Investigation of the Elemental Profiles of *Hypericum perforatum* as used in herbal remedies

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Elemental Profiling of St John's Wort
By J. D. Owen

Abstract

The work presented in this thesis has demonstrated that the use of elemental profiles for the quality control of herbal medicines can be applied to multiple stages of processing. A single method was developed for the elemental analysis of a variety of St John's Wort (*Hypericum perforatum*) preparations using Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES). The optimised method consisted of using 5 ml of nitric acid and microwave digestion reaching temperatures of 185^{o}C. Using NIST Polish tea (NIST INCT-TL-1) the method was found to be accurate and the matrix effect from selected St John's Wort (SJW) preparations was found to be ≤22%. The optimised method was then used to determine the elemental profiles for a larger number of SJW preparations (raw herbs=22, tablets=20 and capsules=12). Specifically, the method was used to determine the typical concentrations of 25 elements (Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Hg, In, Mg, Mn, Mo, Ni, Pb, Pt, Sb, Se, Sr, V, Y and Zn) for each form of SJW which ranged from not detected to 200 mg/g. To further interpret the element profiles, Principal Component Analysis (PCA) was carried out. This showed that different forms of SJW could be differentiated based on their elemental profile and the SJW ingredient used (i.e. extract or raw herb) identified. The differences in the profiles were likely due to two factors: (1) the addition of bulking agents and (2) solvent extraction. In order to further understand how the elemental profile changes when producing the extract from the raw plant, eight SJW herb samples were extracted with four solvents (100% water, 60% ethanol, 80% ethanol and 100% ethanol) and analysed for their element content. The results showed that the transfer of elements from the raw herb to an extract was solvent and metal dependent. Generally the highest concentrations of an element were extracted with 100% water, which decreased as the concentration of ethanol increased. However, the transfer efficiency for the element Cu was highest with 60% ethanol. The solvents utilised in industry (60% and 80% ethanol) were found to preconcentrate some elements; Cu (+119%), Mg (+93%), Ni (+183%) and Zn (+12%) were found to preconcentrate in 60 %v/v ethanol extracts and Cu (+5%) and Ni (+30%). PCA of the elemental profiles of the four types of extract showed that differentiation was observed between the different solvents and as the ethanol concentration increased, the extracts became more standardised. Analysis of the bioactive compounds rutin, hyperoside, quercetin, hyperforin and adhyperforin followed by subsequent Correlation Analysis (CA)

displayed relationships between the elemental profiles and the molecular profiles. For example strong correlations were seen between hyperoside and Cr as well as Quercetin and Fe. This shows potential for tuning elemental extractions for metal-bioactive compounds for increased bioactivity and bioavailability; however further work in needed in this area.

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Abbreviations

A3RT	-	UDP-Rha:anthocyanidin 3-O-glucoside rhamnosyltransferase
AAS	-	Atomic Absorption Spectroscopy
AD	-	Anno Domini
AES	-	Atomic Emission Spectroscopy
BC	-	Before Christ
BPC	-	Bioactive Plant Compound
CA	-	Cluster Analysis
CAM	-	Complementary and Alternative Medicine
CRM	-	Certified Reference Material
CYP	-	Cytochrome P450 enzyme family
DMA	-	Direct Mercury Analyser
DMAPP	-	Dimethylallyl diphosphate
EU	-	European Union
F3GT	-	Flavonol 3-O-glucosyltransferase (EC 2.4.1.91)
FDA	-	Food and Drug Administration
FLS	-	Flavonol synthase (EC 1.14.11.23)
GLP	-	Good Laboratory Practice
GMP	-	Good Manufacturing Practice
GPP	-	Geranyl diphosphate
HF	-	Hydrogen fluoride
HMDE	-	Hanging Mercury Drop Electrode
HPLC	-	High Performance Liquid Chromatography
ICP-MS	-	Inductively Coupled Plasma-Mass Spectrometry
ICP-OES	-	Inductively Coupled Plasma-Optical Emission spectroscopy
KNN	-	K Nearest-Neighbour Analysis
LA-ICP-MS	-	Laser Ablation ICP-MS
LC-ICP-MS	-	Liquid Chromatography ICP-MS
LC-ICP-OES	-	Liquid Chromatography ICP-OES
LDA	-	Linear Discriminate Analysis
LOD	-	Limit of Detection
LOQ	-	Limit of Quantification
MA	-	Marketing Authorisation
MAO	-	Monoamine oxidase
MHRA	-	Medicines and Healthcare products Regulatory Agency
ND	-	Not Detected
PC	-	Principal Component
PCA	-	Principal Component Analysis
ppb	-	Parts per billion
ppm	-	Parts per million
ppt	-	Parts per trillion
RF	-	Radio Frequency

SEM	-	Standard Error of the Mean (95% confidence interval)
SD	-	Standard Deviation
SIMCA	-	Soft Independent Modelling of Class Analogies
SJW	-	St John's Wort
SN	-	Signal to Noise
THR	-	Traditional Herbal Medicines Registration
TLC	-	Thin Layer Chromatography
TMFE	-	Thin Mercury Film Electrode
UHPLC	-	Ultra High Performance Liquid Chromatography
UK	-	United Kingdom
USA/US	-	United States of America
UV	-	Ultraviolet spectroscopy
WHO	-	World Health Organisation
Al	-	Aluminium
As	-	Arsenic
B	-	Boron
Ba	-	Barium
Be	-	Beryllium
Ca	-	Calcium
Cd	-	Cadmium
Co	-	Cobalt
Cr	-	Chromium
Cu	-	Copper
Fe	-	Iron
Hg	-	Mercury
In	-	Indium
Mg	-	Magnesium
Mn	-	Manganese
Mo	-	Molybdenum
Ni	-	Nickel
Pb	-	Lead
Pt	-	Platinum
Sb	-	Antimony
Se	-	Selenium
Sr	-	Strontium
V	-	Vanadium
Y	-	Yttrium
Zn	-	Zinc

1 Introduction

1.1 Brief History of Herbal Medicines

Vegetation across the world has been used for millennia as a staple food source. However, many species of plants have also been utilised for medicinal purposes for thousands of years; these plants are also known as herbal remedies. Such remedies have been described for the treatment of wound healing, diarrhoea and other medical issues. One of the earliest written examples of a herbal medicine document is a Sumerian cuneiform clay tablet dated to around 2100 BC which depicts plant ingredients and instructions on mixing [1]. The next notable publication was the 'Papyrus Ebers' written in archaic phraseology hieroglyphics and dated to about 1500 BC; though the content is believed to be centuries older [2]. Examples of the traditional medicines described by the papyrus (Figure 1.1) include heating a mixture of herbs on a hot brick that allowed sufferers of asthma to breath in the fumes to help relieve their symptoms [3].

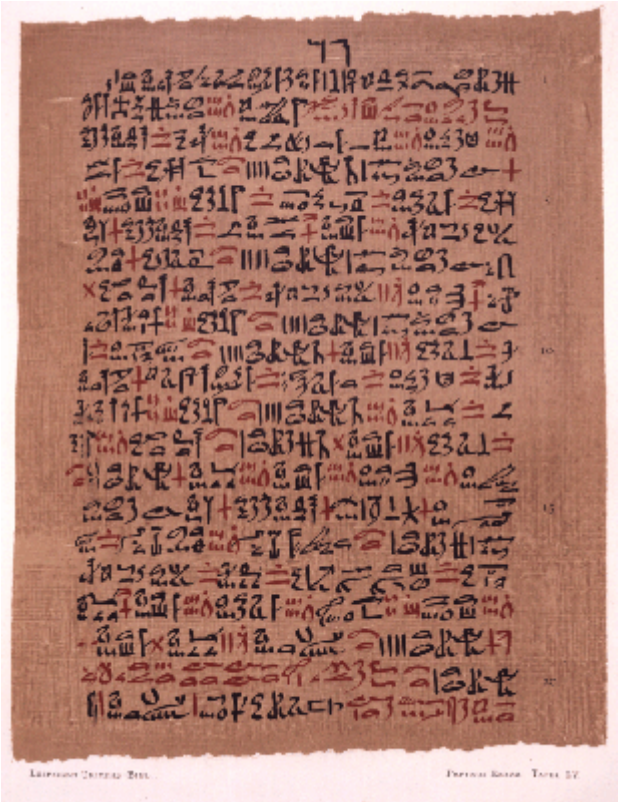


Figure 1.1 A page from the Papyrus Ebers [3]

Other key texts include the Indian Caraka Samhita and Sushruta Samhita [4], the Anglo-Saxon Leechbook of Bald [5] and Culpeper's complete herbal [6]. All of these convey information ranging from how to identify a plant to the ingredients and preparation instructions.

The information and knowledge of herbal remedies has increased from these early texts; however many are still not fully understood. Firstly, the cleanliness of such preparations has improved greatly and many traditional mixtures are no longer utilised due to the discovery of microorganisms. For example, the Papyrus Ebers describes a traditional medicine for wound healing after minor surgery that contains 'Elderberries, uah-corn and cat dung' [2]. Secondly, the use of analytical chemistry has allowed the identification of some of the bioactive constituents in plants that gave a therapeutic effect. From this, manufacturers have been able to separate and purify, or synthesise the compound. Examples include Aspirin originally from willow trees and Digoxin from Foxgloves (*Digitalis purpurea).* Thirdly, herbal remedies are being tested for their effectiveness against their indented use as well as other disorders and the safety of their use. However, herbal remedies are still not fully understood due to their complexity and those which contain more than one herb also need further investigation to understand the synergy between them.

1.2 Herbal Medicines Today

Today, the World Health Organisation (WHO) [7] defines the four types of herbal medicines as:

- **Herbs:** crude plant material such as leaves, flowers, fruit, seed, stems, wood, bark, roots, rhizomes or other plant parts, which may be entire, fragmented or powdered.
- **Herbal materials:** in addition to herbs, fresh juices, gums, fixed oils, essential oils, resins and dry powders of herbs. In some countries, these materials may be processed by various local procedures, such as steaming, roasting, or stir-baking with honey, alcoholic beverages or other materials.
- **Herbal preparations:** the basis for finished herbal products and may include comminuted or powdered herbal materials, or extracts, tinctures and fatty oils of herbal materials. They are produced by extraction, fractionation, purification, concentration, or other physical or biological processes. They also include preparations made by steeping or heating herbal materials in alcoholic beverages and/or honey, or in other materials.
- **Finished herbal products:** herbal preparations made from one or more herbs. If more than one herb is used, the term mixture herbal product can also be used. Finished herbal products and mixture herbal products may contain excipients in addition to the active ingredients. (However, finished products or mixture products to which chemically defined active substances have been added, including synthetic compounds and/or isolated constituents from herbal materials, are not considered to be herbal).

Within Asian and African countries, 80% of their population depend on complementary and alternative medicines (CAM) as their primary form of healthcare [7]. Within developed countries 70-80% of people have used some form of CAM. Herbal remedies are a popular and wide spread form of CAM. In the UK during 2008, the Medicines and Healthcare products Regulatory Agency (MHRA) carried out a survey about herbal medicines use and perception [8]. The report found that 35% of adults had used herbal medicines and of those (who had used herbal medicines in the previous two years), 89% felt most herbal medicines were safe to take. Due, in part, to these views on herbal remedies the UK spent £136 million on herbal medicines in 2009 [9]. The global herbal supplements and remedy market is forecast to reach US$ 107 billion by the year 2017 [10].

1.3 Safety of Herbal Medicines

Many people who use herbal remedies believe they are a safer form of medication because they are 'natural' [8, 11]. However this is sometimes not the case. There have been some instances where the chemical properties of a herbal remedy have rendered it unsafe and as such it is no longer sold. One example of this includes the herbal remedy Ephedra, also known as Mu Huang. The main constituent is ephedrine, which causes elevated heart rate and blood pressure. Persons in America were taking the supplement as an aid to lose weight where the Food and Drug Administration (FDA) found a link between Mu Hang usage and a number of deaths. Therefore the sale of this herbal remedy was subsequently banned in 2004 [12]. Another example occurred due to the substitution of a herbal remedy with another species. Between 1990 and 1993 in Belgium a number of women (over 80 individuals) on a specific slimming program were given capsules containing the species *Aristolochia fungchi* rather than the label claim *Stephania tetrandra* [13, 14]. This resulted in progressive inflammation of the kidneys as well as terminal or preterminal renal failure; many of the people affected required renal transplants [15]. Cases of the same species adulteration were also observed for women from Germany and France [16]. A possible cause for this substitution may be due to the similarity of their Asian names; guang fangji (*Aristolochia fungchi*) and fangji (*Stephania tetrandra*) [16]. These effects were due to aristolochic acids contained within the plants of genus *Aristolochia*; these compounds have also been linked to urothelial cancer [14]. Similar health effects were seen with the species *Aristolochia pistolochia* [17]. Since then a number of species from this genus have been prohibited in the UK [18] and USA [19]. Another example of harmful compounds ingested from herbal remedies includes podophyllotoxin from bajiaolian (*Dysosma pleianthum*) [20]. Infusions and food preparations prepared with bajiaolian caused cases of neurotoxicity; one example is that of a 33 year old woman

who lost sensitivity to touch and deep tendon reflexes as well as abnormal liver function and gastrointestinal upset through ingestion of bajiaolian made with chicken soup. Full recovery took 8 months after two days ingestion of the soup [20, 21]. Other examples of herb safety arise from contamination with microorganisms [22], allergic reactions [16], interactions with other medicines [23], adulteration [24, 25] and metal contamination [26]. For example, an allergic reaction was observed with a 42 year old man who developed progressive renal failure and lupus-like syndrome after the ingestion of Yohimbine (from the yohimbe tree) [27]. Cases of metal contamination include a 5 year old boy from Italy who was given traditional Indian medicine (unknown pill and powder ingredients to prevent removal of his second eye) and suffered from arsenic poisoning [26]. A similar example of a 5 year old boy from China was observed whereby the child suffered from mercury poisoning from traditional Chinese medicine for treatment of mouth ulcers [26]. A 4 month old boy from China (fed numerous herbs from birth for minor aliments) developed cough, fever and vomiting which was due to Pb poisoning from herbal pills 'Po Ying Tan' [21]. As well as adulteration of herbal remedies with different or lower quality herbs (for example, ginseng (Pananx ginseng) has been adultered with cheaper and lower quality species [28]); synthetic compounds such as caffeine, aspirin, diazepam and paracetamol have been used [16]. A case study of a 51 year old woman who had been taking Tung Shueh pills (traditional Chinese medicine) for abdominal pain developed renal problems due to inflamed kidneys [21]. Examination of the pills found that they had been adultered with diazepam and mefenamic acid. Another example of synthetic compounds being used in herbal medicines includes traditional medicines enriched with aminopyrine and phenylbutazone [29]. These caused the suppression of white blood cells which in turn caused severe bacterial sepsis and in one case death [29].

In addition to these safety issues, some herbal medicines have been able to interact with synthetic mainstream medicines. Numerous drug-herb interactions have been reported [30] with some examples displayed in Table 1.1. One infamous interaction is between the herbal remedy St John's Wort (*Hypericum perforatum*) and some oral contraception medication which has resulted in unwanted pregnancy [31] due to the interaction of its bioactive compounds with cytochrome P450 (CYP) 3A4 enzymes [32].

Table 1.1 Examples of herb-drug interactions

Herb	Drug	Adverse Reaction of Taking both
St John's Wort (*Hypericum perforatum*)	Digoxin	Lowers blood concentration of digoxin
Ginkgo (*Ginkgo biloba*)	Warfarin	Bleeding
Garlic (*Allium sativum*)	Chlorpropamide	Hypoglycaemia
Kava (*Piper methysticum*)	Alprazolam	Sedation

1.4 Regulation of Herbal Medicines

The regulation associated with herbal medicines has increased over the last 25 years. For example, the World Health Organization (WHO) conducted a survey on member states and found that in 1991 only 27 member states had some form of regulation on herbal medicines whilst in 2003 this had increased to 83 member states [33]. This is due to safety issues, as explained in the previous section, highlighting the need for quality control of such substances. Such regulations are also being consistently updated with the development of instrumentation. The introduction of regulations and organisations to report adverse effects (e.g. to the Food and Drug Administration (FDA), World Health Organisation (WHO) or the Medicines and Healthcare products Regulatory Agency (MHRA)) has facilitated quality monitoring and if needed, for herbs to be banned if severe health hazards are noted.

The first type of quality control a herbal medicine must adhere to is its herbal remedy monograph. This can be found in their countries Pharmacopoeia (e.g., US Pharmacopoeia, European Pharmacopoeia and British Pharmacopoeia) or the WHO monographs [34]. From this, numerous consumed herb species have a monograph that states basic quality limits including but not limited to foreign organic matter, total ash, microbiological contamination, pesticide residues and heavy metals before human consumption. In 2005, the WHO found that 24% of the member states had national Pharmacopoeias which included herbal medicines or 58% of member states used another Pharmacopoeia in the absence of their own [33]. For those who do not have a national Pharmacopoeia, the three most popular are the European, British and US Pharmacopoeias [33]. However, in some cases, the Pharmacopoeias can differ between countries allowing for confusion. For example with Valerian (*Valeriana officinalis*) extracts the European Pharmacopoeia [35] recommends a minimum of 0.25% valerenic acid whilst the US Pharmacopoeia [36] requires a minimum of 0.3% valerenic acid. Therefore Valerian extracts produced outside of the US may not meet the requirements needed for sale in the US. In some cases, monographs are missing in certain

Pharmacopoeias. For example, the monograph for Passion flower (Passiflora incarnata) is available in the European Pharmacopoeia [35], but is not present in the US Pharmacopoeia [36].

During the manufacture of herbal remedies the Pharmacopoeia guidelines, as well as other practices are followed. These include Good Laboratory Practice (GLP) and/ or Good Manufacturing Practice (GMP) which involves providing a paper trail throughout the production of the herbal medicine [33, 37] to ensure quality procedures are implemented and all analyses/ manufacture or other aspect of production can be accounted for. Following on from a herbs monograph or manufacturing processes, the commercial sale of the remedy must also follow other regulations which are country specific.

To be able to sell a herbal product in the UK one of three criteria must be fulfilled. The first is that a herbal remedy can be sold through a licensed herbalist in which the product is prescribed following a consultation. The second is through a marketing authorisation (MA) which is obtained by providing full clinical trial evidence of safety. The third option is a traditional herbal medicines registration (THR). The THR scheme was brought into effect in 2005 whereby herbal medicines could obtain a THR if they could prove safe use for 30 years with at least 15 years usage within the EU. This allowed the sale of such herbs without the extensive clinical trial data needed for a MA.

A common theme amongst the sales regulations in the UK and US are factors such as correct labelling of the herbal remedies and the types of 'claims' they are allowed to use (e.g. medical, health, nutrient) [37, 38]. Such regulations and guidelines described are being utilised by many countries, but there are some countries, mostly undeveloped, which do not have such systems in place [33].

1.5 Chemical Characterisation of Herbal Medicines

The compounds utilised for medicinal purposes in herbal remedies are produced by the plants for a variety of functions. Some compounds are essential to a plant's metabolism whilst others are produced as by-products of metabolism (known as secondary metabolites). The functions of secondary metabolites vary greatly and can often contribute to the colour and fragrance of flowers or involved in defence against herbivores. Usually the compounds selected for characterisation of the plant are done so as they are either specific to that species or genus of plants. Examples of compounds monitored for quality stated by monographs include: silymarin content in Milk Thistle (*Silybum marianum*) seeds [34], different ginsenosides in Radix Ginseng (*Panax ginseng*) [34]

whereas Passion flower (*Passiflora incarnata*) is assessed by its vitexin content [34]. Such analyses are usually carried out firstly using Thin Layer Chromatography (TLC) to assess and identify the herb. Following this, the standardised extract is analysed by liquid chromatography to quantify the compounds and ensure the concentrations agree with the monograph. In some cases, due to harvest variation, a batch may be too high or too low in concentration for the selected compounds. In industry this is overcome by mixing different batches together to correct the selected compound levels. Elemental constituents are also found in herbs and some are monitored which will be discussed in the next section.

1.6 Elements in Herbal Medicines

In addition to molecular constituents, a diverse range of elements are also found in herbal medicines. The concentrations of elements within plants are highly influenced by the medium in which the plant is grown. Factors such as soil type, temperature, aeration, elemental content, pH and water content can affect the available nutrients for the plant [39, 40]. There is also a large difference between plant species due to genotype and the biochemical processes different plants utilise in relation to elements [39]. This can include factors such as selectivity for certain ions, stage of development, root properties and the release of organic compounds by the plant or microorganisms to free elements (e.g. chemicals secreted by plants/microorganisms to allow easier uptake of nutrients) [39, 40].

1.6.1 Toxic and Non-essential Elements

The monitoring of toxic metals by regulators is of great interest in order to prevent the harmful effects associated with their ingestion. In some cases, the presence of metals in herbal remedies has resulted in As, Hg or Pb poisoning [21, 26]. At present, manufacturers for the UK market test for selected metals ensuring they are not over the recommended limit [41]. This however usually only considers the more toxic elements Cd, Hg or Pb unless a monograph specifically indicates the analysis for particular elements. However, new regulations introduced by the US Pharmacopoeia [42] will increase the number of elements to include limits for As, Ir, Os, Pd, Pt, Rh, Cr, Mo, Ni, V and Cu (Table 1.2) in medications. The majority of these elements are being monitored due to their use as catalysts in the synthesis of medical compounds whilst others are absorbed into plant tissue *via* the plants root or leaf system.

The toxic element As has been found in many different herbs but little is known about its biochemical function [40, 43]. However, there is evidence that this element might be essential in very small amounts for animals [44] but is mostly known for its toxic effects [45, 46]. The speciation of As should also be noted as As (V) is less toxic in comparison to As (III) [47]. Cadmium is not required biologically for plants but is readily introduced *via* the root and leaf systems [40, 43]. In high concentrations, Cd can cause stunting of growth and chlorosis in plants [43]. There is no clear evidence for the essentiality of Cr in plants but Cr added to Cr-deficient soils has shown to increase the growth and yields of plants such as maize, wheat, rye and potatoes [39]. The speciation of Cr is also noted as Cr (III) is less toxic in comparison to Cr (VI). Mercury is not an essential element to plants; however, it can be absorbed and stored in plant tissues [39, 43]. The toxicity of Hg compounds increases from elemental Hg < ionic Hg < organic-Hg compounds [48]. Lead uptake within plants can originate from the atmosphere or soil. Before Pb was removed from petrol, large amounts of Pb particulates would be in the air and as such could become deposited onto plant surfaces [49]. Atmospheric deposition has been shown to be a major contributor for some elements, including Pb, in certain species [49, 50] or within urban areas [49]. For example, Dalenberg and Driel [50] found that 73-95% of the Pb concentration found in the leafy material of *L. multiflorum*, carrots, spinach, wheat grain and wheat straw was attributed to atmospheric deposition. Although the concentrations of Pb can vary between plants, the element has not been shown to be essential [43].

In addition to these elements entering a plant *via* the root or leaf system, other routes of toxic element contamination can occur from human interaction. For example, such elements may be incorporated accidentally during the manufacturing process *via* machinery, or the addition of bulking agents as well as improper storage. Contamination could also occur from known adulteration. Toxic elements such as As, Hg and Pb have caused poisonings in the past from the ingestion of herbal remedies [21, 26]. For example, in many Asian herbal medicines it has been common to add cinnabar (mercury (II) sulphide) [51] or realgar (arsenic sulphide) [52]. The limits by which the toxic elements discussed in herbal medicines must be below can be found in Table 1.2 and Table 1.3.

Table 1.2 Default elemental limits in oral drugs from US Pharmacopoeia

Element	Oral daily dose PDE[a] for drug products (µg/day)	Oral daily dose PDE[a] for drug products with excipients (µg/day)
Cadmium	25	2.5
Lead	5	0.5
Inorganic arsenic[b]	1.5	0.15
Inorganic mercury[b]	15	1.5
Iridium	100	10
Osmium	100	10
Palladium	100	10
Platinum	100	10
Rhodium	100	10
Ruthenium	100	100
Chromium	*	*
Molybdenum	100	10
Nickel	500	50
Vanadium	100	100
Copper	1000	100

[a] PDE = Permissible daily exposure based on a 50 kg person
[b] Speciation may be used
*Not a safety concern

Table 1.3 British Pharmacopoeia [53] concentration limits for Cd, Hg and Pb

Element	Limit
Cd	1.0 ppm
Hg	0.1 ppm
Pb	5.0 ppm

Other elements that are found in plants that are considered not to be essential or are questionable (some benefits in plants seen when present, but not confirmed) include Al, Ba, Be, Co, Sr, Y, V (and possibly Ni).

Aluminium is under investigation as it has been shown that in low concentrations the element can have a beneficial effect on plant growth [54]. Cobalt is essential for microorganisms in fixing N_2 but its essentiality is under investigation amongst higher plants as it may aid chlorophyll formation [40]. The essentiality of Ni in all plants is under investigation as some reports suggest beneficial effects on growth in its presence; however it is considered essential for higher plants [43]. Strontium is not utilised by plants but its uptake is due to its similarity to Ca ions [43].

1.6.2 Essential Elements

Many elements are essential to plants (Table 1.4); however, within herbal medicines these elements are not monitored (with the recent exception of Ni and Cu with the US Pharmacopoeia, Table 1.2). Calcium is present in large concentrations in plant cells [39] as it is used in numerous plant functions including alleviation of toxic metal effects [55, 56]. Copper is involved with enzymes for processes such as photosynthesis, carbohydrate and nitrate metabolism as well as disease resistance [43]. Iron is involved in many metabolic processes such as photosynthesis (very concentrated in the chloroplasts) and metabolism of nucleic acids [43]. Magnesium activates many enzymes and is a constituent of chlorophyll [39]. Manganese is utilised in functions such as photosynthesis and nitrogen assimilation [43] and Mo is applied within nitrogen metabolism [43]. Zinc is involved in several functions such as RNA and ribosome formation, membrane permeability and is essential for the catalytic activity of various enzymes [43].

Table 1.4 Examples of essential element use by plants

Element	Examples of element concentrations[1,2] in food crops (mg/kg)	Biological use
Ca	Wheat, grain: 29-92	Numerous; including alleviation of toxic metals and structural roles
B	Wheat, grain: ~0.69 Rye, grains: ~4.3 Carrot, root: ~9.9 Apple, fruit: ~8.3	Production of flavonoids
Cu	Wheat, grain: 17-50 Rye, grains: 34-43 Potato, tubers: 3-6.6	Numerous; including enzymes for photosynthesis, carbohydrate and nitrate metabolism and disease resistance
Fe	Wheat, grain: 1.3-10 Barley, grain: 4-15 Carrot, root: 4-8.4	Numerous; metabolic processes such as photosynthesis and nucleic acid production
Mg	Wheat, grain: 580-1791	Numerous; activates enzymes and a constituent of chlorophyll
Mn	Wheat, grain: 16-103 Rye, grains: 10-87 Carrot, root: 9-28 Apple, fruit: 1.3-1.5	Numerous; photosynthesis and nitrogen assimilation
Mo	Wheat, grain: 0.2-2.4 Kidney bean, seeds: 0.9 -1.6 Tea, leaves: 0.2-0.3 Sugar beat, pods: 0.45-0.75	Nitrogen metabolism
Ni	Wheat, grain: 0.17-0.67 Barley, grain: 0.1-0.67 Cucumber, fruits: 1.3-2.0	For some higher plants is a component of urease
Zn	Wheat, grain: 23-37 Rye, grains: 29-31 Tomato, fruit: 17-26 Lettuce: 44-73	Numerous; RNA and ribosome formation, membrane permeability and enzymes

[1] Please note this can vary greatly between species and plant growth origin
[2] Data sources adapted from [40, 43, 57]

1.6.3 Hyper-accumulators

Hyper-accumulators are plants that actively take up certain elements in a high concentration compared to that of the growth medium. In order for a plant to be classified as an accumulator or hyper-accumulator it must be able to absorb an element(s) above a certain level per gram of mass. A few examples of element levels can be seen in Table 1.5 and some plant species with the elements they accumulate are indicated in Table 1.6.

Table 1.5 Examples of hyper-accumulation concentrations for some elements (adapted from Krämer 2010 [58])

Element	Hyper-accumulation concentration criterion ($\mu g\ g^{-1}$)
Sb	>1000
As	>1000
Cd	>100
Co	>1000
Cu	>1000
Pb	>1000
Mn	>10 000
Ni	>1000
Se	>1000
Zn	>10 000

Table 1.6 Examples of plants that accumulate elements

Herb	Common Name	Therapeutic Use	Element	Class
Melastoma malabathricum L.	Malabar melastome	Diarrhoea, hemorrhoids, wounds, toothache	Al	Accumulator
Streptanthus polygaloides	Milkwort jewelflower	Not used medicinally	Ni	Hyper-accumulator
Thlaspi caerulescens	Alpine pennygrass	Not used medicinally	Cd	Hyper-accumulator
Pteris vittata	Chinese brake	Not used medicinally	As	Hyper-accumulator
Hypericum perforatum	St John's Wort	Depression and anxiety	Cd	Accumulator
Bulbostylis mucronata	Not applicable	Not used medicinally	Cu	Hyper-accumulator
Sopubia metallorum	Not applicable	Not used medicinally	Co	Hyper-accumulator

Note: Not applicable = no common name known

Depending on the element undergoing hyper-accumulation, this could be beneficial or harmful to human health. There are three theories as to why plants accumulate certain metals in high quantities. The first is that the plants use this mechanism as a form of defence known as the 'elemental defence' hypothesis [59]. This is where the plants accumulate the metals in order to deter herbivore predators such as insects. Examples of this can be seen in alpine pennycress

(*Thlaspi caerulescens*) with Cd [60], milkwort jewelflower *(Streptanthus polygaloides)* with Ni [61] and Chinese brake fern (*Pteris vittata*) with As [62]. The 'trade off' hypothesis explains that some plants utilise such elements as a defence mechanism and in doing so reduces the production of organic defences as a way to save energy. Examples of this have been seen with alpine pennycress (*Thlaspi caerulescens*) and Zn [63] and milkwort jewelflower (*Streptanthus polygaloides*) with Ni [64]. Another reason for the accumulation of certain elements is known as the 'joint effects' hypothesis. This is where the elements in conjunction with the organic defences work together in order to enhance the overall protection of the plant. An example of this has been shown in experiments on larvae of *Plutella xylostella* [65]. The combination of organic defence molecules such as either tannic acid, atropine or nicotine with Ni at certain concentrations statistically decreased the number of larvae reaching the pupal stage compared to either bioactive or Ni alone for the majority of concentrations used.

There is considerable interest in hyper-accumulators due to the potential benefits they could bring. For example, such plants could be used to 'clean up' contaminated land in a more environmentally friendly process [66, 67]. Another interesting avenue using phytoextraction is using plants to specifically 'mine' rare elements for commercial purposes [68].

1.6.4 Links between Elemental and Bioactive Components

As mentioned previously, bioactive compounds can work synergistically to improve a plant's defence system against herbivores [65]. In addition to this, many metals are constituents of enzymes or organelles of the plant [39, 40, 43]. For example, Mn^{2+} is a main component of enzymes arginase (part of the urea cycle) and phosphotransferase (phosphorylation) [43] whereas Zn is part of many enzymes (proteinases, peptidases and phosphohyrolases) [40].

Other aspects of metals and bioactive molecules interacting include the colour of flowers [69, 70]. For example, the vivid blue of a cornflower is from a 'superpigment' known as protocyanin (Figure 1.2) [70]. This pigment consists of four metal ions (Fe^{3+}, Mg^{2+}, and two Ca^{2+}) which are complexed with six anthocyanin molecules and six flavone (apigenin 7-*O*-glucuronide-4'-*O*-(6-*O*-malonyl-glucoside)) molecules.

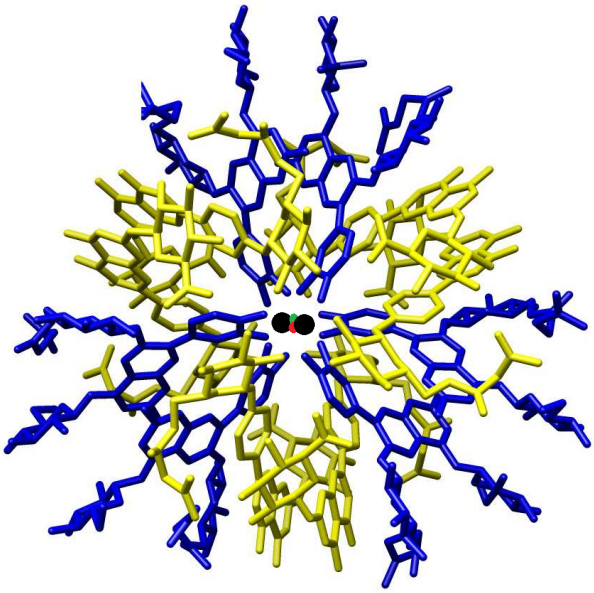


Figure 1.2 Protocyanin molecule; blue = anthocyanin, yellow = flavone glycoside, spheres: red= Fe^{3+}, green =Mg^{2+}; black= Ca^{2+}

Adapted by permission from Macmillan Publishers Ltd: [Nature] Reference [70], copyright (2005)

1.6.5 Medication Interactions with Elements

The elements found in herbal medicines can potentially interact with drugs if taken simultaneously. For example, tetracyclines should not be taken with Ca, Fe, Sr and/or Zn supplements [71, 72] as they can bind to these metals altering their bioactivity (Figure 1.3). Calcium salts can reduce the absorption of medications such as Bisphosphonates, Ciprofloxacin, Corticosteroids, Eltrombopag and Levothyroxine and can increase hypercalcaemia with thiazides and related diuretics [71]. Iron can reduce the absorption of medications including Bisphosphonates, Ciprofloxacin, Entacapone, Levofloxacin, Mycophenolate, Norfloxacin and Penicillamine as well as antagonise the hypotensive effect of methyldopa [71]. Zinc can reduce the absorption of medications including Ciprofloxacin, Levofloxacin, Moxifloxacin, Norfloxacin and Ofloxacin.

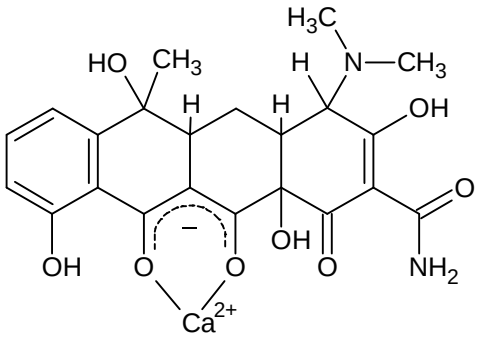


Figure 1.3 Example of tetracycline complexed with Ca, adapted from [73]

1.6.6 Chemical Characterisation of Elements

The analysis of elements can be carried out with a number of different instruments. Flame Atomic absorption spectroscopy (AAS) and flame atomic emission spectroscopy (AES) are able to analyse concentrated samples (above 100ppb) down to approximately 1 ppb level. The advantage of these instruments is their simplicity and also low cost. However, as a flame is used for atomisation and excitation, the temperatures utilised will be 3000 – 4000 K, which can result in chemical interferences such as refractory compounds. Refractory compounds cause chemical interference by emitting/absorbing larger bands compared to that produced by the individual atom; therefore a lower signal is obtained resulting in a lower concentration reading. The formation of these compounds can be overcome by the introduction of releasing agents. Another disadvantage is the low number of elements that can be analysed simultaneously and the large amount of sample needed for analysis. A graphite furnace AAS is able to detect elements down to a ppb levels and uses a much smaller amount of sample. However, this method can also only measure a limited number of elements at a time. Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP) is able to detect elements down to ppb levels and is able to measure multiple elements simultaneously. The high temperature (~10,000 K) allows analysis of the majority of elements without the need for a releasing agent. However, one disadvantage is the large amount of sample needed for analysis and an increase in start-up costs. Inductively Coupled Plasma - Mass Spectrometry is able to detect several elements simultaneously down to low ppt levels within very fast analysis time. However, it can suffer greatly from isobaric interferences and has a very high start-up cost compared to other instruments. Figure 1.4 exhibits the detection ranges for these instruments.

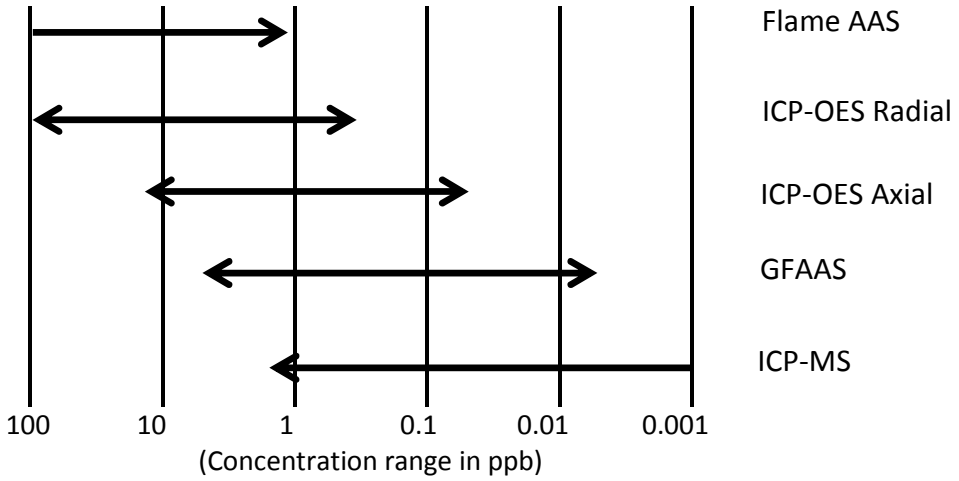


Figure 1.4 Dynamic range of some elemental analysis techniques

Until recently, Pharmacopoeias stated wet chemistry methods only for the determination of metals. For example, one heavy metals limit test; Method A (Figure 1.5) from the European Pharmacopoeia [35] involves the comparison of colour between the sample and a standard solution. This can be problematic due to variation between the eyesight of different people and the prevalence of colour-blindness but can be overcome by using a UV/Vis spectrometer.

Method A (Ph. Eur. method 2.4.8)

Test solution 12 mL of the prescribed aqueous solution of the substance to be examined.

Reference solution (standard) A mixture of 10 mL of *lead standard solution (1 ppm Pb) R* or *lead standard solution (2 ppm Pb) R*, as prescribed, and 2 mL of the prescribed aqueous solution of the substance to be examined.

Blank solution A mixture of 10 mL of *water R* and 2 mL of the prescribed aqueous solution of the substance to be examined.

To each solution, add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min.

System suitability: The reference solution shows a slight brown colour compared to the blank solution.
Result: Any brown colour in the test solution is not more intense than that in the reference solution.
If the result is difficult to judge, filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

Figure 1.5 Method A for heavy metals from European Pharmacopoeia

1.6.7 Statistical Approaches

Due to the number of elements present at different concentrations, the use of chemometric approaches has been extremely powerful for the interpretation of multidimensional data (such as chemical and metal profiles of plant material). Examples of statistical tools used can include principal component analysis (PCA), cluster analysis (CA), linear discriminate analysis (LDA) and K nearest neighbours (KNN). The use of such analyses has allowed underlying patterns to be discovered in large data sets and in some cases can qualitatively differentiate between samples. For example Ni *et al.* [74] subjected different wavelength data collected by High Performance Liquid Chromatography (HPLC) of Cassia seeds (*C. obtusifolia* and *C. tora L*.) to fuzzy clustering analysis (FC) and soft independent modelling of class analogies (SIMCA). The results found that the samples could be differentiated based on the species as well as if the samples underwent roasting or not (i.e. sample preparation). Xie *et al.* [75] analysed different Liuwei Dihuang pills by HPLC and found that using PCA enabled the differentiation of the samples by manufacturer. Fan *et al.* [76] were able to differentiate between samples of Danshen Dropping pill from adulterants *S. Miltiorrhiza* and *P. Notoginseng* using HPLC profiles with PCA. The application of such methods to the metal content of plant species has also allowed the differentiation of species [77-79], manufacturer [80, 81] and growth origin [80-82]. These studies show the potential for an alternative route of quality control in which fingerprints or profiles are utilised.

Principal Component Analysis (PCA) is often initially used in comparison to other data models as it is an unsupervised method. Unsupervised methods carry out the analysis with no input from the analyst as to how the data should be grouped or categorised. However, supervised models (e.g. CA and LDA) do require more input from the analyst by either changing parameters until the desired groupings are achieved, or by creating an example or training model for the actual model to reference and learn and thus be able to group new data. However, supervised models can induce bias from over-supervision. PCA is also commonly used before supervised analysis in order to examine how data is grouped and for some analyses (e.g. SIMCA), the PCA model is further utilised by that method.

Principal Component Analysis (PCA) is a statistical method for the analysis of multi-dimensional data. It works by reducing the data by grouping a large number of variables in a data set to a smaller number. Variables are usually the different measurements or parameters obtained from an earlier lab experiment e.g. concentrations of analytes; different absorbances of analytes, solubility, moisture etc. for each sample. Therefore, if each sample were to have 30 variables noted, it would have a 30 dimensional graph that would not be able to be generated and visualised. PCA would

group these variables to allow the creation of 2 or 3 dimensional data for easier visualisation (Figure 1.6). These groups are also known as principal components.

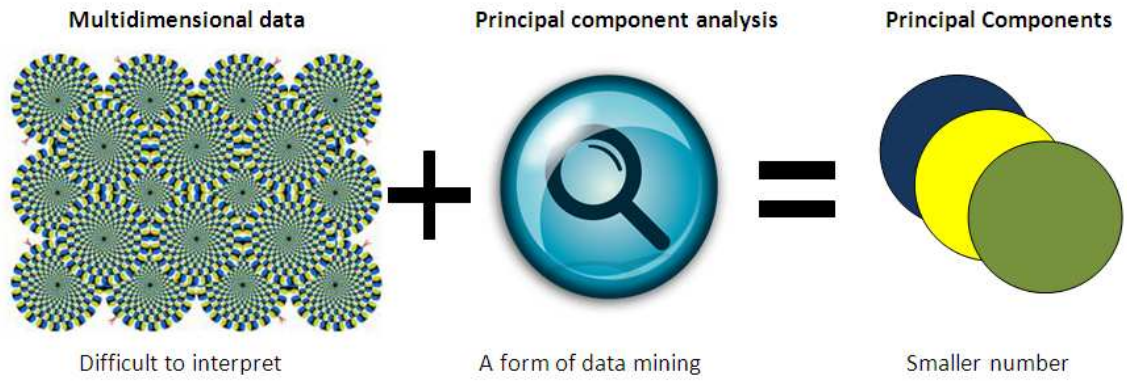


Figure 1.6 Data reduction using PCA

This reduction aids with data analysis as some multivariate data may be so large it can be difficult to view relationships or patterns contained within. Principal components (PC) are linear combinations of the original variables. The first principal component (PC1) accounts for the most variance seen in the data, the second principal component (PC2) accounts for the second largest variance and so carries on orthogonal to further components. Therefore, when significant correlation occurs in the data, the number of useful PCs is much lower in number than the original number of variables. Once the principal components have been formed, usually PC1 and PC2 (sometimes PC3 as well) are compared with one another to see if further patterns or relationships can be found.

1.7 St John's Wort (*Hypericum perforatum*)

1.7.1 Use of St John's Wort

One herb of particular interest due to its popularity is *Hypericum perforatum* (Figure 1.7). *Hypericum perforatum,* otherwise known as St John's Wort (SJW) is used in the treatment of mild to moderate depression [83]. However, it has also been shown to have anti-inflammatory and anti-bacterial effects [84]. Remedies such as SJW, which are used to help with sleeping problems and stress, have seen a growth in sales in the UK; it is believed to be caused by the recession where large scale job losses and financial security have been an issue [9]. During 2008/2009 the UK population spent £4 million alone on SJW [85]. Comparison of sales from June 2011 and June 2012 indicated that sales of SJW increased by 115% [86]. SJW is publicly available in many forms including the raw herb, tablets, capsules and tinctures without the need for prescription, thus methods to characterise these products as a whole and how the various manufacturing and formulation process affect the chemical profile would be very beneficial for identification and quality control purposes.

Figure 1.7 *Hypericum perforatum* flower by J. D. Owen

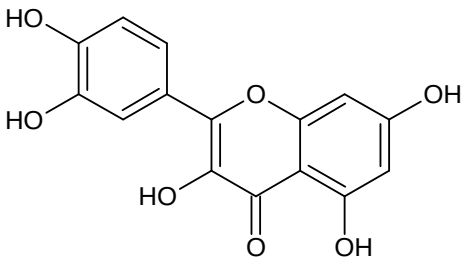
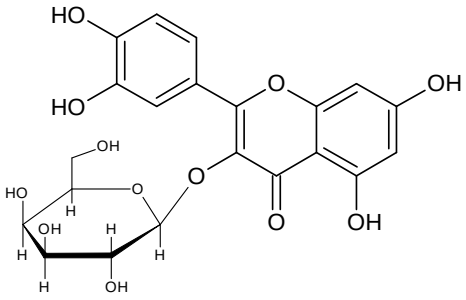
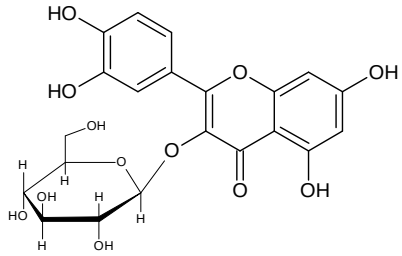
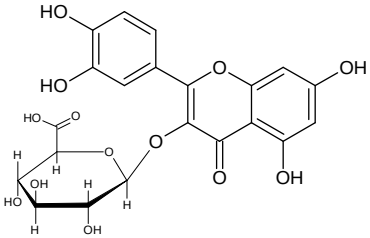
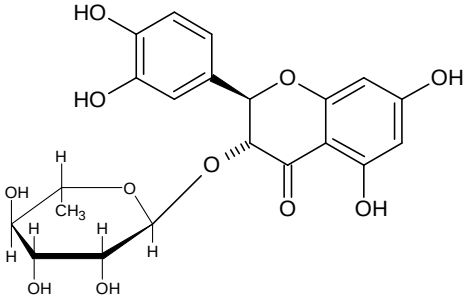
1.7.2 Molecular Analysis of St John's Wort

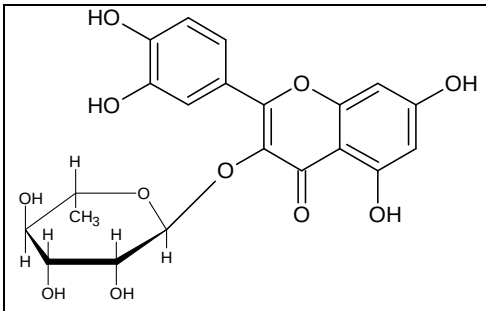
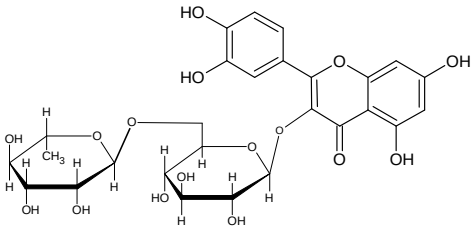
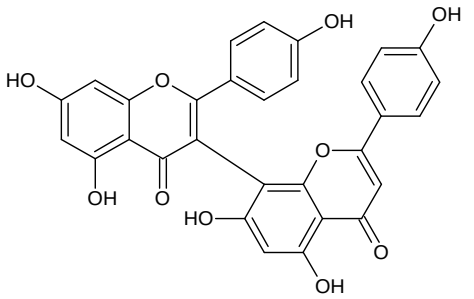
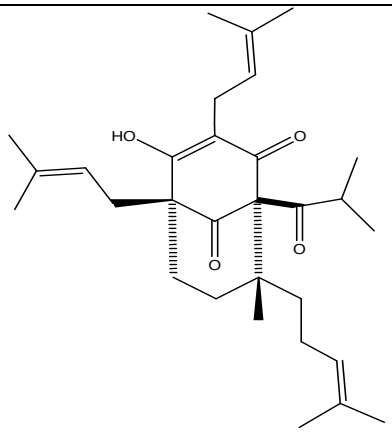
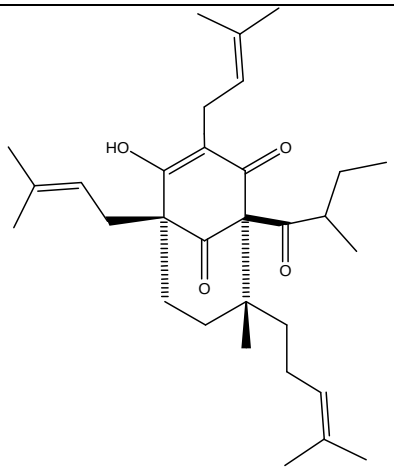
1.7.2.1 Common Molecular Constituents

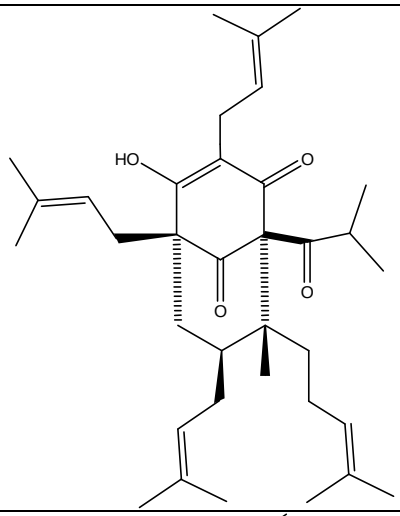
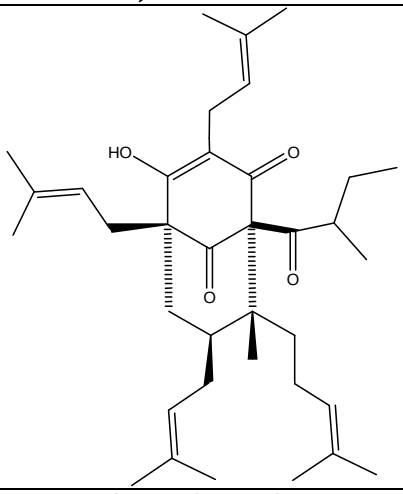
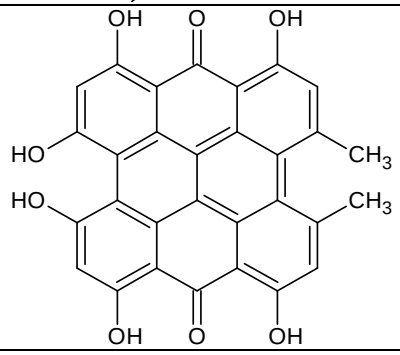
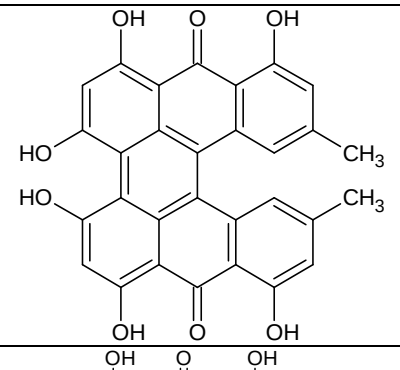
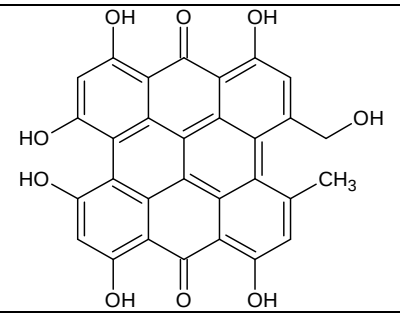
There are many different types of molecular constituents present in SJW. The most common are noted in Table 1.7. The main constituents found in the *Hypericum* genus include hyperforin, hypericin and pseudohypericin, are found in higher concentrations within the species *Hypericum perforatum*. Originally it was thought hypericin was the major compound responsible for the therapeutic effect towards depression. However, more recent studies have found that the major contributor is hyperforin [84, 87, 88], although Hypericin does play a less substantial part in an anti-depression effect with pseudohypericin. Other compounds present within SJW include flavonoids such as rutin and quercetin. These flavonoids possess antioxidant and anti-inflammatory properties.

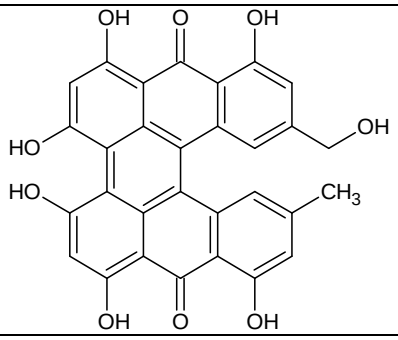
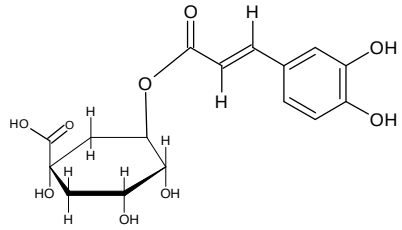
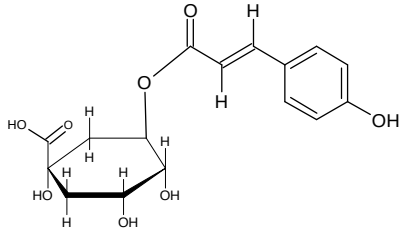
The analysis of SJW constituents has been carried out for many years. The most common form of analysis is HPLC. Usually two separate methods are utilised for the analysis, one method for flavonoids and hyperforin whilst another is used for hypericins [86].

Table 1.7 Common constituents found in St John's Wort

Structure[1]	Chemical Information[2]		Beneficial Properties	Usual Concentrations in SJW
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Quercetin $C_{15}H_{10}O_7$ 302.2 Da Flavonoid, flavanol 4444051 - 117-39-5	Antioxidant [89, 90] Anti-inflammatory [91]	0.3 – 1.3 mg/g dried plant [92] 1.01 – 1.76 mg/g dried plant [93] 0.8 – 3.2 mg/g dried plant [94]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Hyperoside $C_{21}H_{20}O_{12}$ 464.4 Da Flavonoid; flavonol glycoside 4444962 Hyperin, Quercetin 3-β-D-galactoside 482-36-0	Antioxidant [95] Anti-fungal [96]	18.5 – 19.6 mg/g dried plant [92] 5.41 – 22.28 mg/g dried plant [93]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Isoquercitrin $C_{21}H_{20}O_{12}$ 464.4 Da Flavonoid; flavonol glycoside 4444361 Quercetin 3-β-D-glucoside 482-35-9	Antioxidant [97, 98] Anti-inflammatory [99]	0.06-0.12% [100] 0.3% [101] 2442 g/g dried weight biomass [102]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Miquelianin $C_{21}H_{18}O_{13}$ 478.4 Da Flavonoid, flavonol glucuronide 18699310 Quercetin 3-*O*-β-D-glucuronide 22688-79-5	Antioxidant [103]	Identified in *H.perforatum* but not quantified [104, 105]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Dihydroquercitrin $C_{21}H_{22}O_{11}$ 450.4 Da Flavonoid, flavonol 106533 Astilbin 29838-67-3	Insecticidal [106] Antioxidant [107]	Identified in *H.perforatum* but not quantified [104]

	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Quercitrin $C_{21}H_{20}O_{11}$ 448.4 Da Flavonoid, flavonol 4444112 Quercetin 3-O-α-L-rhamnoside 522-12-3	Antioxidant [108] Anti-inflammatory [108]	1.2 – 3.3 mg/g dried plant [92] 1.22 – 3.98 mg/g dried plant [93]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Rutin $C_{27}H_{30}O_{16}$ 610.5 Da Flavonoid; flavonol glycoside 4444362 Quercetin-3-rutinoside 153-18-4	Antioxidant [109, 110] Anti-inflammatory [91]	9.8 – 21.1 mg/g dried plant [92] 0 – 1.86 mg/g dried plant [93] 2 – 17 mg/g dried plant [94]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	I3,II8-Biapigenin $C_{30}H_{18}O_{10}$ 538.5 Da Biflavonoid - - 101140-06-1	Antiviral [111] Anti-inflammatory [112]	0.7 – 3.6 mg/g dried plant [92] 1004 g/g dried weight biomass [102]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Hyperfirin $C_{30}H_{44}O_4$ 468.7 Da Phloroglucinol 28283929 - 927684-15-9		Identified in *H.perforatum* but not quantified [104]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Adhyperfirin $C_{31}H_{46}O_4$ 482.7 Da Phloroglucinol - - 927684-17-1		Identified in *H.perforatum* but not quantified [104]

	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Hyperforin $C_{35}H_{52}O_4$ 536.8 Da Phloroglucinol 16736597 - 11079-53-1	Anti-depressant, anti-biotic and anti-tumoral [84, 87]	0.006 – 1.32 % in plant [113] 5.46 mg/g [114] 7400 g/g dried weight biomass [102]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Adhyperforin $C_{36}H_{54}O_4$ 550.8 Da Phloroglucinol - - 143183-63-5		1470 g/g dried weight biomass [102]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Hypericin $C_{30}H_{16}O_8$ 504.4 Da Naphthodianthrone 4444511 - 548-04-9	Anti-depressant [84, 105]	0.04 – 0.25 % in plant [113] 0.44 – 4.06 mg/g dried plant [93] 2.7 – 3.47 mg/g [114] 620 g/g dried weight biomass [102]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Protohypericin $C_{30}H_{18}O_8$ 506.5 Da Naphthodianthrone 4590166 - 548-03-8		80 g/g dried weight biomass [102]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Pseudohypericin $C_{30}H_{16}O_9$ 520.4 Da Naphthodianthrone 4445065 - 55954-61-5		0.23 – 3.53 mg/g 3.54 mg/g [114] 839 g/g dried weight biomass [102]

	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Protopseudohypericin $C_{30}H_{18}O_9$ 522.5 Da Naphthodianthrone 4590328 - 54328-09-5		79 g/g dried weight biomass [102]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	Chlorogenic acid $C_{16}H_{18}O_9$ 354.3 Da Hydroxycinnamic acid 1405788 Chlorogenate 327-97-9	Anti-obesity [115]	0 – 1.86 mg/g dried plant [93] 1181 g/g dried weight biomass [102]
	Name: MF: Mass: Type: CS- ID: Synonym: CAS №:	3-O-Coumaroylquinic acid $C_{16}H_{18}O_8$ 338.3 Da Hydroxycinnamic acid 4955867 - 1899-30-5		Identified in *H.perforatum* but not quantified [104]

[1] Structures drawn in ChemSketch (ACD Labs)
[2] MF = Molecular formula, Mass = Average mass, CS-ID = ChemSpider ID number

1.7.2.2 Quality Control

The quality control for *Hypericum perforatum* in relation to its bioactive compounds investigates the quantities of flavonoids, hypericins and hyperforin. The British/European Pharmacopoeia [35, 116] states for dried extracts of SJW that:

- Total hypericins, expressed as hypericin: 0.10 percent to 0.30 percent (anhydrous extract);
- Flavonoids, expressed as rutin: minimum 6.0 percent (anhydrous extract);
- Hyperforin: maximum 6.0 percent (anhydrous extract) and not more than the content stated on the label.

In the British and European Pharmacopoeias the compounds used to monitor the quality of SJW include: hypericin, pseudohypericin, rutin, hyperforin, hyperoside, isoquercetin, quercirtoside, quercetin and biapigenin.

In contrast, the monitoring of SJW using the US Pharmacopoeia [117] only monitors hypericin, pseudohypericin and hyperforin in the SJW and uses oxybenzone as an internal standard.

Concentrations of all three compounds must fall between 90.0%–110.0% relative to the oxybenzone; the flavonoids rutin and hyperoside are used in the identification process only.

1.7.3 Elemental Analysis of St John's Wort

1.7.3.1 Known Elemental Constituents

Previous studies [118-145] investigating the elemental content of SJW raw plant and preparations demonstrate the varied elemental profile of SJW where the elements Cd, Cu, Fe, Mn, Pb and Zn were found in concentrations in the range of 0.04-20 μg/g, 4-200 μg/g, 6-1300 μg/g, 8-450 μg/g, ≤0.1-20 μg/g and 10-200 μg/g, respectively. The concentration of other elements in SJW samples such as Al, As, B, Ba, Ca, Co, Cr, Hg, Mg, Mo, Ni, Sb, Sn, Sr and V has also been reported in the literature [119, 125, 126, 129, 131, 142, 145] with techniques such as ICP-OES, ICP-MS, FAAS, FAES, GFAAS and anodic stripping voltammetry. SJW is also known to be an accumulator of the element Cd [66, 120] which is known to be toxic in high concentrations [146].

The elements present in SJW raw herbs mostly enter the plant tissue *via* the presence of the elements in the growth medium. However, during processing elemental contamination can occur in a number of ways from the mechanical processing, incorrect storage and the addition of bulking agents.

1.7.3.2 Quality Control

Elemental quality control of SJW is still rather limited. The British and European Pharmacopoeias [35, 53] specify the analysis of all herbal remedies to be tested for a minimum of Cd, Hg and Pb (Table 1.3). Whereas the US Pharmacopoeia states that no more than 50 μg/g of heavy metals should be present using Method II regarding heavy metals [147] which is a colour-comparison wet chemistry method despite the new guidelines brought in for oral drugs which is based on atomic spectroscopy (Table 1.2). This method does not include the quantification of Hg.

1.7.4 Links between Elements and Bioactive Compounds

The binding of metal ions with bioactive compounds has been shown to modify the biological effects compared to the organic constituents alone. One such effect is the production of the bioactive compounds. In regards to SJW, the presence of Cr (0.01mM) resulted in an increase in the production of protopseudohypericin (+135%), hypericin (+38%) and pseudohypericin (+5%). Higher concentrations of Cr (0.1 mM) resulted in a further increase of protopseudohypericin (+167%), but

the amounts were similar for hypericin (+25%) and pseudohypericin (+5%) [148] when compared to using 0.01mM Cr. However, in the presence of Ni, an opposite relationship was observed. A concentration of 25 mM or 50 mM Ni caused the levels of hypericin and pseudohypericin to significantly decrease by 21- and 15-fold, respectively, whilst hyperforin production fell below limits of detection [149]. A second relationship noted between elements and bioactive compounds is an alteration to bioactivity. For example, flavonoids such as rutin and quercetin have been shown to bind to metal ions (see Chapter 5, Table 5.1) and as a result the functions such as antioxidant [109, 150] and anti-inflammatory [109] properties increased compared to the flavonoid alone or in some cases became pro-oxidant [109]. A third relationship between elements and bioactive compounds is bioavailability. It was found that chickens fed an element rich diet in the presence of herbal remedies were able to uptake more elements into their tissues [151]. Interestingly, the type of herbal medicine influenced the concentrations of different metals to different tissues. For example, Sage significantly increased levels of Cu, Fe, Mn, and Zn in chicken liver whereas St. John's Wort and Small-flowered Willowherb did not. On the other hand, the presence of SJW significantly increased the concentrations of Zn in chicken legs [151]. These studies suggest metal-bioactive compound complexes may be more bioavailable, but more research is needed.

1.7.5 Statistical Approaches

A few studies have used chemometrics to investigate the elemental content of SJW, but these studies are more focused on other plant species or plants found in polluted areas. In a study by Ražić and co-workers [142] the elements Cu, Zn, Mn, Fe, K, Ca, Mg, Al, Ba and B were examined in twenty-six medicinal herbs of which one was SJW. Positive correlations between metal concentrations were found using PCA (e.g. Al and Fe correlated at the 0.01 significance level). Moreno-Jiménez and co-workers [136], monitored the elements Cd, Cu, Fe, Mn and Zn in 25 different plant species grown in a polluted mining area which included *Hypericum perforatum* ($n \leq 12$) as well as other plants from different families such as *Digitalis thapsi, Salix atrocinerea* and *Cytisus scoparius*. The multivariate data was analysed using correlation analysis as well as PCA and showed that Cd and Zn uptake was the greatest variant between species, Cu and Fe uptake was more homogeneous and Mn uptake was independent of pollution. This suggests that the uptake of certain elements is controlled by the plant whereas others are not and is species dependant.

1.8 Aim of Study

The aim of this study is to assess if the elemental profile of SJW could be used as a quality indicator. In order to use elemental profiling as a quality indicator several aspects need to be investigated; therefore the specific objectives of this study are to:

- Develop an accurate method for the elemental profiling of various SJW preparations;
- Analyse a large number of SJW preparations to determine the underlying elemental patterns;
- Evaluate the metal transfer properties of SJW when preparing formulated products from the herb;
- Develop a method to identity and quantify SJW bioactive constituents;
- Compare the elemental profile with the molecular profile to assess correlation and possible biomarker identification.

2 Method Development for the Elemental Analysis of *Hypericum perforatum* (St John's Wort) Preparations

2.1 Introduction

The analysis of herbal remedies for elemental purposes is generally done so in order to determine if the concentrations of such elements are harmful to health. This is especially true for toxic elements such as As, Cd, Hg and Pb [35, 53, 117]. Several incidents have been reported whereby persons have come to harm through metal poisoning *via* the ingestion of herbal medicines [21, 26, 152, 153]. For example, two 5 year old boys (one from Italy, the other from China) were poisoned by As and Hg respectively [26] due to the ingestion of herbal medicines. These as well as other elements can enter the plant *via* a number of instances; the element could be present in the growth medium and taken-up through natural growing purposes *via* the roots or introduced *via* air pollution in addition to manufacturing/processing. During manufacturing, the addition of toxic elements may be accidental or on purpose. Accidental contamination could occur from poor storage or improper following of Good Manufacturing Practices (GMP) whereas known addition of elements could be through bulking agents or as an active ingredient. For example, in many Asian herbal medicines it has been common to add cinnabar (mercury (II) sulphide) [51] or realgar (arsenic sulphide) [52].

Recent studies show the examination of elements with plants could be used in other fields of research in addition to monitoring levels of toxic elements in items for food consumption. This includes the exploitation of a plants elemental up-take to increase nutritional enrichment [154], for cleaning contaminated land [155], to increase the production of secondary metabolites or Bioactive Plant Compounds (BPCs) of interest [148, 149, 156] or possibly improve bioactivity or absorption of elements and BPCs into the body via complexing [151]**.** However, there are several challenges when analysing plant material for elemental content. The most profound being the lack of certified reference material (CRMs) for trace elements in herbal remedies. The majority of CRMs for trace analysis that are available are either not used for general food consumption (e.g., peach, apple or tomato leaves), are used as bases for foods (e.g., oats, wheat, barley), or are popular fruit, vegetables or salads (e.g., potato, cucumber, lettuce, sprouts). Trace element CRMs for true herbal medicines are very limited (e.g., dandelion, clover, pansy, ginkgo biloba). Elements are often present at trace levels in herbal medicines and due to this great care must be taken in order to analyse these reproducibly and without introducing contamination. The herbal remedy, St John's Wort (SJW) is of interest as it is a known metal accumulator [118-145] and is known to contain numerous bioactive compounds [98, 104, 127]. This herb is also popular within Europe and the USA

[9, 85] as it is utilised for its anti-depressant therapeutic effect [83, 101]. A number of studies [118-145] have investigated the elemental content of SJW plant and/or preparations in which concentrations of Cd, Cu, Fe, Mn, Pb and Zn have been found in the range of 0.04-20 μg/g, 4-200 μg/g, 6-1300 μg/g, 8-450 μg/g, ≤0.1-20 μg/g and 10-200 μg/g, respectively. Other studies have monitored the concentrations of Al, As, B, Ba, Ca, Co, Cr, Hg, Mg, Mo, Ni, Sb, Sn, Sr and V [119, 125, 126, 129, 131, 142, 145]. For a full summary of such studies, please see

Table **10.5**, Appendix 10.2.

The techniques employed by these studies vary greatly. For example, there were 16 studies that utilised AAS [118, 120-122, 125, 127, 128, 130, 132, 133, 136, 137, 140, 142, 143, 157], 4 that applied GFAAS [120, 125, 126, 131], 6 that used ICP-OES [126, 129, 138, 139, 142, 145] with an equal number using ICP-MS [119, 123, 124, 131, 141, 144] in addition to 3 studies that applied AES [125, 128, 142] for the determination of elemental content in SJW. A smaller number of studies employed Hanging Mercury Drop Electrode (HMDE) [135], Thin Mercury Film Electrode (TMFE) [139], Direct Mercury Analyser (DMA) [134] or LA-ICP-MS [119] analysis for the elemental determination. Thus, with different instruments of analysis being utilised it is also inherent that many different sample preparation techniques have also been exploited. This includes the acid used to digest the SJW material; varying in aspects of volume and composition (e.g., a single acid such as HNO_3 to a mixture of several acids) as well as the technique of the digestion; varying in length of time, temperature and equipment (e.g., hotplate or microwave). In addition to this, several of the studies [118, 120, 122, 123, 132, 133, 138, 143, 157] examined 5 elements or less in the SJW samples. Also noted is that some of the observed studies [122, 126, 130, 136, 158, 159] contribute data based on SJW grown in a single country or region which, on their own, do not allow full interpretation of these elemental concentrations due to soil and climate constrictions in addition to the majority of the studies examining only the raw herb.

As discussed earlier, the lack of a SJW reference material is an issue for its elemental validation. As such, where these studies have used reference materials to aid validation the material used differs greatly. Some studies have opted for plant based CRMs such as tomato leaves [119, 134, 142], peach leaves [119], spinach leaves [119] hay powder [121] tea leaves [144, 160] mixed polish herbs [160] grass [135] and Rosa plant [135] whereas other studies have utilised reference materials which are not plant-based including soil [134], dogfish muscle tissue [134] and lobster hepatopancreas tissue [134]. Therefore, although the studies above have investigated element

concentrations in SJW, they are often limited by the number of elements, number of samples and geographic location. There are also extremely large discrepancies in the consistency of analysis between these studies, which make it difficult to correlate the information gathered for further interpretation.

In this study, a single method was developed using ICP-OES in order to obtain the concentrations of 25 elements (i.e., Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Hg, In, Mg, Mn, Mo, Ni, Pb, Pt, Sb, Se, Sr, V, Y and Zn) in a range of SJW preparations including raw herb, tablet and capsule form.

