. **Biological response testing**

Characterisation of the potential biological effects of an herb is recommended by several regulatory agencies such as the European Medicines Agency (EMA) to serve as a more biologically useful way to establish quality standards. Biological response testing may include cell line testing, animal testing and genomic response testing.
One of the simplest ways to characterise the biological effects of an herb is by assessing some overall pharmacological property like DPPH free radical scavenging activity, oxygen radical absorbance capacity (ORAC), Folin-Ciocalteu reducing capacity, total phenols, nitric oxide production by the Griess assay and Fe$^{3+}$ reducing antioxidant power. This is particularly applicable to plant extracts that containing phenols, which are known to be potent free radical scavengers.$^{38-40}$

1.6. The compound-based approach to herbal quality evaluation using Endoherb™

The most commonly used approach in HM QC is to identify, quantitate and monitor the concentration of key analytes. The assumption is made that if a sufficient number of bioactive analytes are identified, then the quantitation of these will provide a reliable measure of product quality and hence enable the manufacturer to produce a consistent product.

Section 4.4.2.2. of the ARGCM guidelines recommends the analysis of at least one unique analyte from each constituent herb part in an herbal formulation.$^{20}$ This is to prevent adulteration of the herbal formulation with different herb parts or with a different herb altogether. The analytes selected should ideally contribute to the therapeutic effect of the formulation. 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 analysed should contribute to the effect of the formulation.$^{20}$
1.6.1. A new herbal formulation for the treatment of endometriosis

Endometriosis is characterised by the growth and proliferation of endometrial tissue outside of the uterus and is commonly associated with chronic inflammation, pelvic pain and infertility.\textsuperscript{41,42} It is estimated to affect 0.5 – 5 % of fertile women and 25 – 40 % of infertile women around the world.\textsuperscript{41,43} The aetiology and pathogenesis of this disease is poorly understood, and while medical and surgical treatment are effective for the short-term amelioration of the associated chronic pelvic pain, recurrence is common.\textsuperscript{44}

Endoherb\textsuperscript{TM} is an herbal formulation developed by Dr Alex Liew, a researcher at the University of Western Sydney’s Centre for Complementary Medicine Research (CompleMED), for the treatment of endometriosis-related pain. In a small, double blind clinical trial, Endoherb\textsuperscript{TM} was shown to have significant benefits for relieving symptoms in women with laparoscopy-diagnosed endometriosis. Endoherb\textsuperscript{TM} provided marked pain relief and improved quality of life without unpleasant side effects.\textsuperscript{45} The herbs used and their percentage composition in the Endoherb\textsuperscript{TM} formulation is presented in Table 1.1. For consistency, the herbs used in this formulation are referred to by their pinyin name.

1.6.2. Extraction solvents

In HM, herbs are usually consumed as a water decoction, though aqueous alcoholic extracts are also used using wines and spirits. In this work, three different extraction solvents were used for the preparation of the Endoherb\textsuperscript{TM} extract, these are: pure water, 35 % aqueous ethanol and 95 % aqueous ethanol. A water extraction was used for the aforementioned clinical trial.
<table>
<thead>
<tr>
<th>Pinyin name</th>
<th>Chinese name</th>
<th>Percent in formulation (%)</th>
<th>Latin pharmaceutical name</th>
<th>Latin botanical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aiye</td>
<td>艾叶</td>
<td>4</td>
<td><em>Folium artemisiae argyi</em></td>
<td><em>Artemisia argyi</em> Levl. et Vant.</td>
</tr>
<tr>
<td>Baishao</td>
<td>白芍</td>
<td>8</td>
<td><em>Radix paeoniae alba</em></td>
<td><em>Paeonia lactiflora</em> Pall.</td>
</tr>
<tr>
<td>Baizhu</td>
<td>白术</td>
<td>8</td>
<td><em>Rhizoma atractylodis macrocephalae</em></td>
<td><em>Atractylodes macrocephala</em> Koidz.</td>
</tr>
<tr>
<td>Chishao</td>
<td>赤芍</td>
<td>8</td>
<td><em>Radix paeoniae rubra</em></td>
<td><em>Paeonia lactiflora</em> Pall.</td>
</tr>
<tr>
<td>Chuanxiong</td>
<td>川芎</td>
<td>6</td>
<td><em>Rhizoma chuanxiong</em></td>
<td><em>Ligusticum chuanxiong</em> Hort.</td>
</tr>
<tr>
<td>Danggui</td>
<td>当归</td>
<td>10</td>
<td><em>Radix angelicae sinensis</em></td>
<td><em>Angelica sinensis</em> (Oliv.) Diels</td>
</tr>
<tr>
<td>Fuling</td>
<td>茜草</td>
<td>10</td>
<td><em>Poria</em></td>
<td><em>Poria cocos</em> (Schw.) Wolf</td>
</tr>
<tr>
<td>Guizhi</td>
<td>桂枝</td>
<td>8</td>
<td><em>Ramulus cinnamomi</em></td>
<td><em>Cinnamomum aromaticum</em> Nees</td>
</tr>
<tr>
<td>Mudanpi</td>
<td>牡丹皮</td>
<td>8</td>
<td><em>Cortex moutan</em></td>
<td><em>Cinnamomum cassia</em> Presl (synonym)</td>
</tr>
<tr>
<td>Shudahuang</td>
<td>熟大黄</td>
<td>6</td>
<td><em>Radix et rhizoma rhei</em></td>
<td><em>Paeonia suffruticosa</em> Andr.</td>
</tr>
<tr>
<td>Shudihuang</td>
<td>熟地黄</td>
<td>12</td>
<td><em>Radix rehmanniae praeparata</em></td>
<td><em>Rehmannia glutinosa</em> Libosch.</td>
</tr>
<tr>
<td>Taoren</td>
<td>桃仁</td>
<td>6</td>
<td><em>Semen persicae</em></td>
<td><em>Prunus persica</em> (L.) Batsch</td>
</tr>
<tr>
<td>Zhigancao</td>
<td>炙甘草</td>
<td>6</td>
<td><em>Radix et rhizoma glycyrrhizae praeparata cum melle</em></td>
<td><em>Glycyrrhiza uralensis</em> Fisch.</td>
</tr>
</tbody>
</table>

(Note: Pharmaceutical and botanical names are based on the Pharmacopoeia of the People's Republic of China.)
1.6.3. **Analyte selection**

The first step in the QC of an HM is to decide on the set of analytes to monitor. If the QC results are to reasonably reflect the quality of the medication, then this selection process needs to be justifiable and systematic. Besides considering the pharmacological activity and concentration of the analyte, factors such as regulatory compliance and the analyte uniqueness in the context of the formulation are also considered. Finally, the practicality of the task in terms of the number of analytes, availability of the pure standards and analysis are taken into account.

1.6.3.1. **Substances that may be used in listed medicines in Australia**

The ‘Substances that may be used in Listed Medicines’ publication by the TGA lists the herbs and chemicals eligible for listing on the ARTG. The list includes the approved role of the substance as an active (A), excipient (E), and/or component (C) ingredient. Based on the herbs listed Table 1.1, the Endoherb™ formulation is not eligible to be a listed medicine, principally because it contains taoren, which contains amygdalin. The accepted therapeutic use of the herbs and major constituents are presented in Table 1.2. Substances marked as C are not approved as substances for use in their own right and can only be used in conjunction with an approved source. This table was used as a preliminary guide for the selection of potential analytes.
<table>
<thead>
<tr>
<th>Pinyin name</th>
<th>Latin botanical name</th>
<th>Use</th>
<th>Notes</th>
<th>Active chemicals in herb</th>
<th>Accepted use</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aiye</td>
<td>Artemisia argyi Lev. et Vant.</td>
<td>A, E</td>
<td>Oil derived from this species is a Customs Prohibited Import.</td>
<td>Ligustilide</td>
<td>C</td>
<td>Topical use only.</td>
</tr>
<tr>
<td>Baishao</td>
<td>Paeonia lactiflora Pall.</td>
<td>A, E</td>
<td></td>
<td>Ferulic acid</td>
<td>E, C</td>
<td></td>
</tr>
<tr>
<td>Baizhu</td>
<td>Atractylodes macrocephala Koidz.</td>
<td>A, E</td>
<td></td>
<td>Ligustilide</td>
<td>C</td>
<td>Topical use only.</td>
</tr>
<tr>
<td>Chishao</td>
<td>Paeonia lactiflora Pall.</td>
<td>A, E</td>
<td></td>
<td>Ferulic acid</td>
<td>E, C</td>
<td></td>
</tr>
<tr>
<td>Chuanxiong</td>
<td>Ligusticum chuanxiong Hort.</td>
<td>A, E</td>
<td></td>
<td>Cinnamaldehyde</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Danggui</td>
<td>Angelica sinensis (Oliv.) Diels</td>
<td>A, E</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fuling</td>
<td>Poria cocos (Schw.) Wolf</td>
<td>A, E</td>
<td></td>
<td>Rhein</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Guizhi</td>
<td>Cinnamomum aromaticum Nees</td>
<td>A, E</td>
<td>If an oil, the concentration of this must not exceed 0.001%. Cassia oil is a mandatory component.</td>
<td>Cinnamaldehyde</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Mudanpi</td>
<td>Paeonia suffruticosa Andr.</td>
<td>A, E</td>
<td>Not permitted if the plant part contains leaf. Hydroxyanthracene derivatives calculated as rhein are a mandatory component.</td>
<td>Rhein</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Shudahuang</td>
<td>Rheum palmatum L.</td>
<td>A, E</td>
<td>Only <em>Prunus dulcis</em> var. <em>dulcis</em> seed is permitted. Amygdalin and Hydrocyanic acid are mandatory components.</td>
<td></td>
<td>C</td>
<td>Listed medicines must not contain any amygdalin.</td>
</tr>
<tr>
<td>Shudihuang</td>
<td>Rehmannia glutinosa Libosch.</td>
<td>A, E</td>
<td></td>
<td>Amygdalin</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Taoren</td>
<td>Prunus persica (L.) Batsch</td>
<td>A, E</td>
<td></td>
<td>Glycyrrhizic acid</td>
<td>E, C</td>
<td></td>
</tr>
<tr>
<td>Zhigancao</td>
<td>Glycyrrhiza uralensis Fisch.</td>
<td>A, E</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.6.3.2. Pharmacopoeia references

Only 6 out of the 13 herbs in the Endoherb™ formulation have a BP monograph for their analysis.⁴⁸ These herbs are gancao, dahuang, guizhi, danggui, baishao and chishao (as *P. lactiflora*). The Pharmacopoeia of the People’s Republic of China (P-PRC) has a wider breadth of coverage for HM than the BP, and was therefore used in this project as the reference for the remaining herbs.⁴⁶ As each pharmacopoeia reference is for the individual herb and not the formulation, the references were only used as a guide for analyte selection. Based on the BP and P-PRC monographs, Table 1.3 presents the chemicals selected for analysis in the Endoherb™ formulation.
Table 1.3: The analytes recommended for analysis in the respective herb based on the British Pharmacopoeia (BP) and Pharmacopoeia of the People’s Republic of China (P-PRC) recommendations.

<table>
<thead>
<tr>
<th>Pinyin name</th>
<th>Selected analyte(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baishao</td>
<td>Paeoniflorin</td>
<td>BP, P-PRC</td>
</tr>
<tr>
<td></td>
<td>Paeonol</td>
<td>BP</td>
</tr>
<tr>
<td>Chishao</td>
<td>Paeoniflorin</td>
<td>BP, P-PRC</td>
</tr>
<tr>
<td></td>
<td>Paeonol</td>
<td>BP</td>
</tr>
<tr>
<td>Danggui</td>
<td>Ligustilide</td>
<td>BP</td>
</tr>
<tr>
<td></td>
<td>Ferulic acid</td>
<td>BP, P-PRC</td>
</tr>
<tr>
<td></td>
<td>Heptane</td>
<td>BP</td>
</tr>
<tr>
<td></td>
<td>Linoleic acid</td>
<td>BP</td>
</tr>
<tr>
<td>Mudanpi</td>
<td>Paeonol</td>
<td>P-PRC</td>
</tr>
<tr>
<td>Shudahuang</td>
<td>Rhein</td>
<td>BP, P-PRC</td>
</tr>
<tr>
<td></td>
<td>Chrysophanol</td>
<td>BP, P-PRC</td>
</tr>
<tr>
<td></td>
<td>Physcione</td>
<td>BP, P-PRC</td>
</tr>
<tr>
<td></td>
<td>Emodin</td>
<td>BP, P-PRC</td>
</tr>
<tr>
<td></td>
<td>Aloe-epomodin</td>
<td>BP, P-PRC</td>
</tr>
<tr>
<td>Shudihuang</td>
<td>Catalpol</td>
<td>P-PRC</td>
</tr>
<tr>
<td>Taoren</td>
<td>Amygdalin</td>
<td>BP</td>
</tr>
<tr>
<td>Zhigancao</td>
<td>Glycyrrhizic acid</td>
<td>BP, P-PRC</td>
</tr>
</tbody>
</table>

1.6.3.3. A novel approach to analyte selection

Since there is no pharmacopoeia recommendation for the selection of an analyte for aiye, baizhu, chuanxiong, fuling and guizhi, analytes were chosen based on published literature. Criteria were developed within CompleMED to examine all the reported analytes of each constituent herb in a formulation for their importance in the action towards the targeted disease. This is done to reduce the number of analytes required for QC monitoring to a manageable quantity, while maintaining their usefulness in reflecting herbal quality in terms of outcome for the consumer.

