PHARMACEUTICAL ANALYSIS AND ASPECTS OF THE QUALITY CONTROL OF ST JOHN'S WORT PRODUCTS

by

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ABSTRACT

Most complementary medicines contain a multitude of chemical components, some of which are claimed to contribute to the biological activity of such products. Use of complementary medicines for preventative and therapeutic purposes is increasing rapidly worldwide. Unfortunately, although control of these products is essential to ensure quality, safety, and efficacy, the quality control of most herbal preparations is currently poor to non-existent, with little or no safety and efficacy data required to support the marketing and use of these products. The objective of this study was therefore to develop suitable analytical methods to qualitatively and quantitatively analyse the relevant components (rutin, isoquercitrin, hyperoside, quercitrin, quercetin, kaempferol, hypericin, pseudohypericin and hyperforin) in St John's Wort dosage forms for quality control purposes.

A gradient HPLC method using a Luna 5 : m C_{18} (2) 150 x 2.00mm internal diameter (i.d.) column and UV detection, was developed for the separation of six of the relevant flavonoid compounds in St John's Wort, namely rutin, isoquercitrin, hyperoside, quercitrin, quercetin and kaempferol. The development process involved a systematic investigation of gradient conditions, flow rate, and temperature. This method was subsequently applied to assay selected commercially available St John's Wort products. This system provided the necessary accuracy, precision and reproducibility and was associated with several advantages when compared to using standard bore (4.60 mm i.d.) HPLC columns. The method developed is currently the only known method that separates all six relevant flavonoids in a reasonable run time (less than 20 minutes). It is also one of the few methods that has sufficient separation between rutin, isoquercitrin and hyperoside.

A qualitative method for the fingerprinting of flavonoid components was also developed, using capillary electrophoresis (CE). CE is a rapidly growing powerful analytical technique for the separation of charged compounds. Micellar electrokinetic chromatography (MEKC) is a very powerful electrophoretic technique that is capable of selectively resolving both neutral and ionic solutes in a single run. A MEKC method suitable for the separation and determination of various flavonoid constituents used as marker compounds in *Hypericum perforatum* was developed. Investigations into the effect of pH, ionic strength, applied voltage and capillary dimensions on separation were performed. The optimised method was then applied to qualitatively analyse various

St John's Wort products on the market. This method was found to be advantageous in that it was simple, cost-effective, required minimal sample preparation and utilised very small quantities of sample.

Due to the vast differences in chemical properties between the various marker and active components in St John's Wort, it was necessary to develop separate analytical methods for the flavonoids and for the other three relevant compounds (hypericin, pseudohypericin and hyperforin). An isocratic HPLC method using a Luna 5:m C_{18} (2) 150 x 2.00mm (i.d.) column and UV detection was developed for the separation of hypericin, pseudohypericin and hyperforin. The development process involved a systematic investigation of buffer molarity, mobile phase composition, pH, flow rate, and temperature. This method was subsequently applied to assay selected commercially available St John's Wort products on the South African market. This system also provided the necessary accuracy, precision and reproducibility, as well as the advantages associated with the use of a narrow bore column as opposed to the use of the more commonly used wider bore columns. This method was validated and used to quantitate these three compounds in various commercial St John's Wort products.

By applying this method to liquid chromatography – tandem mass spectrometry (LC-MS-MS), qualitative analyses of the same products was performed to obtain confirmation of the quantitative HPLC results. Mass spectrometry is a powerful detection tool that is more selective and specific than many detection systems used with HPLC. Natural medicines usually constitute a multitude of constituents with much potential interference. In this regard LC-MS-MS is a powerful tool, with its ability to unequivocally identify target analytes regardless of the presence of interferences or complex matrices. ESI-MS-MS was used for the qualitative analysis of the content of the naphthodianthrones and hyperforin in the respective tablet products assayed with HPLC. LC-MS-MS analyses were performed in order to identify the constituents and to verify the specificity of the HPLC method.

High inter-product and inter-batch variability was observed for all nine compounds assayed. These quantitative results were confirmed with the respective qualitative analyses. This study confirms the need for strict quality control of herbal medicinal products commercially available to consumers.

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ARTG	Australian Register of Therapeutic Goods
AUC	area under the concentration-time curve
AUFS	absorbance units full scale
CDs	cyclodextrins
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focussing
CITP	capillary isotachophoresis
CMWG	Complementary Medicines Working Group
CMC	critical micelle concentration
CNS	central nervous system
COMT	catechol O-methyl transferase
CRH	corticotrophin releasing hormone
CZE	capillary zone electrophoresis
DA	dopamine
DSHEA	Dietary Supplement Health and Education Act of 1994
EMEA	European Medicines Evaluation Agency
EOF	electro-osmotic flow
ERP	Expedited Registration Procedure
ESCOP	European Scientific Co-operative on Phytotherapy
ESI	electrospray ionisation
ESP	electrospray
FASI	field amplified sample injection
FDA	Food and Drug Administration
FD&C Act	Federal Food, Drug and Cosmetic Act
GABA	gamma amino butyric acid
GMP	Good Manufacturing Practice
GPV	gradient proportioning valve

HIV	Human Immunodeficiency Virus
HMP	herbal medicinal product
HPB	Canada's Health Protection Bureau
HPLC	high performance liquid chromatography
Hypf	hyperforin
HY	hypericin
HYNa	monosodium salt of hypericin
Hd	hyperoside
ICH	international convention on harmonization
ID	internal diameter
IL-6	interleukin-6
Iqr	isoquercitrin
K	kaempferol
LOD	limit of detection
LOQ	limit of quantitation
MAO	monoamine oxidase
MAOIs	monoamine oxidase inhibitors
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry
PsHY	pseudohypericin
Q	quercetin
Qr	quercitrin
RSD	relative standard deviation
R	rutin
SDS	sodium dodecyl sulphate
SJW	St John=s Wort
SOPs	standard operating procedures
SSRIs	selective serotonin reuptake inhibitors
TCAs	tricyclic antidepressants
TGA	Therapeutic Goods Administration
TSP	thermospray

US	United States
USP	Unites States Pharmacopoeia
UPS	uninterrupted power supply
UV	ultraviolet
UV/VIS	ultraviolet-visible
WHO	World Health Organisation
5-HT	serotonin

"	alpha
$	beta
(	gamma
8	lambda
S	omega
:A	microampere
:g	microgram
:l	microliter
:m	micrometer
cm	centimeter
g	gram
hr	hour
kV	kilovolt
L	liter
M	molar
mbar	millibar
mg	milligram
min	minute
ml	millilitre
mm	millileter
mM	millimolar
nm	nanometer
Pa	Pascal

s	second
V	volt
W	Watt
° C	degrees Celsius

CHAPTER ONE

ST JOHN'S WORT: THE HERBAL MEDICINE

1.1 INTRODUCTION

1.1.1 HERBAL MEDICINES

Since ancient times, medicinal herbs have been used to prevent and treat various diseases and ailments. Hippocrates, a physician who lived 2400 years ago, taught that "nature is the healer of all diseases", and this concept has long been the core of indigenous medicine in many cultures. With the development of analytical technology late in the 19th Century, came the efforts to identify and isolate the active chemical moieties in medicinal plants, so that more potent, safe, efficacious and high quality drugs could be developed. These discoveries enabled the quality process of conventional medicine to begin, and from this evolved the conventional medicines of today.

Currently, the FDA (Food and Drug Administration) in the United States (US), and other drug regulatory authorities, require that a pre-marketing multistep approval process, including the assessment of quality, must be undertaken before any conventional medicine can be marketed, and also proof of both safety and efficacy must be provided. Conventional medicines must have known active chemical moieties that can be analysed quantitatively and qualitatively. Due to differences between herbal and conventional medicines, legislation and control of each group of medicines differ, with herbal products generally being treated as some form of dietary supplement. Consequently the quality control of most herbal preparations is currently poor to non-existent, with little or no safety and efficacy data required to support marketing and use of these products. This is much cause for concern, especially since over the past two decades there has been a tremendous world-wide increase of interest and utilisation of these herbal medicines for preventative and therapeutic purposes.

On a global scale, it has been estimated that 80% of the world's population (four billion people) use some form of herbal medicine as part of their primary health care [1]. In the US total expenditure on herbal medicines in 1997 was estimated at US$ 2700 million. In Australia, Canada and the United Kingdom expenditure was estimated at US$ 80 million, US$ 2400 million and US$ 2300 million respectively. This illustrates the significance of the amount of herbal medicines used [2].

In the United States, St John's Wort has been reported to consistently rank amongst the 3 most popular herbal products, annual sales of this herb increasing from about US$20 million in 1995 to US$200 million in 1997. St John's Wort is also popular in Germany, with annual sales of about US$ 55 million in 1997. Sales of St John's Wort in Germany are claimed to outnumber that of all other anti-depressants combined, and the herbal remedy is claimed to outsell fluoxetine (Prozac) more than seven to one [3,4].

Unfortunately, a general belief amongst conventional medical practitioners is that herbal preparations are residues from the past and that they are largely ineffective and harmless, whilst many patients believe that herbals are often miraculous in their effects and that they are "natural" and therefore safe. Both are misconceptions. Apart from the fact that herbal preparations can be toxic and may interact with medicines being administered concurrently, there have been reports of plant mis-identification, mis-labelling, adulteration, contamination, and lack of standardisation of such dietary supplements. This clearly leaves a lot to be desired with respect to quality, safety and efficacy [5].

1.1.2 DEFINITIONS

Herbal Preparations and Products:

The World Health Organisation (WHO) defines herbal preparations as herbal materials which have been subject to extraction, fractionation, purification, concentration or other physical or biological processes [6]. These medicines contain plant parts or plant material in the crude or processed state as active ingredients and may contain excipients or inert ingredients [7]. Similarly, the European Medicines Evaluation Agency (EMEA) defines herbal medicinal products as medicinal products containing exclusively herbal drugs or herbal drug preparations as active substances. Herbal drugs being plants or plant parts in an unprocessed state which are used for a medicinal or pharmaceutical purpose whereas combinations of plants or plant parts with chemically defined active substances are not considered herbal medicines.

Conventional and Orthodox Medicines:

The South African Medicines and Related Substances Control Act, Act 101 of 1965
defines medicine in the following terms:
"Medicine" means any substance or mixture of substances used or purporting to be suitable for use or manufactured or sold for use in-

(a) the diagnosis, treatment, mitigation, modification or prevention of disease, abnormal physical or mental state or the symptoms thereof in man; or

(b) restoring, correcting or modifying any somatic or psychic or organic function in man and includes any veterinary medicine.

This definition does not apply to any alternative or complementary medicine.

Complementary and Alternative Medicines:

Complementary and alternative medicines are terms used to describe medical practices and therapies that complement, supplement or provide alternatives to modern conventional medicine. These medicines are currently considered to be outside the sphere of conventional medicines. "Complementary medicine" has been defined as "any substance or mixture of substances, originating from a plant, mineral or animal used, manufactured or sold for use in complementing the healing power of a human or animal body." This includes substances generally referred to as Western herbal medicine, African traditional medicine, traditional Chinese medicine, traditional Dutch medicine, homeopathic, ayurvedic, aromatherapeutic medicines and food supplementation [8].

Nutraceutical:

This term was apparently first used by DeFelice in 1991, where he defined a nutraceutical as "any object that may be considered a food or a part of food and provides medical or health benefits, including the prevention and treatment of disease". Canada's Health Protection Bureau (HPB), and others, more recently define the term as "a product produced from food but sold in pills, powders (potions) and other medical forms not generally associated with food and demonstrated to have a physiological benefit or provide protection against chronic disease" [9].

Dietary Supplement:

The FDA defines dietary supplements under the Dietary Supplement Health and Education Act of 1994 (DSHEA). A dietary supplement:

$ "Is a product (other than tobacco) that is intended to supplement the diet that bears or contains one or more of the following dietary ingredients: A vitamin, a mineral, a herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total daily intake, or a concentrate, metabolite, constituent, extract, or combinations of these ingredients.

$ Is intended for ingestion in pill, capsule, tablet, or liquid form.

- **$** Is not represented for use as conventional food or as the sole item of a meal or diet.
- **$** Is labeled Adietary supplement" [10].

Standardised Extract:

A standardised extract is an herbal extract that the manufacturers assure to contain a specific amount of active ingredient(s) or marker compound(s), which are found in the whole herb. The quality of a standardised extract therefore, should be based on assays of selected key compounds, either those for which pharmacologic activity is known, and/or those that serve as markers for the source material and biologically active components [8].

Active Marker:

A constituent of a herbal product with pharmacological activity, or activity that otherwise contributes to the efficacy of the product.

Active Principle:

A constituent that has clinical activity.

Analytical Marker:

A constituent of a herbal product without any pharmacological activity yet can be used as a relevant marker for quality control [11].

Negative Marker:

A constituent that has a negative impact, for example, a toxin [11].

Phytopharmaceutical:

Plant medicine

Functional Food:

Functional foods are considered to be a sub-class of nutraceutical products, however the HPB consider functional foods to be a completely separate class of products. The American Dietetic Association defines functional foods as "any modified food or food ingredient that may provide a health benefit beyond the traditional ingredients it contains". The HPB, alternatively, considers a functional food to be "similar in appearance to conventional foods, consumed as part of a usual

diet, and has demonstrated physiological benefits and/or reduces the risk of chronic diseases beyond basic nutritional functions" [9].