2.2 Method

2.2.1 Materials

2.2.1.1 Reagents, Standards and Samples

High-purity (99.99% trace metal basis) nitric acid 70% (Sigma-Aldrich, Gillingham, UK), high purity 37% trace metal grade hydrochloric acid (Sigma-Aldrich, Gillingham, UK), high purity 35%, trace metal grade hydrogen peroxide (Sigma-Aldrich, Gillingham, UK) and high purity ammonium fluoride (Sigma-Aldrich, Gillingham, UK) was used for determining the optimum acid mixture for the digestion of samples and the recovery of trace metals. Elemental stock solutions, 1000 ppm of Al, As, B, Ba, Cd, Co, Pb, Mg, Mn, Mo, Ni, In and Hg (Fisher, Loughborough, UK), Be and Pt (VWR, Lutterworth, UK), Ca, Cr, Cu, Fe, Sb, Se, Sr and Zn (Merck, Feltham, UK), V (Sigma-Aldrich, Gillingham, UK) and Y (Acros organics, Geel, Belgium) were used to prepare calibration standards and ICP-OES optimisation solution. Certified reference material NIST Polish tea (NIST INCT-TL-1) for trace metals was used within method development and validation. Samples of St John's wort (*Hypericum perforatum*) including raw herbs, tablets and capsules were sourced from retail and internet suppliers (Table 3.1).

2.2.1.2 Instrumentation

Acid digestion was carried out using a Mars Xpress microwave (CEM Corporation, Middle Slade, UK) with Teflon digestion vessels. Elemental analysis was carried out using a Varian 710-ES ICP-OES with SPS3 autosampler.

2.2.1.3 Labware Pre-treatment

All labware was acid washed overnight with 4M Nitric acid created from a 1 in 4 dilution of 70% nitric acid (reagent grade, Fisher, Loughborough, UK) with deionised water (Purite, Select Analyst R1.5, Oxon, UK). Labware was then rinsed thoroughly with deionised water and dried before use.

2.2.2 ICP-OES Parameter Optimisation

Parameter optimisation was carried out by ICP-OES as recommended in the instrument manual [161]. A solution of 1 ppm As, Co, Se and Pb in 2% HNO_3 was analysed at wavelengths 193.696 nm, 238.892 nm, 220.353 nm and 196.026 nm, respectively. Firstly the power was optimised by calculating the signal to noise (SN) value (Equation 1) of the selected elements for the Radio Frequency (RF) powers: 1.1, 1.2, 1.3 and 1.4 kW. The setting that produced the optimum SN value was a RF power of 1.4. Following this, the nebuliser pressure was optimised by calculating the SN value with nebuliser pressures 180, 200, 220 and 240 kPa with a RF power of 1.4 kW. Please note as this calculation involves subtraction rather than division, it is an SN value rather than SN ratio.

$$\text{Signal to Noise Value} = \text{maximum signal intensity} - \text{maximum noise intensity}$$

(Equation 1)

The comparison of two types of nebuliser was carried out. The Conikal nebuliser is a general purpose nebuliser in ICP whereas the SeaSpray nebuliser is able to cope with higher sample salts. The limits of detection were determined with each nebuliser over three days. A blank sample of 2% HNO_3 was run 10 times and the concentration was calculated *via* a one point calibration with 1ppm multi-element standard (Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Hg, In, Mg, Mn, Mo, Ni, Pb, Pt, Sb, Se, Sr, V, Y and Zn) [161].

2.2.3 Quantification - Non-weighted Regression vs. Weighted

As the elemental concentration in herb samples are often in the low ppm range and requires using the low end of the calibration range for ICP-OES, both a non-weighted and weighted regression were explored. The cumulative calibration error in the lowest three standards (0.01 ppm, 0.025 ppm and 0.05 ppm) was compared using a weighted regression line and a non-weighted regression line. Microsoft Excel (2007) was used for regression calculations and t-tests. Weighted regression calculations utilised can be found in Miller and Miller (2010) [162].

2.2.4 Initial Validation Studies

2.2.4.1 Studies using different acid mixtures

The optimum acid for digestion and element recovery was assessed using five acid mixtures and the NIST tea (INCT-TL-1). The acid mixtures (Table 2.1) were selected based on those recommended by CEM (mixture 2, 3 and 4), seen commonly in literature [77, 79, 82, 121, 130, 136, 141, 160, 163-169] (mixture 1, 2, 4 and 5) or stated in the British pharmacopeia [41] (mixture 5) for the digestion of plant material.

Table 2.1. Summary of acid mixtures

Acid mixture	Type of acid and volume
1	5 ml nitric acid
2	2 ml of water, 8 ml nitric acid and 2 ml hydrogen peroxide
3	2 ml of water, 8 ml nitric acid, 2 ml hydrogen peroxide and 200 mg ammonium fluoride.
4	2 ml of water, 8 ml nitric and 2 ml hydrochloric acid
5	15 ml nitric acid

Microwave acid digestion of samples was carried out on a Mars Xpress microwave (CEM Corporation, Matthews, USA) using the protocol set out in Table 2.2.

Table 2.2 MarsXpress microwave settings

Step	Program setting
1	Heat over 12 minutes to 160 °C
2	Hold at 160 °C for 2 minutes
3	Heat to 175 °C over 2 minutes
4	Hold at 175 °C for 2 minutes
5	Heat to 185 °C over 2 minutes
6	Hold at 185 °C for 15 minutes
7	Allowed to cool

2.2.4.2 Elemental Transfer Loss

To assess the impact of transferring the samples between multiple vessels during sample preparation, the five acid mixtures were spiked with known concentrations. All acid mixtures were spiked with 10 ppm Ca, 5 ppm Mg, 1 ppm Al and Fe, 0.2 ppm Mn, and 0.1 ppm As, B, Ba, Be, Cd,

Co, Cr, Cu, Hg, In, Li, Mo, Ni, Pb, Pt, Sb, Se, Sn, Sr, Ti, V, Y and Zn. The samples then underwent microwave digestion (400W) then diluted 1:10 with deionised water, centrifuged at 9000 rpm for 45 minutes and syringe filtered (0.22 µm). The samples were then analysed on the ICP-OES.

2.2.4.3 Analysis of CRM NIST Polish Tea

Microwave digestion of samples was carried out using a CEM Mars Xpress microwave. Approximately 0.4 g of NIST Polish tea was digested in triplicate using each acid mixture and the microwave program at 400W then allowed to cool. The samples were then diluted 1:10 with deionised water, centrifuged at 9000 rpm for 45 minutes and syringe filtered (0.22 µm). The samples were then analysed on the ICP-OES.

2.2.4.4 Analysis of St John's Wort Sample

St John's Wort (SJW) samples were digested using acid mixture 1 and 3. Approximately 0.4 g of a SJW raw herb sample was digested in each acid mixture in triplicate. The samples were then diluted 1:10 with deionised water, centrifuged at 9000 rpm for 45 minutes and syringe filtered (0.22 µm). The samples were then analysed on the ICP-OES.

2.2.4.5 Confirmation of Glass Leaching

To confirm if acid mixture 3 was leaching elements from glass, the CRM NIST tea was prepared in triplicate in glass volumetric flasks as well as plastic certified DigiPrep tubes. Approximately 0.4 g of the NIST Polish tea was digested in each acid mixture in triplicate. The samples were then diluted 1:10 with deionised water, centrifuged at 9000 rpm for 45 minutes and syringe filtered (0.22 µm). The samples were then analysed using ICP-OES.

2.2.4.6 Microwave Power Setting

Microwave acid digestion of samples was carried out on a CEM Mars Xpress microwave. Originally the power setting of the microwave was 400W and this was compared to 1600W using NIST tea. Approximately 0.4 g of the NIST Polish tea was digested in each acid mixture in triplicate, using the microwave programme on each power setting. The samples were then diluted 1:10 with deionised water, centrifuged at 9000 rpm for 45 minutes and syringe filtered (0.22 µm). The samples were then analysed on the ICP-OES.

2.2.5 Validation-Accuracy

2.2.5.1 NIST CRM and Spiked Recovery

Validation of the method was carried out using NIST certified reference material (CRM) Polish tea (INCT-TL-1) and spike recovery methods. The NIST reference was certified for Al, B, Ba, Ca, Cu, Fe, Mg, Mn, Ni, Sr and Zn. To validate the remaining metals or those below detection limits, the NIST reference was artificially enriched with 0.5 ppm As, Be, Cd, Co, Cr, Hg, In, Mo, Pb, Pt, Sb, Se, V and Y prior to acid digestion. Approximately 0.4 g of the NIST Polish tea was microwave digested (1600W) in acid mixture 1 in triplicate. The samples were then diluted 1:10 with deionised water, centrifuged at 9000 rpm for 45 minutes and syringe filtered (0.22 µm). The samples were then analysed using ICP-OES.

2.2.5.2 Standard Addition

Each type of SJW sample (i.e., dry herb, capsule, and tablet) was evaluated using the standard addition method. The samples were digested using acid mixture 1 at 1600W. A single point standard addition method was used to evaluate the matrix effects of the different preparations. In this case, the samples were artificially enriched with elements at concentrations equal or greater than five times the expected elemental concentration [170]. The standards added were 2.5 ppm of As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Hg, In, Pb, Pt, Mg, Mo, Ni, Sb, Se, Sr, V, Y and Zn; 5 ppm of Fe and Mn; 25 ppm for Al; 60 ppm of Mg and 225 ppm Ca.

2.3 Results and Discussion

2.3.1 ICP-OES Parameter Optimisation

Optimization was carried out on the Varian 710-ES ICP-OES using a solution of 1 ppm As, Co, Pb and Se. The RF power controls the magnetic field around the plasma helping to contain its shape while the nebuliser pressure aids the flow of sample through the nebuliser. The background intensities of the selected elements were subtracted from the signal intensity (Equation 1) to give a SN value. The power setting that produced the optimum SN value (Table 2.3) for all elements was an RF power of 1.4 kW. Elements As and Co obtained their optimum at 1.3 kW however there was no decrease in SN value for these at a setting of 1.4 kW; therefore, 1.4 kW was applied for the following nebuliser pressure optimisation. Higher RF powers were not investigated as this was the limitation of the instrument. For elements As and Pb the optimum nebuliser pressure was 180 kPa whereas for Co and Se it was 220 kPa and 200 kPa respectively. As the majority of elements had a higher SN value

at 180 kPa as well as As and Pb being toxic elements (monitored by Pharmacopoeias and known to cause metal poisonings through herbal remedies), the nebuliser pressure of 180 kPa selected. Following this study, the parameters of the ICP-OES were changed from the default settings to those listed in Table 2.4. Optimisation of the ICP-OES allows the limits of detection to become more sensitive in comparison to its default settings, thus allowing lower concentrations of elements to be detected that would otherwise be considered below detection limits.

Table 2.3. SN values from optimisation of Varian ICP-OES

Power	**SN value**			
(kW)	**As (193.696 nm)**	**Co (238.892 nm)**	**Pb (220.353 nm)**	**Se (196.026 nm)**
1.0	33 500	1 570 000	249 600	25 400
1.1	39 700	1 780 000	283 000	29 700
1.2	46 200	2 000 000	337 800	34 900
1.3	51 500	2 180 000	381 900	38 800
1.4	51 500	2 180 000	383 500	39 100
Pressure	**SN Value**			
(kPa)	**As (193.696 nm)**	**Co (238.892 nm)**	**Pb (220.353 nm)**	**Se (196.026 nm)**
180	56 900	2 380 000	435 100	43 200
200	55 700	2 390 000	426 900	44 100
220	56 400	2 420 000	426 900	43 400
240	56 200	2 420 000	426 900	43 400

Table 2.4. Optimised parameters for Varian 710 ICP-OES

Parameter	**Value**
Power (kW)	1.40
Plasma argon flow (L/min)	15.00
Auxiliary argon flow (L/min)	1.50
Nebulizer pressure (kPa)	180

The limits of detection (LOD) were calculated for two different nebulisers, the Conikal and SeaSpray. The nebuliser is a key part of the ICP in which turns the liquid samples into a fine aerosol for analysis. The Conikal is a general purpose nebuliser whilst the SeaSpray has an extended tip which allows the nebuliser to be more robust with samples containing high salts, thus less prone to blocking. The results (Table 2.5) show that overall there is little difference between the two

nebulisers with the majority of elements. However, the LOD was significantly lower ($p<0.05$) for Mn and Zn with the SeaSpray nebuliser and for Pb with the Conikal. Therefore, as more elements have a lower LOD with the SeaSpray nebuliser and due to its engineering it is less liable to blockage, this nebuliser was utilised for all future analyses.

Table 2.5 Limits of detection (LOD) of two different nebulisers

		Limit of Detection (ppb)[1,2]			
Element	**Wavelength (nm)**	**SeaSpray**	**±1SD**	**Conikal**	**±1SD**
Al	396.152	5.1	0.5	5.1	0.4
As	188.98	20	4	22	2
B	249.772	7	2	9	1
Ba	455.403	0.21	0.03	0.17	0.01
Be	234.861	0.20	0.02	0.3	0.1
Ca	396.847	1.9	0.1	1.2	0.6
Cd	214.439	0.5	0.1	0.5	0.1
Co	228.615	3.0	0.5	3.5	0.9
Cr	267.716	1.5	0.1	1.7	0.3
Cu	327.395	4.5	0.7	5	1
Fe	238.204	1.2	0.1	1.6	0.3
Hg	184.887	5	1	7	1
In	230.606	23	3	24	6
Mg	279.553	0.24	0.05	0.15	0.04
Mn	257.61	0.23	0.01*	0.28	0.01
Mo	202.032	2.6	0.6	2.7	0.8
Ni	231.604	3.4	0.6	4	1
Pb	220.353	10.8	0.8	8.0	0.3*
Pt	203.646	31	4	34	4
Sb	217.582	29	3	28	5
Se	196.026	32	3	45	14
Sr	407.771	0.06	0.01	0.06	0.01
V	292.401	3.2	0.2	4	1
Y	371.029	0.6	0.1	0.6	0.2
Zn	213.857	0.63	0.03*	1.1	0.1

[1] LOD = S.D. X 3 [2] n=30 *$p<0.05$ t test

2.3.2 Quantification - Non-weighted Regression vs. Weighted

Calibration curves for the 25 elements in the typical concentration ranges for SJW samples were compared using a weighted *vs.* non-weighted regression. Weighted regressions are often used in routine analyses as well as trace analysis, however it is not noted in the previous studies examining SJW which type of calibration is utilised (Table 10.2 to

Table **10.5**, Appendix 10.2). As the concentration of many elements would fall in this range for ICP-OES, the calibration errors associated with the lowest concentration standards above the LOQ were compared (0.01 ppm, 0.025 ppm and 0.05ppm for the majority of elements however: 0.025 ppm, 0.05 ppm and 0.1ppm for elements Cr and In, 0.05 ppm, 0.1 ppm and 0.5ppm for elements B, Cu, Hg, Mo, Ni and Y followed by 0.5 ppm and 1ppm for Pt, As, Sb and Se). The results (Table 2.6) show that for the majority of elements, the weighted regression line has less error in the calculation of concentrations at these low concentrations in comparison to a non-weighted regression line. For example, with a weighted regression Al has 10% uncertainty whereas Be has 3%, Cd has 4% and Sr has 10% whereas with a non-weighted graph these values are 36%, 27%, 16%, and 46% respectively. This is because in weighted regression lines the line passes more closely to the data points of lower concentration with less associated error which in turn gives more realistic confidence limits for sample concentrations. In contrast non-weighted lines assume all data points have equal error [162]. Some elements however (B, Fe, Se and Zn) have a lower uncertainty with a non-weighted calibration.

Table 2.6. Comparison of calibration error between weighted and non-weighted regression lines

		Total Cumulative Error %[1,2]	
Element	**Wavelength**	**Weighted**	**Non-weighted**
Al	396.152 nm	10	36
As	188.980 nm	1	1
B	249.772 nm	28	13
Ba	455.403 nm	11	25
Be	234.861 nm	3	27
Ca	370.602 nm	14	26
Cd	214.439 nm	4	16
Co	228.615 nm	4	3
Cr	267.716 nm	3	6
Cu	327.395 nm	2	3
Fe	238.204 nm	26	23
Hg	184.887 nm	2	3
In	230.606 nm	8	19
Mg	278.142 nm	34	37
Mn	257.610 nm	4	18
Mo	202.032 nm	2	2
Ni	231.604 nm	3	4
Pb	220.353 nm	2	2
Pt	203.646 nm	3	3
Sb	217.582 nm	4	4
Se	196.026 nm	3	2
Sr	407.771 nm	10	46
V	292.401 nm	3	3
Y	371.029 nm	7	32
Zn	213.857 nm	87	42

[1] Cumulative (out of 300%) for the three lowest concentration standards above LOQ

2.3.3 Microwave Digestion

2.3.3.1 Selection of Acid Mixture

2.3.3.1.1 Elemental Transfer Loss

Before the analysis of herbal material, a control experiment was conducted to determine the impact of multiple container transfers, MW digestion process, and filtering. Each of the acids were artificially enriched with known concentrations of elements and carried through the experimental

protocol. The results (Table 2.7) illustrate that for the majority of elements the transfer loss is similar across the different acid mixtures (less than 10%). However, it was notable that in comparison to the other acid mixtures, acid mixture 5 generally had the lowest recovery of elements for most of the acids. Despite this being the same as acid mixture 1 and only differing in the volume used, it is believed that this reduction in recovery is due to the limited times the acid digestion vessel could be rinsed in comparison to that used for acid mixture 1 (i.e., 5 ml HNO_3 into 50 ml allows the vessel to be rinsed with up to 45 ml, whilst 15 ml HNO_3 into 50 ml allows the vessel to be rinsed with up to 35 ml). Also noted is the recovery of B, as 0.1 ppm is below the LOQ for B the error is quite high, however it is exceptionally high with acid mixture 3. This may be due to the presence of hydrogen fluoride (HF) in the mixture leaching B from the boro-silica glass. Thus giving an inaccurate result as the blank acid is also high in B. Examining the different acids for recovery of elements shows that acid mixture 1, 2 and 4 are similar. Acid mixture 1 has good recoveries and also lower standard deviation of all elements (1SD of ≤10%). The levels of Pt reported across all acids are high; this is due to 0.1 ppm being below the LOQ. This study has shown that there is no significant loss of elements during transfer between containers, but to ensure this is kept to a minimum adequate rinsing is needed.

Table 2.7. Summary of elemental loss due to sample transference

Element	1	±1SD	2	±1SD	3	±1SD	4	±1SD	5	±1SD
Al	95.1	0.5	94.5	0.5	89	15	94.3	0.8	87.6	0.2
As	94	6	97	4	86	8	96	5	87	2
B	100	10	80	10	42	1170	90	20	110	30
Ba	93.2	0.5	96.2	0.6	95	2	95	1	90.1	0.3
Be	92	2	92.4	0.7	90	2	89.1	0.7	86.7	0.7
Ca	99.2	0.6	98	2	97.4	0.8	99	1	94.7	0.2
Cd	93	2	95	1	93	2	91	1	90.8	0.9
Co	95	1	97.0	0.6	95	2	92.5	0.5	91	2
Cr	96	2	98.6	0.6	96	1	95.2	0.3	97.8	0.7
Cu	97	3	100	4	100	2	100	3	98	1
Fe	100	3	95	3	94	1	97	1	95	1
Hg	93	2	95	2	96	1	95.0	0.7	89	2
In	94	7	91	6	91	5	93	3	95	3
Mg	92.9	0.9	92.0	0.9	90	1	89.3	0.2	86	1
Mn	94.8	0.5	96.5	0.6	95	2	95	1	90.4	0.4
Mo	95	2	99	2	98	2	95.9	0.9	96	1
Ni	95	1	96	1	95	2	94.3	0.2	92	1
Pb	95	3	95	1	94	3	95	5	92	3
Pt	106	8	110	10	115	10	114	1	100	10
Sb	88	2	92	4	92	5	92	6	86.3	0.8
Se	91	5	95	4	91	5	92	8	88	3
Sr	93.7	0.6	96	1	95	2	97	3	93.3	0.3
V	95	2	99.6	0.5	97	2	95.3	0.7	96	1
Y	99	2	100.8	0.7	99	2	100.1	0.6	98.0	0.9
Zn	95	4	95	3	85	4	95	9	87.8	0.9

Note: some errors are large as 0.1 ppm is below or close to LOQ for some elements (B, Pt and Se). SD = Standard Deviation. Acid mixture: 1 = 5 ml HNO_3, 2 = 2 ml of H_2O, 8 ml HNO_3 and 2 ml H_2O_2, 3 = 2 ml of H_2O, 8 ml HNO_3, 2 ml H_2O_2 and 200 mg NH_4F, 4 = 2 ml of H_2O, 8 ml HNO_3 and 2 ml HCl and 5 = 15 ml HNO_3.

2.3.3.1.2 Analysis of CRM NIST Polish Tea

The NIST tea was analysed in triplicate in each of the five acid mixtures to assess element recovery with a certified reference material (CRM). Other studies that have utilised CRMs have used tea [144], hay powder [120, 121], tomato leaves [119, 134, 142], peach leaves [119], bush twigs [145], fish protein [166], milk powder [166], grass [135] and Rosa plant [135] due to the lack of a SJW CRM. The NIST Polish tea was utilised as it contains leaves, like SJW, and is the closest to resemble a medicinal herb. The concentrations of the elements obtained with each acid were compared to the certified values. The solutions for all acids were visually clear indicating good digestion. The results (Table 2.8) show that acid mixtures 1 and 3 recovered the most Al of the five mixtures whereas acid mixture 5 recovered the least. For B, most acid mixtures are similar, however, acid mixture 3 shows

to have greatly elevated concentrations of B (Figure 2.1); this is likely to be due to the HF produced in the acid mixture causing leaching of B from the glassware. For Ba recovery, all acid mixtures are similar (36 – 39 mg/kg). Acid mixture 1 had the highest recoveries for elements Mg, Mn and Ni with similar recoveries for elements Ca, Cu, Sr and Zn compared to other acids. Acid mixture 3 had the highest recoveries for B and Fe, acid mixture 4 had highest recovery for Ca. Overall, of the acid mixtures tested, acid mixture 1 was chosen for further investigations as it had the highest recovery for the majority of elements and no recovery values below 80%.

Table 2.8. Recovery of elements of NIST tea with each acid mixture

		Concentration obtained with acid mixtures[1]									
Element	Certified value	1	±1SD	2	± 1SD	3	± 1SD	4	± 1SD	5	± 1SD
Al	0.229 ± 0.028 wt%	0.193	0.009	0.2	0.2	0.183	0.006	0.17	0.02	0.163	0.005
B	26 mg/kg	21	1	25	1	80	50	29	1	30	1
Ba	43.2 ± 3.9 mg/kg	37.6	0.3	36.7	0.2	39	3	37	4	36.2	0.2
Ca	0.582 ± 0.052 wt%	0.538	0.002	0.519	0.003	0.520	0.003	0.539	0.004	0.52	0.06
Cu	20.4 ± 1.5 mg/kg	20	1	18.98	0.09	18.8	0.4	20	2	19.9	0.8
Fe	432 mg/kg	380	10	370	20	470	20	430	50	360	30
Mg	0.224 ± 0.017 wt%	0.203	0.000	0.193	0.001	0.193	0.001	0.19	0.02	0.187	0.001
Mn	0.157 ± 0.011 wt%	0.145	0.002	0.141	0.001	0.140	0.001	0.15	0.02	0.142	0.001
Ni	6.12 ± 0.52 mg/kg	5.40	0.05	4.97	0.06	5.10	0.07	5.1	0.6	5.03	0.05
Sr	20.8 ± 1.7 mg/kg	18.7	0.1	18.158	0.008	19.6	0.1	19	2	18.1	0.1
Zn	34.7 ± 2.7 mg/kg	32.4	0.3	30.7	0.6	29.6	0.5	32	3	30	2

[1] units same as those for certified values. SD = Standard Deviation. Acid mixture: 1 = 5 ml HNO_3, 2 = 2 ml of H_2O, 8 ml HNO_3 and 2 ml H_2O_2, 3 = 2 ml of H_2O, 8 ml HNO_3, 2 ml H_2O_2 and 200 mg NH_4F, 4 = 2 ml of H_2O, 8 ml HNO_3 and 2 ml HCl and 5 = 15 ml HNO_3

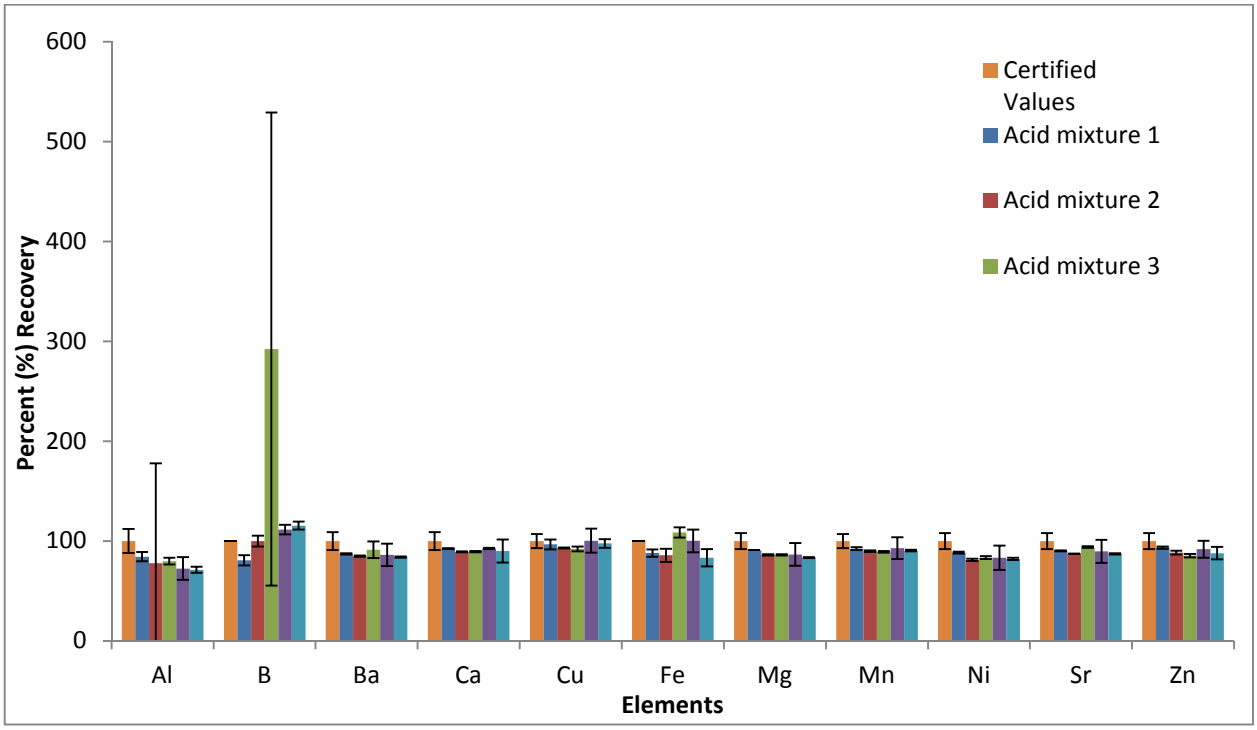


Figure 2.1 Recovery of elements of NIST tea with each acid mixture (error bars ±1SD)

Note: Acid mixture: 1 = 5 ml HNO_3, 2 = 2 ml of H_2O, 8 ml HNO_3 and 2 ml H_2O_2, 3 = 2 ml of H_2O, 8 ml HNO_3, 2 ml H_2O_2 and 200 mg NH_4F, 4 = 2 ml of H_2O, 8 ml HNO_3 and 2 ml HCl and 5 = 15 ml HNO_3

2.3.3.1.3 Analysis of St John's Wort samples

Although the NIST tea CRM is similar in nature to SJW herb, differences due to silicate content may occur. SJW is a combination of flowers, leaves and some stalk, and is therefore a tougher sample to digest than the NIST Polish tea. Also, the silica content [171] can affect the elements recovered; this is due to elements such as Al and Fe being bound to the silica which is not as readily digested in most acids. Acid mixture 1 was chosen as it gave the highest recovery values for the most elements and acid mixture 3 was chosen to see if the silica content of SJW affected the results obtained as the small amount of HF produced would be able to digest the silica contained. The results (Figure 2.2) show that overall the majority of elements did not differ significantly between the two acids; however, there was a significant difference (t-test, $p \leq 0.05$) with elements Al, B and Fe. This is likely due to the presence of silica in the SJW plant material [172]. The HF in acid mixture 3 is strong enough to break down such silica within the plant material however, this also has the disadvantage of potentially introducing contamination into the sample though glass leaching (such as B). Therefore, as the majority of elements are similar between both acids and to prevent damage to glassware within the ICP-OES, acid mixture 1 was chosen for future investigations with SJW. To develop a technique for routine analysis, the use of HF is not ideal as it can have serious

consequences from accidental exposure. It is highly corrosive and readily absorbed by skin which can lead to cardiac arrest. Therefore if using HF consistently or in large concentrations, precautions such as calcium gluconate should be rubbed into hand and arms as a barrier and a person trained in first aid with oxygen tanks should be present. Thus as the method was developed for routine use, and is not considering elements trapped in silicates the HNO_3 was utilised.

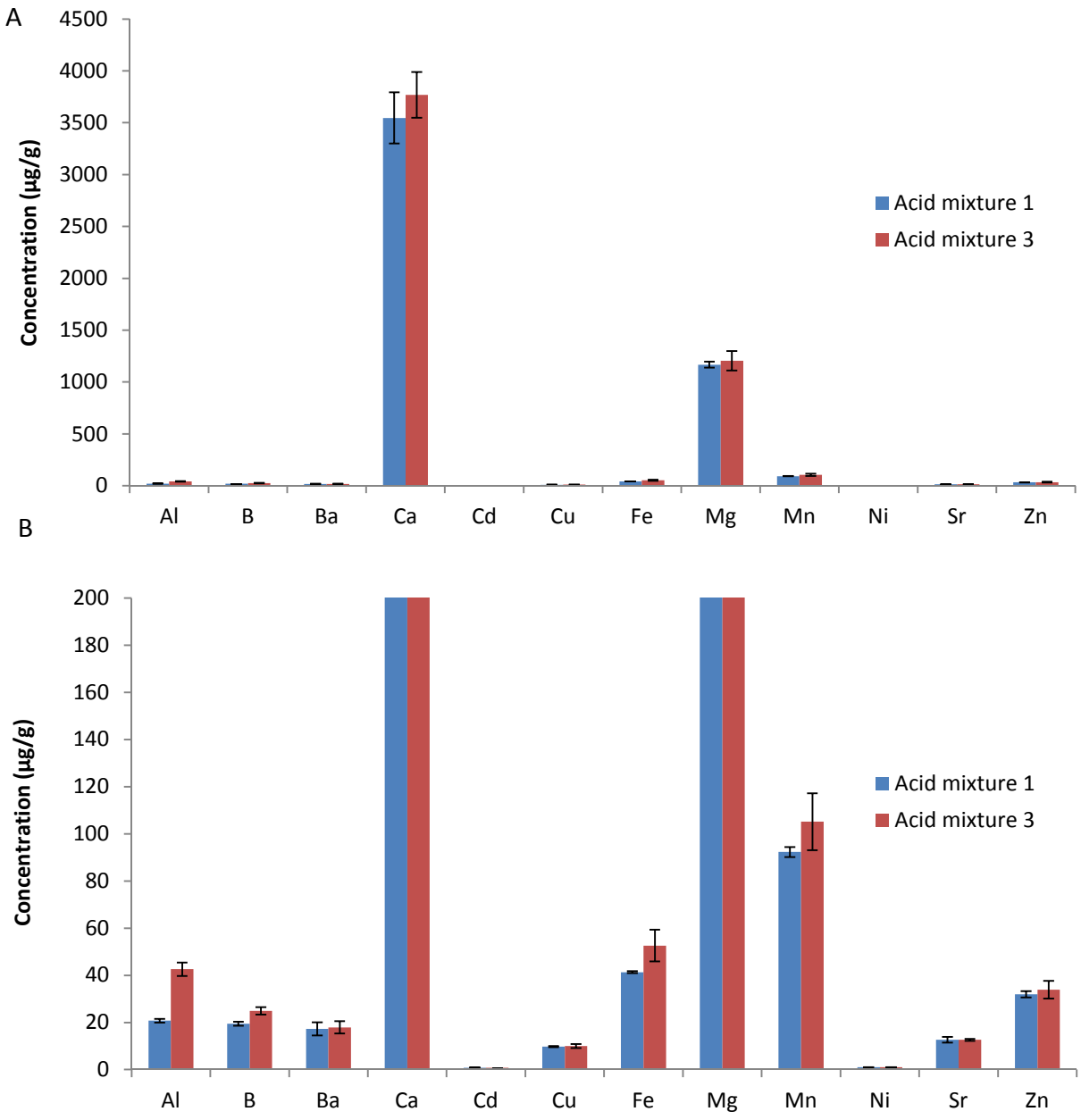


Figure 2.2. (A) Comparison of acid mixture 1 and acid mixture 3 with the digestion of a SJW herb on full y-axis (B) y-axis limited to 400 µg/g (error bars ±1SD).

Note: Acid mixture: 1 = 5 ml HNO_3 and 3 = 2 ml of H_2O, 8 ml HNO_3, 2 ml H_2O_2 and 200 mg NH_4F

2.3.3.1.4 Confirmation of Glass Leaching

To confirm that acid mixture 3 was leaching elements from glassware, the NIST polish tea was prepared in acid mixture 3 within both glass and plastic volumetric containers. The results (Figure 2.3) show that when the samples are prepared with acid mixture 3 in glass volumetric containers, there is a marked decrease in the recovery of B in comparison to the samples prepared in plastic containers (0.09 ± 6 μg/g compared to 27.6 ± 0.6 μg/g (±1SD) respectively). This is due to the amount of B found in the blank being greatly increased in the presence of glass compared to plastic (1.3 ppm compared to 0.06 ppm). This shows that leaching of this element occurs and as a result, when correcting for the blank in the concentration calculations, a greater amount of B is subtracted. The element Cu shows a significant difference between containers (t test, p=0.05). When the samples are prepared in plastic, the Cu recovered is reported 18.0 ± 0.4 μg/g compared to 20 ± 1 μg/g (±1SD) when prepared in glass. This is due to the amount of Cu found in the blank being increased in the presence of the plastic compared to glass (0.01 ppm compared to 0.002 ppm). This shows that leaching of this element occurs in the plastic container. The element Sr also shows a significant difference between containers (t test, p=0.05). When the samples are prepared in plastic, the Sr recovered is reported 18.5 ± 0.1 μg/g compared to 18.0 ± 0.1 μg/g (±1SD) when prepared in glass. This is due to the amount of Sr found in the blank being increased in the presence of the glass compared to plastic (not detected compared to 0.001 ppm). This shows that leaching of this element occurs in the glass container. The other elements show no significant difference between preparation in either glass or plastic volumetric containers. This shows that during the short time of the sample being made to volume during the dilution stage, acid mixture 3 is able to leach elements, particularly B and a smaller amount of Sr from the boro-silica glass. Therefore due to the induced contamination and the damage caused to glassware, this acid mixture was no longer utilised.

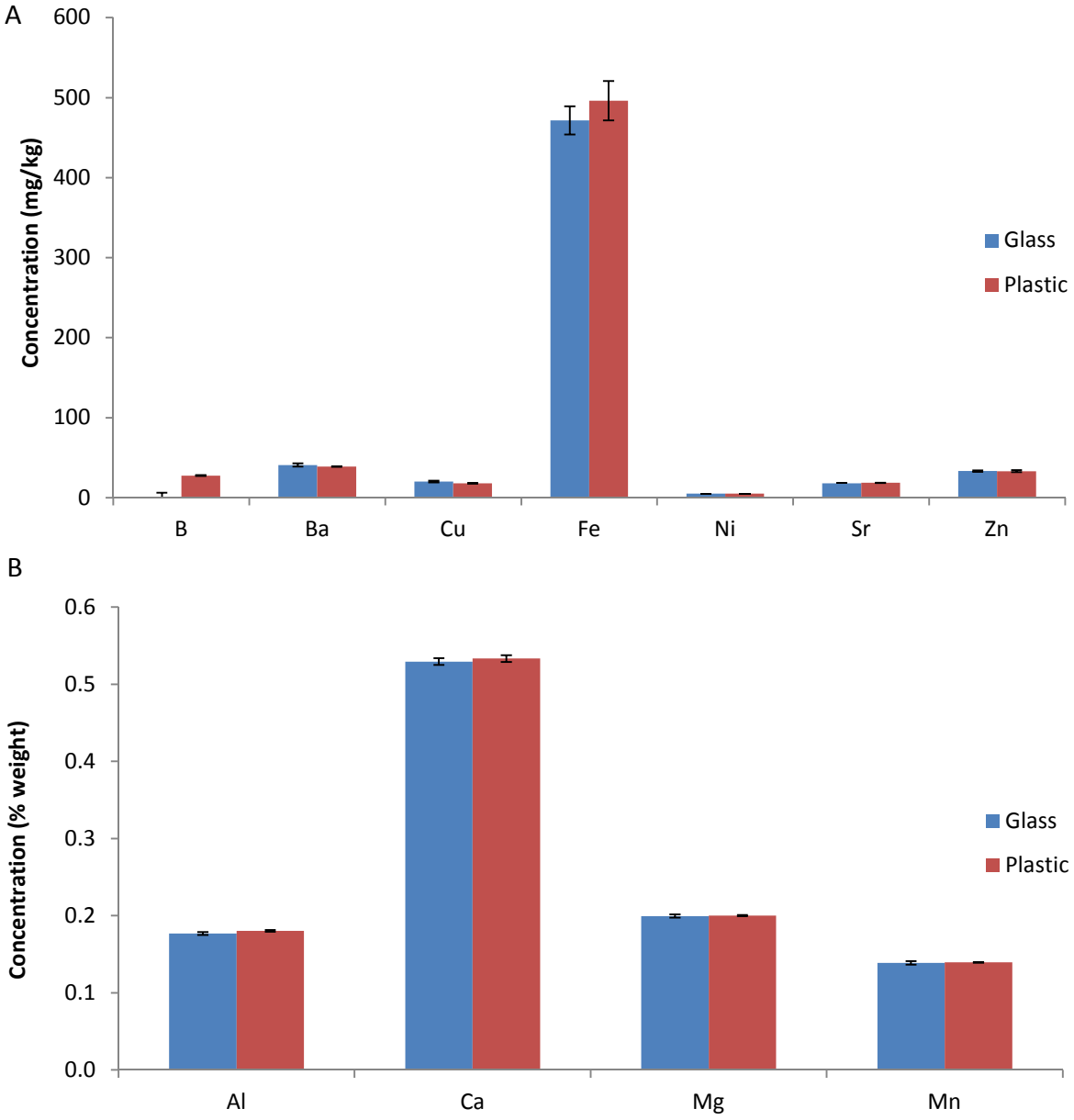


Figure 2.3. (A) Comparison of elements (mg/kg) between glass and plastic volumetric container (B) comparison of elements (% weight) between glass and plastic volumetric container (±1SD).

Experiments have been carried out on a 400 W power setting and a comparison to the 1600 W power was investigated to see if this aided digestion. Results of the NIST tea digested at the two power settings (Table 2.9) show that overall there is no statistical difference between the power settings with six of the elements (Al, Ca, Cu, Mn, Ni and Zn). This may be due to the microwave monitoring the temperature of the samples. Once the solutions reach the required temperatures, the computer system in the microwave automatically would reduce the percentage of the power used (i.e., from 100% 400 W to 60% 400 W). However, a significant difference was seen with the 1600 W setting for B, Ba, Fe, Mg and Sr. For these elements, this difference may be due to the

samples reaching the higher temperature quicker during the ramping stages with 1600 W in comparison to the 400 W setting.

Table 2.9. Comparison of microwave power settings with NIST tea

		Experimental values[1]	
Element	**Certified value**	**Power: 400 W**	**Power: 1600 W**
Al	0.229 ± 0.028 wt%	0.193 ± 0.009	0.192 ± 0.001
B	26 mg/kg	21 ± 1	25 ± 2*
Ba	43.2 ± 3.9 mg/kg	37.6 ± 0.3	40.2 ± 0.1*
Ca	0.582 ± 0.052 wt%	0.538 ± 0.002	0.534 ± 0.003
Cu	20.4 ± 1.5 mg/kg	20 ± 1	23 ± 3
Fe	432 mg/kg	380 ± 14	410 ± 10*
Mg	0.224 ± 0.017 wt%	0.2030 ± 0.0005	0.206 ± 0.002*
Mn	0.157 ± 0.011 wt%	0.145 ± 0.002	0.144 ± 0.001
Ni	6.12 ± 0.52 mg/kg	5.40 ± 0. 05	5.3 ± 0.1
Sr	20.8 ± 1.7 mg/kg	18.8 ± 0.1	19.14 ± 0.08*
Zn	34.7 ± 2.7 mg/kg	32.4 ± 0.3	32.1 ± 0.1

[1] units same as those for certified vales, ±1SD
*t test: significant at $p<0.05$

As SJW samples can contain tough parts of stalks within the sample compared to the NIST tea and because the majority of elements were recovered more efficiently with the higher power setting, the 1600 W power was utilised for further experiments.

2.3.4 Method Validation

2.3.4.1 NIST CRM and Spiked Recovery

Accuracy validation was carried out using NIST certified reference material (CRM) Polish tea (INCT-TL-1) and spike recovery methods. The NIST reference was certified for Al, B, Ba, Ca, Cu, Fe, Mg, Mn, Ni, Sr and Zn. To validate the remaining metals or those below detection limits, the NIST reference was artificially enriched with As, Be, Cd, Co, Cr, Hg, In, Mo, Pb, Pt, Sb, Se, V and Y prior to acid digestion. The results (Table 2.10) show that elements As, Cd, Co, Cr, Cu, Hg, Mn, Mo, Pt, Se, V and Y had a recovery greater than 95%, elements B, Ba, Be, Ca, Fe, In, Mg, Pb, Sb, Sr and Zn had recoveries greater than 90%, elements Al and Ni had recoveries greater than 84%. All elements have good recoveries and are compliant with recommended recoveries of ± 20% for elemental analysis [36]

Table 2.10. Recovery of elements with NIST tea and spiked recovery

Element	Certified value or spike amount	Experimental value[1]	Recovery %
Al	0.229 ± 0.028 wt%	0.192 ± 0.001	83.8 ± 0.4
As	Spiked with 0.5 ppm	0.506 ± 0.003	101.1 ± 0.6
B	26 mg/kg	25 ± 2	95 ± 7
Ba	43.2 ± 3.9 mg/kg	40.2 ± 0.1	93.1 ± 0.3
Be	Spiked with 0.5 ppm	0.464 ± 0.003	92.8 ± 0.7
Ca	0.582 ± 0.052 wt%	0.534 ± 0.003	91.8 ± 0.6
Cd	Spiked with 0.5 ppm	0.480 ± 0.003	95.9 ± 0.7
Co	Spiked with 0.5 ppm	0.482 ± 0.003	96.4 ± 0.6
Cr	Spiked with 0.5 ppm	0.496 ± 0.003	99.2 ± 0.6
Cu	20.4 ± 1.5 mg/kg	23 ± 3	110 ± 10
Fe	432 mg/kg	410 ± 10	96 ± 3
Hg	Spiked with 0.5 ppm	0.49 ± 0.01	98 ± 2
In	Spiked with 0.5 ppm	0.460 ± 0.003	91.9 ± 0.5
Mg	0.224 ± 0.017 wt%	0.428 ± 0.003	85.7 ± 0.6
Mn	0.157 ± 0.011 wt%	0.206 ± 0.002	91.8 ± 0.8
Mo	Spiked with 0.5 ppm	0.144 ± 0.001	91.5 ± 0.6
Ni	6.12 ± 0.52 mg/kg	0.489 ± 0.004	97.8 ± 0.8
Pb	Spiked with 0.5 ppm	5.3 ± 0.1	87 ± 2
Pt	Spiked with 0.5 ppm	0.466 ± 0.003	93.3 ± 0.6
Sb	Spiked with 0.5 ppm	0.53 ± 0.01	106 ± 3
Se	Spiked with 0.5 ppm	0.472 ± 0.001	94.5 ± 0.3
Sr	20.8 ± 1.7 mg/kg	0.52 ± 0.02	103 ± 4
V	Spiked with 0.5 ppm	19.14 ± 0.08	92.0 ± 0.4
Y	Spiked with 0.5 ppm	0.495 ± 0.003	99.1 ± 0.6
Zn	34.7 ± 2.7 mg/kg	0.490 ± 0.004	98.0 ± 0.7

[1] Unit same as certified or spiked unit. (±1SD)

2.3.4.2 Standard Addition

Matrix effects of different preparations were evaluated using standard additions with a SJW raw herb, tablet and capsule preparation. The samples were artificially enriched with each element at concentrations equal to or greater than five times the sample concentration [170]. The results (Table 2.11) show that for the SJW raw herb, capsule and tablet, the weighed calibration results agree within, on average, 13%, 20% and 22% respectively of the standard addition results. Studies that have analysed SJW samples using ICP-OES do not use standard addition [125, 126, 139, 142] as the method of calibration and do not mention if the calibration used is weighted or non-weighted.

Table 2.11. SJW metal concentrations obtained using standard addition *vs.* weighted calibration

	Standard addition			Weighted calibration		
	Herb	Capsule	Tablet	Herb	Capsule	Tablet
Element	(μg/g ± 1SD)	(μg/g ± 1SD)	(μg/g ±1SD)	(μg/g ±1SD)	(μg/g ± 1SD)	(μg/g ±1SD)
Al	188 ± 2	61 ± 6	28.5 ± 0.4	170 ± 30	51 ± 1	24.4 ± 0.4
B	35.4 ± 0.3	22 ± 2	23.1 ± 0.4	29 ± 1	17 ± 1	15.6 ± 0.5
Ba	11.0 ± 0.1	0.62 ± 0.06	0.91 ± 0.01	9.6 ± 0.4	0.44 ± 0.05	0.78 ± 0.09
Ca	6190 ± 60	94000 ± 8000	-	6000 ± 100	87000 ± 2000	-
Cd	1.11 ± 0.01	0.17 ± 0.02	0.095 ± 0.001	0.95 ± 0.02	0.11 ± 0.01	0.05 ± 0.01
Cr	0.55 ± 0.01	3.0 ± 0.3	0.476 ± 0.007	0.43 ± 0.05	2.48 ± 0.02	0.25 ± 0.05
Cu	7.65 ± 0.07	16 ± 1	9.66 ± 0.06	6.9 ± 0.4	14.6 ± 0.6	10.4 ± 0.2
Fe	168 ± 2	91 ± 8	-	160 ± 30	78 ± 1	-
Mg	1730 ± 20	93 ± 8	-	1510 ± 70	60 ± 10	-
Mn	124 ± 1	12 ± 1	16.0 ± 0.2	115 ± 3	10.9 ± 0.2	15.4 ± 0.2
Ni	1.66 ± 0.02	2.2 ± 0.2	1.84 ± 0.03	1.30 ± 0.04	1.65 ± 0.09	1.50 ± 0.02
Sr	21.7 ± 0.2	28 ± 2	6.5 ± 0.1	17.80 ± 0.04	21.8 ± 0.5	5.64 ± 0.04
Zn	31.1 ± 0.3	44 ± 4	31.5 ± 0.5	25.0 ± 0.6	35 ± 1	25.1 ± 0.6

Note: ±1SD = 1 Standard Deviation

2.4 Conclusions

The analysis of several acid mixtures showed that 5 ml HNO_3 was the better of the five mixtures investigated through transfer and recovery studies. Although some elements were recovered in greater quantity with acid mixture 3, this mixture contained HF which was able to digest silicates in the samples. However, as a result, the HF also causes leaching from the glassware used within the preparation and thus potentially damaging to the glassware utilised in the ICP-OES. The microwave power was also investigated and was found that 1600 W was slightly better with some element recovery. Therefore, the 5 ml HNO_3 at a 1600 W setting was chosen to undergo further validation studies. Element recovery using NIST polish tea and spiked recovery studies showed that the method achieved recovery of ≥ 90% for 22 of the elements and ≥84% for all 25 elements. Therefore a simple and optimised method was developed in order to collect the elemental profiles of SJW preparations to assess their use as a tool for quality control.

3 Elemental Analysis of St John's Wort Preparations

3.1 Introduction

Herbal medicines are chemically complex and in many cases the pharmacological effect is a result of interactions between multiple chemical constituents. In the last decade herbal medicine regulation has changed dramatically [42, 173, 174] improving many aspects of quality control; yet challenges still remain to reduce differences between products sold of the same medicinal herb ensuring similar therapeutic effects.

One area that has received limited attention is the monitoring of elemental species for the quality control of herbal products. These products are often standardised according to key molecular constituents, yet herbs also contain a diverse range of essential and non-essential elements. Many herbal plants of medicinal interest are known accumulators or hyper-accumulators of metals [59, 66, 155, 175-177], meaning they actively uptake and accumulate certain metals in high concentrations in comparison to the concentration in the surrounding growth medium. Elements can also be added or removed *via* processing and formulating the raw herb into commercial products. In recent years, much attention has been paid to the presence of toxic elements such as As, Cd, Hg and Pb in herbal products due to the adverse effects they can cause [21, 26]. Consequently, manufacturers are recommended to ensure "heavy metals" fall within recommended limits [41, 178]. The remaining elemental composition, however, is potentially overlooked and underutilised. Firstly, the essential element composition has inherent nutritional value. The form of the metal found in herbs is often more bioavailable than metal salts used in supplements; thus, the accumulation properties of herbs could be exploited to provide key sources of elements. For example, herbs that accumulate Se or Mn could improve deficiencies in these elements. Deficiencies in these elements have been linked to cardiovascular disease [179, 180]. Secondly, elements play a key role in the expression of secondary metabolites, or bioactive plant compounds (BPC), such as polyphenols and flavonoids. Studies have shown that production of secondary metabolites could be tuned by elemental exposure [148, 149, 156]. Thirdly, many BPCs are natural metal chelators where these complexes have been shown to improve metal absorption [151] and alter BPC pharmacological activity [109, 150]. Lastly, the elements found in herbal medicines can potentially interact with drugs if taken simultaneously, altering their bioactivity. In summary, the metal accumulation properties of plants and the addition of elements during processing and formulation have a number of implications for health and product quality, which should be investigated.

One medicinal herb that is of particular interest due to its popularity and metal accumulation properties is *Hypericum perforatum,* otherwise known as St John's Wort (SJW). It is used in the treatment of mild to moderate depression [83] and is also noted for its anti-inflammatory and anti-bacterial effects [84]. The SJW constituents have shown a number of biological interactions in relation to depression. For example, MAO-inhibition (Monoamine oxidase) has been demonstrated for the flavonoids quercetin and luteolin, whereas amentoflavone have affinity for the δ-opioid receptor, hypericin has affinity for the δ-receptor and the reuptake of serotonin can be inhibited by hyperforin [105]. In 2008/2009, the UK spent £4 million on SJW products [85]. Currently, SJW is standardised in Europe according to three groups of pharmacologically active ingredients hypericins, hyperforin and flavonoids (e.g. rutin) [116]. An advantage to using elemental fingerprints for quality control is the greater stability of metals in comparison to molecular constituents, which are subject to oxidation and photo-degradation over time. A number of studies [118-145] have investigated the elemental content of SJW raw plant and preparations in which concentrations of Cd, Cu, Fe, Mn, Pb and Zn are usually found in ranges of 0.04-20 μg/g, 4-200 μg/g, 6-1300 μg/g, 8-450 μg/g, ≤0.1-20 μg/g and 10-200 μg/g, respectively. Other studies have looked at elements such as Al, As, B, Ba, Ca, Co, Cr, Hg, Mg, Mo, Ni, Sb, Sn, Sr and V [119, 125, 126, 129, 131, 142, 145]. Also, the addition of Ni and Cr in the growth medium has been shown to affect the production of BPCs in SJW. For example, a 15-20 fold decrease in the production of hypericin and pseudohypericin was observed when SJW was exposed to 50 mM Ni [149]. On the other hand, SJW exposed to 0.1 mM Cr showed increased production of protopseudohypericin (+167%), hypericin (+25%) and pseudohypericin (+5%) compared to untreated SJW [148]. A number of BPCs found in SJW, such as rutin, quercetin, and hypericin, have also been shown to complex metals *ex-situ* and in some cases affecting the bioactivity [109, 150, 151, 181, 182]. The extent to which this happens in SJW is largely unknown. Although some studies have investigated the elemental content of SJW, they are often limited by the number of elements and/or breadth of samples investigated. A gap remains on how the elemental profiles can be fully utilized, therefore, before the elemental profile of SJW can be exploited further, 'normal' concentration ranges for a selection of elements needs to be determined for both the herb and preparations and this information further interpreted.

Chemometric approaches have been extremely powerful for the interpretation of multidimensional data, as metal profiles of plant material has allowed the differentiation of species [77-79], manufacturer [80, 81] and origin [80-82]. Two studies [150, 155] were outlined in section 1.7.5 regarding SJW and multivariate analysis. In order to determine the feasibility of using elemental profiles for SJW as a mean for quality control, a large number of elements and samples from a large

geographic area must be investigated to establish a typical range and potential variability for the elements monitored.

In this study, the elemental profile was obtained for 54 SJW products including dry herb (n=22), tablets (n=20) and capsules (n=12). Twenty-five elements (i.e., Al, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Hg, In, Mg, Mn, Mo, Ni, Pb, Pt, Sb, Se, Sr, V, Y and Zn) were monitored using ICP-OES. The elemental profiles were also subjected to PCA to identify underlying patterns from the multivariate data. Following this the PCA model was optimised and examined for robustness.

3.2 Method

3.2.1 Materials

A variety of SJW dry herbs, tablets and capsules were purchased through high street retailers and Internet sources. A summary of all samples is shown in (Table 3.1). All labware was acid washed overnight with 4M Nitric acid and rinsed thoroughly with deionised water before use. High-purity HNO_3 70% (99.99% trace metal basis) (Sigma-Aldrich, Gillingham, UK) was used for microwave digestion and preparation of 2% HNO_3 solutions. Elemental stock solutions (1000 ppm) of Al, As, B, Ba, Cd, Co, Pb, Mg, Mn, Mo, Ni, In and Hg (Fisher, Loughborough, UK); Be and Pt (VWR, Lutterworth, UK); Ca, Cr, Cu, Fe, Sb, Se, Sr and Zn (Merck, Feltham, UK); V (Sigma-Aldrich, Gillingham, UK); and Y (Acros organics, Geel, Belgium) were used to prepare calibration standards.

3.2.2 Inductively Coupled Plasma – Optical Emission Spectroscopy Analysis

Elemental analysis was carried out using a 710 ICP-OES (Varian, Mulgrave, Australia) axial spectrometer fitted with a SeaSpray nebuliser and SPS3 autosampler. The wavelengths for each element as well as the instrument parameters used are summarized in Chapter 2. Limits of quantification were calculated (LOQ = standard deviation of the blank x 10) for each wavelength by analysis of a 2% HNO_3 blank (n=40) on three separate days [161] (please see Chapter 2). The calibration standards for the majority of elements were 1, 0.5, 0.1, 0.05, 0.025 and 0.01 ppm with the exception of Al (20, 10, 5, 1, 0.5, 0.25 and 0.1 ppm), Ca (50, 20, 10, 2, 1, 0.5 and 0.2 ppm), Fe (5, 2, 1, 0.2, 0.1, 0.05 and 0.02 ppm) Mg (15, 10, 5, 1, 0.5, 0.25 and 0.1 ppm) and Mn (2, 1, 0.2, 0.1, 0.05 and 0.02 ppm). Concentrations were calculated using a weighted regression.

3.2.3 Sample Preparation

Dry herb samples were ground using a Precelly's homogeniser (Bertin Technologies, Aix-en-Provence, France). The contents of capsule samples were removed from the capsule case; tablet samples were ground using an agate pestle and mortar then sieved (1 mm mesh) to remove any outer coating. The samples were dried (40^{o}C) overnight in an oven (8000 psi) and then stored in desiccators at room temperature before analysis. The sample (0.4 g) was weighed by difference and digested with 5 ml high purity nitric acid *via* a CEM MARS Xpress microwave at 1600W. The samples were then diluted 10:1 with deionised water, centrifuged for 45 minutes at 9000 RPM and filtered using 0.22 µm syringe filter (Millipore, Watford, UK) prior to analysis. All samples were prepared in triplicate.

Table 3.1. Summary of SJW samples

Sample[1]	Amount of extract (mg)[2]	Amount of ground herb (mg)	Amount of extract per tablet/capsule (%)[3]	Country of origin	Ingredients (in addition to *Hypericum perforatum* plant or extract)
H1	-	whole	-	Poland	-
H2	-	whole	-	Poland	-
H3	-	whole	-	Poland	-
H4	-	whole	-	Poland	-
H5	-	whole	-	Poland	-
H6	-	whole	-	UK	-
H7	-	whole	-	Hungary	-
H8	-	whole	-	Belgium	-
H9	-	whole	-	Chile	-
H10	-	whole	-	Hungary	-
H11	-	whole	-	Hungary	-
H12	-	whole	-	Albania	-
H13	-	whole	-	Eastern Europe	-
H14	-	whole	-	Hungary	-
H15	-	whole	-	Bulgaria	-
H16	-	whole	-	Poland	-
H17	-	whole	-	Spain	-
H18	-	whole	-	Poland	-
H19	-	whole	-	Poland	-
H20	-	whole	-	UK	-
H21	-	whole	-	Bulgaria	-
H22	-	whole	-	Bulgaria	-
T1	300	-	30	Europe and USA	Unavailable
T2	300	-	30	Europe and USA	Unavailable
T3	425	-	40	Europe, North and South America	Coating: hypromellose, sucrose, talc, calcium carbonate E170, tragacanth, acacia, liquid glucose (dry substance), titanium dioxide E171, iron oxide hydrate E172 (yellow iron oxide), vanillin, beeswax white, carnauba wax, shellac. Tablet core: Maltodextrin, silica colloidal anhydrous, microcrystalline cellulose, croscarmellose sodium, sodium starch glycolate (Type A), magnesium stearate.
T4	300	-	39	China	Contains: , lactose, talc, sucrose, calcium carbonate, cellulose, acacia, titanium dioxide, silicon dioxide, shellac, kaolin, magnesium stearate, iron oxide, polyoxyethylene sorbitan monooleate, beeswax, carnauba wax.
T5	17	-	6	China	Contains: calcium carbonate, microcrystalline cellulose, stearic acid, maltodextrin, magnesium stearate, silicon dioxide.
T6	-	330	0	Poland	potato starch, silicon dioxide (E551)
T7	40-73	-	14 - 26	Switzerland	Contains: microcrystalline cellulose, maise starch, soya polysaccharide, hydrogenated cottonseed oil.
T8	425	-	40	Europe, North and South America	Coating: hypromellose, sucrose, talc, calcium carbonate E170, tragacanth, acacia, liquid glucose (dry substance), titanium dioxide E171, iron oxide hydrate E172 (yellow iron oxide), vanillin, beeswax white, carnauba wax, shellac. Tablet core: Maltodextrin, silica colloidal anhydrous, microcrystalline cellulose, croscarmellose sodium, sodium starch glycolate (Type A), magnesium stearate.

T9	340	-	41	Sothern Europe	Contains: di calcium phosphate, cellulose, croscarmellose sodium, Hydroxypropylmethylcellulose, silicon dioxide, steric acid, titanium dioxide, magnesium stearate, glycerin, iron oxides.
T10	340	-	68	China	Contains: microcrystalline cellulose, hypromellose, magnesium stearate, titanium dioxide, silicon dioxide, stearic acid, crosamellose, sodium, talc, yellow iron oxide, glycerol, carnauba wax.
T11	300	-	32	China	Contains: calcium carbonate, microcrystalline cellulose, maltodextrin, magnesium stearate, hydroxypropylmethylcellulose, silicon dioxide, glycerol.
T12	500	-	57	China, France and Italy	Contains: di calcium phosphate, microcrystalline cellulose, sodium carboxymethylcellulose, hydroxypropylmethylcellulose, black iron oxide, red iron oxide, yellow iron oxide, titanium dioxide, magnesium stearate, silicon dioxide.
T13	340	-	40	Europe and USA	Contains: di calcium phosphate, cellulose, croscarmellose sodium, Hydroxypropylmethylcellulose, silicon dioxide, steric acid, titanium dioxide, magnesium stearate, glycerin, iron oxides.
T14	170	-	41	UK	Contains: cellulose, maltodextrin, croscarmellose sodium, hypromellose, silicon dioxide, magnesium stearate, stearic acid, talc.
T15	340	-	41	UK	Contains: di calcium phosphate, cellulose, croscarmellose sodium, hypromellose, maltodextrin, silicon dioxide, magnesium stearate, steric acid, colours (titanium dioxide, iron oxide), talc.
T16	170	-	34	Albania and Morocco	Contains: di calcium phosphate, cellulose, croscarmellose sodium, hypromellose, maltodextrin, silicon dioxide, magnesium stearate, steric acid, titanium dioxide, glycerol, yellow iron oxide, talc, carnauba wax.
T17	340	-	45	China	Contains: di calcium phosphate, microcrystalline cellulose, maltodextrin snowflake, stearic acid, silica, magnesium stearate.
T18	334	-	40	Chile	Contains: Dicalcium Phosphate, Microcrystalline Cellulose, Croscarmellose Sodium, Silicon Dioxide, Magnesium Stearate, Stearic Acid Tablet coating: Hypromellose, Iron Oxide Yellow (E172), Titanium Dioxide (E171), Purified Talc.
T19	300	-	99	USA	Contains: di calcium phosphate, cellulose, steric acid, silicon dioxide.
T20	400	100	41	China and Eastern Europe	Contains: acacia, microcrystalline cellulose, stearic acid, magnesium stearate.
C1	-	300	0	Eastern Europe and the Balkans	Pure powdered herb placed in capsule casing
C2	-	350	0	Bulgaria	Pure powdered herb placed in capsule casing
C3	150	-	46	Chile	Contains: microcrystalline cellulose, silicon dioxide, magnesium stearate, stearic acid, maltodextrin.
C4	150	-	40	France and China	Contains: maltodextrin, magnesium silicate, magnesium stearate, silicon dioxide.
C5	300	-	53	France and China	Contains: di calcium phosphate, magnesium stearate, silicon dioxide, stearic acid.
C6	300	-	50	China	Contains: rice starch, magnesium stearate, silica.
C7	300	100	65	USA	Contains: magnesium stearate, beta carotene, ascorbic acid.
C8	150	-	82	France & China	Contains: maltodextrin, magnesium silicate, magnesium stearate, silica.
C9	300	-	53	Poland and Albania	Contains: rice starch, magnesium stearate, silica.
C10	-	500	0	Unknown	Contains: maltodextrin, magnesium stearate.
C11	300	-	66	Unknown	Unavailable
C12	140	-	37	Unknown	Contains: maltodextrin, silica, hydroxypropylmethylcellulose, magnesium stearate.

[1] H = dry herb, T = tablet, C = capsule.

[2] Amounts given are per 1 tablet or capsule.

[3] Percent weight of extract in each singular tablet/capsule (based on average weight).

3.2.4 Statistical Analysis

Correlation analysis was carried out on the elemental profile of dry SJW using Excel 2007 (Microsoft) to see if relationships exist between the elements. Principal component analysis (PCA) was carried out using the *Unscrambler* X (CAMO) software. Elements with concentration values below the LOQ were removed from the dataset and remaining values were ratio normalised prior to analysis. All of the data associated with a given element across all samples was concatenated to give a single point on the loadings plot. This gave 16 descriptors in total. The relative position (x-coordinate) of each descriptor on the first principal component was established. Points with similar values on PC1 are indicative of two elements explaining the total variance of the dataset in the same manner, the inference being that this is an over-representation of information in the dataset. Having identified the two descriptors with the most similar values on PC1, an analysis of the magnitude of their y-coordinate values on the loadings plot i.e. their contribution to explaining the variance in the dataset according to the PC2 vector was carried out. The individual descriptor in the pair which had the y-coordinate value closest to zero was removed from the dataset. The PCA analysis was then regenerated using the reduced dataset. This process was repeated until the percentage variance being explained by the first two components remained constant (i.e. the maximum amount of noise had been removed from the dataset). A qualitative appraisal of the scores plot associated with the PCA was also carried out after each reiteration to ensure that removal of elemental data did not have a deleterious effect on the separation of dry herbs *vs.* formulated products on the scores plot.

3.3 Results and Discussion

3.3.1 Elemental Analysis of SJW Samples

The concentrations of 25 elements were determined for 54 SJW samples including dry herbs, tablets and capsules (Table 3.2). For all three types of SJW, sixteen elements (i.e. Al, B, Ba, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Pt, Sr, Y and Zn) had concentrations above the calculated Limits of Quantification (LOQ), whereas four elements were below the LOQs (i.e. Be, Co, Pb and V) and five elements below Limits of Detection (LOD, i.e. As, Hg, In, Sb and Se). The levels of Cd, Cr and Pb were below recommended daily intake values [71, 146, 183].

The elements found in highest concentrations (38 – 4870 μg/g) for the dry herbs (Table 10.6 and Table 10.7, Appendix 10.3) were in the order Ca>Mg>Fe>Mn>Al>Zn. For Ca, obtained results (2611-9533 μg/g) are higher in concentration (up to 10 times) in comparison to previous SJW studies of dry herb [125, 128, 145]. Calcium is present in large concentrations in plant cells [39] and Ca ions are used in numerous plant functions including alleviation of toxic metal effects [55, 56]. The results for Mg (789-1869 μg/g), Fe (38-756 μg/g), Mn (59-261 μg/g), Al (20-373 μg/g), and Zn (23-64 μg/g) agreed with previous studies [118, 120, 121, 125-127, 129, 130, 136, 142, 145]. Magnesium is also an essential element for plants as it activates many enzymes and is also a constituent of chlorophyll [39]. Iron is used in the production of chlorophyll and aids enzyme systems in plants [39]. Aluminium function in plants is unclear [39], although it was shown that low concentrations can have a beneficial effect on growth [54]. Aluminium is usually noted for its toxicity to plants with the most recognized effect being the reduction of root growth [39, 55, 184]. Zinc is an essential component of many proteins in plants [185]. Elements found in concentrations between 13 and 28 μg/g were in the order B>Sr>Cu>Ba. Strontium uptake in plants is influenced by the Ca content of the soil [186] and is an essential element for higher plants [47]. Levels of Sr in dry herbs found, agree with other studies [129, 144]. Copper is utilised for plant processes such as photosynthesis, protein metabolism, and respiration [39]. Elements found in the lowest concentrations (0.01 – 2 μg/g) were in the order Y<Mo<Cr<Cd<Pt<Ni. Chromium may be an essential element in plant growth [39] and has been shown to increase production of bioactive constituents in SJW [148]. Cadmium is not essential for plants [39, 187] and usually low levels are integrated into plant material from water uptake and growth medium.

Some plants however, including SJW, are Cd accumulators [66, 188]. Cadmium concentrations found during this investigation agree with previous studies [120, 121, 123, 126, 135, 139, 140]. As far as we are aware, this is the first time Pt has been reported in *Hypericum perforatum*. Platinum is used in alloys for machinery [189], which may be a potential source of contamination.

Table 3.2 Summary of Concentrations found in SJW preparations[1]

Element	Raw herb (µg/g) (n=22)	Capsule (µg/g) (n=12)	Tablet (µg/g) (n=20)
Al	101 (20 – 373)	76 (4 – 399)	85 (BLQ – 858)
As	ND	ND	ND
B	28 (16 – 47)	19 (ND – 42)	14 (BLQ – 40)
Ba	13 (3 – 22)	5 (0.3 – 17)	2 (0.5 – 6)
Be	ND	ND	BLQ
Ca	4 870 (2 611 – 9 533)	9 690 (406 – 93 124)	69 113 (299 – 199 067)
Cd	0.8 (BLQ – 1.7)	0.4 (ND – 1.8)	0.07 (ND – 0.49)
Co	BLQ	BLQ	BLQ
Cr	0.3 (ND – 1.4)	0.9 (ND – 2.4)	2 (ND – 5)
Cu	14 (5 – 117)	19 (9 – 83)	8 (BLQ – 20)
Fe	145 (38 – 756)	173 (18 – 747)	174 (1 – 628)
Hg	ND	ND	ND
In	ND	ND	ND
Mg	1 473 (790 – 1 870)	1 400 (949 – 2 334)	1 729 (406 – 3 527)
Mn	113 (59 – 261)	53 (4 – 240)	18 (2 – 85)
Mo	0.5 (ND – 1.5)	BLQ	BLQ
Ni	2 (ND – 5)	2 (BLQ – 3)	1 (ND – 3)
Pb	BLQ	BLQ	ND
Pt	1.4 (ND – 17.1)	3 (ND – 19)	3 (ND – 15)
Sb	ND	ND	ND
Se	ND	ND	ND
Sr	15 (9 – 30)	7 (1 – 21)	22 (1 – 84)
V	BLQ	BLQ	BLQ
Y	0.01 (ND – 0.3)	0.05 (ND – 0.3)	0.3 (ND – 0.9)
Zn	38 (23 – 64)	40 (17 – 60)	27 (7 – 57)

[1] Average value (range low – high), ND = not detected, BLQ = below limits of quantification.