The analytes are ranked in importance and selected according to the following criteria.
• Concentration of the analyte in the herb
• Uniqueness of the analyte to the herb
• Physical and chemical properties of the analyte
• Commercial 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
• Traditional use of the herb
• The part of the plant used in the formulation, such as the seed, root or rhizome

These criteria were used to create a numerical ranking system from 0 – 6 as presented in Table 1.4. The higher the number, the more important the analyte is for monitoring purposes.
Table 1.4: The ranking system used to select analytes for the compound-based analysis of Endoherb™.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Details</th>
</tr>
</thead>
</table>
| 6       | Analyte has highest bioactivity related to major symptoms of disease for which there is sufficient evidence to support its activity ($\geq 2$ studies)  
AND  
Analyte is present in high yield in the herb  
AND  
Traditional/other use of the herb and its therapeutic action supports the activity of the analyte  
AND  
Bioavailability of analyte and/or its metabolites are known to be bioavailable  
OR  
Analyte may be toxic and needs to be screened to comply to safety limits |
| 5       | Analyte has high bioactivity related to major symptom/s of the disease for which there is sufficient evidence to support its activity ($\geq 2$ studies)  
AND  
Analyte is present in high yield in the herb  
AND  
Traditional/other use of the herb and its therapeutic action supports the activity of the analyte |
| 4       | Analyte has bioactivity related to a symptom (major/minor) of the disease, however there is only 1 study to support this action  
AND  
Analyte is present in high yield in the herb  
AND  
Traditional/other use of the herb and its therapeutic action supports the activity of the analyte |
| 3       | Analyte has bioactivity related to a symptom (major/minor) of the disease, however there is only 1 study to support this action  
AND  
Analyte is present in low yield in the herb  
AND  
Traditional/other use of the plant maybe be related to action of the analyte |
| 2       | Analyte’s activity is indirectly related to symptom of disease  
OR  
Analyte is present in too low a yield in the herb for it to be screened  
OR  
Analyte’s activity is not supported by traditional and therapeutic use of herb |
| 1       | Analyte’s activity is not related to indication of the disease of interest or no commercial source of standard is available. |
| 0       | There are no bioactivity studies currently available for this analyte |
This ranking system was also used to rank the previously identified analytes for comparison, as presented in Table 1.5. It is desirable to restrict the number of analytes to 1-2 per herb since this is a 13-herb formulation and the analysis would be impractically large otherwise. In total, 12 analytes were chosen for analysis. This number of analytes is probably the close to the maximum number a industry QC lab can routinely cope with.\(^5\)

The herbs baishao, chishao and mudanpi are of the same genus, *Paeonia*, and are consequently too similar for a unique analyte to be found to differentiate them. Instead, paeoniflorin and paeonol, which are common to the *Paeonia* genus are analysed. Similarly ligustilide is analysed, although it is common to both chuanxiong and danggui.
Table 1.5: The analytes chosen in the Endoherb™ formulation, based on British Pharmacopoeia (BP), Pharmacopoeia of the People’s Republic of China (P-PRC), Therapeutic Goods Administration (TGA), and Hong Kong Department of Health (DOH) recommendation and the ranking system-based literature references.

<table>
<thead>
<tr>
<th>Pinyin name</th>
<th>Selected analyte(s)</th>
<th>Structure</th>
<th>Rank</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aiye</td>
<td>Carveol</td>
<td><img src="image" alt="Carveol Structure" /></td>
<td>3</td>
<td>Bensky^{12}</td>
</tr>
<tr>
<td>Baishao</td>
<td>Paeoniflorin</td>
<td><img src="image" alt="Paeoniflorin Structure" /></td>
<td>6</td>
<td>BP^{48}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P-PRC^{46}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tang^{13}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wagner^{51}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wagner^{52}</td>
</tr>
<tr>
<td></td>
<td>Paeonol</td>
<td><img src="image" alt="Paeonol Structure" /></td>
<td>5</td>
<td>BP^{48}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bensky^{12}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tang^{15}</td>
</tr>
<tr>
<td>Baizhu</td>
<td>Atractylenolide III</td>
<td><img src="image" alt="Atractylenolide III Structure" /></td>
<td>3</td>
<td>Bensky^{12}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tang^{15}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wagner^{51}</td>
</tr>
<tr>
<td>Chishao</td>
<td>Paeoniflorin</td>
<td>See baishao</td>
<td>6</td>
<td>BP^{48}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P-PRC^{46}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wagner^{52}</td>
</tr>
<tr>
<td></td>
<td>Paeonol</td>
<td>See baishao</td>
<td>5</td>
<td>BP^{48}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bensky^{12}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tang^{15}</td>
</tr>
<tr>
<td>Chuanxiong</td>
<td>Tetramethyl-pyrazine</td>
<td><img src="image" alt="Tetramethyl-pyrazine Structure" /></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ligustilide</td>
<td><img src="image" alt="Ligustilide Structure" /></td>
<td>5</td>
<td>Bensky^{12}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tang^{15}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wagner^{53}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DOH^{57}</td>
</tr>
<tr>
<td>Danggui</td>
<td>Umbelliferone</td>
<td><img src="image" alt="Umbelliferone Structure" /></td>
<td>3</td>
<td>Anuj^{55}</td>
</tr>
<tr>
<td></td>
<td>Ligustilide</td>
<td>See chuanxiong</td>
<td>5</td>
<td>BP^{48}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wagner^{56}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DOH^{57}</td>
</tr>
<tr>
<td>Pinyin name</td>
<td>Selected analyte(s)</td>
<td>Structure</td>
<td>Rank</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------</td>
<td>-----------</td>
<td>------</td>
<td>-----------</td>
</tr>
<tr>
<td>Fuling</td>
<td>Pachymic acid</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>3</td>
<td>Bensky\textsuperscript{12} \n Gapter\textsuperscript{58}</td>
</tr>
<tr>
<td>Guizhi</td>
<td>Cinnamaldehyde</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>3</td>
<td>TGA\textsuperscript{47} \n P-PRC\textsuperscript{59} \n Tang\textsuperscript{13} \n BP\textsuperscript{48}</td>
</tr>
<tr>
<td>Mudanpi</td>
<td>Paeoniflorin</td>
<td>See baishao</td>
<td>6</td>
<td>DOH\textsuperscript{60}</td>
</tr>
<tr>
<td></td>
<td>Paeonol</td>
<td>See baishao</td>
<td>5</td>
<td>P-PRC\textsuperscript{46} \n DOH\textsuperscript{60}</td>
</tr>
<tr>
<td>Shudahuang</td>
<td>Rhein</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>3</td>
<td>BP\textsuperscript{48} \n P-PRC\textsuperscript{46} \n Tang\textsuperscript{13} \n DOH\textsuperscript{61}</td>
</tr>
<tr>
<td>Shudihuang</td>
<td>Catalpol</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>4</td>
<td>P-PRC\textsuperscript{46} \n Tang\textsuperscript{13} \n Wagner\textsuperscript{62}</td>
</tr>
<tr>
<td>Taoren</td>
<td>Amygdalin</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>5</td>
<td>BP\textsuperscript{48} \n Anuj\textsuperscript{55}</td>
</tr>
<tr>
<td>Zhigancao</td>
<td>Glycyrrhizic acid</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>6</td>
<td>BP\textsuperscript{48} \n P-PRC\textsuperscript{59} \n Tang\textsuperscript{13} \n DOH\textsuperscript{63}</td>
</tr>
</tbody>
</table>
1.6.4. Development and validation of analytical methods of analysis for the selected analytes

Analytical method validation is defined as the process of demonstrating that the analytical procedure is suitable for its intended purpose, for example, identification, determination of impurities, assay of active or other ingredients. Based on the flowchart presented in Figure 1.2, the first step is to develop the analytical method for Endoherb™ and to test and characterise its performance through the validation process. The validation parameters outlined in Section 1.6.5 are adapted from the European Union Commission decision of 12 August 2002 implementing Council Directive 96/23/EC. Once the analytical method is developed, it can be used to analyse different sources of the herb or herbal formulation. A full method validation involves inter-laboratory testing with typically ≥ 7 laboratories participating. This undertaking is usually carried out by an agency or organisation like the Association of Official Analytical Chemists.
1.6.5. Method validation parameters

1.6.5.1. Identity confirmation

Analyte identification is achieved by comparing the retention times of sample and standard peaks. If the PDA is used, identity confirmation is achieved by comparing the UV-Vis spectra of the standard and sample peaks to determine their match. Comparison of the UV-Vis spectra is informative if the spectrum has multiple peaks for several points of evaluation. For the MS detector, spectral comparison is more reliable. In the case of LC-MS, electrospray ionisation (ESI) coupled to a tandem MS (MS/MS)
enables the identification of the molecular ion, which can then be fragmented by collision-induced dissociation to form distinct daughter ions. In the case of GC-MS, the molecular ion is usually fragmented by electron impact (EI), generally forming many ion fragments. The MS spectra of sample and standard peaks can then be compared for the presence of these ions as well as their relative intensities. Additionally, if the observed fragments can be reasonably rationalised to the presumed structure, additional confidence is gained that the assumed chemical structure is correct.

1.6.5.2. Accuracy (recovery)

Accuracy is assessed to determine whether the analytical method gives results that are close to the true value. In this study accuracy is determined from fortification (spiking) recoveries carried out at the 50, 100 and 200 % levels. Fortification is carried out using a mixed standard fortification solution, where the ratio of the concentration of the standards corresponds to their relative concentrations in the un-fortified sample as shown in Figure 1.3. 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 %, to give fortification levels of 50, 100 and 200 % respectively. After adding the fortification solution the solvent is allowed to evaporate before starting the analysis. Each fortification level and the un-fortified sample are determined with $n = 7$ replicates to give a total of 28 analyses.

Figure 1.3: The equation used to calculate recovery.

$$
\text{recovery} = \left( \frac{\text{fortified value} - \text{unfortified value}}{\text{amount of analyte added}} \right) \times 100 \%
$$
1.6.5.3. Precision

The precision of an analytical method is defined as the degree of agreement between a set of replicate results. Precision is assessed for both the sample and standards. This agreement is expressed in terms of the standard deviation (SD) and relative standard deviation (RSD) of \( n = 7 \) replicate sample preparations and analyses. This technique determines the total precision of the method, incorporating the contributing uncertainties from sample preparation and instrumental analysis.

1.6.5.4. Linearity

Instrumental linearity refers to the ability of the detector to produce results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of the chemical standard. Method linearity, that is the linearity starting from extraction to instrumental analysis, is tested over a range in which the analyte concentration can reasonably be expected to lie – this typically encompasses from 50 % of an expected lowest value to 100 % above an expected highest value. Method linearity tests can reveal, for example, if the extraction solvent has reached saturation for the analyte.

1.6.5.5. Detection limits

The limit of detection (LOD) is defined as the lowest amount of analyte that can be detected by a method within a specified degree of accuracy and reproducibility. The method LOD is typically defined as three times the SD of a set of replicate (typically \( n = 7 \)) extractions and analyses.
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 ten times the SD of a set of replicate (typically \( n = 7 \)) extractions and analyses.

LOD and LOQ are calculated statistically in this way as both the sample preparation and instrumental uncertainties are accounted for. Determination of the detection limits using the signal-to-noise ratio of a chromatogram only determines the instrumental detection limit. If the LOD is determined from the SD of replicates of multiple injections of the same solution, the uncertainty also incorporates the error contribution from the autosampler.

1.6.5.6. Stability

Stability refers to the decrease in analyte concentration (decomposition) in both the standard and sample under specified storage conditions as a function of time. This is determined by analysing the samples at specific time intervals using the validated analytical method. The samples and standards are discarded when the analyte peak area has decreased \( \geq 2\% \). Typically stability is assessed by storing the samples and standards at room temperature (25 °C), in a fridge (4 °C) and in a freezer (-20 °C). Generally analyte stability is lower in the sample than in the standard.
1.7. The pattern-based approach to herbal quality evaluation using *Equisetum arvense* L.

1.7.1. A pattern-based approach to assessing herb quality

Pattern-based chromatographic profiling is increasingly being used as a supplementary tool in assessing herbal medicine quality.\(^{65-67}\) The pattern-based approach considers all detectable constituents of a given herbal material to establish a characteristic chromatographic profile without necessarily identifying or quantifying all the constituents. The pattern-based approach may be used to determine the similarity of the chromatographic profile of a new batch of herb compared to a reference batch. This method employs some subjectivity in judging the degree of similarity or difference between the profiles.

The pattern-based methodology is generally applied to the individual herbs in a mixture rather than the mixture itself because it is difficult to identify the component herb(s) that may be responsible for a mixture chromatographic profile that deviates significantly from a target profile. It is a reasonable expectation that by using individual herbs that have a comparable profile to a reference, it is more likely that a mixture will meet its target profile.

1.7.2. Rationale for analysing *E. arvense*

It was originally intended that the pattern-based approach be applied to one of the herbs used in Endoherb\(^{\text{TM}}\). Unfortunately we were unable to obtain a sufficient number of samples from different manufacturers of any of the individual herbs used in order to conduct a statistically meaningful comparison. However we were able to obtain 13
sources of the extract of the herb *E. arvense*, so this herb was used for this pattern-based application to quality control.