1.1.3 QUALITY, SAFETY AND EFFICACY OF HERBAL PRODUCTS

1.1.3.1 Introduction

Quality, as defined by German drug law, is the status of a drug, which is determined either by identity, purity, content, and other chemical, physical or biological properties, or by the manufacturing process. This definition should also be applied to phytopharmaceuticals. Bauer [7] stated that reproducible efficacy and safety of phytopharmaceuticals are based on reproducible quality. However, herbal medicines are generally much more complex than synthetic drugs and utilising the quality criteria for synthetic drugs, in this case, poses many difficulties.

Phytopharmaceuticals are nearly always complex mixtures of many different constituents, and are therefore prone to much variation. Concentrations and even the presence or absence of various constituents will depend greatly on variables such as different growth and climatic, harvesting, drying and storage conditions. The polarity of the solvent used and method of extraction, alongside the stability of the constituents will also affect the variability of concentrations of constituents from one herbal product to another. These processes and variables need to be kept as constant as possible, to ensure quality, safety, and batch-to-batch reproducibility.

There are other problems with herbal products:

$ The active principle(s) may not yet be known, in which case it is difficult to standardize the product

$ Selective analytical methods may not yet have been developed

$ Reference compounds may not be commercially available

Of concern as well is the variation between different plants, implying that collections from individual plants may vary greatly, and different cultivars of the same species do exist in some cases.

1.1.3.2 Quality Criteria

The quality criteria that have been suggested for herbal medicinal products (HMPs) are:

$ Clear botanical identification of the plant material

$ Proof of absence or proof of contents below official limits with respect to undesired toxic

components and toxicologically significant impurities, such as heavy metals, pesticides, radioactivity, bacteria or fungi.

- **$** Quantitation of active principles or marker compounds
- **$** Validation and control of all production steps [7,12]

1.1.3.2.1 Botanical Identification

These should include macroscopic and microscopic botanical characteristics, chromatographic methods for at least 2 reference markers or constituents and qualitative chemical reactions for identification [12].

1.1.3.2.2 Quantitation

The quantitation of active constituents, or markers, requires that adequate analytical methods be developed and applied. Depending upon the current knowledge of the active principles of the plant, different methods may need to be applied in order to establish relevant quantitative criteria for quality.

Phytopreparations can be divided into 2 categories:

- **$** Those extracts or preparations in which the active principle are known and accepted, and
- **$** Those extracts or preparations in which the active principle(s) are not known.

These 2 groups each have to be handled differently in characterising the preparation in question.

According to the European guidelines, standardisation of an herbal medicine falling into the former category can be done by "normalisation". Normalisation refers to the adjustment of total content of active principle(s) in the herbal medicine, after quantitation, by either adding inert material to the herbal medicine, or by blending the product with a highly concentrated batch.

When the active components are not known, or still being investigated, the total native extract is regarded as the active principle, and marker compounds are required for quality control. In this case, the preparations or extracts should not be normalised on marker compounds, as these compounds are not necessarily related to therapeutic activity. Marker compounds alternatively provide an analytical handle on reproducible processing of the plants, batch-related in-process quality control and batch-to-batch consistency.

1.1.4 LEGISLATION OF HERBAL MEDICINALS

1.1.4.1 The United States of America

In the US, herbal medicines or nutraceuticals are regulated as dietary supplements. For decades, the FDA regulated dietary supplements as foods under the Federal Food, Drug and Cosmetic Act (FD&C Act). However, with the passage of the DSHEA of 1994, Congress amended the FD&C Act to include several provisions that apply only to dietary supplements. The DSHEA set up a new framework for FDA regulation of dietary supplements. It also created an office to co-ordinate research on dietary supplements, and it required an independent dietary supplement commission to be initiated to report on the use of claims in dietary supplement labelling.

The FDA recognises that its requirement for pre-market review of dietary supplements is less than those for the other products it regulates, which means that responsibility for checking the safety of dietary supplements and determining the truthfulness of the label claims falls in the hands of consumers and manufacturers [10,13].

1.1.4.2 Europe

Currently, the regulatory framework in most of Europe makes it fairly easy to market herbal medicines, especially those with a long history of use. This is due to 2 main reasons:

i) Substances are approved under the "doctrine of reasonable certainty"

ii) The time to regulatory approval is relatively short

The doctrine of reasonable certainty contends that, in the absence of scientific evidence to the contrary, a substance's historical use is a valid measure of safety and efficacy [9].

The German Minister of Health established an expert committee in 1978 called the Commission E, whose job it was to review herbal drugs and preparations from medicinal plants. Germany, which is currently viewed by many as a leader in control of herbal medicines, have now published the Commission E Monographs, which is the result of this committee, and presently contain approximately 400 monographs on natural products, much of this information being derived from historic use [9,14].

1.1.4.3 Asia

Asian countries such as Japan, China, and India have a long history of herbal medicinal use, and utilise herbal remedies almost as much as conventional medicine. In Japan, the definition, licensing and approval of functional foods is regulated by the Nutrition Approvement Act, which in turn is administered by the Japan Health Food and Nutrition Association. Pakistan however has a problem in that herbal products are aggressively advertised and freely available on the market, but there is no herbal drug law in place to control these medicines.

1.1.4.4 Australia

In Australia, herbal medicines fall under the term "therapeutic goods". A therapeutic good is broadly defined in Australian legislation as a product for use in humans that is represented in any way to be, or is likely to be taken for a therapeutic purpose.

The Therapeutic Goods Act 1989 provides a national framework for the regulation of therapeutic goods in Australia, and ensures their quality, safety and efficacy. Before any product with therapeutic claims can be marketed, the product must be entered on the Australian Register of Therapeutic Goods (ARTG). The Therapeutic Goods Act 1989 contains the requirements for the inclusion of any therapeutic goods in the ARTG.

Control of the supply of therapeutic goods is performed by the Therapeutic Goods Administration (TGA), who administers the provisions of the Therapeutic Goods Act 1989, and also exerts control over therapeutic goods through 3 avenues:

- **$** Pre-market assessment
- **$** Licensing of manufacturers, and
- **$** Post-market vigilance.

Pre-market assessment:

Non-prescription items such as complementary medicines are considered as being low-risk items, and as such are assessed solely for quality and safety (not efficacy).

Licensing of manufacturers:

All manufacturers of therapeutic goods must be licensed, and their processes must comply with principles of Good Manufacturing Practice (GMP).

Postmarket Vigilance:

This includes different activities, such as problem reporting, monitoring activities and laboratory testing to ensure compliance to legislation [15].

1.1.4.5 South Africa

In January 1997, a Complementary Medicines Working Group (CMWG) was appointed and given the responsibility to develop an appropriate registration system for complementary medicines. A unique system was developed, comprising two routes of registration:

i) A full registration application, addressing "toxicity issues, safety and efficacy through pre-clinical and clinical evidence, as well as quality".

ii) A registration through listing procedure, known as the Expedited Registration Procedure (ERP) or Registration through Listing Procedure.

The ERP is based on the Australian system for complementary medicines and allows for many different disciplines or categories of complementary medicines. This is in contrast to a health system dealing only with herbals and/or nutritional supplements, as in the US for example, or legislation only catering for anthroposophy, homeopathy and herbals, as in Germany.

A call up notice for all medicines frequently referred to as complementary medicines in terms of the Medicines and Related Substances Control Act, 1965 (Act 101 of 1965) was issued in February 2002. The categories of the medicines frequently referred to as complementary medicines include: anthroposophical medicines; aromatherapeutic medicines; ayurvedic medicines; Chinese traditional medicines; energy substances; homeopathic medicines; nutritional substances that purport to have therapeutic or medicinal effects; western herbal medicines; Unani-Tibb medicines; combination homeopathic/flower essence; combination complementary medicines. The issue of control of African Traditional Medicines is being considered separately. The call up process was instituted as a primary step towards registration of these medicines, and manufacturers were required to submit the required information by August 22.

The data compiled from this call up will assist in the compilation of an audit of all such products available on the market [16].

1.2 HISTORY OF ST JOHN'S WORT

1.2.1 NAME

The name St John's Wort refers to the plant *Hypericum perforatum L.* [17]. The USP NF monograph defines St John's Wort as consisting of the dried flowering tops or aerial parts of *Hypericum perforatum* Linné (Fam Hypericaceæ), gathered shortly before or during the flowering season, and containing not less than 0.04% of total hypericins, calculated as hypericin [18].

There are many theoretical explanations regarding the plant's name:

- The name is derived from a belief that the red spots on the leaves were the blood of St John the Baptist - the spots were thought to appear on the anniversary of the saint's beheading (said to be August 29)
- The plant blooms brightest on St John the Baptist's birthday (June24).
- Sleeping with a piece of the plant under one's pillow causes St John the Baptist to appear in a dream, according to legend, and St John would then give his blessing and prevent the dreamer or a loved one from dying in the upcoming year.
- The name results from the English tradition of throwing the flowers into a bonfire on the eve of St John's Day.
- According to legend, from the 6th Century, St Columbo always carried a piece of the plant as a token of his respect for St John [3].

It is believed that the official name (*Hypericum perforatum L.*) was derived from the Greek word *yper* for "upper" and *eikon* for "image" which translates as "over and icon", or "over an apparition". It is said that the plant was placed over religious icons as protection against evil spirits, witches, or other supernatural beings. Supposedly they would take 1 "whiff" of the unpleasant-smelling plant and would immediately depart. The name perforatum, Latin for "perforated" refers to the perforations that appear on the leaves when held up to the light.

Figure 1.1 is an illustration of the St John's Wort flowers.

Figure 1.1. Photograph of typical St John's Wort flowers. [19]

1.2.2 USES

Hypericum extracts in the past have been regarded as a universal remedy. In ancient Greece, the herb was used to treat many ailments, including sciatica and poisonous reptile bites. Since Greek and Roman times it has been more popular as a nerve tonic for mood and temperament [20].

In the 16th Century, Paracelsus recommended its use for melancholia, nervous disturbances, abdominal and uro-genital pains, ulcerated burns and lesions [21].

Homoeopathically, SJW has been used for pains, asthma, bites, brachial neuralgia, concussion, bruises, bunions, compound fractures, corns, diarrhoea, coxalgia, gunshot wounds, impotence,

hydrophobia, hypersensitivity, meningitis, neuralgia, rheumatism, whooping cough, tetanus, to name some of the indications.

In Russia and Europe, SJW has a history of use very much along the lines of those above. Specifically, in Europe it was used traditionally in restorative treatment for melancholic conditions, depression and the convalescence following concussion and other trauma, as well as anxiety [20,21].

During the 1970's and 80's, SJW research was concentrated on its weedy properties, and experimentation was focused on development of herbicides and biological controls. This was mainly due to the fact that SJW in many parts of Europe and North America was considered a weed and a pest as it causes photosensitization of livestock if consumed [22,23]. During the late 1980's and 90's research began to focus on the medicinal properties of SJW [24]. Today, SJW preparations are a popular herbal remedy widely extolled as an alternative to synthetic antidepressants**,** and are currently proclaimed by many to be nature's "Prozac". Its use is primarily in the treatment of mild to moderate depression, and in Germany is licensed for the treatment of depression, anxiety and insomnia.

Other avenues of current research include:

- The anti-viral effects of hypericin and pseudohypericin
- Wound-healing effects
- Antineoplastic activity

1.3 CULTIVATION

1.3.1 DESCRIPTION

SJW is a shrubby, aggressive, perennial weed that reproduces by seeds, and by runners from the base of the stem. It generally grows to a height of between half a metre and 1m, and a width of about 30cm. The flowers have 5 petals, are star-shaped and yellow, although the lower petals are yellow to yellow-brown. Situated around the margin of the back of the petals, are glands that appear as red-black dots, about 0.5-1mm in diameter. The leaves are small, elliptical to oblong, oppositely set without stalks and most commonly a green to pale green colour. They are punctuated by numerous glands, also approximately 0.5-1mm in diameter, which appear translucent when held up to the light. The stems are pale green, cylindrical, and hollow, with the distinct characteristic of faint, longitudinal, opposing ribs, which distinguishes *H. perforatum* from other species [3,24].

The dried herb should consist mainly of the flowering tops, including the leaves, unopened buds and flowers, and the powder should be a green to yellow-brown colour, with the yellow colour becoming more pronounced as the percentage of flowers and unopened buds increase [25].

The flowering tops contain the highest concentrations of naphthodianthrones and flavonoids, followed by the uppermost leaves and flowers during the blooming period. According to many herbalists, it is the translucent "perforations" on the leaves and the black-red spots on the petals that contain most of the active ingredients [25,26].

1.3.2 DISTRIBUTION

SJW is native to Europe, and is thought to have been introduced into eastern North America by European settlers in 1793. The plant then spread to the western side of the continent much later, probably around the 1940's. In Canada, the plant is widely distributed, with the greatest concentrations in the southern portions of British Colombia, Ontario, and Quebec. Showing great distribution world-wide, SJW can also be found in Asia, North and South Africa and Australia [27].

SJW is an aggressive invader of grazed lands especially where over-grazing has occurred. Eradication is difficult because of the plant's extensive root system and long-lived seeds. The plant tends to grow rapidly, but is fairly short-lived, usually lasting only 5 or 6 years. The most ideal soil

is fairly dry and well-drained soil with a pH of about 6 or 7, although the plant can tolerate pH ranges of 4,3-7,6. Dappled shade or full sunlight tends to be preferred [24].