Products of SJW, capsule (Table 10.8, Appendix 10.3) and tablet forms (Table 10.9 and Table 10.10, Appendix 10.3), were also analysed to establish notable changes to the elemental profile as a result of processing and formulation. The elements found in highest concentrations (40 – 9 690 µg/g) for the capsules (Table 3.2) were in the order of Ca>Mg>Fe>Al>Mn>Zn. For the tablet forms, the elements found (Table 3.2) in highest

concentrations (22 – 69 113 μg/g) were in the order of Ca>Mg>Fe>Al>Zn>Sr. A steady increase in Ca was observed when comparing the dry herb (4 870 μg/g), capsule (9 690 μg/g) and tablet form (69 113 μg/g). The increase in Ca content for the capsules and tablets is likely due to the addition of excipients such as calcium carbonate and di-calcium phosphate as stated on their label claim, which are used as bulking agents. Values obtained for Ca are higher than those seen in previous studies for tablets/capsules [119, 125]. A small increase was observed for Mg when comparing the dry herb (1473 μg/g) and capsule (1400 μg/g) content to the tablet forms (1729 μg/g). The increase in Mg content for the tablets is likely to be due to excipient addition of magnesium stearate and magnesium silicate. The results agree with those found by Bu et al (2012) [119] for Mg in capsules. An increase in the average Fe concentration was also observed when comparing the dry herb (145 μg/g) to the formulated products (173 & 174 μg/g). Iron oxides are used as a colouring for tablet coatings, however in this study much care was taken to remove these. An increase of Fe could also be due to contamination through processing whereby Fe is a major component of stainless steel [190]. The levels of Fe in tablets agree with the range reported by Kalny *et al.* (2012) [131]. The elements found in midrange average concentrations were B, Cu, Sr, and Ba for capsules (5-19 μg/g); and Ba, Cu, B, and Mn for tablets (2 and 18 μg/g). The levels of Ba and Cu in the capsules and tablets agree with previous studies [119, 125, 131]. Elements found in the lowest average concentrations were Y, Cd, Cr, Ni, and Pt for capsules (0.05 – 3 μg/g); and Cd, Y, Pt, Ni, and Cr for tablets (0.05 – 2 μg/g). Levels of Cd, Cr and Ni in the capsules and tablets agree with previous studies [119, 126, 131]. Although the concentration of Ca and Mg increased in the formulated products, a number of elements (i.e. Al, B, Ba, Cd, and Mn) decreased in concentration from 25-75% of that found in the dry herb samples. For example, a steady decrease was observed for Mn when comparing the raw herb (113 μg/g), capsule (53 μg/g) and tablet forms (18 μg/g). Also, Ba decreased from an average concentration of 13 μg/g to 2-5 μg/g in the SJW formulated products. One element, Mo, was found in the dry herb samples above the LOQ but not in the capsules and tablets analysed in this study. These decreases could be due to a combination of two main factors. Firstly, a majority of the formulated products in this study contained the dry alcoholic extract of SJW and not the dry herb (Table 1) [113, 191]. The extraction process would only transfer those elements that are released from the bulk plant material and in a soluble form. The element concentration in the extract (μg/g) could potentially increase or

decrease depending on the extraction efficiency and the amount of extract recovered. Secondly, the addition of excipients when formulating would act as a diluent and further decrease the elemental concentration. Thus, an element could be concentrated *via* the extraction process, but then diluted by the addition of excipient and have similar concentrations in herb and tablet form.

3.3.2 Application of Statistics to SJW Elemental Profiles

Correlation between the 16 elements (i.e. those above calculated LOQs) in SJW dry herbs was investigated to determine the relationship between elements. The correlation matrix (Pearson's) between the elements (Table 3.3) shows that there are several positive correlations between elements. A correlation between Ca and Sr was observed with a value of 0.6458 and this relationship is well documented [39] as Sr ions often replace some Ca ions. Therefore as the concentration of Ca increases, Sr will increase as well. Other correlations found (such as Al with Ca, Al with Fe, B with Mn as well as Ca with Cr) may be due to soil conditions. As soil becomes more acidic, more elements are taken up by a plant and other factors such as soil type, moisture content and the plants root surface properties can result in synergic (or antagonistic) relationships [40].

Table 3.3 Correlation matrix of elements monitored in SJW dry herbs [1, 2]

	Al	*B*	*Ba*	*Ca*	*Cd*	*Cr*	*Cu*	*Fe*	*Mg*	*Mn*	*Mo*	*Ni*	*Pt*	*Sr*	*Y*	*Zn*
Al	1															
B	**0.5806**	1														
Ba	-0.2167	-0.1877	1													
Ca	**0.8406**	**0.7668**	0.0312	1												
Cd	0.3915	0.4938	0.4935	**0.6711**	1											
Cr	**0.6530**	0.3624	0.0953	**0.6698**	**0.5388**	1										
Cu	0.1308	-0.0638	-0.1494	-0.0081	-0.1850	0.4135	1									
Fe	**0.9215**	**0.6380**	-0.1963	**0.7934**	0.3315	**0.6215**	0.1902	1								
Mg	0.4035	**0.5468**	-0.4930	0.2967	-0.2901	-0.1327	0.1385	0.4259	1							
Mn	0.3876	**0.6835**	0.1549	**0.5793**	**0.5962**	0.2370	-0.2280	0.4117	0.1076	1						
Mo	0.0600	-0.2139	-0.2712	-0.0837	-0.4132	0.1414	**0.5751**	0.0859	0.2192	-0.3522	1					
Ni	**0.5706**	0.4383	0.3453	**0.6237**	**0.6984**	**0.6829**	-0.0276	**0.5303**	-0.0855	0.3226	-0.2494	1				
Pt	**0.7535**	**0.5104**	-0.0491	**0.6509**	0.2758	0.4905	-0.0391	**0.9048**	0.2817	0.3546	-0.0862	**0.5009**	1			
Sr	**0.6348**	0.3299	0.3829	**0.6458**	**0.6717**	**0.6254**	-0.0495	0.4686	-0.0656	0.4234	-0.2172	**0.7340**	0.3839	1		
Y	**0.7262**	**0.5136**	-0.1245	**0.6511**	0.3244	0.3403	-0.0764	**0.7590**	0.3304	0.3186	-0.1238	0.3680	**0.7801**	0.3921	1	
Zn	**0.5396**	**0.6029**	0.0098	**0.6302**	0.2207	0.4477	0.0972	**0.6207**	0.4258	**0.5749**	-0.0162	0.3195	**0.6314**	0.4264	0.3806	1

[1]Correlations are noted in bold text

[2]Elements that were BLQ in all samples were removed (As, Be, Co, In, Hg, Pb, Sb, Se and V).

As correlations between elements were found in SJW, the elemental profiles of all 54 SJW samples were subjected to Principal Component Analysis (PCA) to establish any underlying patterns of the multi-dimensional dataset. Elements that had concentrations below the LOQs for all samples were removed from the dataset (e.g. As, Be, Co, In, Se, Sb, Pb, V and Hg). A PCA was carried out using the remaining 16 elements. The first two principal components accounted for 57% of the variance (Figure 3.1). A 95% confidence interval ellipse was also applied to the data set. Despite the samples being of a different form (raw herb, tablet or capsule) and from various geographic areas, 91% of the St John's wort samples were within the 95% confidence limit. A general trend observed was the separation of the raw herb samples from the processed samples. One tablet and three capsule samples (i.e., T6, C10, C1, and C2) grouped closely with the herb samples; these samples were observed to contain dry herb only (Table 3.1). As five samples fell outside the ellipse (i.e., H15, T5, T19, C1 and C2), they were treated as outliers to avoid skewing the model (i.e., samples that are outliers/very different from the others will cause the model to focus on this difference rather than the underlying patterns heeding investigation). These samples possessed higher concentrations of Al, B, Fe, Mn, Ni, Sr and/or Pt compared to the other samples. These five samples were removed from the dataset and the data was renormalized accordingly.

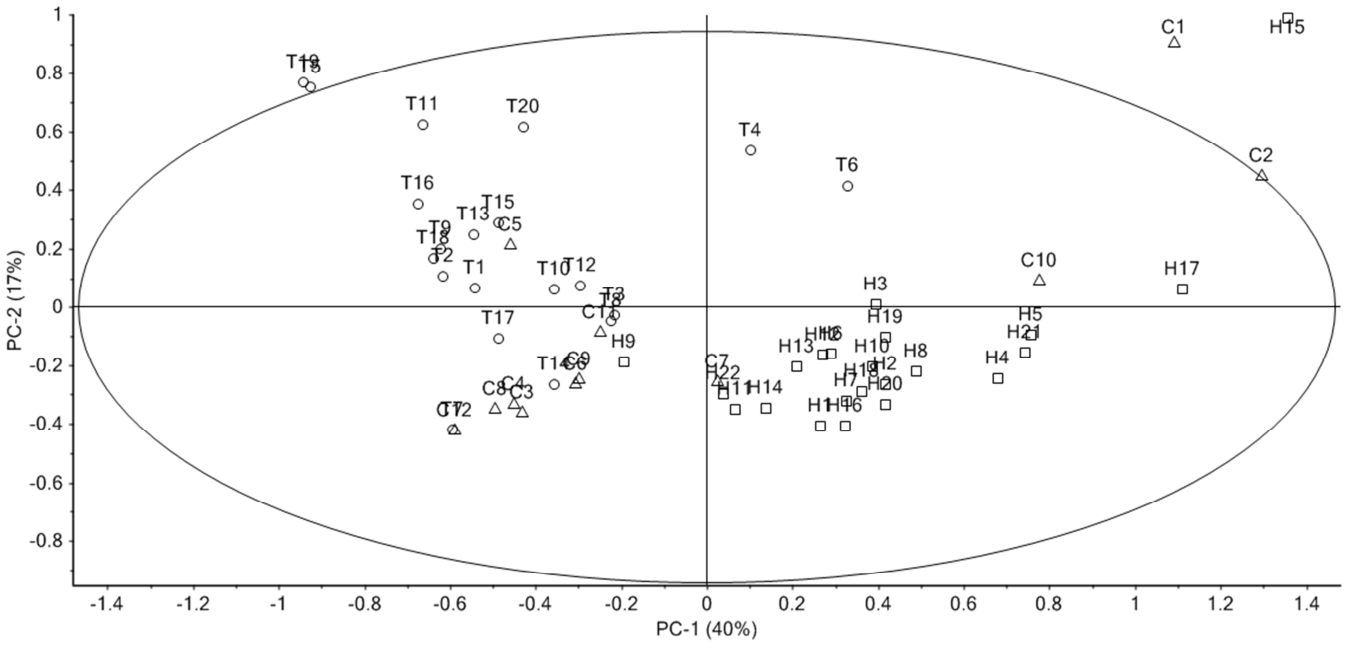
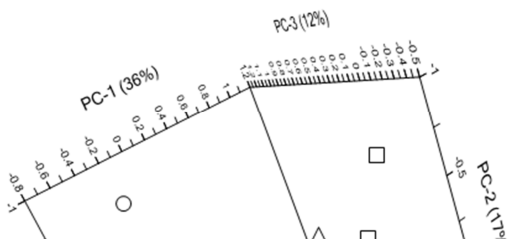


Figure 3.1 Two-dimensional PCA plot (PC1 *vs.* PC2) using 16 elements found in 54 SJW samples with a 95% confidence ellipse applied. The samples H15, T5, T19, C1 and C2 were outside the 95% confidence ellipse and considered outliers in comparison to the rest of the dataset.

A PCA was carried out on the remaining 49 SJW samples and the results are shown in Figure 3.2. A 3D plot using the first three principal components (Figure 3.2 B), which represented 65% total variance, shows delineation between the raw herb and formulated products and some general delineation between tablets and capsules with a small amount of overlap. The separation is primarily along principal component 1 (PC1) which has high positive loadings for B, Ba, Cd, Mn, Ni and Zn as well as high negative loadings for Ca, Cr and Y (Figure 3.2 A). This shows that the herb samples have higher values for B, Ba, Cd, Ni, Zn and Mn and lower values for Ca, Cr and Y in comparison to the processed samples. As mentioned previously, formulated products often contain excipients containing calcium such as calcium carbonate (or talc) and di-calcium phosphate, which may contribute to these differences. On principal component 2 (PC2) there is a positive loading for Al, Cu, Fe, Ni and Mo and a high negative loading for Ca, Cr, Y and Sr. The loadings on principal component three (PC3) included a high positive loading for Al, Fe, Mg, Ni and Pt. Those samples that were not clearly separated were investigated in more detail to determine the cause, if any, for their miss-grouping. Again, C10 and T6, which grouped closely to the raw herb samples, are composed of ground herb only and contained no extract or added excipients according to their label claim. Also, C7 was found to contain a mixture of ground herb and alcoholic extract, therefore explaining why this sample is positioned between the capsule and dry herb clusters. The capsule C5 is clustered closely with the tablets and its label claim indicates the presence of bulking agents more closely linked to those used for tablets as compared to the other capsules. Moreover, the levels of Ca, Mg and Sr are more comparable to those of the tablets than the other capsules.

A

B



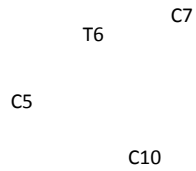


Figure 3.2. (A) 2D loading plot of PC1 & PC2 and (B) 3D plot of PC1, PC2 & PC3 using 16 elements from 49 SJW samples (squares = herbs, circles = tablets and triangles = capsules)

As there is clear delineation between the raw herb and formulated products, it was of interest to assess if excipients containing Ca and Mg were the main cause of delineation or if other factors influenced the separation. The elements Ca and Mg were removed from the dataset and using the remaining 14 elements (i.e. Al, B, Ba, Cd, Cr, Cu, Fe, Mn, Mo, Ni, Pt, Sr, Y and Zn) and 49 samples, a new PCA was constructed (Figure 3.3). The results show delineation is clear between the herb samples and the processed SJW but less delineation is seen between the tablets and capsules. Thus it appears the differentiation of the raw herbs from the processed samples can occur based on the other elements. The removal of Ca and Mg also indicated that these elements can vary between the tablet samples as without Ca and Mg the tablet samples are grouped closer together. From Table 3.1, the tablets have both Mg and Ca containing excipients, while the capsules have primarily Mg excipients. This is echoed in the loadings (

Figure 3.2 A), which show greater Ca loading for the tablets than for the capsules. The delineation that remains between the herb and formulated products may be influenced by other factors than elements introduced *via* excipients and that an underlying fingerprint from SJW can be monitored even when mixed with excipients to form a formulated product. It is also important to note, that

the two main explanations for the difference in the elemental fingerprints are 1) the use of extracts *vs.* raw herb and 2) the effect that would occur upon addition of the excipients. Most of the formulated samples contain methanol or ethanol extracts as the extraction process is used to concentrate bioactive metabolites from remaining plant material. As a result, there seems to be a significant change in the elemental profile that may be due to this extraction process [132, 133, 137, 143]. For example, Sulibursk a and Kaczmarek [143] prepared hot water infusions of herbs including SJW. Their study found that elements Zn, Cu, Mg and Ca had extraction efficiencies on average between 30.9-47.2% and Fe had 12.4% from the original herb. Konieczyński and Wesołowski [132] examined the water extractable Mg, Mn and Cu in herbal remedies including SJW. This study found that Mn extraction was very low compared to the original herb (<10%) whereas Mg and Cu had better extraction efficiencies (~40% and ~30% respectively). Another study by the same authors [133] examined Fe and Zn and found in the majority of samples <6% of Fe was extracted (8 samples were <6% and 3 samples were between 15-82%) and Zn was extracted by approximately 30%. Helmja *et al.*, [127] extracted SJW in ethanol as well as water and observed that the water extract, extracted between 10-25% Zn, Mn, Co, and Cr whereas the levels extracted by ethanol were 10-25 times lower. These studies indicate that the elements are extracted to different extents depending on the metal and also the solvent used. The PCA results suggest that by monitoring the elemental profile, not only can the quality be assessed based on the elemental composition of the product in comparison to other products, but this can also be used to decipher the processing, or lack of, that has been applied to the medicinal product. The herbal extracts are produced to concentrate and standardise certain compounds, i.e. hypericins and hyperforin in SJW, however deciphering whether the formulated products contains extract or dry herb is challenging as analysis by HPLC or MS would require an extraction step; which may defeat the original objective. The use of elemental profiling does not require this extraction step and would look at the total metal composition.

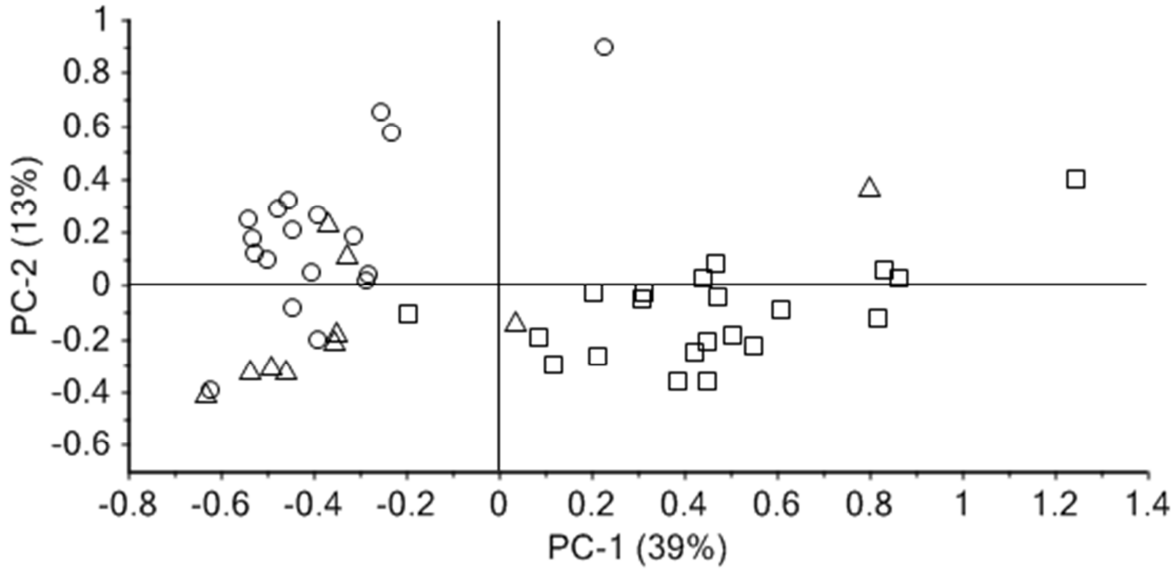


Figure 3.3. 2D PCA of 49 SJW samples with 14 elements. Squares = herbs, circles = tablets and triangles = capsules. Three PCs with 65% total variance.

If a method such as this is to be used for assessing the quality/form of SJW by the herbal industry or regulatory agencies, it is of interest to determine the least amount of elements needed for delineation (i.e., to reduce time and cost of analysis). To simplify the method and to eliminate redundancies, elements were removed logically from the PCA to determine the minimum number of elements needed for delineation of the samples. This may allow for a preliminary model to be developed which can be a template for the determination of quality of SJW forms using the elemental composition. Elements closely correlated with one another on PC1 were compared according to their loading values and the element that gave redundant information was removed. The numerical values were compared between elements, with those with the closest relationship examined further. The element found to have the least contribution to the PCA was removed from the data set (i.e. the lowest loading values across the first 3 PCAs with most importance being assigned to PC1 followed by PC2 then PC3) (Table 3.4). The initial PCA indicated that Al and Mo had the closest loading values (a difference of 0.0009) on PC1. Of these two elements, Mo had a higher loading on PC2, therefore Al was removed from the data set. Al was considered to be redundant information when compared to Mo. A new PCA was produced with the remaining elements and the same method was applied until factors such as no close correlation between two elements, a decrease in the total variance, reduced differentiation of samples or the data set is no longer orthogonal became apparent. In total 9 elements were removed from the PCA in the following order: Al, Mg, Cr, Pt, Mo, Cu, Mn, Zn and B. The values seen in Table 3.4 show the loading difference between closely related elements increased as elements were removed. For PCA 11, the

removal of Fe resulted in the groupings becoming more dispersed, thus this element was left in the dataset and no further elements were removed.

Table 3.4. Difference in loading values between elements correlated on PC1

PCA №	Elements correlated	Numerical difference	Element Removed
1	Al + Mo	0.0009	Al
2	Cu + Mg	0.0017	Mg
3	Cr + Y	0.0027	Cr
4	Cu + Pt	0.0114	Pt
5	Sr + Mo	0.0218	Mo
6	Cu + Sr	0.0370	Cu
7	Cd + Mn	0.0532	Mn
8	Ni + Zn	0.0772	Zn
9	B + Ni	0.0891	B
10	Fe + Sr	0.1005	-

The remaining 7 elements (i.e. Ba, Ca, Cd, Fe, Ni, Sr and Y) were then used to produce a new PCA (Figure 3.4) for the 49 samples. The exclusion of 9 elements reduced the associated noise such that the first three PCs represented 85% of the total variance whilst retaining delineation between the three sample types. In addition, the processed samples that contained raw herb (C10, C7 and T6) still grouped according to their composition. Thus, using 7 key elements, an indication of the sample composition (extract or raw herb) can be determined. More work is needed to investigate the changes in the elemental profile of SJW when extracts are produced.

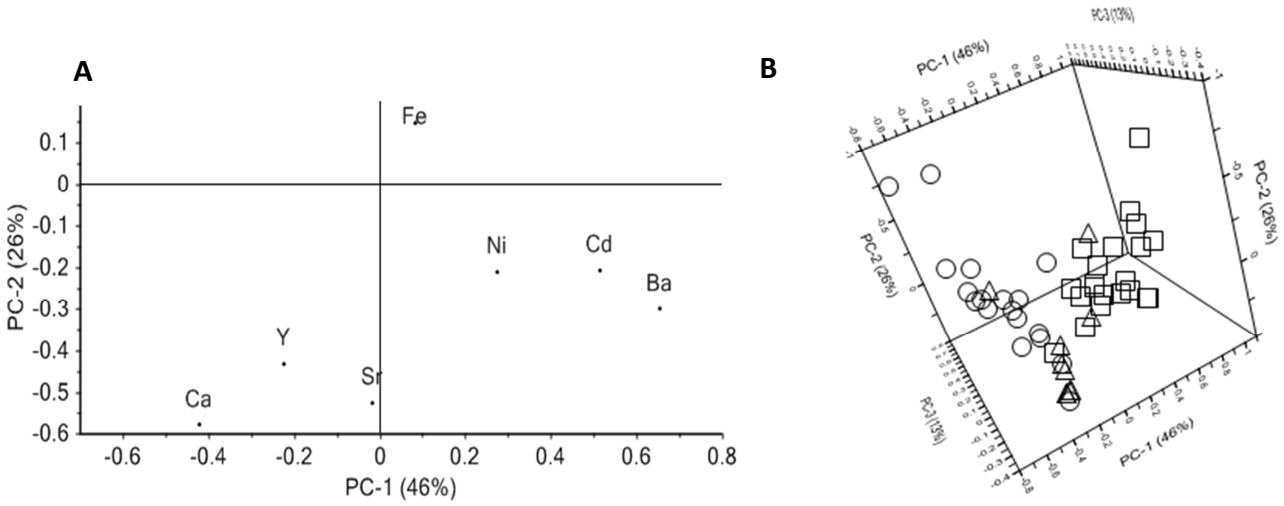


Figure 3.4. (A) 2D loading plot of PC1 & PC2 and (B) 3D plot of PC1, PC2 & PC3 using 7 elements from 50 SJW samples (squares = herbs, circles = tablets and triangles = capsules).

3.3.2.1 Investigation of the Robustness of the PCA Classification

It is important to ensure this PCA classification is robust and not greatly affected by any single sample. From the original PCA (Figure 3.1), sample H17 close to the 95% confidence ellipse limit and a higher loading on PC1 compared to T11 and T20. In order to see the influence of this sample, the PCA was processed without this sample. Following the sample process of examining the loadings for element correlations, a PCA was produced (Figure 3.5) from 48 samples and 10 elements (B, Ba, Ca, Cd, Mn, Mo, Ni, Sr, Y and Zn) with 77% total variance with the first three PCs. The figure shows delineation of the three types of sample as seen from Figure 3.4 when H17 was retained in the dataset. From comparing this PCA (48 samples) with the original optimised PCA (49 samples) we can see that elements Ba, Ca, Cd, Ni, Sr and Y are inherent for both PCAs. This shows that although a sample included in the original PCA could be considered as a near-outlier data point, there was no evidence of this point skewing the model indicating the robustness of the PCA constructed.

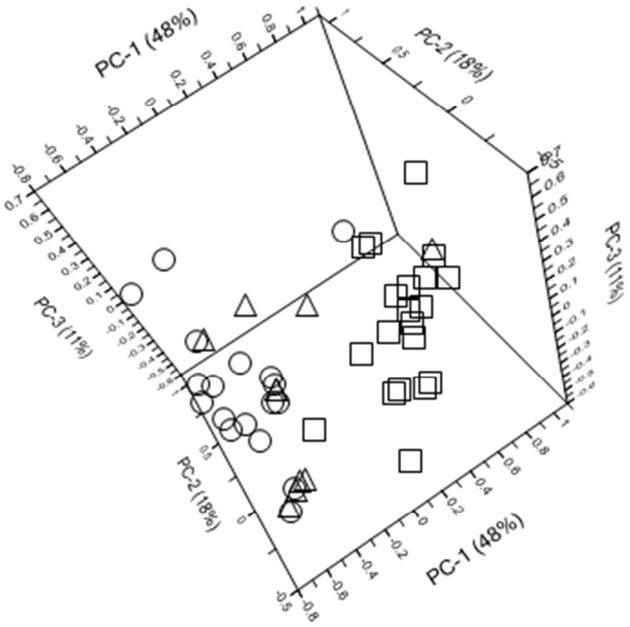


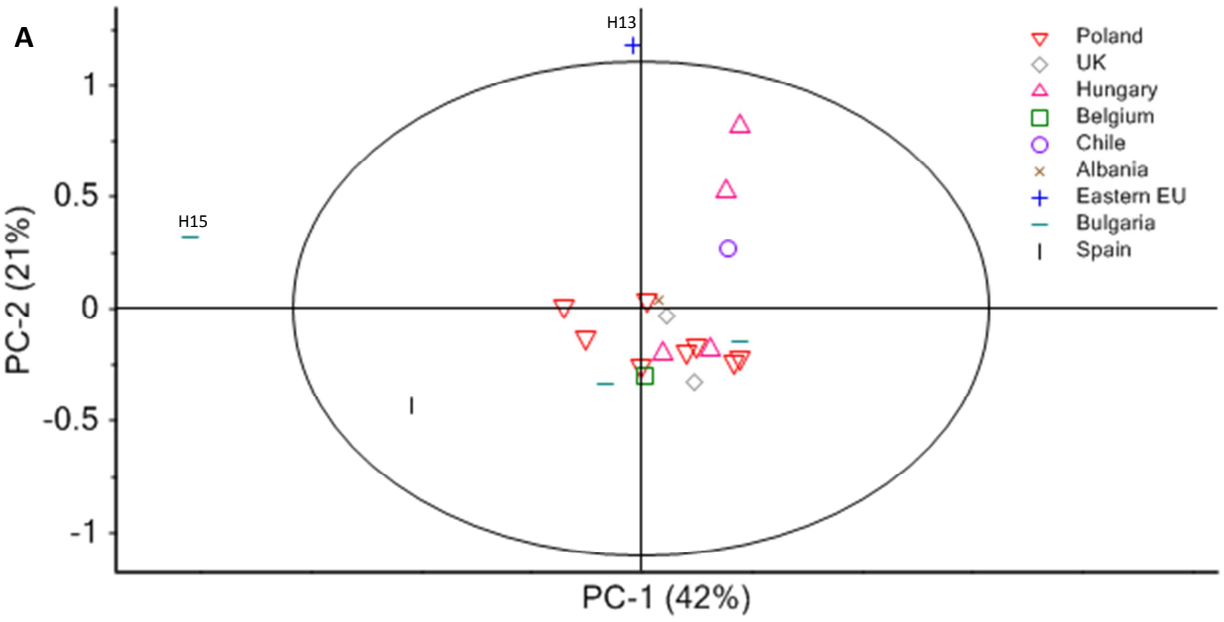
Figure 3.5. 3D PCA of 48 SJW samples with 10 elements. Squares = herbs, circles = tablets and triangles = Capsules. Three PCs with 77% total variance.

3.3.2.2 Investigations of SJW Origin and Identity

In other studies looking at herbal material, it was shown that PCA could be used to identify sample geographic origin [81, 82, 192, 193]. Moreda-Piñeiro *et al.* [82] found that the analysis of tea (*Camellia sinensis*) leaves for elements (i.e. Al, Ba, Ca, Cd, Co, Cr, Cu, Cs, Mg, Mn, Ni, Pb, Rb, Sr, Ti, V and Zn) with PCA was able to differentiate the teas by their Asian or African origin. Another study

by Anderson and Smith [192] was able to discriminate raw pistachios by their growth origin (Iran, Turkey or California) with elements Ba, Be, Ca, Cu, Cr, K, Mg, Mn, Na, V, Fe, Co, Ni, Cu, Zn, Sr, Ti, Cd, and P.

Therefore, this was investigated for SJW using the dry herb samples which were from locations across the world (Table 3.1). The total metal content of SJW dry herbs was analysed using PCA. The initial PCA (Figure 3.6) shows that there is some general grouping of samples from Poland (red down-facing triangles). However, it was found that herb samples H13 and H15 were considered outliers in comparison to the other herbs with a 95% ellipse. This was because they had much higher levels of Cu, Al, B or Ca compared to other herb samples. These two samples were removed from the data set and a new PCA was carried out. This showed (Figure 3.6) that the Polish herbs still retained a small amount of grouping; however, these had now formed two general groups. The two UK samples (grey diamonds) are present in the negative aspect of PC1 and those of Hungarian origin (pink up-facing triangles) are also in the same region. The sample of Albanian origin is similar to Polish herbs whilst the sample of Belgium origin is similar to a Bulgarian sample. Overall, these PCAs show that potentially this kind of analysis could be utilised for geographic origin identification, however, in order to confirm this, a large number of samples from each locality would be needed to firstly represent the locality and to secondly increase the sample to variable ratio. To account for variances of samples within the same locality; information on soil type would be useful.



B

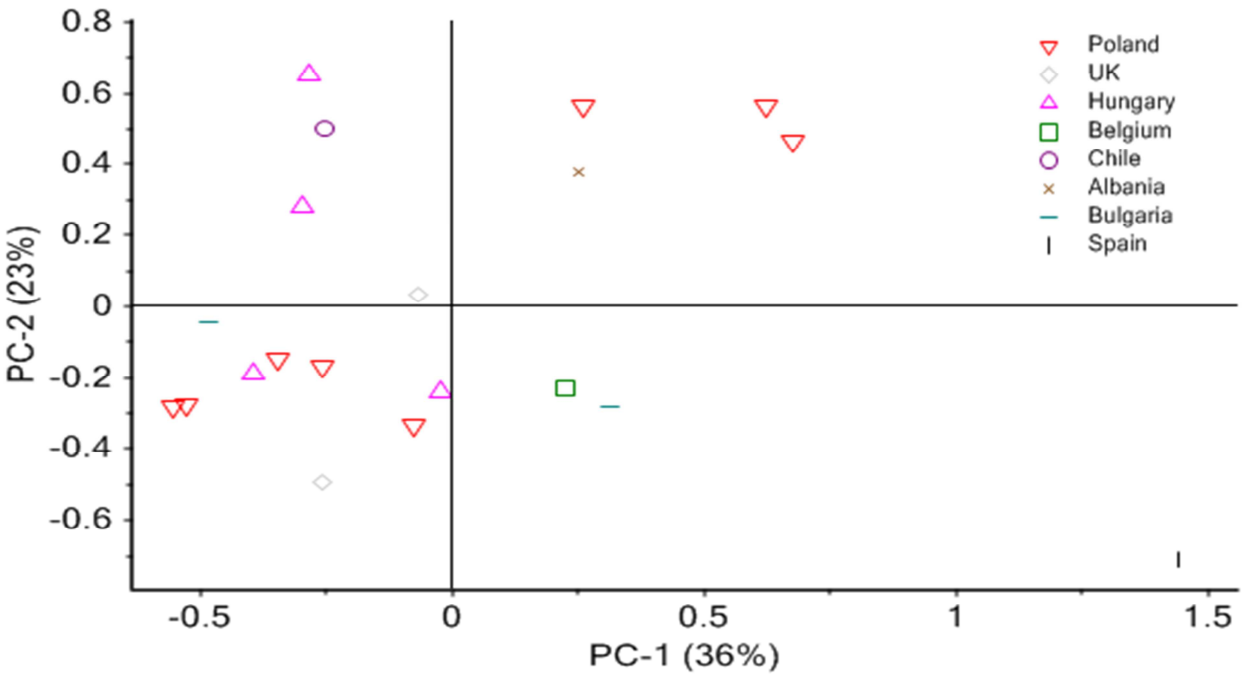


Figure 3.6. PCA of (A) all 22 SJW raw herb samples with 16 elements with 95% confidence ellipse and (B) SJW raw herbs without H13 and H15

3.3.2.3 Preliminary Investigation with Different Plant Species

In order to see if elemental profiling could be used to differentiate plant species, a PCA was carried out on the SJW capsules and capsules of Ginger, Milk thistle and Ginseng (Good 'n' natural, Ronkonkoma, USA). Capsules were chosen for this preliminary investigation as their elemental profiles undergo a large change from the original herb to become a standardised product. Thus, if standardised capsules are able to be differentiated despite the addition of bulking agents, then perhaps the herbs (with more variability), would also be able to be differentiated. All SJW capsules with the exception of C1, C2 and C10 were utilised in the PCA. These capsules (C1, C2 and C10) were removed to ensure the comparison was between true processed samples only as earlier investigations identified these capsules as containing raw herb only. The values were ratio normalised before analysis and the PCA consists of a total of 12 capsules (8 being SJW) with 19 elements (i.e. Al, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Pb, Pt, Sr, V, Y and Zn).

The results (Figure 3.7) show that there is some differentiation between the samples. The SJW sample C5 appears drastically different from the other SJW samples. This is because it contains some raw herb as well as the extract whereas the other SJW samples contain just extract. The PCA also shows that the Ginseng and Ginger capsules are clearly separated from the SJW groups whereas the Milk thistle is closer to the SJW samples. This is interesting as despite all the samples undergoing extraction and further processing, there are still elemental differences between them

to allow differentiation of the other herbs from the SJW. Ginseng and Ginger are particularly differentiated well; this may be due to these herbal remedies being root based as opposed to flower/leaf based with SJW. Milk thistle is seed based and is not as clearly differentiated from the SJW. On PC1, the loadings describe that Ginger and Ginseng are defined due to their higher than average concentrations of Ca, Cr, Mg and Sr and lower than average Cu content. The Milk thistle on the other hand is defined by its higher than average Al and Mn as well as lower than average Zn content on PC2.

Although this preliminary study only investigates a small number of samples, it shows the potential that herbal species could be identified in their processed form if a full model was constructed. For example, plant families Asteraceae, Apiaceae, Fabaceae and Lamiaceae were separated by their elemental profile (using elements B, Zn, Fe, Na, Mg, Ca and K) with PCA [77] and black, green and oolong tea were differentiated using elements Zn, Mn, Mg, Cu, Al, Ca, Ba and K [78]. Therefore, as the processed forms showed some differentiation between species and examples using raw herbs have been shown possible with other plants [77, 78], this method could potentially be used for species identification. Although this and the noted studies have shown this is possible between different families of plants, if this type of analysis could differentiate between plants of the same family (e.g. *Hypericum perforatum vs. Hypericum balearicum, Hypericum calycinum, Hypericum olympicum* etc.) then this information would be very useful in quality control.

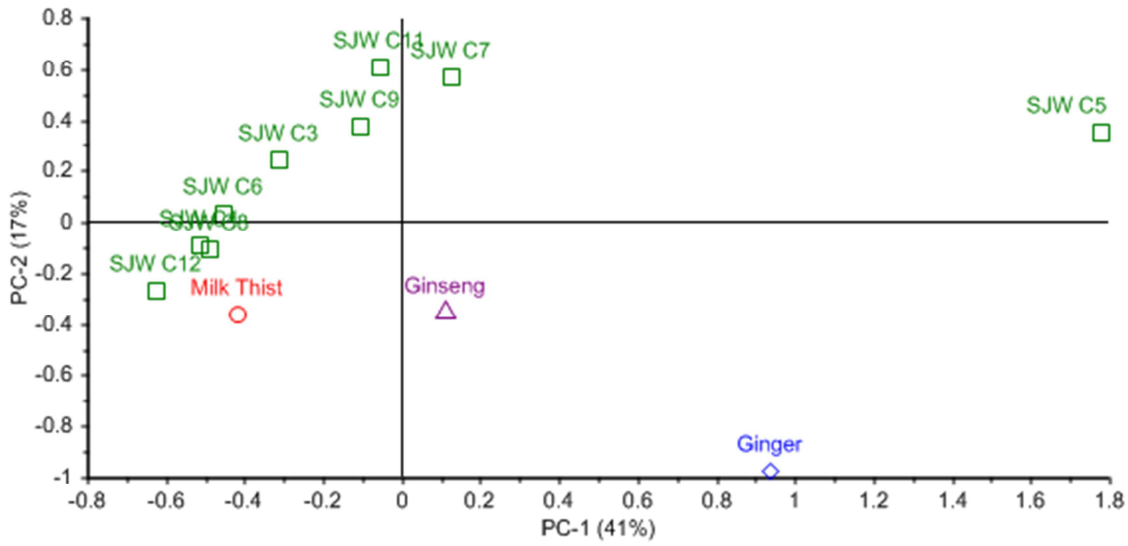


Figure 3.7 PCA of SJW capsules with Ginger, Ginseng and Milk thistle capsules

3.4 Conclusions

This study has determined the 'normal' range of 16 elements (i.e. Al, B, Ba, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Pt, Sr, Y and Zn) in St John's wort raw herb and processed preparations. Nine elements were either below LOQ or LOD. For the toxic elements As, Pb, Hg and Cd, all samples were within recommended daily allowances (if the products were taken within the dosage recommendations on the label claim). The application of PCA to the elemental profiles for the SJW samples clearly differentiated the raw herb samples from the processed samples with some general differentiation between tablets and capsules. A reduction in essential elements B, Ba, and Mn seems to occur after formulation either due to factors such as the extraction process or powder dilution. A reduction in Cd and Ni was also observed. Higher levels of Ca and Mg found in processed forms were expected, but higher levels of Cr, Y, and Sr were also found. The PCA model was able to be optimised and reduced the 16 quantified elements to a minimum of 7 (Ba, Ca, Cd, Fe, Ni, Sr and Y) that still facilitated differentiation between SJW samples. Removal of a near-outlier data point showed that the PCA method is robust. Results indicated sample forms (herb, tablet and capsule) were differentiated by a change in the elemental profile due to excipient addition, dilution, and/or the extraction process. This initial study indicated that SJW samples can be classified by formulation using the elemental fingerprint. There is also evidence to suggest that this fingerprint may also classify based on a change due to the extraction process, yet, even with this processing, the identity of the herb may be feasible. Further investigations should focus on the extraction process as well as obtaining other similar herbal samples to confirm classifications of identity.

In addition to this, the preliminary investigation that compared SJW capsules to capsules containing milk thistle, ginseng and ginger indicated that this type of analysis with PCA could potentially be used for species identification. However, the results obtained in this study were inconclusive due to number of samples analysed. Therefore, to assess the use of this method for species identification, future investigations need to increase the number of samples and also investigate with the raw herbs.

4 Elemental Analysis of St John's Wort Extracts

4.1 Introduction

St John's Wort is commonly available in many forms of preparation. The raw herb is often utilised to make teas and tinctures that can be homemade or bought commercially. These, in addition to the majority of tablets and capsules available on the market, utilise St John's Wort (SJW) in an extracted form. In teas, the SJW constituents are extracted in water, for tinctures the plant is extracted in ethanol, and the dried extract (i.e., used in tablet and capsule formulations) is obtained by extraction in ethanol or methanol. Therefore the majority of SJW products that are used by consumers are of an extracted form. SJW extracts, as opposed to the raw herb, are utilised more frequently because during the extraction process the bioactive constituents responsible for the therapeutic effect become concentrated and can be isolated. This process also aids with standardising the formulated products [191, 194, 195]. Although it has been reported that the elemental form can influence the bioactivity of these constituents [109, 150, 196], little is understood regarding the extraction process for elements present in the raw herb. In addition, initial studies (Chapter 3) indicate that the extraction process may have a large, yet predictable impact on the elemental fingerprint of SJW products. Understanding the changes in element composition after extraction may further assist with the quality control of these products. The extraction of elements is of interest as some elements have been shown to form complexes with

bioactive compounds such as flavonoids [90, 109, 150, 181, 196, 197] and hypericins [198, 199] which are found in SJW. The complexation of metal ions to these bioactive compounds *in vitro* has shown to affect their bioactivity properties [109, 196] as well as their bioavailability [151]. To date, such complexes have not been identified in herbal remedy extracts. However, if such complexes occur naturally (i.e., in the herb and extract), it could lead to herbal medicines being artificially enriched with certain elements to aid disease treatment (e.g., Se and heart disease [180]). Also, the amount of herb extract used in manufacturing could be reduced while maintaining the same level of bioactivity and new options for quality control could be utilised. However, before these further avenues can be examined, the relationship between the elements transferred and extraction solvent used needs to be understood.

Although elements have been analysed in the infusions [125, 126, 131-133, 143, 157, 200], herbs [125-129, 133, 135, 136] or manufactured versions of SJW [119, 125, 126], little has been explored on the effect of the type of extraction solvent on the elemental content in extracts of SJW. More emphasis has been placed on the extraction of the bioactive molecules; however, it is known that elements are also transferred in the extraction process [127, 137]. In addition to the formation of complexes, a particular concern is the speciation of the elements extracted. It has been shown that for elements such as As and Cr certain forms of these elements are safer than others (e.g., Cr (VI) is more toxic than Cr (III) [45]). The speciation of elements could aid pharmaceutical companies that have to comply with the new USP elemental limits [36] within their products. For example, if a product has just surpassed a limit it could be proven through speciation that the element is of the safer form (e.g., Cr (VI) rather than Cr (III)).

The limited studies that investigate the elemental content of SJW extracts are primarily water extracts of SJW [125, 126, 131-133, 143, 157, 200] with fewer studies investigating alcoholic extracts [127, 137]. Gomez *et al.* [125, 126] , Oledzka and Szyszkowska [157], Kalny *et al.* [131] as well as Suliburska and Kaczmarek [143] examined hot water extractions of SJW herb for elements including Al, Ba, Ca, Cd, Co, Cu, Cr, Fe, Pb, Mg, Mn, Ni, V and Zn. Gomez *et al.* [125, 126] prepared two teas of SJW using boiling water and found that of the elements analysed (Cd, Co, Pb, Al, Cr, Fe, V, Ca, Cu, Mg, Mn, Zn and Ni), all concentrations were below that of the original herb. These studies found that, compared to the original herb, Ca, Cu, Ba, Zn, Mg, Mn and Ni had extraction efficiencies between 17-74%, whereas elements Cd and Fe had extraction efficiencies between 7.8-8.4% and 8-23% respectively. Elements Cr and Pb were not detected in infusions. Konieczyński and Wesołowski [132] examined the water extractable Mg, Mn and Cu in herbal remedies including SJW. This study found that Mn extraction was very low compared to the original herb (<10%) whereas Mg and Cu

had higher extraction efficiencies (~40% and ~30% respectively). A similar study by the same authors [133] examined elements Fe and Zn which found on average <6% Fe was extracted (with 3 high samples 15-82% compared to other 8 samples) and Zn was extracted on average by 30%.

Two studies investigated the elemental content of alcoholic extracts. Naeem *et al.* [137] prepared methanol extracts of various Hypericum species including *Hypericum perforatum*. The extracts were then analysed for elements Ni, Cr, Cu, Pb, Cd, Co and Fe whereby 0.069 ± 0.007 mg/g, 0.054 ± 0.004 mg/g, 0.210 ± 0.004 mg/g, 0.0460 ± 0.0001 mg/g, 0.005 ± 0.001 mg/g, 0.054 ± 0.009 mg/g and 0.318 ± 0.009 mg/g was found respectively. Helmja *et al.* [127] extracted SJW in ethanol as well as water and observed that the water extract, extracted between 10-25% Zn, Mn, Co, and Cr whereas the ethanol extracted levels 10-25 times lower.

The majority of these studies are limited as they examine three or fewer SJW samples with the exception of Koineczynski *et al.* [133], whom investigated eight. All samples within these studies use SJW from only one country of origin, thus consistency between herbal samples still needs investigating. Another limitation of the previous studies mentioned is that only concentrations of 100% water, ethanol or methanol are investigated as extraction solvents. For commercial formulations, these solvents are used in concentrations of 60% and 80% ethanol or methanol as they have been shown to extract more bioactive constituents at these percentages [191, 194, 195]. To be able to understand the mechanism of metal transfer, systematic studies are needed using different solvent conditions.

In this study, fourteen elements from those identified as being present in SJW from Chapter 3 (Al, B, Ba, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Sr and Zn) were monitored in different SJW extracts. A total of eight SJW raw herbs from different localities (Table 4.1) were used to prepare extracts using 100% water, 60 %v/v ethanol, 80 %v/v ethanol and 100% ethanol to identify transfer relationships in a systematic way.

4.2 Method

4.2.1 Materials

Eight SJW dry powdered herbs were purchased through high street retailers and internet sources. A summary of all samples is shown in Table 4.1. Due to the large amount of sample needed for this study, new SJW samples were purchased in addition to those stated in Chapter 3. All labware was acid washed overnight with 4M nitric acid and rinsed thoroughly with deionised water before use.

High-purity nitric acid 70% (99.99% trace metal basis, Sigma-Aldrich, Gillingham, UK) was used for hotplate digestion and preparation of 2% HNO_3 solutions. Elemental stock solutions (1000 ppm) of Al, As, B, Ba, Cd, Co, Pb, Mg, Mn, Mo, Ni, In and Hg (Fisher, Loughborough, UK); Be and Pt(VWR, Lutterworth, UK); Ca, Cr, Cu, Fe, Sb, Se, Sr and Zn(Merck, Feltham, UK); V(Sigma-Aldrich, Gillingham, UK); and Y (Acros organics, Geel, Belgium) were used to prepare calibration standards. Extractions of samples were carried out using mixtures of HPLC grade water (Fisher, Loughborough, UK) and absolute ethanol (Fisher, Loughborough, UK) in %v/v. Whatman cellulose filter paper (grade 1) and Whatman glass microfiber filter paper (GF/A) were used in the filtering stage of sample preparation.

Table 4.1. Summary of SJW powdered samples obtained

Sample Name	Sample Number	Species	Country of Origin
Herb 1	H10[1]	*Hypericum perforatum*	Hungary
Herb 2	H17[1]	*Hypericum perforatum*	Spain
Herb 3	H23	*Hypericum perforatum*	Hungary
Herb 4	H24	*Hypericum perforatum*	Hungary
Herb 5	H25	*Hypericum perforatum*	Poland
Herb 6	H26	*Hypericum perforatum*	Hungary
Herb 7	H27	*Hypericum perforatum*	Czech Republic
Herb 8	H29	*Hypericum perforatum*	USA

[1]SJW dry herb used in the initial investigation (Chapter 3)

4.2.2 Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES)

Elemental analysis was carried out using a 710 ICP-OES (Varian) axial spectrometer fitted with a Seaspray nebuliser and SPS3 auto sampler. Please see Chapter 3 for full ICP-OES parameters and wavelengths used.

4.2.3 Method Development

4.2.3.1 Filter Paper Comparison

In order to determine the effect of filtering on the elemental profile, the element leaching and retention was examined during the filtering process. A volume of 20 ml 60 %v/v ethanol was filtered through two types of filter paper (Whatman cellulose and Whatman glass microfiber) in triplicate and compared to a non-filtered solution. A volume of 20 ml 60 %v/v ethanol spiked with known quantities of elements (0.8 ppm for elements As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, Pb, Mg, Mo, Ni, In, Hg, Pt Sb, Se, Sr, Y and Zn, 8 ppm for Al and 1.6 ppm for Mn) was also filtered through the two types of paper (Whatman cellulose and Whatman glass microfiber) in triplicate and compared to non-filtered solution. The resulting solutions were dried down on a hotplate followed by acid digestion with 5 ml HNO_3. The sample was then diluted 1:10 with deionised water, centrifuged at 9000 rpm for 15 minutes and filtered (0.2 µm) before ICP-OES analysis.

4.2.3.2 Extraction Time

Approximately 1 g of sample (herb 2) was weighed by difference into an amber jar (50mm x 100 mm). To the jar was added 20 ml of 60 %v/v ethanol. The samples were then stirred for 1, 2, 4, 8 or 24 hrs with an x-bar magnetic stirrer at 450 rpm. Following this the samples were filtered (Whatman grade 1 cellulose filter paper). Following filtration, 4 ml was transferred from the Buchner flask into an amber vial. The remaining sample solution was transferred to a tall beaker with watch glass and rinsed with an additional 5 ml 60 %v/v ethanol. The solution was evaporated to dryness on a hotplate. Following this, 5 ml high purity HNO_3 was added to the beaker and acid digestion was carried out until no NO_2 fumes were visible. The samples were then diluted 1:10 with deionised water, centrifuged at 9000 rpm for 15 minutes and filtered (0.2 µm). Samples were then analysed *via* ICP-OES.

4.2.3.3 Validation

Validation of the hotplate digestion was carried out using the certified reference material NIST Polish tea (NIST INCT-TL-1). Approximately 0.2 g was weighed by difference and digested with 5 ml high purity HNO_3 *via* hotplate digestion in triplicate. The NIST tea was also spiked with known quantities of elements to assess those elements not certified or below limits of detection (As, Be, Cd, Co, Hg, In, Mo, Pb, Pt, Sb, Se, V and Y). The sample was then diluted 1:10 with deionised water, centrifuged at 9000 rpm for 15 min and filtered (0.2 µm) before ICP-OES analysis.

4.2.4 SJW Sample Preparation

Approximately 2 g of sample was weighed by difference into an amber jar (50 mm x 100 mm) with 20 ml of the selected extraction solvent (100 % water, 60 %v/v ethanol, 80 %v/v ethanol or 100 %v/v ethanol). The samples were stirred for 1 hour with an x-bar magnetic stirrer at 450 rpm, and filtered (Whatman grade 1 cellulose filter paper). Once filtered, 4 ml was transferred from the Buchner flask into an amber vial for future liquid chromatography (LC) analysis (see Chapter 5). The remaining sample solution was transferred to a tall beaker with watch glass and rinsed with an additional 5 ml of solvent. The solution was evaporated to dryness on a hotplate. Following this, 5 ml high purity HNO_3 was added to the beaker and acid digestion was carried out until no NO_2 fumes were visible. The samples were then diluted 1:10 with deionised water, centrifuged at 9000 rpm for 15 minutes and filtered (0.2 µm). Samples were then analysed *via* ICP-OES. Samples were prepared in triplicate unless further repeats were needed (n = 4-5) to improve precision.

4.2.5 Statistical Analysis

The solvent extraction data of the dried extracts were subjected to correlation analysis (CA) using Microsoft Excel (2007) to see if relationships exist between the elements in each extraction solvent. Principal component analysis (PCA) was utilised on the elemental profiles produced by the four types of extraction solvent as well as the concentrations elements found in the original herb. Data was normalised using ratio normalisation before undergoing PCA and was carried out using the Unscrambler X (CAMO) software.

4.3 Results and discussion

4.3.1 Method Development

4.3.1.1 Filter Paper Comparison

Cellulose and glass microfiber filter paper was compared in order to determine the optimum filter paper that would not introduce elemental contamination to samples as well as not retain elements on the paper. Firstly, 20 ml of 60 %v/v ethanol was filtered through each type of filter paper in triplicate and compared to unfiltered solution. A 60 %v/v ethanol solution was chosen for this study as it is the most popular ethanol concentration used by extract manufacturers of SJW [201]. The results (Figure 4.1) show that five of the elements of interest (Al, Ca, Fe, Mg, and Zn) were detected in the control experiment of unfiltered 60 %v/v ethanol solution. These elements may come from the solvents used as they are not of high purity grade. When the solution was filtered through

cellulose paper, no significant element transference was observed compared to the unfiltered solution. When the solution was filtered through the glass microfiber paper, the solutions had significant differences in the concentrations of Ba (0.32± 0.03 ppm), Ca (0.4 ± 0.1 ppm), Mg (0.085 ± 0.004 ppm) and Sr (0.004 ± 0.001 ppm) (t test $p<0.05$) compared to the unfiltered solution. Therefore of the two filter papers, it was found that the cellulose filter paper did not introduce contamination that was statistically significant when compared to the unfiltered solution, whereas the glass microfiber filter paper did.

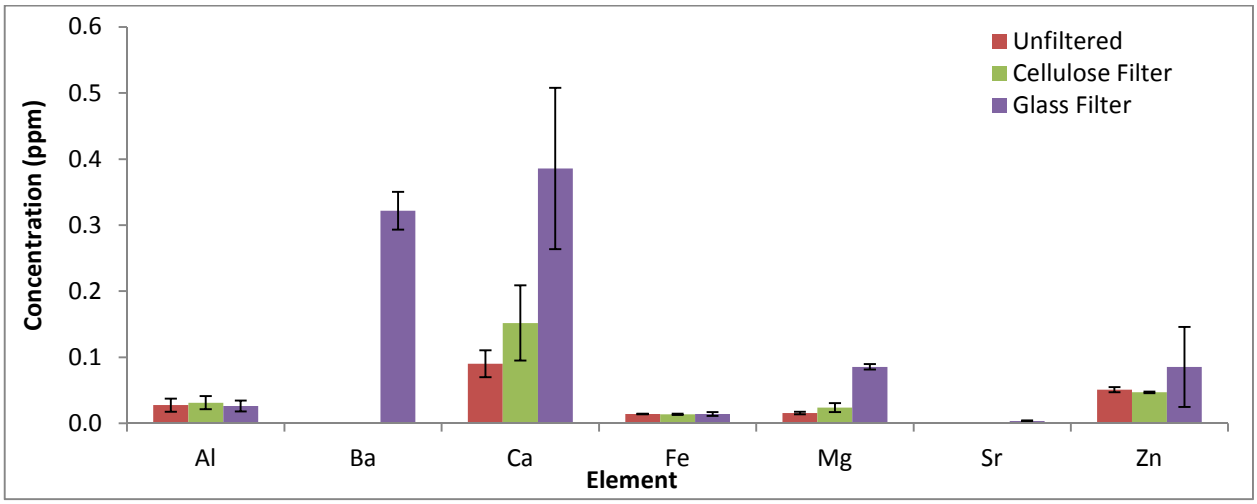


Figure 4.1 The element concentration of unfiltered, cellulose filtered and glass fibre filtered 60 %v/v ethanol solution. Uncertainty is ±1SD

The filter papers were also assessed for their retention of elements during filtration. For this the 20 ml 60 %v/v ethanol was enriched with known quantities of each element and the recovery examined and compared to an unfiltered spiked solution. The results (Figure 4.2) show that for the majority of elements, recoveries were greater than 90% however; Hg and Sb had recoveries of 1-17% and 18-42%, respectively. The poor recovery of Hg is likely due to the digestion method used, which was not in a closed vessel, allowing elemental loss from volatilisation. However, due to the extended period on the hotplate (up to 4 hours) in concentrated HNO_3 before dilution, Sb recovery is greatly reduced possibly due to the formation of oxides (Sb_2O_3 and Sb_2O_5) [202]. To overcome this formation of antimony oxides, HCl could be added to form *aqua regia* or a mixture of nitric and tartaric acid, however, as Sb has not been detected in SJW, this was not carried out. Calcium shows a recovery greater than the spiked value. This may be due to the solvents used not being of high purity grade and thus introducing some contamination. A high recovery of Ba was observed with the glass microfiber filter paper which is introduced from the filter paper as contamination. The low and variable recovery of B is due to B leaching from the borosilicate beakers during acid digestion. The recoveries of Mo are consistently lower (71-84%) across filtered and unfiltered samples, this is

due to the prolonged length of time on the hotplate whereby Mo oxidises to MoO_3 [203]. In the total digestions of SJW herb Mo is only found in very small quantities (0.4 - 1.5 µg/g), therefore it is unlikely this element will be seen in quantities above LOQ. Based on these results, there are no noticeable concentration decreases due to filter paper retention. As discussed above, many of the decreases are due to the method of hotplate digestion and these elements will not be used for further analysis (i.e., B, Hg, and Sb). Thus, the cellulose filter was used for further studies.

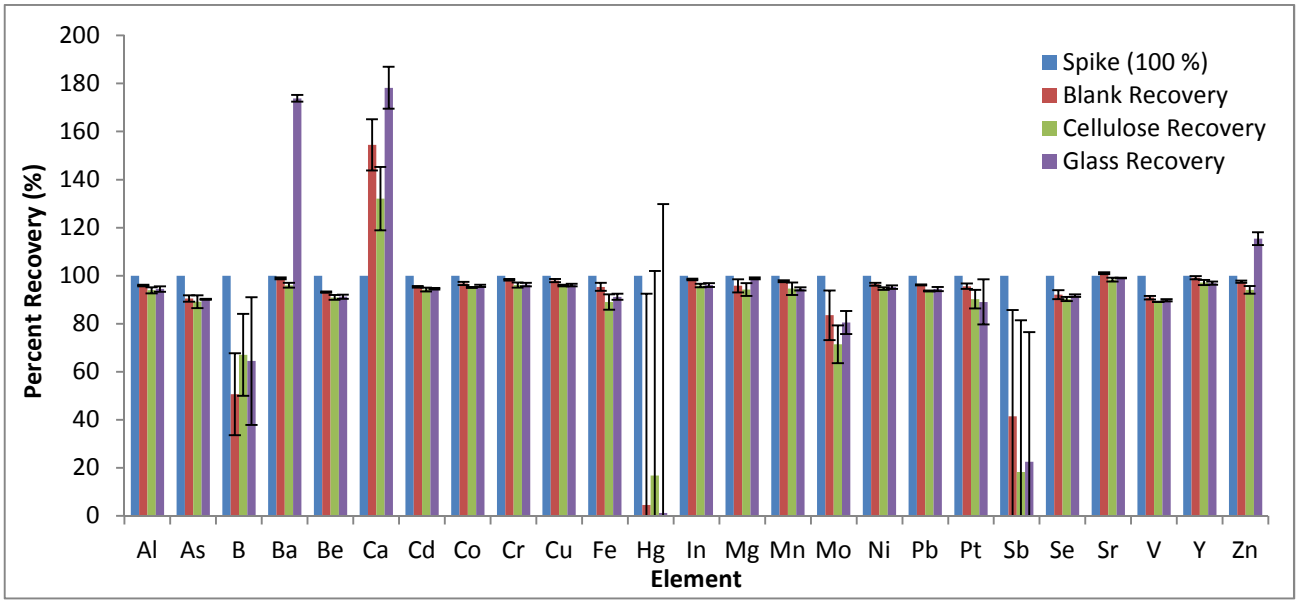


Figure 4.2 Comparison of unfiltered (blank) and filtered (cellulose or glass fibre) element enriched 60 %v/v ethanol solution. Uncertainty is ±1SD.

4.3.1.2 Extraction Time

To establish the optimum extraction time, a powdered sample of SJW (herb 2) was extracted in 60 %v/v ethanol for 1, 2, 4, 8 and 24 hours. The solution of 60 %v/v ethanol was chosen for this study as it is the most popular concentration of ethanol used by extract manufacturers of SJW. Of the 25 elements investigated only 14 were detected (Table 4.2). As the levels detected for most elements are either close to the LOD or not above the LOQ, the associated error is high. Also noted for the 4 hour extraction, one of the triplicates is visually high compared to the other two samples – however, Dixons Q-test does not determine this as an outlier (p=0.05) and thus these values were left in the dataset. Further inspection of the results showed that there were no significant increases in element concentration as the extraction time increased. Therefore an extraction time of 1 hour was chosen for further investigations.

Table 4.2. Concentration of elements in SJW transferred during extraction for 1, 2, 4, 8 and 24 hrs

Element	Concentration (µg/g)[1]				
	1 hour	**2 Hour**	**4 Hour**	**8 Hour**	**24 Hour**
Al	0.8 ± 0.4	1.04 ± 0.07	0.9 ± 0.3	2.0 ± 0.4	0.3 ± 0.1
As	-	-	-	-	-
B	10 ± 3	8 ± 1	8 ± 2	12 ± 4	5.6 ± 0.6
Ba	0.07 ± 0.01	0.13 ± 0.08	0.14 ± 0.07	0.3 ± 0.2	0.06 ± 0.01
Be	-	-	-	-	-
Ca	190 ± 20	221 ± 5	210 ± 70	240 ± 20	210 ± 30
Cd	0.023 ± 0.003	0.025 ± 0.009	0.02 ± 0.02	0.02 ± 0.01	0.006 ± 0.008
Co	0.13 ± 0.06	0.2 ± 0.1	0.11 ± 0.07	0.2 ± 0.1	0.07 ± 0.02
Cr	0.04 ± 0.05	0.07 ± 0.02	0.04 ± 0.03	0.080 ± 0.007	0.006 ± 0.009
Cu	3.7 ± 0.1	4.0 ± 0.1	3 ± 1	4.0 ± 0.1	4.08 ± 0.09
Fe	1.0 ± 0.2	1.23 ± 0.04	1.0 ± 0.3	1.6 ± 0.4	0.67 ± 0.03
Hg	-	-	-	-	-
In	-	-	-	-	-
Mg	364 ± 9	380 ± 10	370 ± 80	390 ± 20	402 ± 5
Mn	8.0 ± 0.8	8.8 ± 0.1	9 ± 2	9.3 ± 0.6	8.6 ± 0.7
Mo	-	-	-	-	-
Ni	1.14 ± 0.03	1.19 ± 0.01	1.1 ± 0.4	1.30 ± 0.03	1.40 ± 0.05
Pb	-	-	-	-	-
Pt	-	-	-	-	-
Sb	-	-	-	-	-
Se	-	-	-	-	-
Sr	0.25 ± 0.03	0.300 ± 0.005	0.3 ± 0.1	0.35 ± 0.08	0.26 ± 0.05
V	-	-	-	-	-
Y	-	-	-	-	-
Zn	7.3 ± 0.2	8 ± 1	6 ± 3	7.8 ± 0.3	7.3 ± 0.3

[1]uncertainty is reported as ±1SD, µg of element /g of original dry herb. - = Below limit of quantification.

4.3.1.3 Validation

To assess the accuracy of the hotplate digestion method for liberation of elements from herbal material, the NIST Polish tea CRM was analysed using the method. Elements Al, B, Ba, Ca, Cr, Cu, Mg, Mn, Ni, Sr and Zn were validated using the certified values whereas the other elements (As, Be,

Cd, Co, Hg, In, Mo, Pb, Pt, Sb, Se, V and Y) were validated using spiked recovery methods. The results (Table 4.3) show that the majority of elements had recoveries ≥ 90% whereas Al and Zn had recoveries ≥88%. Iron had a low recovery of 60 ± 20% due to the incomplete digestion of silicates in the sample. This could be overcome by the addition of a few drops of HF, however this would then lead to the damage of glassware utilised within the ICP-OES. The recovery of B is exceedingly high in comparison to other elements as the acid digestion stage of the sample preparation causes leaching of B from the borosilicate glass used. The cause of the high recovery of Cr (132 ± 9 %) is unknown. The values reported for Hg, Sb and Mo have a better recovery with this analysis as the hotplate digestion only took 2 hours compared to 4 hours.

In conclusion, this shows that the hotplate digestion method used can effectively digest SJW plant material and recover most elements of interest. It is important to note that the herbal extracts will not have silicates and thus will not suffer from low Fe recovery in this manner. Although the Cr recovery is high, it is reproducible, thus comparing between extraction methods should still give us an indication of relative transfer, but absolute values may have an associated error. As the extracts will be prepared in borosilicate glass beakers, the method is not fit to quantify B.

Table 4.3. Recovery of elements from hotplate digestion of NIST tea

Element	Certified Value or Spike amount	Experimental Value[1,2]	% Recovery[2]
Al	0.229 ± 0.028 wt%	0.200 ± 0.001	87 ± 0.7
As	Spiked with 0.5 ppm	0.49 ± 0.02	97 ± 4
B	26 mg/kg	35.7 ± 0.5	137 ± 1
Ba	43.2 ± 3.9 mg/kg	42.22 ± 0.07	97.7 ± 0.2
Be	Spiked with 0.5 ppm	0.47 ± 0.03	94 ± 5
Ca	0.582 ± 0.052 wt%	0.545 ± 0.001	93.6 ± 0.1
Cd	Spiked with 0.5 ppm	0.47 ± 0.02	95 ± 4
Co	Spiked with 0.5 ppm	0.49 ± 0.02	98 ± 4
Cr	1.91 ± 0.22 mg/kg	2.22 ± 0.07	116 ± 3
Cu	20.4 ± 1.5 mg/kg	21 ± 1	103 ± 5
Fe	432 mg/kg	280 ± 20	64 ± 8
Hg	Spiked with 0.5 ppm	0.53 ± 0.04	106 ± 7
In	Spiked with 0.5 ppm	0.45 ± 0.02	91 ± 4
Mg	0.224 ± 0.017 wt%	0.203 ± 0.002	91 ± 1
Mn	0.157 ± 0.011 wt%	0.1490 ± 0.0002	94.9 ± 0.1
Mo	Spiked with 0.5 ppm	0.50 ± 0.02	99 ± 4
Ni	6.12 ± 0.52 mg/kg	5.6 ± 0.3	92 ± 6
Pb	Spiked with 0.5 ppm	0.47 ± 0.01	94 ± 3
Pt	Spiked with 0.5 ppm	0.48 ± 0.02	96 ± 4

Sb	Spiked with 0.5 ppm	0.46 ± 0.02	91 ± 5
Se	Spiked with 0.5 ppm	0.48 ± 0.02	96 ± 3
Sr	20.8 ± 1.7 mg/kg	19.82 ± 0.05	95.3 ± 0.2
V	Spiked with 0.5 ppm	0.50 ± 0.02	101 ± 4
Y	Spiked with 0.5 ppm	0.49 ± 0.03	99 ± 5
Zn	34.7 ± 2.7 mg/kg	30.9 ± 0.6	89 ± 2

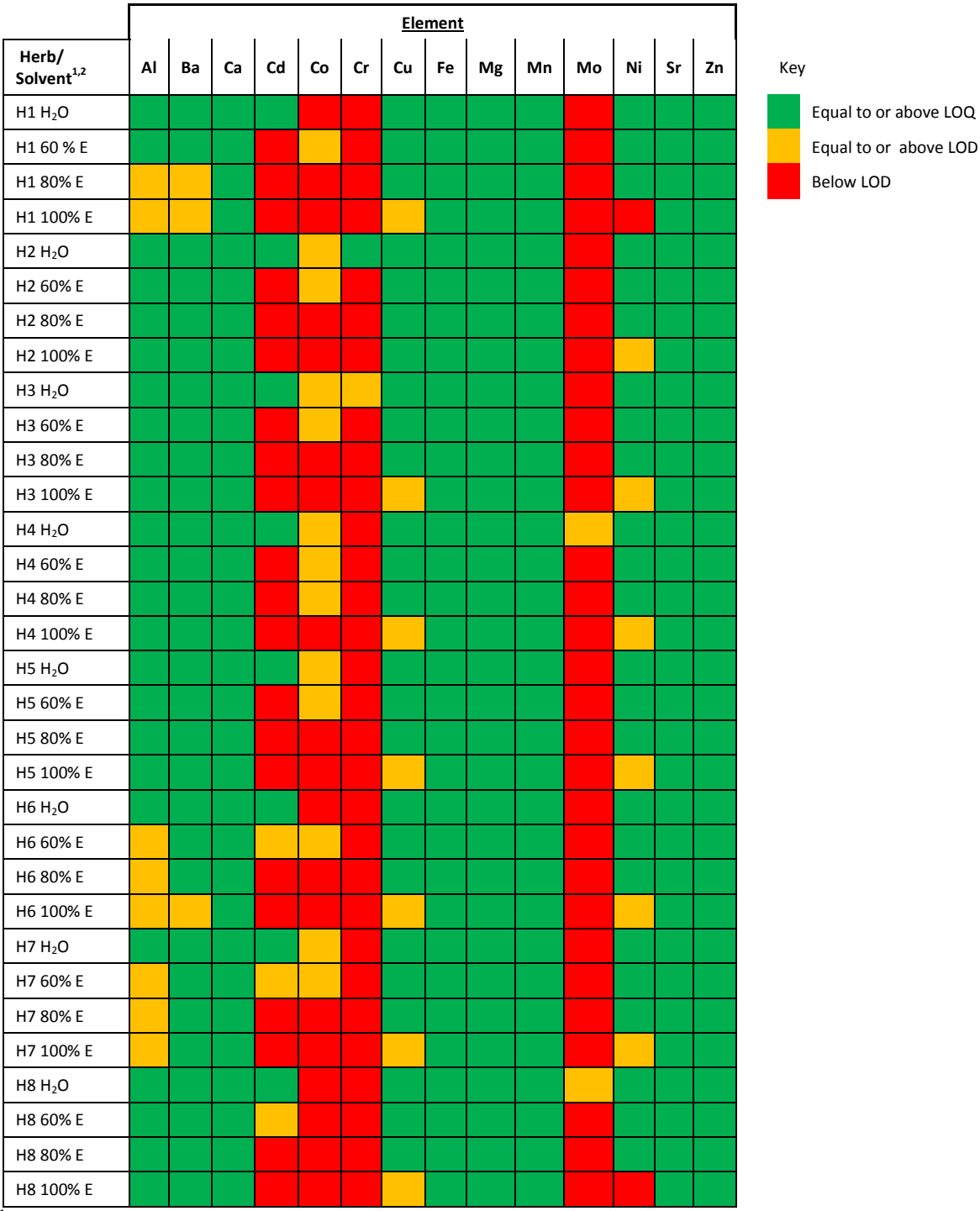
[1] Units are same as those stated for certified/spiked value
[2] Uncertainties are reported to ±1SD

4.3.2 Elemental Analysis of St John's Wort Extracts

The majority of SJW herbal preparations that are available or prepared by the public are generally in an extracted form. Common forms being herbal infusions or teas prepared by water as well as SJW tablets or capsules. The majority of the manufactured forms use a dried alcoholic extract where the original extraction is usually carried out with 60% ethanol, 80% ethanol or 80% methanol [191, 194, 195, 201]. The most prevalent amongst manufacturers being 60% ethanol. Thus, to explore how elements are transferred during the extraction process this study investigated the 60 %v/v and 80 %v/v ethanol solutions but also 100% water and 100 %v/v ethanol to further decipher overall trends from the extraction process.