In this study *E. arvense* was used as a model to observe the effect of worldwide cultivation on phytochemical profile, genomic profile and pharmacological activity. *E. arvense* was selected because it is distributed worldwide and the *Equisetum* species plus its hybrids are widely reported to possess extensive morphological, morphometric and chemotypical variation.\(^{68}\) It is used in traditional medicine as a hypoglycaemic, diuretic, anti-inflammatory, antioxidant, vasorelaxant and haemostatic\(^{69-74}\) and contains flavonoids, styrylpyrones, phenyl carboxylic acids, alkaloids and silica.\(^{68,75}\)

### 1.7.3. Chemometrics

The pattern-based approach to the assessment of herbal raw material quality using chromatographic profiling is being increasingly utilised, however natural variations in the starting material make the comparison somewhat ambiguous.\(^{65,76}\) The term chemometrics has been used since the 1970s to describe the application of mathematical (generally statistical) methods of analysis on chemical data.\(^{77}\) Statistical techniques such as principal component analysis (PCA) and hierarchical cluster analysis (HCA) are commonly used in chemometrics as they help to greatly reduce the complexity of chromatographic profiles to a more user friendly form, such as a 2-dimensional plot.\(^{78}\) Pre-processing of the profile data is generally required prior to statistical analysis in order to minimise the ‘garbage in, garbage out’ principle, thereby reducing the contribution of meaningless information (such as instrumental noise) to the final result.\(^{79}\)
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. 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. The ‘Stats’ package included in R contains many of the commonly used statistical techniques such as PCA and HCA.

PCA works by explaining a large number of highly correlated variables such as various peak height measurements using a small number of uncorrelated variables called principal components (PCs). 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-based techniques can be used in conjunction with PCA to aid in the classification of sample groups. K-nearest neighbour clustering (KNN), for example, can be used to categorise samples into a user-defined number of groups.

1.7.3.1. Phytomics similarity index

The Phytomics Similarity Index (PSI) is a statistical method proposed by Tilton et al that uses the linear correlation coefficient (r) to compare two chromatographic profiles. This technique is unique to others in that it compares the chromatographic profiles based on the ratio of each peak intensity (n) with each of the other (n-1) peak in the same chromatogram.
This process was used as a starting point for subsequent PCA analysis. The rationale for using the ratio information instead of simply the peak intensity values is that the ratio provides measurement relative to each of the other peaks in the chromatogram. This is used in an attempt to perform a more holistic analysis.

**1.7.4. Genomic authentication**

A range of genomic techniques can be used to authenticate plants based on species-specific variations in the DNA sequences of various chloroplast and nuclear regions.$^{33}$ PCR-based methods only require minute amounts of DNA and can be applied to fresh and dried plant parts.

DNA barcoding is a tool that has the potential to allow rapid and unequivocal identification of any land plant.$^{35,82-84}$ DNA barcodes consist of a standardised short sequence of DNA often between 400 and 800 base pairs long. While DNA barcoding has proven to be considerably more demanding in plants than in animals, where a single locus has proved sufficient, a concerted effort of scientists worldwide has led to the identification of four regions that can be used for DNA barcoding of land plants with reasonable success. The three plastid markers *rbcL*, *matK* and intergenic spacer *trnH–psbA* have proved to be effective in a wide range of land plants. In addition, the nuclear ribosomal marker ITS, which has been used for the genomic authentication of medicinal plants, has also proved suitable for species discrimination in a large number of plants.$^{33}$

**1.7.5. Chemically based antioxidant assessment**

Due to the highly variable nature of the flavonoid and phenyl carboxylic acid content of *E. arvense* extracts, and the correlation of phenolic content to radical scavenging
capacity, the radical scavenging capacity can be used as a basic and preliminary measure of pharmacological activity.\textsuperscript{38,39} It should be noted that this data is limited to the chemical sense and should not be used \textit{in lieu} of a cell line-based or \textit{in vivo} assay.\textsuperscript{39}

It is important to assess the biological effect of a complementary medicine as part of a strict QA regime. This is necessary as extracts may be chemically characterised as equivalent, though they 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.\textsuperscript{19}

The two main methods by which a compound can function as an antioxidant are hydrogen atom transfer (HAT) and electron transfer (ET).\textsuperscript{39} This study assesses the radical scavenging capacity of the \textit{E. arvense} extracts using both HAT and ET mechanisms.

HAT reactions such as ORAC are kinetic based methods, whereby fluorescein and the antioxidant being measured compete for peroxyl radicals generated by the thermal decomposition of 2,2’-azobis(2-amidinopropane) hydrochloride (AAPH).\textsuperscript{85} The stronger the antioxidant is, the longer the substrate takes to be degraded.

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 rapid and simple antioxidant assay that is commercially available. In its oxidised form DPPH has an intense purple colour ($\lambda_{\text{max}}$ 515 nm) and when it is reduced it becomes yellow ($\lambda_{\text{max}}$ 320 nm), the colour change being proportional to the antioxidant concentration.
Both the ORAC and DPPH methods use gallic acid as a reference for antioxidant capacity. That is, these assays measure how much better (or worse) the *E. arvense* extracts are at being antioxidants than gallic acid. An on-line LC-PDA DPPH assay was also used to detect the chemicals that may contribute the bulk antioxidant capacity of the extract.

### 1.7.6. Structural elucidation

While it is not a requirement to identify or quantify chemicals using the pattern-based method, the use of LC-MS, LC-DPPH-PDA and chemometrics provided a convenient starting point for the identification of pharmacologically active chemicals.

### 1.8. Aims

1. Use a systematic method for the selection of analytes for the quantitative analysis of Endoherb™.
2. Develop and validate an analytical method for the quantitation of the selected analytes in the dried Endoherb™ extract prepared from the pure aqueous, 35 % aqueous ethanol and 95 % aqueous ethanol extraction solvents.
3. Use the validated analytical method to evaluate the extraction efficiencies of the three solvents tested.
4. To develop TLC, LC-PDA and LC-MS methods to chromatographically profile and characterise the *E. arvense* extracts for phytochemical variability.
5. Use chemometric methods to statistically analyse the chromatographic profiles and determine the variability of the *E. arvense* extracts.
6. Use chemical antioxidant assays as a rapid and simple method for assessing the pharmacological variability of *E. arvense* extracts.
CHAPTER 2.

EXPERIMENTAL FOR ENDOHERB™
2.1. Preparation of Endoherb™ extracts

Three powdered extracts of approximately 200 g were produced by The Centre for Phytochemistry and Pharmacology, Southern Cross University (Lismore, NSW). The extracts were prepared using 95 % aqueous ethanol, 35 % aqueous ethanol, and water as the extraction solvent to investigate the effect of extraction solvent polarity on the quantity of the analytes in the final extract. All extracts were stored at room temperature in a desiccator over phosphorus pentoxide.

2.1.1. Sample preparation of the individual raw herbs for mixing

Each dried plant material was ground to ≤ 500 µm and then mixed in the in the ratio given in Table 1.1 to produce 2 kg of the mixture. Dried plant material was supplied by Beijing Tong Ren Tang (Sydney, Australia). The extracts were then prepared as follows.

2.1.2. Preparation of the aqueous ethanol extracts

For the 95 % aqueous ethanol extract, 2 kg of the herbal mixture was placed in a 50 L stainless steel rocking extraction vessel fitted with a filtration head. 20 L of 95 % ethanol was added and the mixture allowed to steep with a gentle rocking motion for 24 h. The 20 L of extract was then filtered through 3 × Whatman No. 1 filter papers. The extract was then concentrated using a 20 L rotary evaporator. The yield for this extract was lower than the desired 10 : 1 extraction ratio, so another 20 L of 95 % ethanol was added to the herb residue and steeped for a further 24 h, filtered, concentrated and combined with the first extract. The extract was then placed on a freeze drier as a final concentration step to yield 221.5 g of dried extract.
For the 35 % aqueous ethanol extract, the same procedure was followed except that 35 % ethanol was used instead of 95 % ethanol and only one aliquot of extraction solvent was required. The procedure produced 201.1 g of dried extract.

2.1.3. Aqueous extract

2 kg of the herbal mixture was placed in a 50 L stainless steel rocking extraction vessel. 20 L of water was added and allowed to steep with a heating element inserted into the extraction vessel. The extract was heated at 65 °C for 2 h with regular stirring. A filter head was then fitted to the extraction vessel and allowed to rock for 3 h to improve extraction. The extract was then filtered and dried as for the aqueous ethanol extracts to give 326.3 g of dried extract.

2.2. Equipment

2.2.1. 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 dissolve samples.

2.2.2. LC-PDA and LC-MS instrumentation

The LC-PDA analysis of glycyrrhizic acid, paeoniflorin, and rhein was performed on a Varian Prostar system comprising of 2 × 210 single pumps, a column valve module 500, a 430 auto-sampler, and a 335 PDA detector with a ‘9×0 mm’ analytic flow cell (Varian Inc., Australia). Solvents were degassed using a model AF DG2 in-line degasser (Waters, USA). The system was controlled using Varian Star Workstation version 6.20.
The column was a Luna C18 (150 × 4.6 mm, 5 µm) equipped with a C18 SecurityGuard (3 × 4.6 mm, 5 µm) guard column (Phenomenex, Australia).

The LC-MS analysis of amygdalin and pachymic acid was performed on the same Varian LC-PDA system except that the column eluent was passed through a splitter which diverted 20 % of the flow to a Varian 1200L triple quadrupole mass spectrometer and 80 % to the PDA detector.

2.2.3. GC-MS instrumentation

The GC-MS system used for the analysis of carveol, cinnamaldehyde, tetramethylpyrazine and umbelliferone was an Agilent 7890A GC with a 5975C inert XL EI/CI mass selective detector (MS) and CombiPal autosampler (Agilent, Australia). The column was a HP-5MS (30 m × 0.25 mm ID, 0.25 µm; J&W scientific, USA). The GC-FID system was the same as used for the GC-MS, though the column was connected to a FID. Helium was used as the carrier gas.

2.3. Reagents

Acetonitrile was of LC grade (Mallinckrodt Chemical Ltd., UK). Ethanol (95 %), methanol and formic acid (90 %) were reagent grade (Biolab, Australia). Air, argon, helium, hydrogen and nitrogen were of ultra-high purity grade (Coregas, Australia). Purified water (> 18 MΩ cm) was obtained from an Elga Purelab Prima and Purelab Ultra high purity water system (Biolab, Australia).

Amygdalin (98.6 %), catalpol, (-)-carveol and (+)-carveol (60 : 40 % mixture of isomers, 99.4 %), trans-cinnamaldehyde (99.4 %), glycyrrhizinic acid (93.4 %) and tetramethylpyrazine (99.9 %) were of primary grade (Sigma-Aldrich, Australia).
Atractylenolide III (99.5 %), ligustilide (98.8 %), paeonol (100 %), paeoniflorin (98.7 %), pachymic acid (97.9 %), rhein (99.7 %) and umbelliferone (100 %) were of secondary grade (Phytomarker Ltd., Tianjin, China). The primary grade standards have purity and spectroscopic characterisation; the secondary grade standards have purity by LC only. The results presented in the results section have been corrected for standard purity.

2.3.1. 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.

2.4. Preparation of fortification and mixed standard calibration solutions

2.4.1. Amygdalin, glycyrrhizic acid and paeoniflorin mixed fortification solution

A mixed fortification solution containing 2000 µg/mL amygdalin, 4000 µg/mL glycyrrhizic acid and 1000 µg/mL paeoniflorin were prepared by weighing 10.0, 20.0 and 5.0 mg of the respective standard into a 5 mL volumetric flask and adding approximately 3 mL of 50 % aqueous methanol before sonicating for 5 min or until the solid has dissolved. The solution was allowed to cool before making up to volume with 50 % aqueous methanol.
2.4.2. *Amygdalin, glycyrrhizic acid and paeoniflorin mixed standard solutions*

An intermediate mixed standard calibration solution was prepared by diluting 50 µL of the mixed fortification solution to 1000 µL with 50 % aqueous methanol to give a 20-fold dilution of the mixed fortification solution. This intermediate mixed standard was diluted 1, 1/5, 1/10, 1/50 and 1/100 to give the mixed working standard solutions.

2.4.3. *Pachymic acid and rhein mixed fortification solution*

A mixed fortification solution containing 3000 µg/mL pachymic acid and 1700 µg/mL rhein was prepared by weighing 30.0 and 17.0 mg of the respective standards into a 10 mL volumetric flask and adding approximately 7 mL of 95 % ethanol before sonicating for 5 min or until the standards have dissolved. The solution was allowed to cool before making up to volume with 95 % ethanol.

2.4.4. *Pachymic acid and rhein mixed standard solutions*

An intermediate mixed standard calibration solution was prepared by diluting 50 µL of the mixed fortification solution to 1000 µL with 95 % aqueous methanol to give a 20-fold dilution of the mixed fortification solution. This intermediate mixed standard was diluted 1, 1/5, 1/10, 1/50 and 1/100 to give the mixed working standard solutions.

2.4.5. *Atractylenolide III, ligustilide and paeonol mixed fortification solution*

A mixed fortification solution containing 4000 µg/mL atractylenolide III, 5500 µg/mL ligustilide and 18,200 µg/mL paeonol was prepared by adding 20.0 mg, 27.5 mg and 91.0 mg of the respective standards into a 5 mL volumetric flask containing approximately 3 mL ethanol. Ethanol was first placed in the flask because ligustilide is
volatile. The flask was loosely stoppered and sonicated until the solid just dissolved before cooling and making up to volume with 95 % ethanol.

2.4.6. Atractylenolide III, ligustilide and paeonol missed standard solutions

An intermediate mixed standard calibration solution was prepared by diluting 25 µL of the mixed fortification solution to 1000 µL with 95 % aqueous methanol to give a 40-fold dilution of the mixed fortification solution. This intermediate mixed standard was diluted as shown in Table 2.1 to give the mixed working standard solutions.

Table 2.1: Preparation of working standard solutions from the intermediate standard.