1.3.3 HARVESTING

Collection of SJW takes place when blooming occurs. This varies with location, elevation, and the local climate, but in the Pacific north-west and eastern states of America this occurs from June through to September. The flowering tips can be harvested once a minimum of 4 flowers are opened, at a stem length of 20cm and should be gathered immediately before to immediately after opening. Harvest can be repeated 3-4 times at 3-5 day intervals, with the best harvesting time before midday. The parts that should be collected include the flowers, buds, middle to top leaves, and secondary stems. Harvesting of the lower leaves results in a significant lowering of the concentrations of the key constituents of SJW, as the broadest range and highest concentration of the flavonoids and naphthodianthrones occur in the top portion of the plant, - usually between 30-60cm from the base of the plant [28-30].

Plants can reach heights of 30-40cm during the first year of growth, but second year plants provide the largest yield. SJW is usually cultivated for 2-3 years at a time.

The harvested plant parts should be protected from light and dried rapidly after harvest to preserve the content of the secretory glands, as it has been reported that drying *H. perforatum* in sunlight can result in as much as 80% hypericin (HY) loss due to oxidation [25, 28]. Fresh plant material needs to be processed as soon as possible after harvesting. Drying the plant material at 70°C for 10 hours has been found to preserve all the constituents, but plants need to be dried in the shade to prevent oxidation. Plants can be air-dried in bundles or on drying flats during warm weather or with the help of moderate artificial heat at 30-40ºC [31]. The highest concentration of HY and pseudohypericin (PsHY) has been observed after microwave drying [32].

Variability of plant material and ultimately, concentrations of primary constituents will occur according to numerous parameters. These include different growth, harvest, drying and storage conditions, varietal and species differences, handling practices, moisture, altitude and the different plant parts harvested.

1.3.4 STORAGE

The naphthodianthrones and phloroglucinols are highly photosensitive and protection of *Hypericum* from light is an important factor to consider in order that these compounds are preserved. HY content has been shown to drop significantly in powdered extract, tablet and juice preparations when stored at temperatures of 60° C for more than 6 weeks. Dry extracts stored at 20° C were found to remain stable for at least one year [25].

1.4 ACTIVE CONSTITUENTS AND THEIR CHEMICAL PROPERTIES

1.4.1 ACTIVE CONSTITUENTS

Over 50 active constituents have been recorded [33]. The major ones are listed below:

$ The naphthodianthrones (make up 0.03-3.0% of total plant): These include hypericin, pseudohypericin, protohypericin, protopseudohypericin and cyclopseudohypericin. Also included in this category are the chemical relatives of hypericin such as isohypericin, hypericodehydrodianthrone, emodinanthrone and more distant relatives including frangula-emodin, and anthranol.

$ The flavonoids (constituting 11.71% of the leaves and 7.4% of the stalks):

Flavonols - including quercetin (2%), kaempferol, luteolin, myricetin.

Flavonol glycosides - including hyperoside (0.7-1.1%), also known as hyperin, quercitrin (0.3-0.524%) and isoquercitrin (0.3%), luteolin, rutin (0.3%).

Biflavones - including amentoflavone, also known as I3',II8-biapigenin (0.01-0.05% in flowers), and I3,II8-biapigenin (0.1-0.5%).

$ The phloroglucinols and their derivatives (up to 3%): including hyperforin, adhyperforin, and leucocyanidin.

$ Proanthocyanidins and procyanidins (12% of aerial portions of plant): These consist mostly of dimers, trimers, tetramers and high polymers of catechin and epicatechin.

$ Tannins (6.5-15%)

$ Coumarins: Umbellifone and scopoletin

$ Essential oils (0.06-0.35% of plant): These mainly consist of the monoterpenes (0.05-3.0% of the plant)- including the pinenes, myrcene, limonene, and also the sesquiterpenes - caryophyllene and humulene. The saturated hydrocarbon 2-methyl octane would also fall into this category.

$ Amino acids: Cysteine, GABA, glutamine, leucine, lysine, ornithine, proline, and threonine.

- â-sisterol.
- The xanthones (0.128%): However these are mainly found in the roots, and the roots should not be utilised in St John's Wort preparations.
- Phenolic carboxylic acids: chlorogenic, caffeic (0.1%), p-coumaric, ferulic, isoferulic, gentisic, and cinnamic acids.
- Other organic acids: isovalerianic, lauric, myristic, nicotinic (0.12% in leaves), palmitic and stearic acids.
- Carotenoids and other ingredients: including vitamin C, choline, pectin, phlobaphene and rhodan.
- Cytokines [25]

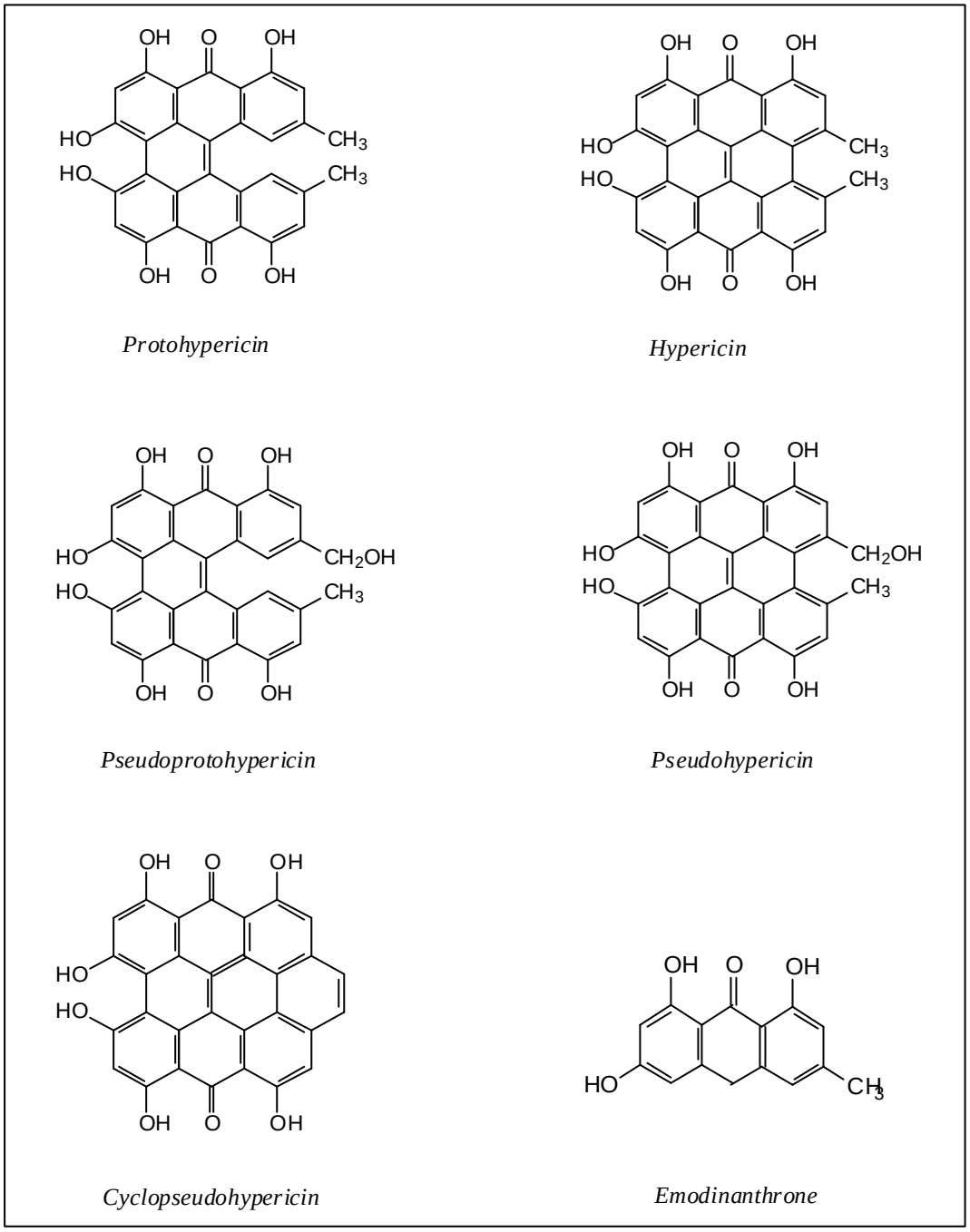
The most biologically active compounds are currently believed to be the naphthodianthrones, flavonoids, and phloroglucinols. At present, standardisation of *Hypericum perforatum* extracts is by quantitation of hypericin. Although this might offer no guarantee of pharmacological equivalence between SJW products, it is thought that there is good correlation between the amount of this substance and those of the flavonoids and phloroglucinols, which are also considered to be active constituents of SJW [34].

1.4.2 CHEMICAL PROPERTIES OF ACTIVE CONSTITUENTS

1.4.2.1 The Naphthodianthrones

The hypericins are thought to be derived from anthranoid metabolism. Emodinanthrone, which can be seen in Figure 1.2, is thought to be the precursor [35]. The protonaphthodianthrones, protohypericin and protopseudohypericin represent partially cyclic precursors of the naphthodianthrones, which upon irradiation with visible light, convert efficiently into their naphthodianthrone analogues [36]. These naphthodianthrone structures are also illustrated in Figure 1.2. The ratio of protonaphthodianthrones to naphthodianthrones in the fresh plant and in the extract may vary considerably between 1:5 and 5:1, depending on the extent of the photoconversion that took place during the preparation of the extract [21, 37].

Figure 1.2. Chemical structures of the naphthodianthrones and their supposed anthrone precursor [35].



Pseudohypericin (PsHY) is usually present in 2 - 4 fold higher concentrations than hypericin (HY). In some extracts, this ratio may be 10:1. The concentration of HY and PsHY in *H. perforatum* ranges from 0.03 to 0.3%, depending on the developmental stage of the plant. Greater amounts of these two are localised in the flowers.

A significant variation in concentration of HY and PsHY has been observed in individual plants, even of the same clone. It appears that narrow-leafed plants have higher concentrations of the naphthodianthrones than those with broad leaves do [35].

Cyclopseudohypericin, also structurally illustrated in Figure 1.1 is thought to be an oxidation product of pseudohypericin.

HY and its analogues are slightly acidic compounds. They are freely soluble in MeOH as well as pyridine and other organic bases, yielding cherry red solutions with red fluorescence. PsHY and HY both absorb visible light with a maximum absorption at 588nm. They are highly fluorescent in methanol when exposed to ultraviolet (UV) light. HY and PsHY are soluble in alkaline solutions, - below pH 11.5, the solutions are red, and above pH 11.5, the solutions are green with red fluorescence. The naphthodianthrones, especially HY, are almost insoluble in water at ambient temperature. However, it has been found that more than 40% of HY and PsHY present in the herb is extractable when using water heated between 60 and 80ºC [21,35].

HYs first pKa value is 1.7, and second pKa is 12.0. It forms monobasic salts with inorganic bases between a pH of 4 and 11, which are soluble in polar solvents. The monobasic salts disperse in water, producing light transparent, non-fluorescent high molecular weight aggregates. HY is used in most biological assays and clinical trials as its monosodium salt (HYNa). In the plant, however, PsHY and HY occur mainly as potassium salts [21].

Electrochemical studies show that HY and its salts have electron-accepting as well as electron-donating properties, and are, thus, both oxidising and reducing agents. HY and its salts are photodynamically active. Upon irradiation with visible light, HY has been found to oxidise tryptophan and it is thought that this occurs via the participation of singlet oxygen in the oxidation reaction (Type II mechanism). The photosensitising effect of HY was also reported to oxidise fatty acids. The *in vivo* photosensitising effect of HY causes skin erythema and oedema - a phenomenon known as hypericism. Hypericism is derived to a large extent from generation of singlet oxygen. The generation of singlet oxygen from the lowest excited triplet state of HY in alcohol solution, in micelles or in liposomes, has been confirmed by laser spectroscopy (Type II mechanism). It has been shown that HY or HYNa in organic solvents can also photo-reduce oxygen to superoxide radicals (Type I mechanism).

HY is an extremely potent photosensitizer as numerous investigations have documented, and it is therefore necessary to conduct experimental work with HY and PsHY in conditions that prevent photoactivation of the compound [21]. Another fact to bear in mind is that PsHY is unstable at high pH [38].

1.4.2.1.1 Stability

Wirtz [39] performed studies on the stability of HY and PsHY in solution. Solutions of HY and PsHY in methanol and methanol-pyridine (99:1 v/v) were stored in the dark at -20ºC, 4ºC, and at room temperature, as well as in light at room temperature. He discovered that the degradation of the naphthodianthrones roughly follows first order kinetics.

HY was stable over a time period of 140 days at all three temperatures where the solutions were stored in the dark. Light exposure led to the loss of between 21 to 29% of the initial HY amount within 140 days. The addition of pyridine did not appear to influence the stability of HY.

The degradation of pseudohypericin was only prevented by storage at -20ºC in the dark. Exposure of PsHY to light for 140 days at room temperature left only about 20% of the initial amount unchanged after 140 days.

Studies performed on extract solutions illustrated that storage conditions at reduced temperature and in the dark largely prevented degradation. (-20ºC and 4ºC) [39].