The elemental concentrations were determined for each extract for each herb and will be discussed in the following sections. For all herbs, of the 16 elements, 14 elements were detected in samples. Table 4.4 shows which elements in which extract were above limits of detection (LOD) and limits of quantification (LOQ). Eleven elements were consistently above LOQ (i.e., Al, B, Ba, Ca, Cu, Fe, Mg, Mn, Ni, Sr and Zn) for 100% water solutions. Elements Cd, Co, Cr and Mo were below LOQs for most extracts and below LODs for 80 %v/v and 100 %v/v ethanol extracts (Table 4.4). The average weights of the dried extracts produced by each solvent (Table 4.5) shows that the 60 %v/v ethanol and 80 %v/v ethanol solvents produce more dried extract than the other solvents, this may be due to the increased bioactive compounds extracted in the concentrations [191, 194, 195]. The differences in extraction efficiency, dried extract concentration and comparisons to the original raw herb concentration with each solvent are described by element.

Table 4.4 Summary of concentrations of elements within different extracts of eight SJW herbs



[1] H1= herb 1, H2 = herb 2, H3 = herb 3, H4 = herb 4, H5 = herb 5, H6 = herb 6, H7 = herb 7, H8 = herb 8.

[2] H_2O = 100% water, 60%E = 40:60 water: ethanol, 80% E = 20:80 water: ethanol and 100% E = 100% ethanol.

Table 4.5 Comparison of dried extract weights from different solvents

Extraction Solvent	Number of Extractions (n)	Average weight (mg ±1SD)
100 % water	26	137 ± 21
40:60 water: ethanol	27	245 ± 54
20:80 water: ethanol	31	220 ± 45
100% ethanol	34	107 ± 25

4.3.2.1 Aluminium

Aluminium function in plants is unclear [39], although it has been shown that in low concentrations the element can have a beneficial effect on growth [54]. Within humans however, abnormally high concentrations of the element has been linked to Alzheimer's disease [204] and Osteomalacia [205] (softening of bones due to defective mineralisation).

The results in Figure 4.3 A illustrate a general trend in the Al concentration despite the SJW samples being from various geographical locations and collection processes being variable. The results show that the highest concentrations of aluminium are extracted with 100% water (0.9 - 3.7 µg/g of original herb). These concentrations are lower compared to Gomez *et al.* [126] which may be due to the extraction process using boiling water rather than room temperature and larger volume (200 ml compared to 20 ml). These levels decrease on average by 65% when extracted with 60 %v/v ethanol (0.5 to 1.1 µg/g of original herb). Across the three ethanol concentrations there was no noticeable difference in the aluminium amount extracted between the eight herbs. These results show that between the solvents, 100% water contained the most aluminium compared to the ethanol solvents. However analysis of samples H1 (80 %v/v and 100%), H6 (60%v/v, 80 %v/v and 100%), H7 (60%v/v, 80 %v/v and 100%) for Al yielded values below the LOQ and these data are only used to indicate a general trend between the extraction solvents used. Comparing these values to the total concentrations of aluminium in the original herb (Figure 4.3 B), water had an extraction efficiency average (±1SD) of 1.6 ± 0.3 % from the original herb. Whereas 0.6 ± 0.4 %, 0.5 ± 0.2 % and 0.5 ± 0.2 % was extracted, respectively, for the 60 %v/v, 80 %v/v, and 100% ethanol solutions.

These results show that the higher the ethanol concentration used within the extraction solvent, the less total aluminium is transferred from the original plant into the extract.

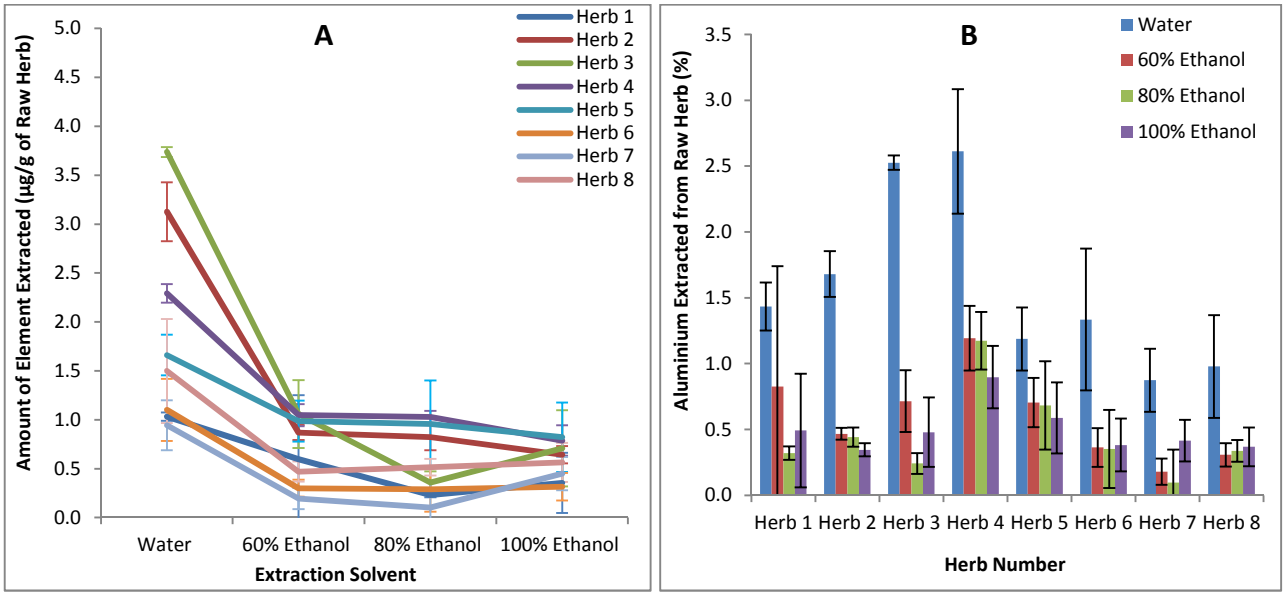


Figure 4.3 (A) Extraction of Al from SJW powdered herbs in different solvents and (B) percent of Al extracted from original raw herb. Uncertainty is ±1SD.

When considering the amount of Al in the dried extract (Figure 4.4 A), it can be seen that the 100% water extract has the highest concentration of the element. The water dried extracts contain between 15-47 µg/g Al whereas the 60 %v/v, 80 %v/v, and 100% ethanol dried extracts contain1-10 µg/g, 0.9-11 µg/g and 5-26 µg/g, respectively. This is likely due to free aluminium and aluminium salts being able to move more freely in the water compared to the ethanol. Polyphenolic compounds such as flavonoids can be extracted in water [206]. These compounds (e.g., rutin and quercetin) which are found in SJW have been shown to bind to metals [109, 150]. Therefore is it possible some of the Al found in the water extracts are also within a bound form. A study that examined Al in water infusions from black tea (*Camellia sinensis*) leafs has shown that, of the identifiable Al, mostly polyphenolic bound Al (30.0 ± 2.1%) and cationic (14.5 ± 1.6 %) Al was present [206]. Flavonoids are more readily extracted from the plant material with solvents such as methanol and ethanol compared to water [194, 195, 207]. Therefore, the high concentrations of Al seen in the water are probably a mixture of bound, free and Al salts whereas the ethanol extracts may contain predominantly bound Al.

Comparison of the dried extract concentrations to that of the total concentration of the original raw herb (Figure 4.4 B) shows that all four types of dry extract contain less Al than the original herb. On average, 23% of Al concentration is retained in the dry water extract. For the ethanolic

solutions, this drops to 10% or less. This illustrates that no preconcentration occurs with this element within dried extracts due to the extraction process.

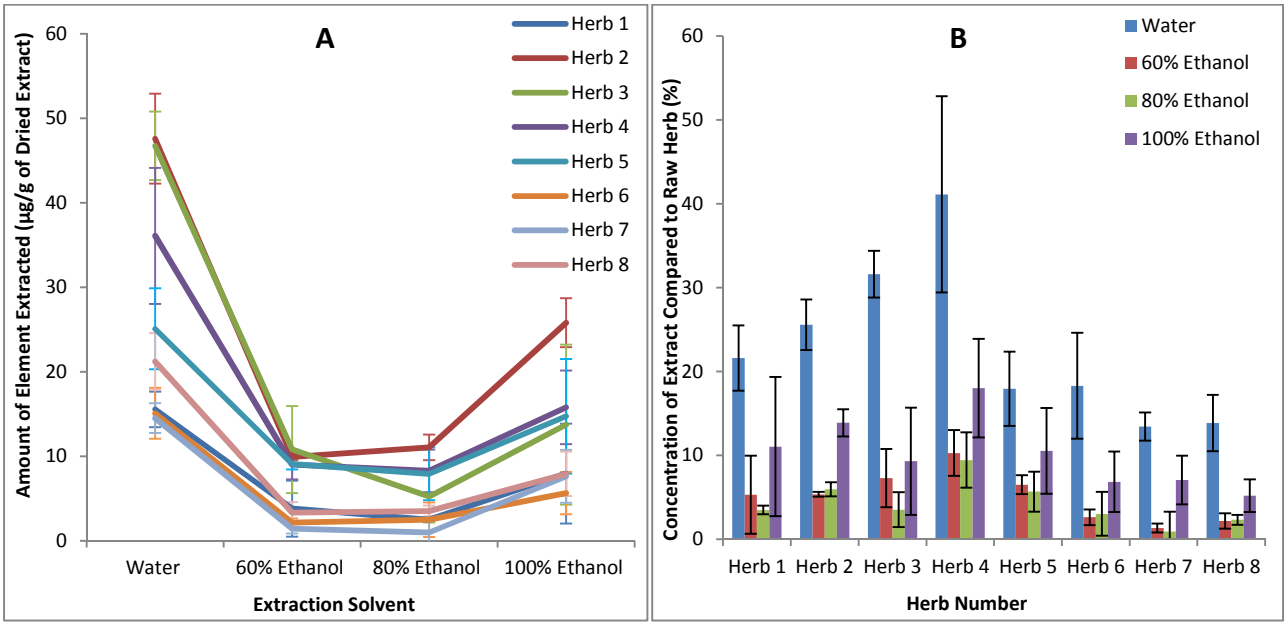


Figure 4.4 (A) Concentration of Al in dried extracts (B) Comparison of Al concentration in dry extract to dry herb. Uncertainty is ±1SD.

4.3.2.2 Barium

Although Ba has been found in several plant species, the element is not essential [40]. However, Ba intake can cause accumulation in the skeleton as well as renal failure in humans through *hypokalaemia* (low plasma potassium levels; due to a transfer of potassium from extracellular to intracellular compartments via the K^+-channel of the Na–K pump in the cell membranes becoming blocked thus causing kidney weight to increase and lesions) [208]. Also, accumulation of Ba in the eyes has been noted (with pigmented areas such as the iris, sclera and choroid accumulating the highest concentrations) [208]. In the region of Kiating, China, Ba poisoning caused the endemic 'pa ping' disease [209] which caused nausea, vomiting and diarrhoea as well as paralysis or death in some cases.

The extraction of different SJW powdered herbs with four solvents illustrate a general trend in Ba concentration despite varied geographical origin and collection processes being variable. The results (Figure 4.5 A) show that the highest concentrations of Ba are extracted with 100% water (0.7-2.3 µg/g of original herb). These levels decrease on average by 92% when extracted with 60 %v/v ethanol (0.05-0.20 µg/g of original herb). Across the three ethanol concentrations the 60 %v/v ethanol solvent extracted on average 37% more barium compared to the 80 %v/v ethanol solvent. There was no noticeable difference in the Ba amount extracted between 80 %v/v and 100%

ethanol. All eight herbs showed this trend, with the exception of Herb 5. This is due to high levels in one sample from the 80 %v/v ethanol extraction (0.13 µg/g compared to other two samples 0.03-0.05 µg/g). These results show that between the solvents, 100% water contained the most Ba compared to the ethanol solvents. However analysis of samples H1 (80 %v/v and 100% ethanol) and H6 (100% ethanol) for Ba yielded values below the LOQ and these data are only used to indicate a general trend between the extraction solvents used. Comparing these values to the total concentrations of Ba in the original herb (Figure 4.5 B), water had an extraction efficiency average (±1SD) of 5.2 ± 0.7% from the original herb. Whereas 0.4 ± 0.1%, 0.5 ± 1.5% and 0.3 ± 0.1% was extracted respectively for 60 %v/v, 80 %v/v, and 100% ethanol solutions. These results show that the higher the ethanol concentration used within the extraction solvent, the less total Ba is transferred from the original plant into the extract.

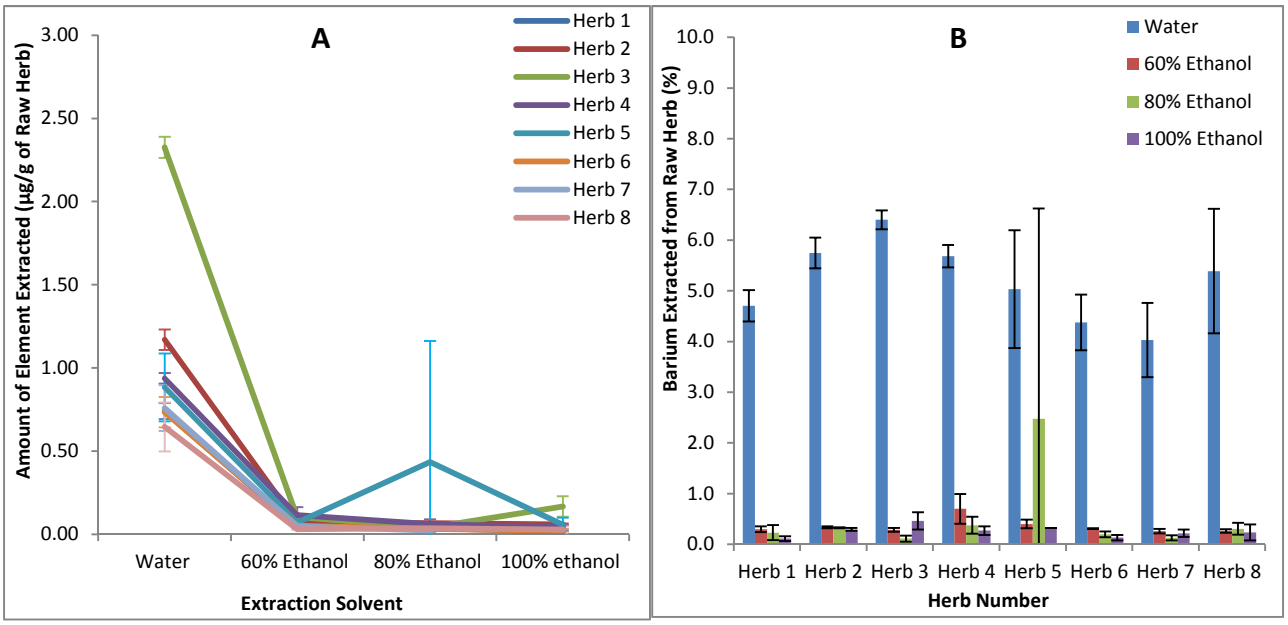


Figure 4.5 (A) Extraction of Ba from SJW powdered herbs in different solvents and (B) Percent of Ba extracted from original raw herb. Uncertainty is ±1SD.

When considering the amount of Ba in the dried extract (Figure 4.6 A), it can be seen that the 100% water dried extract had a significantly higher concentration of the element compared to the ethanol dry extracts. The water dry extracts contain between 9-29 µg/g Ba whereas the 60 %v/v, 80 %v/v, and 100% ethanol dry extracts contain 0.4-1.0 µg/g, 0.3-0.9 µg/g and 0.4-1.8 µg/g, respectively. There is no significant difference between the ethanol extracts and this indicates a decrease on average of 95% from 100% water to ethanol extracts. This is likely to be due to Ba salts having more affinity for water compared to the ethanol solvents.

Comparison of the dried extract concentrations to that of the total concentration of the original raw herb (Figure 4.6 B) shows that all four types of extract contain less Ba than the original herb. This illustrates that no preconcentration occurs with this element within dried extracts due to the extraction process. Interestingly, the concentration of Ba in the dry herb is close to that of the water dry extract (75 ± 7%). These results are slightly higher than that for SJW hot water infusions (56.6 ± 0.2%) by Kalny *et al.* [131], which may be due to stirring being used.

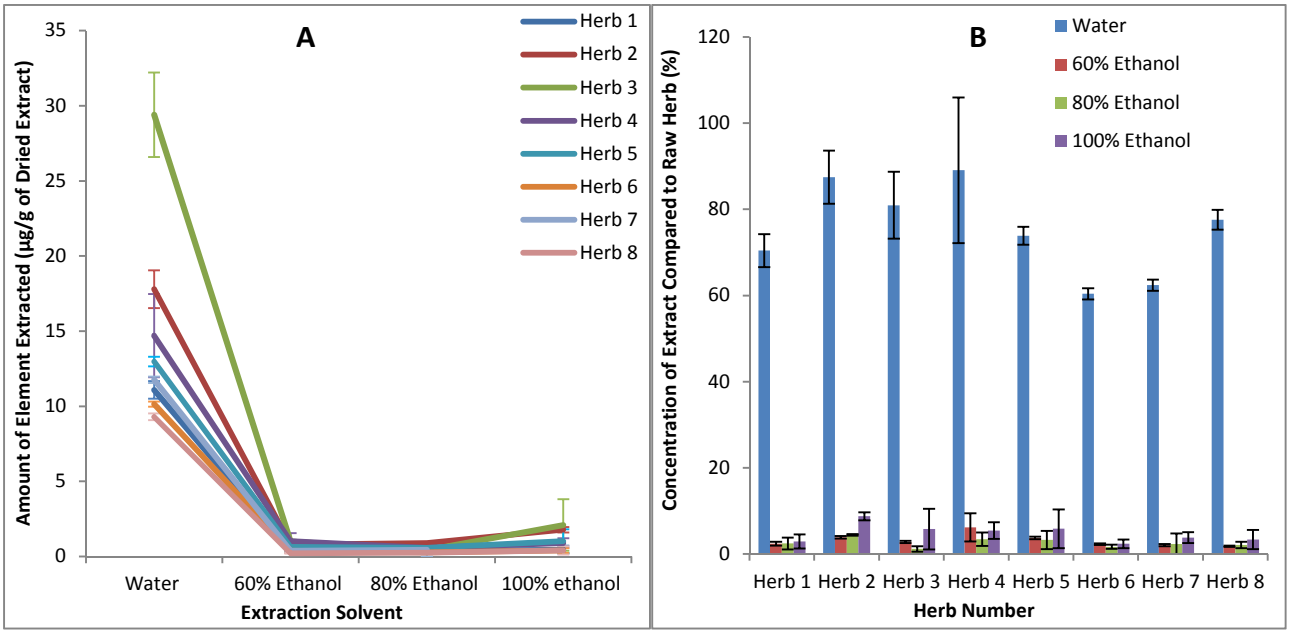


Figure 4.6 (A) Amount of Ba in dried extracts (B) Comparison of Ba extract concentration to original herb concentration. Uncertainty is ±1SD.

4.3.2.3 Calcium

Calcium is an essential element in plants and is present in large concentrations in plant cells [39] as it is used in numerous plant functions including alleviation of toxic metal effects [55, 56]. In humans a lack of Ca can induce rickets [210].

The extraction of different SJW powdered herbs with four solvents illustrate a general trend in Ca concentration despite varied geographical origin and collection processes being variable. The results (Figure 4.7 A) show that the highest concentrations of Ca are extracted with 100% water (740-1600 µg/g of original herb). These concentrations are lower compared to other studies (180 – 400 mg/g) [143, 157] but higher than the concentrations (648 ug/g – 712 ug/g) reported by Gomez *et al.* [125]. This may be due to the extraction process using a large volume of boiling water (100 – 250 ml) compared to small volume (20 ml) at room temperature. These levels decrease on average

by 77% when extracted with 60 %v/v ethanol (120-430 µg/g of original herb). The concentrations further decrease by 80% with the 80 %v/v ethanol solvent (24-114 µg/g of original herb) and continues to decrease by 61% with 100% ethanol (11-30 µg/g of original herb). These results show that between the solvents, 100% water contained the most Ca compared to the ethanol solvents. Comparing these values to the total concentrations of Ca in the original herb (Figure 4.7 B), water had an extraction efficiency average (± 1SD) of 20 ± 3% from the original herb. Whereas 4.4 ± 0.6%, 0.8 ± 0.3% and 0.3 ± 0.1% was extracted, respectively, for the 60 %v/v, 80 %v/v, and 100% ethanol solutions. These results show that the higher the ethanol concentration used within the extraction solvent, the less total calcium is transferred from the original plant into the extract.

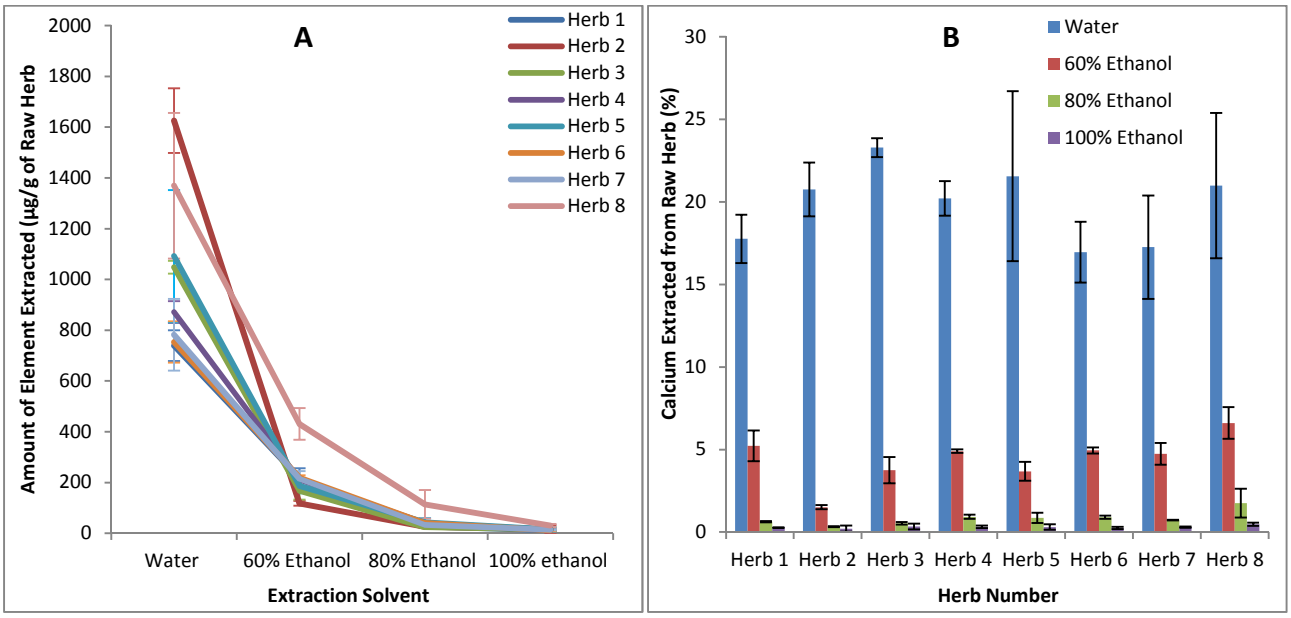


Figure 4.7 (A) Extraction of Ca from SJW powdered herbs in different solvents and (B) Percent of Ca extracted from original raw herb. Uncertainty is ±1SD.

When considering the amount of Ca in the dried extract (Figure 4.8 A), it can be seen that the 100% water extract has a significantly higher concentration of the element compared to the ethanol extracts. The water extracts contain between 10-24 mg/g calcium whereas the 60 %v/v, 80 %v/v and 100% ethanol solutions contain 1.3-2.9 mg/g, 0.3-0.6 mg/g and 0.2-0.5 mg/g, respectively. This is likely to be due to free Ca and Ca salts being able to move more freely in the water compared to the ethanol solvents.

Comparison of the dried extract concentrations to that of the original raw herb (Figure 4.8 B) shows that the water extract contains more Ca per gram whilst the three ethanol extracts contain less Ca than the original herb. This illustrates that preconcentration of Ca occurs in the dried water extract

up to 3 times. Water dry extracts are not currently used by manufacturers or consumers for tablet or capsule formulations. The ethanol extractions show no preconcentration of this element due to the extraction process.

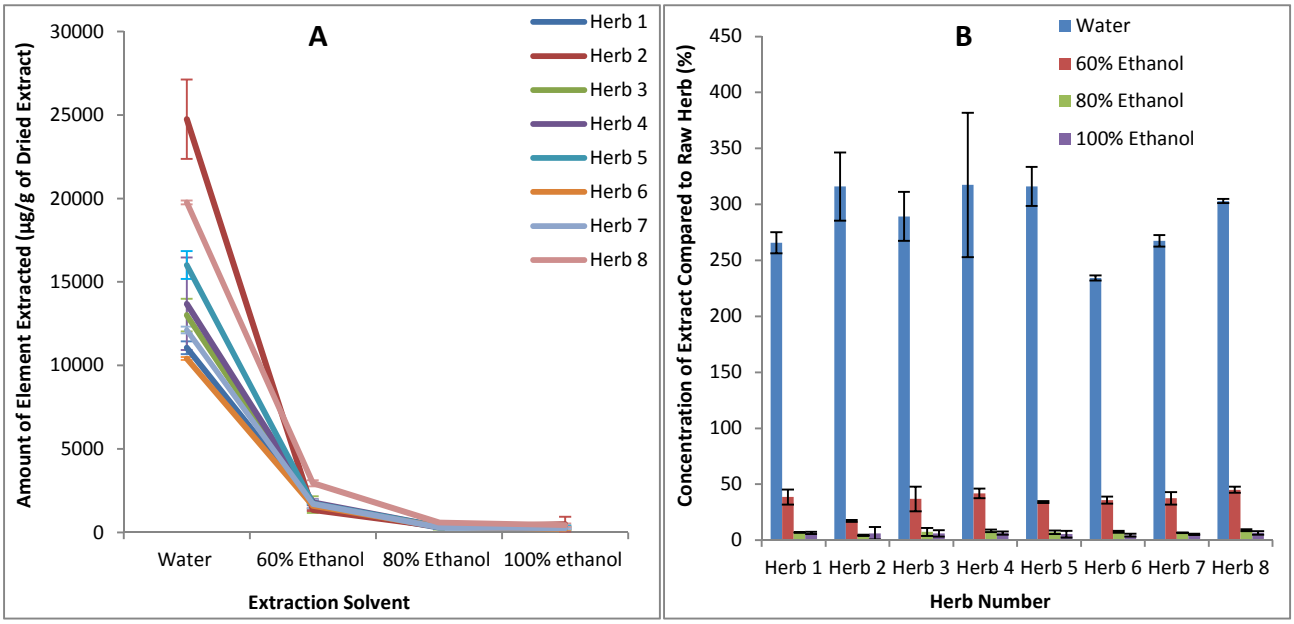


Figure 4.8 (A) Amount of Ca in dried extracts (B) Comparison of Ca extract concentration to original herb concentration. Uncertainty is ±1SD.

4.3.2.4 Cadmium

In high concentrations Cd is toxic to plants [40] and its essentiality is under investigation as it has been shown that Cd is involved with unknown enzymes that induce cysteine and methionine synthesis in soybeans [211]. Cadmium is also known to be toxic to humans [212]. The species *Hypericum perforatum* are known hyper-accumulators of Cd [66]. Cadmium has been shown to complex with flavonoids such as quercetin and in doing so, increased its anti-bacterial properties compared to quercetin alone [213].

The extraction of different SJW powdered herbs with four solvents illustrated a general trend despite varied geographical origin. Cadmium levels were above the LOQ for samples H1, H2, H3, H4 and H5 in 100% H_2O whereas H6, H7 and H8 were below the LOQ (Table 4.6). All other herbal extracts measured were below the LOD for Cd using 60 %, 80 % and 100 %v/v ethanol. The levels of Cd found in the water extraction are lower compared to Gomez *et al.* [126], this may be due to the water used in this extraction was at room temperature whereas Gomez *et al.* used boiling water.

Table 4.6 Cadmium transferred from SJW raw herbs in water[1]

Herb №	Water µg/g[2] ± 1SD
Herb 1	0.04 ± 0.01
Herb 2	0.16 ± 0.01
Herb 3	0.07 ± 0.02
Herb 4	0.0625 ± 0.0002
Herb 5	0.05 ± 0.02
Herb 6	0.046 ± 0.004
Herb 7	0.03 ± 0.01
Herb 8	0.02 ± 0.01

[1] µg of Cd transferred/ g of original raw herb

Comparing these values to the total concentrations of Cd in the original herb (Figure 4.9), water had an extraction efficiency average of 20 ± 3% from the original herb which agrees with Kalny *et al.* [131]. These results show that the higher the ethanol concentration used within the extraction solvent, the less total Cd is transferred from the original plant into the extract. When considering toxic elements such as Cd that may have been taken up by a plant due to industrial pollution, only a small fraction would be extracted from the dry herb when preparing a formulated herbal product.

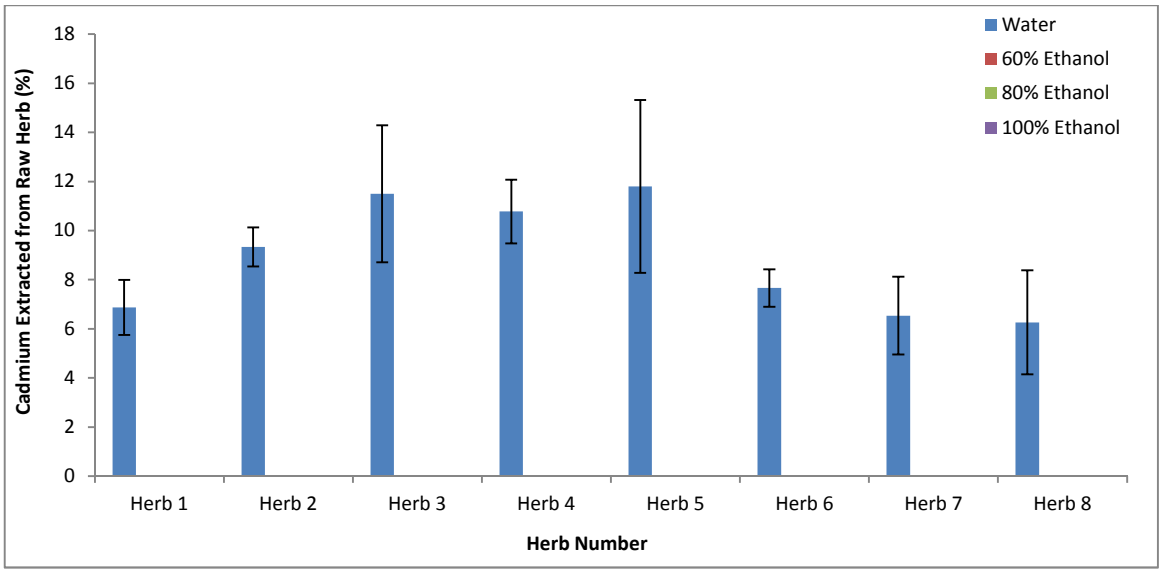


Figure 4.9 Percent of Cd extracted from original raw herb. Uncertainty is ±1SD.

When considering the amount of Cd in the dried extract (Table 4.7), it can be seen that the 100% water extract has a significantly higher concentration of the element compared to the ethanol extracts. The water extracts contain between 0.3-2.5 µg/g Cd whereas the ethanol extraction

concentrations are below the LOD. This is likely to be due to free Cd or Cd salts being able to move more freely in the water compared to the ethanol solvents.

Table 4.7 Amount of Cd in dried water extracts

Herb №	Total µg/g ± 1SD	Water µg/g ± 1SD
Herb 1	0.561 ± 0.004	0.57 ± 0.03
Herb 2	1.73 ± 0.02	2.5 ± 0.3
Herb 3	0.57 ± 0.02	0.9 ± 0.2
Herb 4	0.58 ± 0.07	1.0 ± 0.2
Herb 5	0.445 ± 0.001	0.8 ± 0.1
Herb 6	0.59 ± 0.02	0.63 ± 0.02
Herb 7	0.47 ± 0.02	0.47 ± 0.05
Herb 8	0.34 ± 0.01	0.30 ± 0.04

[1] **ND = Below LOD, Uncertainty is represented by ±1SD.**

Comparison of the dried extract concentrations to that of the original raw herb (Figure 4.10) shows that the water extract contains more Cd per gram whilst the ethanol extracts contain less Cd than the original herb as the concentrations fall below the LOD. This illustrates that although preconcentration occurs in the dried water extract, this form of extract is not knowingly used by manufacturers or consumers. The ethanol extractions show no preconcentration of this element due to the extraction process; however, those extracts manufactured with a lower percentage of ethanol (<60%) could contain more Cd than those produced with a higher ethanol percentage. Although only approximately 2% of Cd is transferred from the total, the amount of dry extract recovered was small resulting in a higher concentration in the dry water extract. Thus, depending on where the SJW is collected and if in a polluted area, the method of preparing the extract may be used to reduce the level of Cd in the final dry extract.

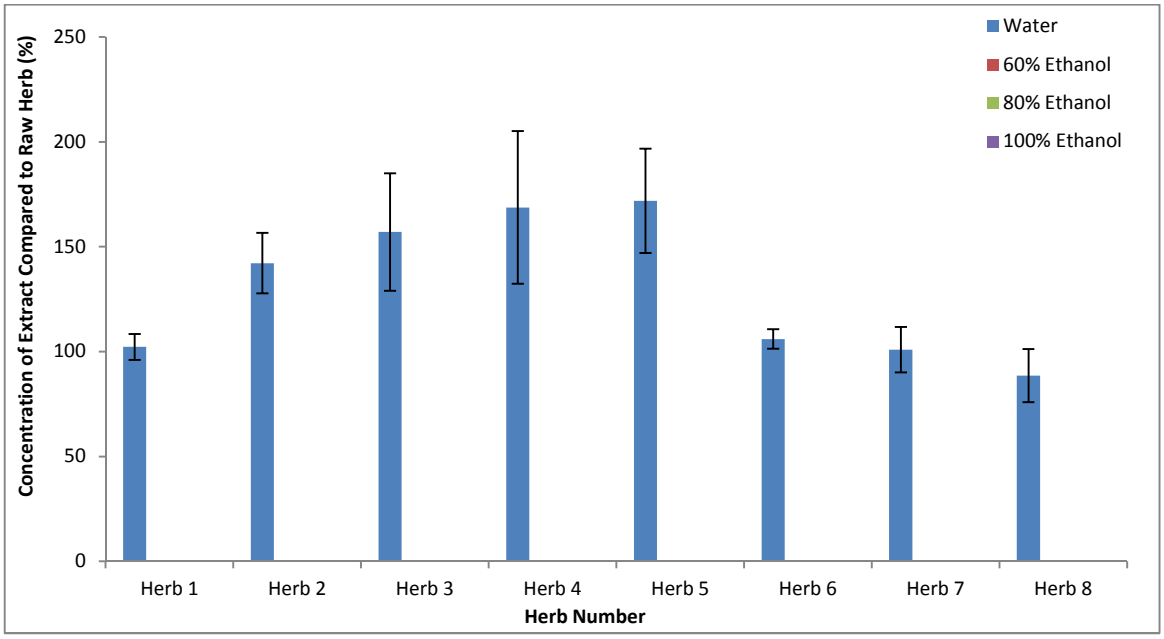


Figure 4.10 Comparison of Cd extract concentration to original herb concentration. Uncertainty is ±1SD.

4.3.2.5 Cobalt

Cobalt is essential for microorganisms in fixing N_2 but its essentiality is questionable amongst higher plants as it may aid chlorophyll formation [40]. Within humans Co is an essential element and is a constituent of Vitamin B_{12}. Cobalt has been shown to complex with flavonoids such as quercetin and rutin [214] and in doing so increased anti-oxidant properties compared to quercetin alone [215].

All values obtained for Co were below the LOQ, therefore these results are only utilised to observe the general trend between solvents. Cobalt levels were above the LOD for herbs H2, H3, H4, H5 and H7 for 100% water. Herbs H1, H2, H3, H4, H5, H6 and H7 were above the LOD with 60 %v/v ethanol (Table 4.8) and herb H7 with 100% ethanol. All other herbs or solvents used were present in concentrations below LODs. Comparing the values for herb H7 to the total concentrations of Co in the original herb, water had an extraction efficiency of 22 ± 5% and 60 %v/v ethanol had an efficiency of 24 ± 5%. These results show that of all the solvents utilised, 60 %v/v ethanol transfers the most Co from the original herb. This was due to the original dry herbs of samples H1, H2, H3, H4, H5 and H6 having Co concentrations below the LOD, however, upon extraction with 60 %v/v ethanol, the concentrations were above the LOD.

Table 4.8 Cobalt transferred from SJW raw herbs in different solvents from original herb

Herb №	100% Water[2] µg/g[1] ± 1SD	%transfer efficiency	60 %v/v Ethanol[2] µg/g[1] ± 1SD	%transfer efficiency	80 %v/v Ethanol[2] µg/g[1] ± 1SD	% transfer efficiency
Herb 1	ND	UN	0.12 ± 0.02	UN	ND	ND
Herb 2	0.126 ± 0.004	UN	0.10 ± 0.03	UN	ND	ND
Herb 3	0.12 ± 0.01	UN	0.12 ± 0.04	UN	ND	ND
Herb 4	0.14 ± 0.02	UN	0.17 ± 0.02	UN	0.09 ± 0.02	UN
Herb 5	0.13 ± 0.02	UN	0.12 ± 0.02	UN	ND	ND
Herb 6	ND	UN	0.11 ± 0.01	UN	ND	ND
Herb 7	0.10 ± 0.02	21 ± 5	0.12 ± 0.01	24 ± 5	ND	ND
Herb 8	ND	ND	ND	ND	ND	ND

[1] µg of Co/ g of original raw herb
[2] ND = Below LOD, UN = Unknown due to original herb below LOD

Comparison of the dried extract concentrations to that of the original raw herb (Table 4.9), herb H7 shows that the dry water extract has a concentration 3 times that of dry herb and the 60 %v/v ethanol dry extract has a concentration 2 times that of the dry herb. Interestingly, the 60 %v/v dry ethanol extract preconcentrated Co enough to be detectable in the extracts of all herbs except for levels found for herb 8 which were below the LOD. This is similar for the 100% water extracts of H2, H3, H4 and H5, whereby concentrations of Co are detected The results indicate that that preconcentration occurs in the 60 %v/v ethanol solvent, however, due to the very low levels of Co found the analysis should be carried out with more sample or an alternative instrument (such as GFAAS or ICP-MS) in order to determine the true pattern of extraction for this element. These levels are much lower than those found by Naeem *et al.* [137] (53.8 ± 0.9 µg/g). This may be due to the extraction time as this study was carried out over 60 minutes whereas Naeem *et al.* extraction was over 3 days.

Table 4.9 Concentration of Co in dried extract and the comparison to total Co in original herb

	100% Water[2]		60 %v/v Ethanol[2]		80 %v/v Ethanol[2]	
Herb №	**µg/g[1] ± 1SD**	**%transfer efficiency**	**µg/g[1] ± 1SD**	**%transfer efficiency**	**µg/g[1] ± 1SD**	**% transfer efficiency**
Herb 1	ND	UN	1.0 ± 0.2	UN	ND	ND
Herb 2	0.92 ± 0.08	UN	1.2 ± 0.4	UN	ND	ND
Herb 3	1.7 ± 0.5	UN	1.2 ± 0.2	UN	ND	ND
Herb 4	2.2 ± 0.6	UN	1.5 ± 0.1	UN	0.8 ± 0.2	UN
Herb 5	1.9 ± 0.4	UN	1.07 ± 0.03	UN	ND	ND
Herb 6	ND	UN	0.83 ± 0.06	UN	ND	ND
Herb 7	1.6 ± 0.05	340 ± 70	0.9 ± 0.1	190 ± 50	ND	ND
Herb 8	ND	ND	ND	ND	ND	ND

[1] ND = Below LOD, UN = Unknown due to original herb below LOD. Uncertainty is represented by ±1SD.

4.3.2.6 Chromium

Chromium is not an essential element to plants [40] but is essential for humans as it potentiates insulin action [216] and also effects cholesterol synthesis [43]. However, the speciation of Cr is important. The most common form, Cr (III), is useful biologically, however, in high concentrations can be harmful. In comparison, Cr (VI) is much more toxic and is a carcinogen [43]. Chromium is able to bind to flavonoids such as quercetin [217].

Only sample H2 extracted by 100% H_2O was above the LOQ. Therefore these results are only utilised to observe the general trend between solvents. Chromium levels were below the LOD for all ratios of ethanol solvents (Table 4.10). Comparing these values to the total concentrations of Cr in the original herb, water had an extraction efficiency average of 8% from the original raw herb. These results show that of all the solvents utilised, 100% water transfers the most Cr from the original herb.

Table 4.10 Chromium transferred from SJW raw herbs in different solvents from original herb

	100% Water[2]	
Herb №	**µg/g[1] ± 1SD**	**%transfer efficiency**
Herb 1	ND	ND
Herb 2	0.11 ± 0.02	8 ± 2
Herb 3	0.05 ± 0.02	8 ± 3
Herb 4	ND	ND
Herb 5	ND	ND
Herb 6	ND	ND
Herb 7	ND	ND
Herb 8	ND	ND

[1] µg of Cr/ g of original raw herb
[2] ND = Below LOD

When considering the amount of Cr in the dried extract (Table 4.11), it can be seen that the 100% water extract has a higher concentration of the element compared to the ethanol extracts. The water extracts with levels above the LOD contain 0.7-1.7 µg/g Cr whereas the ethanol extracts concentrations were below LOD. This is likely to be due to Cr salts being able to move more freely in the water compared to the ethanol solvents.

Comparison of the dried extract concentrations to that of the original raw herb shows that the water extract contains similar or more Cr per gram. The ethanol extractions show less Cr than the original sample. This shows that although preconcentration occurs in the dried water extract, this form of extract is not knowingly used by manufacturers or consumers.

Table 4.11 Concentration of Cr in dried extract and the comparison to total Cr in original herb

	100% Water[1]	
Herb №	**µg/g ± 1SD**	**Extract to Total %**
Herb 1	ND	ND
Herb 2	1.7 ± 0.3	120 ± 20
Herb 3	0.7 ± 0.2	110 ± 30
Herb 4	ND	ND
Herb 5	ND	ND
Herb 6	ND	ND
Herb 7	ND	ND
Herb 8	ND	ND

[1] ND = Below LOD, Uncertainty is represented by 1SD

4.3.2.7 Copper

Copper is an essential element in both plants and humans. In plants the element is involved with enzymes for important processes such as photosynthesis, carbohydrate and nitrate metabolism as well as disease resistance [43]. In humans Cu forms the basis of several metaloenzymes and is involved in haemoglobin synthesis [43, 216].

The extraction of different SJW powdered herbs with four solvents illustrate a general trend despite varied geographical origin. The results (Figure 4.11 A) show that the highest concentrations of Cu are extracted with 60 %v/v ethanol (2.5-3.7 µg/g of original herb). These levels decrease on average by 40% when extracted with 100% water (1.7-3.0 µg/g of original herb). The concentrations further decrease by 45% with the 80 %v/v ethanol solvent (1.0-1.8 µg/g of original herb) and continues to decrease by 73% with 100% ethanol (0.3-0.6 µg/g of original herb). These results show that between the solvents, 60 %v/v ethanol transferred the most Cu compared to the other solvents.

The 100% ethanol extracts for samples H1, H3, H4, H5, H6, H7 and H8 are below the LOQ for Cu, therefore these results are only utilised to observe the general trend between solvents. Also noted is that Cu does not follow the general transfer pattern of the other elements in which 100% water extracts the most with a downwards trend towards 100% ethanol. Copper is a particularly good element at forming metal complexes with bioactive compounds such as flavonoids rutin and quercetin [109, 150, 214] and has been shown to increase antioxidant and anti-inflammatory properties. Copper is able to form strong complexes due to its small ionic radius and also ligand field effects [218]. Complexes of Cu with the bioactive compounds may be a reason for this increased transfer at 60 %v/v ethanol. The levels found in the water extract are much lower than Suliburska *et al.* (0.6 mg/g) [143], and slightly lower than those found by Konieczynski *et al.* (3.6 µg/g) [132]. This may be due to boiling water being used in their extraction compared to room temperature water.

Comparing these values to the total concentrations of Cu in the original herb (Figure 4.11 B), water had an extraction efficiency average (±1SD) of 19 ± 3% from the original herb. Whereas 26 ± 3%, 11 ± 2% and 3.0 ± 0.4% was extracted for the 60 %v/v ethanol, 80 %v/v ethanol and 100% ethanol solutions, respectively. These results show that the optimum transfer of Cu is not with 100% water or 100% ethanol, but towards a lower percentage of ethanol. The extraction efficacy of water is lower than that of other studies [131, 143, 157] as they report an efficiency of 47%, 53% and 54% with water. This may be due to boiling water being used in their extraction compared to room temperature.

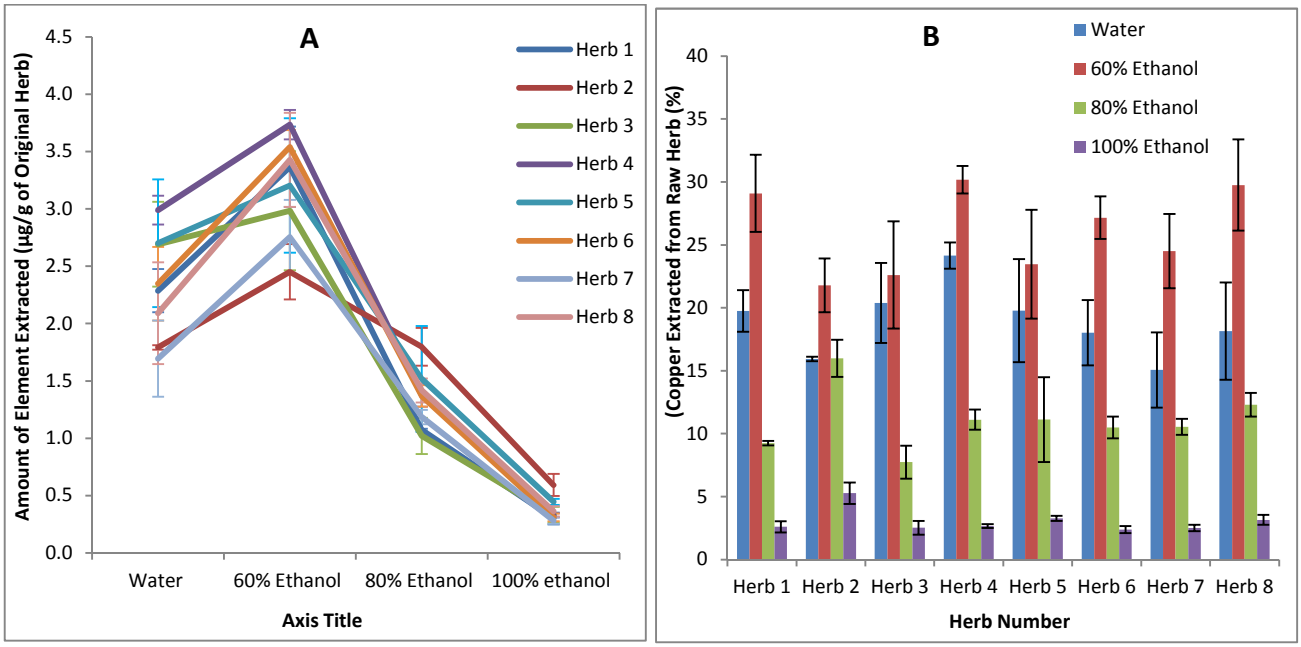


Figure 4.11 (A) Extraction of Cu from SJW powdered herbs in different solvents and (B) Percent of Cu extracted from original raw herb. Uncertainty is represented by ±1SD.

When considering the amount of Cu in the dried extract (Figure 4.12 A), it can be seen that the 100% water extract has a higher concentration of the element compared to the ethanol extracts. The water extracts contain between 26-47 µg/g whereas the 60 %v/v ethanol, 80 %v/v ethanol and 100% ethanol solutions contain 22-32 µg/g, 10-25 µg/g and 5-18 µg/g, respectively. This is likely to be due to free Cu and Cu salts being able to move more freely in the water compared to the ethanol solvents. Although previously the transfer efficiency was better with the 60 %v/v ethanol, this shows that in the dried extract more Cu is found in the 100% dry water extract compared to the ethanol extracts. Assessment of the dried extract concentrations to that of the original raw herb (Figure 4.12 B) shows that the water extract, 60 %v/v ethanol and 80 %v/v ethanol contains more Cu per gram whilst the 100% ethanol extract contains less than the original herb. Although preconcentration occurs in the dried water extract, this form of extract is not knowingly used by manufacturers or consumers. The 60 %v/v ethanol solvent is a popular choice of manufacturers during their extraction of bioactive compounds from SJW as it has been shown that this percentage of alcohol to water is able to extract more bioactive compounds from the plant than a higher or lower percentage [191, 207]. As Cu complexes with flavonoids such as rutin and quercetin have shown altered bioactivity in solution studies [91, 109, 150], this could have implications for the therapeutic dose. Therefore, this study indicates that products that use a dried extract originally produced with 80 %v/v or less ethanol, the concentrations of Cu could be higher than in the original raw material and potentially contain the presence of flavonoid-Cu complexes that could alter potency. The levels of Cu in the ethanol extracts are lower than those reported for methanol

extracts by Naeem *et al.* (210 µg/g) [137]. This may be due to the difference in extraction time where they macerated over 3 days compared to stirring for 1 hour.

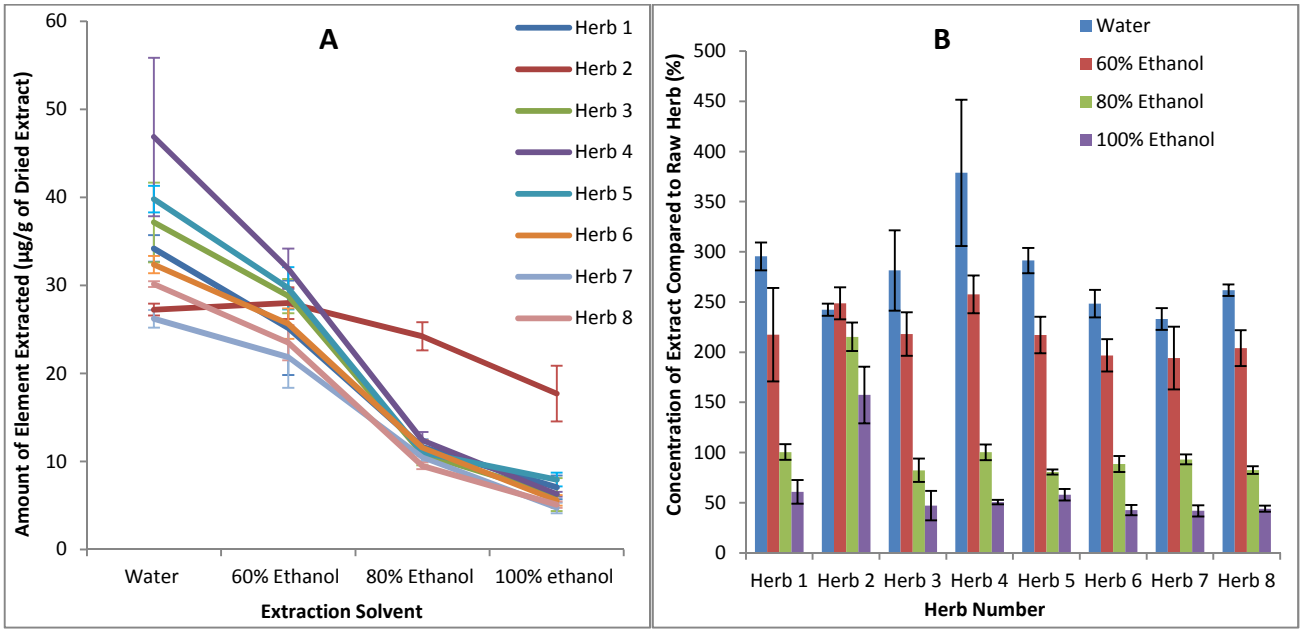


Figure 4.12 (A) Amount of Cu in dried extracts (B) Comparison of Cu extract concentration to original herb concentration. Uncertainty is represented by ±1SD.

4.3.2.8 Iron

Iron is an essential element for plants as it is involved in maybe metabolic processes such as photosynthesis (very concentrated in the chloroplasts) and metabolism of nucleic acids [43]. Iron is also essential to humans as it plays a role in several enzymes and is the main constituent in haemoglobin [43]. Iron has been shown to bind to compounds such as chlorogenic acid [219], rutin [109, 150, 220, 221] and quercetin [109, 150, 221] and has been shown to increase the antioxidant capacity of such compounds as well as show some pro-oxidant activity [109]. The extraction of different SJW powdered herbs with four solvents illustrate that six of the eight herbs follow a general trend. The results (Figure 4.13 A) show that the highest concentrations of Fe are extracted with 100% water (1-3 µg/g of original herb). These concentrations are lower compared to Gomez *et al.* [126], this may be due to the water used in this extraction was at room temperature with 20 ml whereas Gomez *et al.* used 200 ml boiling water. Concentrations with water agree with those found by Konieczynski *et al.* [118] (1.7-7.3 µg/g). Iron levels decrease on average by 70% when extracted with 60 %v/v ethanol (0.6-2.7 µg/g of original herb) with little difference seen between the 80 %v/v and 100% ethanol solvents. These results show that between the solvents, 100% water contained the most Fe compared to the ethanol solvents. The concentrations for herb H1 in 60 %v/v ethanol appear high as two samples of the five analysed are particularly high (8.6 and 2.9 µg/g compared to

other three samples 0.5 – 0.8 µg/g). Due to two samples being high, the Dixons Q-test does not discriminate the 8.6 µg/g as an outlier; therefore to reduce uncertainty in the value more replicates would be needed. A similar occurrence is seen for Herb 8 with 80 %v/v ethanol where one sample is higher (3.2 µg/g) compared to the others (0.6-1.0 µg/g). Linking these values to the total concentrations of Fe in the original herb (Figure 4.13 B), water had an extraction efficiency average (±1SD) of 1.6 ± 0.6% from the original herb. This is lower than that reported by Kalny *et al.* (17%) [131] and Oledzka and Szyszkowska (7.8%) [157]. Whereas 0.8 ± 1%, 0.5 ± 0.4% and 0.4 ± 0.3% was extracted respectively for the 60 %v/v, 80 %v/v, and 100% ethanol solutions. These results show that the 80 %v/v and 100% ethanol solutions used appear to extract similar concentrations of Fe.

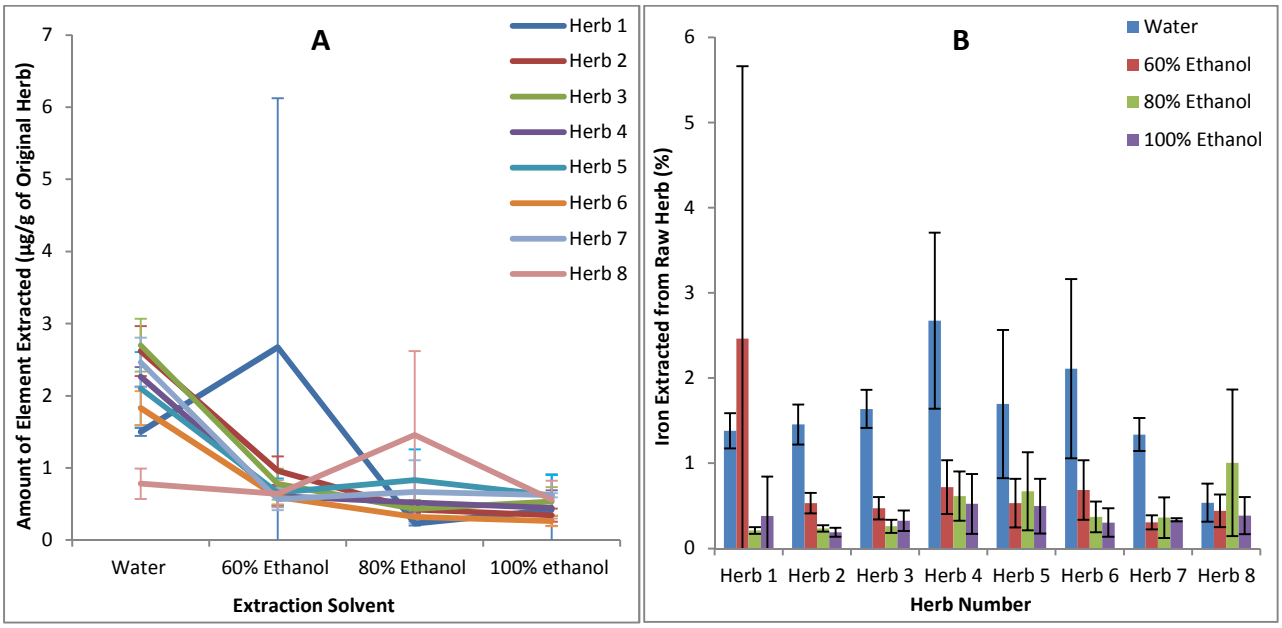


Figure 4.13 (A) Extraction of Fe from SJW powdered herbs in different solvents and (B) Percent of Fe extracted from original raw herb. Uncertainty is represented by ±1SD.

When considering the amount of Fe in the dried extract (Figure 4.14 A), it can be seen that the 100% water extract has a higher concentration of the element compared to the ethanol extracts. The water extracts contain between 11-40 µg/g whereas the 60 %, 80 % and 100 %v/v ethanol solutions contain 4-11 µg/g, 3-10 µg/g and 4-12 µg/g, respectively. This is likely due to Fe salts being able to move more freely in the water compared to the ethanol solvents.

Comparison of the dried extract concentrations to that of the total concentration of the original raw herb (Figure 4.14 B) shows that all extracts contain less Fe per gram than the original herb. This illustrates that preconcentration does not occur through the extraction process with Fe. This may be due to Fe being bound within silica structures which causes the element to become less mobile.

Levels of Fe found in the ethanol extracts are lower than that reported by Naeem *et al.* (318 µg/g) [137]. This may be due to the extraction being carried out over 3 days as opposed to 60 minutes.

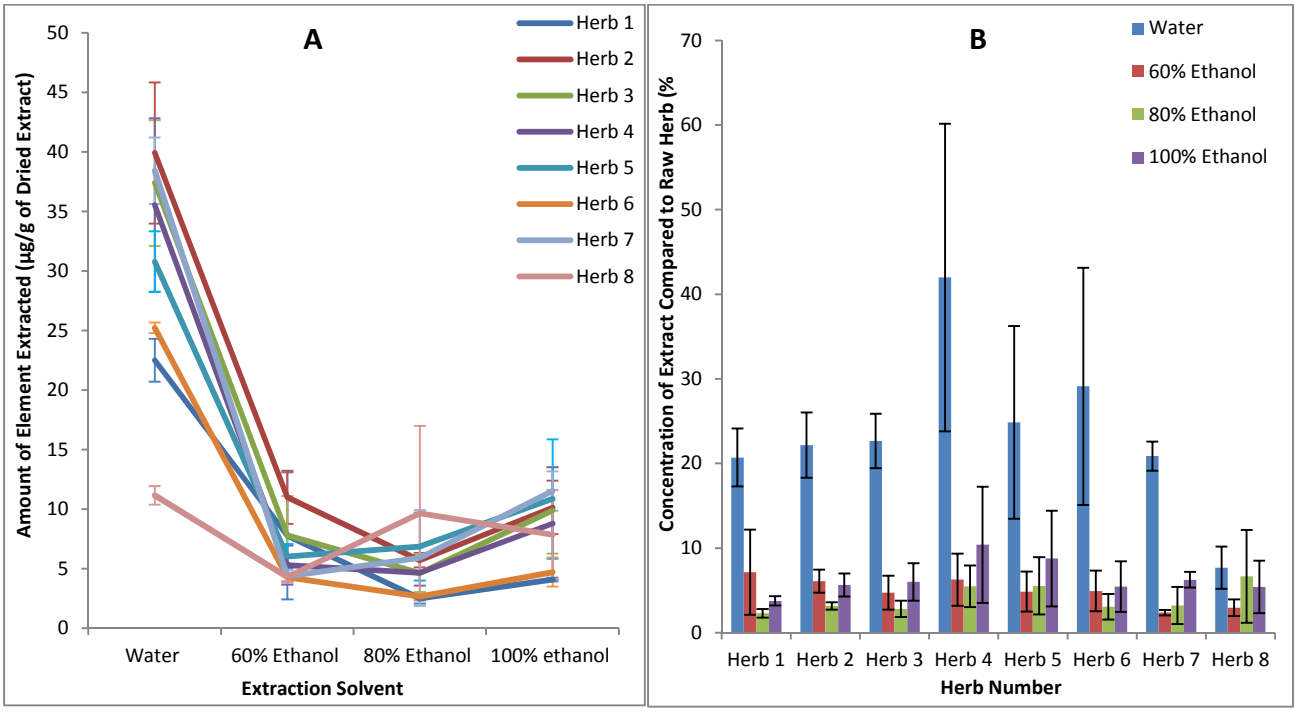


Figure 4.14 (A) Amount of Fe in dried extracts (B) Comparison of Fe extract concentration to original herb concentration. Uncertainty is represented by ±1SD.

4.3.2.9 Magnesium

Magnesium is an essential element within plants as it activates many enzymes and is a constituent of chlorophyll [39]. Magnesium is also essential in humans as it is involved in many biological processes including intestinal absorption, energy metabolism and cell proliferation [222].

The extraction of different SJW powdered herbs with four solvents illustrate a general trend despite varied geographical origin. The results (Figure 4.15 A) show that the highest concentrations of Mg are extracted with 100% water (410-590 µg/g of original herb). These concentrations are lower than those reported by Gomez *et al.* (123-371 mg/g) [125]. These levels decrease on average by 24% when extracted with 60 %v/v ethanol (226-561 µg/g of original herb). The concentrations further decreased on average by 71% with 80 %v/v ethanol (61-200 µg/g of original herb) followed by a decrease of 79% with 100% ethanol (12 to 40 µg/g of original herb). These results show that between the solvents, 100% water contained the most Mg compared to the ethanol solvents. Herb 8 follows a slightly different pattern, as it appears to show little difference between the 100% water and 60 %v/v ethanol solvents before the levels of Mg fall with the 80 %v/v and 100% ethanol.

Comparing these values to the total concentrations of Mg in the original herb (Figure 4.15 B), water had an extraction efficiency average (±1SD) of 36 ± 5 % from the original herb. This is lower than that reported by Oledzka and Szyszkowska (67%) [157]. Whereas 25 ± 3%, 7 ± 1% and 1.5 ± 0.2% was extracted respectively for the 60 %v/v, 80 %v/v, and 100% ethanol solutions. These results show that the levels of Mg do not differ significantly between 100% water and 60 %v/v ethanol for most herbs, but a large decrease is seen between the 60 %v/v and 80 %v/v ethanol across all herbs.

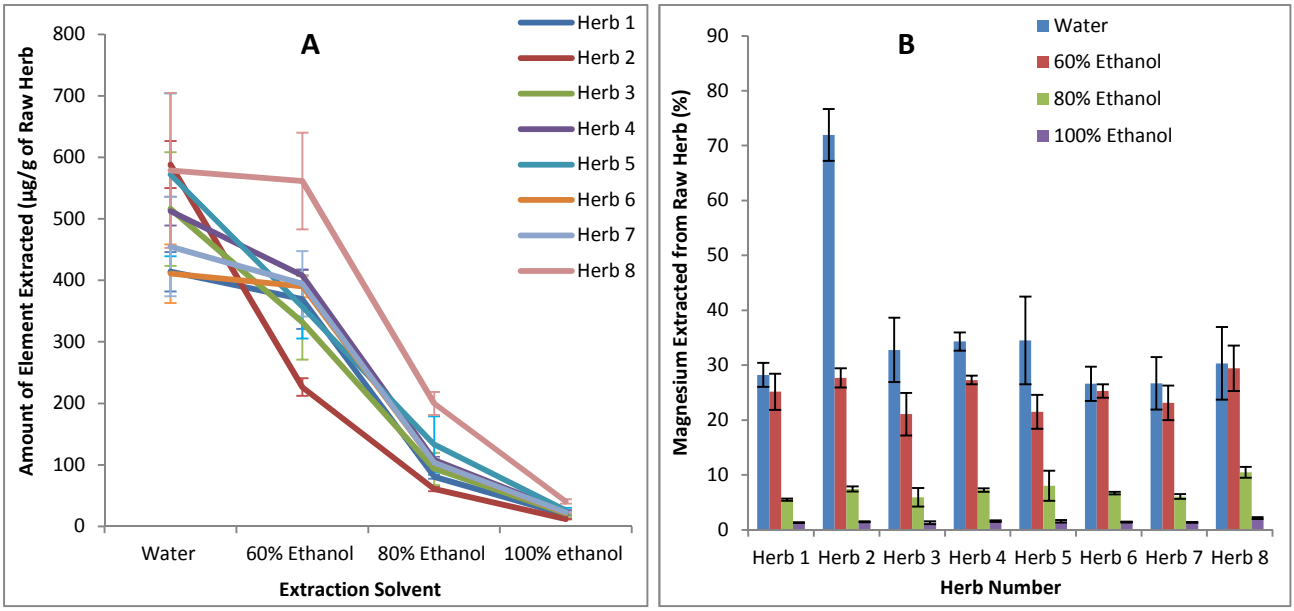


Figure 4.15 (A) Extraction of Mg from SJW powdered herbs in different solvents and (B) Percent of Mg extracted from original raw herb. Uncertainty is represented by ±1SD.

Examination of the amount of Mg in the dried extracts (Figure 4.16 A) show that the 100% water extract has a significantly higher concentration of the element compared to the ethanol extracts. The water extracts contain between6-9 mg/g Mg whereas the 60 %v/v, 80 %v/v, and 100% ethanol solutions contain 1.0-3.8 mg/g, 0.4-1.3 mg/g and 0.2-0.6 mg/g, respectively. This is likely to be due to free Mg and Mg salts being able to move more freely in the water compared to the ethanol solvents.

Comparison of the dried extract concentrations to that of the total concentration of the original raw herb (Figure 4.16 B) shows that the 100% water and 60 %v/v ethanol extracts contain more Mg per gram, whilst the other ethanol extracts contain less Mg, than the original herb. This illustrates that although preconcentration occurs in the dried water extract, this form of extract is not knowingly used by manufacturers or consumers. The 60 %v/v ethanol solvent is used by manufacturers to produce dried extracts of SJW. This study has demonstrated with this extraction process preconcentration of Mg occurs with an increase of 2-fold compared to the original herb.

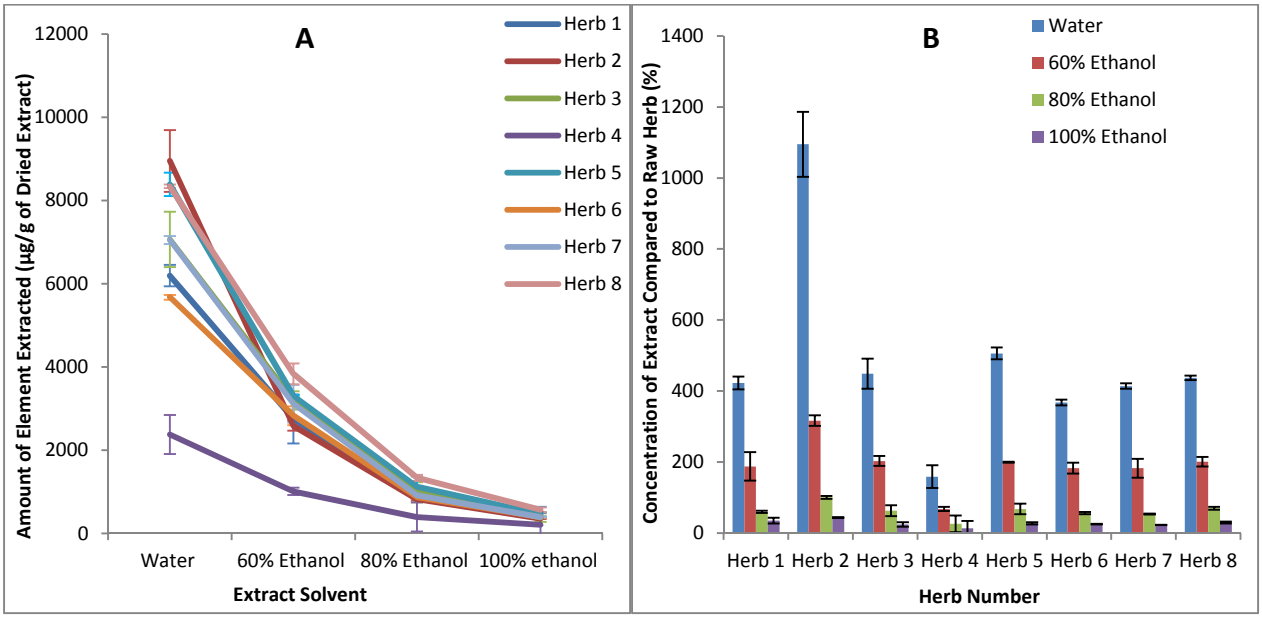


Figure 4.16 (A) Amount of Mg in dried extracts (B) Comparison of Mg extract concentration to original herb concentration. Uncertainty is represented by ±1SD.

4.3.2.10 Manganese

Manganese is essential to plants and is utilised in functions such as photosynthesis and nitrogen assimilation [43]. It is also essential within humans as it is involved with several enzymes and also aids gene expression and DNA stabilisation [43]. Manganese has been shown to complex with quercetin [213] and chlorogenic acid [223].