<table>
<thead>
<tr>
<th>Fold dilution</th>
<th>Volume of standard (µL)</th>
<th>Volume of 95 % ethanol (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/100</td>
<td>100</td>
<td>900</td>
</tr>
<tr>
<td>1/5</td>
<td>200</td>
<td>800</td>
</tr>
<tr>
<td>1/2.5</td>
<td>400</td>
<td>600</td>
</tr>
<tr>
<td>1/1.7</td>
<td>600</td>
<td>400</td>
</tr>
<tr>
<td>1/1.25</td>
<td>800</td>
<td>200</td>
</tr>
<tr>
<td>1 (no dilution)</td>
<td>1000</td>
<td>0</td>
</tr>
</tbody>
</table>

2.4.7. Carveol, cinnamaldehyde, tetramethylpyrazine and umbelliferone mixed fortification solution

A mixed fortification solution containing 287 µg/mL carveol, 278 µg/mL cinnamaldehyde, 100 µg/mL tetramethylpyrazine and 400 µg/mL umbelliferone was prepared by adding 3 µL (or 2.87 mg), 2.5 µL (or 278 mg), 1.0 mg and 4.0 mg of the respective standards into a 10 mL volumetric flask containing approximately 7 mL of 95 % ethanol. The flask was loosely stoppered and sonicated for approximately 5 min or till the solid is just dissolved. After cooling the solution was made up to volume with 95
% ethanol. The 95 % ethanol was first added to the flask, as carveol and cinnamaldehyde are volatile.

2.4.8. Atractylenolide III, ligustilide and paeonol standard solutions

An intermediate mixed standard calibration solution was prepared by diluting 25 µL of the mixed fortification solution to 1000 µL with 95 % aqueous methanol to give a 40-fold dilution of the mixed fortification solution. This intermediate mixed standard was diluted as shown in Table 2.1 to give the mixed working standard solutions.

2.5. Sample preparation

Two different extractions were used, utilising different solvents to selectively extract particular analytes. For the analysis of amygdalin, glycyrrhizic acid and paeoniflorin, 50 % aqueous methanol was used as the extraction solvent, while 95 % aqueous ethanol was used for the others.

200 mg of the dried extract was weighed into a 25 mL volumetric flask, approximately 20 mL of the extraction solvent added and the mixture sonicated for 30 min. The solution was allowed to cool before making up to volume with the extraction solvent. The sample was passed through a 0.45 µm PVDF syringe filter into a 1.5 mL autosampler vial for LC analysis.

2.6. Chromatographic conditions

Separate LC methods were used for the determination of amygdalin, glycyrrhizic acid, paeoniflorin, pachymic acid and rhein. GC-MS was used for the simultaneous determination of carveol, cinnamaldehyde, tetramethylpyrazine and umbelliferone. GC-
FID was used for the simultaneous determination of atractylenolide III, ligustilide and paeonol.

### 2.6.1. Analysis of amygdalin by LC-MS

The initial mobile phase composition was 88 % solvent A and 12 % solvent B, maintained for 10 min before changing to 95 % solvent B for 5 min to wash the column before returning to the initial composition for 5 min to equilibrate the column for the next analysis. The injection volume was 10 µL. The MS was operated using (-)-ESI with a drying gas temperature of 350 ºC, capillary voltage of -60 V, needle voltage of -4850 V, shield voltage of -400 V and a detector voltage of 1500 V. Q1 and Q3 both operated with a peak width of 3 AMU and a scan time of 2 s. The [M-H]⁻ ion (m/z 456) was monitored for quantitation and two transitions are monitored for qualification (m/z 456→119, 25 V and m/z 456→323, 13 V).

### 2.6.2. Analysis of glycyrrhizic acid by LC-PDA

The initial mobile phase was 58 % solvent A and 42 % solvent B, maintained for 10 min before changing to 95 % solvent B for 5 min to wash the column before returning to the initial composition for 5 min to equilibrate the column for the next analysis. The injection volume was 10 µL. The glycyrrhizic acid peak was monitored at 254 nm.

### 2.6.3. Analysis of paeoniflorin by LC-PDA

The initial mobile phase was 85 % solvent A and 15 % solvent B, maintained for 10 min before changing to 95 % solvent B for 5 min to wash the column before returning to the initial composition for 5 min to equilibrate the column for the next analysis. The injection volume was 10 µL. The paeoniflorin peak was observed at 230 nm.
2.6.4. Analysis of pachymic acid by LC-MS

The mobile phase was 20 % solvent A and 80 % solvent B. The injection volume was 10 µL. The MS was operated using (-)-ESI with a drying gas temperature of 300 ºC, capillary voltage of -100 V, needle voltage of -5000 V, shield voltage of -275 V and a detector voltage of 1500 V. Q1 and Q3 both operated with a peak width of 3 AMU and a scan time of 2 s. The [M-H]⁻ ion (m/z 528) was monitored for quantitation and two daughter ions were monitored for qualification (m/z 528→465, 46 V and m/z 528→467, 45 V).

2.6.5. Analysis of rhein by LC-PDA

The initial mobile phase was 55 % solvent A and 45 % solvent B, maintained for 10 min before changing to 95 % solvent B for 5 min to wash the column before returning to the initial composition for 5 min to equilibrate the column for the next analysis. The injection volume was 10 µL. Rhein was monitored at 430 nm.

2.6.6. Analysis of carveol, cinnamaldehyde, tetramethylpyrazine and umbelliferone by GC-MS

The autosampler was programmed for a 1 µL injection, 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 ºC using a split ratio of 20:1. The column pressure was programmed to maintain a constant flow of 1.5 mL/min. The initial oven temperature is held at 100 ºC for 1 min and then increased to 220 ºC at a rate of 10 ºC/min and held at 220 ºC for 2 min. The oven temperature was then increased to 250 ºC for 2 min after the run to clean the column. The MS transfer line was maintained at 250 ºC, the EI source at 230 ºC and the quadrupole at 150 ºC.
Quantitation was performed in selected ion monitoring (SIM) mode, monitoring the product ion shown in Table 2.2.

Table 2.2: Analytes and their ions monitored by GC-MS.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Product ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atractylenolide III</td>
<td>147, 215, 230</td>
</tr>
<tr>
<td>(+)-Carveol</td>
<td>84, 109, 134</td>
</tr>
<tr>
<td>(-)-Carveol</td>
<td>84, 109, 134</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>77, 103, 131</td>
</tr>
<tr>
<td>Ligustilide</td>
<td>148, 161, 190</td>
</tr>
<tr>
<td>Paeonol</td>
<td>108, 151, 166</td>
</tr>
<tr>
<td>Tetramethylpyrazine</td>
<td>54, 95, 136</td>
</tr>
<tr>
<td>Umbelliferone</td>
<td>105, 134, 162</td>
</tr>
</tbody>
</table>

2.6.7. *Atractylenolide III, ligustilide and paeonol using GC-FID*

The operating conditions for the GC, autosampler and column are the same as those used in Section 4.5. The FID was set at 300 ºC with a H₂ flow of 30 mL/min, air flow of 400 mL/min and He makeup flow of 25 mL/min.

2.6.8. *MS signal optimisation*

Prior to MS detection, several parameters were optimised to maximise ion formation, ion trajectories as well as ion fragmentation.

For LC-MS, these were determined by direct infusion of standard solutions (approximately 100 µg/mL in methanol) into the ESI interface at 20.0 µL/min. The ESI parameters optimised include needle voltage, shield voltage, capillary voltage, drying gas temperature and ionisation mode (ESI(+) or ESI(-)).
Once the optimum conditions for maximum molecular ion signal were determined, fragmentation experiments were performed to produce transition products for quantification and identity confirmation.

The transitions measured for GC-MS analysis were determined by injecting 1 μL of a 20 μg mL⁻¹ solution of chemical reference and determining the most intense and highest m/z transitions for each chemical.
CHAPTER 3.

EXPERIMENTAL FOR *E. ARVENSE*
3.1. Samples

3.1.1. Sources of E. arvense

The non-standardised E. arvense extracts sourced from the USA \((n = 7, 4 : 1\) extract ratio, dicalcium phosphate excipient); China \((n = 3, 5 : 1\) extract ratio, glucose excipient); Europe \((n = 2, 5 : 1\) extract ratio, lactose monohydrate excipient); and India \((n = 1, 4 : 1\) extract ratio, dicalcium phosphate excipient) were provided by LIPA Pharmaceuticals Ltd (NSW, Australia). The authenticity of the extracts was established by phytochemical comparison against reference extracts prepared from authenticated E. arvense plants with the traceability documents provided by each manufacturer. Where the raw material used to produce the extracts were available, these were used for genomic authentication.

3.1.2. Sample preparation

The excipient was removed from the commercial extracts to minimise sample variability due to different excipients used and their different extract-to-excipient ratios. Approximately 4 g of each commercial extract was weighed into a 250 mL conical flask and 250 mL of 80 % aqueous methanol was added. The mixture was sonicated for 1 h with occasional stirring and then centrifuged at 4000 g for 5 min to pellet out the insoluble excipient. The supernatant was filtered through a 0.45 µm PVDF syringe filter and the filtrate evaporated to dryness under vacuum at 60 °C to remove the methanol and the residue freeze-dried for 12 h to remove the remaining water. The resultant solid was stored at 4 °C if not used on the same day.
3.2. Phytochemical profiling

3.2.1. TLC

A CAMAG (Muttenz, Switzerland) TLC system equipped with a sample applicator and visualisation chamber was used with Merck (Darmstadt, Germany) silica gel 60 F$_{254}$ TLC plates (20 cm × 10 cm) for TLC profiling. The TLC profiling method was from Wagner et al, using a mobile phase of ethyl acetate : formic acid : glacial acetic acid : water (100 : 11 : 11 : 26 mL).\textsuperscript{86}

Working solutions of each extract were made by dissolving 50 mg of the purified sample in 1 mL of 80 % methanol. The solution was sonicated briefly to dissolve the extract and then filtered through a 0.45 µm PVDF syringe filter. 2 µL bands were applied to the plate.

To visualise the flavonoid and phenyl carboxylic acid profile, the plate was developed in natural products (diphenylboric acid 2-aminoethyl ester) and polyethylene glycol 4000 (PEG) reagent (NPPEG) and viewed under 366 nm light.

To visualise chemicals that scavenge the 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical, the plate was developed in DPPH reagent (200 µg/mL in ethanol) and visualised in white light. Chemicals that scavenge the DPPH radical appear yellow.

3.2.2. LC–PDA and LC-MS

A Varian (California, USA) ProStar system equipped with a 430 autosampler, 335 photodiode array detector (PDA) and 1200L quadrupole MS/MS detector was used for the LC profiling. An Alltech (Queensland, Australia) Prevail C18 column
Experimental for *E. arvense*

(150 mm × 4.6 mm, 5 µm) with a Phenomenex (California, USA) Security C18 guard column (2 mm × 4 mm, 5 µm) was used for analytical separation.

Working solutions of each extract were prepared by dissolving 50.0 mg of the purified sample in 1 mL 80 % methanol and briefly sonicating it to dissolve the extract before filtering through a 0.45 µm PVDF syringe filter.

The LC analysis was carried out using a 10 µL injection volume and a mobile phase flow rate of 1 mL/min consisting of 0.1 % aqueous formic acid (mobile phase A) and acetonitrile (mobile phase B). The mobile phase program was 10 % B for 10 min with a linear increase to 50 % B between 10 - 63 min. The column was then washed with 100 % B for 10 min and equilibrated with starting mobile phase for 10 min between each analysis.

The eluate was split to send 80 % to the PDA and 20 % to the MS. PDA chromatograms are acquired at 280 nm. The MS was set to acquire in the (−)-ESI mode, scanning between 70-700 AMU using a nebulisation gas temperature of 400 °C at 19 psi, needle voltage -3900 V at 15 µA, shield voltage -400 V, capillary voltage -100 V, and MS detector at -1700 V.

3.3. Chemometric analysis

Software supplied by the ‘R Project for Statistical Computing’ was used for the data processing and statistical analysis. Specific packages used with R are detailed as follows.87

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 was done to minimise sample variation due to instrumental factors.

PCA was used together with KNN to differentiate samples and highlight the chemical components potentially responsible these differences using the ‘stats’ package included with R. PCA was first conducted on the corrected chromatograms and the results are plotted using the first two PCs. KNN was then applied to the first two PCs in order to highlight samples that cluster together. Three groups were specified for the KNN, based on the country of origin of the sample: 1) USA, 2) China/Europe and 3) India. The group-specific peaks and their corresponding UV and MS spectra were compiled and compared those in the literature to tentatively identify the compounds.

Using the chromatogram correction technique outlined, the average number of peaks detected was determined using the usual techniques of TLC, LC-PDA and LC-MS to estimate their detection power. The determination of the statistical significance (p < 0.05) between the analytical techniques was measured by one-way ANOVA with a Tukey post-test using GraphPad Prism 5.0d for Mac OS X.

3.4. Genetic authentication

Genomic DNA was extracted and purified from the dried stem using a Qiagen DNeasy mini plant mini kit (Victoria, Australia) following the manufacturer’s instructions except that water was used instead of buffer AE. The loci used for genomic authentication are the chloroplast genes matK (Maturase K) and rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) as specified by the Consortium for the Barcode of Life (CBoL). For the PCR amplification of matK, the primers ATACCCCATTTTATTCATCC in the forward direction and
Experimental for E. arvense

TACTTTTATGTTTACGAGC in the reverse direction were used as recommended by the Royal Botanic Gardens, Kew. For the PCR amplification of rbcL the primers ATGTCACCACAACAGAGACTAAAGC in the forward direction and GTAAAATCAAGTCCACCCRCG in the reverse direction were used as recommended by CBoL. The iProof high-fidelity DNA polymerase PCR kit from Bio-Rad (NSW, Australia) was used for PCR amplification as per the manufacturer instructions for a 50 µL reaction with 35 cycles of the following temperature program: initial denaturation 98 °C, 60 s; denaturation 98 °C, 30 s; annealing 53 °C, 40 s; extension 72 °C, 40 s; final extension 72 °C, 5 min. The PCR products were purified using the Qiagen QIAquick PCR Purification Kit according to the manufacturer’s instructions except that water was used instead of buffer AE. PCR products were sequenced at the Australian Genome Research Facility Ltd (NSW, Australia). Data processing was performed using the program Geneious™.