1.4.2.2 The Flavonoids

The flavonoids are believed to be capillary protectants. This group of compounds includes the flavonols, the flavonol glycosides and the biflavones [40].

1.4.2.2.1 The Flavonols

These compounds are the aglycone forms of the flavonol glycosides, and include quercetin (Q), kaempferol (K), luteolin and myricetin. Of these four, Q is by far the most common, being the aglycone of the flavonoid compounds of interest in SJW. It is thought that the small amount of Q found in the herbal product SJW is due to the hydrolysis of quercetin glycosides during drying and processing [35].

Quercetin:

This is available as the dihydrate, and becomes anhydrous at 95-97ºC. The powder is yellow in colour. One gram dissolves in 290 ml absolute alcohol, in 23 ml boiling alcohol and is practically insoluble in water. It is soluble in glacial acetic acid and in aqueous alkaline solutions and yields a yellow solution. The alcoholic solutions taste very bitter. Quercetin has UV absorbance maxima at 258 and 375nm [40].

Kaempferol:

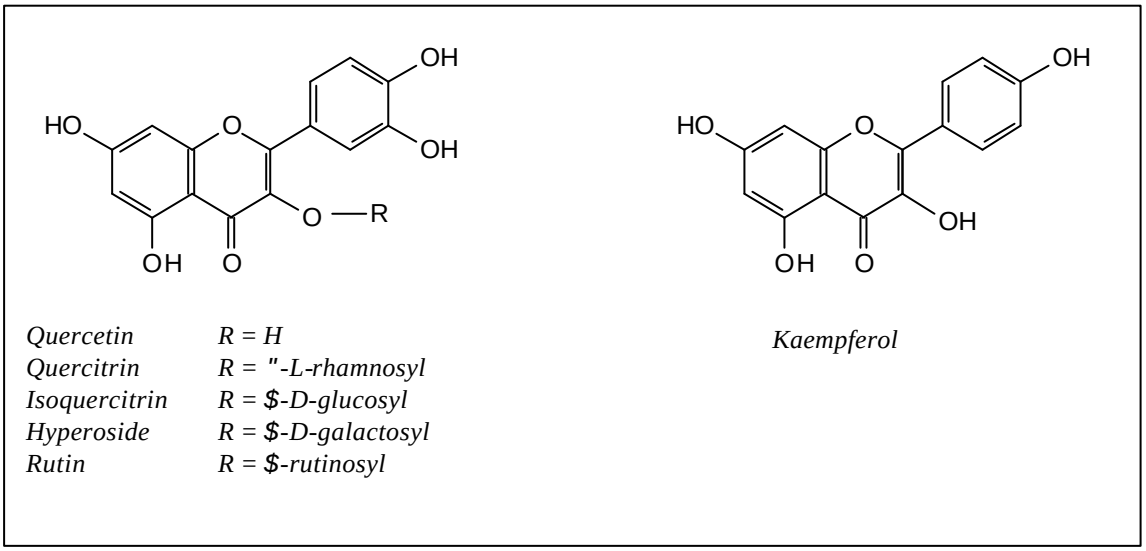
K has the appearance of a light yellow powder, or of yellow needles. It is slightly soluble in water, and soluble in hot alcohol, ether and alkalies. It has a melting point of between 276 and 278ºC and UV absorbance maxima at 265 and 365nm [40].

The structures of Q and K can be seen in Figure 1.3.

1.4.2.2.2 The Flavonol Glycosides

This group of compounds, with quercetin as the aglycone, constitute the major group of compounds in *H. perforatum*. The structures of these compounds are illustrated in Fig 1.3 below.

Figure 1.3. Diagrammatic representation of the flavonols and flavonol glycosides [35].



Quercetin *R = H*
Quercitrin *R = "-L-rhamnosyl*
Isoquercitrin *R = $-D-glucosyl*
Hyperoside *R = $-D-galactosyl*
Rutin *R = $-rutinosyl*

Kaempferol

Quercitrin:

Quercitrin (Qr) occurs as yellow crystals at room temperature, with a melting point between 176 and 179° C and UV absorption maxima of 350 and 258nm. It is practically insoluble in ether and cold water, moderately soluble in hot water and soluble in alcohol. Qr is soluble in aqueous alkaline solutions yielding an intense yellow colour. On exposure to air these solutions are oxidised to yield a brown colour [40].

Isoquercitrin:

Isoquercitrin (Iqr) has the appearance of yellow needles with a melting point between 225 and 227ºC. It has UV absorbance maxima at 257 and 369nm. It is practically insoluble in cold water, sparingly soluble in boiling water; soluble in alcohol and soluble in alkaline solutions yielding a deep yellow coloured solution [40].

Hyperoside:

Hyperoside (Hd) also has the appearance of yellow needles. It is practically insoluble in cold water, sparingly soluble in boiling water, soluble in alcohol and in alkaline solutions yielding a deep yellow colour as isoquercitrin [40].

Rutin:

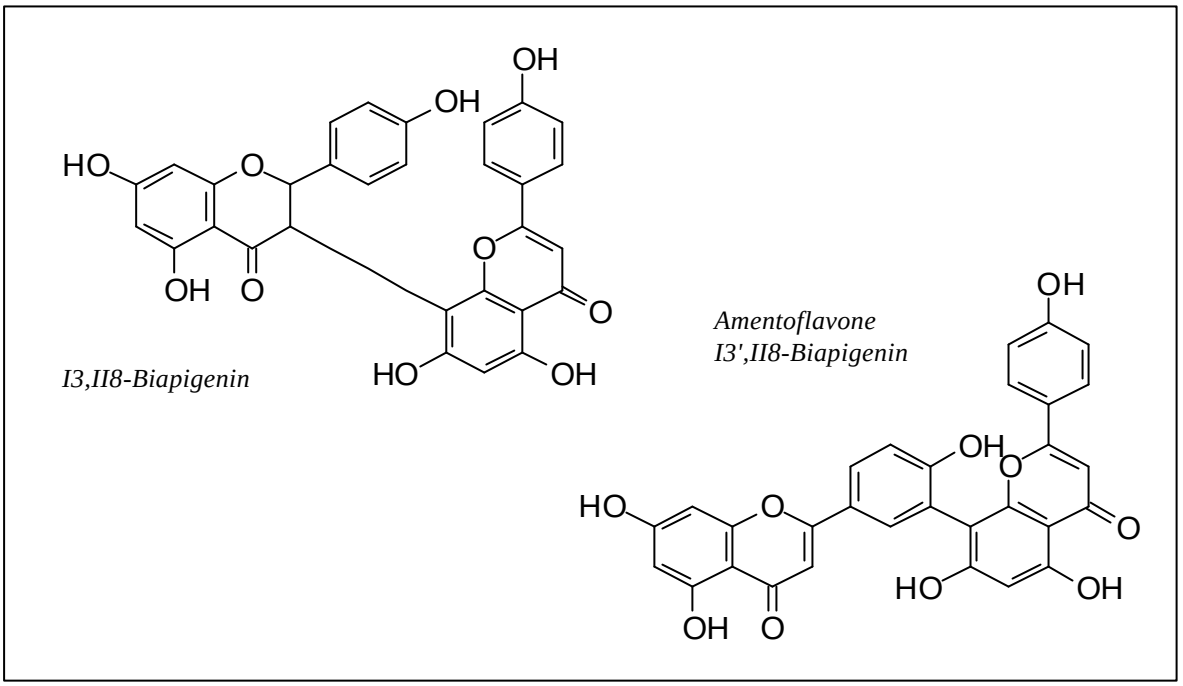
Rutin (R) is available as pale yellow needles at room temperature, gradually darkening on exposure to light. The crystals contain water and become anhydrous after 12 hours at 110º C and 10 mm Hg. One gram of R dissolves in about 8 litres of water, 200ml boiling water and 7ml boiling methanol. R is soluble in pyridine, formamide and alkaline solutions and is slightly soluble in alcohol, acetone and ethyl acetate. R is practically insoluble in chloroform, ether, benzene and petroleum solvents [40].

1.4.2.2.3 The Biflavones

Two compounds in this comparatively uncommon group of dimeric flavones have been detected in SJW. These biflavones include amentoflavone (I3',II8-biapigenin) and I3,II8-biapigenin, and are found exclusively in the buds and blossoms of the plant [35].

The structures of the biflavone compounds are illustrated in Figure 1.4.

Figure 1.4. Diagrammatic representation of the biflavones [35].

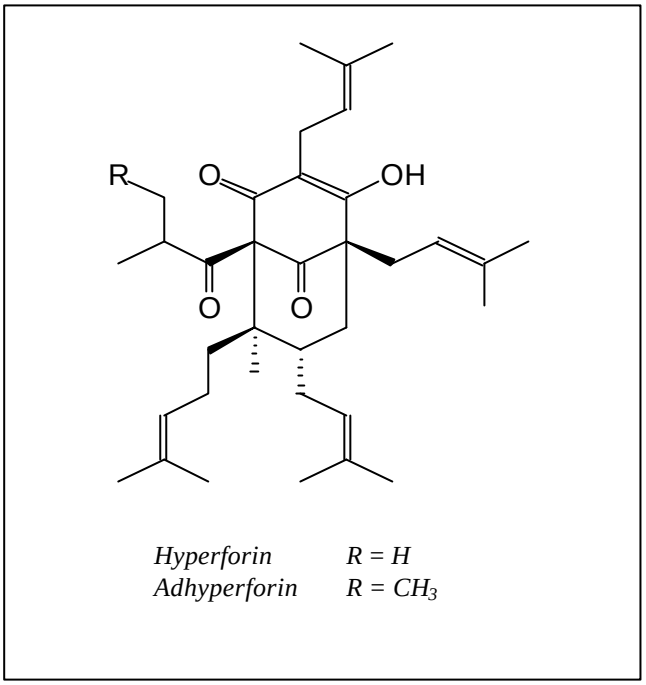


1.4.2.3 The Phloroglucinols

The phloroglucinol derivatives (Figure 1.5) are believed to be some of the main constituents of the dried herb *Hypericum perforatum L.* Two closely related compounds have been found, hyperforin (Hypf), which is the major phloroglucin, and adhyperforin. Both compounds occur exclusively in the reproductive parts of the plant. It has been reported that the amount of both compounds increases from about 2% Hypf and 0.2% adhyperforin in the unripe fruits to 5% Hypf and 2% adhyperforin in the ripe fruits [35].

Hypf and adhyperforin are acylphloroglucinol-type compounds, which consist of a phloroglucinol skeleton substituted with lipophilic isopren chains (Figure 1.5).

Figure 1.5. Diagrammatic representation of the phloroglucinols [35].



Hyperforin $R = H$
Adhyperforin $R = CH_3$

The phloroglucinols are lipophilic, unstable towards heat and light either on storage or in solution, and very susceptible to oxidation. It was believed that Hypf was chemically too unstable to play a role as a pharmacologically relevant constituent of SJW preparations. However, Erdelmeier [41] reports that if appropriately selected and dried SJW herb is used, both lipophilic and hydroacoholic extracts may contain the relevant percentages of Hypf.

The stability of isolated Hypf in solution and solid form was investigated in several antioxidant systems. It was reported that Hypf showed greater stability in polar solvents than in non-polar solvents, and was much more stable in pH 2.0 methanolic solution than in pH 12 solution. It has also been reported that degradation under light exposure can be diminished in methanol by acidification (pH 2.0), and degradation under light exposure at pH 12.0 results in total decomposition within 30 days. However, when kept in the dark, hyperforin is stable in acid and alkaline methanolic solutions [39,42].

1.5 PHARMACOLOGIC ACTIVITY

SJW is widely renown for its antidepressant properties. Its efficacy when used to treat "mild to moderate depressive states" has been confirmed by the European Scientific Co-operative on Phytotherapy (ESCOP). It is also used and being investigated for a wide range of other pharmacological activities [43].

1.5.1 WOUND-HEALING, ANTIBACTERIAL AND ANTI- INFLAMMATORY EFFECTS

The flowers and buds soaked in oil and exposed to sunlight for several weeks produce red oil that exhibits the above effects upon topical application. It appears that Hypf and the tannins are responsible for the wound healing and Hypf on its own exhibits antibacterial effects. The anti-inflammatory properties are thought to be due to the flavonoids [3,44,45].

1.5.2 VASCULAR ACTIVITY

The procyanidins exhibit vascular activity in the form of advanced vasodilation, and this has been known to increase coronary blood flow in porcine and guinea pig arteries [46].

1.5.3 ANTIVIRAL ACTIVITY

HY and PsHY have appeared to be effective antiviral agents in some investigations - active against a broad range of viruses and retroviruses. HY has demonstrated antiviral activity *in vitro* against herpes simplex virus types I and II, para influenza virus type III, vesicular stomatitis virus, Epstein-Barr virus and human cytomagalovirus. Important to note though is that many agents are effective *in vitro*, but show little or no activity *in vivo.* Studies suggest that lipid-enveloped viruses and retroviruses such as the influenza virus, Herpes simplex virus and HIV (Human Immunodeficiency Virus) are inactivated, whereas unenveloped viruses such as adenoviruses and polioviruses are resistant to HY and PsHY [3].

There are 2 proposed mechanisms for the antiviral activity:

i) Direct viral inactivation of mature and properly assembled viruses, probably due to the interference with release of active enzymes

ii) Interference with assembly or processing of intact virions from affected cells.