The extraction of different SJW powdered herbs with four solvents illustrate a general trend despite varied geographical origin. The results (Figure 4.17 A) show that the highest concentrations of Mn are extracted with 100% water (12-47 µg/g of original herb). These concentrations are much lower than those reported by Gomez *et al.,* (108-121 µg/g). These levels decrease on average by 68% when extracted with 60 %v/v ethanol (5-10 µg/g of original herb). The concentrations further decreased on average by 81% with 80 %v/v ethanol (0.8-1.9 µg/g of original herb) followed by a decrease of 71% with 100% ethanol (0.2-0.6 µg/g of original herb). These results show that between the solvents, 100% water contained the most Mn compared to the ethanol solvents.

Comparing these values to the total concentrations of Mn in the original herb (Figure 4.17 B), water had an extraction efficiency average of 26 ± 4% from the original herb. This is lower than that reported by Oledzka and Szyszkowska (58.7%). Whereas 8.3 ± 0.9%, 1.6 ± 0.4% and 0.4 ± 0.1% was extracted respectively for the 60 %v/v, 80 %v/v, and 100% ethanol solutions.

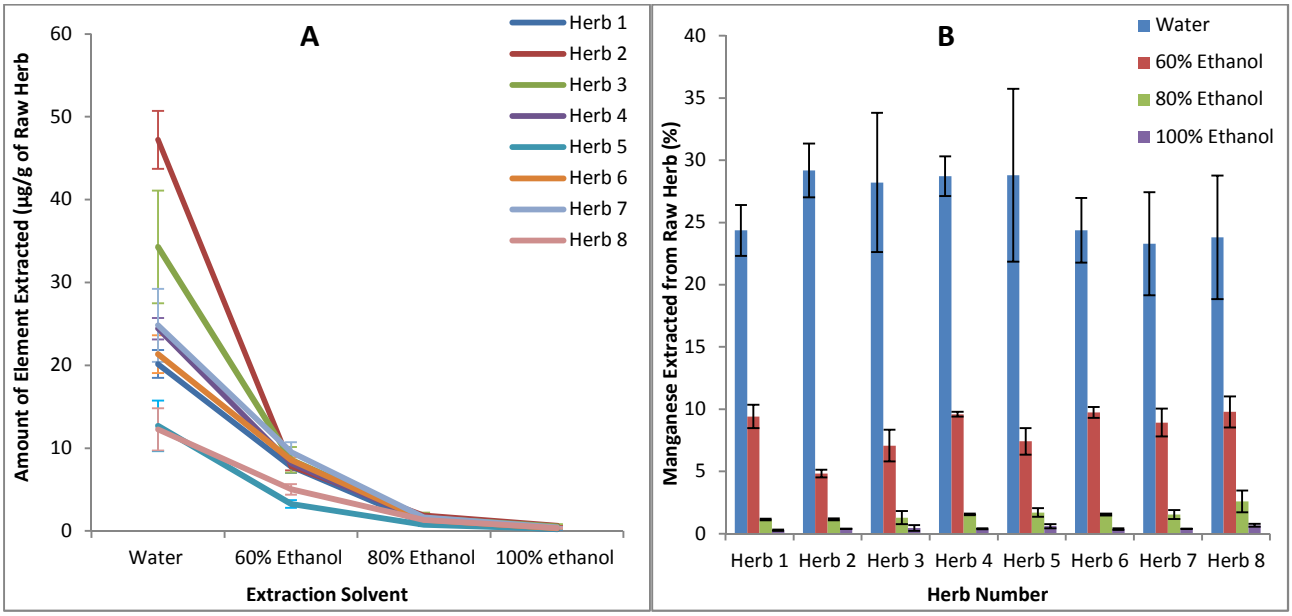


Figure 4.17 (A) Extraction of Mn from SJW powdered herbs in different solvents and (B) Percent of Mn extracted from original raw herb. Uncertainty is represented by ±1SD.

Examination of the amount of Mn in the dried extracts (Figure 4.18 A); show that the 100% water extract has a significantly higher concentration of the element compared to the ethanol extracts. The water extracts contain 177-719 µg/g whereas the 60 %v/v, 80 %v/v, and 100% ethanol solutions contain 30-89 µg/g, 6-25 µg/g and 5-19 µg/g, respectively. This is likely to be due to free Mn and Mn salts being able to move more freely in the water compared to the ethanol solvents.

Comparison of the dried extract concentrations to that of the total concentration of the original raw herb (Figure 4.18 B) shows that the 100% water extract contains more Mn per gram, whilst the other ethanol extracts contain less Mn, than the original herb. This illustrates that although preconcentration occurs in the dried water extract, this form of extract is not knowingly used by manufacturers or consumers. Interestingly, the Mn concentration seen in the 60 %v/v ethanol extract is on average 80% that found in the original herb. Therefore dried extracts produced with a solvent with lower ethanol: water potentially could be preconcentrated with Mn.

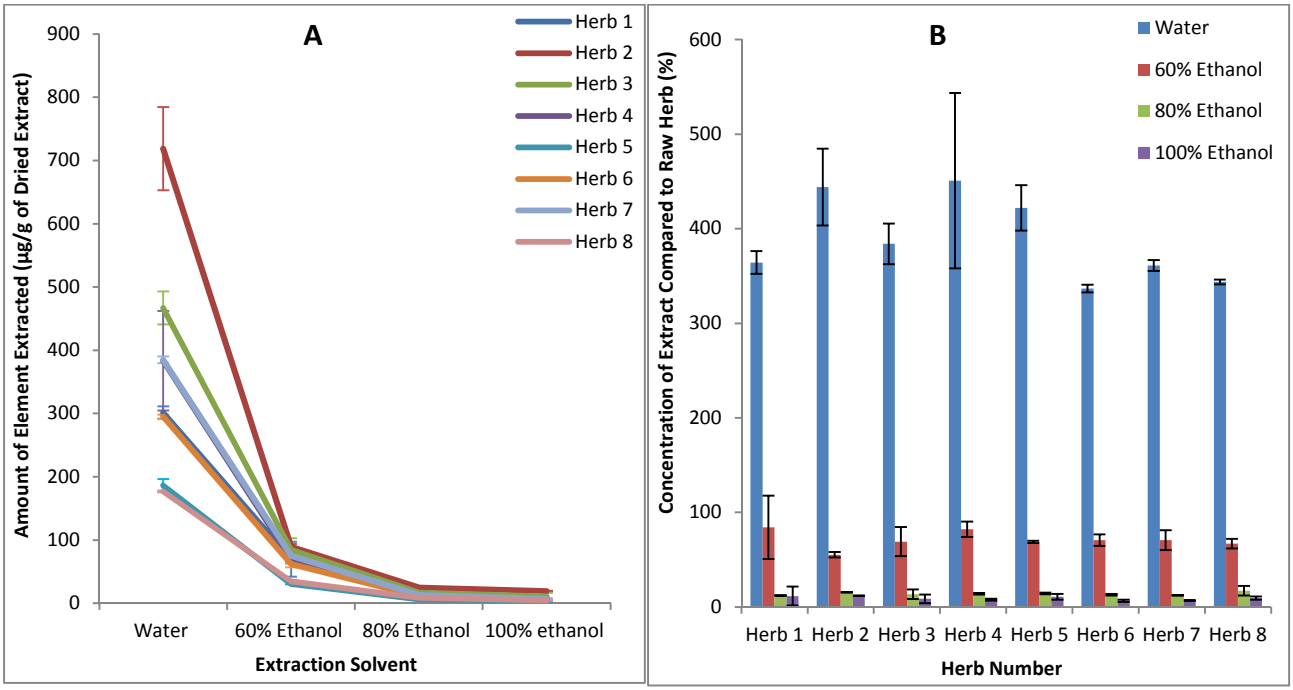


Figure 4.18 (A) Amount of Mn in dried extracts (B) Comparison of Mn extract concentration to original herb concentration. Uncertainty is represented by ±1SD.

4.3.2.11 Molybdenum

Molybdenum is essential to plants and is utilised within nitrogen metabolism [43]. This element is also an essential micronutrient in humans and is present in many enzymes, including those involved in the metabolism of purines and fats [43]. Molybdenum has been shown to complex with quercetin [224, 225].

All values obtained for Mo were below the LOQ, therefore these results are only utilised to observe the general trend between solvents. Concentrations of Mo were above the LOD for herbs H4 and H8 in 100% water. For all other herbs or solvents used the Mo concentration was below the LOD. Comparing these values (Table 4.12) to the total concentrations of Mo in the original herb, water had extraction efficiency of 28 ± 5% and 9 ± 3% respectively for H4 and H8.These results show that of all the solvents utilised, 100% water transfers the most Mo from the original herb.

Table 4.12 Molybdenum transferred from SJW raw herbs in 100% water

	100% Water[2]	
Herb №	**µg/g[1] ± 1SD**	**% transfer efficiency**
Herb 1	ND	ND
Herb 2	ND	ND
Herb 3	ND	ND
Herb 4	0.09 ± 0.01	28 ± 5
Herb 5	ND	ND
Herb 6	ND	ND
Herb 7	ND	ND
Herb 8	0.09 ± 0.03	9 ± 3

[1] µg of Mo/ g of original raw herb
[2] ND = Below LOD

Comparison of the dried extract concentrations to that of the total concentration of the original raw herb (Table 4.13) shows, for herbs H4 and H8, that the water extract contains more Mo per gram than the original herb. So far as we are aware, dried down water extracts are not used by manufacturers or consumers.

Table 4.13 Concentration of Mo in dried extract and the comparison to total Mo in original herb

	Total[1]	100% Water[1]	
Herb №	**µg/g ± 1SD**	**µg/g ± 1SD**	**Extract to Total %**
Herb 1	0.46 ± 0.02	ND	ND
Herb 2	ND	ND	ND
Herb 3	ND	ND	ND
Herb 4	0.33 ± 0.02	1.5 ± 0.5	400 ± 100
Herb 5	ND	ND	ND
Herb 6	0.38 ± 0.03	ND	ND
Herb 7	ND	ND	ND
Herb 8	1.04 ± 0.06	1.3 ± 0.2	130 ± 20

[1] ND = Below LOD

4.3.2.12 Nickel

The essentiality of Ni in all plants is under investigation as some reports suggest beneficial effects on growth in its presence; it is considered essential for higher plants [43]. Nickel is essential for humans and is utilised in fat metabolism [43]. Nickel has been shown to complex with quercetin and rutin [214].

The extraction of different SJW powdered herbs with four solvents illustrate a general trend despite varied geographical origin. The results (Figure 4.19 A) show that the highest concentrations of Ni are extracted with 100% water (0.6-2.3 µg/g of original herb). These concentrations are lower than those reported by Gomez *et al.,* (4-6 µg/g) [125].These levels decrease or are similar when extracted with 60 %v/v ethanol (0.6-1.8 µg/g of original herb). The concentrations then decrease on average by 59% with 80 %v/v ethanol (0.3-0.8 µg/g of original herb) with all of the herbs being below LOQ when 100% ethanol is used. Samples H7 and H8 had levels of Ni below the LOQ for 80 %v/v ethanol, therefore these are only utilised to observe the general trend between solvents These results show that between the solvents, 100% water generally contained the most Ni compared to the ethanol solvents.

Comparing these values to the total concentrations of Ni in the original herb (Figure 4.19 B), water had an extraction efficiency average of 36 ± 6% from the original herb. This is lower than that reported by Kalny *et al.,* (74%) [131]. Whereas 34 ± 4%, 14 ± 2% and 29 ± 0.8% was extracted respectively for the 60 %v/v and 80 %v/v ethanol solutions

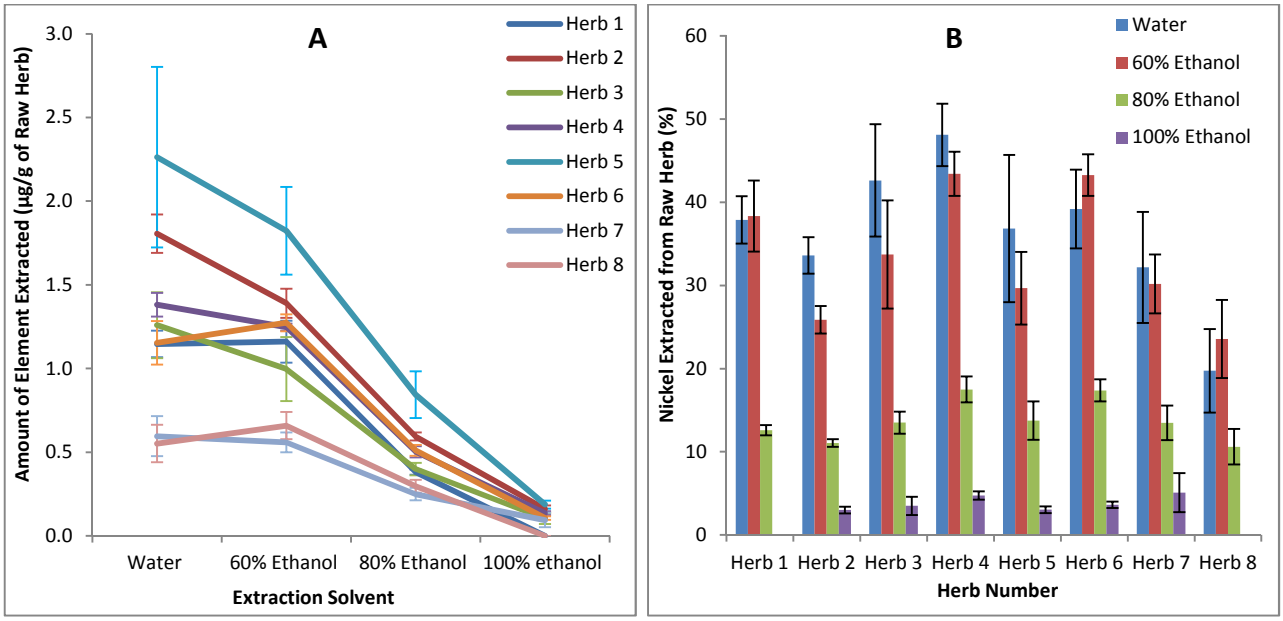


Figure 4.19 (A) Extraction of Ni from SJW powdered herbs in different solvents and (B) Percent of Ni extracted from original raw herb. Uncertainty is represented by ±1SD.

Examination of the amount of Ni in the dried extracts (Figure 4.20 A); show that the 100% water extract has a higher concentration of the element compared to the ethanol extracts. The water extracts contain between 8-33 µg/g Ni, whereas the 60 %v/v and 80 %v/v ethanol solutions contain 4-17 µg/g and 2-8 µg/g respectively. Concentrations for Ni in herbs H1 and H8 were below the LOD

while all other samples were below the LOQ for 100% ethanol. This is likely to be due to Ni salts being able to move more freely in the water compared to the ethanol solvents. The ethanol extracts are lower than that reported by Naeem *et al.* [137] (68.5 µg/g), which may be due to the extraction time being over 3 days rather than the 60 minutes utilised in this study.

Comparison of the dried extract concentrations to that of the total concentration of the original raw herb (Figure 4.20 B) shows that the 100% water, 60 %v/v and 80 %v/v ethanol extracts contain more Ni per gram than the original herb. Although preconcentration occurs in the dried water extract, this form of extract is not knowingly used by manufacturers or consumers. There is however, approximately a preconcentration of +180% in the 60 %v/v ethanol dry extract and +30% in the 80 %v/v ethanol extracts. Therefore potentially dried extracts produced with these solvents or with a lower ethanol percentage would contain more Ni per gram compared to the original herb. Overexposure to Ni can cause gastrointestinal upset, giddiness, headache, weariness and possible reproductive toxicity [48], thus the choice of extraction solvent can reduce the amount of Ni transferred to the final extract.

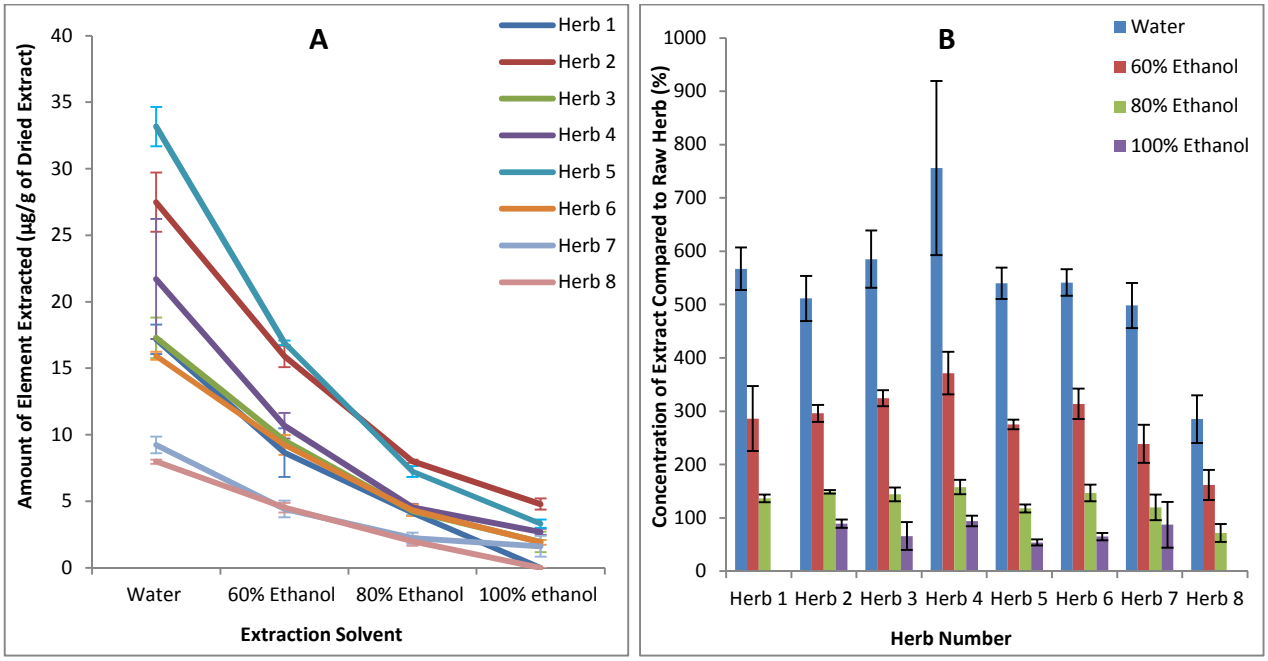


Figure 4.20 (A) Amount of Ni in dried extracts (B) Comparison of Ni extract concentration to original herb concentration. Uncertainty is represented by ±1SD.

4.3.2.13 Strontium

Strontium is not utilised by plants but its uptake is due to its similarity to Ca ions [43] and therefore it is readily found in plants. In humans, the biochemistry of Sr is little understood but it is needed in small quantities to ensure calcification of teeth and bones [43].

The extraction of different SJW powdered herbs with four solvents illustrate a general trend despite varied geographical origin. The results (Figure 4.21 A) show that the highest concentrations of Sr are extracted with 100% water (1.7-4.6 µg/g of original herb). These levels decrease on average by 87% with 60 %v/v ethanol (0.2-0.4 µg/g of original herb). The concentrations further decrease on average by 78% with 80 %v/v ethanol (0.03-0.08 µg/g of original herb) with similar levels when 100% ethanol is used (0.03-0.06 µg/g of original herb). These results show that between the solvents, 100% water contained the most Sr compared to the ethanol solvents.

Comparing these values to the total concentrations of Sr in the original herb (Figure 4.21 B), water had an extraction efficiency average of 11 ± 2% from the original herb. Whereas 1.4 ± 0.4% and 0.3 ± 0.2% and 0.2 ± 0.1% was extracted respectively for the 60 %v/v, 80 %v/v, and 100% ethanol solutions.

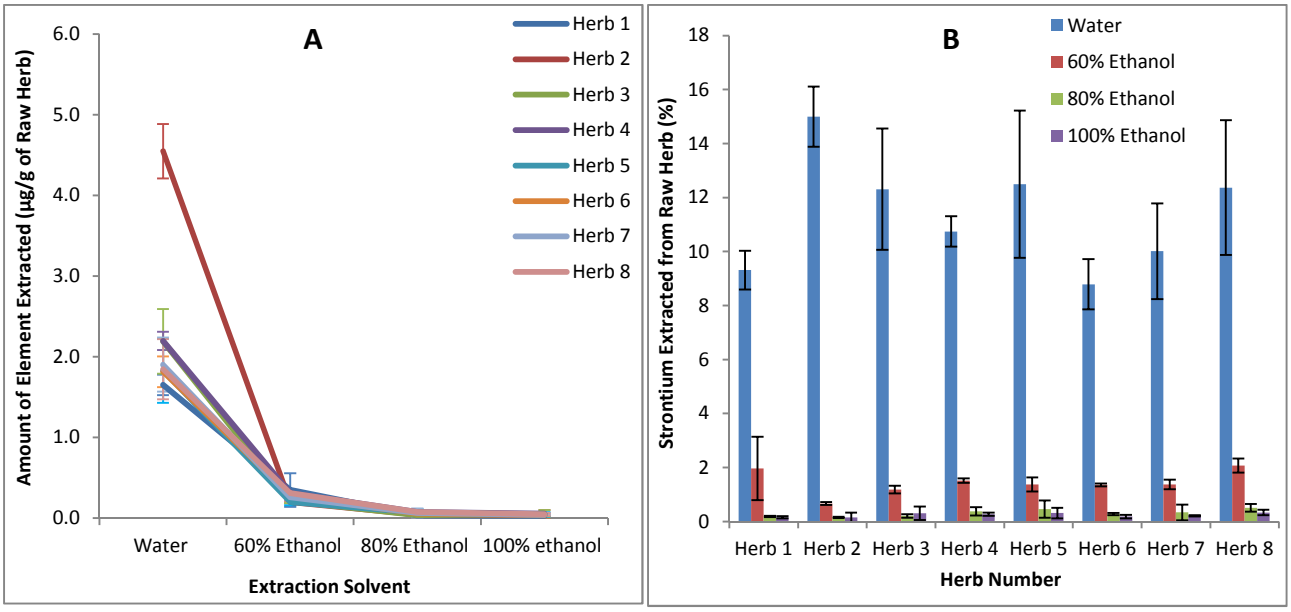


Figure 4.21 (A) Extraction of Sr from SJW powdered herbs in different solvents and (B) Percent of Sr extracted from original raw herb. Uncertainty is represented by ±1SD.

Examination of the amount of Sr in the dried extracts (Figure 4.22 A); show that the 100% water extract has a higher concentration of the element compared to the ethanol extracts. The water extracts contain between 25-69 µg/g Sr whereas the 60 %v/v, 80 %v/v, and 100% ethanol solutions

contain 1.9-2.7 µg/g, 0.4-0.7 µg/g and 0.7-1.4 µg/g, respectively. This is likely to be due to Sr salts being able to move more freely in the water compared to the ethanol solvents.

Comparison of the dried extract concentrations to that of the total concentration of the original raw herb (Figure 4.22 B) shows that the 100% water extracts contain more Sr per gram than the original herb. This illustrates that although preconcentration occurs in the dried water extract, this form of extract is not knowingly used by manufacturers or consumers. The ethanol extracts contain less Sr per gram in comparison to the raw herb indicating no preconcentration of this element occurs due to the extraction process.

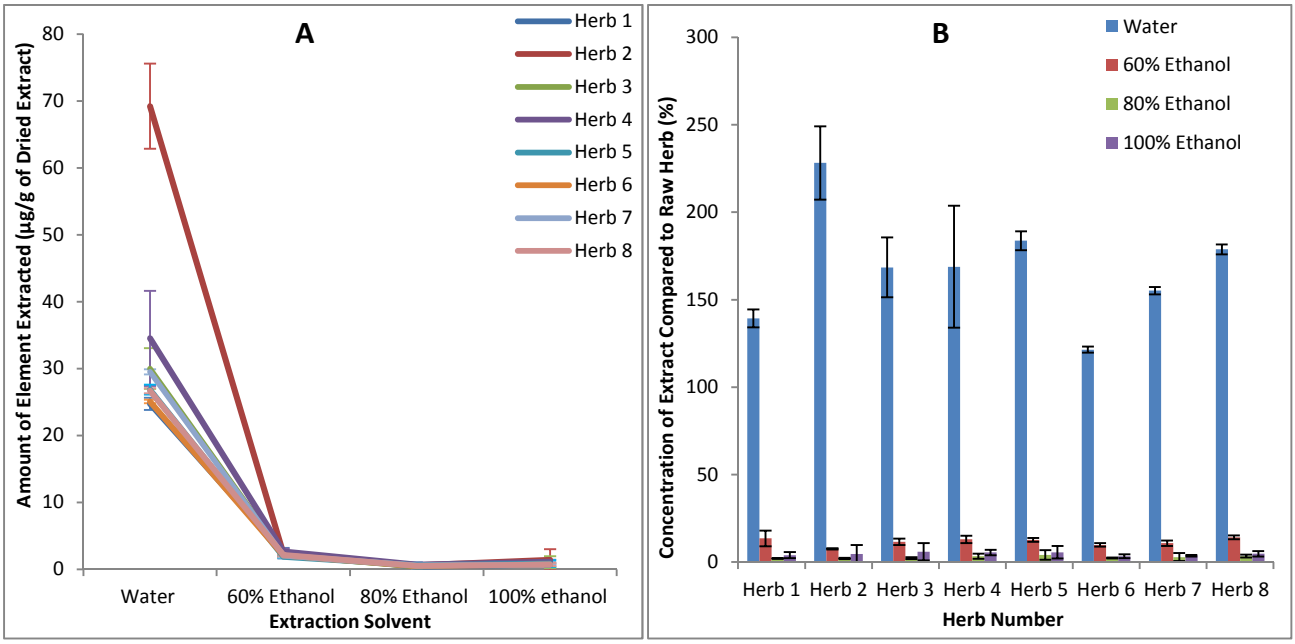


Figure 4.22 (A) Amount of Sr in dried extracts (B) Comparison of Sr extract concentration to original herb concentration. Uncertainty is represented by ±1SD.

4.3.2.14 Zinc

Zinc is an essential element within plants which is involved in several functions such as RNA and ribosome formation, membrane permeability and various enzymes [43]. Within humans, zinc is also essential as it is involved with several metabolic processes with DNA, proteins and carbohydrates in order to grow, develop and reproduce [43]. Zinc has been shown to bind to flavonoids quercetin and rutin and was shown to increase anti-oxidant properties compared to flavonoids alone [150].

The extraction of different SJW powdered herbs with four solvents illustrate a general trend despite varied geographical origin. The results (Figure 4.23 A) show that the highest concentrations of zinc

are extracted with 100% water (7.5-10.7 µg/g of original herb). These results agree with those reported by Konieczynski *et al.* (6.3-49.3 µg/g) but are lower than Gomez *et al.* (88-114 µg/g) [125]. Zinc concentrations decrease on average by 42% with 60 %v/v ethanol (3.2-7.6 µg/g of original herb), further decrease on average by 67% with 80 %v/v ethanol (1.2-2.0 µg/g of original herb) and by 56% when 100% ethanol is used (0.5-1.3 µg/g of original herb). These results show that between the solvents, 100% water contained the most zinc compared to the ethanol solvents.

Comparing these values to the total concentrations of zinc in the original herb (Figure 4.23 B), water had an extraction efficiency average of 24 ± 4% from the original herb. This agrees with values reported by Oledzka and Szyszkowska (17%) [157] but is lower than those reported by Kalny *et al.* (66%) [131]. Whereas 14 ± 2% and 4 ± 1% and 1.9 ± 0.7% was extracted respectively for the 60 %v/v, 80 %v/v, and 100% ethanol solutions.

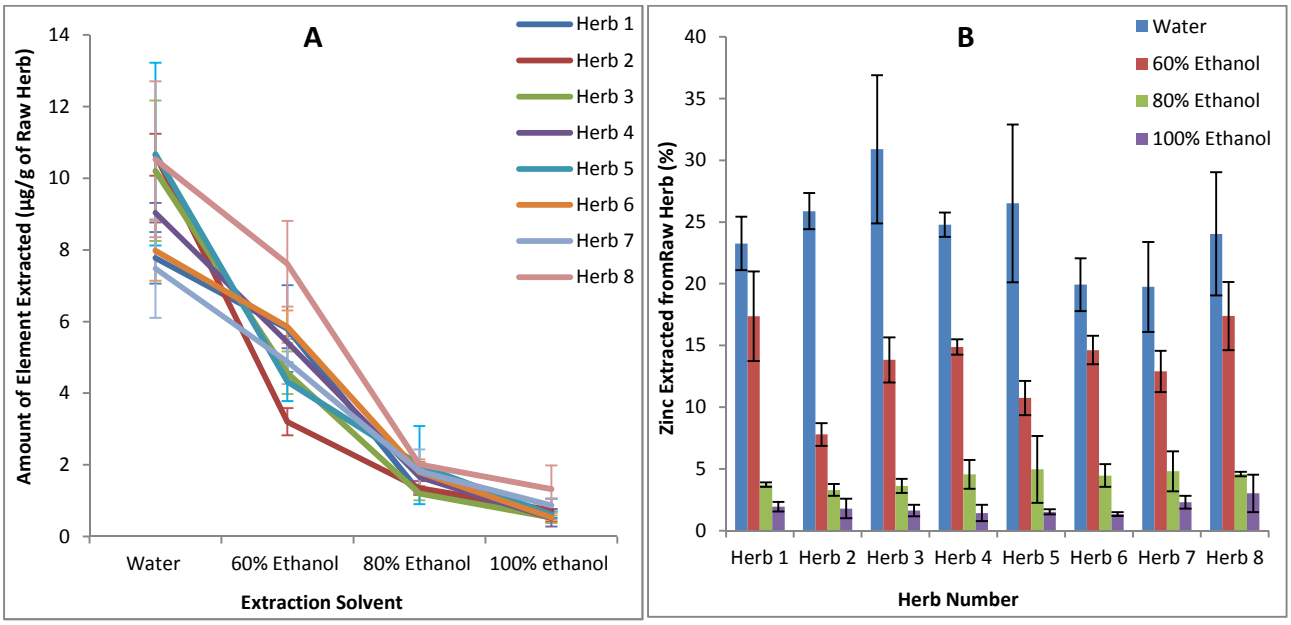


Figure 4.23 (A) Extraction of zinc from SJW powdered herbs in different solvents and (B) Percent of zinc extracted from original raw herb. Uncertainty is represented by ±1SD.

Examination of the amount of zinc in the dried extracts (Figure 4.24 A) show that the 100% water extract has a higher concentration of the element compared to the ethanol extracts. The water extracts contain between 110-162 µg/g whereas the 60 %v/v, 80 %v/v and 100% ethanol solutions contain 39-52 µg/g, 12-24 µg/g and 10-22 µg/g, respectively. This is likely to be due to zinc salts being able to move more freely in the water compared to the ethanol solvents.

Comparison of the dried extract concentrations to that of the total concentration of the original raw herb (Figure 4.24 B) shows that the 100% water and 60 %v/v ethanol extracts contain more zinc per gram than the original herb. Although preconcentration occurs in the dried water extract, this

form of extract is not knowingly used by manufacturers or consumers. The 60 %v/v ethanol extracts are preconcentrated on average by +10% compared to the original raw herb. The higher percentages of ethanol solvents do not show preconcentration of zinc. Therefore, these results show that extracts produced with lower percentages of ethanol will contain more zinc than those with a high percentage and could also preconcentrate the element.

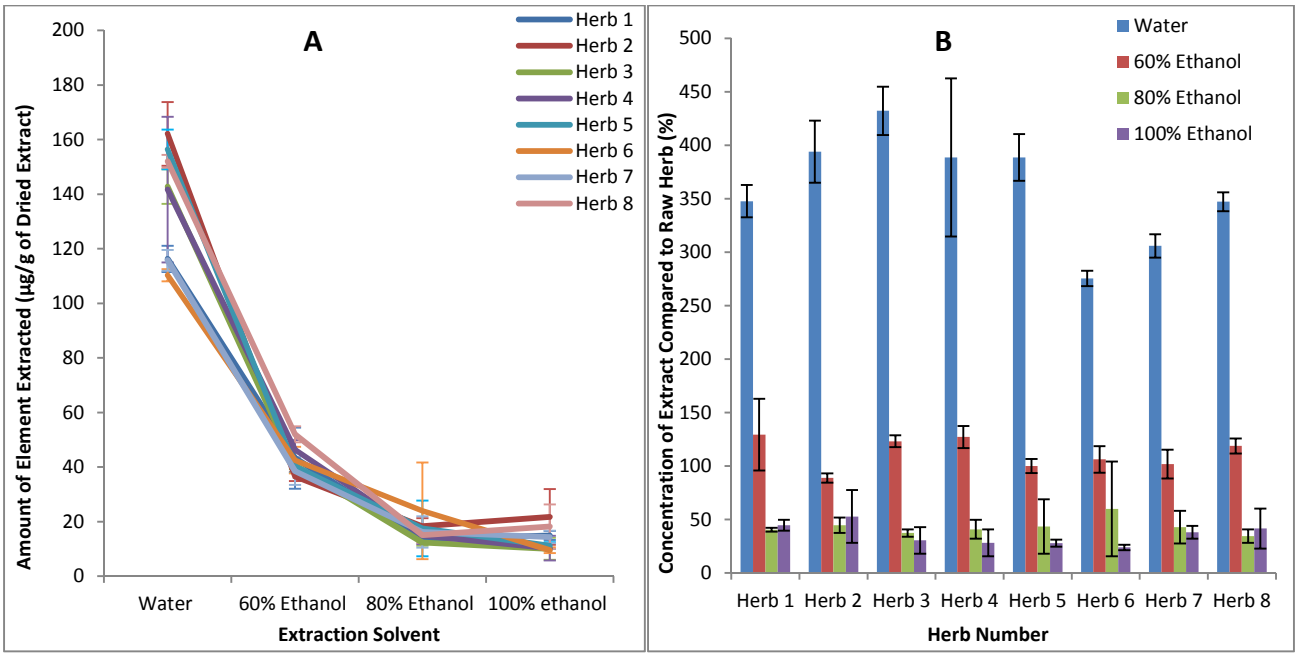


Figure 4.24 (A) Amount of zinc in dried extracts (B) Comparison of zinc extract concentration to original herb concentration. Uncertainty is represented by ±1SD.

4.3.2.15 Comparison of All Extraction Results for All Solvents

From examining the extraction trends of elements between different solvents it has become apparent that all metals do not extract in the same manner. The majority of elements followed the general trend of less elements being extracted as the concentration of ethanol increased. Copper followed a different trend to the other elements. It was found that, of the solvents used, more Cu was extracted with the 60 %v/v ethanol compared to the 100% water, then as the ethanol concentration increased above 60 %v/v, then concentrations transferred decreased. This trend may possibly be seen with Co; however, to investigate this fully more sample would be needed in the initial extract to ensure this element is detected well or the use of a GFAAS.

It appears elements Ba, Ca and Sr follow a similar trend as a large amount is transferred with the 100% water which then significantly decreases for the ethanol extractions. Calcium and Sr are

known to be closely correlated due to their similar ionic radius [39]. The other elements Al, Fe, Mg, Mn and Zn follow a more gradual decline in element transfer as ethanol concentration increases.

The majority of elements were found to preconcentrate in the dried water extract with the exception of Al, Ba and Fe. The lack of preconcentration for Al and Fe may be due to these elements being bound to silica thus affecting their mobility. Fe is usually extracted in a lower percentage than other major elements in SJW (such as Mg and Cu), with the exception of that found by Naeem et al [137]. This may be due to the extraction technique which involved maceration over three days. The other studies [125, 126, 131-133, 143, 157, 200] extraction methods took 60 minutes or less and usually did so without agitation to the solution. The extended length of time and maceration may be able to release Fe from plant structures that a hot water infusion cannot. Elements Cu (+118%), Mg (+94%), Mo (+121%), Ni (+173%) and Zn (+21%) (and possibly Co) were found to preconcentrate in 60 %v/v ethanol extracts. Elements Cu (+9%) and Ni (+21%) also preconcentated in 80 %v/v extracts with no elements preconcentrating in 100% ethanol extractions. The elements that preconcentrate in the 60 %v/v and 80 %v/v ethanol are of interest as this shows that by carefully selecting the extraction solvent, the quantity of elements that are transferred can be influenced. This could aid enrichment of herbal extracts for nutritional value as well as prevent the preconcentration of elements that may cause harm. This also suggests that extracts prepared with these solvents could potentially be identified based on their elemental profile before further dilution/preconcentration from further processing (e.g. addition of bulking agents). In order to see if this was possible, the results underwent further statistical analysis.

4.3.3 Statistical Analysis of Different Solvents

4.3.3.1 Correlation Analysis

Correlation Analysis (CA) was carried out to determine if there were any relationships between elements in each solvent solution. In Chapter 3 it was hypothesised that the main differences in the elemental fingerprint between products and dry herbs was as a result of solvent extraction and not due to excipient addition. Please see Table 4.14 for clarification of correlation terms.

Table 4.14 Correlation term definitions

Correlation Term	P value range
Weak positive correlation	**0.46 – 0.50**
Positive correlation	**0.50 – 0.79**
Strong positive correlation	**0.80 – 1.00**
Weak negative correlation	**-0.46 – -0.50**
Negative correlation	**-0.50 – -0.79**
Strong negative correlation	**-0.80 – -1.00**

The CA of 100% water extracts (Table 4.15) show that there were 34 correlations between elements (p is greater than 0.5 and less than -0.5). Of these correlations, 1 was negatively correlated whilst 33 were positively correlated. A total of 9 correlations had a strong positive correlation (p ≥0.8) which were in order of highest to lowest; Cd-Sr, Cd-Cr, Cr-Sr, Cr-Mn, Cd-Mn, Mn-Sr, Co-Fe, Ca-Zn and Al-Ba. The negatively correlated elements were Cu-Mg. Also noted were 2 weak correlations where 0.46 ≤ p <0.5 or -0.5 < p ≤ -0.46 of which both were positive. There were no strong negative correlations between the elements in the 100% water extraction.

Table 4.15 Correlation analysis of elements in eight herbs extracted with 100% water[1]

	Al	Ba	Ca	Cd	Co	Cr	Cu	Fe	Mg	Mn	Mo	Ni	Sr	Zn
Al	1													
Ba	0.8163	1												
Ca	0.5321	0.0759	1											
Cd	0.7439	0.3854	0.6842	1										
Co	0.6514	0.5237	0.2538	0.5033	1									
Cr	0.7750	0.5377	0.7115	0.9287	0.3821	1								
Cu	0.2449	0.2093	-0.3056	-0.1307	0.3602	-0.3375	1							
Fe	0.5630	0.5798	0.0371	0.5819	0.8463	0.5041	0.0945	1						
Mg	0.0724	0.0627	0.5823	0.2272	-0.0917	0.4373	-0.6663	-0.1163	1					
Mn	0.7186	0.5513	0.4431	0.8771	0.4890	0.8969	-0.2610	0.7224	0.0991	1				
Mo	0.0582	-0.2351	0.1834	-0.2074	-0.0060	-0.2967	0.4208	-0.3801	-0.4566	-0.2772	1			
Ni	0.4595	0.2180	0.3190	0.5884	0.5594	0.3786	0.4311	0.4240	0.1063	0.2446	-0.2548	1		
Sr	0.6762	0.2774	0.7815	0.9654	0.4571	0.9231	-0.2905	0.5134	0.2938	0.8742	-0.1043	0.4199	1	
Zn	0.6920	0.3303	0.8358	0.5319	0.4970	0.5294	0.1718	0.1090	0.4231	0.2433	0.2861	0.5368	0.5412	1

[1]Dark green = strong positive correlation, green = positive correlation, red = strong negative correlation, pink = negative correlation, orange = weak correlation.

The elements Cd, Cr and Mo were removed from the CA of 80 %v/v ethanol extracts as all herbs were below the LOD for this ethanol concentration. These elements were removed as there was no variation between the samples (all below the LOD) and therefore render these elements as redundant. The CA of 60 %v/v ethanol extracts (Table 4.16) show that there were 27 correlations between elements (p ≥0.5 or ≤-0.5). A reduction of 10 correlations compared to the water analysis. Of these correlations, 12 were negatively correlated whilst 15 were positively correlated. Four correlations had strong positive correlations (p ≥0.8) which were Al-Ba, Ca-Zn, Ba-Cu and Al-Cu. Two of these element correlations (Al-Ba and Ca-Zn) were seen as a strong correlation in the previous 100% water CA. Also noted was 1 weak positive correlation (where p is between 0.46 and 0.5). Many of the correlations seen with the 60 %v/v ethanol extracts are different or transformed compared to that of the water CA. For example, in the water CA, no significant correlation was observed between Ca – Co whereas with the 60 %v/v ethanol CA, Ca– Co transforms to a strong negative correlation. There were two strong negative correlations between the elements in the 60 %v/v ethanol extraction which included Ca-Co and Mg-Sr.

Table 4.16 Correlation analysis of elements in eight herbs extracted with 60 %v/v ethanol[1]

	Al	Ba	Ca	Co	Cu	Fe	Mg	Mn	Ni	Sr	Zn
Al	1										
Ba	0.8895	1									
Ca	-0.3081	-0.4475	1								
Co	0.5963	0.7960	-0.8298	1							
Cu	0.8577	0.8612	-0.3256	0.6945	1						
Fe	0.6321	0.4566	-0.5349	0.4290	0.3382	1					
Mg	-0.3127	-0.5912	0.4085	-0.7196	-0.6176	-0.1143	1				
Mn	0.2257	0.4633	-0.6531	0.5702	0.0752	0.5661	-0.4195	1			
Ni	0.7317	0.5356	-0.5422	0.5722	0.7259	0.6034	-0.2434	0.0082	1		
Sr	0.1599	0.3406	-0.0879	0.3629	0.3321	0.2288	-0.8158	0.4283	-0.0495	1	
Zn	-0.2794	-0.3128	0.8696	-0.6172	-0.0928	-0.5493	0.0370	-0.5600	-0.5050	0.2527	1

[1]Dark green = strong positive correlation, green = positive correlation, red = strong negative correlation, pink = negative correlation, orange = weak correlation.

The elements Cd, Cr and Mo were removed from the CA of 80 %v/v ethanol extracts as all herbs were below LOD for this ethanol concentration. These elements were removed as there was no variation between the samples (all below LOD) and therefore render these elements as redundant. The CA of 80 %v/v ethanol extracts (Table 4.17) show that there were 15 correlations between elements (p ≥0.5 or ≤-0.5). A reduction of 12 correlations compared to the 60 %v/v ethanol analysis. Of these correlations, 1 was negatively correlated whilst 14 were positively correlated. Six correlations had strong positive correlations (p ≥0.8) which were in order of highest to lowest; Al-

Ba, Ba-Cu, Cu-Mn, Al-Ni, Ca-Fe and Ba-Ni. Of these element correlations, Al-Ba was also seen as a strong positive correlation in the previous 100% water and 60 %v/v ethanol CA. Also noted was 1 weak positive correlation. Many of the correlations seen with the 80 %v/v ethanol extracts are different or transformed compared to that of the 60 %v/v ethanol CA. For example, in the 60 %v/v ethanol CA, no correlation of Al-Sr is seen but with the 80 %v/v ethanol a positive correlation is observed. There were no strong negative correlations between the elements in the 80 %v/v ethanol extraction.

Table 4.17 Correlation analysis of elements in eight herbs extracted with 80 %v/v ethanol[1]

	Al	*Ba*	*Ca*	*Co*	*Cu*	*Fe*	*Mg*	*Mn*	*Ni*	*Sr*	*Zn*
Al	1										
Ba	**0.8508**	1									
Ca	0.0120	-0.3277	1								
Co	0.3490	0.1735	-0.0170	1							
Cu	**0.7111**	**0.8638**	-0.2098	-0.0256	1						
Fe	0.1663	0.0163	**0.8105**	-0.1160	-0.0755	1					
Mg	-0.3104	-0.3962	**0.5646**	-0.7792	-0.2999	**0.6020**	1				
Mn	*0.4774*	**0.6866**	-0.2698	-0.0682	**0.8486**	-0.1367	-0.3017	1			
Ni	**0.8322**	**0.8091**	-0.3261	-0.0107	**0.7117**	-0.1450	-0.2065	0.4041	1		
Sr	**0.6349**	**0.5860**	0.1327	**0.5916**	0.3715	0.3527	-0.4125	0.1257	0.3834	1	
Zn	-0.0006	0.0160	-0.0723	-0.1770	0.2300	-0.2027	-0.0376	0.0327	0.2653	0.2487	1

[1]Dark green = strong positive correlation, green = positive correlation, red = strong negative correlation, pink = negative correlation, orange = weak correlation.

The elements Cd, Cr, Co and Mo were removed from the CA of 100% ethanol extracts as the concentrations of these elements were below the LOD for all herbs for this ethanol concentration. Due to this, there is no variation between samples and therefore render these elements as redundant. The CA of 100% ethanol extracts (Table 4.18) show that there were 26 correlations between elements ($p \geq 0.5$ or ≤ -0.5). An increase of 11 correlations compared to the 80 %v/v ethanol analysis. Of these correlations, 2 were negatively correlated whilst 24 were positively correlated. Five correlations had strong positive correlations ($p \geq 0.8$) which were in order of highest to lowest; Al-Sr, Ca-Zn, Cu-Mn, Al-Cu and Al-Ni. Of these element correlations, the Cu-Mn and Al-Ni were also seen as a strong positive correlation in the 80% v/v extract, whilst the Al-Cu in the 60% v/v CA and Ca-Zn in the 60% v/v extraction CA. Many of the correlations seen with the 100% ethanol extracts are different or transformed compared to that of the 80 %v/v ethanol CA. For example, in the 80 %v/v ethanol CA, no correlation of Al-Ca is seen but with the 100% ethanol a positive correlation is observed. There were two negative correlations; Mg-Ni and Mg-Sr in the 100% ethanol extraction.

Table 4.18 Correlation analysis of elements in eight herbs extracted with 100 % ethanol[1]

	Al	*Ba*	*Ca*	*Cu*	*Fe*	*Mg*	*Mn*	*Ni*	*Sr*	*Zn*
Al	1									
Ba	0.7421	1								
Ca	0.6114	0.2968	1							
Cu	0.8740	0.5435	0.6860	1						
Fe	0.4994	0.5644	0.2157	0.2213	1					
Mg	-0.4480	-0.3763	0.2196	-0.1759	-0.2914	1				
Mn	0.7512	0.6610	0.5828	0.8793	0.1651	-0.2142	1			
Ni	0.8451	0.6242	0.2527	0.7393	0.5483	-0.6027	0.5425	1		
Sr	0.9528	0.7785	0.5561	0.7958	0.4133	-0.5925	0.7796	0.7765	1	
Zn	0.4165	0.0789	0.8808	0.6508	0.1268	0.3495	0.6147	0.1136	0.3368	1

[1]Dark green = strong positive correlation, green = positive correlation, red = strong negative correlation, pink = negative correlation, orange = weak correlation.

The differences displayed by the CAs of the solvents used in extraction indicate that the extraction solvent changes the relationships between the elements extracted. However, due to the large number of correlations it is difficult to fully interpret these relationships therefore these results, combined with the total concentrations of each element for all herbs were subjected to Principal Component Analysis (PCA).

4.3.3.2 Principal Component Analysis

The correlation analysis of the four solvents showed that each type of dried extract exhibited different elemental relationships. In order to interpret this further and to see if dried extracts could be clearly differentiated based on their extraction solvent, the data, combined with the total concentrations of elements from the original plant was subjected to Principal Component Analysis (PCA). The concentration values were ratio normalised prior to analysis. The results (Figure 4.25 A) show that the original herb and different extracts can be differentiated based on their elemental content using 14 elements (i.e., Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Sr and Zn) The ellipses shown are used to visualise the groupings. The total variance of the first two principal components (PC) without optimisation is 77%. The loadings (Figure 4.25 B) show that PC1 has positive loadings for all variables. PC2 loadings however show high positive loadings for Al, Ba and Fe with lower positive loadings for Cd, Cr, Mo and Sr. Large negative loadings were seen on PC2 for Co, Cu, Mg, Ni and Zn with smaller negative loadings for Ca and Mn. With regards to PC1 loadings, this shows that the original herb samples and the water extracted samples have higher than average values for all elements whereas the ethanol extracts have lower than average levels for these elements. Along

PC1 there is some overlap between 100% water extracts and total concentration, total concentration and 60 %v/v ethanol extracts and between 80%v/v and 200% ethanol extracts. Along PC2 the total concentration are differentiated from the 100% water and 60 %v/v ethanol extractions are due to the original herbs having higher than average values for Al, Ba, Cd, Mo, Cr, Fe and Sr and lower than average values for Cu, Mg, Co, Ni and Zn. The 100% water and 60 %v/v ethanol extracts being *vice versa*. The main elements causing this separation are Al, Ba, Cr and Fe. The 80 %v/v and 100% ethanol extracts are also separated based on these elements, but to a lesser extent.

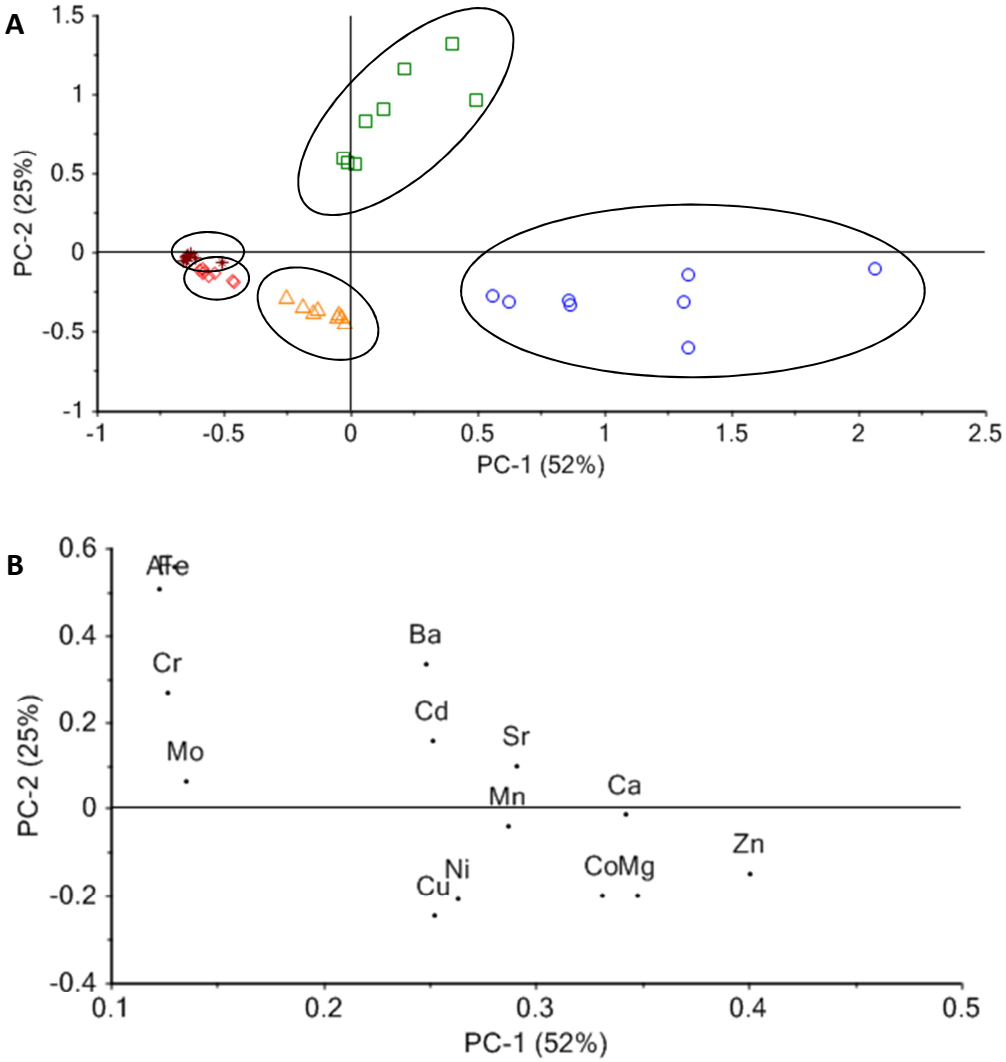


Figure 4.25 (A) PCA of eight herbs with 14 elements. Square = original Herb, circle = 100% water extraction, triangle = 60 %v/v ethanol extraction, diamond = 80 %v/v ethanol extraction and star = 100% ethanol extraction. (B) 2D loadings for PCA.

4.4 Conclusions

The elemental fingerprint of SJW was shown to alter when extracted depending on the solvent used. With this extraction process, all elements (with the exception of Cu), were transferred in higher concentrations when extracted with 100% water. This indicates that SJW taken as a tea infusion would contain the most variety of elements before further processing is introduced (e.g., addition of excipient *via* the production of tablets/capsules). However, Cu was found to be transferred in higher concentrations with 60 %v/v ethanol. Further interpretation found that all elements examined, with the exception of Al, Ba and Fe, became preconcentrated in dried water extracts. This type of extract is not knowingly used by consumers or manufacturers; however, this study suggests the possibility of tuning the metal content for future applications. The 60 %v/v ethanol solvent however, is used extensively by manufacturers in the production of dried extracts of SJW as it is shown to be the optimum percentage of alcohol for the extraction of hypericins. With this extraction solvent, the elements Cu, Mg, Ni and Zn preconcentrate in the extract compared to the original herb. The 80 %v/v ethanol solvent is also utilised by industry and from this study has shown to preconcentrate Cu and Ni. The preconcentration of elements observed in this study could be of benefit to nutritional disorders caused by a deficiency of these elements. For example, a deficiency in zinc with humans can cause retardation of growth, diarrhoea, failure of appetite and behavioural changes. A deficiency in Cu can cause hypopigmentation of hair and skin, osteoporosis and vascular abnormalities. Severe Ni deficiency can cause depressed growth and haematopoiesis. Therefore by controlling the percentage of ethanol in the extraction process it is possible to modify the extraction to also extract and concentrate these elements. Thus allowing added nutritional value but also reducing the amount of toxic elements (Cd, Cr etc.) available for consumption.

The principal component analysis demonstrated that the elemental fingerprint changes in a predictable manner with the solvents used regardless of where the sample was obtained from or cultivated. This study shows the extraction solvent plays a key role in the concentrations of elements contained in a dried extract. However, only one kind of extraction method was utilised. Other methods such as Soxhlet and sonication should be utilised for further studies to understand their impact on the elemental profile. In addition to this, other solvents such as methanol or chloroform should also be investigated. Ultimately, this process could be used to determine whether dry herb or type of dry extract has been used in a product. Other considerations would be to use other extraction solvents, such as methanol which is also used in industry, to see in different solvent extractions can also be identified.

5 Investigations of Bioactive Compounds in St John's Wort

5.1 Introduction

Quality control of St John's Wort (*Hypericum perforatum*) in relation to its bioactive properties is normally monitored by assessing the compounds rutin, hypericin, pseudohypericin and hyperforin [36, 116]. Rutin is a natural antioxidant found in many species of plant [93, 110, 226, 227]. It has been shown to be an effective anti-inflammatory [91] and is also an antioxidant [228]. Rutin has been shown to increase the antioxidant activity of ascorbic acid as synergism was found when the two compounds were present together in radical scavenging experiments (in homogeneous aqueous solutions, ufasome and erythrocyte ghost preparations) [229] and is also a metal chelator [109, 150, 220, 230-232]. The antioxidant properties of rutin can be increased eight fold by the complexation with Cu [109]. Hypericin, pseudohypericin and hyperforin are compounds produced by the *Hypericum* genus of plants, of which, *Hypericum perforatum* contains the highest concentrations (There are over 400 species within the genus *Hypericum*, of which only a small number have been studied. Of those studied, *Hypericum perforatum* has the highest concentration of these compounds). These compounds are only readily found in this family of plants, however, hypericin has now been shown to exist in a species of fungus [233] and has been detected in fossilised crinoids [234]. Originally it was thought hypericin was the major bioactive component to cause the therapeutic effect against depression. Further research has shown that although it does play a part in the treatment of depression, the compound hyperforin actually produced the largest therapeutic effect [88]. The compounds adhyperforin [235] and pseudohypericin also contribute to the therapeutic effect. Hyperforin has also been shown to be antibacterial, an anti-proliferate (stops growth) and pro-apoptotic (induces cell death) towards some cancer cells as well as other possible beneficial properties [84]. Hyperoside is also a flavonoid that possesses antifungal [96] and antioxidant properties [95]. It differs from the structure of rutin by the substitution of the sugar group as galatose rather than rutinose. The most common molecular constituents in SJW and their relative amounts can be seen in Table 1.7, Chapter 1.

The analysis of the bioactive compounds within SJW is generally completed using HPLC or UHPLC [92, 93, 159, 236-240]. In order to analyse these compounds they firstly have to be extracted from the SJW sample. The extraction of bioactive compounds from SJW has been investigated in other studies with a number of different extraction solvents [113, 191, 207, 241-243]. Generally these studies found that of the solvents tested, ethanol and methanol were able to extract a higher concentration of hypericins and flavonoids. Also noted was that these solvents in either 60% v/v or

80% v/v with water were the optimum ratios which are reflected by the products of SJW produced commercially. The concentrations of the bioactive compounds within SJW can vary significantly depending on soil type and water content, growth climate and genetics [92, 93, 127, 237, 244, 245]. The variation has also been shown to be different between seasons for the same crop as well as the time of harvest [237, 245]. The storage post-harvest of the herb is also of importance as inadequate storage can greatly reduce the concentrations of these bioactive constituents as many are sensitive to light, pH and temperature [244, 246, 247]. Examples of the concentration ranges these bioactive compounds have been quantified can be seen in Table 1.7, Chapter 1. The HPLC methods utilised generally use C18 columns [92, 93, 207, 236, 237] but differ in their mobile phase preparation and type of analysis. For example Ari *et al.* employ a gradient method using aqueous 5 mM ammonium acetate (Mobile phase A) and acetonitrile (Mobile phase B), Çirak et al. utilise a gradient method with 0.1% trifluoroacetic acid in water (Mobile phase A) and 95:5 of acetonitrile to 0.1% trifluoroacetic acid in water (Mobile phase B) whereas Couceiro et al. uses an isocratic method with 0.1 mol triethyl ammonium acetate(Mobile phase A) and acetonitrile (33:67, v/v) (Mobile phase B). However, many studies that examine the bioactive content of SJW use buffers as part of their mobile phase composition. Therefore, in order to identify and characterise the peaks from UHPLC analysis on an LC-MS, a method would need to be developed that did not utilised buffers as these can cause serious blockages to the electrospray sample induction system and high background noise on the LC-MS.

Some studies have shown that there is a link between the expression of secondary metabolites in SJW and the elements in the growth medium. For example, a 15-20 fold decrease in the production of hypericin and pseudohypericin was observed when SJW was exposed to 50 mM Ni [149]. On the other hand, SJW exposed to 0.1 mM Cr showed increased production of protopseudohypericin (+167%), hypericin (+25%) and pseudohypericin (+5%) compared to untreated SJW [148]. As well as elements influencing the production of secondary metabolites, metals ions can form complexes with them and alter their bioactivity (Table 5.1).

Table 5.1 Literature findings of secondary metabolites complexed with metal ions

Bioactive Constituent	Metal Complexes in literature	Effect if tested [1,2]
Rutin	Cu	Increases antioxidant capacity with DPPH test [150], LDL oxidation [197] Increases anti-inflammatory capacity [109]
	Fe	Increases antioxidant capacity with DPPH test [150] Can be pro-oxidant [109]
	Al	Increases antioxidant capacity with DPPH test [150]
	Zn	Increases antioxidant capacity with DPPH test [150]
	Co	N/A [214]
	Ni	N/A [214]
	Sn	N/A [248]
Quercetin	Cu	Increases antioxidant capacity with DPPH test [150], LDL oxidation [197]
	Fe	Increases antioxidant capacity with DPPH test [150]
	Al	Increases antioxidant capacity with DPPH test [150]
	Zn	Increases antioxidant capacity with DPPH test [150]
	Ni	N/A [214]
	Co	N/A [214]
Hypericin	Al	N/A [198]
	Fe	N/A [198]
	Cu	N/A [198]
	Gd	N/A [198]
	Tb	N/A [198]
Chlorogenic acid	Cu	N/A [219], Can be Prooxidant [223]
	Mn	N/A [219]
	Zn	N/A [219]
	Fe	N/A [219]

[1]DPPH = 1,1-diphenyl-2-picrylhydrazyl radical scavenging method, LDL = Low-density lipoprotein,
[2]N/A = No biological assay performed.

In addition to altering bioactivity and production of bioactive compounds, metal complexes can also affect the bioavailability of the compound. In a study where chickens were fed a mineral rich diet with the addition of a herbal remedy, those fed a mineral rich diet with St John's Wort had increased levels of Zn in the liver and decreased levels of Mn in the leg meat [151].

Although a number of SJW bioactive constituent-metal complexes have been characterised (Table 5.1) they are all prepared from the standards of the bioactive constituent and metal. The extent of bioactive-metal complexes in SJW samples (i.e., *in situ*) has not yet been studied. In order to see if such complexes exist naturally in the extracts of SJW, an Ultra High Performance Liquid Chromatography (UHPLC) method to determine the presence of the rutin-Cu complex will be investigated. In addition to this, the concentrations of the flavonoids rutin, hyperoside and quercetin as well as hyperforin and adhyperforin will be quantified in order to see if there is a relationship between extracted bioactive compounds and metal concentration (see Chapter 4).

Therefore, in this thesis, a method was developed that was able to be utilised on LC-MS as well as UHPLC in order to allow quantification and characterisation of these compounds.

5.2 Method

5.2.1 Materials

Eight SJW dry powdered herbs were purchased through high street retailers and internet sources. A summary of all samples is shown in Table 4.1, Chapter 4. All labware was acid washed overnight with 4M nitric acid and rinsed thoroughly with deionised water before use. Extractions of samples were carried out using mixtures of HPLC grade water (Fisher, Loughborough, UK), HPLC grade methanol (Fisher, Loughborough, UK) and absolute ethanol (Fisher, Loughborough, UK). Whatman cellulose filter paper (grade 1) was used in the filtering stage of sample preparation whereas 0.2 µm syringe filters (Sigma-Aldrich, Gillingham, UK) were used before UHPLC/HPLC analysis.

Standards of rutin (Fisher, Loughborough, UK), quercetin (Sigma-Aldrich, Gillingham, UK), hyperforin/adhyperforin (Schwabe Pharma, Karlsruhe, Germany) and hyperoside (Schwabe Pharma, Karlsruhe, Germany) were utilised for method development and identification or quantification. The mobile phase for LC analysis used HPLC grade water (Fisher, Loughborough, UK), HPLC grade acetonitrile (Fisher, Loughborough, UK) and formic acid (Fisher, Loughborough, UK).

5.2.2 Instruments

Several instruments were utilised during these studies. A Varian Cary 1G UV/Vis spectrometer was used to monitor the formation of rutin-Cu complexes. A Perkin Elmer 200 EP DAD UHPLC with autosampler was used for the method development and analysis of rutin-Cu complexes as well as liquid extracts of SJW. The Perkin Elmer Flexar UV/Vis HPLC with autosampler and Varian ProStar 500 DAD HPLC with ProStar 410 autosampler were used to assess method transferability. A Varian ProStar 210 LC- Varian 1200L quadrupole MS/MS with ProStar 410 autosampler was utilised for confirmation of a rutin-Cu complex formation as well as characterisation of SJW peaks. A Perkin Elmer LC oven 101 was used for temperature control. The columns utilised were either a Phenomenex Kinetex™ (2.6 µm C18 100 Å, 100 x 4.6 mm) LC column or a Phenomenex Luna® (3 µm C18 100 Å, 150 x 4.6 mm) LC column.

5.2.3 Rutin – Copper Complex Study

5.2.3.1 Rutin - Copper Complex Formation

In order to assess the optimum reflux time needed to form a rutin-Cu complex, approximately 0.1 g of rutin was dissolved in 100 ml methanol to prepare the rutin sample. A $CuCl_2$ solution was prepared by 0.1 g in 100 ml methanol. These were then mixed in a 1:1 molar ratio. The 1:1 mixture was then refluxed over a period of 4 hours whereby a 1 ml aliquot was removed every 30 minutes to determine optimum reflux time for complex formation. The complex formation was monitored using UV-Vis and Mass Spectrometry. The sample was scanned between wavelengths 200-800 nm. Mass spectrometry was carried out using direct injection of 20 µl/second, in positive mode with mass scan between 50-1500 m/z single quadrapole.

5.2.3.2 Investigating a Chromatographic Method for the Monitoring of Rutin-Cu Complex

Development of an LC method for the rutin Cu complex was investigated using a gradient method where mobile phase A was 0.1 %v/v formic acid in water and mobile phase B was 0.1 %v/v formic acid in acetonitrile unless otherwise stated. Mobile phases were sonicated for 30 minutes prior to use. For a full list of methods with mobile phase composition and gradient parameters used during method development please see Appendix 10.4. Approximately 66mg of rutin was dissolved in 100 ml methanol, filtered using a 0.2µm syringe filter and run on UHPLC (Perkin Elmer). Methods 001 to 002, Appendix 10.4.

5.2.4 Method Development for the Analysis of SJW Extracts

The mobile phases used were; mobile phase A: HPLC grade water with 0.1 %v/v formic acid, mobile phase B: HPLC grade acetonitrile with 0.1 %v/v formic acid unless otherwise stated. Mobile phases were sonicated for 30 minutes prior to use. A Phenomenex Kinetex™ (2.6 µm C18 100 Å, 100 x 4.6 mm) LC Column or Phenomenex Luna® (3 µm C18 100 Å, 150 x 4.6 mm) LC Column was used. For full list of gradient methods with mobile phase ratios, flow rates and ramp times please see appendix 10.4.

5.2.4.1 Preliminary Analysis of SJW and Column Comparison

Approximately 1 g of SJW herb (H10) was sonicated with 10 ml of 60 %v/v ethanol. The samples were centrifuged at 8000 rpm for 20 minutes, and then syringe filtered (0.22 µm) before LC analysis (methods 003 to 005, appendix 10.4). Two types of column were compared by analysing the SJW extract, as well as rutin and quercetin standards. The Phenomenex Kinetex™ column was compared

to that of a Phenomenex Luna® column. The same basic method (methods 006 and 007, appendix 10.4) was used across both columns with the flow rate being adjusted using a Luna column to compensate for particle size. Following this, optimisation was carried out to allow the separation of the flavonoids, rutin and hyperoside.

5.2.4.2 Improving Retention Time Consistency with Temperature Control

During large sequences the retention time (Rt) of peaks increased by up to 15 minutes then returned to normal as the sequence progressed. In order to see if this was due to a drop in temperature as the sequence was running over night, the same sequence (method 008, Appendix 10.4) was analysed in the presence of an external column oven (Perkin Elmer 101); at a temperature of 30 ± 3°C.

5.2.4.3 Reducing Run time

Previous adjustments to the method to allow separation of flavonoids resulted in the run time being 145 minutes per sample (method 008). In order to save time and mobile phase, the method was examined closely to reduce the run time to less than 100 minutes by increasing the initial aqueous mobile phase to 92% and editing the gradient step to reach 79:21 A:B compared to 73:27 A:B (method 009 and 010, appendix 10.4).

5.2.5 Method Validation

For method validation experiments, the mobile phase A was HPLC grade water with 0.1 %v/v formic acid and mobile phase B was HPLC grade acetonitrile with 0.1 %v/v formic acid. The full list of gradient methods with mobile phase volume ratios and ramp times are shown in appendix 10.4. Mobile phases were sonicated for 30 minutes prior to use. A Phenomenex Luna® (3 µm C18 100 Å, 150 x 4.6 mm) LC Column was used at a flow rate of 1 ml/min.

5.2.5.1 UHPLC; Consistency Between Injections

An extraction took place with 1 g SJW H10 in 10 ml 60 %v/v ethanol which was sonicated for 30 minutes. The sample was centrifuged at 8000 rpm for 20 minutes then syringe filtered. The sample was run by UHPLC for ten times using method 009, appendix 10.4 to determine the consistency of the method.

5.2.5.2 UHPLC; Characterisation and Calibration of Reference Standards

Rutin standards of concentration 0.016 mg/ml, 0.033 mg/ml, 0.066 mg/ml, 0.099 mg/ml, 0.148 mg/ml, 0.222 mg/ml, 0.248 mg/ml, 0.371 mg/ml, 0.495 mg/ml and 0.99 mg/ml were prepared in 60

%v/v ethanol and run in triplicate on the UHPLC with Method 010, appendix 10.4 to quantify rutin *via* calibration graph.

Separate to the rutin calibration ran in triplicate, several new compound stocks solutions were prepared. This included rutin (1.064 mg/ml), hyperoside (0.662 mg/ml), quercetin (1.60 mg/ml) and hyperforin/adhyperforin (0.841 mg/ml; 0.660mg hyperforin, 0.181mg adhyperforin) prepared in 60 %v/v ethanol. Following this, a multi-standard was created with all compounds by taking 2 ml of each solution and diluting to 10 ml with 60 %v/v ethanol. These were run on UHPLC for identification of peaks by retention time (Rt) and on LC-MS for identification by Rt and m/z. Following this, subsequent dilutions were made using multi-component standard 1 and all standards were examined on UHPLC (single injection). The resulting concentrations for each standard are shown in Table 5.2.