3.5. Antioxidant assays

3.5.1. Chromatographic DPPH assay

Chromatographic peaks that scavenged the DPPH radicals were determined by introducing DPPH reagent (40 µg/mL in 60 % A and 40 % B) into the post-column eluent using a third pump (0.6 mL/min) and reacting the solution in a coil (5.0 m × 0.5 mm) based on the work by Bandoniene et al, as presented in Figure 3.1. The PDA detector was set to monitor at 280 nm for the chromatogram and 515 nm for the degradation of the DPPH radical.
Figure 3.1: A schematic of the online DPPH instrumentation.

3.5.2. DPPH radical scavenging assay

A method adapted from Blois et al and Molyneux et al was used to estimate the DPPH radical scavenging capacity of the *E. arvense* extracts compared to a gallic acid standard. All reagents are prepared in 80 % aqueous methanol. The gallic acid standard curve was made by diluting a gallic acid stock (3 mM) to give 0.3, 0.6, 0.9 and 1.5 mM working standards. Samples are prepared by dissolving 1.0 mg of the extract in 10 mL 80 % aqueous methanol which was also the solvent used as the reagent blank. 180 µL of the DPPH reagent (250 µM) was pipetted into each microtitre plate well used. In triplicate, 20 µL of each working standard, sample or blank was pipetted into the DPPH reagent to make a total volume of 200 µL in each well. To correct for sample absorbance (i.e. absorbance not due to the DPPH), sample blanks were also prepared in triplicate by adding 180 µL of 80 % aqueous methanol to the well and adding 20 µL of sample. The plate was vortexed at 700 rpm for 30 min in the dark prior to measuring the absorbance at 515 nm. The sample antioxidant scavenging capacity is reported as the gallic acid equivalent.
3.5.3. Oxygen Radical Absorbance Capacity assay

The oxygen radical absorbance capacity (ORAC) assay measures the ability of *E. arvense* extracts to protect fluorescein from degradation by peroxyl radicals using the method described in the BMG LABTECH (Ortenberg, Germany) application note 148 using gallic acid as the reference standard. All reagents were prepared in pH 7.4 phosphate buffer (10 mM). The gallic acid standard curve was prepared by diluting the gallic acid stock (200 µM) to give 12.5, 25, 50 and 100 µM working standards. Samples were prepared by dissolving 1.0 mg of extract in 10 mL of 80% aqueous methanol which was also the solvent used as the reagent blank. 150 µL fluorescein (10 nM) and 25 µL of either gallic acid standard, sample or blank was pipetted into each microtitre plate well used for analysis. This solution was vortexed for 30 min at 37 °C before rapidly adding 25 µL of the radical generator 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH, 240 mM) to each well and measuring the plate every 90 s (excitation 485 nm, emission 520 nm). The areas under the signal degradation curves of the samples were compared to the gallic acid standard and the results are given as the gallic acid equivalent.

3.5.4. Structural elucidation

Retention time, UV-Vis and MS spectra of the chromatographic peaks identified as being active using the LC-DPPH-PDA assay were compared to the literature for tentative structural elucidation.
CHAPTER 4.

RESULTS AND DISCUSSION FOR ENDOHERB™
4.1. Analyte selection

4.2. Extract production

The three Endoherb™ extracts were deliquescent, making accurate weighing and sampling difficult. The 95 % ethanol extract was the most difficult to work with as it did not dry to a solid, presumably because it contained oils. Both the 35 % and water extracts could be dried and powdered for homogeneous sampling when desiccated. The extraction method used involved soaking the powdered herb in water or aqueous ethanol and is therefore a relatively mild method of extraction. The extraction method approximates home extraction where the sliced or ground-up herbs are soaked in spirits. Home extraction by water usually involves boiling the herb to produce the decoction.

4.3. Quantitation of the selected analytes

4.3.1. Sample preparation

Many solvents were trialled in order to maximise the analyte extraction efficiency, and ideally, to perform a simultaneous extraction. Using water in combination with either methanol or ethanol caused a white cloudy solution to form in the ethanolic extracts of Endoherb™ instead of the clear coloured liquid obtained through extraction with pure ethanol. The cloudiness is probably due to non-polar components that are dispersed in the solvent but are not soluble in it. Consequently two extraction solvents: 50 % aqueous methanol and 95 % aqueous ethanol were needed to extract the high and low polarity analytes separately.
4.3.2. *Analytical method development*

The variety of chemical groups and hence chemical properties of the analytes present in the mixture made their analysis in a single analytical run difficult. Thus several methods had to be used for their analysis. The techniques of LC-PDA, LC-MS, GC-FID and GC-MS were employed. The use of chromatographic techniques appropriate to the chemicals analysed removed the need for long and involved sample preparation involving SPE or solvent-solvent partitioning.

A summary of the methods of analysis and the rationale for their use is presented in Figure 4.1.

![Fractionation Scheme](image)

*Figure 4.1: The fractionation scheme used for the analysis of the analytes.*

Chromatographic methods were developed for all analytes except catalpol. All the methods showed good resolution of the analyte peak and satisfactory signal-to-noise for their accurate and reproducible quantitation. Representative chromatograms of the chemical standards and herbal extracts are presented in Figure 4.2 - Figure 4.8.
Results and discussion for Endoherb™

Figure 4.2: Representative LC-PDA chromatograms of the glycyrrhizic acid standard (red) and the 95 % ethanolic extract (black). $\lambda_{\text{det}} = 254$ nm.

Figure 4.3: Representative LC-PDA chromatograms of the paeoniflorin standard (red) and the 95 % ethanolic extract (black). $\lambda_{\text{detection}} = 230$ nm.
Results and discussion for Endoherb™

Figure 4.4: Representative LC-PDA chromatograms of the rhein standard (red) and the 95 % ethanolic extract (black). $\lambda_{det} = 430$ nm.

Figure 4.5: Representative LC-MS chromatograms of the amygdalin standard (red) and the 95 % ethanolic extract (black).
Results and discussion for Endoherb™

Figure 4.6: Representative LC-MS chromatograms of the pachymic acid standard (red) and the 95 % ethanolic extract (black).

Figure 4.7: Representative GC-FID chromatograms of the mixed standard (red) and the 95 % ethanolic extract (black).
MS detection was required for most of the chemicals markers as they were present at low concentrations in the extract (< 1 mg/g). LC-PDA detection could not detect many of the analytes even if they had chromophore as it was not specific enough to separate potentially hundreds of other analytes unless an impractically long run (up to 2 h per sample) was used. MS can provide a means of specific detection of these analytes even without complete resolution from other peaks. GC-MS was used for the more volatile analytes as the significantly decreased peak width compared to LC made their analysis more specific and rapid.

Catalpol was unable to be measured with the available instrumentation, as it was too polar to show satisfactory retention on a C18 column and it has no satisfactory chromophore for sensitive and specific detection using a PDA detector. Catalpol was also not detected in any of the extracts using the MS detector during method
development. The result of this is that the herb Shudihuang is not measured in the final formulation. A possible reason for why catalpol has not been detected is that Shudihuang is honey baked; therefore the cells may not be being ruptured during the extraction process, especially when the honey coating is not soluble in 35 % or 95 % ethanol extraction solvent. It was also not possible to grind finely due to its stickiness.

Tetramethylpyrazine is below the limit of detection in the extracts tested. Even though tetramethylpyrazine was not detected, method validation was still conducted on this chemical, as the substance may be present in other sources of the herb.

It was necessary to use LC-PDA for the analysis glycyrrhizic acid, paeoniflorin and rhein as matrix effects prevented their accurate quantitation using the ESI source. The presence of strong chromophores, high concentration, and the robustness of the LC-PDA system negated the necessity to use LC-MS to analyse these chemicals.

4.3.3. Analytical method validation

All the methods showed good resolution of the analyte peak with a satisfactory signal-to-noise and good precision. Method validation was completed on all analytes except catalpol. A summary of the method validation parameters tested is presented in Table 4.2 - Table 4.6.

4.3.3.1. Analyte identity confirmation

Reasonable identity confirmation was achieved for those analytes analysed by LC-PDA by comparison of the UV-Vis spectra of the peaks obtained from the sample extract and standard solutions. Each analyte displayed a characteristic spectrum with a $\lambda_{\text{max}}$ together with at least one other peak. The sample and standard spectrum show good overlap as
Results and discussion for Endoherb™

can be observed in Figure 4.9 - Figure 4.11. Identity confirmation was supported by LC-MS as discussed in the next paragraph and Table 4.1.

![Graph showing UV spectra comparison](image)

**Figure 4.9:** Comparison between the standard (red) and sample (black) UV spectra of glycyrrhizic acid.
Figure 4.10: Comparison between the standard (red) and sample (black) UV spectra of paeoniflorin.

Figure 4.11: Comparison between the standard (red) and sample (black) UV-Vis spectra of rhein.

Identity confirmation for the chemicals markers analysed by LC-MS and GC-MS was achieved with greater confidence than by comparison of the UV-Vis spectrum, namely
Results and discussion for Endoherb™

by comparing the m/z of the ions obtained, as well as their relative abundances with those obtained for the standard. Additional confidence is gained if one is able to rationalise the observed ions to an assumed chemical structure. The relative intensities for the analytes in the standard and samples fall within acceptable tolerances.\textsuperscript{64} The MS identity confirmation results are presented in Table 4.1.
### Table 4.1: A comparison of the MS spectra of the analytes determined by LC-MS and GC-MS and their proposed fragmentation pattern.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Standard</th>
<th>Sample</th>
<th>Percentage difference (%)</th>
<th>Permitted tolerance (%)</th>
<th>Pass / Fail</th>
<th>Proposed fragmentation pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdalin</td>
<td>456(100)</td>
<td>456(100)</td>
<td>±0</td>
<td>±20</td>
<td>Pass</td>
<td><img src="image" alt="Amygdalin Fragmentation" /></td>
</tr>
<tr>
<td></td>
<td>323(9)</td>
<td>323(5)</td>
<td>±44</td>
<td>±50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>119(7)</td>
<td>119(4)</td>
<td>±43</td>
<td>±50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atractylenolide III</td>
<td>147(100)</td>
<td>147(100)</td>
<td>±0</td>
<td>±20</td>
<td>Pass</td>
<td><img src="image" alt="Atractylenolide Fragmentation" /></td>
</tr>
<tr>
<td></td>
<td>215(10.3)</td>
<td>215(9.9)</td>
<td>±5</td>
<td>±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>230(10.6)</td>
<td>230(10.2)</td>
<td>±4</td>
<td>±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carveol</td>
<td>84(51.8)</td>
<td>84(50.0)</td>
<td>±4</td>
<td>±10</td>
<td>Pass</td>
<td><img src="image" alt="Carveol Fragmentation" /></td>
</tr>
<tr>
<td></td>
<td>109(100)</td>
<td>109(100)</td>
<td>±0</td>
<td>±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>134(4.1)</td>
<td>134(4.3)</td>
<td>±5</td>
<td>±50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>77(61.1)</td>
<td>77(55.6)</td>
<td>±9</td>
<td>±15</td>
<td>Pass</td>
<td><img src="image" alt="Cinnamaldehyde Fragmentation" /></td>
</tr>
<tr>
<td></td>
<td>103(100)</td>
<td>103(100)</td>
<td>±0</td>
<td>±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>131(95.5)</td>
<td>131(90.9)</td>
<td>±5</td>
<td>±10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 4.1: Continued

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Standard Sample</th>
<th>Percentage difference (%)</th>
<th>Permitted tolerance (%)</th>
<th>Proposed fragmentation pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycyrrhizin acid</td>
<td>193(87) 351(100)</td>
<td>±20</td>
<td>±20</td>
<td>Pass</td>
</tr>
<tr>
<td>Ligustilide</td>
<td>148(78.0) 161(100)</td>
<td>±2</td>
<td>±0</td>
<td>Pass</td>
</tr>
<tr>
<td>Pachymic acid</td>
<td>528(00)</td>
<td>±20</td>
<td>±20</td>
<td>Pass</td>
</tr>
<tr>
<td>Paeoniflorin</td>
<td>121(100) 327(10)</td>
<td>±20</td>
<td>±50</td>
<td>Pass</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Analyte</th>
<th>Percentage difference (%)</th>
<th>Permitted tolerance (%)</th>
<th>Proposed fragmentation pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>193</td>
<td>Glycyrrhizin acid</td>
<td>±20</td>
<td>±20</td>
<td>Pass</td>
</tr>
<tr>
<td>148</td>
<td>Ligustilide</td>
<td>±20</td>
<td>±0</td>
<td>Pass</td>
</tr>
<tr>
<td>528</td>
<td>Pachymic acid</td>
<td>±20</td>
<td>±20</td>
<td>Pass</td>
</tr>
<tr>
<td>121</td>
<td>Paeoniflorin</td>
<td>±20</td>
<td>±50</td>
<td>Pass</td>
</tr>
</tbody>
</table>
### Table 4.1: Continued

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Standard</th>
<th>Sample</th>
<th>Percentage difference (%)</th>
<th>Permitted tolerance (%)</th>
<th>Pass / Fail</th>
<th>Proposed fragmentation pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paeonol</td>
<td>108(7.4)</td>
<td>108(7.1)</td>
<td>±4</td>
<td>±50</td>
<td>Pass</td>
<td><img src="image" alt="Fragmentation pattern" /></td>
</tr>
<tr>
<td></td>
<td>151(100)</td>
<td>151(100)</td>
<td>±0</td>
<td>±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>166(34.9)</td>
<td>166(36.4)</td>
<td>±4</td>
<td>±15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhein</td>
<td>182(43)</td>
<td>182(43)</td>
<td>±0</td>
<td>±25</td>
<td>Pass</td>
<td><img src="image" alt="Fragmentation pattern" /></td>
</tr>
<tr>
<td></td>
<td>239(72)</td>
<td>239(70)</td>
<td>±3</td>
<td>±20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetramethylpyrazine</td>
<td>54(43.8)</td>
<td>54(41.1)</td>
<td>±6</td>
<td>±10</td>
<td>Pass</td>
<td><img src="image" alt="Fragmentation pattern" /></td>
</tr>
<tr>
<td></td>
<td>95(100)</td>
<td>95(100)</td>
<td>±0</td>
<td>±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>136(42.3)</td>
<td>136(45.6)</td>
<td>±8</td>
<td>±50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Umbelliferone</td>
<td>105(51.0)</td>
<td>105(48.1)</td>
<td>±6</td>
<td>±15</td>
<td>Pass</td>
<td><img src="image" alt="Fragmentation pattern" /></td>
</tr>
<tr>
<td></td>
<td>134(100)</td>
<td>134(100)</td>
<td>±0</td>
<td>±10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>162(87.8)</td>
<td>162(84.4)</td>
<td>±4</td>
<td>±10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.3.2. Accuracy

The 50, 100 and 200 % spike recovery results for the 35 % ethanol extract of the Endoherb™ mixture are presented in Table 4.2. Considering all the analytes, the average recovery was 104.2 % (range 79.0 to 151.9 %) with an average RSD of 10.7 % (range 1.7 to 29 %).