Investigations have also shown that virucidal activity in some instances is enhanced in a dose-dependant manner by visible light. It is believed that photo-activation of HY plays a part in the antiviral activity.

In other research, HY and PsHY have demonstrated inhibition of animal retroviruses *in vivo* in mice, showing that when these compounds interact with the infecting particles shortly after *in vivo* administration, the disease is completely prevented. This retrovirus animal model is used to evaluate compounds as possible therapeutic agents against HIV. HY was evaluated with the carbonyl groups removed, which resulted in a significant decrease in anti-retroviral activity. This implies that the same parts of the molecule involved in the photodynamic mechanism may play a role in antiviral activity.

In a Phase I clinical trial designed to evaluate the safety and anti-retroviral activity of HY in 30 HIV-infected patients, HY caused significant photo-toxicity and had no anti-retroviral activity in the limited number of patients studied. There is therefore need for more investigations before any conclusions can be drawn [3,47].

1.5.4 ANTINEOPLASTIC ACTIVITY

HY and PsHY are thought to inactivate protein kinase C *in vitro*. The growth of glioma cell lines has appeared to be inhibited by HY, and HY has been found to be a potent inducer of cell death due to inhibition of protein kinase C. HY also appears to inhibit the growth of lung cancer and skin cancer *in vitro*. Investigators have used HY in combination with laser therapy for photodynamic treatment of cancer [3].

1.5.5 ANTIDEPRESSANT ACTIVITY

1.5.5.1 Antidepressant Mechanisms of Action

Amphetamine, developed in the1930's, was the first drug to be found effective in elevating mood in endogenous depression. Amphetamines cause the release of norepinephrine (NE) from the nerve ending and inhibit its re-uptake, thereby increasing the concentration of NE in the synaptic gap. From this the 'amine hypothesis' of endogenous depression was formulated, which suggests that depression is due to a deficiency of the neurotransmitters NE and serotonin (5-HT). These neurotransmitters are actively secreted into the synapses by neurones, and then taken up by receptors at the post-synaptic neurone [33].

There are 3 basic groups of synthetic anti-depressants at present:

i) The Tricyclic Antidepressants (TCAs):

These cause an increase in the availability of NE and 5-HT at the post-synaptic receptor. TCAs can interact with many other drugs, most importantly, the monoamine oxidase inhibitors (MAOIs). A reaction resembling atropine toxicity may be precipitated if any 2 drugs within these 2 classes are taken less then 10-14 days apart.

ii) The Monoamine Oxidase Inhibitors:

MAO is an enzyme that plays an important role in the regulation of the metabolic degradation of NE and epinephrine. MAO also oxidatively deaminates 5-HT. MAOIs form stable complexes with MAO, thereby irreversibly inactivating the enzyme, leading to an increase in intracellular amine concentrations. The inhibition of MAO causes an increase in available tyramine, which can have major implications when it comes to the clinical toxicity of this class of drugs, especially when foods containing tyramine are taken in conjunction with the MAOIs. MAOIs have a high side-effect profile and potential for drug interactions, and so have more recently been replaced by a newer generation of antidepressant medications, the Selective Serotonin Reuptake Inhibitors (SSRIs) [48].

iii) The Selective Serotonin Re-uptake Inhibitors:

SSRIs selectively block the re-uptake of 5-HT into pre-synaptic nerve terminals, resulting in an increase in concentration of 5-HT in the synaptic gap, and more 5-HT available for action on the post-synaptic receptors. SSRIs have the same benefit in therapy as the TCAs, but because of their selectivity, side effects of the antidepressant therapy tend to be reduced dramatically [49,50].

1.5.5.2 St John's Wort as an Antidepressant

Many pharmacological studies have been conducted to ascertain the mechanism of action of *Hypericum perforatum* extract and many believe that it exhibits action similar to one or more of the synthetic antidepressants. However, the evidence supporting the underlying pharmacological mechanisms of action for the effects of *Hypericum* extract is inconclusive at present. There are 3 basic probabilities:

i) The mechanism is similar to that of the synthetic antidepressants (TCAs / MAOIs / SSRIs)

ii) The antidepressant action is a result of an additive effect of some combination of various pharmacological mechanisms that individually are not substantial enough to yield clinical antidepressant effects.
iii) The mechanism is as yet unknown.

The various possible mechanisms each present possible concerns and implications:
i) Action mimicking MAOIs or SSRIs may lead to drug interactions with other agents with similar mechanisms and even cause certain side effects with various foods (e.g. MAOIs and tyramine-containing foodstuffs).
ii) Plasma concentrations of other medications may be affected by actions on metabolic enzymes.
iii) If the actions are distinct from that of the synthetic antidepressants, then this could provide novel targets for drug discovery efforts.

The most active constituent of *H. perforatum* exhibiting antidepressant properties, was commonly thought to have been HY, - the compound that has been used to standardise commercially available preparations. However, recent research indicates that Hypf rather than HY may have more of a part to play in the antidepressant activity of the extract [20,51,52].

Initial studies carried out demonstrated that HY inhibited monoamine oxidase (MAO). However, it was then discovered that it was not the HY, but other constituents (flavonoids and xanthones) that were present in the extract that accounted for the inhibition of MAO [20,53,54]. There have been many investigations conducted since this discovery to ascertain the effects of *Hypericum* whole extract or fractions of the extract on MAO and COMT (catechol O-methyl transferase). These have showed that the weak inhibition of MAO was inadequate to explain the antidepressant effect of *Hypericum* and that at estimated clinically relevant doses there was no significant inhibition of COMT either by *Hypericum* whole extract, or HY alone. It was concluded that the antidepressant effect of *Hypericum* relies neither on MAO inhibition alone, nor on COMT inhibition alone to any appreciable extent [55].

Further *in vitro* investigations have suggested that SJW acts as a SSRI and that *H. perforatum* extract in high concentrations modulates the expression of 5-HT receptors by reducing their availability, resulting in impaired 5-HT uptake. However, it is thought that the high concentrations necessary to inhibit serotonin uptake would be too high to be clinically achieved. A possibility to consider is that brain concentrations of the active ingredients of St John's Wort might rise with the

chronic dosing that characterises its use and this may accentuate any SSRI effects. This, of course, would be assuming that the "actives" cross the blood-brain barrier. It appears that most of the investigations conducted in this arena, have been *in vitro*, and it has been said that it is not known whether the active constituents from *H. perforatum* extract do cross the blood-brain barrier. Other investigators have found, though, that after treatment with HY, there is an increase in urinary metabolites of 5-HT and NE, which would imply an ability to cross the blood-brain barrier, and an action mimicking that of the TCA's [55,56].

In another study [55], an HY-free extract was used in a study and the concentration of 5-HT in the CNS increased on dosage, which suggested that the extract may have some 5-HT inhibitory properties, but these not on account of the presence of HY. This was confirmed in a later study where the affinity of HY for various receptor and uptake sites was tested. The results illustrated that HY did not display significant (not greater than 25%) inhibition of 5-HT, NE, or dopamine (DA) uptake sites nor did it have high affinity for most receptors.

From the above it can be seen that many of the results obtained thus far are contradictory with respect to the active ingredients and their mechanism of action. Additional studies are necessary to determine the exact pharmacological mechanism(s) of the antidepressant effect.

Another possible contributing mechanism to the antidepressant effects of *H. perforatum* may involve GABA (Gamma amino butyric acid). In a recent study of a receptor screen of a crude extract of SJW, it was suggested that SJW crude extract has a high affinity for $GABA_A$ and $GABA_B$ receptors. The source of this affinity, if present, was not thought to be from HY, but rather from the GABA present in the *H. perforatum* (0.7mg/g) - assuming it survives the extraction process - or some other unidentified constituent [55]. However in a study conducted by Müller and Schäfer, such high affinity was not observed [57].

There are additional possible antidepressant actions of *H. perforatum* that have been noted. One study found that SJW has an effect on melatonin secretion and that this action is shared by synthetic antidepressants (e.g. amitryptiline and desipramine). Melatonin reportedly has important effects on regulation of sleep, biological rhythms and sexual activity. *H. perforatum* has also been reported to increase dopamine (DA) turnover, possibly by inhibiting the enzyme dopamine â-hydroxylase causing an increase in the concentration of DA [55,58].

The biflavonoids have been reported to have a partial agonistic effect on benzodiazepine receptors *in vitro* - explaining the use of *H. perforatum* as an anxiolytic. The authors of this study discounted this clinical effect, though, due to a lack of *in vivo* binding, which indicates rapid metabolism, or lack of penetration of the blood brain barrier. Amentoflavone, however, has been shown to bind to brain benzodiazepine receptors exhibiting activity equivalent to diazepam, but weaker [35,59].

There are hypotheses of non-central nervous system origins and causes of depression. Some researchers have postulated that the primary site of action of *H. perforatum* may actually lie outside the central nervous system (CNS) [60]. These researchers suggest that the site of action of the plant could be the lymphocytes or the monocytes of the immune system, and that this would cause modulation of cytokine expression e.g. interleukin-6 (Il-6). Interleukin-6-induced stimulation of corticotrophin releasing hormone (CRH), adrenocorticotrophic hormone or cortisol secretion may lead to depression. Therefore, inhibition of Il-6 by *H. perforatum* extract could exert an antidepressant effect by regulation of CRH concentrations [55].

The number of known compounds with pharmacologic activity in St John's Wort is steadily increasing. In spite of this, a single compound demonstrating strong activity in depression and an acceptable dosage is still lacking. At present, much of the data is inconclusive and conflicting as to the precise mechanism of action of *Hypericum perforatum* as an antidepressant. *H. perforatum* extracts have only weak activity in assays related to the mechanisms of synthetic antidepressants, that is inhibition of MAO, COMT or 5-HT uptake. As well as this, involvement of the immune system in the antidepressant effects of St John's Wort is, as yet, merely a hypothesis. It is postulated and seems most likely that the clinical efficacy of SJW could be attributable to the combined contribution of several mechanisms, each one too weak to account for the overall effect on its own.

1.6 ADVERSE EFFECTS, INTERACTIONS AND CONTRAINDICATIONS

1.6.1 ADVERSE EFFECTS

In phase 1 studies, HY was seen to induce serious cutaneous phototoxicity [61], and in the 1970's, the Federal Drug Administration classified SJW as "unsafe" because of reports of grazing animals developing phototoxicity after eating large amounts of *H. perforatum*. The phototoxicity results

from ingestion of *H. perforatum* and subsequent sun exposure. External contact with *H. perforatum* has not been seen to cause phototoxicity.

The incidence of adverse reactions reported in clinical trials varies. Most common reported adverse effects are emotional vulnerability, fatigue, pruritus, weight gain, gastrointestinal discomfort, allergic reactions, restlessness, dry mouth and dizziness [62].

1.6.2 DRUG INTERACTIONS

An obvious concern for drug interactions would be that of the additive effect of SJW with any one of the conventional antidepressant drugs. In patients concurrently taking SSRIs and *H. perforatum* extract, serotonin syndrome may occur. Effects observed here may include hypertension, mental status changes, hyperthermia, muscle rigidity, tremor and restlessness [63,64]. The concurrent use of MAOIs and *H. perforatum* may result in additive effects of the antidepressant actions, and even toxicity, and with MAO being inhibited. Prescription MAOIs interact with anaesthetics, antidiabetic agents, $-blockers, dextromethorphan, guanethidine, levodopa, meperidine, sympathomimetics, and l-tryptophan, as well as tyramine-containing foods and beverages. It is not known whether SJW has these drug and food interactions [63].

St John's Wort has been seen to induce the cytochrome-P450 enzyme system, and possibly the P-glycoprotein. Drug-interaction studies have provided evidence to suggest that SJW induces CYP3A4, CYP1A2 and CYP2C9 enzymes [64]. As well as this, *in-vitro* studies have shown that SJW extracts inhibit CYP2D6, CYP2C9, CYP3A4, CYP1A2 and CYP2C19 enzyme activity. Specifically, hyperforin is a competitive inhibitor of CYP2C9 and CYP3A4 activity and a non-competitive inhibitor of CYP2D6 activity. Hyperforin has also been seen to markedly induce CYP3A4 expression as well as being a potent ligand for the pregnane-X receptor, which is a receptor that regulates the expression of CYP3A4 mono-oxygenase. These effects could result in decreased blood levels and therefore therapeutic effects of medicines metabolised by these enzymes in the liver. Some of the medicines that would be affected would include indinavir and other protease inhibitors, HIV non-nucleoside reverse transcriptase inhibitors, warfarin, cyclosporin, oral contraceptives, digoxin and theophylline. With the induction of metabolic enzymes, abrupt discontinuation of SJW may result in a rise of interacting medicines and possible toxicity. Alternatively, switching between SJW preparations may lead to varying levels of induction because of the different concentrations of active ingredients in the different preparations.

SJW preparations may also interact dangerously with the 'tryptan'' drugs, or drugs used to treat migraine, and anti-convulsant medication (phenytoin, carbamazepine, phenobarbitone) [64,65].

1.6.3 CONTRAINDICATIONS

SJW has been shown to possess uterotonic and possibly abortifacient properties, and should therefore be avoided by pregnant women [53,64].