Table 5.2 Concentrations of rutin, hyperoside, quercetin, hyperforin and adhyperforin in multi-component standards

	Concentration mg/ml				
Standard Name	**Rutin**	**Hyperoside**	**Quercetin**	**Hyperforin[2]**	**Adhyperforin[2]**
Stock[1]	1.064	0.662	1.600	0.660	0.181
Multi-1	0.213	0.132	0.320	0.132	0.036
Multi-2	0.142	0.088	0.213	0.088	0.024
Multi-3	0.095	0.059	0.142	0.059	0.016
Multi-4	0.063	0.039	0.095	0.039	0.011
Multi-5	0.032	0.020	0.047	0.020	0.005

[1] Stock contains one compound only

[2] Concentrations based on original weight and area ratio of peaks Hyperforin: Adhyperforin 78.5:21.5

5.2.6 Transferability to Other LC Systems

In order to assess the transferability of the method, samples of St John's Wort extracts were analysed on different HPLC systems. A SJW extract was prepared by sonicating 1 g of SJW herb (H17) for 15 minutes in 60 %v/v ethanol. This extract was then filtered (0.2 µm) *via* syringe and analysed several times using method 008 (appendix 10.4). The mobile phases used were 0.1 %v/v formic acid in water (A) and 0.1 %v/v formic acid in acetonitrile (B) unless otherwise stated. The mobile phases were sonicated for 30 minutes prior to use. For a full list of methods with mobile phase ratios and ramp times please see Appendix 10.4 (The Varian ProStar 500 is a DAD HPLC with

autosampler and Perkin Elmer Flexar is a UV/Vis HPLC with autosampler were utilised). A detector wavelength of 280 nm was utilised.

5.2.7 Analysis of SJW Extracts

5.2.7.1 Analysis of SJW Extracts

Please see full extraction method in Chapter 4. Eight herbs of SJW were extracted in 60 %v/v ethanol in triplicate, of which 4 ml were obtained for liquid chromatography purposes. From this stock, 1 ml was filtered (0.22 µm) into an amber vial for UHPLC analysis. In addition to this, herb 7 and herb 8 were also extracted with 100% water, 80 %v/v ethanol and 100% ethanol to compare the effect of different solvents on the extraction of molecular constituents. All samples were run within 24 hours of initial extraction unless otherwise stated. Phenomenex Luna® (3 µm C18 100 Å, 150 x 4.6 mm) LC Column, Mobile phase A: HPLC grade water with 0.1 %v/v formic acid, mobile phase B: HPLC grade acetonitrile with 0.1 %v/v formic acid. A flow rate of 1 ml/min with Method 010, Appendix 10.4 was utilised.

5.3 Results and Discussion

5.3.1 Rutin – Copper Complex Study

5.3.1.1 Rutin – Copper Complex Formation

Preparation of rutin in methanol and copper chloride in methanol was carried out followed by UV-Vis analysis. These solutions were then mixed in a 1:1 molar ratio and also subjected to UV-Vis analysis at room temperature. The results (Figure 5.1) show that the solution containing both the rutin and Cu have a different spectrum compared to its individual counterparts. Upon addition of Cu, new peaks at 285 nm and 420 nm appear. The new peaks are most likely due to the Cu complexing with the rutin (the main bioactive constituent); however there is some debate over the most favourable site of binding [150, 197, 221, 231, 249]. Most studies report that the Cu ions bind to the B ring of the rutin *via* the catechol structure (two hydroxyl groups) as well as the 4-oxo-5-hydroxyl group [150, 197]. Other studies suggest binding can occur with the rutinose moiety [221] or with the 7-hydroxyl group [231]. These studies also showed that the rutin-Cu complex can be in different ratios (metal ion: rutin molecule) including, but not limited to 1:1, 1:2 and 3:2. The bathochromic shift seen around 420nm is consistent with the Cu ion binding to the catechol group

(Figure 5.2 A) whilst the bathochromic shift at 285 nm is attributed to binding with the 4-oxo-5-hydroxyl group (Figure 5.2 B) [197, 231].

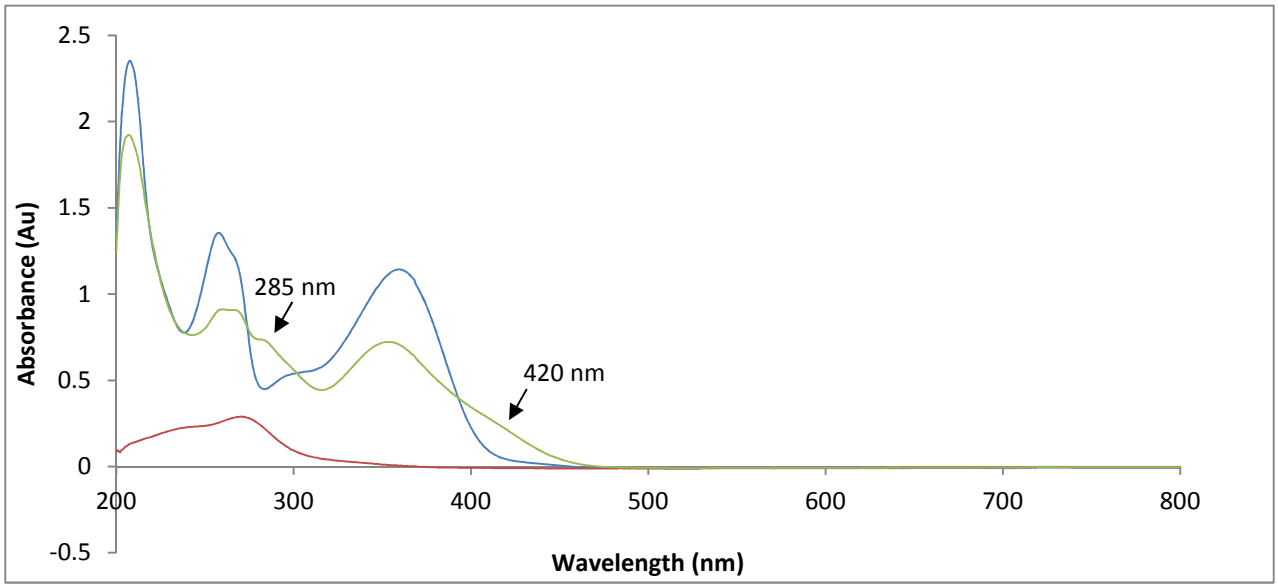


Figure 5.1 UV-Vis spectra of rutin (blue), $CuCl_2$ (red) and Rutin-Cu (green) in methanol.

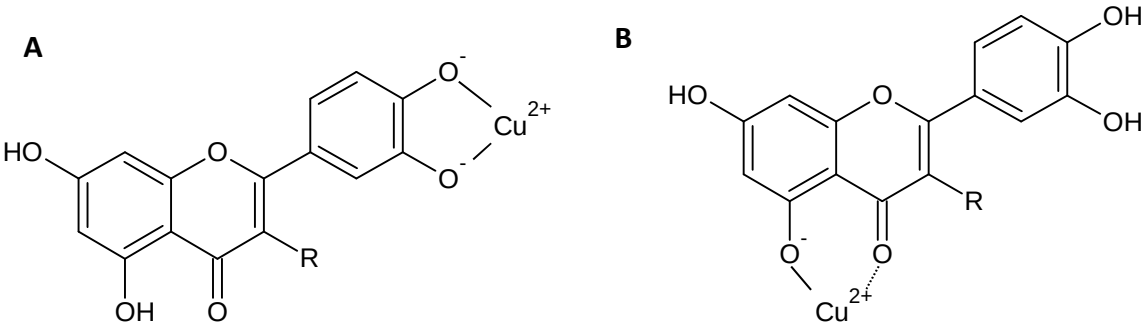


Figure 5.2 (A) Cu complexed at catechol group on rutin and (B) Cu complexed at 4-oxo-5-hydroxyl group on rutin. R = Rutinose moiety.

The preparation of 1:1 mM Rutin to Cu was prepared at room temperature and then subjected to reflux over several hours. In order to determine the optimum time of reflux for Rutin-Cu complexation, a sample of 1 ml was taken from the reflux every 30 minutes over a total of 4 hrs and run on a UV/Vis spectrometer. The results (Figure 5.3) show that the solution at room temperature after initial mixing had a high absorbance at 420 nm and 285 nm which increased when refluxed for 30 minutes. However, beyond 30 minutes the mixture seemed to oscillate in intensity in these regions. All further reflux solutions remained below the 30 minute maximum absorbance seen at

420 nm. The second highest intensity for these regions was observed for the solution prepared at room temperature which underwent no reflux (RT 0 min), whilst the lowest was for the reflux sample of 210 minutes. No absorbance was detected above 500 nm.

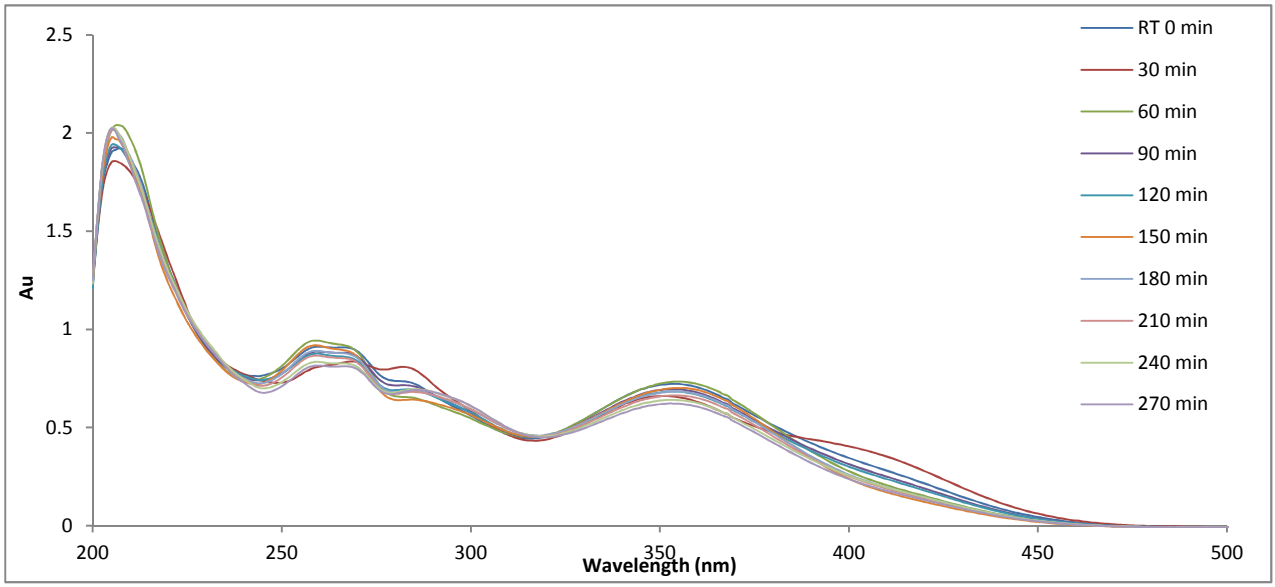


Figure 5.3 UV-Vis spectra of a mixture of rutin and $CuCl_2$ refluxed for different times. RT = Room temperature.

All samples were also analysed by direct injection mass spectrometry in order to confirm the formation of the rutin-Cu complex. The results (Figure 5.4) show that the room temperature and early reflux samples do contain the rutin-Cu complex (672 m/z), but also the presence of rutin-Na (633 m/z) and rutin-K (649 m/z). As the length of time increases for the reflux, the appearance of mass 303 m/z occurs. This indicates the breakdown of rutin into quercetin (quercetin 302 m/z). Mass 326 m/z is quercetin–Na, whereas other masses that appear after reflux such as 363 m/z and 385 m/z suggest the presence of quercetin-Cu and quercetin-Cu-Na fragments, respectively. Therefore, as the room temperature mixture produced rutin-Cu complex with minimal degradation to quercetin, this method of preparation was chosen for future investigations.

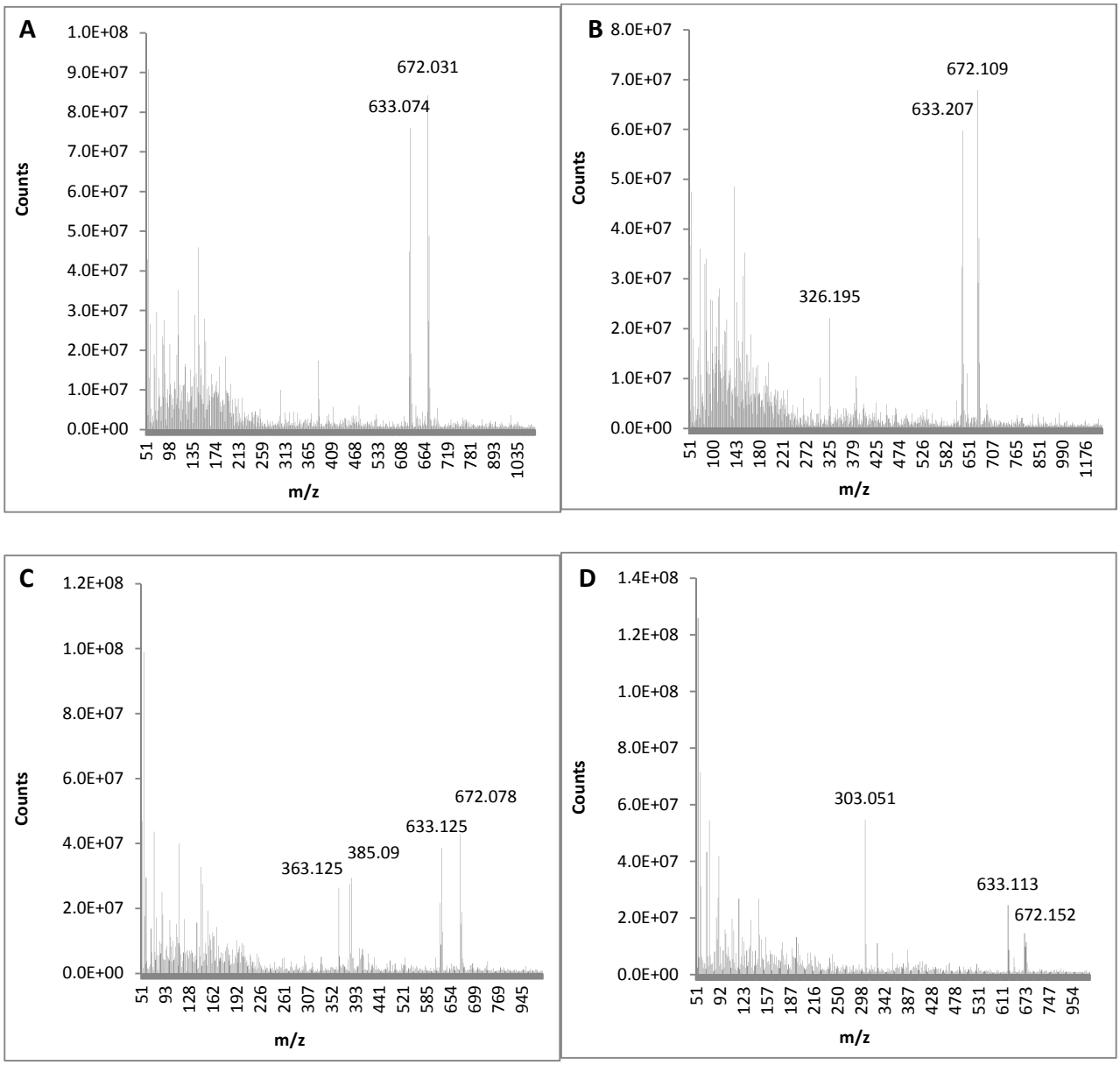


Figure 5.4 Mass spectra collected by direct injection of a Rutin-Cu complex solution at (A) room temperature-0 min (B) 30 min (C) 180 min and (D) 210 min of reflux.

5.3.1.2 Investigating a Chromatographic Method for the Monitoring of Rutin-Cu Complex

In order to determine if UHPLC can be used for monitoring of rutin-Cu complex, a standard of rutin was run as a control. The first method used for the analysis of rutin by UHPLC (in appendix 10.4, method 001) showed that the rutin standard co-eluted with the solvent front (Figure 5.5). From this, several methods were utilised to ensure the rutin peak was fully resolved from the solvent front. It was found that by increasing the starting aqueous mobile phase from 55% to 80% allowed the separation of rutin (in appendix 10.4, method 002).

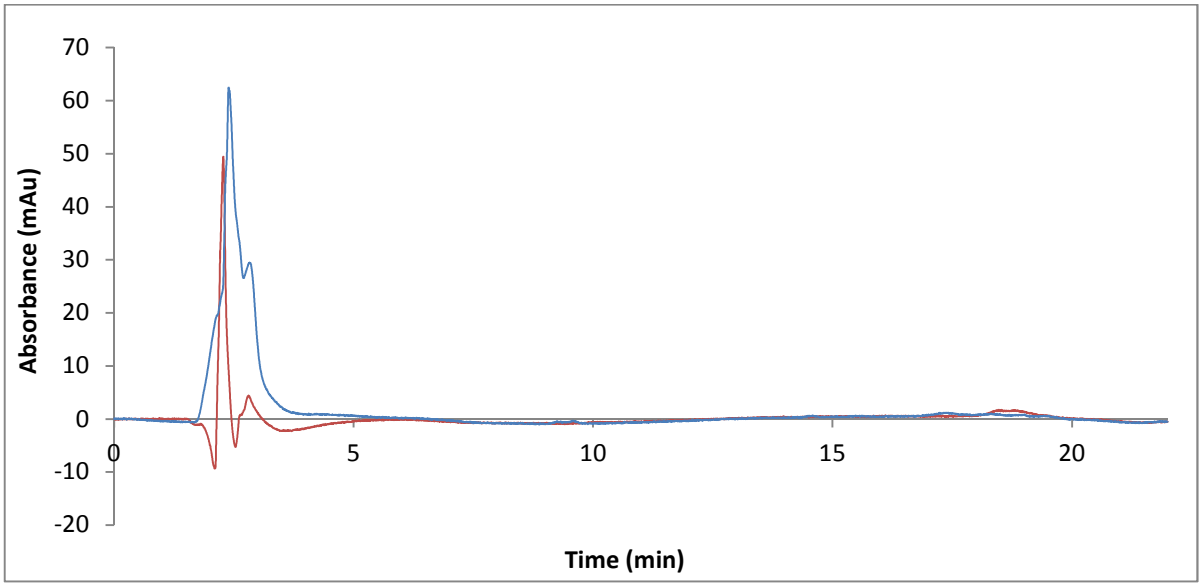


Figure 5.5. Rutin standard (blue) and methanol (red) run using UHPLC and method 001, appendix 10.4, (λ = 280 nm)

Using method 002, appendix 10.4; methanol, $CuCl_2$ in methanol and rutin-Cu complex reconstituted in methanol was analysed by UHPLC (Figure 5.6). These results showed that the rutin peak had shifted by several minutes in the presence of Cu indicating the presence of another chemical species, perhaps the rutin-Cu complex. However, later studies with LC-MS showed this to actually be a fragment of rutin whereby the sugar groups glucose and rhamnose (together known as rutinose) had become detached *via* a heterolytic cleavage [250] and thus quercetin remained.

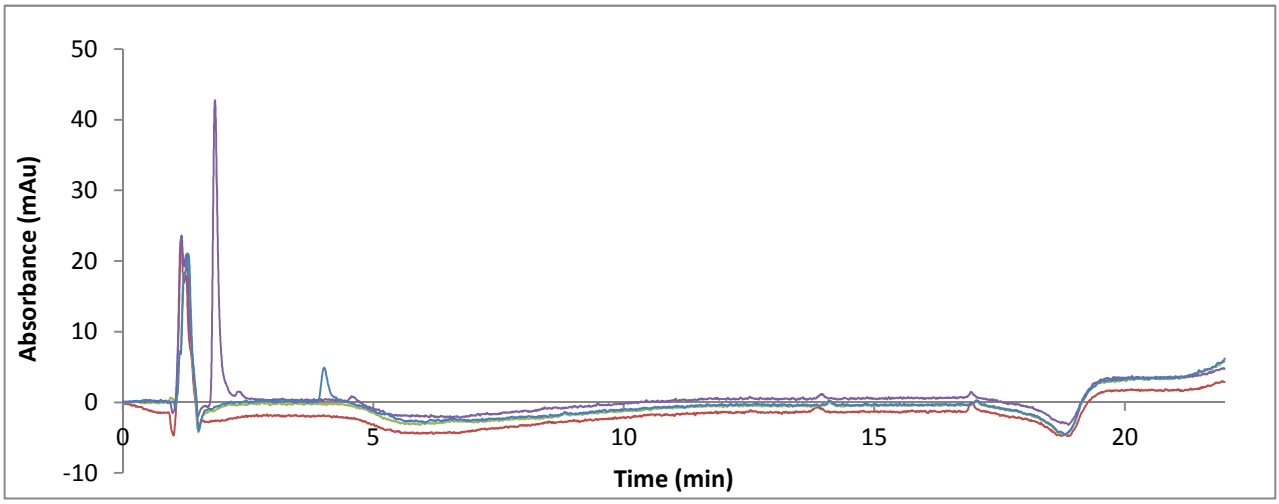


Figure 5.6. UHPLC chromatograms of methanol (red), $CuCl_2$ (green), rutin (purple) and Rutin-Cu (blue) complex using method 002, appendix 10.4 (λ = 280 nm)

As rutin-Cu has a distinct absorption at 420 nm, the PDA results were analysed for any absorption at 420 nm. Closer inspection of this wavelength on UHPLC showed that there was no significant increase in absorbance was observed associated with the complexation of rutin to Cu [109]. Although a visible change in colour is observed on the addition of $CuCl_2$ to rutin, the UHPLC was unable to detect any additional peaks. In order to see if the rutin-Cu complex was affected by the mobile phase, the sample was injected directly into a mass spectrometer. The rutin-Cu complex was dissolved in the following solutions: methanol, mobile phase with formic acid and mobile phase without formic acid whereby the mobile phase A: B ratio was 83:17. The results confirmed that although the rutin-Cu complexes were present in the methanol solutions, if made in the UHPLC mobile phase with formic acid (83:17 water 0.1 %v/v formic acid: ACN 0.1 %v/v formic acid), the complexes were no longer visible by direct injection mass spectrometry (Figure 5.7). When made in the same mobile phase ratio without the formic acid, the complexes were present but to a much lesser extent (approximately 4%) of that exhibited in methanol. These results indicated that although the rutin-Cu complex was injected on the UHPLC column, once in contact with the mobile phase the rutin-Cu complex would dissociate due to the low pH (mobile phase A with formic acid pH 2.6)[251] and also possible incompatibility with the mobile phases as when no formic acid was utilised the rutin-Cu counts still decreased. As the extracts would only contain a small fraction of rutin-Cu complex, this method was unsuitable for its detection.

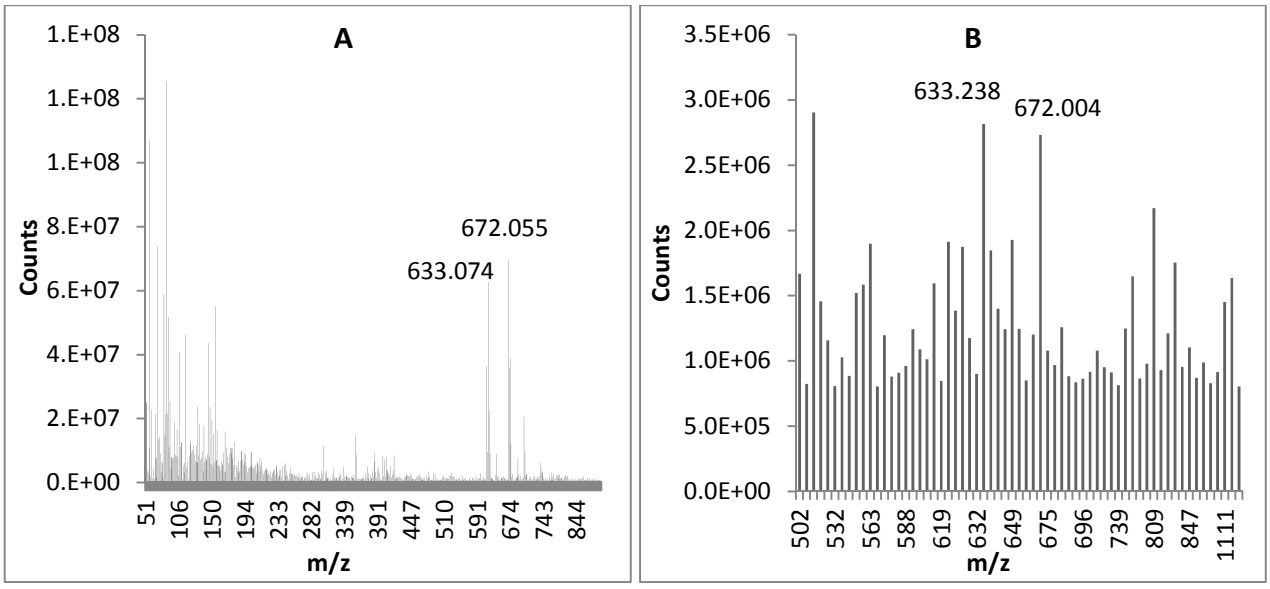


Figure 5.7 Mass Spectra collected by the direct injection of (A) rutin-Cu in methanol and (B) rutin-Cu in UHPLC mobile phase with no formic acid

These results show that in order to analyse such metal complexes by UHPLC a different mobile phase would be required, possibly buffer or methanol based [109, 197]. Also, another factor to be

considered is the UHPLC components. In order to have sharp peaks for such complexes the tubing should not be metal as the molecules could also interact with this. For future studies this could be done with the correct tubing (e.g. Teflon) and mobile phase. A more suitable instrument would be a LC-ICP-MS, which is fitted with inert tubing which could separate the metal complexes within the LC column and then also identify which ones are present.

5.3.2 Method Development for the analysis of SJW extracts

5.3.2.1 Preliminary Analysis and Column Comparison

SJW extracts in methanol were utilised for initial method development to determine a method that would separate out SJW molecular constituents. The first injection of SJW liquid extract using an adapted method from the rutin-Cu complex study (Method 003, appendix 9.4) showed that the majority of compounds detected with wavelength 280 nm co-eluted with the solvent peak as well as within the first 20 minutes of the run (Figure 5.8). By increasing the aqueous mobile phase by 5% in the beginning of the run and also the hold time to 5 minutes (004, appendix 10.4), it increased the Rt of the majority of the compounds and thus separated them from the solvent front. However, there was still a large amount of co-elution of these compounds (Figure 5.9).

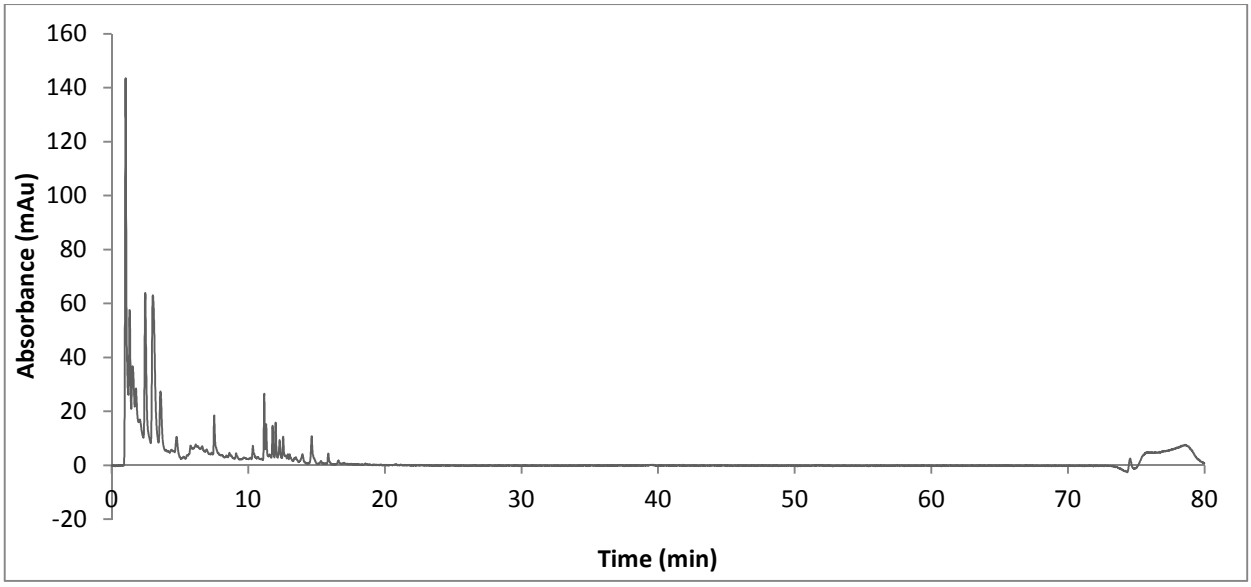


Figure 5.8. A chromatogram of SJW methanol extract (λ = 280 nm) using method 003 (appendix 10.4), Kinetix C18 column.

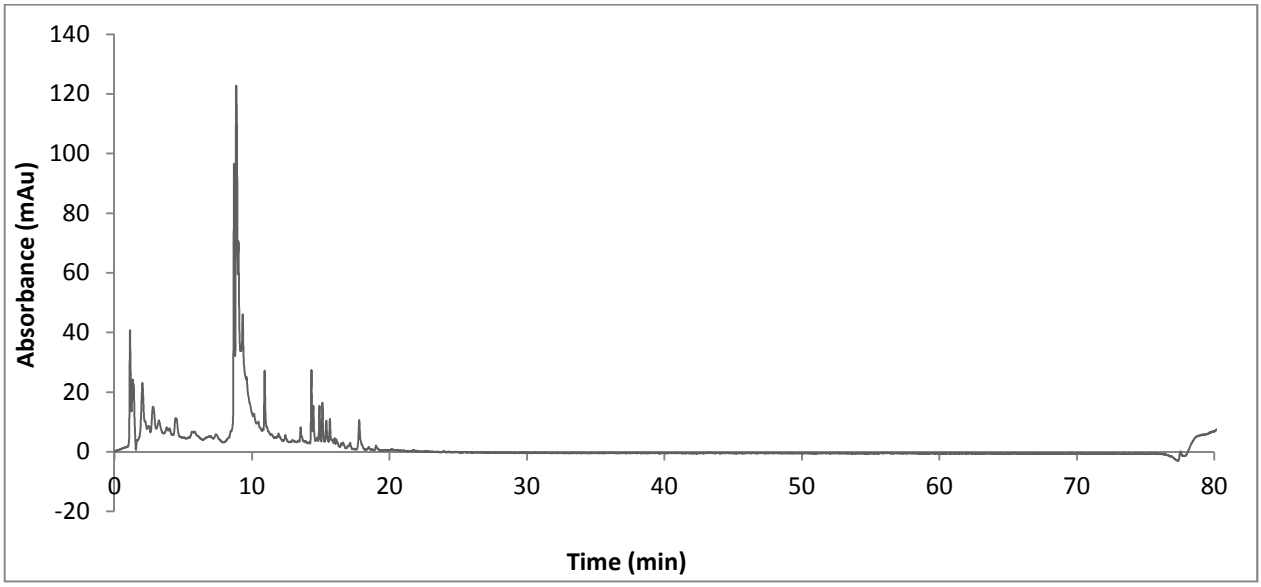


Figure 5.9. Chromatogram (λ = 280 nm) of SJW methanol extract using Method 004 (appendix 10.4) Kinetix C18 column. Shows increase of aqueous mobile phase increases the Rt of some compounds and removes some from solvent front.

The introduction of a slower gradient step from 83:17 to 55:45 (instead of going to 0:100 H_2O: ACN) helped with the separation of compounds by slowing down the introduction of ACN onto the column (Figure 5.10). The separation was assessed with various methods with the gradient step however, separation of the compounds did not occur.

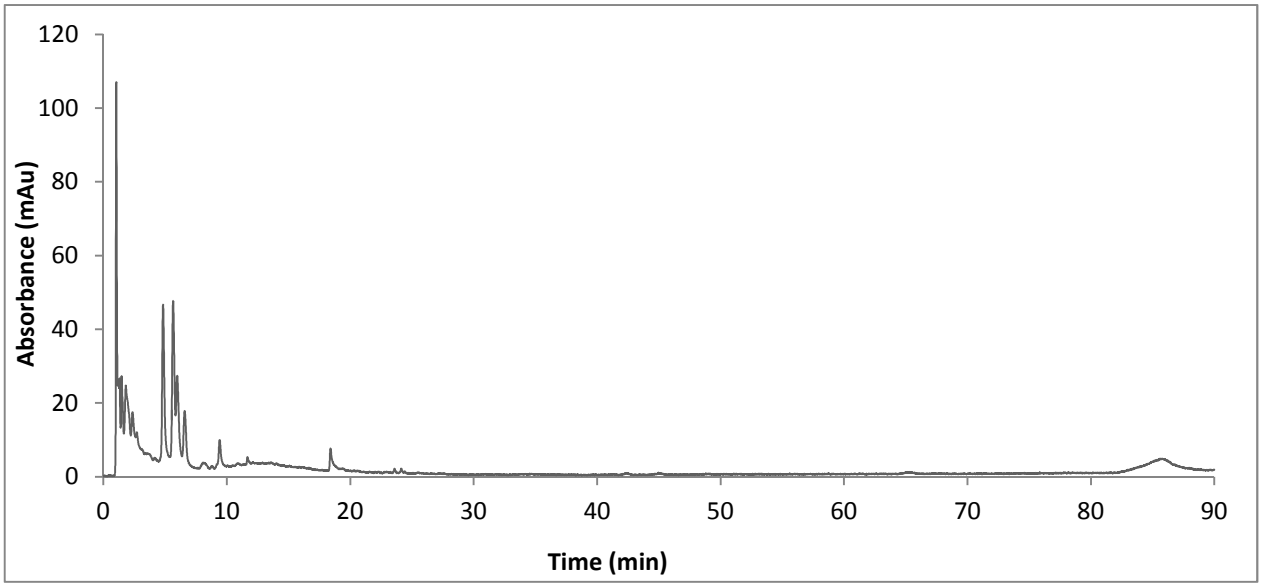


Figure 5.10 A chromatogram of SJW extract in methanol with additional gradient step. (Method 005, appendix 10.4, λ = 280 nm) Kinetix C18 column.

Therefore, following these initial injections and method development, the comparison of two columns was carried out with the rutin and quercetin standards as well as a SJW extract. The Phenomenex Kinetex™ (2.6 µm C18 100 Å, 100 x 4.6 mm) LC Column was compared to that of a Phenomenex Luna® (3 µm C18 100 Å, 150 x 4.6 mm) LC Column, with the same basic method (method 006 and 007, appendix 10.4) with only the flow rate being adjusted due to the larger particle size of the Luna column. The results show that the chromatograms produced using the Luna column (Figure 5.12) had less background interference from the changing gradient of the mobile phases in comparison to the Kinetix column (Figure 5.11). The results also show, that despite the low signal from the SJW sample (due to degradation of compounds), the Luna column also appeared to separate and begin to resolve some of the compounds better in comparison to the Kinetix. Peak width can be assessed with the rutin sample as there is no baseline interference from a change in gradient. This shows that the peak width is 0.8 min with a Kinetix column and 0.6 min with the Luna column. Therefore, taking this information into account, the Luna column was then chosen for future analyses as the baseline was less affected by the changing mobile phase, and this column appeared to give better separation of the compounds in the SJW samples and also give a slightly better peak width.

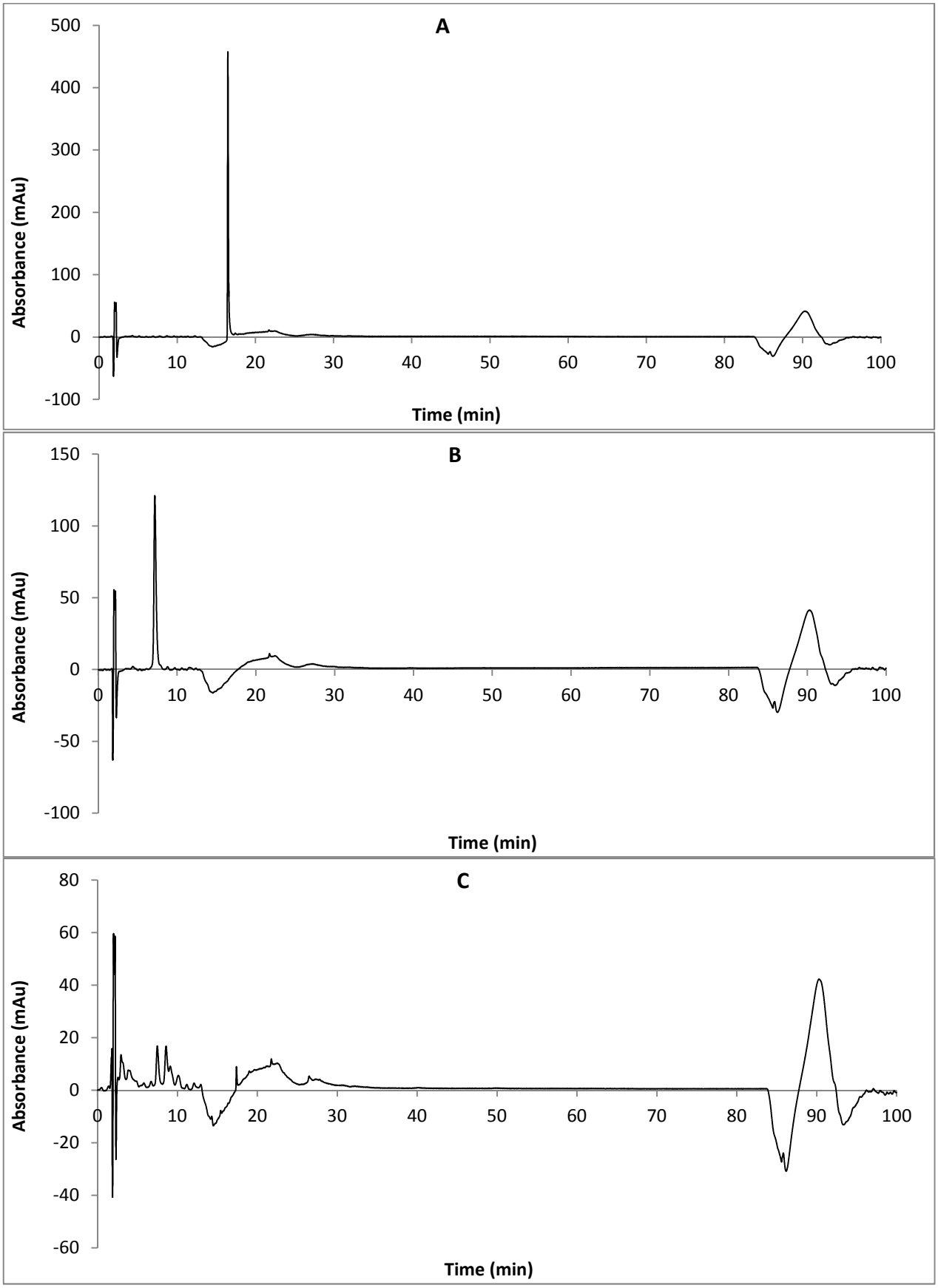


Figure 5.11. Chromatograms of (A) Quercetin, (B) Rutin and (C) SJW extract using Phenomenex Kinetix Column (method 006, appendix 10.4, λ = 280 nm)

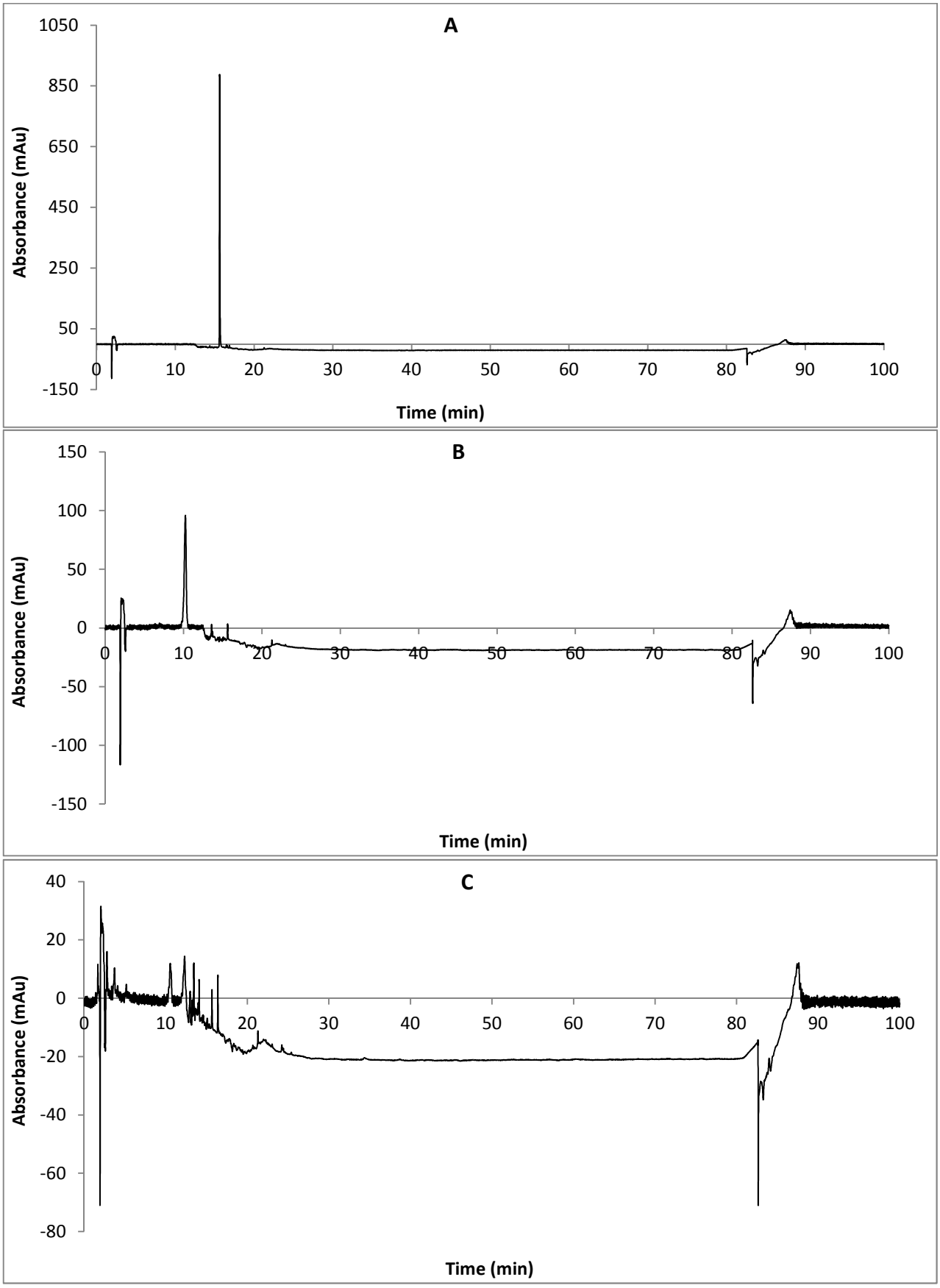


Figure 5.12. Analysis of samples using Phenomenex Luna Column (A) Quercetin, (B) Rutin and (C) SJW extract (method 007, appendix 10.4, λ = 280 nm)

It is worth noting that if the future method was not being utilised on LC-MS for compound identification, the Kinetix column may be able to give the same if not better resolution. The reason for its poor performance here is the low flow rate to ensure the pressure doesn't exceed 3000 psi in order to not exceed pressure limits on the LC-MS. If using for UHPLC analysis only, this column could be used for better separation of SJW extracts by increasing the flow rate and thus using it as a true UHPLC system.

Following this, method development was carried out on the Luna column at a flow rate of 1 ml/min and 60 %v/v ethanol extracts of SJW. The 60 %v/v ethanol was utilised as the extraction solvent as it is the most common extraction solvent used by industry [201]. Several methods were utilised in the method development stage which focused on the separation of the flavonoids. It was noted that the hypericins present in SJW (hypericin and pseudohypericin) absorb at 590 nm. However, throughout the analyses no peaks were observed at 590nm. As hypericin standards are very expensive for a small amount (£145 for 1 mg, Sigma Aldrich), the decision was made to focus on the flavonoids within SJW that could be quantified (rutin, hyperoside and quercetin). Through various methods, adjusting the ratios of the 3-step gradient and increasing the initial aqueous mobile phase, a SJW method was developed that allowed the separation of the flavonoid peaks (Figure 5.13).

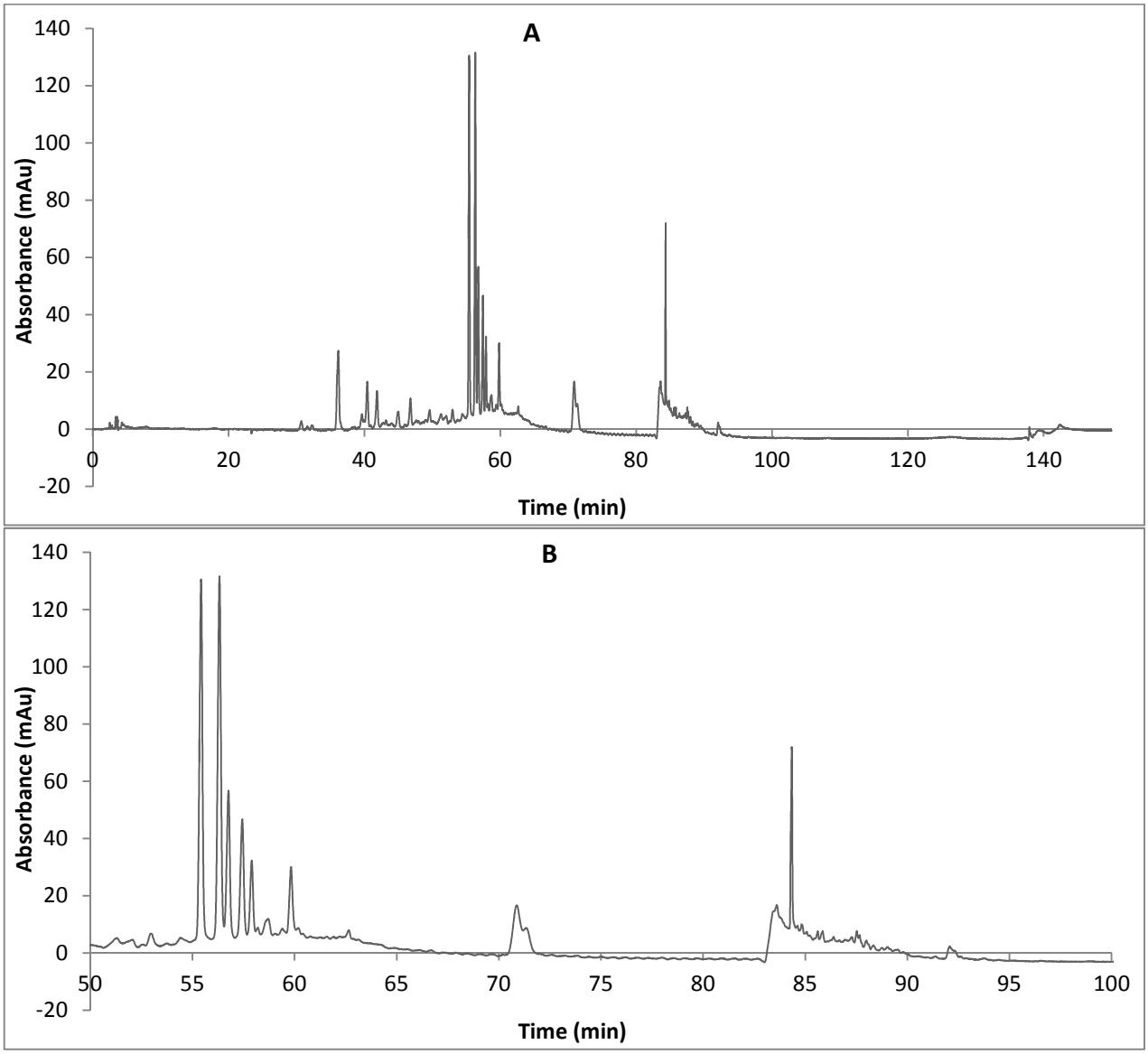


Figure 5.13 Separation of SJW peaks in 60 %v/v ethanol. (A) Full chromatogram and (B) Expanded view of chromatogram. (method 008, appendix 10.4).

Replicates of rutin standards (concentrations between 0.134–1.204 mg/ml, 15 samples x 95 minutes each) were run to evaluate variation of retention time (Rt). It was seen that the Rt varied (Figure 5.14) by up to 15 minutes as the sequence progressed. For example, the first chromatogram gave a retention time of approximately 48 minutes for rutin. As the sequence progressed this increased to 62 minutes then as the sequence progressed further the Rt returned towards 50 minutes. In order to see if this was due to a temperature decrease as the sequence was running over night, the same sequence was analysed in the presence of an external column oven (Perkin Elmer 101) at 30 ± 3 °C. The results show that in the presence of the column oven, the Rt no longer shifts. Therefore this suggests that the ambient room temperature can have a large influence on this analysis. In order to prevent this, the column oven was utilised for further analyses at 30 ± 3 °C.

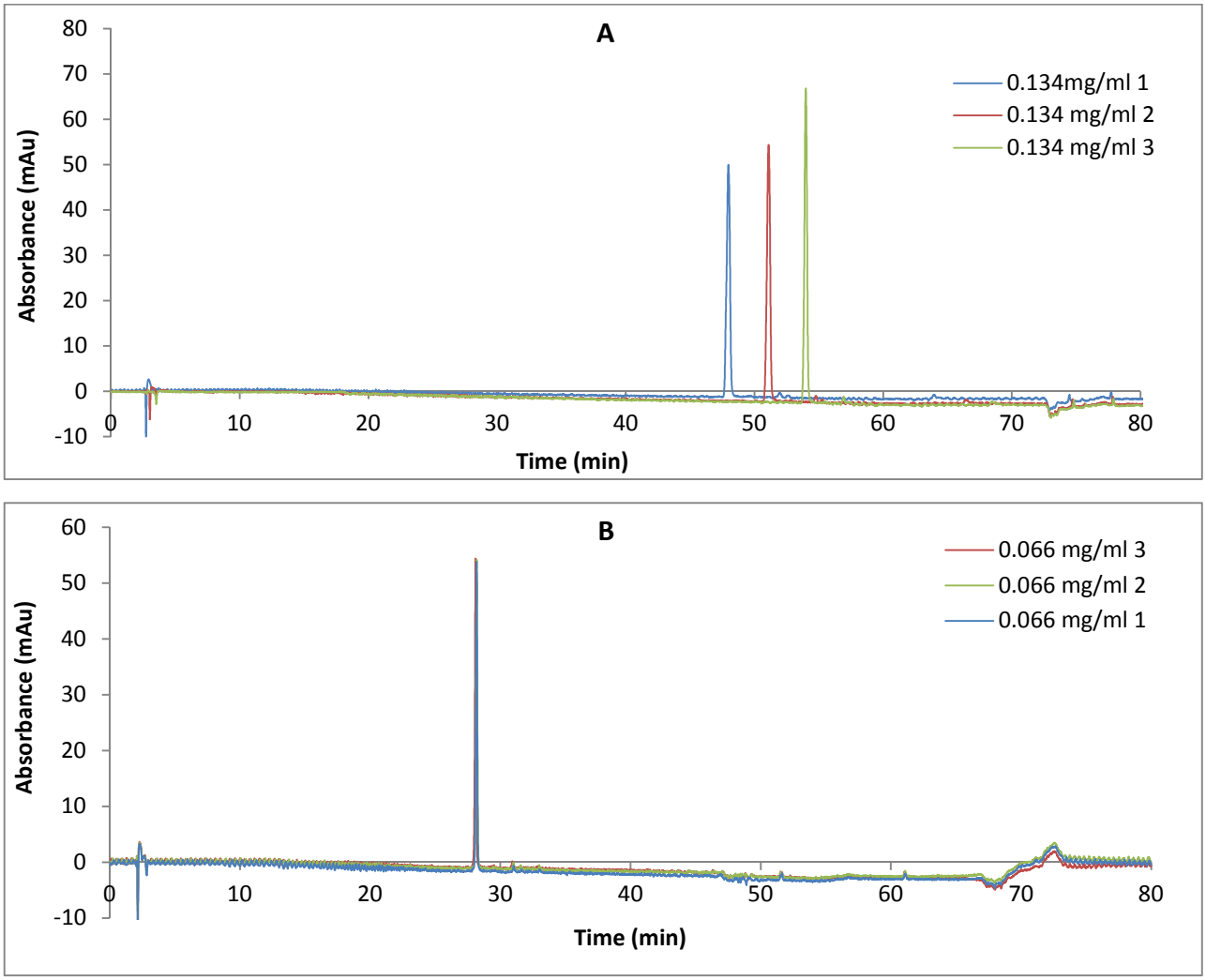


Figure 5.14 Subsequent injections of rutin standard (A) showing retention time drift before column oven is fitted (B) no retention time drift with column oven (λ = 280 nm)

5.3.2.2 Reducing Run Time

Throughout the method development the length of time the run takes has been very long (>120 minutes) in order to achieve the flavonoid separation. The final SJW method took 145 minutes per sample (method 008). Due to time constraints and wanting to reduce the amount of mobile phase used, the gradient utilised was further examined. The original gradient changed from 90:10 H_2O: ACN to 73:27 over 45 minutes. By altering the starting aqueous mobile to 92:08 with a gradient to 79:21 over 18 minutes, this region of the method was able to be reduced by 27 minutes whilst retaining the separation of the rutin and hyperoside. Quercetin was also well resolved however, some flavonoid peaks (Figure 5.15) began to co-elute once more. However, the peaks required (rutin, hyperoside, quercetin, hyperforin and adhyperforin) were separated and resolved. To further reduce the run time, the gradient step to 05:95 H_2O: ACN was reduced by 5 minutes and the

holding time with this ratio reduced by 30 minutes. Overall this new method saved 55 minutes per sample and still retained important flavonoid separation.

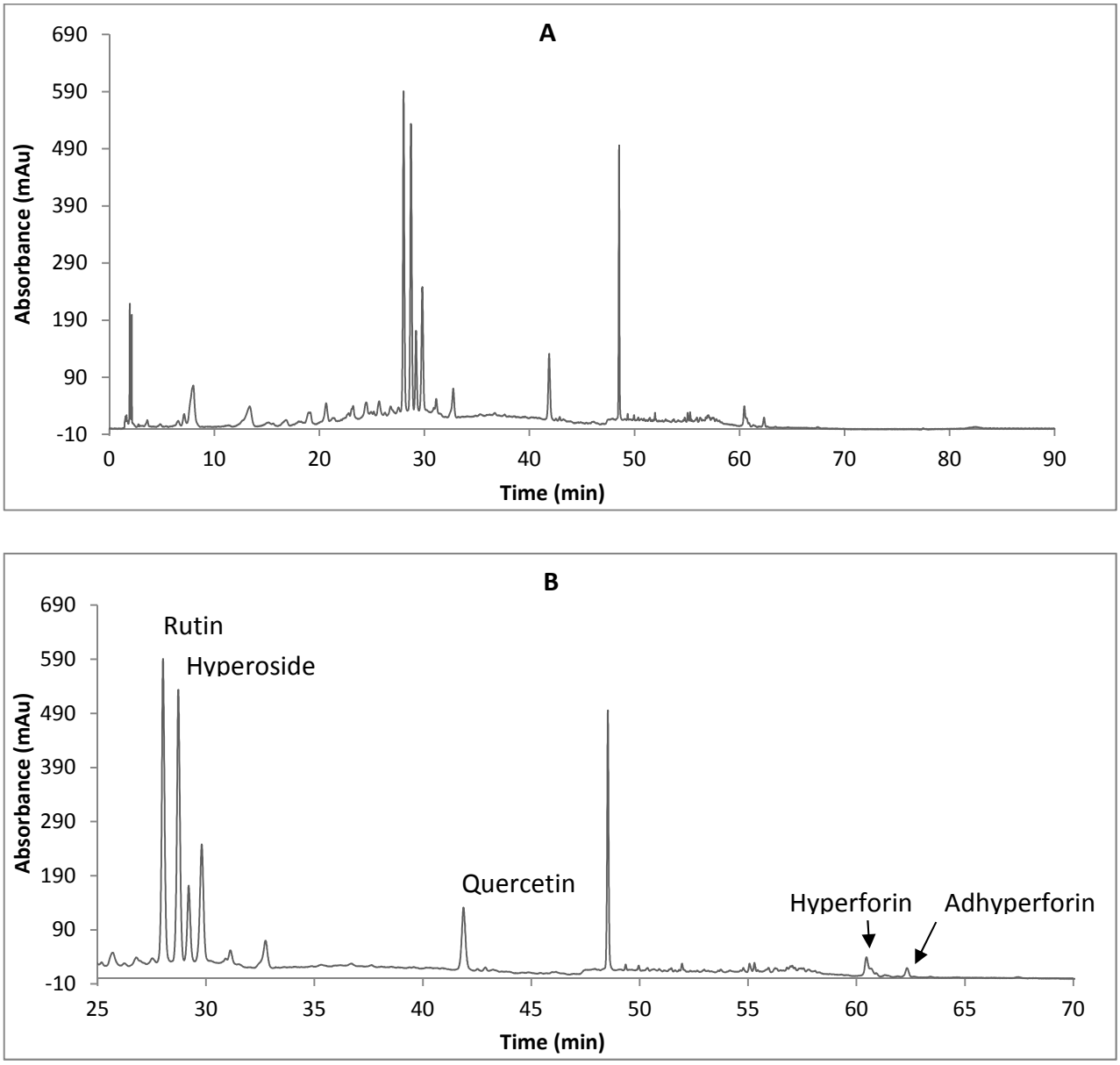


Figure 5.15 SJW 60 %v/v ethanol liquid extract (A) Full chromatogram and (B) an expanded view (25-70 mins) of the region of interest (method 010, appendix 10.4, λ = 280 nm)

5.3.3 Validation

5.3.3.1 UHPLC Consistency

A 60 %v/v ethanol extraction of SJW was prepared and run 10 times on the UHPLC in order to test the consistency of the UHPLC over a period of time (13 hours). The overlaid chromatograms (Figure 5.16) show very good consistency between injections. Closer inspection of three peaks (i.e., rutin, hyperoside and quercetin) showed good consistency between injections. Rutin had an Rt standard deviation of 0.05% and a peak area deviation of 1% across 10 injections. Hyperoside had an Rt deviation of 0.6% and peak area deviation of 0.8% whereas quercetin had an Rt deviation of 0.03% and a peak area deviation of 1.7 % across 10 injections of the same sample. Therefore this technique shows good consistency between samples over a prolonged period of time.

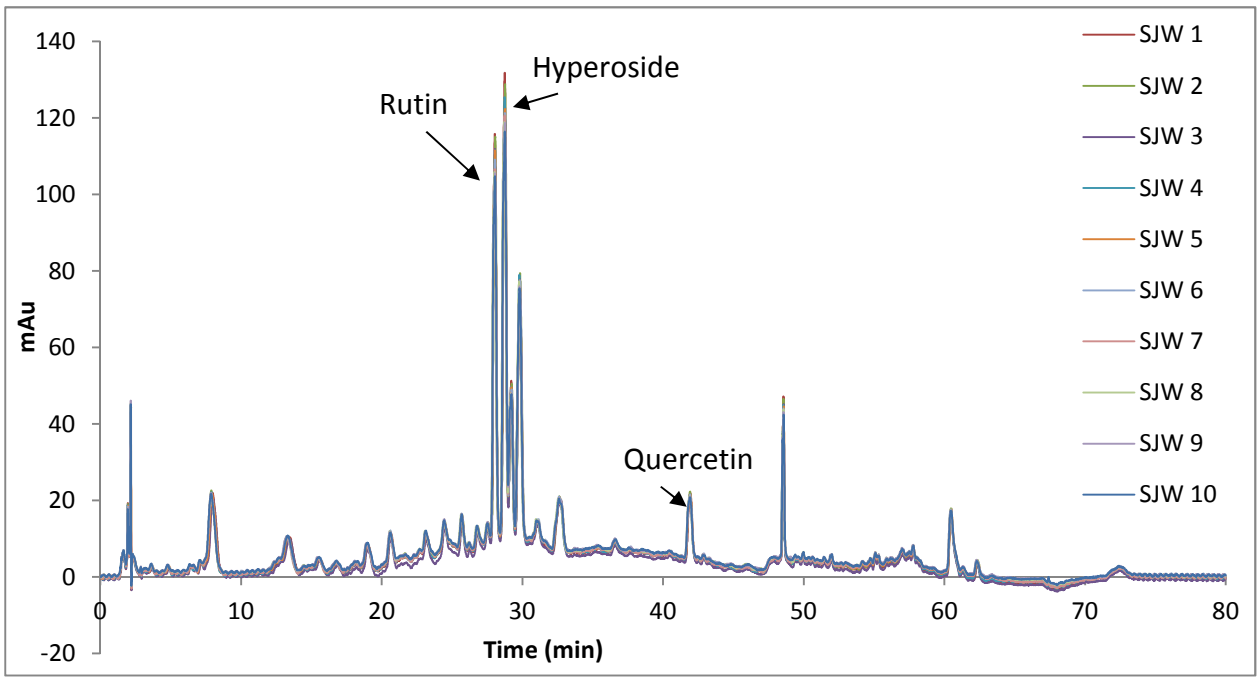


Figure 5.16 Overlay of 10 chromatograms of same SJW sample. Method 009, appendix 10.4

5.3.3.2 Characterisation and Calibration of Reference Standards

The run time of the rutin calibration takes 13 hours to complete and as such would be highly impractical to run every day. Therefore, a rutin calibration was run in triplicate so that rutin QCs could be run with samples to cut down on UHPLC run time. The rutin calibration standards were prepared at ten different concentrations (0.016-0.99 mg/ml) and run on UHPLC (monitored at 280nm). The calibration curve (Figure 5.17) has a correlation coefficient of 0.999 and had less that 2% deviation between injections.

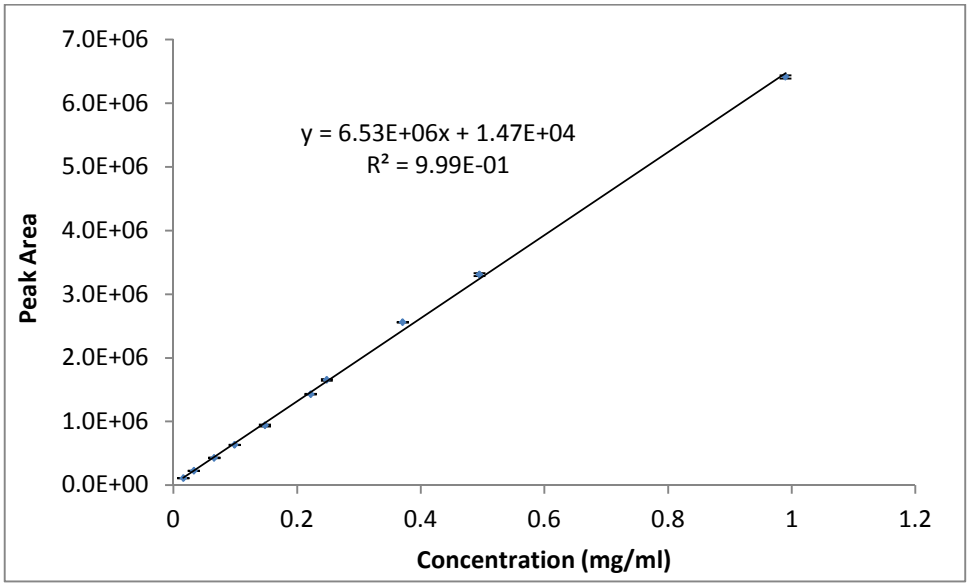


Figure 5.17 Calibration curve of rutin on UHPLC (Λ = 280nm)

In order to confirm the identification of peaks by retention time (Rt), standards of rutin, hyperoside, quercetin and hyperforin/adhyperforin were run on the UHPLC. The chromatograms (Figure 5.18) show each peak has a separate Rt and is resolved. In order to identify the two peaks in the hyperforin/ adhyperforin standard and to confirm identity of the compounds, a multi-component standard of all the compounds (multi-standard 1, Table 5.2) was subjected to LC-MS analysis using the same LC method and column (method 010, appendix 10.4).

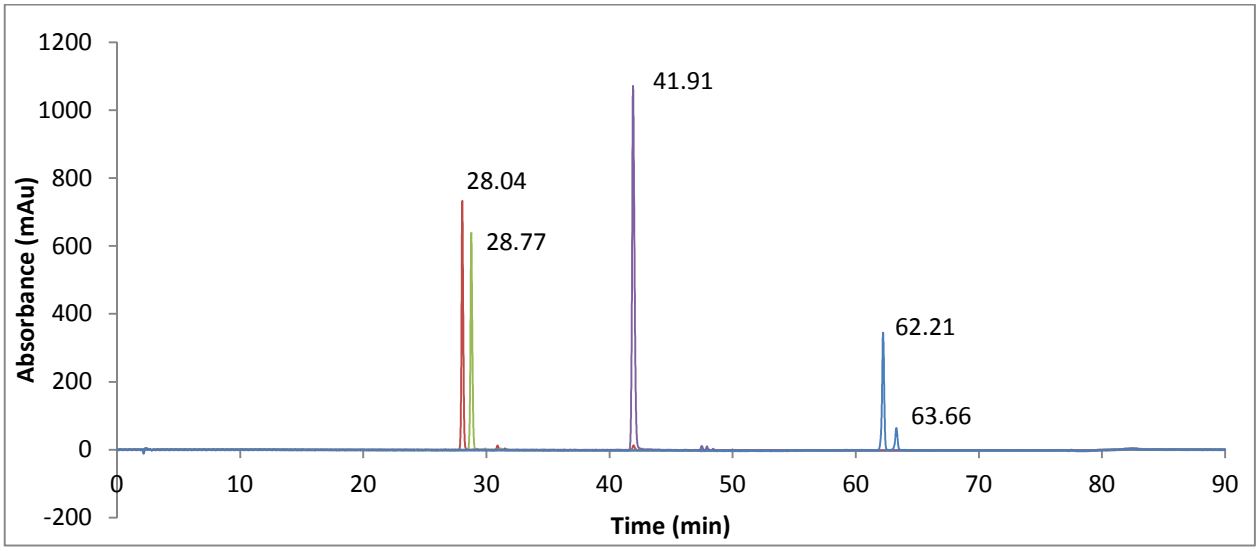


Figure 5.18 Chromatogram Overlay of rutin (red), hyperoside (green), quercetin (purple) and hyperforin/adhyperforin (blue) standards (Λ=280nm).

The analysis of the multi-standard by LC-MS has shown that the retention times between the two instruments (UHPLC and LC-MS) are similar and are usually within 1 minute (Figure 5.19). There is some deviation however seen between the hyperforin and adhyperforin peaks; an additional 3 minutes longer on the LC-MS compared to the UHPLC retention time. The 1 minute separation between hyperforin and adhyperforin is still intact. The LC-MS chromatogram (Figure 5.19) also illustrates the separation of the five compounds in the multi-standard.