Where the percentage recovery decreases with increasing fortification, for example for carveol, cinnamaldehyde, pachymic acid, paeonol, tetramethylpyrazine and umbelliferone, this may be due to the chemical being more difficult to dissolve completely at higher concentrations. Where the percentage recovery increases with an increase in fortification, such as atractylenolide III, glycyrrhizic acid, paeoniflorin and rhein, this may be due to a constant loss of chemical, such as by adsorption onto the surface of glass flasks or the filters used. The loss of a constant amount of analyte is proportionately more significant the lower the analyte concentration.

The umbelliferone measurement showed a significiation over-estimation of the expected concentration. This may be due to uncertainties regarding the purity of the standard since it was a secondary standard. This may be resolved by performing a painstaking purity characterisation of the standard using elemental analysis and thermal analysis of the standard. Purity by LC-PDA is unreliable because impurities without a chromophore (for example water) are not detected. The more common explanation for > 100 % recovery is a coeluting peak, but this is less likely in our case as a MS detector was used and the ion ratio comparison between sample and standard peaks are a good match.
Table 4.2: Summary of recoveries for the analytes.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Technique</th>
<th>Accuracy</th>
<th>50 % level</th>
<th>100 % level</th>
<th>200 % level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygadin</td>
<td>LC-MS</td>
<td>96.5</td>
<td>3</td>
<td>101.1</td>
<td>100.2</td>
</tr>
<tr>
<td>Atractylidenolide III</td>
<td>GC-FID</td>
<td>109.7</td>
<td>7.7</td>
<td>110.7</td>
<td>3.5</td>
</tr>
<tr>
<td>(+)-Carveol</td>
<td>GC-MS</td>
<td>131.1</td>
<td>10.5</td>
<td>79.3</td>
<td>10</td>
</tr>
<tr>
<td>(-)-Carveol</td>
<td>GC-MS</td>
<td>142.3</td>
<td>10.7</td>
<td>107.9</td>
<td>5.7</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>GC-MS</td>
<td>12.5</td>
<td>7.7</td>
<td>112.0</td>
<td>13.1</td>
</tr>
<tr>
<td>Glycyrrhizic acid</td>
<td>LC-PDA</td>
<td>87.9</td>
<td>4.7</td>
<td>96.6</td>
<td>2.8</td>
</tr>
<tr>
<td>Ligustilide</td>
<td>GC-FID</td>
<td>79.8</td>
<td>7.4</td>
<td>107.1</td>
<td>7.5</td>
</tr>
<tr>
<td>Paeoniflorin</td>
<td>LC-MS</td>
<td>114.6</td>
<td>1.9</td>
<td>107.1</td>
<td>3.3</td>
</tr>
<tr>
<td>Paeonol</td>
<td>LC-PDA</td>
<td>87.5</td>
<td>10.9</td>
<td>96.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Rhein</td>
<td>GC-FID</td>
<td>101.5</td>
<td>2.6</td>
<td>94.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Tetramethylpyrazine</td>
<td>GC-MS</td>
<td>93.6</td>
<td>1.7</td>
<td>94.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Umbelliferone</td>
<td>GC-MS</td>
<td>134.1</td>
<td>2.9</td>
<td>99</td>
<td>9.9</td>
</tr>
</tbody>
</table>

a Average and RSD calculated from n = 7 replicates.
4.3.3.3. Precision

Good instrumental and method precision was obtained using all methods of analysis as presented in Table 4.3.

Table 4.3: A summary of the method precision for the analytes.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Technique</th>
<th>Precision (^a)</th>
<th>Amount ± SD (mg/g)</th>
<th>(t_R) ± SD (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdalin</td>
<td>LC-MS</td>
<td></td>
<td>0.85 ± 0.03</td>
<td>7.433 ± 0.008</td>
</tr>
<tr>
<td>Atractylenolide III</td>
<td>GC-FID</td>
<td></td>
<td>1.08 ± 0.05</td>
<td>15.081 ± 0.001</td>
</tr>
<tr>
<td>(+)-Carveol</td>
<td>GC-MS</td>
<td></td>
<td>0.078 ± 0.005</td>
<td>4.462 ± 0.000</td>
</tr>
<tr>
<td>(-)-Carveol</td>
<td>GC-MS</td>
<td></td>
<td>0.007 ± 0.001</td>
<td>4.592 ± 0.004</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>GC-MS</td>
<td></td>
<td>0.146 ± 0.007</td>
<td>5.074 ± 0.000</td>
</tr>
<tr>
<td>Glycyrrhizic acid</td>
<td>LC-PDA</td>
<td></td>
<td>1.80 ± 0.03</td>
<td>6.43 ± 0.02</td>
</tr>
<tr>
<td>Ligustilide</td>
<td>GC-FID</td>
<td></td>
<td>11.2 ± 0.3</td>
<td>11.1622 ± 0.0005</td>
</tr>
<tr>
<td>Pachymic acid</td>
<td>LC-MS</td>
<td></td>
<td>0.46 ± 0.01</td>
<td>8.33 ± 0.02</td>
</tr>
<tr>
<td>Paeoniflorin</td>
<td>LC-PDA</td>
<td></td>
<td>14.5 ± 0.1</td>
<td>8.84 ± 0.03</td>
</tr>
<tr>
<td>Paeonol</td>
<td>GC-FID</td>
<td></td>
<td>8.1 ± 0.1</td>
<td>7.7908 ± 0.0005</td>
</tr>
<tr>
<td>Rhein</td>
<td>LC-PDA</td>
<td></td>
<td>0.119 ± 0.005</td>
<td>8.23 ± 0.03</td>
</tr>
<tr>
<td>Tetramethylpyrazine</td>
<td>GC-MS</td>
<td></td>
<td>0.003 ± 0.001 (^b)</td>
<td>3.08 ± 0.01 (^b)</td>
</tr>
<tr>
<td>Umbelliferone</td>
<td>GC-MS</td>
<td></td>
<td>0.013 ± 0.001</td>
<td>11.561 ± 0.000</td>
</tr>
</tbody>
</table>

\(^a\) Average and RSD calculated from \(n = 7\) replicates.
\(^b\) Not determined in the unfortified sample, value determined from the 50 % fortification.

4.3.3.4. Limits of detection

The LOQs are sufficiently low to quantify the concentrations of these chemicals that can be reasonably expected in the ethanolic extracts of Endoherb\textsuperscript{TM}, as presented in Table 4.4.
Table 4.4: Summary of the analytical method detection and quantification limits for the analytes.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Technique</th>
<th>Detection limits $^a$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LOD (mg/g)</td>
<td>LOQ (mg/g)</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>LC-MS</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Atractylenolide III</td>
<td>GC-FID</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>(+)-Carveol</td>
<td>GC-MS</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>(-)-Carveol</td>
<td>GC-MS</td>
<td>0.003</td>
<td>0.01</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>GC-MS</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>Glycyrrhizic acid</td>
<td>LC-PDA</td>
<td>0.09</td>
<td>0.3</td>
</tr>
<tr>
<td>Ligustilide</td>
<td>GC-FID</td>
<td>1</td>
<td>3.2</td>
</tr>
<tr>
<td>Pachymic acid</td>
<td>LC-MS</td>
<td>0.04</td>
<td>0.1</td>
</tr>
<tr>
<td>Paeoniflorin</td>
<td>LC-PDA</td>
<td>0.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Paeonol</td>
<td>GC-FID</td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>Rhein</td>
<td>LC-PDA</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>Tetramethylpyrazine</td>
<td>GC-MS</td>
<td>0.003 $^b$</td>
<td>0.009 $^a$</td>
</tr>
<tr>
<td>Umbelliferone</td>
<td>GC-MS</td>
<td>0.003</td>
<td>0.012</td>
</tr>
</tbody>
</table>

$^a$ Average and RSD calculated from $n = 7$ replicates.

$^b$ Not determined in the unfortified sample, value determined from the 50 % fortification.

4.3.3.5. **Linearity**

The calibration curves show good linearity with correlation coefficients ($r^2$) > 0.995 as presented in Table 4.5.
Table 4.5: Summary of the calibration curve linearity for the analytes.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Technique</th>
<th>Linearity Range (µg/mL)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdalin</td>
<td>LC-MS</td>
<td>1 - 100</td>
<td>0.996</td>
</tr>
<tr>
<td>Atractylenolide III</td>
<td>GC-FID</td>
<td>10.5 - 105</td>
<td>0.9998</td>
</tr>
<tr>
<td>(+)-Carveol</td>
<td>GC-MS</td>
<td>0.4 - 4</td>
<td>0.9995</td>
</tr>
<tr>
<td>(-)-Carveol</td>
<td>GC-MS</td>
<td>0.4 – 4</td>
<td>0.9994</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>GC-MS</td>
<td>0.12 – 12</td>
<td>0.996</td>
</tr>
<tr>
<td>Glycyrrhizic acid</td>
<td>LC-PDA</td>
<td>2 - 211</td>
<td>0.9992</td>
</tr>
<tr>
<td>Ligustilide</td>
<td>GC-FID</td>
<td>12 - 120</td>
<td>0.9998</td>
</tr>
<tr>
<td>Pachymic acid</td>
<td>LC-MS</td>
<td>1.5 - 150</td>
<td>0.998</td>
</tr>
<tr>
<td>Paeoniflorin</td>
<td>LC-PDA</td>
<td>10 - 1000</td>
<td>0.9999</td>
</tr>
<tr>
<td>Paeonol</td>
<td>GC-FID</td>
<td>45 - 450</td>
<td>0.9998</td>
</tr>
<tr>
<td>Rhein</td>
<td>LC-PDA</td>
<td>0.8 - 42</td>
<td>0.9994</td>
</tr>
<tr>
<td>Tetramethylpyrazine</td>
<td>GC-MS</td>
<td>0.25 – 2.5</td>
<td>0.9995</td>
</tr>
<tr>
<td>Umbelliferone</td>
<td>GC-MS</td>
<td>1 – 10</td>
<td>0.998</td>
</tr>
</tbody>
</table>

4.3.3.6. Stability

For accurate quantitation, standards and samples need to be prepared freshly on the day of analysis, with the sample extracts being stable for up to 24 h.

4.3.4. Extract comparison

A summary of the analyte concentrations in the various extracts is presented in Table 4.6 and Figure 4.12. The 95 % ethanolic extract contained the highest concentrations of the analytes, followed by the 35 % ethanolic extract, and lastly the aqueous extract as can be observed in Figure 4.12.
Results and discussion for Endoherb™

Table 4.6: Summary of the results obtained for each of the Endoherb™ extracts tested

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Technique</th>
<th>Aqueous extract</th>
<th>35 % ethanol extract</th>
<th>95 % ethanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdalin</td>
<td>LC-MS</td>
<td>0.298 ± 0.002</td>
<td>0.85 ± 0.03</td>
<td>2.078 ± 0.1</td>
</tr>
<tr>
<td>Atractylenolide III</td>
<td>GC-FID</td>
<td>&lt; LOD</td>
<td>1.08 ± 0.05</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>(+)-Carveol</td>
<td>GC-MS</td>
<td>&lt; LOD</td>
<td>0.078 ± 0.005</td>
<td>0.2021 ± 0.0002</td>
</tr>
<tr>
<td>(-)-Carveol</td>
<td>GC-MS</td>
<td>&lt; LOD</td>
<td>0.007 ± 0.001</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td>GC-MS</td>
<td>&lt; LOD</td>
<td>0.146 ± 0.007</td>
<td>0.512 ± 0.07</td>
</tr>
<tr>
<td>Glycyrrhizic acid</td>
<td>LC-PDA</td>
<td>0.6 ± 0.1</td>
<td>1.80 ± 0.03</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>Ligustilide</td>
<td>GC-FID</td>
<td>&lt; LOD</td>
<td>11.2 ± 0.3</td>
<td>8.4 ± 1.5</td>
</tr>
<tr>
<td>Pachymic acid</td>
<td>LC-MS</td>
<td>&lt; LOD</td>
<td>0.46 ± 0.01</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>Paeoniflorin</td>
<td>LC-PDA</td>
<td>11.6 ± 0.1</td>
<td>14.5 ± 0.1</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>Paeonol</td>
<td>GC-FID</td>
<td>&lt; LOD</td>
<td>8.1 ± 0.1</td>
<td>11.4 ± 0.6</td>
</tr>
<tr>
<td>Rhein</td>
<td>LC-PDA</td>
<td>&lt; LOQ</td>
<td>0.119 ± 0.005</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Tetramethylpyrazine</td>
<td>GC-MS</td>
<td>&lt; LOD</td>
<td>&lt; LOD</td>
<td>&lt; LOD</td>
</tr>
<tr>
<td>Umbelliferone</td>
<td>GC-MS</td>
<td>0.05 ± 0.05</td>
<td>0.013 ± 0.001</td>
<td>0.0242 ± 0.0001</td>
</tr>
</tbody>
</table>
Figure 4.12: A comparison of the amount of chemical determined in each of the different extracts.
CHAPTER 5.