1.7 PHARMACOKINETICS

As yet, there is very little data describing the pharmacokinetics of HY and PsHY, hyperforin, or any of the other constituents of SJW. In order to optimise dosage, dosage interval and consequently therapy, information concerning the rate and amount of absorption, half-life, clearance, volume of distribution and dose-dependence of plasma levels of the major compounds in SJW is mandatory. However, it has been noted that investigations into the pharmacokinetics of individual substances of natural medicines may have limited value in phytotherapy, as the resultant inferences concerning efficacy of the whole product may only be partially applicable [43,66,67].

1.7.1 DOSAGE

To date there are no dose-finding studies, so the question of extract dosage remains open, and the minimum dosage that guarantees a therapeutic effect is not known [43]. In general, 0.9-2.7mg of hypericin per day is the recommended dosage for adults with mild to moderate depression. This equates to approximately 300-900mg of *Hypericum perforatum* extract (standardised to 0,3% hypericin) per day. The most common dosages appear to be 300mg of the extract 2-3 times daily. The constituents of *H. perforatum* cannot be standardised in teas [53].

1.7.2 PHARMACOKINETICS

The dianthrone derivatives HY and PsHY were chosen as active markers in three of the pharmacokinetic studies quoted below. HY and PsHY appear to be good indicator compounds for *Hypericum perforatum* as they are easily measured quantitatively and are specific to this plant. Chatterjee *et al*. [93] also performed studies on SJW extracts to ascertain the oral bioavailability of Hypf from Hypericum extracts in rats and human volunteers. The results from this study are reported in Section 1.7.2.2.

1.7.2.1 Hypericin and Pseudohypericin

Despite the structural similarities between these 2 naphthodianthrones, they elicit substantial pharmacokinetic differences [67].

In this study, the single- and multiple-dose pharmacokinetics of HY and PsHY were studied. Pharmacokinetics reported for single dosing are as follows:

Table 1.1. Single dose pharmacokinetic parameters reported by Staffeldt et al. [67]

Pharmacokinetic Parameter	**HY**	**PsHY**
Elimination half-life (hrs)	24.8 - 26.5	16.3 - 36.0
k_{el} (hr^{-1})	$2.62x10^{-2}$ - $2.79x10^{-2}$	$1.93x10^{-2}$ - $4.25x10^{-2}$

HY showed a remarkably prolonged median lag-time, ranging from between 2 to 2.6 hours, as compared to PsHY (0.3 to 1.1 hours).

Both compounds reached steady state after 4 days with multiple dosing, and elimination half-lives were found to be 28.0 and 23.5 hours respectively for HY and PsHY. The area under the concentration-time curve (AUC) is a measure of the amount of substance appearing in the plasma and available to exert an effect. A non-linear increase in AUC with increase in dose was reported, and this was shown to be statistically significant for HY [67].

Kerb [68] performed further studies using the same dose of extract with the same dosage intervals, and the results obtained were comparable, differing only slightly.

Pharmacokinetics reported are as follows:

Table 1.2. Pharmacokinetics of hypericin and pseudohypericin reported by Kerb et al. [68]

Pharmacokinetic Parameter	**HY**	**PsHY**
Elimination half-life (hrs)	43.1	24.8
k_{el} (hr^{-1})	$1.61x10^{-2}$	$2.79x10^{-2}$
Clearance (Cl) (ml/min)	9.2	43.3
Volume of distribution (V) at SS (L)	19.7	39.3

Kerb illustrated that upon administration of 3 different single doses of *H. perforatum* extract, HY has approximately double the elimination half-life of PsHY. This was reflected in the presence of HY in the systemic circulation 72 hours after administration, and PsHY was not detectable at this time. HY was also seen to have a 2 hour lag time before appearing in the systemic circulation, whilst the lag time for PsHY was 0.4hours. These figures are comparable to those obtained by Staffeldt [67] in an earlier study on the pharmacokinetics of HY and PsHY. The terminal elimination half-life of HY increased significantly with higher doses, but this was not so with PsHY [68].

Brockmöller [69] also studied the pharmacokinetics of PsHY and HY, and the results from these studies for the most part confirm the kinetics already found. The naphthodianthrones were found to be low-clearance drugs with half-lives of about 40 hours for HY and 25 hours for PsHY. In contrast to Staffeldt's findings, however, was that kinetic parameters measured at three different dose levels followed linear pharmacokinetics for HY and PsHY. HY illustrated a prolonged lag time here too, which is mechanistically not understood yet.

1.7.2.2 Hyperforin

In a study of the bioavailability of Hypf from *H. perforatum* extracts, Hypf illustrated linear pharmacokinetics up to 600mg of extract. Increasing the dose of extract to 900 and 1200mg resulted in lower C_{max} (maximum concentration) and AUC values than those expected from linear extrapolation of data from lower doses [93].

Table 1.3. Pharmacokinetics of hyperforin as reported by Chatterjee et al. [93]

Pharmacokinetic Parameter	**Hyperforin**
Elimination half-life (hrs):	9
k_{el} (hr^{-1}):	$7.70x10^{-2}$
Clearance (Cl) (ml/min): Dose: 300mg extract (14.8mg hyperforin)	199.3
600mg extract (29.6mg hyperforin)	238.2
1200mg extract (59.2mg hyperforin)	340.3

It` has also been reported that in a repeated dose study, despite the high half-life and retention time in plasma, Hypf did not accumulate in circulation [69].

1.7.3 DISCUSSION

To date, few studies have investigated the pharmacokinetics of HY, PsHY and Hypf, or any of the other supposed active components of SJW. In contrast to this lack of kinetic knowledge, the consumption of SJW-based therapies is increasing rapidly, which is a concern. One concern that arises out of the findings reported, is that Kerb found that there was a non-linear increase in the AUC and maximum plasma concentration in proportion to the administered dosage. This implies that regulation of therapeutic blood levels may be difficult, as a small increase in dosage could cause excessive increases in blood levels. This may pose a problem where *H. perforatum* preparations are not standardised, and patients change preparations [68].

There does appear to be a substantial amount of agreement of findings between the few studies performed on the pharmacokinetics of HY and PsHY. This is promising and serves to give insight and assist in the dosing of patients with SJW. The study performed by Chatterjee *et al*. [93] is also interesting and helpful in terms of SJW administration. However, there is still a lot of investigation that needs to be done in establishing the pharmacokinetics of these and other supposed actives in SJW, and in determining the therapeutic windows of the active(s).

CHAPTER TWO

QUALITATIVE ANALYSIS OF THE FLAVONOIDS USING CAPILLARY ELECTROPHORESIS

2.1 INTRODUCTION

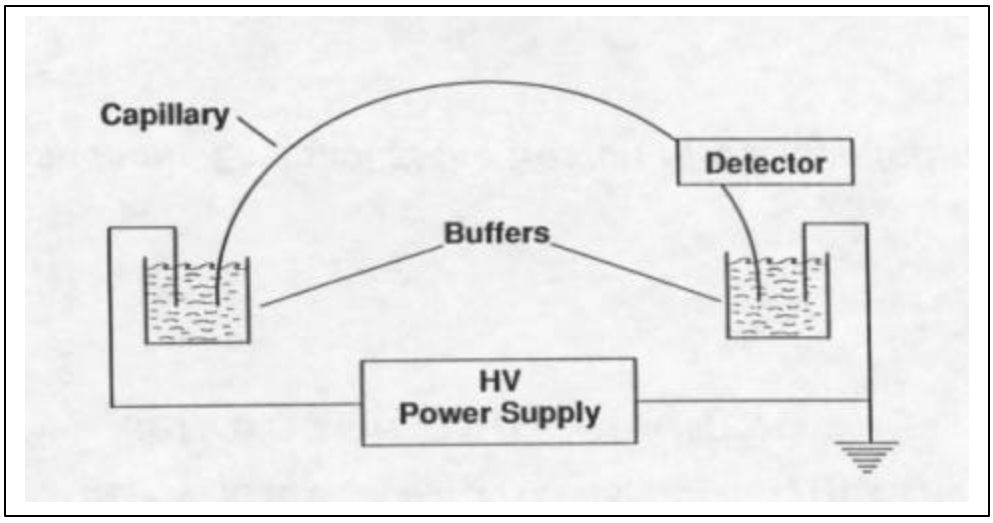
Capillary electrophoresis (CE) is an analytical technique in which efficient separation of components in a small sample volume is obtained by differential migration of charged species in an electric field. Once a sample solution is introduced to the separation system, the components of the sample are induced to move under an applied potential and separate into spatially discrete zones of separate substances according to the differences in their effective mobilities.

CE comprises numerous modes of operation that have dramatically different operative and separative characteristics. These modes include capillary zone electrophoresis, micellar electrokinetic chromatography, capillary gel electrophoresis, capillary electrochromatography, capillary isotachophoresis and capillary isoelectric focussing [70,71].

2.1.1 CAPILLARY ZONE ELECTROPHORESIS (CZE)

CZE is currently the most commonly used mode of CE due to its simplicity and versatility. The separation of analytes takes place in a narrow bore (inner diameter of 25 to 75 μm) fused silica capillary and is dependent upon the differential electrophoretic migration of the ionic species in the electrophoretic buffer, which is the medium for separation. As illustrated in Figure 2.1, the ends of the capillary are placed in separate buffer reservoirs and the capillary filled with buffer. Electrodes positioned in the buffer reservoirs make contact between the high voltage power supply and the capillary contents. Most often the sample is loaded onto the capillary at the anodic end of the capillary, an electric field applied across the capillary and detection of analytes then takes place at the opposite end of the capillary directly through the capillary wall.

Figure 2.1. Diagrammatic representation of a basic capillary electrophoresis system [72].



Separation is dependent on the differential electrophoretic migration of the ionic species in the electrophoretic buffer. The effective mobility of the ionic species is dictated by their charge-to-mass ratio at a specific pH. However, the total velocity of the ions in the capillary when an electric field is applied is dependent upon the sum of the electroosmotic flow (EOF), which is the bulk flow of liquid in the capillary, as well as their respective mobilities. Cations with the largest charge-to-mass ratio migrate first, followed by cations with smaller ratios, neutral substances, then anions with small and then the largest charge-to-mass ratios. Anions, although electrophoretically attracted toward the anode, are swept toward the cathode with the EOF, and separate out from the cations and neutral molecules. It is possible to alter the charge-to-mass ratio of many ions by altering the pH of the buffer medium, affecting ionization and therefore electrophoretic mobility.

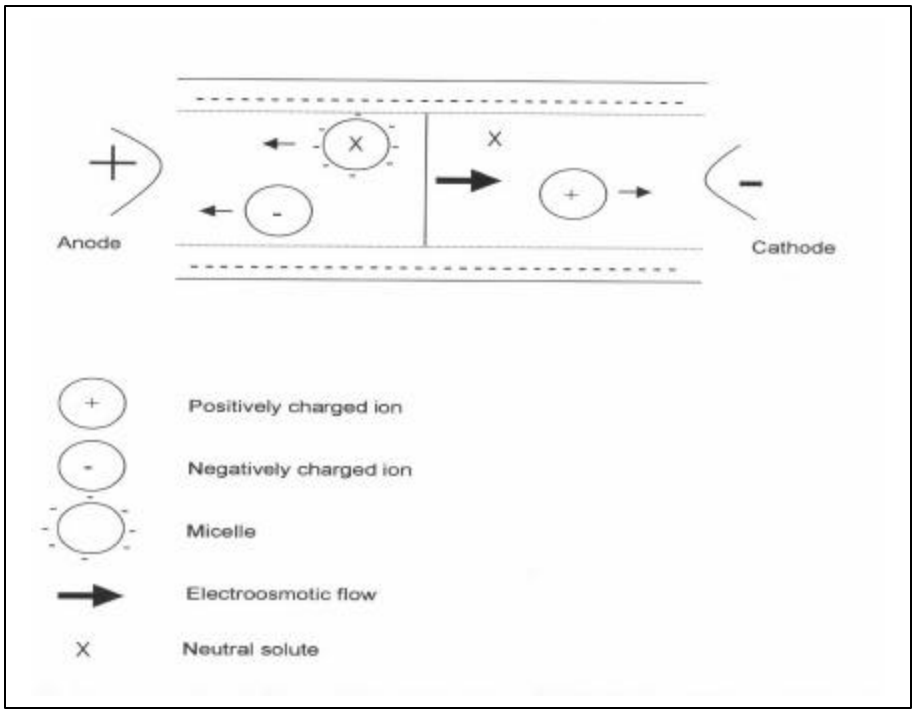
A significant feature and advantage of CZE is the flow profile of the liquid in the capillary, which is beneficial in terms of resolution. This is discussed further in Section 2.2.2 [70,71].

2.1.2 MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

MEKC is a useful technique because it provides a method that can be used to resolve both charged and neutral molecules in a single run. In MEKC, ionic surfactants are incorporated into the buffer at concentrations above the critical micelle concentration (CMC).

The most commonly used surfactants in MEKC are anionic surfactants, and sodium dodecyl sulphate is the most popular of this group. Charged micelles migrate either with or against the EOF, dependent upon their charge. In the case of anionic surfactants, the negatively charged head groups tend to orientate themselves on the outer surface edges of the micelle, with the hydrophobic tail groups orientating themselves toward the centre of the micelle. These anionic micelles are attracted to the anode, but due to the bulk flow movement of the EOF, they move toward the cathode, although at a slower rate than that of the EOF.

Figure 2.2. Diagrammatic representation of MEKC.