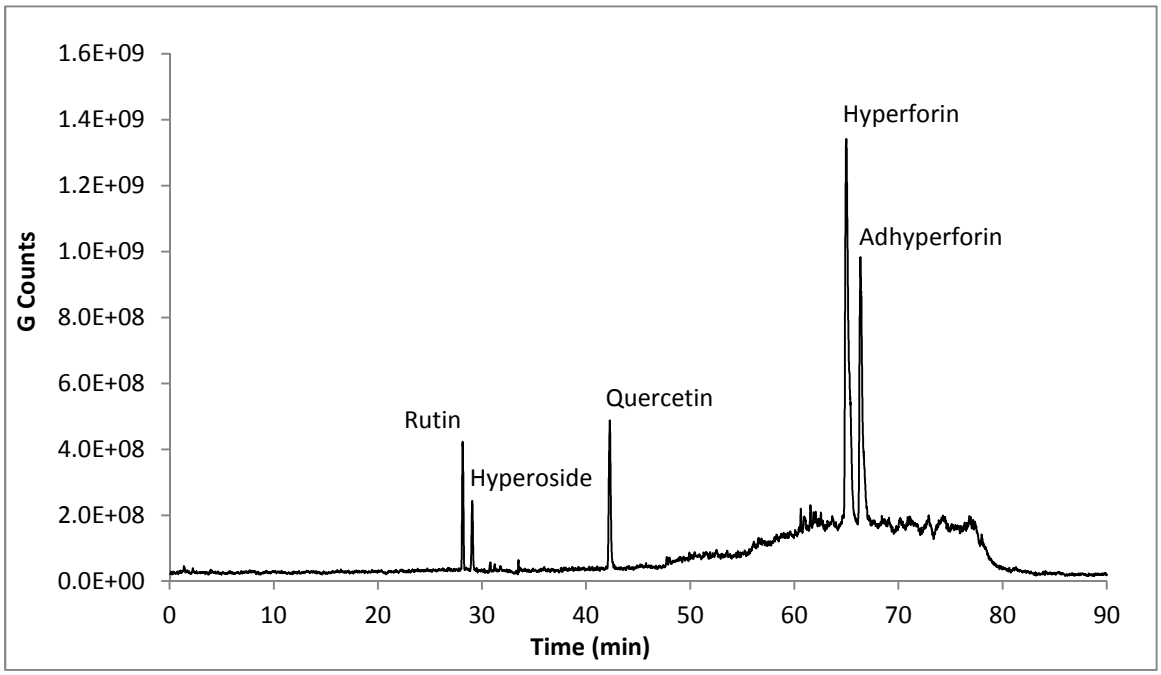


Figure 5.19 LC-MS chromatogram of multi-standard 1

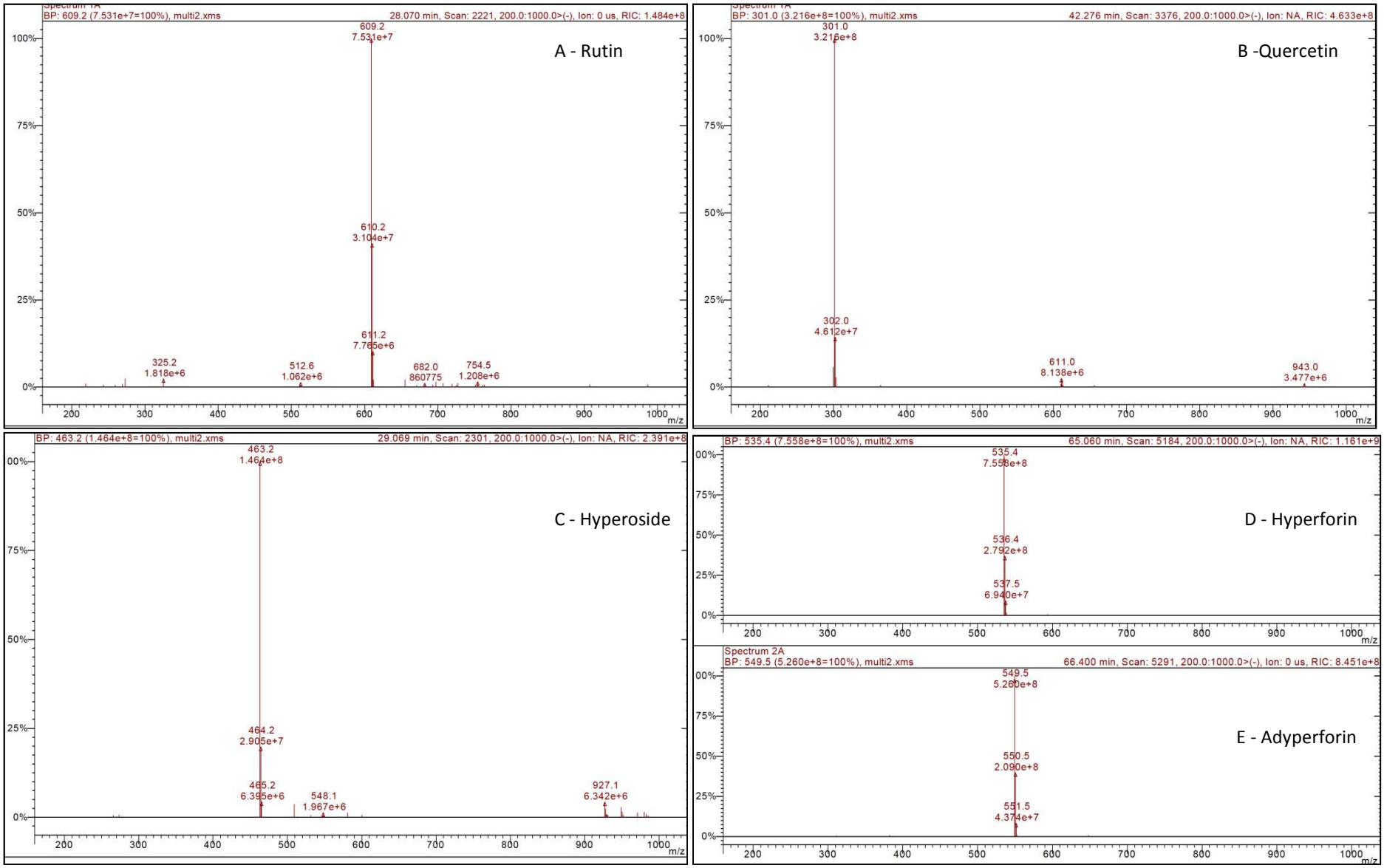


Figure 5.20 Mass spectrum of LC peak associated with (A) rutin, (B)quercetin (C) hyperoside (D) hyperforin (E) Adhyperforin

The mass spectrum of these peaks (Figure 5.20) show that all compounds are of high purity. The hyperoside spectrum (Figure 5.20 B) shows the existence of a small amount of a hyperoside dimer at mass 927 m/z formed during the electrospray ionisation (ESI) process. Following the LC-MS confirmation of the peaks the two peaks resulting from the hyperforin/adhyperforin standard could now be identified as hyperforin being the first peak with an Rt of 62 minutes, followed a minute later by adhyperforin.

Table 5.3 Comparison of UHPLC and LC-MS retention times

Compound	UHPLC Rt (min)	LC-MS Rt (min)	Mass (m/z)	LC-MS $[M-H]^-$
Rutin	28.04	28.07	610	609.2
Hyperoside	28.77	29.07	464	463.2
Quercetin	41.91	42.28	302	301.1
Hyperforin	62.21	65.06	536	535.4
Adhyperforin	63.66	66.04	550	549.5

Calibration curves were constructed for rutin (0.032-1.064 mg/ml), hyperoside (0.02-0.66 mg/ml), quercetin (0.047-1.600 mg/ml), hyperforin (0.02-0.66 mg/ml) and adhyperforin (0.005-0.181 mg/ml) (Table 5.2). Comparison of this new rutin calibration to the previous calibration shows there is only a 1% deviation from the original trend line (Figure 5.21) and is thus a robust method. This shows the parameters of the UHPLC are consistent as there was only a 1% deviation when each calibration was run 3 weeks apart. Therefore, the new calibrations for these other compounds are able to be utilised to quantify hyperoside, quercetin, hyperforin and adhyperforin.

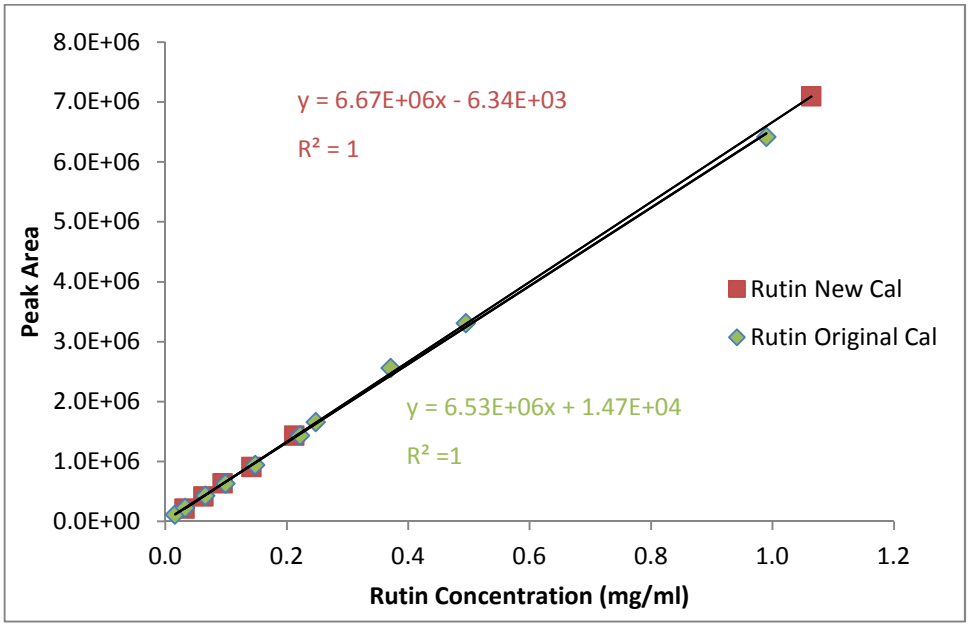


Figure 5.21 Comparison of original rutin calibration and new calibration

As the hyperforin/ adhyperforin came as one standard, the weights of each compound had to be calculated in order to perform a calibration. The original weight used to make the stock solution of these compounds was 8.41 mg in 10 ml of 60 %v/v ethanol. From taking the ratio of the hyperforin peaks to the adhyperforin peaks across the different dilutions, it was found that the average ratio of hyperforin: adhyperforin was 78.5:21.5. Therefore, from the original weight, there is 0.660 mg hyperforin and 0.181 mg adhyperforin. Therefore the stock consisted of 0.66 ± 0.01 mg/ml hyperforin and 0.181 ± 0.004 mg/ml adhyperforin (± 2%). All calibration graphs (Figure 5.22) had a correlation coefficient higher than 0.999.

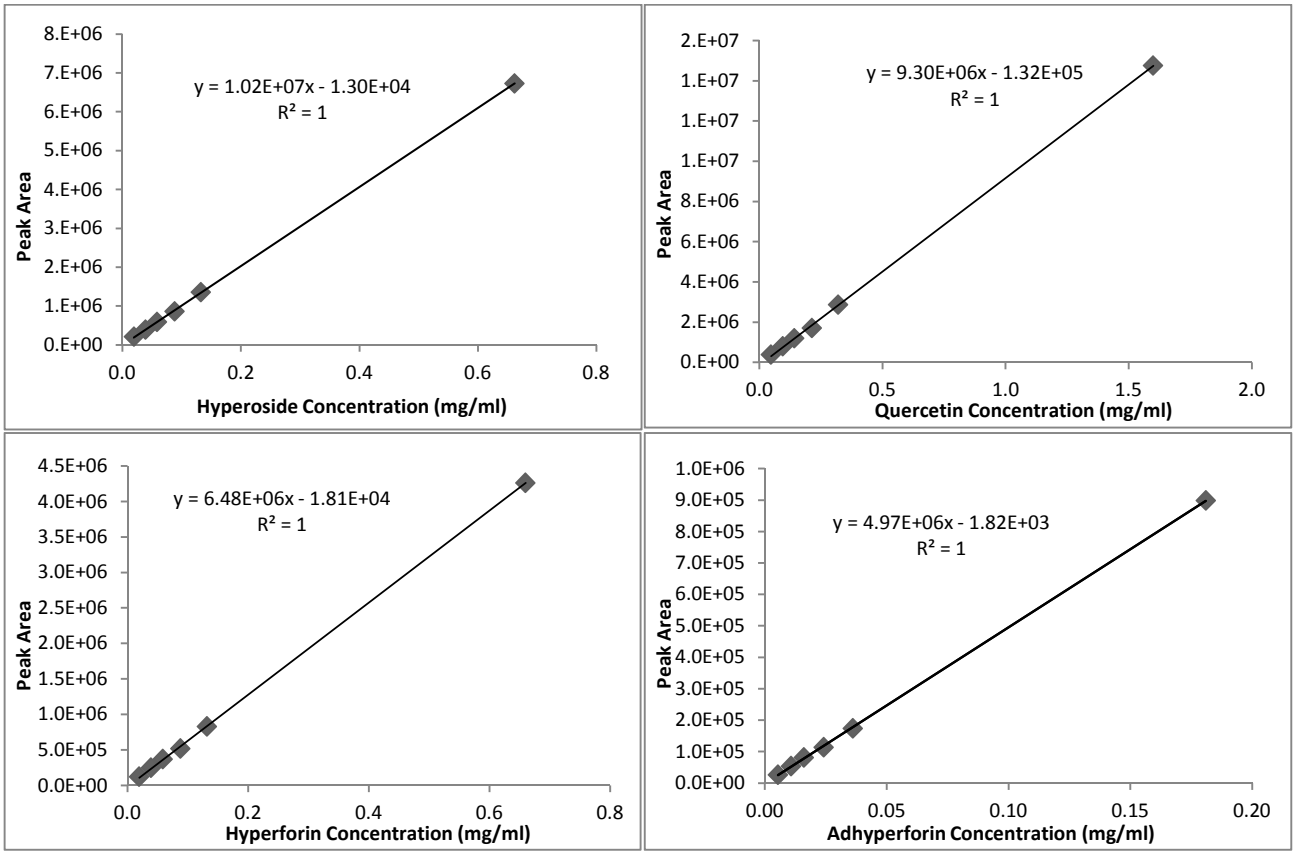


Figure 5.22 Calibration graphs for (A) hyperoside, (B) quercetin, (C) hyperforin and (D) adhyperforin.

5.3.4 Transferability to Other LC Systems

5.3.4.1 Varian ProStar 500

Several SJW extract samples were run on the Varian ProStar 500 HPLC to assess transferability of the method. The results show that the majority of the flavonoids were resolved on the new instrument, but a large amount of fronting is visible on the chromatograms (Figure 5.23) despite the column, guard column and mobile phases being the same. Fronting is usually caused by overloading the column or incompatibility with the solvent; however, this is not seen on other systems with the same column. A new column of the same brand and dimensions also showed fronting therefore indicating the fronting was not due to column or guard column breakdown. Therefore one possible reason this may have occurred is the longer sample tube on the instrument between the injection port and the detector (tube is an extra 30 cm longer on Varian ProStar 500 compared to Perkin Elmer UHPLC), however this issue usually causes peak broadening rather than fronting. Therefore a partial blockage in the system or a joint that isn't completely flush may have caused the fronting.

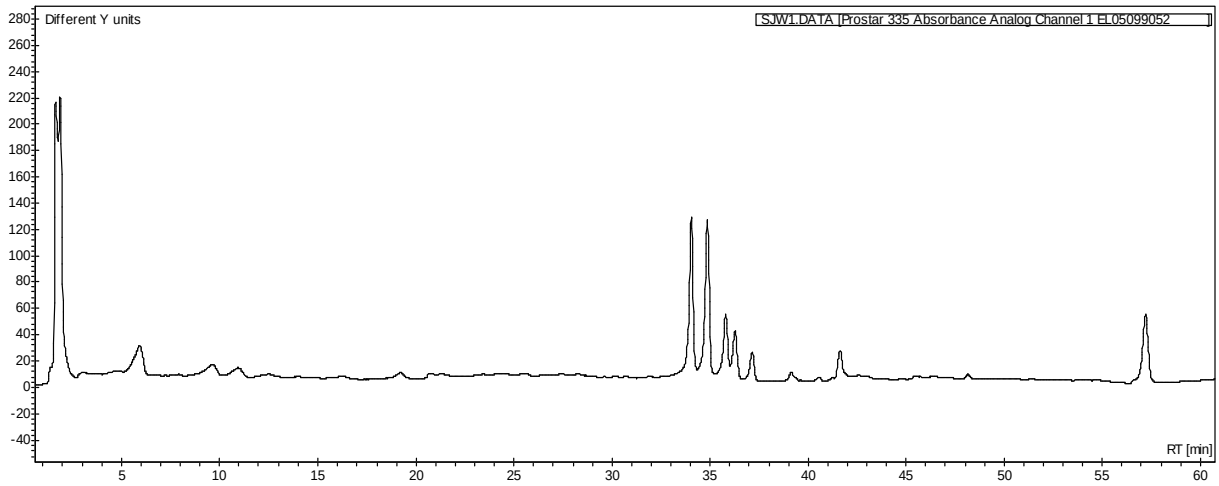


Figure 5.23. Expanded view of SJW extract run on Varian ProStar 500 (λ=280nm). Method 008, appendix 10.4

Following this the sample was injected consecutively and assessed for consistency. Over eight injections, it was found the the flavonoid region over time was progressively eluting at a shorter retention time and shifts by 15 minutes (Figure 5.24). As the column oven was utilised to prevent temperature change, this is due to the instrument inadequately mixing the mobile phases. Therefore, instead of a consistent gradient with each injection, the instrument was increasing the amount of acetonitrile slightly with each injection. This in turn causes the flavonoids to elute quicker and also lose peak resolution. Also noted is the solvent peak area increasing with each injection. This shows that all the compounds that elute before the flavonoid region are now coeluting with the solvent front. This analysis shows the importance of ensuring that the gradient system you have chosen for the method is able to do so consistently. It also notes that issues may arise, such as fronting, that may not be seen on other instruments.

In conclusion, this particular LC system could not be used for the analysis of SJW extracts as it lacked peak resolution, injection consistency and also exibited artifacts not seen by other instruments. A method would have to developed that would be able to run isocratically on this instrument, however, separation of such closely related flavoniods without a gradient system would be difficult.

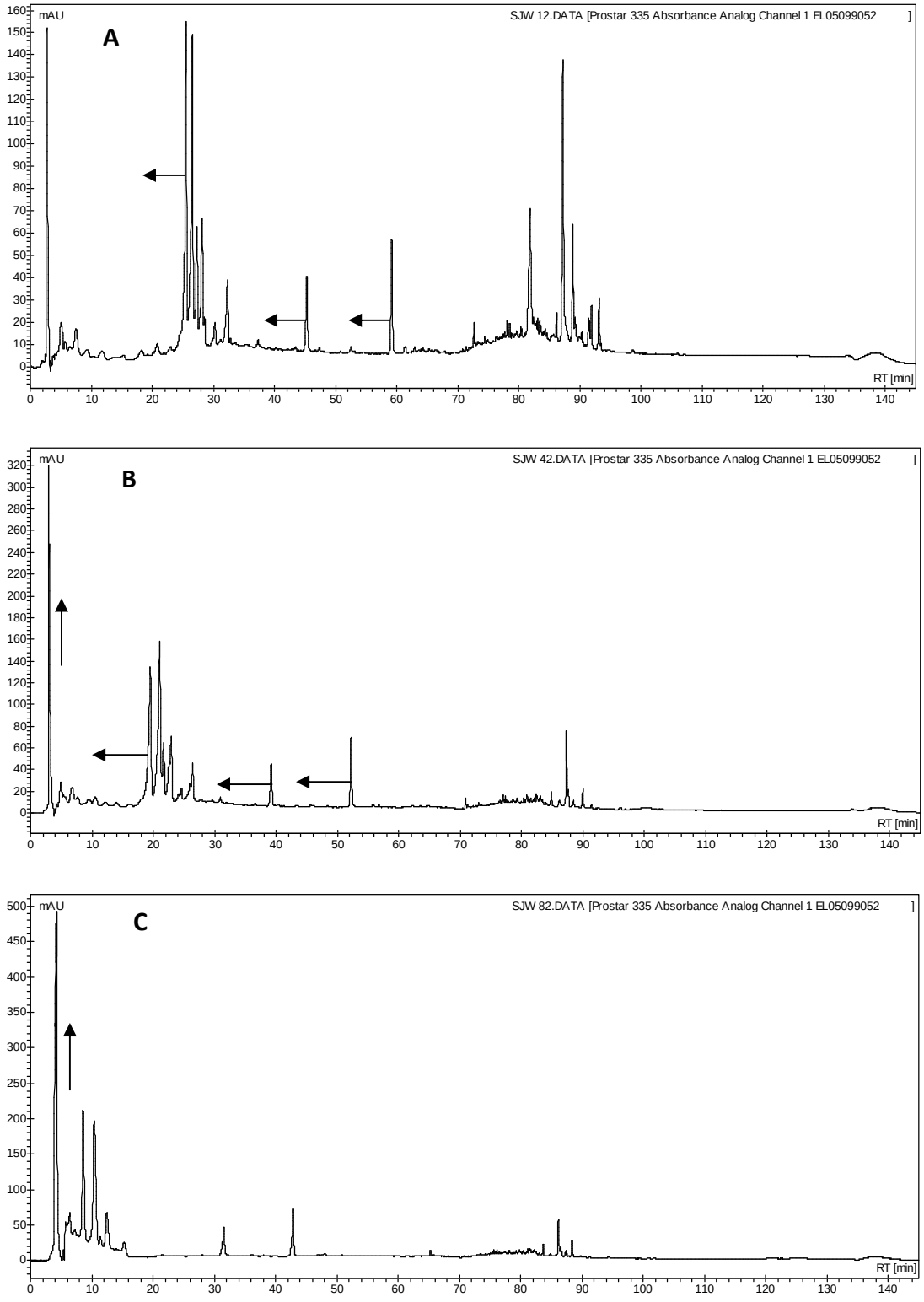


Figure 5.24. Analysis of SJW extract on Varian ProStar 500 HPLC (A) first injection, (B) fifth injection and (C) eighth injection. Arrows shows Rt drift of 15 minutes over the injections and an increase in compounds eluting with solvent front. Method 008, appendix 10.4

5.3.4.2 Perkin Elmer Flexar

A SJW 60 %v/v ethanol extract was also analysed on a Perkin Elmer Flexar HPLC. Firstly the sample was injected 10 times in order to assess the consistency of the instrument. The results (Figure 5.25) show that between injections the retention times and peak areas are very consistent. However, most notable with the chromatograms is the decrease in peak resolution in comparison to the Perkin Elmer UHPLC and initial runs with the Varian ProStar. Despite the samples being run on the same method as the Varian ProStar and Perkin Elmer UHPLC; the flavonoids in the region of interest were overlapping extensively on the Perkin Elmer Flexar. Some method development was carried out to see if minor changes to the gradient could resolve the peaks however, results showed (Figure 5.26) that although rutin could be separated from the other flavonoids, of the methods investigated, the flavonoids themselves were not resolved. Although this instrument shows very good consistency between injections over a long period time, the separation is not satisfactory and more method development would be needed. Thus, this shows the difficulty of transferring optimised methods to other instruments, especially methods used for natural products.

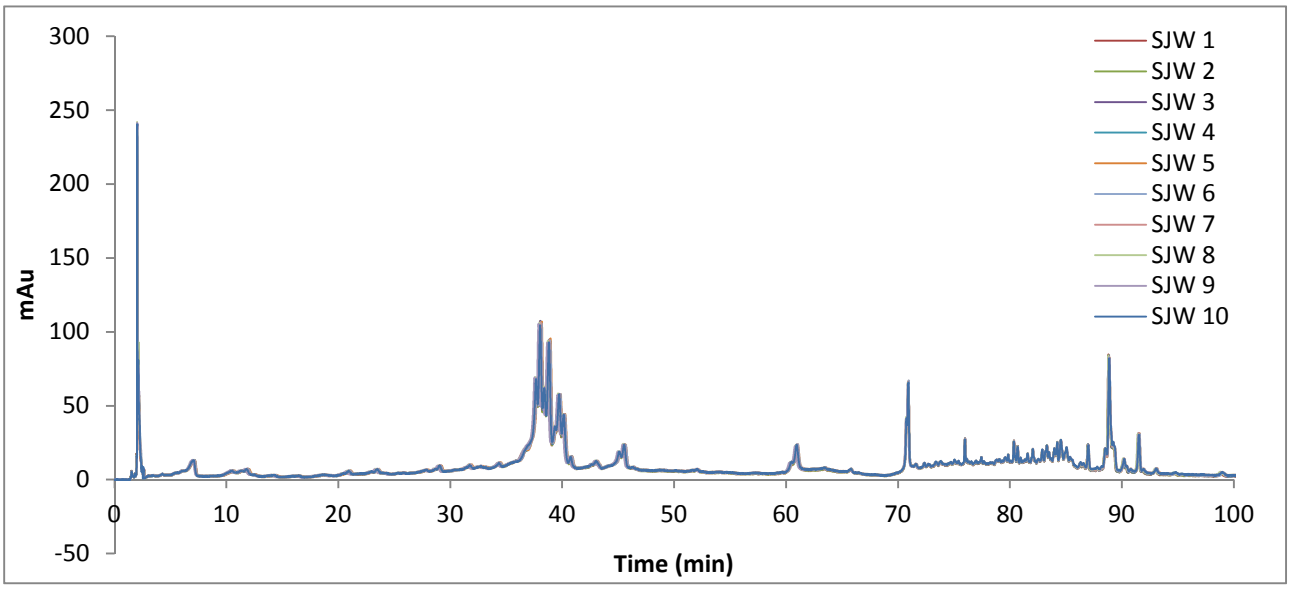


Figure 5.25 Overlay of 10 injections of SJW 60 %v/v ethanol extract on Perkin Elmer Flexar HPLC

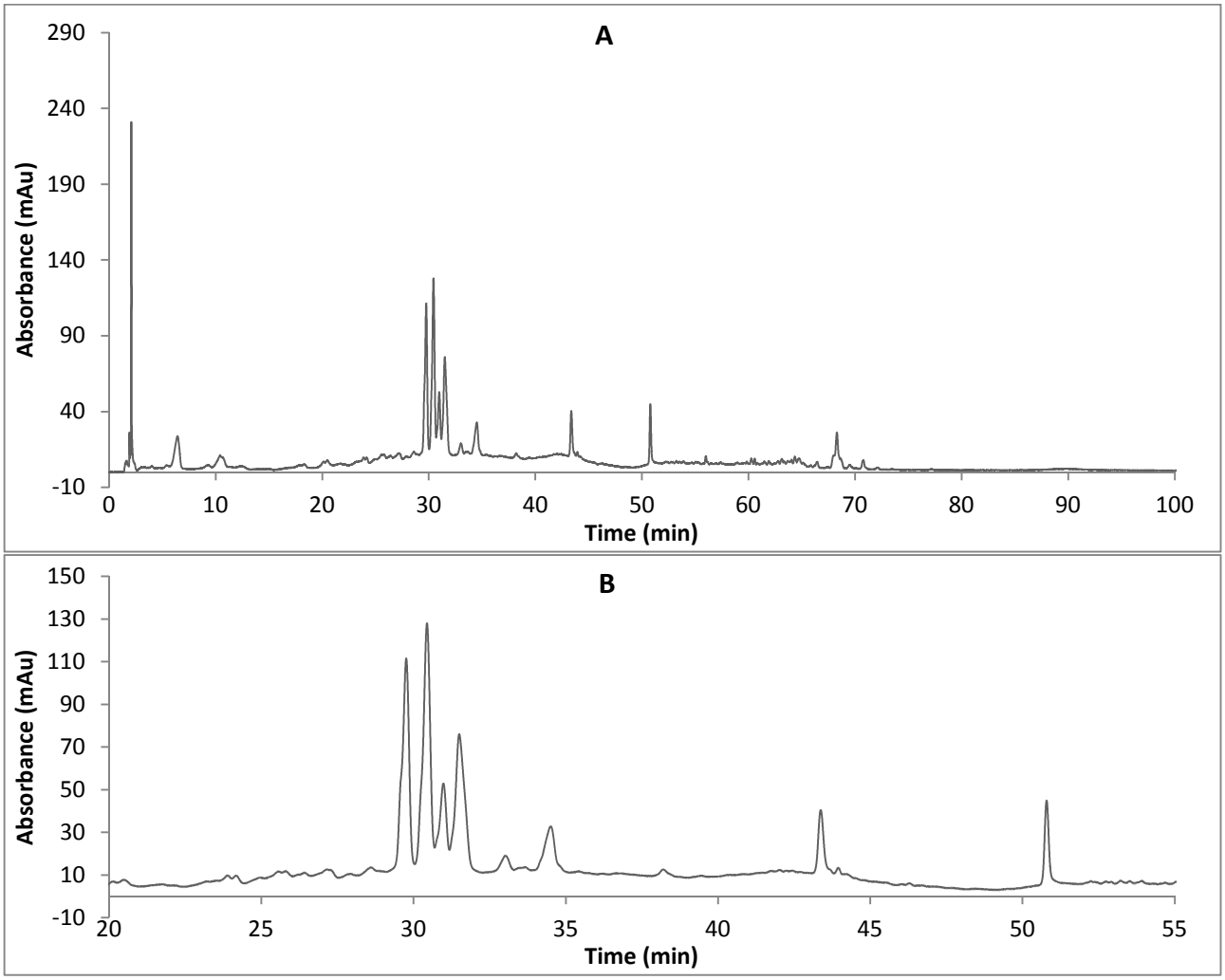


Figure 5.26 (A) Full chromatogram of SJW 60 %v/v ethanol on Perkin Elmer Flexar (method 008) and (B) expanded view of flavonoid region

5.3.5 Analysis of SJW extracts

The developed and validated method (010, appendix 10.4) was used to analyse SJW solvent extracts to determine the presence and levels of key bioactive constituents which are proposed to have interactions with metal constituents. Eight samples of powdered SJW were extracted with 60 %v/v ethanol and subjected to UHPLC analysis. The 60 %v/v ethanol solvent was used as it was found this particular concentration of solvent is favoured by industry [201] as this percentage extracts the most bioactive constituents [191, 194, 195]. The flavonoids rutin, hyperoside, quercetin as well as hyperforin and adhyperforin were quantified.

5.3.5.1 Rutin

Rutin is a natural antioxidant present in many plant species. It has been shown in studies that it acts as a metal chelator, as it can bind to metals such as Cu [109, 150, 197, 220, 230-232], Fe [109, 150, 220], Al [150, 230], Zn [150, 252] and Mn [252]. Studies have shown complexation of rutin to metal ions can have a profound effect on its antioxidant capacity. For example, when rutin is complexed with Cu, the antioxidant capacity increased eight-fold compared to rutin alone [109]. However, when the compound was complexed with Fe, it mostly showed a two fold increase in antioxidant capacity but in some instances, also showed some pro-oxidant capacity [109].

Using the calibration curve the LOD of rutin was calculated to be 0.010 mg/ml and the LOQ was 0.029 mg/ml. The extraction of rutin from the original dried herb varied between samples (Figure 5.27 A). The lowest concentration of rutin was extracted from herb 5 (2.2 ± 0.8 mg/g original herb) with approximately 7 – 9 mg/g of original herb for the majority of the other herbs. These levels agree with Çirak *et al.* [93] who extracted rutin with 95% ethanol *via* shaking. The levels also agree with those reported by Bagdonaite *et al.* [92] when a Soxhlet extraction with chloroform/methanol was used; however, concentrations were higher when compared to a maceration extraction with methanol by the same authors [92]. The amount of rutin was also calculated in relation to the dried extract (Chapter 4). The results (Figure 5.27 B) show that herb 5 also had the lowest rutin concentration (16 ± 8 mg/g) whereas herb 2 had the highest rutin concentration (70 ± 10 mg/g).

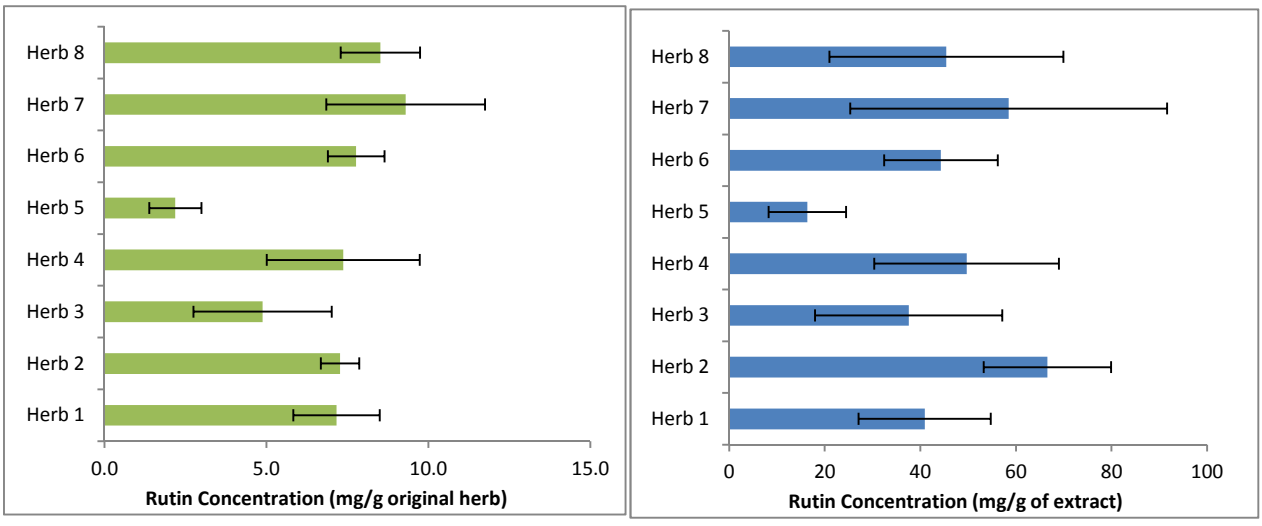


Figure 5.27 (A) Amount of rutin extracted per original herb (B) Amount of rutin per dried extract. Uncertainty is reported as ±1SD

5.3.5.2 Hyperoside

Hyperoside is a flavonoid found in several species of plants and is also known as an antioxidant. It has been shown to have a greater reducing power than rutin, which is possibly due to the smaller sugar group attached [98]. The amount of hyperoside extracted from the original dried herb is shown in Figure 5.28 A. Using the calibration the LOD of hyperoside was calculated to be 0.006 mg/ml and LOQ was 0.019 mg/ml. The lowest concentrations of hyperoside was extracted from herbs 2 and 8 (2.0 ± 0.2 mg/g original herb and 2.1 ± 0.5 mg/g original herb respectively) with the majority of the other herbs approximately 4 – 6 mg/g of original herb was extracted. These levels agree with those reported by Çirak *et al.* [93] for extracts prepared by shaking with 96% ethanol, but are lower than those reported by Bagdonaite *et al.* [92] for extraction with 96% ethanol *via* maceration. The amount of hyperoside was calculated in relation to the dried extract (Chapter 4). The results (Figure 5.28 B) show that herbs 2 and 8 also have the lowest amount of hyperoside in the dried extract (19 ± 4 mg/g and 11 ± 6 mg/g) whereas the other herbs are consistent between 28 and 36 mg/g.

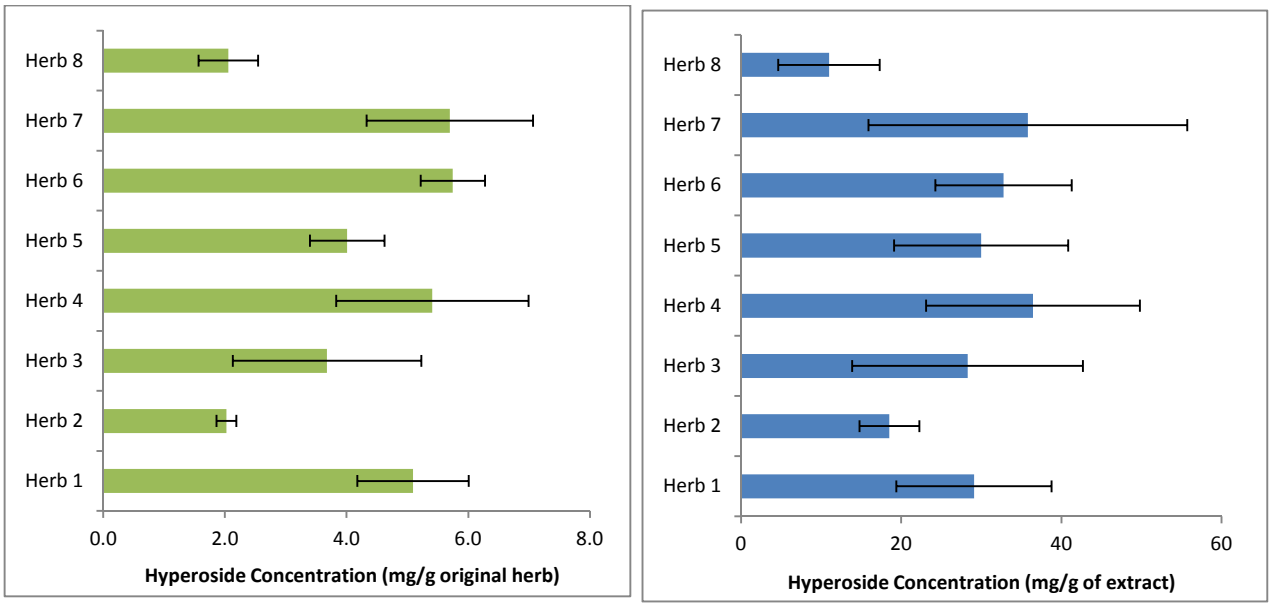


Figure 5.28 (A) Amount of hyperoside extracted from original herb (B) Amount of hyperoside in dried extract. Uncertainty is reported as ±1SD

5.3.5.3 Quercetin

Quercetin is a flavonoid found in many species of plants. It has been shown in studies that it acts as a metal chelator as it can bind to metals such as Cu [109, 150, 197, 232], Fe [150, 181], Al [150, 253] and Zn [150]. Quercetin was found to have a greater reducing power than rutin and hyperoside, probably due to the lack of a sugar group [98].

Using the calibration the LOD of quercetin was calculated to be 0.031 mg/ml and LOQ was 0.094 mg/ml. The extraction of quercetin from the original dried herb was relatively consistent between samples (Figure 5.29 A) and ranged between 1.1 – 1.9 mg/g of original herb. These amounts agree with those reported by Bagdonaite *et al.* [92] and Çirak *et al.* [93] for extractions in 96% ethanol *via* maceration or shaking. The amount of quercetin was calculated in relation to the dried extract (Chapter 4). The results (Figure 5.29 B) also show consistency between the herbs in the dried extract with a range of 17.4 – 13.0 mg of quercetin/g of extract.

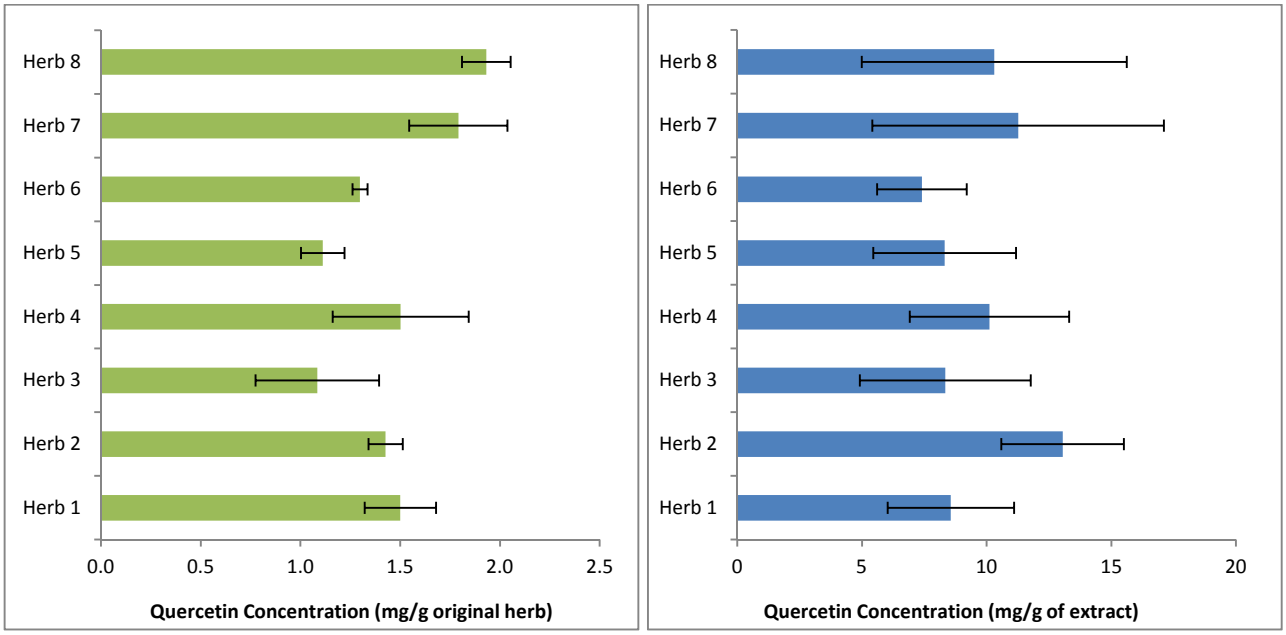


Figure 5.29 (A) Amount of quercetin extracted from original herb (B) Amount of quercetin in dried extract. Uncertainty is reported as ±1SD

5.3.5.4 Hyperforin

Hyperforin is a major constituent of *Hypericum perforatum*. It has recently been shown to be the main active constituent, rather than hypericin, to give a therapeutic affect for depression [88]. The compound has also shown antibacterial properties as well as anti-proliferate and pro-apoptotic effects towards some cancer cells as well as other possible beneficial properties [84]. It has only been found in the *Hypericum* genus of which Hypericum perforatum contains the highest concentrations.

Using the calibration curve the LOD of hyperforin was calculated to be 0.01 mg/ml and the LOQ was 0.03 mg/ml. The extraction of hyperforin from the original dried herb varied between samples (Figure 5.30 A). The concentration of hyperforin in Herb 2 fell below LOD therefore is not reported; whereas the most hyperforin was extracted from herb 5 with 1.3 ± 0.1 mg/g of original herb. These levels

agree with those found by Helmja *et al.*, [127] from ethanol extracts produced by sonication. The amount of hyperforin was also calculated in relation to the dried extract (Chapter 4). The results (Figure 5.30 B) show that herbs 7 and 8 have very similar amounts of hyperforin (2.0 ± 1 mg/g) whilst herb 5 has the highest concentration of hyperforin in the dried extract (10 ± 3 mg/g). These results show that with a 60 %v/v ethanol extraction, herb 5 dried extract contains approximately twice as much hyperforin compared to the other two highest hyperforin extracts; herb 4 and 6. Herb 3 hyperforin concentration was below the LOQ at 0.026 mg/ml.

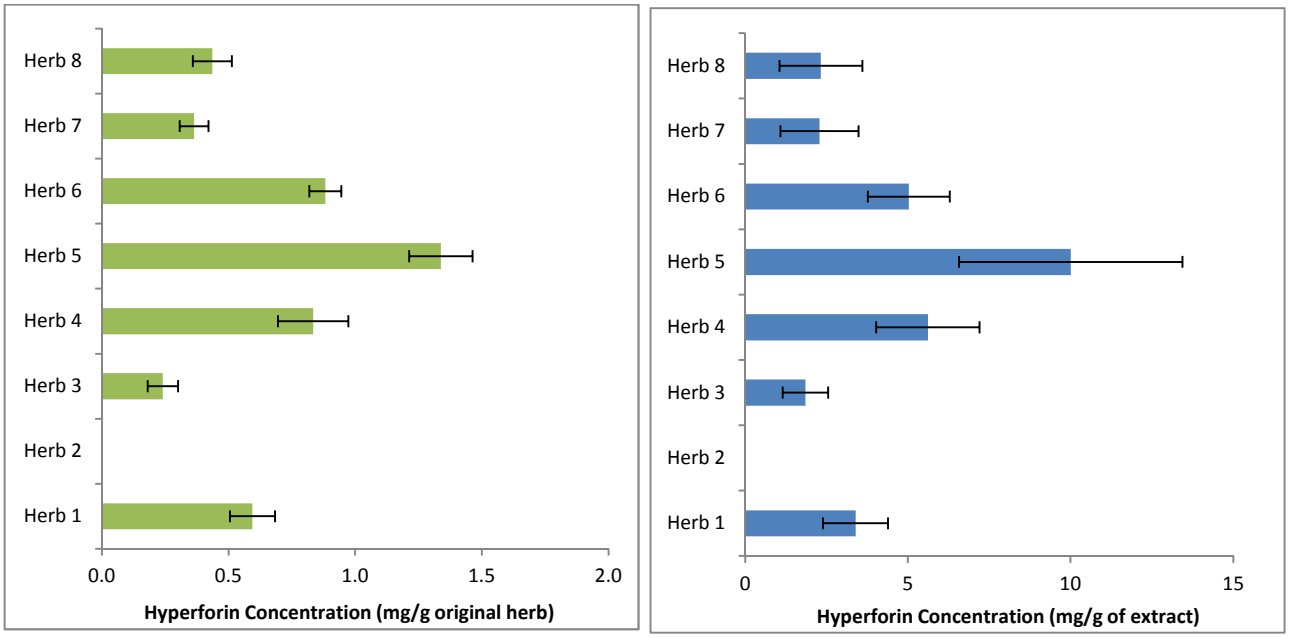


Figure 5.30 (A) Amount of hyperforin extracted from original herb (B) Amount of hyperforin in dried extract. Uncertainty is reported as ±1SD

5.3.5.5 Adhyperforin

Adhyperforin differs from hyperforin by replacement of a methyl group in the isopropyl moiety with an ethyl group. It has been shown to have similar effect to hyperforin with regards to inhibiting the uptake of dopamine, serotonin and noradrenaline [84].

Using the calibration the LOD of adhyperforin was calculated to be 0.002 mg/ml and LOQ was 0.007 mg/ml. The extraction of adhyperforin from the original dried herbs varied between samples (Figure 5.31 A). Herb 2 adhyperforin concentration fell below the LOD therefore is not reported, whereas the most adhyperforin was extracted from herb 5 with 1.02 ± 0.07 mg/g of original herb. The amount of hyperoside was calculated in relation to the dried extract (Chapter 4) and corrected for the entire 20 ml extract. The results (Figure 5.30 B) show that the majority of extracts had 1 to 3 mg/g adhyperforin

whilst herb 5 has the highest concentration with 8 ± 3 mg/g. These results show that with a 60 %v/v ethanol extraction, herb 5 dried extract contains approximately twice as much adhyperforin compared to the other two highest adhyperforin extracts; herbs 4 and 6. Herbs 3, 7 and 8 adhyperforin concentrations are below LOQ at 0.005 mg/ml, 0.005 mg/ml and 0.006 mg/ml respectively.

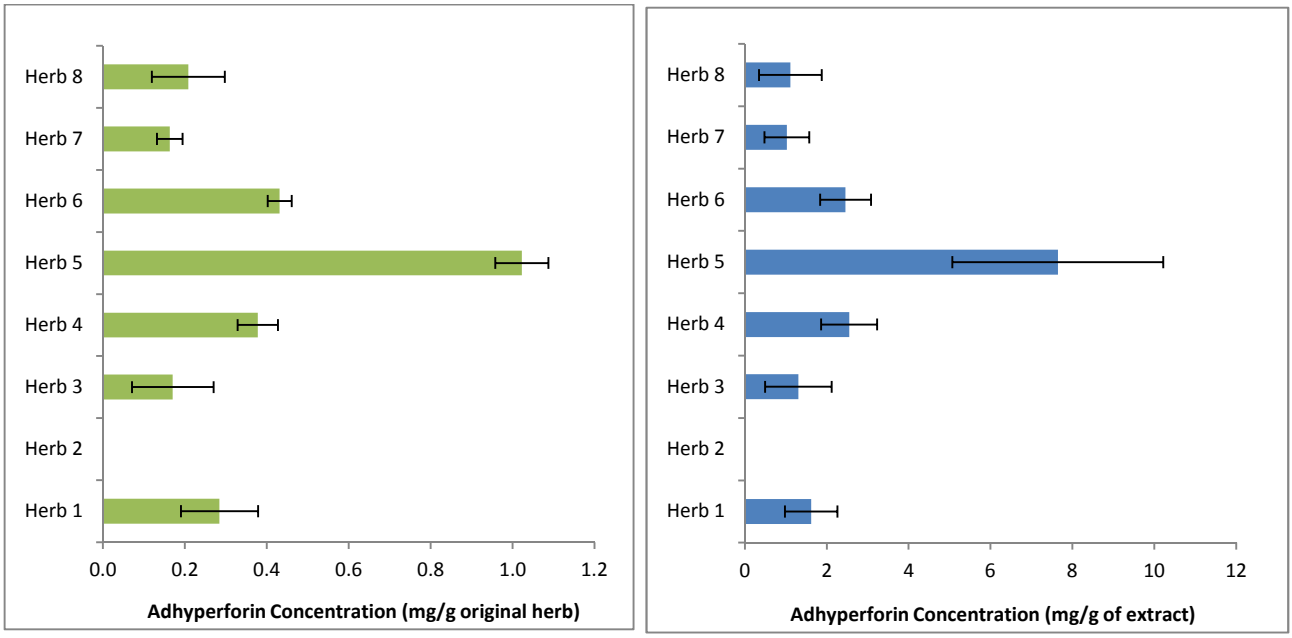


Figure 5.31 (A) Amount of adhyperforin extracted from original herb (B) Amount of adhyperforin in dried extract. Uncertainty is reported as ±1SD

5.4 Conclusions

The study of rutin and Cu complexes showed that this combination of flavonoid and metal easily forms and can do so at room temperature. However, although complexation can occur in methanol, it was found that on introduction of a 0.1 %v/v formic acid mobile phase (either 0.1 %v/v formic acid in water or acetonitrile), the majority of complex present dissociates due to the low pH. Without the presence of formic acid, it was found a small amount of complex still remained; therefore the mobile phase is unsuitable for analysis of the rutin-Cu complex. In order to thoroughly investigate these complexes, a method would need to be developed in which the mobile phases are as neutral as possible, buffer based or methanol based. Another consideration is the tubing in the instrument. The UHPLC used in this analysis has metal tubing, and thus the complexes could interact with it and form much broader peaks. To overcome this, an inert system fitted with Teflon tubing is recommended. An LC-ICP-MS would be the most suitable instrument for the analysis of metal complexes as it could potentially separate the uncomplexed and complexed rutin and also provide estimates for the concentrations complexed. As the complexation occurred in methanol at room temperature, this leads to the conclusion that in methanol extracts of SJW in industry, such flavonoid-metals complexes may occur in the extracts. Previous work (chapter 4) had shown that the similar solvent ethanol was able to extract and pre-concentrate Cu when 60 %v/v ethanol is used. Therefore, although this avenue of research could not be fully investigated, it does show there is a possibility of these complexes being present in SJW extracts. This gives for the future an exciting opportunity to identify which are present and in what quantities.

The analysis of the flavonoid content in eight herbs of SJW showed that they are readily transferred from the plant in 60 %v/v ethanol. The results also showed that generally, there is little variation in quercetin concentration between SJW samples and that rutin and hyperoside concentrations are also similar. On the other hand, the concentrations of hyperforin and adhyperforin varied, sometimes by twice as much, between the herbs and in some cases the observed concentrations were below LOQ (therefore only general trends could be illustrated for these). Comparison of these values to other studies is difficult as the bioactive constituents can vary drastically depending on factors such as geographical origin [92, 93], harvesting time [92, 237, 245] and the extraction process used [113, 191, 241].

6 Analysis of Combined Elemental and Chemical Profiles

6.1 Introduction

The interaction of bioactive compounds and metal ions has been discussed in previous sections. Such complexes have been shown to have three main relationships with bioactive compounds. The first is the production of such bioactive compounds. Studies with the plant *Hypericum perforatum* have shown that, the presence of Cr in the growth medium exhibited an increase of the production of some hypericins [148], however, in the presence of Ni, an opposite relationship was observed [149]. A second relationship noted between elements and bioactive compounds is an alteration in bioactivity. For example, flavonoids such as rutin and quercetin have been shown to bind to metal ions (see Chapter 5, Table 5.1) and as a result the functions such as antioxidant [109, 150] and anti-inflammatory [109] properties increased compared to the flavonoid alone or in some causes became pro-oxidant [109]. A third relationship between elements and bioactive compounds is bioavailability. It was found that chickens fed an element rich diet in the presence of herbal remedies were able to uptake more elements into their tissue [151]. Interestingly, the type of herbal medicine depicted the concentrations of different metals to different tissues. For example, Sage significantly increased levels of Cu, Fe, Mn, and Zn in chicken liver whereas St. John's Wort and Small-flowered Willow herb did not. On the other hand, the presence of SJW significantly increased the concentrations of Zn in chicken legs and Mn in chicken liver [151].

The relationships with elements and bioactive compound production could be utilised to optimise the production of hyperforin and other hypericins to increase the production yield each year. Such elemental relationships with bioavailability and bioactivity could be utilised in order to make herbal medicines more potent, thus allowing for less product being contained in a dosage. However, in order to do this more information is needed on these interactions between elemental content and the bioactive compound content in addition to the normal concentrations of such elements within the herbal remedies.

In this thesis, Chapter 3 acquired the concentrations of 25 elements in raw herb, tablet and capsule formulations of SJW (n=54). From this, the elemental profiles of some water and ethanolic extracts were examined to see how the elements change from raw herb to extracted form. This illustrated that extraction solvent can greatly influence the elements extracted and could therefore be used for the preconcentration of certain elements. Chapter 5 then utilised UHPLC in order to quantify some bioactive compounds contained within some SJW samples. This chapter will focus on correlation

analysis in order to indicate if there are any relationships between the bioactive compounds studied and the elements investigated. The bioactive compounds rutin, hyperoside, quercetin, hyperforin and adhyperforin were compared to the elements in the original herb as well as those in the dried extract. However, it is noted that the information generated will be strictly for qualitative purposes. This is due to the low number of samples (8 herbs), therefore data that fell below LOQ was also utilised.

6.2 Method

6.2.1 Materials

For all materials utilised please see materials sections in Chapter 3, Chapter 4 and Chapter 5.

6.2.2 Elemental Analysis

The elemental profile of eight SJW herbs was collected for total and extracted SJW using Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES), please see Chapter 3 and Chapter 4 for full experimental details.

6.2.3 Chemical Analysis

The content of flavonoids rutin, hyperoside and quercetin as well as hyperforin and adhyperforin were determined using UHPLC for eight herb samples of SJW. Please see Chapter 5 for full experimental details.

6.2.4 Statistical Analysis

Correlation analyses (CA, Pearson's) were carried out on the combined data of total metals, extracted metals and bioactive values for eight SJW herbs, using Excel 2007 (Microsoft).

6.3 Results and Discussion

6.3.1 Elemental Analysis Summary

The elemental concentrations were determined for eight SJW in both the raw herb (please see Chapter 3) as well as an ethanolic extract (please see Chapter 4). An overview of the elements in the raw herbs is presented in Table 6.1. All eight herbs had concentrations of the elements As, Be, Hg, In, Sb and Se below the LOD. Herbs 7 and 8 were the only samples to have concentrations of Co above LOD whereas herb 2 was the only sample to have detectable levels of Pb and V. Yttrium was detected in herb samples 2, 5 and 7 and was above LOQ for herb 8.

Table 6.1 Summary of element content in eight SJW raw herbs

Element	Concentration (μg/g)[1,2]							
	Herb 1	Herb 2	Herb 3	Herb 4	Herb 5	Herb 6	Herb 7	Herb 8
Al	72 ± 9	186 ± 7	148 ± 3	90 ± 20	140 ± 20	80 ± 20	108 ± 4	150 ± 30
As	ND	ND	ND	ND	ND	ND	ND	ND
B	23.8 ± 0.2	32.5 ± 0.5	21.8 ± 0.4	24 ± 4	23.5 ± 0.6	23.8 ± 0.6	24 ± 1	33 ± 1
Ba	15.8 ± 0.1	20.3 ± 0.1	36.3 ± 0.4	16.5 ± 0.3	17.6 ± 0.2	16.8 ± 0.2	18.8 ± 0.2	12.0 ± 0.2
Be	ND	ND	ND	ND	ND	ND	ND	ND
Ca	4160 ± 30	7830± 40	4500 ± 10	4310 ± 60	5070 ± 90	4450 ± 10	4530 ± 40	6520 ± 10
Cd	0.561 ± 0.004	1.73 ± 0.02	0.57 ± 0.02	0.58 ± 0.07	0.445 ± 0.001	0.59 ± 0.02	0.47 ± 0.02	0.34 ± 0.01
Co	ND	ND	ND	ND	ND	ND	*0.5 ± 0.1*	1.8 ± 0.3
Cr	*0.27 ± 0.01*	1.40 ± 0.05	0.63 ± 0.02	*0.33 ± 0.04*	0.93 ± 0.06	*0.35 ± 0.09*	0.55 ± 0.09	3 ± 1
Cu	11.6 ± 0.2	11.25 ± 0.03	13 ± 1	12.4 ± 0.1	13.7 ± 0.3	13.0 ± 0.6	11.3 ± 0.3	11.5 ± 0.2
Fe	110 ± 20	180 ± 20	165 ± 2	80 ± 30	120 ± 60	90 ± 40	184 ± 7	150 ± 50
Hg	ND	ND	ND	ND	ND	ND	ND	ND
In	ND	ND	ND	ND	ND	ND	ND	ND
Mg	1467 ± 9	818 ± 7	1570 ± 20	1490 ± 30	1658 ± 3	1540 ± 30	1700 ± 20	1900 ± 30
Mn	82.8 ± 0.3	161.8 ± 0.6	121.5 ± 0.2	85 ± 1	44.1 ± 0.7	87.6 ± 0.5	107 ± 1	51.5 ± 0.1
Mo	*0.46 ± 0.02*	ND	*0.46 ± 0.06*	*0.33 ± 0.02*	ND	*0.38± 0.03*	ND	*1.04 ± 0.06*
Ni	3.03 ± 0.08	5.37 ± 0.08	2.95 ± 0.07	2.9 ± 0.2	6.1 ± 0.2	2.9 ± 0.1	1.85 ± 0.09	2.8 ± 0.4
Pb	ND	*1.8 ± 0.2*	ND	ND	ND	ND	ND	ND
Sb	ND	ND	ND	ND	ND	ND	ND	ND
Se	ND	ND	ND	ND	ND	ND	ND	ND
Sr	17.74 ± 0.07	30.33 ± 0.08	17.8 ± 0.1	20.5 ± 0.3	14.6 ± 0.1	20.7 ± 0.2	19.0 ± 0.1	14.93 ± 0.09
V	ND	*0.48 ± 0.02*	ND	ND	ND	ND	ND	ND
Y	*0.07 ± 0.01*	*0.18 ± 0.01*	ND	ND	*0.08 ± 0.01*	ND	*0.07 ± 0.01*	ND
Zn	33.4 ± 0.5	41.2 ± 0.6	33.0 ± 0.9	36 ± 1	40 ± 1	40.1 ± 0.7	37.9 ± 0.6	44.9 ± 0.9

[1]ND = Below LOD, *italic* = Below LOQ

[2] Uncertainty reported ±1SD

An overview of the elements in the 60 %v/v ethanolic dried extracts is presented in Table 6.2. For full discussion on extraction efficiencies and trends seen with different elements and extraction solvents please see Chapter 4.

Table 6.2 Summary of element content in eight SJW ethanolic extracts

Element in Extract	Concentration (µg/g)[1,2]							
	Herb 1	**Herb 2**	**Herb 3**	**Herb 4**	**Herb 5**	**Herb 6**	**Herb 7**	**Herb 8**
Al	4 ± 3	9.9 ± 0.4	11 ± 5	9 ± 1	9 .1± 0.7	2.2 ± 0.5	1.4 ± 0.6	3 ± 1
Ba	0.38 ± 0.07	0.78 ± 0.06	1.01 ± 0.09	1.0 ± 0.5	*0.65 ± 0.05*	0.38 ± 0.04	0.38 ± 0.05	0.22 ± 0.02
Ca	1600 ± 300	1350 ± 70	1700 ± 500	1800 ± 200	1730 ± 40	1600 ± 100	1700 ± 300	2900 ± 200
Cd	ND	ND	ND	ND	ND	ND	ND	ND
Co	*1.0 ± 0.2*	*1.2 ± 0.4*	*1.2 ± 0.2*	*1.5 ± 0.1*	*1.07 ± 0.03*	*0.83 ± 0.06*	*0.9 ± 0.1*	ND
Cr	ND	ND	ND	ND	ND	ND	ND	ND
Cu	25 ± 5	28 ± 2	29 ± 2	32 ± 2	30 ± 2	26 ± 2	22 ± 3	23 ± 2
Fe	8 ± 5	11 ± 2	8 ± 3	5 ± 2	6 ± 1	4.3 ± 0.3	4.4 ± 0.5	4.3 ± 0.4
Mg	2800 ± 600	2600 ± 100	3200 ± 200	1010 ± 90	3310 ± 20	2800 ± 200	3100 ± 500	3800 ± 300
Mn	70 ± 30	89 ± 5	80 ± 20	70 ± 7	30.3 ± 0.3	62 ± 5	80 ± 10	34 ± 3
Mo	ND	ND	ND	ND	ND	ND	ND	ND
Ni	9 ± 2	15.9 ± 0.8	9.6 ± 0.4	11 ± 1	16.1 ± 0.2	9.2 ± 0.8	4.4 ± 0.6	4.5 ± 0.6
Sr	2.4 ± 0.8	2.3 ± 0.1	2.1 ± 0.3	2.7 ± 0.4	1.9 ± 0.2	2.0 ± 0.2	2.1 ± 0.3	2.1 ± 0.2
Zn	40 ± 10	37 ± 2	41 ± 1	46 ± 4	40 ± 2	43 ± 5	39 ± 5	52 ± 3

[1]ND = Below LOD, *italic* = Below LOQ
[2] Uncertainty reported ±1SD

6.3.2 Chemical Analysis Summary

The eight samples of SJW herbs that underwent extraction with 60 %v/v ethanol were also subjected to UHPLC analysis to quantify flavonoids rutin, hyperoside, quercetin as well as hyperforin and adhyperforin. A summary of the concentrations found in relation to the dried extract are summarised in Table 6.3. For full extraction details and discussion, please see Chapter 5.

Table 6.3 Summary of Bioactive Content in Eight SJW Ethanolic Extracts

Herb №	Compound in Extract (µg/g)[1,2]				
	Rutin	**Hyperoside**	**Quercetin**	**Hyperforin**	**Adhyperforin**
1	40 ± 10	29 ± 9	9 ± 3	3 ± 1	1.6 ± 0.6
2	70 ± 10	19 ± 4	13 ± 2	ND	ND
3	40 ± 20	28 ± 14	8 ± 3	*1.9 ± 0.7*	*1.3 ± 0.8*
4	50 ± 20	40 ± 10	10 ± 3	6 ± 2	2.5 ± 0.7
5	16 ± 8	30 ± 10	8 ± 3	10 ± 3	8 ± 3
6	40 ± 10	33 ± 9	7 ± 2	5 ± 1	2.5 ± 0.6
7	60 ± 30	40 ± 20	11 ± 6	2 ± 1	*1.0 ± 0.6*
8	50 ± 20	11 ± 6	10 ± 5	2 ± 1	*1.1 ± 0.8*

[1]ND = Below LOD, *italic* = Below LOQ
[2] Uncertainty reported ± 1SD

6.3.3 Correlation Analysis

6.3.3.1 Correlation of Original Herb Elements with Bioactive Compounds

The bioactive compound content underwent correlation analysis with the elemental content of the original herb. Please see Table 4.14 for correlation definitions and colour coding. The results (Table 6.4) show there are several strong correlations. Please note that correlations observed with elements Co, Cr and Mo cannot be fully interpreted as these elements were below LOQ in majority of samples and are therefore only semi-quantitative.

Table 6.4 Correlation analysis of total element concentrations in original herb to bioactive compounds[1]

	Rutin	*Hyperoside*	*Quercetin*	*Hyperforin*	*Adhyperforin*
Al	0.1348	-0.6972	0.5362	-0.3411	-0.1157
Ba	-0.0796	0.1619	-0.1433	-0.2743	-0.1505
Ca	0.4231	**-0.8114**	0.7120	-0.4434	-0.2999
Cd	0.5916	-0.2849	0.6471	-0.4901	-0.3958
Co	0.1133	-0.6729	0.2300	-0.2497	-0.2458
Cr	0.0870	**-0.9120**	0.3726	-0.2634	-0.1675
Cu	-0.7941	0.3567	-0.7582	0.7103	0.7361
Fe	0.3934	-0.3731	0.6291	-0.6149	-0.4129
Mg	-0.5061	0.0835	-0.5054	0.3771	0.3151
Mn	0.7182	-0.0141	0.5560	-0.7318	-0.6638
Mo	0.0015	-0.5327	-0.1171	-0.0864	-0.2031
Ni	-0.3560	-0.2456	0.0996	0.3896	0.5588
Sr	0.7527	-0.0904	0.6426	-0.5350	-0.5156
Zn	0.1320	-0.5771	0.3516	0.0592	0.0993
Rutin	1	-0.1462	0.7741	-0.7990	**-0.8546**
Hyperoside		1	-0.3624	0.4330	0.3078
Quercetin			1	-0.5837	-0.5206
Hyperforin				1	**0.9615**
Adhyperforin					1

[1]Dark green = strong positive correlation, green = positive correlation, red = strong negative correlation, pink = negative correlation, orange = weak correlation.

Rutin showed positive correlations with the elements Cd, Mn and Sr and negative correlations with Cu and Mg. One possible reason for the negative correlation with total Cu may be due to the enzyme F3GT (EC 2.4.1.91 - flavonol 3-O-glucosyltransferase). This enzyme is involved in the production of rutin from quercetin (Figure 6.1) and has been shown to become inhibited by up to 96% in the presence of Cu^{2+} [254]. An investigation of rutin in *Zucchini cotyledons* found that Cu^{2+} ions decreased rutin production [255].

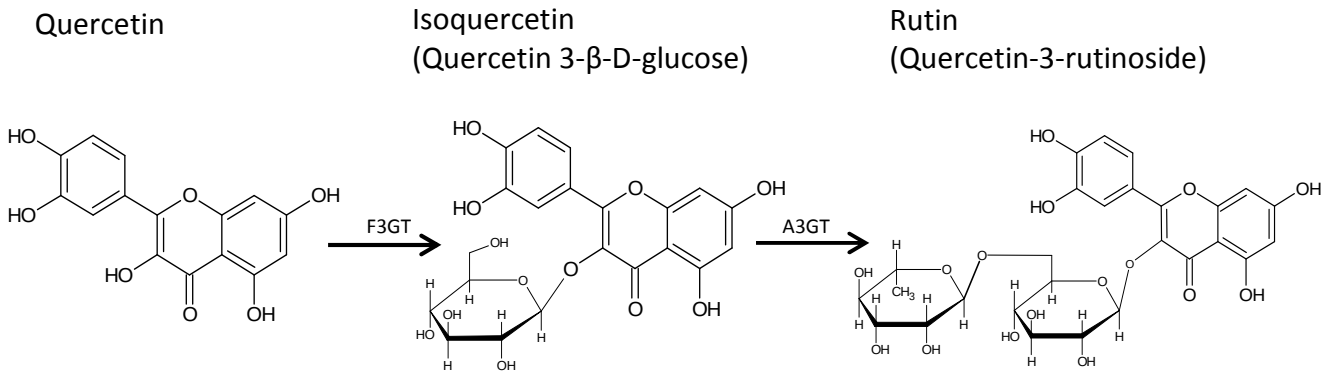


Figure 6.1 Biosynthesis of rutin production from quercetin. F3GT = flavonol 3-O-glucosyltransferase, A3RT = UDP-Rha: anthocyanidin 3-O-glucoside rhamnosyltransferase. Adapted from [256]

Quercetin exhibited positive correlations with elements Al, Ca, Cd, Fe, Mn and Sr. The compound also showed negative correlations with Cu and Mg. The quercetin-Fe correlation may be due to the enzyme involved in quercetin biosynthesis (Figure 6.2); FLS (EC 1.14.11.23 - flavonol synthase) requires Fe^{2+} for activation [257, 258].

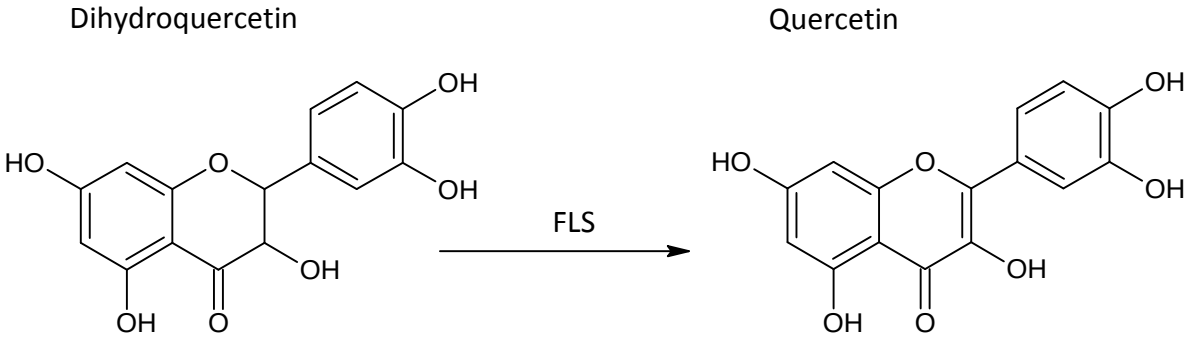


Figure 6.2 Biosynthesis of quercetin production from dihydroquercetin. Adapted from [256]

The positive correlation seen with quercetin-Al could possibly be linked with the detoxification of Al. Kidd *et al.*, [259] found that some varieties of maize may use flavonoids, including quercetin, in conjunction with Si to detoxify Al.

Hyperoside displayed very strong negative correlations with Ca and Cr followed by negative correlations with elements Al, Co, Mo and Zn. Tirillini *et al.*, investigated the effect of increased Cr in growth medium on the production of hypericin, pseudohypericin and protohypericin [148]. The levels of Cr detected in the plants in this study (0.3-3.0 µg/g) are much lower than those reported for the untreated leaves (9 ± 3 µg/g) in the Tirillini *et al.*, study [148]. As half the samples are below LOQ it is difficult to determine the relationship between Cr and hyperoside. Therefore growth studies which expand the number of compounds investigated with Cr enriched medium would be needed.

Hyperoside displays negative correlations with the elements studied and differs from those seen with rutin and quercetin despite having a similar chemical structure. Therefore, hyperoside may follow a different biosynthesis route compared to that of quercetin and rutin.

Hyperforin showed a positive correlation with Cu and negative correlations with the elements Fe, Mn and Sr. A weak negative correlation was observed with Cd. The biosynthesis of hyperforin (Figure 6.3) is not fully understood but a proposed route has been suggested from studies [87, 260, 261].

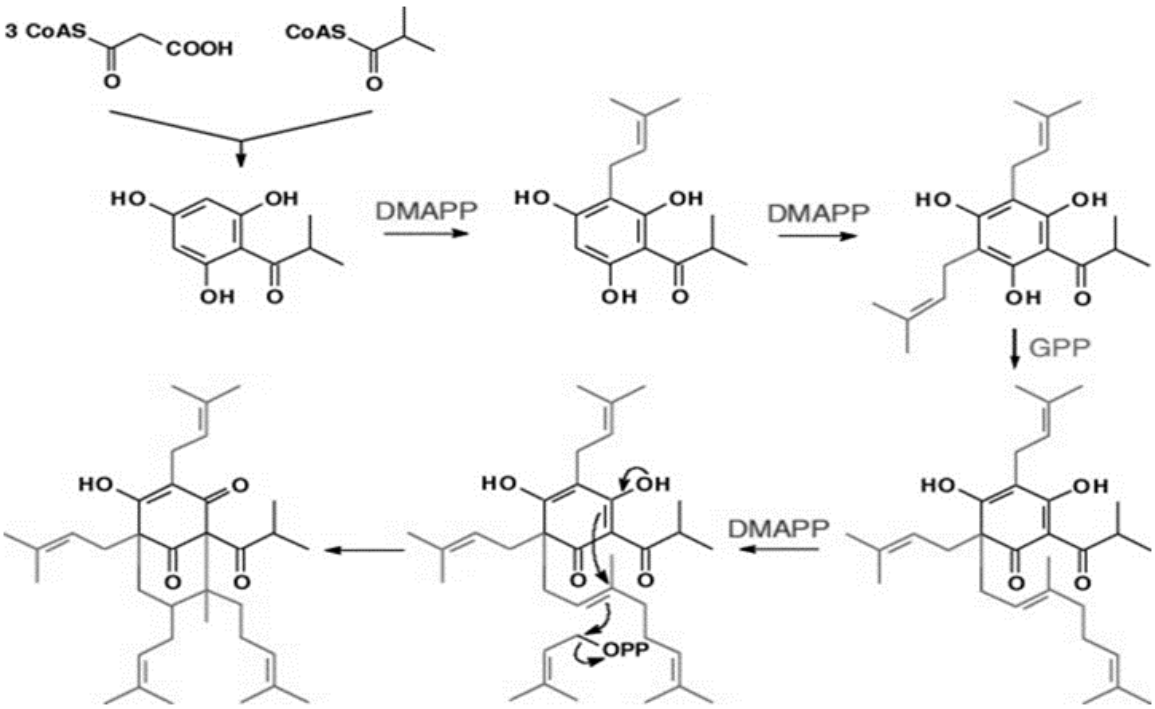


Figure 6.3 Proposed hyperforin biosynthetic pathway (reproduced with permission from [87]). DMAPP - dimethylallyl diphosphate, GPP - geranyl diphosphate and PP - diphosphate.