RESULTS AND DISCUSSION FOR *E. ARVENSE*
5.1. Phytochemical profiling

Significant quantitative and qualitative differences were observed in the profiles obtained using TLC, LC-PDA and LC-MS, especially in relation to phenyl carboxylic acids and flavonoids.

The flavonoid and phenyl carboxylic acid TLC profile, while being useful as a rapid profiling technique, was insufficient at revealing the complex nature of the chemical variation between samples, as presented in Figure 5.1. The TLC profile was on average able to resolve $9 \pm 3$ peaks, however only a single peak was detected in the India 13 sample. The TLC profile was however sufficient to indicate a general quantitative difference in phenyl carboxylic acid and flavonoid concentration between, forming three general groups: 1) the USA extracts 2) the European and Chinese extracts 3) the Indian extract. Due to the poor sensitivity of the TLC technique, it is difficult to conclude whether any qualitative differences between the samples exist.
Results and discussion for E. arvense

Figure 5.1: Chromatographic characterisation of the E. arvense extracts using TLC stained with Natural Products and Polyethylene Glycol reagent view under 366 nm UV light.

The LC-PDA chromatogram acquired at 280 nm contained $35 \pm 7$ peaks, triple the number of peaks contained in the TLC profile, as presented in Figure 5.2. The same general trend in phenyl carboxylic acid and flavonoid concentration in the extracts from different was similar to that in the TLC. The LC-PDA profile has the added benefit of acquiring the UV-Vis spectrum of each peak, which can be useful in downstream process such as structural elucidation. Clear qualitative differences also exist between the samples, especially in regards to the Indian sample, detectable due to the increased sensitivity of the LC-PDA technique over TLC.
Results and discussion for *E. arvense*

Figure 5.2: Chromatographic characterisation of the *E. arvense* extracts using LC-PDA viewed at 280 nm. (Note: The annotation a represents chicorium acid).

The LC-MS technique detected on average 43 ± 8 peaks, displaying both qualitative and quantitative differences between the extracts as shown in Figure 5.3. The same general trend in phenyl carboxylic acid and flavonoid concentration between countries of origin was similar to that in the TLC.
Figure 5.3: Chromatographic characterisation of the *E. arvense* extracts using LC-MS. (Note: The annotation *a* represents chicoric acid).

The increase in the number of peaks obtained by on each profiling technique is presented in Figure 5.4.
Figure 5.4: The number of peaks detected in the TLC, LC-PDA and LC-MS chromatograms using the msProcess peak detection software. (Note: * represents a statistical significance of $p < 0.05$).

The detection of peaks in a chromatogram is crucial for both qualitative and quantitative analyses, because the amount of information increases as more peaks are detected. The comparison of the different profiling techniques is useful information for industry, where TLC is the profiling method of choice for herbal authentication. TLC is straightforward to perform but the information it provides is somewhat limited. The LC-MS is much more informative but the cost of the instrumentation is more than double that of the TLC and involves much more maintenance and is more costly to operate.

5.2. Chemometric analysis

5.2.1. Chromatographic processing

The package msProcess was successfully used to remove instrumental noise, remove baseline drift, identify peaks, and remove peak retention time variations to accurately
Results and discussion for *E. arvense*

Quantify peak height. Although it is desirable to use peak area for accurate quantitation using LC, peak height was used due to a limitation of the software. Satisfactory identification of peaks was obtained using a trial and error method, resulting in the parameters presented in Table 5.1.

Table 5.1: The parameters used for chromatographic processing and a brief outline of their function.

<table>
<thead>
<tr>
<th>Process</th>
<th>Parameter</th>
<th>Value</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>msDenoise</td>
<td>function</td>
<td>wavelet</td>
<td>Decreases the amplitude of the wavelet coefficients resulting in a denoised version of the original series.</td>
</tr>
<tr>
<td>msDenoise</td>
<td>thresh.scale</td>
<td>5</td>
<td>Amplifies or attenuates the threshold values at each decomposition level to obtain a smoother or rougher result.</td>
</tr>
<tr>
<td>msNoise</td>
<td>function</td>
<td>spline</td>
<td>Smooths the series based on a spline.</td>
</tr>
<tr>
<td>msDetrend</td>
<td>function</td>
<td>supsmu</td>
<td>Estimates the baseline by fitting a smooth curve to the local minima within the span parameter.</td>
</tr>
<tr>
<td>msDetrend</td>
<td>span</td>
<td>0.1</td>
<td>The fraction of the observations in the span of the running lines smoother.</td>
</tr>
<tr>
<td>msPeak</td>
<td>function</td>
<td>simple</td>
<td>Performs peak detection via a simple local maxima search.</td>
</tr>
<tr>
<td>msPeak</td>
<td>snr.thresh</td>
<td>10</td>
<td>The signal to noise threshold. Only the local maxima whose signal to noise level is above this value will be recorded as peaks.</td>
</tr>
<tr>
<td>msAlign</td>
<td>function</td>
<td>cluster</td>
<td>Clusters peaks using one-dimensional hierarchical clustering and uses distances between peak locations as the similarity measure.</td>
</tr>
<tr>
<td>msAlign</td>
<td>snr.thresh</td>
<td>10</td>
<td>The peaks with signal-to-noise ratio larger than this value will be used to construct the common set of peak classes.</td>
</tr>
<tr>
<td>msAlign</td>
<td>mz.precision</td>
<td>0.01</td>
<td>A numeric value, used to construct the threshold when performing clustering.</td>
</tr>
<tr>
<td>msQuantify</td>
<td>function</td>
<td>intensity</td>
<td>The measure used to identify candidate peaks is the maximum intensity value found over a pre-defined span of the corresponding peak class.</td>
</tr>
</tbody>
</table>

An illustration of processes involved in the chromatographic processing is presented in Figure 5.5.
Results and discussion for *E. arvense*

Figure 5.5: A representative chromatogram of the China 8 sample indicating various corrections performed using msProcess.

A) The original chromatogram. B) The black line represents the noise detected in the chromatogram. The red line represents the average. C) The black line represents the de-noised chromatogram and the red line represents the detected baseline drift. D) The black line represents the chromatogram with the baseline drift removed; the red circles represent the detected peaks. E) The corrected chromatogram converted into centroid form.

Once each individual chromatogram is processed, they are compared to each other to determine a set of representative peaks present in most of the chromatograms, as
presented in Figure 5.6. From this, a table of intensity values was produced that was used for the subsequent statistical analysis. Ultimately, 107 intensity values were obtained for each chromatogram.

Figure 5.6: The chromatographic peak alignment of the *E. arvense* chromatograms.

(Note: Red circles represent where a peak is detected in each chromatogram. Where that peak is identified in most chromatograms, the peak height is measured in all chromatograms at the average retention time, represented by the vertical orange lines).

5.2.2. Statistical analysis

The multivariate statistical techniques of PCA and KNN were successfully used to identify differences and similarities between the different *E. arvense* extracts based on peak intensity. KNN was used to identify grouping obtained using PCA, by highlighting samples that were classified into the 3 groups. PCA was also used to determine which
chromatographic peaks and therefore phytochemicals are responsible for the observed differentiation.

As presented in Figure 5.7, grouping in the scores plot (left) is observed, which is consistent with the country of origin, that is: USA (red), China / Europe (blue), and India (green). KNN clustering represented by coloured circles proved very effective in sample classification. Based on the similar proximity of chromatographic peaks in the loadings plot (right) to the sample groups in the scores plot, the peaks responsible for sample differentiation were identified. Three representative peaks have been highlighted in the same colours as the sample groups to indicate the groups they affect. Chicoric acid is highlighted in Figure 5.2 and Figure 5.3 (annotation a) as a representative example of how this peak is detected only in the India 13 sample by visual examination, and confirmed as a differentiating variable in the PCA. Other chemicals that have been tentatively identified in the E. arvense extracts based on comparison of LC, UV-Vis and MS data to the literature are presented in Section 5.5.
Results and discussion for *E. arvense*

Figure 5.7: Principal component analysis (PCA) of LC-MS chromatographic peaks identified using msProcess.

The colour and ellipses on the scores plots denote grouping obtained from KNN clustering using 3 specified clusters. The proportion of variance encompassed by each PC is given in brackets. The scores plot (left) is based on the absolute amplitude of all 107 detected peaks, showing that the geographical origin of the extracts is primarily associated with the 3 specified groups (*red* = USA, *blue* = Europe and China, *green* = India). The loadings plot (right) highlights peaks that are representative of the grouping observed in the scores plot (3-hydroxyflavone for USA, methyl caffeoylquinic acid for Europe and China, chicoric acid for India).

As presented in Figure 5.8, the PCA based on the intensity ratios (PSI) showed better delineation between the USA and European extracts. The downside to this method is many more data points are produced (left) which make peak identification difficult.
Results and discussion for *E. arvense*

Figure 5.8: Principal component analysis (PCA) of LC-MS chromatographic peaks identified using msProcess.

The colour and ellipses on the scores plots denote grouping obtained from KNN using 3 specified clusters. The proportion of variance encompassed by each PC is given in brackets. The scores plot (left) is of the 5,671 peaks intensity ratios obtained from the 107 detected peaks using the rational of Tilton *et al* showing the geographical origin of the extracts is primarily associated with the 3 specified groups (red = USA, blue = Europe and China, green = India). The loadings plot (right) contained no significant information.

5.3. Genetic authentication

Both the *matK* and *rbcL* loci were successfully used to authenticate the representative China, Europe and India samples with reasonable certainly. The *matK* locus was better at differentiating *E. arvense* from the other *Equisetum* species than *rbcL*, with a BLAST search of GenBank® yielding between 97.3 – 99.9 % (India and Europe respectively) identical sites to the *E. arvense* database entries using the *matK* products compared to 98.9 – 100 % (Europe and India respectively) for *rbcL*. Although the percentage match using *rbcL* is higher, the percentages are equally shared with other *Equisetum* species,
for example India 13 shared the 100 % match with both *E. fluviatile* and *E. diffusum*. Numerous single nucleotide polymorphisms (SNPs) are present in the *matK* sequence for the India 13 sample, including an insertion between 465-472 bp not present in any other GenBank® entries, which may indicate it is a different species yet to be published in GenBank®. Nucleotide alignments of the China 8, Europe 11 and India 13 *matK* and *rbcL* sequences against other species in the GenBank® database are presented in Figure 5.9 and Figure 5.10.
Results and discussion for *E. arvense*

Figure 5.9: matK DNA barcodes of the original plant material used to produce the China 8, Europe 11 and India 13 extracts compared to other *Equisetum* species entries in the GenBank database.

(Note: Differences between the sequences are marked with a coloured box (red = A, green = T, blue = C, yellow = G).)
Results and discussion for *E. arvense*

Figure 5.10: *rbcL* DNA barcodes of the original plant material used to produce the Europe 11 and India 13 extracts compared to other *Equisetum* species entries in the GenBank® database. (Note: Differences between the sequences are marked with a coloured box (red = A, green = T, blue = C, yellow = G)).
5.4. Radical scavenging assays

5.4.1. Radical scavenging capacity

Assessment of the total radical scavenging capacity of the extracts was measured using two different antioxidant techniques to account for the different kinetic models involved in antioxidant activity. Figure 5.11 shows a comparison of the antioxidant capacity of the different *E. arvense* extracts. Generally speaking, the ORAC and DPPH results are comparable, indicating that the flavonoids and phenyl carboxylic acids can function in both the HAT and ET mechanisms. The China 8 and USA 7 samples showed the highest antioxidant capacity of the extracts. This is contrary to what is implied by the phytochemical profiling, which indicates that the China and European extracts are similar to each other and distinct from the USA extracts.

![Bar graph showing antioxidant activity of various *E. arvense* extracts using DPPH and ORAC assays.](image)

Figure 5.11: The antioxidant activity of the various *E. arvense* extracts using the DPPH and ORAC assays.
The DPPH-based antioxidant assay was very useful for the assessment of antioxidant activity due primarily to the rapidity of the reaction, as well as being performed in a solvent compatible with the herbal extracts. The major downside of this assay is that the assay may have little biological implication.\(^{39}\)

### 5.4.2. Chromatographic DPPH assay

Due to the rapidity of the reaction, DPPH is useful as a TLC stain. When in the presence of a radical scavenger, the purple DPPH radical is converted to a yellow colour. As presented in Figure 5.12, the Chinese and European extracts contain approximately 5 strongly antioxidant analytes.

Figure 5.12: The antioxidant activity of the various *E. arvense* extracts as profiled using TLC developed in DPPH reagent, viewed under white light. (Note: Pink/purple regions are unreacted DPPH, lighter regions are where the DPPH radical has been scavenged by an antioxidant).

The online LC-DPPH-PDA method was adapted in order to determine the analytes potentially responsible for the antioxidant activity of the extracts. The preliminary
results in tandem with the tentative structural elucidation provide a useful method to expose the chemicals responsible for the purported pharmacological effects. As presented in Figure 5.13, peaks at 280 nm (similar to that in Figure 5.2) that have a DPPH radical scavenging capacity are identified by the corresponding decrease in DPPH absorbance measured simultaneously at 515 nm.