The MEKC system has 2 phases: the micellar phase and the aqueous phase. The micelles are dispersed throughout the aqueous phase. This is illustrated in Figure 2.2 where a single micelle, which represents the micellar phase, is shown. The separation mechanism in MEKC is based upon the differential partitioning of the solute between the micellar and aqueous phases. If the analytes are charged, they migrate according to their electrophoretic mobilities. If they are neutral, they will tend to migrate with the micelles. The extent of solubilisation of a neutral solute molecule by a micelle is dictated by the hydrophobicity of that solute molecule. The more hydophilic the neutral molecule is, the less time it will spend inside the micelle, and the quicker it will migrate as it will migrate more readily with the electroosmotic flow (EOF). On the other hand, the more hydrophobic the neutral

molecule is the more time it will tend to spend solubilised in the micelles, eluting with the micelles. The migration time window is defined by the difference between the migration time of the bulk solution and that of a micelle. In other words, it is the difference between the elution of a neutral hydrophilic molecule and a neutral hydrophobic molecule. All other solutes with intermediate hydrophobicity will migrate within this migration window. A combination of hydrophobicity charges interactions and charge-to-mass ratios all play a part in the separation of ionic and neutral solutes in MEKC.

2.1.3 CAPILLARY ISOTACHOPHORESIS (CITP)

CITP is performed in a discontinuous buffer system and is a moving boundary electrophoretic technique. A combination of two buffer systems is used to form separate sample zones, which move at the same velocity. A steady-state migrating configuration of these sample zones is produced as a result of sample constituents condensing between leading and terminating electrolytes. The electric field in each zone varies, and is self-adjusting to maintain constant velocity (where the velocity equals the product of the mobility and the field). This results in sharp boundaries between the zones, with the lowest field across the zone with the highest mobility. The leading electrolyte in a zone dictates the concentration of that zone, and this concentration remains constant. Ions that diffuse into neighbouring zones experience a velocity change and immediately return to their own zones.

The applications of CITP are limited since separation of anions and cations in a single run is not feasible. As well as this, selection of a suitable buffer system at the desired pH is often difficult.

2.1.4 CAPILLARY ISOELECTRIC FOCUSING (CIEF)

CIEF is a high-resolution technique that is used to separate ampholytes, and does so on the basis of their isoelectric points or pI values. This technique is most commonly used in the separation of peptides and proteins.

A reservoir containing basic solution is placed at the cathodic end of the capillary and reservoir containing acidic solution at the anodic end and the capillary is filled with a mixture of solute and ampholytes. An electric field is applied across the capillary, which results in a pH gradient forming

within the capillary. Upon application of the electric field, the charged ampholytes migrate through the medium until they reach a region where they become uncharged. Migration halts and the ampholytes focus at the position which corresponds to their isoelectric point. This is known as focussing. Once the focussing process is complete and a steady-state is reached, current will no longer be observed. The zones of separated ampholytes are then mobilized by pressure or addition of salt to one of the reservoirs to facilitate migration of the bands past the detection window.

2.1.5 CAPILLARY GEL ELECTROPHORESIS (CGE)

CGE is a size-based separation technique, most often used in the separation of macromolecules such as proteins and nucleic acids. The capillary is filled with a suitable polymer, the analytes are injected onto the capillary and an electric field is applied. The analytes undergo molecular sieving as they migrate through the polymer, larger solutes being hindered more than smaller ones.

Different polymers may be used in CGE, including covalently cross-linked polymers (such as bis-polyacrylamide), hydrogen-bonded polymers (such as agarose) or linear polymer solutions (such as polyacrylamide or methylcellulose). Although the polymer structures of these gels are very different, the mechanism of separation remains the same.

Cross-linked polyacrylamides are characterised by a rigid structure with small pore sizes, and this precludes the use of hydrodynamic sample injection. Linear polymers are essentially polymer solutions and are thus more flexible than the cross-linked gels. Generally the polymer concentration required is inversely proportional to the size of the analyte. Hydrodynamic injection can be used in this case.

Resolution and efficiency in CGE are similar to that in CZE, as both techniques are zonal electrophoretic techniques.

2.2 PRINCIPLES OF CAPILLARY ELECTROPHORESIS

2.2.1 ELECTROPHORESIS [70,71]

The migration of any species in CE under the influence of an applied potential is the result of the electric force that is applied to the molecule, balanced by the molecule's frictional drag. Charged species subjected to an electric field *E* (which is a function of the applied voltage and capillary length), will migrate at a velocity that is largely dependent upon their molecular charge and mass, as well as the bulk flow of the electrolyte and the electrolyte viscosity.

The migration velocity (v_{ep}) of any species is defined by:

$$v_{ep} = m_{ep}E = \frac{m_{ep}V}{L_T} = \frac{ze_0E}{6\pi\eta r} \qquad (2.1)$$

m_{ep} = *absolute electrophoretic mobility* ($cm^2.V^{-1}.s^{-1}$)
E = *electric field strength* ($V.s^{-1}$)
L_T = *total capillary length (cm)*
V = *applied voltage (V)*
z = *charge number*
e_0 = *elemental charge*
η = *Newtonian viscosity (Pa.s)*
r = *Stokes radius (cm)*

The migration velocity is directly proportional to the electric field strength (E), where the absolute mobility (μ_{ep}) is the constant. Therefore,

$$m_{ep} = \frac{ze_0}{6\pi\eta r} \qquad (2.2)$$

These equations (2.1 and 2.2) are applicable to spherical particles migrating through a non-conducting medium. Here the electrophoretic mobility for any species is a physical constant at a given set of conditions, dependent solely upon the viscosity of that medium. The electrophoretic medium however is a conducting medium, and the ionic charge, local electric field and effective radii of the migrating species are altered by the ionic atmosphere of oppositely charged counter-ions in the electrophoretic medium. As a result in an electrolyte of high ionic strength the absolute mobility ($:_{ep}$) of a species will tend to be reduced due to the electrostatic interactions between ions of the electrolyte and between

the analyte and its counterions. The effective mobility will tend to be lower than the absolute mobility in these cases.

There are many factors that act synergistically to determine the electrophoretic mobility of a solute. The pH of the medium will dictate the charge of a solute. The size of the solute then becomes important as the charge to mass ratio plays a role in its mobility. Another factor to be considered is the electric field strength, which is determined by the length of the capillary and the applied voltage. Electrophoretic mobility increases exponentially with the increase in temperature of the medium. This is due to the viscosity of the medium which drops exponentially with increasing temperature.

2.2.2 ELECTRO-OSMOTIC FLOW (EOF)

EOF is the bulk flow of the liquid in the capillary with an applied electric field. The inside of a fused silica capillary is covered with silanol groups, which dissociate to produce the anionic form (SiO^-) especially above a pH of 4. Due to these SiO^- groups, the interior of the capillary tends to be negatively charged which attracts cations. The resultant increase in concentration of cations near the capillary surface forms an electric double layer and creates a potential difference very close to the wall. This potential difference is known as the zeta potential (.). The cations forming the double layer are generally solvated, and when voltage is applied across the capillary, the cations move toward the cathode, pulling with them the bulk solution in the capillary. EOF can be expressed in terms of velocity or mobility by:

$$v_{EOF} = (g./O)E \qquad (2.3)$$

v_{EOF} = EOF velocity
g = dielectric constant

or

$$:_{EOF} = (g./O) \qquad (2.4)$$

$:_{EOF}$ = EOF mobility

[70,71]

The charge on the capillary wall is highly dependent upon pH, which means that EOF is dependent upon pH to a large degree. This is illustrated by a significantly greater EOF at higher pHs where the silanol groups are deprotonated, as opposed to lower pHs, where the silanol groups are protonated.

The EOF controls, to a large degree, the amount of time that solutes remain in the capillary by superposition of flow onto solute mobility. In the presence of electroosmotic flow, the migration time (t) and velocity (v) of a solute are given by:

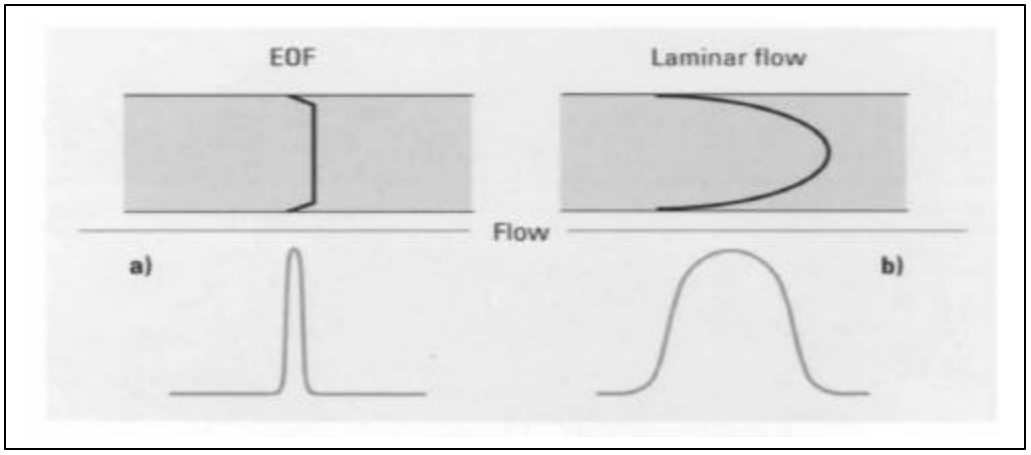
$$v = \frac{(\mu_{eo} + \mu_{ep})V}{L_T} \qquad \mu_{eo} = \text{electroosmotic mobility} \tag{2.5}$$

and

$$t = \frac{L_T^2}{(\mu_{eo} + \mu_{ep})V} \tag{2.6}$$

A benefit of EOF is its characteristic flat flow profile, which results in sharp peaks with high resolution. External pump systems such as those used in liquid chromatography result in laminar or parabolic flow profiles whose peaks tend to be rounded and broad in comparison to the peaks generally obtained in CE. This is illustrated in Figure 2.3.

Figure 2.3. Comparison of EOF and laminar flow profiles ***[71].***



Another advantage of EOF is that it causes the movement of nearly all species, in the same direction regardless of charge. This means that anions, cations, and neutral molecules can be electrophoresed in the same run [71].

EOF in some CE systems can be deleterious. In the case of CGE, CITP and CIEF, electro-osmosis is undesirable. In CZE however, resolution of zones is dependent upon the EOF rate, and in MEKC, a strong EOF towards the cathode is frequently extremely beneficial [70].

2.2.3 DISPERSION EFFECTS

CE is a high-resolution technique that requires the control of sources of dispersion in order to enhance separation. Separation of analytes in CE is based upon differences in solute mobilities and the resolution of two separate zones is dependent upon the length of these zones. Dispersion affects both of these, causing increases in zone length, changes in mobility, disruption of separation, and an overall decrease in system efficiency.

2.2.3.1 Joule Heat

The heat generated by the passage of electrical current through an electrolyte solution is called Joule heat, and this causes an elevation of temperature within the capillary. The high thermal conductivity of fused silica results in more effective heat transfer across the capillary wall than through the electrolyte. In this fashion a temperature gradient is set up. While an absolute rise in temperature in a capillary would generally not be detrimental, heat dissipation through the capillary wall results in higher temperatures in the centre of the capillary than at the walls. This causes non-uniform temperature gradients within the capillary, which gives rise to local changes in viscosity and zone deformation.

The temperature difference between the centre of the capillary and the walls of the capillary is dependent upon the inner radius of the capillary, the thickness of the capillary wall, the thickness of the polyimide coating and the heat transfer coefficient to the surroundings. The magnitude of this temperature gradient is related to a number of parameters as shown in Equation 2.7.

$$W = \frac{d^2 \beta C V^2}{4L} \qquad (2.7)$$

W = rate of heat generated per unit volume ($W.cm^{-3}$)
d = capillary diameter (cm)
V = applied voltage (V)
C = ionic strength (M)
L = total capillary length (cm)
β = molar conductivity ($cm^2.S^1.M^{-1}$)

It is beneficial to use narrow internal diameter capillaries with large outer diameters to assist in heat removal and reduce temperature gradients to a minimum.

Control of temperature is very important as one degree change in temperature can result in a 2 to 3 % change in viscosity, and a 2-3% change in mobility. Temperature increase depends on power (product of voltage and current) generated, and temperature gradients are reduced linearly with a reduction in power. Hence a reduction in applied voltage would cause a proportional decrease in the heat generated. However, as theoretical equations for resolution and efficiency advocate the use of as high electric fields as possible, a decrease in electric field would reduce efficiency and resolution to some extent.

A reduction in capillary diameter would cause a dramatic decrease in current (current is proportional to the cross-sectional area of the capillary and hence the square of the capillary radius), a decrease in power generated and a decrease in temperature gradient. A reduction in the capillary diameter though, would decrease sensitivity and may increase the likelihood of sample adsorption [71].

While joule heating is a problem, many of the commercial instruments do have cooling facilities, either through the use of liquid coolant that fills into the capillary cartridge before the current is applied, or by the use of a fan.