An enzyme believed to be phlorisobutyrophenone dimethylallyltransferase [261] may be responsible for the prenylation of DMAPP (dimethylallyl diphosphate) and the activity was found to be dependent on a divalent cation. The most efficient co-factor was found to be Fe^{2+} with decreasing efficiency with $Mg^{2+} > Zn^{2+} > Cu^{2+} > Ca^{2+} = Mn^{2+} = Co^{2+}$ [259]. However, hyperforin was found to have a negative correlation with the majority of these elements with the exception of Cu. More research would have to be carried out in order to see if the Cu-phlorisobutyrophenone dimethylallyltransferase is more prevalent than other forms due to other elements, like Fe, being utilised by other metal specific co-factors of enzymes (e.g. FLS). Hyperforin content was shown to decrease significantly with increased Ni in the growth medium [149] however, levels of this study cannot be compared to those by Murch *et al.*, as no Ni was detected in the control plants. Adhyperforin displayed a positive correlation with Cu and Ni as well as a negative correlation with elements Mn and Sr. Adhyperforin follows a similar

biosynthesis route to hyperforin and differs from hyperforin by one of the methyl groups in the isopropyl moiety being replaced by an ethyl group. However, it appears to have a positive relationship with Ni concentration compared to that reported of hyperforin [149].

Also noted from the analysis are correlations between the flavonoids themselves. For example a positive correlation is exhibited between rutin and quercetin. This is because quercetin is an aglycone of rutin (Figure 6.1), therefore the more quercetin that is available in a plant, the more rutin could be produced. Hyperoside on the other hand, shows no strong correlations with any of the other compounds monitored. Hyperforin and adhyperforin are very strongly correlated which may be due to their structural similarity with only a $–CH_3$ group difference between them. Strong negative correlations were observed between flavonoids (rutin and quercetin) and the phloroglucinols (hyperforin and adhyperforin).

6.3.3.2 Herb Dried Extracts with Bioactive Compounds

The bioactive compound content underwent correlation analysis with the elemental content of the dried 60 %v/v ethanolic extract. Elements Cd, Mo and Cr were removed from the dataset as all values were below the LOD. Please note that correlations with Co cannot be fully interpreted as these elements were below LOQ and are therefore are for qualitative purposes only. Please see Table 4.14 for correlation definitions and colour coding. The results (Table 6.5) show several strong correlations.

Table 6.5 Correlation Analysis of Extracted Elements to Bioactive Compounds

	Rutin	Hyperoside	Quercetin	Hyperforin	Adhyperforin
E-Al	-0.1891	-0.0623	0.1235	0.0891	0.2132
E-Ba	-0.0175	0.2923	0.0877	0.0443	0.0778
E-Ca	-0.1303	-0.5833	-0.0002	-0.0129	-0.0412
E-Co	0.0259	0.6795	0.0221	0.1860	0.1713
E-Cu	-0.3082	0.2339	-0.1326	0.4184	0.4184
E-Fe	0.2490	-0.2892	0.3995	-0.4218	-0.2691
E-Mg	-0.2942	-0.5290	-0.1450	-0.1122	0.0588
E-Mn	0.6688	0.2538	0.3714	-0.6733	-0.6799
E-Ni	-0.2842	0.0283	0.0378	0.3920	0.5112
E-Sr	0.4776	0.1134	0.3254	-0.2578	-0.4203
E-Zn	-0.1670	-0.3589	-0.2225	0.1049	-0.0287

[1]Dark green = strong positive correlation, green = positive correlation, red = strong negative correlation, pink = negative correlation, orange = weak correlation.

Rutin shows a positive correlation with the element Fe. In other studies, rutin has been shown to complex with Fe [109, 150, 220] however this affinity is less so than other complexes such as rutin-Cu [252]. This may indicate the presence of rutin-Fe complexes, however further analytical work would need to be carried out to confirm this. Quercetin displays no strong correlations with any of the extracted elements whilst hyperoside displays a positive correlation with Mn and negative correlations with Ca and Mg. A negative correlation between hyperforin and Mn is observed whereas adhyperforin exhibits a positive correlation with Ni and a negative correlation with Mn.

6.4 Conclusions

Flavonoids are multifunctional compounds within plants. For example, rutin and quercetin protect against UV-B damage [262] but also as a defence against insects [263]. Due to their multi-functionality their relationships with elements can be complex. However, the CA of the flavonoids with the total elements in the original herbs indicate some possible links previously not reported for SJW. For example, previous studies examined the effect of increased concentrations of Ni and Cr on hypericins/ hyperforin production in SJW [148, 149]. This study found no negative correlations between Ni and hyperforin, but in contrast found a positive correlation between Ni and adhyperforin. Interestingly, a very strong negative correlation is observed for hyperoside with Cr. Also noted were some correlations between flavonoids and elements which could possibly be linked to the enzymes used in their production. For example, the negative rutin-Cu correlation could possibly be due to F3GT being inhibited by Cu ions.

The CA analysis of flavonoids to the metals obtained during the extracts show some correlations. However, as some of the data utilised in these investigations were below the analytes LOQ values, the results obtained are to be used for indicative qualitative purposes. The positive correlations could possibly indicate some complexing between the constituents; however, many of the combinations could form stronger complexes with other elements. In order to access if these elements (e.g. Mn and Sr with rutin) are in a complexed form, mass spectrometry analysis would need to be carried out, using direct injection and SIM.

Overall this study has shown some interesting interactions between elements and flavonoids as well as flavonoids with other flavonoids. However, to ensure the correlations are completely robust the number of SJW samples needs to be increased from eight, and also the concentrations of the extract increased to ensure more data falls above LOQ or another analytical instrument with increased sensitivity utilised (e.g. a UHPLC with a single UV wavelength detector). In order to compare with the other limited studies on elemental influence on bioactive production, the hypericin compounds should also be investigated.

7 Conclusions

The work presented in this thesis has demonstrated that the use of metal fingerprints for the quality control of herbal medicines can be applied to multiple stages of processing. This study suggests that the processing steps, such as extraction and addition of excipients, has a less random and more predictable effect on the elemental profile of SJW. Thus, these trends can be exploited for further assessment of SJW quality. This work has produced a validated and simple method for the analysis of trace metals in SJW, and demonstrated the differences in the metal fingerprint of SJW upon formulation and the consistency between products, determined the elemental transfer trends in solvents of increasing alcohol content (i.e., each element has a different extraction profile, yet the extraction profile is consistent between SJW samples), as well as demonstrating correlations between the elemental and molecular (i.e., flavonoids and phloroglucinol) constituents present in SJW. The major conclusions from the work are highlighted below.

The lack of a certified reference material for trace elements in SJW presented a challenge when an accurate metal profile is desired. Thus, a method for the analysis of trace elements was determined involving validation using NIST polish tea, spiked recovery methods and standard addition. These experiments illustrated good recovery of elements with the NIST tea, however validation with SJW samples showed the presence of silicates when HF was applied. Thus, current certified reference materials may not be similar in the silicate content as SJW samples. However, methods used without HF will need to state the metal content is not from the silicates present. The standard addition also highlights matrix effects with SJW samples as well. In addition, there were significant improvements in the error of the measurement when using a weighted calibration curve to that of a non-weighted curve. All previous studies in the literature investigating elemental content of SJW have used external calibrations for element quantification therefore not considering the matrix effects present. Thus, the validation study highlighted current limitations to using available standard reference materials for SJW, but also the consideration that should be made when making this comparison.

As mentioned, there are several studies that have investigated the elemental content of SJW; however, they were limited by the number of samples, number of elements analysed, geographical origin as well as little continuity between studies. This project was able to investigate a large number of SJW samples in both its raw and processed forms to give a normal range for the concentrations of 25 elements. The samples were also sourced worldwide to avoid localisation of one growth area/country. The results showed that 93% of the SJW samples fell within a 95% confidence interval. This implies that despite the SJW samples being in different forms and from worldwide growth

locations, underlying elemental patterns were still able to define the majority of sample forms. Thus, SJW samples were differentiated based on their elemental profiles. Those samples that overlapped in different groups could be justified. For example, a capsule that grouped with the raw herbs was in fact just raw herb with no added excipients. This could allow manufacturers to confirm their claim for products that are 'organic' rather than using an extract. This analysis can also show if the products are wholly extract, raw herb or a mixture of both. The PCA model used was robust as despite including a sample considered a near- outlier; it was still able to produce the differentiation between SJW raw herbs, tablets and capsules. A PCA constructed without the elements Ca and Mg (common constituents of bulking agents) was created and showed that differentiation between the SJW forms still existed; but less separation was observed between the tablets and capsules. The results from this project suggest that the elemental differences observed between the different SJW forms are due to two main factors. The first being the extraction process of the bioactive compounds from the raw herb and the second being the addition of excipients such as bulking agents. The PCA analysis was also utilised to assess the potential for geographic origin and species identification. These studies were inconclusive and thus studies investigating more samples of SJW raw herb from different growth localities and of different species may be considered. Literatures from other studies (with different plant families) have shown that such identifications could be obtained with PCA, thus this analysis in the future may have potential.

The work investigating SJW elemental profiles indicated that the extraction process played a key role in shaping the elemental profiles of the processed forms. Therefore to understand the effect to the elemental profile, eight SJW herbs were extracted in four different solvents (100% water, 60 %v/v ethanol, 80 %v/v ethanol and 100% ethanol). These solvents were utilised as the 60 %v/v and 80 %v/v ethanol concentrations are used routinely in industry to manufacture extracts whilst the two 100% solutions were utilised to understand the transfer trends. The results of these studies showed that the elemental transfer from the original herb for the majority of elements was small (≤35% of original herb concentration). More interestingly, the results displayed that transfer was solvent and metal dependent. Generally the highest concentrations of an element were extracted with 100% water, which decreased as the concentration of ethanol increased. However, the transfer efficiency for the element Cu was highest with 60 %v/v ethanol. The concentrations in the dried extract was compared to that of the original herb and results showed that preconcentration occurs with all elements with the exception of Al, Ba and Fe. The majority of preconcentration occurred in the dry 100% water extract; however this form is not knowingly used therefore does not cause concern. The solvents utilised in industry however was found to preconcentrate some elements; Cu (+119%), Mg (+93%), Ni

(+183%) and Zn (+12%) were found to preconcentrate in 60 %v/v ethanol extracts and Cu (+5%) and Ni (+30%) preconcentated in 80 %v/v extracts. These results indicate that the selection of solvent plays an important role in elemental extraction as well as bioactive extraction. It also shows the potential that the extraction of elements contained in raw herbs could be tuned for purpose. For example, there are several nutritional disorders due to deficiencies of nutrients (e.g. deficiencies in Se and Mn linked to cardiovascular disease), therefore by selecting an appropriate solvent the elemental concentration could be increased for nutritional purposes and to assist with the formation of metal-bioactive complexes which could increase bioavailability and/or bioactivity. The tuning of elemental extract *via* solvent to decrease the elemental content could lower the transfer of toxic elements as well as decrease drug interactions. Other possibilities for tuning the elemental extraction include the use of plants for 'mining' rare earth metals or cleansing contaminated land in order to retrieve a larger yield.

The elemental profiles produced from the extraction processes underwent PCA with the total concentrations found from the original herbs. The PCA results showed that the extracts produced by each solvent are well differentiated indicating that each solvent type provides a specific and predictable elemental profile. The results also show that as the ethanol content increases, the extract samples become more standardised (i.e., elemental profile has less variation). Therefore these results with the extracted samples show again the potential for tuning the elemental profile of SJW products.

As noted, the elemental concentrations can interact with the bioactive compounds of a plant in numerous ways. Therefore, the elemental and molecular profiles were compared to see if synergy between them could potentially be exploited in using the metal content to predict the bioactive content. The results from correlation analyses suggest that this may only be possible with the total concentrations from the original raw herb as few strong correlations were apparent with the extract values. The results also suggest more biochemical roles of elements within the original plant as correlations found are not consistent with reported metal-flavonoid complexes. The correlation analysis with the extract data were investigated to see if such complexes could be investigated indirectly; however, correlations obtained were of elements that formed weaker complexes (e.g., Mn and Sr with rutin) compared to those reported as strong complexes (e.g., Cu and Fe with rutin).

Overall the investigation of the elemental profiles of SJW raw herbs, tablets, capsules and extracts has shown that the profiles differ greatly between each form, but follow specific trends. The analysis of these profiles by PCA shows the potential for this method to be used for quality control. It can be utilised to assess batch to batch consistency, confirm if the SJW is the raw herb or an extract and if it

is an extract potentially identify the extraction solvent/process that produced it. On the other hand, it also showed that the elemental profile can be tuned for exploitation of metal bioactive complexes, however, further work is needed in this area.

8 Future Work

This project has highlighted a number of routes this research could follow in the future.

The results achieved from the geographical origin experiments were inconclusive; however some grouping was observed (Poland and UK samples). This could be to a number of factors such as the part of the plant utilised, growth year variation and limited number of samples for some localities (e.g. Spain and Chile). To further investigate if this method could be used for the identification of growth origin; a large number of samples with proper paperwork would be required. To do this, a pilot study would be carried out using different regions of a country as a basis. Poland would be a strong candidate as all manufacturers or producers of herbal remedies state the growth region on the label. Therefore a large number of samples could be purchased from different regions of Poland to see if elemental profiling could differentiate between them. This pilot study would allow the investigation of growth origin before going to the expense of different countries around the world. This could be used as a tool to follow Good Manufacturing Practice (GMP) where necessary paperwork is needed to follow a paper trail should any discrepancies occur.

In addition to this, the preliminary experiments with different species showed that despite being in a processed form, the different species of plant-based capsules did not overlap with those of SJW. Therefore there is the potential for the developed method to be utilised in species identification. To confirm this, several different species of medicinal herbs could be purchased and their elemental profiles collected, then included in the PCA model. This could then potentially be utilised to differentiate families of plants (as seen in literature). A true test of compatibility could be carried out between different species of *Hypericum* to see if more closely related plants can be differentiated. It could also be used to check the quality of raw herbs before a manufacturer continues with production. If different forms (tablets and capsules) of these other species are also purchased, investigations could be carried out to see if differentiation between these forms is possible in other plant species. This would confirm or invalidate an 'organic' claim by manufacturers.

The elemental profiles collected from the extracted SJW showed that the extracts could be differentiated by the solvent used. By further investigating different extraction techniques and solvents; the elemental profiles could be assessed to see if extraction solvent, technique and manufacturer can be differentiated. This could be used as a method to check and ensure batch to batch consistency of a product. This could also be useful in cases where a product is of low quality and thus trace back to manufacture source.

The correlation analyses of the elemental and molecular profiles showed there were relationships between the two. Due to this, the analysis of the elements can give a clearer picture to the overall herb than just the concentration of a few bioactive species. To date, only Cr and Ni in growth medium has been assessed in relation to the production of hypericins. These growth studies could be expanded upon to aid the understanding of such relationships. A number of other elements could be investigated, starting with those found from this study (e.g. Al, Ba, Co, Cu, Fe, Mn, Ni) to see if they effect the production of the hypericins and hyperforin production. In addition to the elements, the bioactive compounds could be expanded to include flavonoids such as rutin and quercetin as well as other common constituents. The Results from this project show that the different bioactive compounds have different relationships with the same element. These studies could be carried out on a smaller scale within a green house or much larger using fields. The optimum method for assessing elemental nutrition on the production of bioactive compounds would be to use a hydroponic farming system in which the elements introduced to the plants could be uniquely controlled and also removes considerations such as soil interactions. This information could then be utilised in industry to improve yields of these compounds. From the previous in-depth growth studies or from extensive analysis of many SJW herbs for elemental and chemical profiles; if strong relationships are identified between certain elements and bioactive compounds, those elements could be utilised as an indicator for the concentration of the compound. This would be beneficial to laboratories that do not have extensive equipment. Also, if one particular metal was found to have a very strong relationship, a quick and simple wet chemistry test could be developed to aid those in developing countries who do not have access to laboratories. If proven with SJW, such biomarkers could then be investigated in other herbal remedies.

The analysis of the extracts of SJW has shown that elementals are present in addition to the bioactive constituents. External experiments with standards of rutin and copper chloride also displayed that metal-bioactive compounds are able to form readily at room temperature. Therefore, it is highly likely that such complexes may exist within the herbal extract. However, to be able to detect these in depth method development would be needed on HPLC to ensure the complexes remain stable for the shift in wavelength to be detected. A better instrument to utilise with the investigation of metal-bioactive complexes would be a LC-ICP-MS. This would separate the bioactive compounds from one another and transfer each one separately to the MS system. This would then give information as to which metal ions are complexed to that molecule and the approximate ratios between them. This could firstly identify, through standards, which bioactive complexes could form from mixing singular compounds with various metal ions. Continuing this, multiple bioactive compounds with multiple

metal ions could be mixed to see how they interact (i.e. is there competition for certain metal ions, will some compounds form stable complexes or do they disassociate due to the presence of other bioactive compounds, do complexes form with multiple ions and compounds?). Such experiments could help identify the kinetics of the complexes and also the metal species involved. Following such experiments, a method could be optimised to detect and quantify complexes in true samples by the analysis of herbal extracts or infusions. The speciation is of interest as this would identify if the more toxic or safer forms of an element are present. The MS would also be able to record the element isotopes and thus provide additional variables for the differentiation of samples through isotope ratios. In addition to the physical properties and identification of metal-bioactive complexes in herbal extracts, these could then be isolated and tested for various biological activities such as antioxidant, anti-inflammatory, anti-bacterial and anti-cancer properties. From this, bioavailability could also be assessed by examining these complexes in biological matrixes. These experiments would show which are stable and would survive gut conditions or more likely which reform within the duodenum. If proven to exist in such conditions this could be taken further with cell cultures to assess their bioavailability then ultimately their therapeutic effect. In addition to this, the effect of other herbs or main stream synthetic medications being present could be explored.

These are a few examples and routes the research from this project could progress to now that the normal range of elements in SJW has been identified in addition to the presence of relationships between the bioactive and elemental constituents and how they change with processing.

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10 Appendix

10.1 Element Limits from different Agencies

Table 10.1 Exposure Limits of Different Elements from the European Union Scientific Committee on Food (SCF), US Institute of Medicine(IOM), World Health Organisation (WHO), US Environmental Protection Agency (EPA), US Agency for Toxic Substances and Disease Registry (ATSDR) and European Food Safety Authority (ESFA)[1]

	SCF			IOM		WHO		EPA	ATSDR	ESFA	
Element	UL	NOAEL	RDA	UL	TDI	TWI	UL	RfD	MRL	TWI/ TDI	UL
Al	-	-	-	-	-	7mg/kg/week[2]	-	0.0004 mg/kg-day[3]	1 mg aluminum/kg/day	1 mg/kg/week	-
As[4]	-	-	-	-	-	15µg/kg/week	-	0.0003 mg/kg-day	3 x10-4 mg/kg-day		-
B	-	-	-	20mg/day	88µg/kg/day	-	-	-	0.2 mg/kg-day		-
Ba	-	-	-	-	51 µg/kg/day	-	-	0.2 mg/kg-day	0.2 mg/kg-day		-
Be	-	-	-	-	-	-	-	0.002 mg/kg-day	0.002 mg/kg/day		-
Ca	2500 mg/day	-	1000 mg/day	2500 mg/day	-	-	-	-	-	-	2,500 mg/day
Cd	-	-	-	-	-	7 µg/kg/day	-	0.001 mg/kg-day	0.0001 mg/kg/day	5.8 µg/kg/day	-
Co	-	-	-	-	-	-	-	-	0.01 mg/kg/day		-
Cr	1mg/day	-	25 - 35 µg/day	-	250 µg/day	-	-	1.5 mg/kg-day	0.0009 mg/kg/day[5]		-
Cu	-	10 mg/day	900 µg/day	10 000 µg/day	10 - 12 mg/day	-	-	-	0.01 mg/kg/day		-
Fe	-	-	8 - 18 mg/day	45mg/day	9.7-58.8mg/day	-	-	-	-	-	-
Hg	-	-	-	-	5 µg/kg/day	-	-	-	0.0001 mg/kg/day[6]	4 µg/kg/week	-
In	-	-	-	-	-	-	-	-	-	-	-
Mg	2,500 mg/day,	-	310-420 mg	350mg/day	-	-	-	-	-	-	-
Mn	-	-	1.8-2.3mg	11 mg/day	2.0-5.0 mg/day	-	-	0.14 mg/kg-day	5 x10-3 mg/kg-day		-
Mo	0.01 mg/kg/day	-	45 µg/day	2000 µg/day	0.4 µg/kg/day	-	-	-	-	-	-
Ni	-	-	-	1mg/day	-	-	-	-	-	2.5 µg/kg/week	-
Pb	-	-	-	-	25 µg/kg/day	-	-	-	-	1.5 µg/kg/day	-
Pt	-	-	-	-	-	-	-	-	-	-	-
Sb	-	-	-	-	-	-	0.43 mg/kg	-	-	-	-
Se	300 µg Se/day	-	55 µg/day	400 µg/day	50-200 pg/day	-	-	0.005 mg/kg/day		-	300 µg/day
Sr	-	-	-	-	-	-	-	0.6 mg/kg/day		-	-
V	-	-	-	-	-	-	-	0.009 mg/kg/day[7]		-	0.2-0.3 µg/kg/day
Y	-	-	-	-	-	-	-	-	-	-	-
Zn	-	50 mg/day	8-11 mg/day	40 mg/day	-	-	35-45mg/day	0.3 mg/kg-day	0.3 mg/kg/day	-	25 mg/day

[1] UL = Upper limit, NOAEL = No observable adverse effect limit, RDA – Recommended Daily Allowance, TDI = Total daily intake, TWI = Total weekly intake, RfD = Reference dose for chronic oral exposure, MRL = minimal risk level

[2] Joint FAO/WHO Expert Committee on Food (JECFA)

[3] Aluminium phosphide

[4] Inorganic arsenic,

[5] Cr (VI)

[6] Methyl mercury

[7] Vanadium pentoxide

10.2 Element Concentrations Found in Other Studies

Table 10.2 Summary of Element Concentrations (µg/g unless otherwise stated) Found in *Hypericum perforatum* Products

Type of SJW	Origin	Method	Reference	Al	As	B	Ba	Be	Ca	Cd	Co	Cr	Cu	Fe	Hg	In	Li
Aqueous Extract	Poland	AAS	[133]	-	-	-	-	-	-	-	-	-	-	7.5-228	-	-	-
Aqueous Extract	Poland	AAS	[132]	-	-	-	-	-	-	-	-	-	3.6	-	-	-	-
Aqueous Extract	Poland	GFAAS and ICP-MS	[131]	-	-	-	14.27-14.37	-	-	0.063 - 0.068	-	-	3.65 - 4.65	17.02 - 35.87	-	-	-
Capsule	Unknown	LA-ICP-MS and ICP-MS	[119]	23-31					412-464	0.043-0.059	0.3-0.4	0.25-0.33	8.08-10.2	62.7-82.9			
Liquid extract	Poland	ICP-MS	[124]	-	≤20	-	-	-	-	10 - 30	-	-	190 -270	1060-4880	≤ 20	-	-
Raw Herb	Argentina	GFAAS, AAS and AES	[125]	-	-	-	-	-	105-460	-	-	-	12.8-13.5	-	-	-	0.03-0.05
Raw Herb	Argentina	GFAAS and ICP-OES	[126]	1.23-3.20	-	-	-	-	-	0.05-0.08	0.09-0.33	<0.005	-	7.43-8.79	-	-	-
Raw Herb	Austria and Vienna	AAS	[121]	-	-	-	-	-	-	0.15-0.98	-	-	6.2-10.1	57.1-303	-	-	-
Raw Herb	Austria	GFAAS and AAS	[120]	-	-	-	-	-	-	0.01-0.6	-	-	8.4-11.8	-	-	-	-
Raw Herb	Serbia	AAS	[122]	-	-	-	-	-	-	0.22-1.28	-	-	-	-	-	-	-
Raw Herb	Spain	ICP-MS	[123]	-	0.1 - 0.51	-	-	-	-	0.05 - 1.71	-	-	-	-	-	-	-
Raw Herb/(extracts)	Estonia	AAS	[127]	-	-	-	-	-	-	-	0.1-0.18	0.12 - 0.25	-	-	-	-	-
Raw Herb	Romania	ICP-OES	[129]	<5 - 76	-	-	0.5 - 15.7	-	-	0.1-1.5	-	<1 - 20	-	83 - 288	-	-	-
Raw Herb	Turkey	AAS	[130]	-	-	-	-	-	-	-	-	5.1-5.9	10.1-12.1	448 - 542	-	-	-
Raw Herb	Poland	AAS	[118]	-	-	-	-	-	-	-	-	-	-	6.4-34.5	-	-	-
Raw Herb	Poland	AAS	[132]	-	-	-	-	-	-	-	-	-	12.4	-	-	-	-
Raw Herb	Bulgeria and China	HMDE	[135]	-	-	-	-	-	-	0.22-1.3	-	-	-	-	-	-	-
Raw Herb	Spain	AAS	[136]	-	-	-	-	-	-	6.29-20.32	-	-	5.21-26.50	92.8-119.2	-	-	-
Raw Herb	Unknown	TMFE and ICP-OES	[139]	-	-	-	-	-	-	0.05-1 6	-	-	-	-	-	-	-
Raw Herb	Yugoslavia and R. Srpska	AAS	[140]	-	-	-	-	-	-	0.3-3	-	-	10 - 18	-	-	-	-

Table 10.3 Summary of Element Concentrations (µg/g unless otherwise stated) Found in *Hypericum perforatum* Products Continued

Type of SJW	Origin	Method	Reference	Al	As	B	Ba	Be	Ca	Cd	Co	Cr	Cu	Fe	Hg	In	Li
Raw Herb	Unknown	AAS, AES and ICP-OES	[142]	28-30	-	22.4 - 28.2	11.29 - 16.09	-	0.29 ± 0.01 %	-	-	-	9.6 - 10.76	53 - 59	-	-	-
Raw Herb	Turkey	ICP-MS	[144]	-	-	-	-	-	-	-	0.90-0.98	3.55-3.85	11.8-12.0	1077-1277	-	-	-
Raw Herb	Xinjiang atai	ICP-OES	[145]	167.8	-	-	74.1	-	8.18	-	-	2.03	17.4	99.5	-	-	-
Raw Herb	Poland	GFAAS and ICP-MS	[131]	-	-	-	21.5 - 29.1	-	-	0.11 - 1.51	0.1893 - 0.2071	0.26-0.52	7.47 - 7.89	117 - 187	-	-	-
Raw Herb	Turkey	ICP-OES	[138]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raw Herb/Capsule	Pakistan	AAS and AES	[128]	-	-	-	-	-	192	<0.001	2.6	<0.003	25.4	1020.4	-	-	-
Solid extract	Pakistan and UK	AAS	[137]	-	-	-	-	-	-	4.3-6.3	52.9-62.8	53.4-54.2	210.1-210.7	317.9-319	-	-	-
Tablet	Argentina	GFAAS, AAS and AES	[125]	-	-	-	-	-	111	-	-	-	16.9	-	-	-	0.07
Tablet	Argentina	GFAAS and ICP-OES	[126]	<0.06	-	-	-	-	-	0.26	<0.03	<0.04	-	25.62	-	-	-
Tablet	Poland	GFAAS and ICP-MS	[131]	-	-	-	2.54 - 3.88	-	-	0.664 - 0.782	0.3423 - 0.3585	1.26 - 3.13	4.13 - 5.35	127 - 197	-	-	-
Tablet/Capsule	Unknown	DMA	[134]	-	-	-	-	-	-	-	-	-	-	-	0.002-0.004	-	-
Tablet/Capsule	Unknown	ICP-MS	[141]		0.078-0.828 µg/day	-	-	-	-	0.047-2.115 µg/day		0.219-6.047 µg/day	9.534-34.648 µg/day		ND	-	-
Tinture/Tea	Argentina	GFAAS, AAS and AES	[125]	-	-	-	-	-	81-5210	-	-	-	<5-42.3	-	-	-	0.09-0.29
Tinture/Tea	Argentina	GFAAS and ICP-OES	[126]	5.27-17.65 µg/l	-	-	-	-	-	<0.008-0.24 µg/l	<0.15 µg/l	<0.2 µg/l	-	<0.2-45.09 µg/l	-	-	-
Raw Herb	Poland	AAS	[143]	-	-	-	-	-	4400 - 6070	-	-	-	12.4 - 15.7	108 - 250			
Aqueous Extract	Poland	AAS	[143]	-	-	-	-	-	2.0 - 6.2 mg/100ml	-	-	-	0.01 - 0.02 mg/100ml	0.04 - 0.07 mg/100ml		-	-
Raw Herb	Poland	AAS	[157]	-	-	-	-	-	4100 - 5300	-	-	-	5.3 - 6.8	155 - 161	-	-	-
Aqueous Extract	Poland	AAS	[157]	-	-	-	-	-	8 mg/250ml	-	-	-	4.8 mg/250ml	49 mg/250ml		-	-

Table 10.4 Summary of Element Concentrations (µg/g unless otherwise stated) Found in *Hypericum perforatum* Products Continued

Type of SJW	Origin	Method	Reference	Mg	Mn	Mo	Ni	Pb	Pt	Sb	Se	Sn	Sr	Ti	V	Y	Zn
Aqueous Extract	Poland	AAS	[133]	-	-	-	-	-	-	-	-	-	-	-	-	-	26-214
Aqueous Extract	Poland	AAS	[132]	800	-	-	-	-	-	-	-	-	-	-	-	-	-
Aqueous Extract	Poland	GFAAS and ICP-MS	[131]	-	-	-	1.4 - 1.7	-	-	-	-	-	-	-	-	-	38 - 61
Capsule	Unknown	LA-ICP-MS and ICP-MS	[119]	2251-2713	7.60-10.48		1.00-1.46								0.06-0.2		19.3-25.7
Liquid extract	Poland	ICP-MS	[124]	-	5760-6420	-	-	≤ 680	-	-	-	-	-	-	-	-	2863-3157
Raw Herb	Argentina	GFAAS, AAS and AES	[125]	34.5-112	80.9-91.6	-	0.04-0.12	-	-	-	-	-	-	-	-	-	42.8-46.3
Raw Herb	Argentina	GFAAS and ICP-OES	[126]	-	-	-	-	0.21-0.36	-	-	-	-	-	-	1.63-2.23	-	-
Raw Herb	Austria and Vienna	AAS	[121]	-	33.8-175.0	-	-	0.2-1.0	-	-	-	-	-	-	-	-	20.9-47.5
Raw Herb	Austria	GFAAS and AAS	[120]	-	20.2-50.4	-	-	-	-	-	-	-	-	-	-	-	26.1-42.4
Raw Herb	Serbia	AAS	[122]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raw Herb	Spain	ICP-MS	[123]	-	-	-	-	0.04 - 8.5	-	-	-	-	-	-	-	-	-
Raw Herb/(extracts)	Estonia	AAS	[127]	1823 - 2284	30.6 - 59.8	-	-	0.11 - 0.23	-	-	-	-	-	-	-	-	29-36.1
Raw Herb	Romania	ICP-OES	[129]	-	31 - 219	-	0.5 - 4.9	<0.1 - 3.8	-	-	-	-	<1 - 33	-	-	-	40 - 96
Raw Herb	Turkey	AAS	[130]	-	62.6-5.3	-	8.5-10.3	-	-	-	-	-	-	-	-	-	18.6-20.4
Raw Herb	Poland	AAS	[118]	-	-	-	-	-	-	-	-	-	-	-	-	-	33.9-73.4
Raw Herb	Poland	AAS	[132]	2500	440	-	-	-	-	-	-	-	-	-	-	-	-
Raw Herb	Bulgeria and China	HMDE	[135]	-	-	-	-	1.39-14	-	-	-	-	-	-	-	-	-
Raw Herb	Spain	AAS	[136]	-	134.4-344.5	-	-	-	-	-	-	-	-	-	-	-	24.09-102.73
Raw Herb	Unknown	TMFE and ICP-OES	[139]	-	-	-	-	0.1-19	-	-	-	-	-	-	-	-	-
Raw Herb	Yugoslavia and R. Srpska	AAS	[140]	-	26-226	-	1 - 8	0.5-3.5	-	-	-	-	-	-	-	-	21-56
Raw Herb	Unknown	AAS, AES and ICP-OES	[142]	0.18 ± 0.01 %	154 -156	-	-	-	-	-	-	-	-	-	-	-	29-31

Table 10.5 Summary of Element Concentrations (µg/g unless otherwise stated) Found in *Hypericum perforatum* Products Continued

Type of SJW	Origin	Method	Reference	Mg	Mn	Mo	Ni	Pb	Pt	Sb	Se	Sn	Sr	Ti	V	Y	Zn
Raw Herb	Turkey	ICP-MS	[144]	-	122-132	-	6.21-6.29	0.9-1.44	-	-	-	-	22.1-22.9	-	-	-	-
Raw Herb	Xinjiang atai	ICP-OES	[145]	1.43	57.4	-	-	2.12	-	-	-	-	-	-	-	-	26.7
Raw Herb	Poland	GFAAS and ICP-MS	[131]	-	-	-	1.5 - 2.8	1.38 - 1.78	-	-	-	-	-	-	-	-	62 - 88
Raw Herb	Turkey	ICP-OES	[138]	-	-	-	-	-	-	-	0.018-0.020	-	-	-	-	-	-
Raw Herb/Capsule	Pakistan	AAS and AES	[128]	-	-	-	<0.006	<0.015	-	-	-	-	-	-	-	-	78.2
Solid extract	Pakistan and UK	AAS	[137]	-	-	-	67.8-69.2	46	-	-	-	-	-	-	-	-	-
Tablet	Argentina	GFAAS, AAS and AES	[125]	192	9.7	-	0.49	-	-	-	-	-	-	-	-	-	40.4
Tablet	Argentina	GFAAS and ICP-OES	[126]	-	-	-	-	<0.012	-	-	-	-	-	-	<0.01	-	-
Tablet	Poland	GFAAS and ICP-MS	[131]	-	-	-	1.53 - 2.81	2.33 - 2.85	-	-	-	-	-	-	-	-	158 - 230
Tablet/Capsule	Unknown	DMA	[134]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tablet/Capsule	Unknown	ICP-MS	[141]	-	-	0.279-3.035 µg/day	-	0.068-5.831 µg/day	-	0.003-0.6 µg/day	-	0.013-0.637 µg/day	-	-	0.063-3.513 µg/day	-	16.831-75.810 µg/day
Tinture/Tea	Argentina	GFAAS, AAS and AES	[125]	15476-52890	13.5-322	-	0.51-0.96	-	-	-	-	-	-	-	-	-	11-121.4
Tinture/Tea	Argentina	GFAAS and ICP-OES	[126]	-	-	-	-	<0.06-16.89 µg/l	-	-	-	-	-	-	5.16-30.01 µg/l	-	-
Raw Herb	Poland	AAS	[143]	1920 - 2480	-	-	-	-	-	-	-	-	-	-	-	-	23 - 40
Aqueous Extract	Poland	AAS	[143]	1.0 - 1.6 mg/100ml	-	-	-	-	-	-	-	-	-	-	-	-	0.003 - 0.032mg/100ml
Raw Herb	Poland	AAS	[157]	1100 - 1500	104 - 122	-	-	-	-	-	-	-	-	-	-	-	26 - 34
Aqueous Extract	Poland	AAS	[157]	4.1 mg/250 ml	304 mg/250ml	-	-	-	-	-	-	-	-	-	-	-	88 mg/250ml

10.3 Elemental Concentrations in SJW Preparations

Table 10.6 Concentrations of Elements in SJW raw Herbs (H1 – H11)

									Concentration (µg/g)													
Element	H1	±1SD	H2	± 1SD	H3	± 1SD	H4	± 1SD	H5	± 1SD	H6	± 1SD	H7	± 1SD	H8	± 1SD	H9	± 1SD	H10	± 1SD	H11	± 1SD
Al	20	3	34	3	170	30	31	4	160	20	80	30	29.3	0.4	120	30	133	9	72	9	100	10
As	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	20.98	0.03	25.4	0.6	34	2	37.1	0.7	37.9	0.2	32.0	0.4	26	1	21.0	0.5	20.7	0.4	23.8	0.2	24.3	0.6
Ba	17.7	0.2	12.6	0.2	9.7	0.2	18.8	0.4	10.7	0.1	7.6	0.4	14.2	0.5	17.9	0.9	2.7	0.2	15.8	0.1	8.0	0.1
Be	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ca	3640	80	4100	100	6300	200	5400	200	6600	200	4720	70	3800	300	4780	30	3270	20	4160	30	4300	200
Cd	0.65	0.03	0.89	0.02	1.00	0.02	0.80	0.01	0.97	0.01	0.71	0.04	0.72	0.02	1.16	0.05	*0.07*	*0.01*	0.561	0.004	0.37	0.01
Co	-	-	-	-	-	-	-	-	*0.18*	*0.02*	*0.10*	*0.05*	*0.12*	*0.04*	*0.24*	*0.06*	*0.50*	*0.03*	-	-	-	-
Cr	-	-	-	-	*0.42*	*0.02*	-	-	*0.34*	*0.06*	*0.24*	*0.04*	*0.12*	*0.07*	*0.28*	*0.02*	*0.21*	*0.08*	*0.27*	*0.01*	*0.25*	*0.04*
Cu	9.7	0.1	9.5	0.2	6.7	0.1	11.8	0.5	4.64	0.07	10.6	0.1	9.1	0.2	10.6	0.2	9.6	0.4	11.6	0.2	9.3	0.4
Fe	38	3	66	2	170	40	65	9	210	20	90	20	52	3	130	30	100	20	110	20	140	20
Hg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
In	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mg	1170	20	1400	30	1700	50	1730	20	1610	10	1570	30	1290	80	1270	40	1802	8	1467	9	1670	50
Mn	98	2	133	3	124	3	136	5	261	4	61	1	106	7	78	3	65	1	82.8	0.3	79	3
Mo	*0.34*	*0.02*	-	-	*0.43*	*0.07*	*0.57*	*0.08*	*0.47*	*0.03*	*0.34*	*0.04*	*0.31*	*0.05*	-	-	*0.32*	*0.04*	*0.46*	*0.02*	1.47	0.05
Ni	*0.93*	*0.06*	1.45	0.03	1.40	0.09	1.71	0.04	*1.23*	*0.04*	2.91	0.06	*1.03*	*0.09*	2.04	0.02	-	-	3.03	0.08	*0.93*	*0.05*
Pb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pt	-	-	-	-	*3.3*	*0.9*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Se	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sr	11.2	0.2	11.7	0.1	18.3	0.2	18.4	0.5	17.0	0.1	9.29	0.06	10.9	0.5	21.3	0.8	17.0	0.1	17.74	0.07	11.5	0.4
V	-	-	-	-	*0.40*	*0.02*	-	-	-	-	-	-	-	-	-	-	*0.40*	*0.06*	-	-	-	-
Y	*0.10*	*0.01*	*0.08*	*0.01*	0.26	0.06	-	-	*0.20*	*0.01*	*0.10*	*0.01*	*0.08*	*0.01*	*0.14*	*0.01*	*0.17*	*0.01*	*0.07*	*0.01*	*0.09*	*0.02*
Zn	33.3	0.6	40.9	0.6	29	1	56	2	50	2	36.3	0.3	34	2	29.2	0.7	42.5	0.2	33.4	0.5	37.1	0.3

- Samples below LOD

SD = Standard Deviation

Italic = Below LOQ

Table 10.7. Concentrations of Elements in SJW raw Herbs (H12 – H22)

	Concentration (µg/g)																					
Element	H12	± 1SD	H13	± 1SD	H14	± 1SD	H15	± 1SD	H16	± 1SD	H17	± 1SD	H18	± 1SD	H19	± 1SD	H20	± 1SD	H21	± 1SD	H22	± 1SD
Al	147	6	150	7	84	6	370	30	22	2	186	7	23	3	100	10	52	3	109	3	39	6
As	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	29.5	0.5	26	1	19.7	0.6	47	1	20	2	32.5	0.5	31	1	45	1	19	2	30	1	16	1
Ba	10.2	0.3	8.1	0.5	12.6	0.3	11.7	0.6	18	1	20.3	0.1	12.2	0.7	5.3	0.1	22	4	19	1	8.5	0.2
Be	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ca	6200	60	4800	50	4560	90	9500	1000	3500	300	7830	40	4400	200	5000	100	3700	300	4000	200	2600	300
Cd	0.67	0.02	0.46	0.01	0.376	0.002	1.18	0.02	0.64	0.01	1.73	0.02	0.74	0.03	0.81	0.01	0.72	0.07	0.89	0.03	0.56	0.02
Co	-	-	-	-	-	-	*0.43*	*0.05*	-	-	-	-	-	-	-	-	-	-	*0.45*	*0.03*	-	-
Cr	*0.39*	*0.02*	0.85	0.03	*0.21*	*0.02*	1.02	0.08	-	-	1.40	0.05	-	-	*0.27*	*0.04*	*0.20*	*0.02*	*0.46*	*0.07*	-	-
Cu	8.2	0.3	120	10	9.5	0.6	10.1	0.1	8.9	0.4	11.25	0.03	9.0	0.3	5.5	0.1	12.1	0.5	10.7	0.4	4.9	0.2
Fe	173	3	280	10	105	6	760	80	39	5	180	20	47	2	200	10	65	3	120	10	58	8
Hg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
In	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mg	1740	10	1670	60	1570	30	1860	10	1100	100	818	7	1370	60	1870	30	1290	90	1620	60	790	50
Mn	76.5	0.2	68	2	59.1	0.6	194	1	131	9	161.8	0.6	125	7	148	4	73	4	159	8	76	6
Mo	*0.7*	*0.1*	1.41	0.08	1.16	0.09	*0.54*	*0.07*	*0.30*	*0.05*	-	-	*0.58*	*0.07*	*0.48*	*0.04*	-	-	-	-	-	-
Ni	1.28	0.09	1.38	0.06	1.39	0.03	5.11	0.07	*0.82*	*0.08*	5.37	0.08	*1.20*	*0.09*	*1.35*	*0.05*	2.3	0.3	3.8	0.2	*1.56*	*0.02*
Pb	-	-	-	-	-	-	*1.6*	*0.2*	-	-	*1.8*	*0.2*	-	-	-	-	-	-	-	-	-	-
Pt	-	-	*6.1*	*0.8*	-	-	17	2	-	-	*5*	*1*	-	-	-	-	-	-	-	-	-	-
Sb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Se	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sr	11.8	0.1	13.3	0.9	12.4	0.1	24	2	10.6	0.5	30.33	0.08	11.54	0.08	10.44	0.05	14.3	0.6	20.2	0.4	11.5	0.6
V	-	-	*0.45*	*0.05*	-	-	*0.88*	*0.08*	-	-	*0.48*	*0.02*	-	-	-	-	-	-	-	-	-	-
Y	-	-	*0.09*	*0.01*	0.10	0.02	*0.34*	*0.01*	-	-	*0.18*	*0.01*	-	-	-	-	-	-	*0.14*	*0.01*	-	-
Zn	34.6	0.2	41	1	32.8	0.8	64	2	37	4	41.2	0.6	36	2	30	1	28	2	37	2	23	1

- Samples below LOD
SD = Standard Deviation
Italic = Below LOQ

Table 10.8. Concentrations of Elements in SJW Capsules

	Concentration (µg/g)																							
Element	C1	± 1SD	C2	± 1SD	C3	± 1SD	C4	± 1SD	C5	± 1SD	C6	± 1SD	C7	± 1SD	C8	± 1SD	C9	± 1SD	C10	± 1SD	C11	± 1SD	C12	± 1SD
Al	399	7	160	20	18.8	0.5	31	1	50	3	5.54	0.05	28	5	47.7	0.6	7.0	0.3	144	9	5.7	0.5	4.4	0.3
As	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	39.0	0.6	42	1	15	1	13.0	0.6	17.3	0.2	14.9	0.4	22	1	9.5	0.6	13.4	0.2	30	1	14	1	-	-
Ba	8.5	0.4	17.4	0.2	1.3	0.1	0.30	0.06	0.48	0.02	0.33	0.06	9.8	0.3	0.34	0.08	0.59	0.07	15.7	0.3	0.389	0.001	1.05	0.07
Be	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ca	5640	50	7090	20	560	60	570	30	93000	2000	580	70	1012	9	410	20	592	2	5650	10	615	6	438	5
Cd	1.78	0.01	1.20	0.01	-	-	-	-	*0.12*	*0.01*	*0.07*	*0.01*	*0.153*	*0.004*	*0.07*	*0.01*	*0.072*	*0.003*	0.787	0.003	*0.07*	*0.01*	-	-
Co	*0.55*	*0.05*	-	-	*0.43*	*0.01*	-	-	*0.51*	*0.05*	*0.44*	*0.04*	*0.81*	*0.08*	*0.46*	*0.05*	-	-	-	-	*0.60*	*0.05*	-	-
Cr	1.13	0.01	1.6	0.3	*0.5*	*0.1*	-	-	2.42	0.02	*0.18*	*0.02*	*0.5*	*0.1*	*0.35*	*0.03*	*0.20*	*0.02*	1.32	0.09	2.1	0.2	-	-
Cu	12.9	0.6	10.6	0.1	9.0	0.3	11.0	0.3	14.3	0.4	13.0	0.4	19.2	0.1	9.77	0.08	14.0	0.1	9.1	0.2	16.74	0.04	83	2
Fe	750	10	520	80	18.7	0.4	32	4	70.8	0.4	17.8	0.7	52	2	30.6	0.4	60	10	450	20	39	1	31	5
Hg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
In	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mg	1066	9	1010	10	958	1	1290	30	2330	70	1670	30	1780	20	949	7	1550	30	1960	20	1201	7	1030	10
Mn	81.0	0.2	240	6	16.4	0.2	10.7	0.5	10.5	0.1	16.2	0.1	21.4	0.4	5.78	0.09	16.9	0.1	199.9	0.4	13.35	0.06	4.4	0.2
Mo	*0.56*	*0.09*	*0.73*	*0.03*	-	-	-	-	*0.73*	*0.03*	*0.37*	*0.07*	-	-	-	-	*0.45*	*0.02*	*0.66*	*0.09*	-	-	-	-
Ni	2.65	0.03	2.90	0.07	*1.06*	*0.03*	*1.21*	*0.05*	1.63	0.03	*1.47*	*0.03*	2.30	0.08	*1.265*	*0.005*	1.54	0.05	2.03	0.01	2.9	0.2	*0.6*	*0.1*
Pb	*2.7*	*0.1*	*1.7*	*0.3*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*2.20*	*0.04*	-	-	-	-
Pt	18.7	0.3	14.0	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*5.9*	*0.6*	-	-	-	-
Sb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Se	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sr	16.2	0.4	19.6	0.1	1.8	0.3	0.9	0.2	21.2	0.4	1.1	0.3	7.03	0.05	0.92	0.08	1.12	0.02	13.27	0.06	1.74	0.01	1.2	0.1
V	*0.81*	*0.03*	*0.46*	*0.07*	-	-	-	-	*0.66*	*0.07*	-	-	-	-	-	-	-	-	*0.44*	*0.05*	-	-	-	-
Y	0.33	0.01	*0.171*	*0.001*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*0.14*	*0.02*	-	-	-	-
Zn	60	1	60	1	23	2	29	1	36	2	42.8	0.6	48.9	0.9	25	1	41.84	0.06	54.3	0.7	45.2	0.5	17.2	0.7

- Samples below LOD

SD = Standard Deviation

Italic = Below LOQ

Table 10.9. Concentrations of Elements in SJW raw Tablets (T1 – T10)

	Concentration (µg/g)																			
Element	T1	± 1SD	T2	±1SD	T3	± 1SD	T4	± 1SD	T5	± 1SD	T6	± 1SD	T7	± 1SD	T8	± 1SD	T9	± 1SD	T10	± 1SD
Al	59.36	0.02	48	1	110	10	900	100	110	20	61	6	*1.2*	*0.1*	101	7	47	2	13	1
As	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	13.7	0.4	13.7	0.2	20	1	14.2	0.7	*2*	*2*	37	2	4.8	0.2	21.0	0.6	13.7	0.9	9.28	0.05
Ba	5.0	0.1	1.58	0.05	0.87	0.03	2.5	0.2	0.90	0.08	5.7	0.3	0.51	0.04	0.77	0.02	1.23	0.08	1.29	0.04
Be	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ca	95200	700	99000	2000	8700	200	7600	400	199000	3000	5660	40	300	40	7700	200	77800	200	1250	20
Cd	-	-	-	-	-	-	-	-	-	-	0.49	0.01	-	-	*0.0628*	*0.0003*	-	-	-	-
Co	-	-	-	-	*0.85*	*0.03*	*0.67*	*0.05*	-	-	*0.41*	*0.04*	-	-	*0.66*	*0.03*	-	-	0.48	0.02
Cr	2.23	0.03	2.31	0.03	*0.23*	*0.02*	*0.49*	*0.06*	*0.23*	*0.02*	5	1	-	-	*0.30*	*0.01*	1.75	0.09	0.96	0.08
Cu	5.8	0.1	5.83	0.03	9.27	0.07	10.9	0.2	*1.57*	*0.09*	9.3	0.3	1.99	0.09	8.9	0.2	7.0	0.4	5.90	0.08
Fe	59.9	0.5	79.3	0.6	260	10	630	40	225	9	500	80	1.154	0.004	246	6	63	6	620	50
Hg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
In	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mg	1520	30	1390	10	3100	100	2300	100	3530	30	1660	30	410	20	3050	90	1030	50	1870	10
Mn	7.7	0.1	7.90	0.05	12.0	0.1	13.46	0.07	10.9	0.1	84.5	0.6	3.3	0.1	11.51	0.09	21.9	0.8	12.64	0.05
Mo	*0.64*	*0.03*	*0.85*	*0.09*	-	-	-	-	-	-	*0.5*	*0.1*	-	-	-	-	-	-	-	-
Ni	*1.079*	*0.003*	*0.85*	*0.05*	1.71	0.02	1.38	0.06	*0.60*	*0.05*	3.2	0.6	-	-	1.54	0.02	*0.88*	*0.05*	*1.22*	*0.05*
Pb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pt	-	-	-	-	*5.3*	*0.6*	14.6	0.9	7.0	0.9	*11*	*1*	-	-	*3.9*	*0.4*	-	-	*12.8*	*0.9*
Sb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Se	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sr	23.2	0.1	22.68	0.04	4.4	0.1	6.0	0.1	83.6	0.6	9.3	0.1	0.88	0.06	4.1	0.1	18.1	0.7	8.75	0.07
V	*0.83*	*0.03*	*0.72*	*0.03*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y	*0.19*	*0.01*	*0.20*	*0.02*	-	-	*0.20*	*0.01*	0.461	0.005	*0.07*	*0.01*	-	-	-	-	0.42	0.01	-	-
Zn	19.4	0.9	18.5	0.6	34	1	28.9	0.3	7	1	36	1	11	6	34.8	0.2	20.5	0.8	15.6	0.5

- Samples below LOD

SD = Standard Deviation

Italic = Below LOQ

Table 10.10. Concentrations of Elements in SJW raw Tablets (T11 – T20)

	Concentration (µg/g)																			
Element	T11	± 1SD	T12	± 1SD	T13	± 1SD	T14	± 1SD	T15	± 1SD	T16	± 1SD	T17	± 1SD	T18	± 1SD	T19	± 1SD	T20	± 1SD
Al	26	3	20.8	0.2	27	1	21	1	41.7	0.3	52	1	24	1	31	2	23	2	30	2
As	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	10.7	0.3	17	1	10	2	13.8	0.4	16.35	0.07	11.5	0.2	14	1	9.7	0.3	4	1	13.8	0.2
Ba	2.19	0.04	0.65	0.01	0.78	0.01	0.72	0.03	1.12	0.03	1.3	0.2	0.65	0.04	0.58	0.02	1.00	0.06	4.51	0.07
Be	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*0.047*	*0.003*	*0.029*	*0.001*
Ca	147000	2000	46800	400	85000	2000	1100	40	81300	300	102400	900	51800	300	112000	1000	151000	4000	102290	80
Cd	*0.25*	*0.005*	*0.07*	*0.01*	-	-	-	-	*0.074*	*0.004*	-	-	-	-	-	-	*0.14*	*0.01*	*0.25*	*0.02*
Co	*0.49*	*0.03*	*0.41*	*0.07*	-	-	*0.51*	*0.03*	*0.50*	*0.06*	*0.38*	*0.03*	*0.33*	*0.05*	-	-	-	-	*0.59*	*0.07*
Cr	0.77	0.06	1.13	0.02	1.98	0.05	*0.19*	*0.01*	1.64	0.04	2.1	0.2	1.08	0.02	3.07	0.09	2.91	0.06	2.21	0.02
Cu	7.7	0.2	13.6	0.1	12.1	0.4	10	0.9	9.1	0.2	8.8	0.2	11.4	0.3	5.71	0.02	2.5	0.2	20.0	0.6
Fe	67.8	0.5	260	9	57	1	25	1	90	10	97	5	57	3	43	1	25	1	74	2
Hg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
In	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mg	1750	20	1860	6	948	5	1480	30	1690	20	1263	1	930	10	1017	8	950	30	2790	20
Mn	26.8	0.2	17.31	0.06	15.31	0.09	15.2	0.3	21.9	0.2	18.50	0.02	16.9	0.2	12.3	0.1	2.4	0.1	26.0	0.1
Mo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ni	2.0	0.1	1.572	0.004	*0.86*	*0.07*	1.53	0.01	*1.49*	*0.05*	*1.13*	*0.09*	*1.08*	*0.02*	1.32	0.03	*0.62*	*0.02*	2.19	0.04
Pb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pt	-	-	*6*	*1*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Se	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sr	24.7	0.4	11.7	0.1	25.6	0.2	5.6	0.2	24.5	0.1	25.4	0.2	15.3	0.5	16.97	0.09	54	1	50.4	0.8
V	*0.67*	*0.06*	-	-	-	-	-	-	-	-	-	-	-	-	*0.42*	*0.03*	*0.22*	*0.05*	0.22	0.03
Y	0.906	0.004	*0.24*	*0.01*	0.34	0.01	-	-	0.343	0.003	0.48	0.01	*0.223*	*0.004*	*0.20*	*0.01*	0.92	0.01	0.59	0.01
Zn	21	2	39	2	56.6	0.4	28.4	0.5	27.7	0.7	22	1	34.9	0.3	15.6	0.7	20.8	0.4	45.3	0.8

- Samples below LOD
SD = Standard Deviation
Italic = Below LOQ

10.4 Liquid chromatography Methods

Table 10.11 Details of all the methods and parameters used on the Perkin Elmer UHPLC.

Method Code	Method Name	Mobile Phase A	Mobile Phase B	Flow Rate (ml/min)	Column	Method Details
001	JOwenSJW020312shortSlowA	0.1 %v/v FA in H_2O	0.1 %v/v FA in ACN	0.1	Phenomenex Kinetex™ 2.6 µm C18 100 Å, LC Column 100 x 4.6 mm (PN: 00D-4462-E0)	1) Equilibrate 0.5 minutes A:B, 55:45 2) Hold for 2 minutes; 55:45 3) Over 8 minutes; 0:100 4) Hold for 5 minutes; 0:100 5) Over 1 minute; 55:45 6) Hold for 6 minutes; 55:45
002	060312 JOwen 80:20	0.1 %v/v FA in H_2O	0.1 %v/v FA in ACN	0.2	Phenomenex Kinetex™ 2.6 µm C18 100 Å, LC Column 100 x 4.6 mm (PN: 00D-4462-E0)	1) Equilibrate 0.5 minutes A:B, 80:20 2) Hold for 2 minutes; 80:20 3) Over 8 minutes; 0:100 4) Hold for 5 minutes; 0:100 5) Over 1 minute; 80:20 6) Hold for 6 minutes; 80:20
003	220312 JOwen 80:20 Long SJW	0.1 %v/v FA in H_2O	0.1 %v/v FA in ACN	0.2	Phenomenex Kinetex™ 2.6 µm C18 100 Å, LC Column 100 x 4.6 mm (PN: 00D-4462-E0)	1) Equilibrate 0.5 minutes A:B, 80:20 2) Hold for 2 minutes; 80:20 3) Over 8 minutes; 0:100 4) Hold for 60 minutes; 0:100 5) Over 3 minute; 80:20 6) Hold for 7 minutes; 80:20
004	260312 JOwen 85:15 Long SJW	0.1 %v/v FA in H_2O	0.1 %v/v FA in ACN	0.2	Phenomenex Kinetex™ 2.6 µm C18 100 Å, LC Column 100 x 4.6 mm (PN: 00D-4462-E0)	7) Equilibrate 0.5 minutes A:B, 85:15 8) Hold for 5 minutes; 85:15 9) Over 8 minutes; 0:100 10) Hold for 60 minutes; 0:100 11) Over 3 minute; 85:15 12) Hold for 7 minutes; 85:15
005	020412 JOwen 83:18 to 55:45	0.1 %v/v FA in H_2O	0.1 %v/v FA in ACN	0.2	Phenomenex Kinetex™ 2.6 µm C18 100 Å, LC Column 100 x 4.6 mm (PN: 00D-4462-E0)	1) Equilibrate 0.5 minutes A:B, 83:17 2) Hold for 5 minutes; 83:17 3) Over 15 minutes; 55:45 4) Hold for 60 minutes; 55:45 5) Over 3 minute; 83:17 6) Hold for 7 minutes; 83:17
006	240612 JOwen 83:17 A	0.1 %v/v FA in H_2O	0.1 %v/v FA in ACN	0.2	Phenomenex Kinetex™ 2.6 µm C18 100 Å, LC Column 100 x 4.6 mm	1) Equilibrate 0.5 minutes A:B, 83:17 2) Hold for 10 minutes; 83:17

006					(PN: 00D-4462-E0)	3) Over 10 minutes; 0:100 4) Hold for 60 minutes; 0:100 5) Over 5 minute; 83:17 6) Hold for 15 minutes; 83:17
007	240612 JOwen 83:17 A 1ml/min	0.1 %v/v FA in H_2O	0.1 %v/v FA in ACN	1.0	Phenomenex Luna® 3 µm C18 100 Å, LC Column 150 x 4.6 mm	1) Equilibrate 0.5 minutes A:B, 83:17 2) Hold for 10 minutes; 83:17 3) Over 10 minutes; 0:100 4) Hold for 60 minutes; 0:100 5) Over 5 minute; 83:17 6) Hold for 15 minutes; 83:17
008	261012 2step Grad SJWf	0.1 %v/v FA in H_2O	0.1 %v/v FA in ACN	1.0	Phenomenex Luna® 3 µm C18 100 Å, LC Column 150 x 4.6 mm	1) Equilibrate 0.5 minutes A:B, 90:10 2) Hold for 10 minutes; 90:10 3) Over 40 minutes; 73:27 4) Over 10 minutes; 65:35 5) Hold for 20 minutes; 65:35 6) Over 5 minute; 05:95 7) Hold for 50 minutes; 05:95 8) Over 5 minutes; 90:10 9) Hold for 10 minutes; 90:10
009	220113 SJW Method 2 modified	0.1 %v/v FA in H_2O	0.1 %v/v FA in ACN	1.0	Phenomenex Luna® 3 µm C18 100 Å, LC Column 150 x 4.6 mm	1) Equilibrate 0.5 minutes A:B, 92:08 2) Hold for 10 minutes; 92:08 3) Over 18 minutes; 79:21 4) Hold for 2 minutes; 79:21 5) Over 15 minutes; 65:35 6) Over 10 minutes; 05:95 7) Hold for 20 minutes; 05:95 8) Over 5 minutes; 92:08 9) Hold for 10 minutes; 92:08
010	220113 SJW Method 2 modified b	0.1 %v/v FA in H_2O	0.1 %v/v FA in ACN	1.0	Phenomenex Luna® 3 µm C18 100 Å, LC Column 150 x 4.6 mm	1) Equilibrate 0.5 minutes A:B, 92:08 2) Hold for 10 minutes; 92:08 3) Over 18 minutes; 79:21 4) Hold for 2 minutes; 79:21 5) Over 15 minutes; 65:35 6) Over 10 minutes; 05:95 7) Hold for 10 minutes; 05:95 8) Over 5 minutes; 92:08 9) Hold for 10 minutes; 92:08

Note: H_2O = HPLC grade water, ACN = HPLC grade acetonitrile, FA = formic acid

10.5 The periodic table of elements

1 **H** Hydrogen 1.00794																	2 **He** Helium 4.003
3 **Li** Lithium 6.941	4 **Be** Beryllium 9.012182											5 **B** Boron 10.811	6 **C** Carbon 12.0107	7 **N** Nitrogen 14.00674	8 **O** Oxygen 15.9994	9 **F** Fluorine 18.9984032	10 **Ne** Neon 20.1797
11 **Na** Sodium 22.989770	12 **Mg** Magnesium 24.3050											13 **Al** Aluminum 26.981538	14 **Si** Silicon 28.0855	15 **P** Phosphorus 30.973761	16 **S** Sulfur 32.066	17 **Cl** Chlorine 35.4527	18 **Ar** Argon 39.948
19 **K** Potassium 39.0983	20 **Ca** Calcium 40.078	21 **Sc** Scandium 44.955910	22 **Ti** Titanium 47.867	23 **V** Vanadium 50.9415	24 **Cr** Chromium 51.9961	25 **Mn** Manganese 54.938049	26 **Fe** Iron 55.845	27 **Co** Cobalt 58.933200	28 **Ni** Nickel 58.6934	29 **Cu** Copper 63.546	30 **Zn** Zinc 65.39	31 **Ga** Gallium 69.723	32 **Ge** Germanium 72.61	33 **As** Arsenic 74.92160	34 **Se** Selenium 78.96	35 **Br** Bromine 79.904	36 **Kr** Krypton 83.80
37 **Rb** Rubidium 85.4678	38 **Sr** Strontium 87.62	39 **Y** Yttrium 88.90585	40 **Zr** Zirconium 91.224	41 **Nb** Niobium 92.90638	42 **Mo** Molybdenum 95.94	43 **Tc** Technetium (98)	44 **Ru** Ruthenium 101.07	45 **Rh** Rhodium 102.90550	46 **Pd** Palladium 106.42	47 **Ag** Silver 107.8682	48 **Cd** Cadmium 112.411	49 **In** Indium 114.818	50 **Sn** Tin 118.710	51 **Sb** Antimony 121.760	52 **Te** Tellurium 127.60	53 **I** Iodine 126.90447	54 **Xe** Xenon 131.29
55 **Cs** Cesium 132.90545	56 **Ba** Barium 137.327	57 **La** Lanthanum 138.9055	72 **Hf** Hafnium 178.49	73 **Ta** Tantalum 180.9479	74 **W** Tungsten 183.84	75 **Re** Rhenium 186.207	76 **Os** Osmium 190.23	77 **Ir** Iridium 192.217	78 **Pt** Platinum 195.078	79 **Au** Gold 196.96655	80 **Hg** Mercury 200.59	81 **Tl** Thallium 204.3833	82 **Pb** Lead 207.2	83 **Bi** Bismuth 208.98038	84 **Po** Polonium (209)	85 **At** Astatine (210)	86 **Rn** Radon (222)
87 **Fr** Francium (223)	88 **Ra** Radium (226)	89 **Ac** Actinium (227)	104 **Rf** Rutherfordium (261)	105 **Db** Dubnium (262)	106 **Sg** Seaborgium (263)	107 **Bh** Bohrium (262)	108 **Hs** Hassium (265)	109 **Mt** Meitnerium (266)	110 (269)	111 (272)	112 (277)	113	114				

58 **Ce** Cerium 140.116	59 **Pr** Praseodymium 140.90765	60 **Nd** Neodymium 144.24	61 **Pm** Promethium (145)	62 **Sm** Samarium 150.36	63 **Eu** Europium 151.964	64 **Gd** Gadolinium 157.25	65 **Tb** Terbium 158.92534	66 **Dy** Dysprosium 162.50	67 **Ho** Holmium 164.93032	68 **Er** Erbium 167.26	69 **Tm** Thulium 168.93421	70 **Yb** Ytterbium 173.04	71 **Lu** Lutetium 174.967
90 **Th** Thorium 232.0381	91 **Pa** Protactinium 231.03588	92 **U** Uranium 238.0289	93 **Np** Neptunium (237)	94 **Pu** Plutonium (244)	95 **Am** Americium (243)	96 **Cm** Curium (247)	97 **Bk** Berkelium (247)	98 **Cf** Californium (251)	99 **Es** Einsteinium (252)	100 **Fm** Fermium (257)	101 **Md** Mendelevium (258)	102 **No** Nobelium (259)	103 **Lr** Lawrencium (262)

Figure 10.1 The periodic table of elements

10.6 List of Publications

10.6.1 Papers Undergoing Finalisation for Submission for Publication

Owen J.D.; Kirton, S. B.; Evans, S. E.; Assi, S.; and Stair, J. L., 'Elemental fingerprinting of Hypericum perforatum (St John's Wort) herb and preparations using ICP-OES and chemometrics'.

Owen J.D.; Evans, S. E.; and Stair, J. L., Method Development for the Elemental Analysis of Hypericum perforatum (St John's Wort) Products using ICP-OES and Microwave Digestion

10.6.2 Published abstracts and documents

Owen, J. D.; Evans, S. J.; Stair, J. L., Elemental Profile of Hypericum perforatum (St John's Wort) Preparations Using ICP-OES. *Journal of Pharmacy and Pharmacology* 2010**,** 62, (10), 1206-1207.

Guirguis, A.; Owen, J. D.; Stair, J. L., Presence of metals in herbal extracts. *Pharmaceutical Journal* 2012**,** 289, (7731), 536.

Anjum, K.; Staff, K.; Mistry, T.; Owen, J.; Stair, J. L.; Moss, G. P., The effect of depilation on the percutaneous absorption of aluminium from antiperspirant products. *Journal of Pharmacy and Pharmacology* **2010,** 62, (6), 800-800.

Mistry, T.; Staff, K.; Anjum, K.; Owen, J.; Stair, J. L.; Moss, G. P., The effect of occlusion on the percutaneous absorption of aluminium from antiperspirant products. *Journal of Pharmacy and Pharmacology* **2010,** 62, (6), 799-799.

10.6.3 Oral Presentations

Owen J.D.; Kirton, S. B.; Evans, S. E.; Assi, S.; and Stair, J. L., 'Elemental profiling as a tool for quality control of *Hypericum Perforatum* (St John's Wort)'. American Chemical Society, 244th national meeting, Philadelphia, 19th- 24th August 2012.

Owen J.D.; Kirton, S. B.; Evans, S. E.; Assi, S.; and Stair, J. L., 'Elemental profiling as a tool for quality control of St John's Wort (*Hypericum Perforatum)*'. Young Researchers BioResources, Reading, 3rd July 2012.

10.6.4 Poster Presentations

Owen, J. D.; Evans, S. J.; Assi, S.; Stair, J. L., Elemental Fingerprinting of Hypericum perforatum (St John's Wort) with Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES) and Chemometrics. In *RSC Analytical Research Forum* Nottingham, 2011.

Owen, J. D.; Evans, S. J.; Stair, J. L., Elemental Fingerprinting of Hypericum perforatum (St John's Wort) using ICP-OES. In *RSC Analytical Research Forum*, Loughborough University, 2010.

Owen, J. D.; Evans, S. J.; Stair, J. L., Trace Metal Analysis of *Hypericum perforatum* (St John's Wort) Using ICP-OES. In *Student Forensic Science Conference*, Westminster University, 2010.

Guirguis, A.; Owen, J. D.; Stair, J. L., The effect of extraction conditions on the elemental profile of *Hypericum Perforatum* L. (St John's Wort). In *PharmSci*, Nottingham, 2012.

Mistry, T.; Staff, K.; Anjum, K.; Owen, J. D.; Stair, J. L.; Moss, G., The effect of occlusion on the percutaneous absorption of aluminium from antiperspirant products. In *Perspectives in Percutaneous Penetration*, La Grande Motte, France, 2010.

Mistry, T.; Staff, K.; Anjum, K.; Owen, J. D.; Stair, J. L.; Moss, G., The effect of occlusion on transdermal aluminium absorption after the application of antiperspirant products. . In *UKICRS*, Hertfordshire, UK 2010.

Anjum, K.; Staff, K.; Mistry, T.; Owen, J. D.; Stair, J. L.; Moss, G., The effect of depilation on the percutaneous absorption of aluminium from antiperspirant products. In *Perspectives in Percutaneous Penetration*, La Grande Motte, France, 2010.

Anjum, K.; Staff, K.; Mistry, T.; Owen, J. D.; Stair, J. L.; Moss, G., The effect of depilation on transdermal aluminium absorption after the application of antiperspirant products. . In *UKICRS*, Hertfordshire, UK 2010.

10.6.5 Book Sections

Mistry, T.; Ajum, K.; Owen, J.D.; Stair, J.L.; Wilkinson, S.C.; Staff, K.; Moss, G.P., The percutaneous absorption of aluminium from antiperspirant products. In: Brain, K.R., Chilcott, R. (eds) Advances in the Dermatological Sciences, Royal Society of Chemistry, Cambridge, UK., December 2013.