![Absorbance graph](image)

**Figure 5.13:** A representative chromatogram of the China 8 sample using the on-line DPPH assay.

(Note: The chromatogram at 280 nm is overlayed with the DPPH absorbance at 515 nm. Analytes that have DPPH antioxidant activity are observed as a negative peak at 515 nm).

### 5.5. Structural elucidation of antioxidant peaks

The retention time, as well as the UV-Vis and MS spectra of the peaks observed in the LC-PDA and LC-MS chromatograms respectively was compared to the literature for tentative preliminary structural elucidation as presented in Table 5.2. A representative example of how the structure of dicaffeoyltartaric acid and a genkwanin acetylglucoside were elucidated is presented in Figure 5.14 and Figure 5.15 respectively.
Table 5.2: The tentative structural elucidation of several chemical constituents contained in the *E. arvense* samples. Table kindly supplied by Dr Suresh Govindaraghavan.

<table>
<thead>
<tr>
<th>tR (min)</th>
<th>PDA peaks (nm)</th>
<th>MS peaks (m/z)</th>
<th>Tentative ID</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.6</td>
<td>241, 328</td>
<td>179, 149, 312(100), 312(100)</td>
<td>Caffeoyl tartaric acid isomer</td>
<td>98,99</td>
</tr>
<tr>
<td>6.5</td>
<td>242, 327</td>
<td>149, 225, 312(100)</td>
<td>Caffeoyl tartaric acid isomer</td>
<td>98,99</td>
</tr>
<tr>
<td>9.9</td>
<td>241, 327</td>
<td>148, 179, 311(100)</td>
<td>Caffeoyl tartaric acid isomer</td>
<td>98,99</td>
</tr>
<tr>
<td>11.4</td>
<td>241, 328</td>
<td>135, 179, 225, 367(100)</td>
<td>Methyl caffeoylquinic acid</td>
<td>98,99</td>
</tr>
<tr>
<td>16.6</td>
<td>240, 323</td>
<td>295, 336(100)</td>
<td>Caffeoyl shikimic acid isomer</td>
<td>100</td>
</tr>
<tr>
<td>18.5</td>
<td>254, 315</td>
<td>179, 295, 335(100)</td>
<td>Caffeoyl shikimic acid isomer</td>
<td>100</td>
</tr>
<tr>
<td>19.8</td>
<td>253, 326</td>
<td>650(100)</td>
<td>Quercetin or Protogenkwanin derivative</td>
<td>101</td>
</tr>
<tr>
<td>24.6</td>
<td>231, 283</td>
<td>319, 448(100)</td>
<td>Luteolin glucoside</td>
<td>68,102</td>
</tr>
<tr>
<td>26.6</td>
<td>252, 324</td>
<td>300, 342, 464(100)</td>
<td>Quercetin glucoside</td>
<td>68,101</td>
</tr>
<tr>
<td>28.7</td>
<td>255, 366</td>
<td>271, 579(100)</td>
<td>Apigenin 3-O-glucoside-7-O-rhamnoside</td>
<td>68,103</td>
</tr>
<tr>
<td>30.1</td>
<td>256, 326</td>
<td>431(100), 463, 489</td>
<td>Apigenin glucoside</td>
<td>68,102</td>
</tr>
<tr>
<td>31</td>
<td>235, 269, 330</td>
<td>410(100), 462</td>
<td>Quercetin glucoside</td>
<td>68,101</td>
</tr>
<tr>
<td>31.4</td>
<td>235, 287</td>
<td>301, 610</td>
<td>Quercetin 3-O-glucoside-7-O-rhamnoside</td>
<td>103</td>
</tr>
<tr>
<td>32.9</td>
<td>238, 328</td>
<td>149, 178, 311(100), 473</td>
<td>Dicaffeoyl tartaric acid</td>
<td>104</td>
</tr>
<tr>
<td>34.9</td>
<td>237, 261, 333</td>
<td>445(100), 490</td>
<td>Genkwanin glucoside isomer</td>
<td>68,101</td>
</tr>
<tr>
<td>41.1</td>
<td>288, 353</td>
<td>285(100)</td>
<td>Kaempferol derivative</td>
<td>68,101</td>
</tr>
<tr>
<td>42.7</td>
<td>233, 270, 324</td>
<td>284(100)</td>
<td>Genkwanin glucoside isomer</td>
<td>68,101</td>
</tr>
<tr>
<td>46.8</td>
<td>231, 288</td>
<td>302(100)</td>
<td>Quercetin / Protogenkwanin</td>
<td>101</td>
</tr>
</tbody>
</table>
Results and discussion for *E. arvense*

Figure 5.14: Tentative spectral structural elucidation of dicaffeoyltartaric acid using (A) LC-MS and (B) LC-PDA and (C) the proposed MS fragmentation pattern.
Figure 5.15: Tentative spectral structural elucidation of genkwanin acetylglucoside using (A) LC-MS and (B) LC-PDA and (C) the proposed MS fragmentation pattern.
CHAPTER 6.

GENERAL CONCLUSIONS
6.1. Summary

The past decade has seen an unprecedented growth in the popularity of complementary medicines in Western countries. As the popularity of complementary medicines continues to grow, serious concerns have been raised about their quality and safety. HM is faced with problems of plant misidentification and inconsistent quality. Herbal quality control is a significant challenge due to the large assortment of chemicals found in a natural product and their expected compositional variation. The complexity of applying quality control to multi-herb medicines increases with each additional herb. This study shows how quality control of a complex herbal mixture may be carried out and how the quality and identity of the single herb may be established.

Current regulatory standards predominantly focus on chromatographic methods of analysis for the separation of the complex assortment of chemicals contained in herbal formulations. The compound-based method focuses on the quantitative analysis of one or more chemical constituents of an herb or formulation in order to determine quality and hence product consistency. In contrast, the pattern-based method compares the chromatographic profile of the extract of a new batch with that of a target or reference batch, without necessarily identifying or quantifying the constituents. Similarly new genetic and pharmacological methods are also available to supplement the characterisation of plants.

Importantly, no single technique is adequate for the QA and QC of herbal material. Rather, a multifaceted strategy including qualitative chemical profiles, quantitative analysis of analytes, genetic authentication and pharmacological activity, should be considered for characterising herbs and herbal products.
6.2. Major findings and implications in the application of the compound-based approach to the quality evaluation of Endoherb™

The complex nature of Endoherb™ made it an exemplar model for assessing the compound-based approach to herbal medicine QA. The methodology used in this study has demonstrated how the systematic QC of a complex herbal mixture can be carried out. The process firstly involves applying a logical and systematic method for the selection of the analytes, and then developing a method for their sensitive, specific and accurate analysis. This methodology has wide-ranging implications for the QC of complex herbal mixtures.

6.2.1. A systematic method for the selection of analytes for the quantitative analysis of Endoherb™

A major contribution of this work is the logical and systematic process that has been applied for the careful selection of analytes relevant to the safety and efficacy of the Endoherb™ formulation. The need for such a process is self evident, as a typical multi-herb formula, generally containing between 3 to 20 herbs, could contain many hundreds of analytes, therefore making the total chemical analysis impractical. With no currently established and accepted criteria for the selection of marker chemicals in the QA industry, especially in relation to complex herbal mixtures, this methodology provides a foundation on which some form of informed decision can be made. Accurate chemical characterisation of an HM is achieved by taking into account variables such as the traditional use of the herb, previous research, analyte localisation, bioavailability, uniqueness, concentration, toxicity and commercial availability of the selected analyte.
6.2.2. *A validated analytical method for the quantitation of the selected analytes in the dried Endoherb™ extract prepared from the pure aqueous, 35 % aqueous ethanol and 95 % aqueous ethanol extraction solvents.*

The trustworthiness of any chemical analysis is dependant on the quality of the analytical method used. Considerable time was invested in ensuring that the performance of the developed method was well characterised with regards to specificity, accuracy and precision in quantifying the selected analytes.

The diverse chemical properties of the selected analytes made it difficult to analyse them using a single simultaneous chromatographic method. Several methods were instead used for the specific analysis of these chemical components based on factors such as their polarity, volatility and presence of a chromophore. LC-PDA, LC-MS, GC-FID and GC-MS were employed.

The sample preparation is rapid and simple, utilising sonication as the extraction method. This method of extraction is amenable to batch processing. The substantial differences in the chemistry of the analytes made it necessary to use two extraction solvents: 50 % aqueous methanol and 95 % aqueous ethanol to extract the analytes.

6.2.3. *Evaluation of the extraction efficiencies of the three solvents tested.*

The results of the extraction solvent comparison showed that increasing the organic modifier in the extraction solvent, even for predominantly polar analytes, could increase analyte extraction efficiency. This finding is in contradiction to the traditional methods of preparation used in the TCM industry, which predominantly use aqueous extraction solvents. It is therefore likely that most TCM preparations in the market today have only
a small amount the active components extracted, with the rest of the herb mass left over
going to waste. As discussed in the ‘future work’ section, these results indicate that an
investigation into the extraction efficiency of the individual raw herb should be carried
out, as it is possible that the starting material does not contain the target analyte. This
would require that each individual herb in the formulation be analysed prior to
extraction.

6.3. Major findings and implications in the application of the pattern-based
approach to the quality evaluation of *E. arvense*

Characterisation of herbal authenticity and quality is one of the major challenges facing
the herbal medicine industry, a task made difficult due to fundamental challenges in
species delineation. The pattern-based approach to quality evaluation, namely the use of
chemical fingerprinting as well as DNA barcoding, is conceptually demonstrated here to
be a useful supplement to the existing quality control framework of analyte quantitation.

6.3.1. TLC, LC-PDA and LC-MS methods to chromatographically profile and
characterise the *E. arvense* extracts for phytochemical variability

The usefulness of any profiling method for qualitative or quantitative analysis is
dependent on the resolution and sensitivity of the chromatographic method. A limitation
of the fingerprinting technique is that it does not have the resolving power to
satisfactorily and accurately characterise an extract. Overall, LC-MS was shown to
provide excellent sensitivity, resolution, and reproducibility for the chemical
characterisation of the *E. arvense* extracts. Although a considerably more expensive
instrument for profiling than TLC, the significant increase in information content will
make this a worthwhile addition to herbal chemical profiling. Limitations of the LC-
General conclusions

MS, for example the ionisation source, still limit the chemicals that can be detected with good sensitivity, for example large complex molecules. Other spectral characterisation techniques such as NMR and IR should certainly be included in future fingerprinting methods. A further consideration may be time-of-flight LC-MS for the characterisation of polysaccharides and other large molecules.

6.3.2. Chemometric methods to statistically analyse the chromatographic profiles and determine the variability of the E. arvense extracts

Chromatographic pre-processing of profile data is necessary for subsequent statistical analysis as the instrumental contribution to a chromatographic profile, especially using LC-MS, can make the detection of peaks cumbersome and the statistical inferences inaccurate. The package msProcess was successfully used to remove instrumental noise, remove baseline drift, identify peaks, and remove peak retention time variations to accurately quantify peak height.

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. The novel use of KNN combined with PCA allows for a more objective classification of sample grouping, classifying extracts with minimal subjectivity. As with all statistical analysis, the overall reliability of the result is dependent on the sample size. For a large QC laboratory, the potentially hundreds of batches of product will make this statistical analysis more appropriate. Similarly, more complex statistical analyses such as soft independent modelling of class analogy (SIMCA) may be employed on larger sample sizes.
6.3.3. Chemical antioxidant assays as a rapid and simple method for assessing the pharmacological variability of E. arvense extracts

The chemical measurement of antioxidant capacity of the E. arvense extracts is a biologically useful way, albeit fairly basic and preliminary, to establish quality standards of pharmacological equivalence. Importantly, the results of this study indicate that the implied biological effects of the extracts are not reflected in their chemical profile. This is useful knowledge for industry, where extracts are commonly standardised to contain a particular concentration of a single component purported to be the principal active ingredient, generally to the omission of other chemicals. As it relates to this case, it appears that pharmacologically active chemicals may not be detectable by the chemical analyses used or that many of the analytes detected in the chemical profile may not contribute in a significant way to antioxidant activity.

6.3.4. Genomic authentication methods for the identification of E. arvense starting materials

A significant advancement in herbal authentication process is the application of genomic methods to achieve species delineation. Although there are a large number of molecular techniques becoming available to authenticate plants, such as next generation whole genome sequencing, the ‘traditional’ PCR-based DNA barcoding successfully used the proposed standardised DNA loci of rbcL and matK to confirm the identity of the E. arvense raw materials. As DNA barcoding becomes more standardised and rigorous, it will certainly be an essential tool herbal authentication.

6.4. Future work

The work reported in this thesis has considerable scope for expansion.
With respect to the analysis of the Endoherb™ formulation, the proposed method should be subject to inter-laboratory testing and used to analyse more extracts from different suppliers to determine method ruggedness. As the analyte tetramethylpyrazine and catalpol were unable to be measured in final formulation, the starting material should be analysed prior to being used in the formulation to assure that analyte is present. The accuracy of the umbelliferone result requires significant improvement. The umbelliferone peak should be derivatised for GC analysis in order to remove the excessive peak tailing due to its polarity. A trimethysilyl derivative using for example ethyl trimethylsilylacetae/tetrabutylammonium fluoride should be trialled.105

With respect to the analysis of *E. arvense*, many of the results presented here are preliminary and require further development before forming part of a rigorous QC system. Notably, the identification and characterisation of the potentially pharmacologically active analytes needs to be conducted with at least NMR characterisation. Similarly, other biological measures of pharmacological effect should be investigated, though they need to be simple enough to be used day-to-day in a chemistry QC lab.

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


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