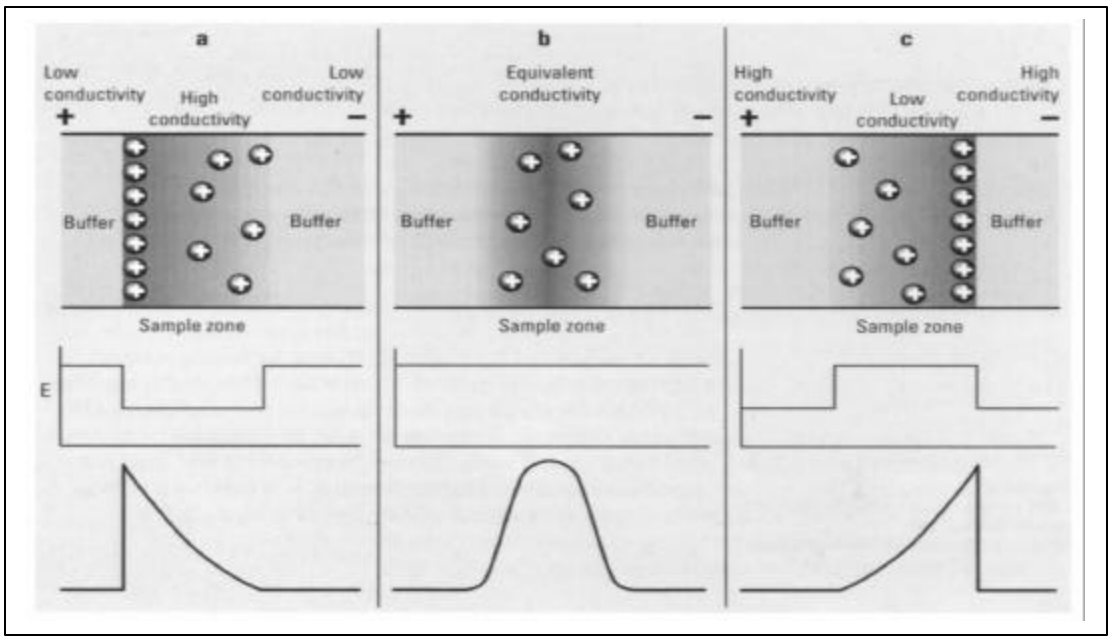
An alternative method that could be used to decrease the electrical current is to decrease buffer ionic strength or concentration. Increased sample adsorption may result from this too. There are a number of ways to minimize this effect. Varying pH of the buffers can minimize adsorption. Other strategies include the so-called “static approach” through permanent modification of the capillary wall using suitable coatings, and the “dynamic approach” using certain buffer additives. With this latter approach, the capillary wall is dynamically modified in each run by masking the charged sites.

2.2.3.2 Electrodispersion

Electrodispersion or electrophoretic dispersion is a result of differences in sample zone and electrolyte conductivity, giving rise to fluctuations in the electric field strength along the length of the capillary. This may manifest in 3 ways:

- distorted peak shapes,
- solute concentration or focussing where the sample is of a lower conductivity than the electrolyte, or solute de-focusing where the sample is of a higher conductivity than the electrolyte,
- temporary isotachophoretic states where there is an excess of a certain ion.

Figure 2.4. Illustration of electrodispersion caused by mismatched sample and buffer conductivities [71].



In Figure 2.4 it can be seen how the differences in conductivity between sample zone and buffer would cause peak shape distortion. These distortions generally always occur, especially with samples containing solutes with a wide range of mobilities. Matching sample and buffer conductivities will eliminate these effects, but they tend to be minimal in comparison to other dispersive effects. Peak shape distortions are only detrimental where resolution is lost [71].

2.2.3.3 Diffusion

Under ideal conditions, longitudinal diffusion (along the capillary), is considered to be the sole contributor to solute zone broadening. Application of higher voltages is beneficial in reducing diffusion as migration time would be decreased and the solute would have less time to diffuse.

2.2.3.4 Adsorption

From simple solute-wall interactions to total adsorption of solute molecules onto the capillary wall, interactions between solute and capillary wall will result in varying degrees of peak distortion. In minor cases of reversible adsorption, band broadening and peak tailing are apparent, while irreversible adsorption prevents the migration of the adsorbed species altogether. The main causes of adsorption onto the fused silica walls are ionic interactions between cations and the negatively charged wall, and

hydrophobic interactions. The larger the surface area to volume ratio of the capillary, which is beneficial in terms of heat dissipation, the greater the likelihood of adsorption.

2.2.3.5 Injection Plug Length

Large sample volumes easily overload CE systems resulting in a loss of resolution and efficiency. With respect to sample overloading, injection plug length is a more critical parameter than volume. The sample plug length should be less than 1 to 2 % of the total length of the capillary, which would equate to an injection length of several millimetres. Restriction on injection plug length should be stringent, especially for larger molecules, and current instrumentation can load these small volumes reproducibly. However under typical conditions, detection limit difficulties often necessitate longer injection plug lengths.

2.3 OPERATING PARAMETERS

2.3.1 CAPILLARY

Some of the ideal properties for a capillary substance would include being chemically, physically and electrically inert, UV-Visible transparent, flexible, robust and inexpensive. One of the main functions of capillaries in CE is to achieve the efficient heat dissipation, which is essential in the use of the high voltages that are applied. The capillary material should also therefore possess the necessary thermal properties. There are however very few materials that possess these necessary properties and are available in the very small dimensions required.

One of the most commonly used capillary materials is fused silica because it possesses most of the above mentioned properties. There are several potential drawbacks in the use of fused silica capillaries. Firstly, the silica surface is characterised by hydroxyl groups that may provide sites for interaction with charged molecules. The second is that capillaries with small diameters are used in order to minimize heating effects. This limits detection sensitivities, especially where optical detection is concerned. Further developments have been made as a consequence of this, which have led to the development of rectangular tubings, coated capillaries, and optically transparent outer coatings.

Fused silica capillaries are fragile and are coated with a protective layer of polyimide coating. To create a detection window, the polyimide coating is removed using an electrical arc, electrically heated wire, or flame. Chemical modification of the inner surface of the capillary is possible by the covalent binding of different substances onto the capillary wall. These coatings are used for a variety of purposes such as to reduce sample adsorption, to change the ionic charge on the capillary wall, or to reverse EOF.

2.3.1.1 Capillary Dimensions

Capillary dimensions have an effect on several factors in CE, and careful selection of these dimensions is necessary to optimise separation. Typical capillary dimensions include an inner diameter of 25 to 75 : m and an outer diameter of 350 to 400 : m. Effective lengths most frequently range from 50-75cm, with the total lengths being anything from 5 to 15cm longer than the effective length, or dependent upon the instrument dimensions.

(a) Capillary Length

In selecting capillary length, a compromise between resolution, efficiency and analysis time is necessary. Resolution is proportional to the square root of the length, therefore longer capillaries result in greater resolution. Analysis time however, is directly related to length, and longer capillaries require longer analysis times.

Production of Joule heat is a concern in CE. An advantage of longer capillaries is that they offer a larger surface area for heat dissipation. According to Ohm's Law, applied voltage is the product of electrical resistance and current. Increase in capillary length causes an increase in electrical resistance and a decrease in current. This is also advantageous in terms of Joule heat, as less current implies less heat generation.

Electric field strength is inversely proportional to the length of the capillary, - the longer the capillary, the less the electric field strength. From equation 2.1, it can be seen that a decrease in electric field strength would mean a decrease in the electrophoretic velocity of a species, and longer analysis times.

(b) Capillary Diameter

Wider internal diameter capillaries (,100 : m) offer shorter analysis times and increased sensitivity to analytes. However, the amount of heat generated and the thermal gradient produced in these capillaries is undesirable. Reduction of the capillary internal diameter increases the efficiency of heat dissipation, and the highest resolution in CE is obtained with very narrow bore capillaries (internal diameter 25 : m). These internal diameters reduce sensitivity and increase the risk of capillary blockage. A compromise therefore between sensitivity and resolution is most often obtained by using capillaries with an internal diameter of 50 : m.

2.3.1.2 Capillary Conditioning

One of the most significant problems and common criticisms of CE is that migration times are often not sufficiently reproducible. These problems with reproducibility stem mainly from the difficulties in maintaining a clean reproducible capillary surface. Variations in capillary surface tend to affect EOF and consequently analyte migration times. Cohen and Grushka [73] observed that the EOF changed perceptibly from one run to another, and this variation was especially prevalent during the first few runs. It is reported that rinsing the capillary between injections aids in keeping the surface of the capillary clean and reproducible. Rinsing with buffer alone results in the most reproducible conditions however, adsorption of sample onto the surface of the capillary, and changes in capillary surface charge often do not permit this. As a result, base conditioning is frequently used. This deprotonates the surface silanol groups and removes any adsorbates from the capillary walls.

A new capillary should be flushed with 1.0M sodium hydroxide solution, followed by 0.1M sodium hydroxide solution, water and then buffer. In between analyses, the last 3 steps are sufficient.

2.3.2 ELECTROLYTE SYSTEM

The electrolyte system in CE is of vital importance as its composition fundamentally determines the migration behaviour of the analytes. Due to the strong dependence of electroosmotic and electrophoretic mobilities on the electrolyte system, careful consideration to several factors is necessary prior to selection of any CE electrolyte system. Some of these would include solubility, stability and degree of ionization of the analyte in the electrolyte, and effect of pH, electrolyte additives, and ionic strength of the electrolyte on the separation.

The selected buffer ideally should possess the following properties:

- Display sufficient buffering capacity for the pH range of choice
- Have low absorbance at the wavelength of detection
- Have sufficiently low mobility to minimize current generation
- Display as limited variation with temperature as possible

A wide range of electrolyte systems have been used in CE to effect the required separations, the majority of these being aqueous buffers. In order to perform the electrophoretic run, analytes need to be soluble in the electrophoretic solution used. For a large number of substances solubility may not be a problem. However, with very hydrophobic compounds, mixed solvents may be required for the analysis.

2.3.2.1 Electrolyte Composition

Maintenance of a constant pH throughout the electrophoretic analytical procedure is essential. The degree of ionization of the analytes, the EOF and many other analytic parameters will vary with fluctuations in pH. The majority of buffer systems have sound capacity to 1 pH unit above or below their respective pK_a value. It is therefore necessary that the pH at which the solutes are ionised is established prior to buffer selection, as this will dictate to a large extent, the choice of buffer.

Electrolytes that possess high buffer capacity and simultaneously exhibit low conductance are ideal as they generate low current. In this case, higher electric fields can be used, and higher resolution obtained [74].

2.3.2.2 Ionic Strength

Ionic strength or buffer concentration has significant influence on many of the parameters defining good separation in CE. These include solute mobilities and migration times, separation efficiency, selectivity and peak resolution. The electrolyte concentration (C) is linked to various parameters in the electrophoretic process according to the following equation:

$$I = \frac{B r^2 g C.}{0}$$

I = *intensity of current*
r = *radius of capillary*
g = *dielectric constant*

(2.8)

It is evident from the above equation that an increase in buffer concentration will cause an increase in electric current, which may result in unwanted Joule heating. It can also be seen that an increase in ionic strength will increase viscosity of the electrolyte. This will decrease analyte and electroosmotic mobilities and also affect separation. The increased ionic strength of an electrolyte will also have an effect on separation, - causing compression of the electrical double layer resulting in a decrease in the zeta potential, and a reduction in EOF.

2.3.2.3 Electrolyte pH

Electrolyte buffer pH is one of the most important parameters to consider in optimisation of separation in CE, as it determines the degree of ionization of species present in the electrolyte system, degree of ionization of the silanol groups of the capillary wall and hence electrophoretic and electroosmotic mobilities.

2.3.2.4 Electrolyte Additives

Incorporation of additives into the CE electrolyte solution offers numerous opportunities to enhance the separation and selectivity of the system. Electrolyte modifiers can be utilised to improve resolution, peak shape and also reproducibility to a degree. They are frequently used in separation of chiral compounds [75].

2.3.2.4.1 Inclusion Complexes

The use of inclusion complexes such as cyclodextrins and crown ethers enhances separation selectivity by the formation of selective complexes between analyte and the inclusion complex molecule. Cyclodextrins (CDs) and crown ethers are useful for the chiral separation of a wide range of enantiomers in CE.

The complexation of analyte and chiral selector (inclusion complex) is dependent upon Van der Waal interactions, solvation effects, hydrophobic interactions, hydrogen bonding and the size and shape of the molecules.

CD's are neutral oligosaccharides composed of six, seven or eight glucopyranose units corresponding to ", $ and (CD. The number of glucopyranose units determine the size of the CD moiety in solution. CDs form a shape like a cone, with hollow hydrophobic cavity of fixed diameter, and a hydrophilic circumference. Selectivity occurs through the inclusion of the hydrophobic portion of the analyte into the cavity and the formation of hydrogen bonds with the hydroxyl moieties around the hydrophilic circumference.

Crown ethers are macrocyclic polyethers also capable of enhancing resolution through an inclusion-complexation mechanism. These macrocyclic polyethers contain electron-donating heteroatoms which form weak dipole-dipole interactions with analyte molecules within the hydrophobic cavity. For a stable complex to be formed, the size of the analyte and dimensions of the cavity should be matched. Consideration of the chemical structure of the analyte, the pH of the electrolyte, organic additives and buffer composition is necessary to optimize the effectiveness of using inclusion complexes.

2.3.2.4.2 Organic Solvents

Organic solvents, either pure or as a mixture with water, are used in CE for many reasons. Organic solvents may increase the solubility of analytes, they may influence the effective mobility of analyte, the electroosmotic mobility and EOF, and often enhance the separation selectivity in CE. CE methods which solely utilise the effective mobilities of analytes to effect separation such as CZE, use organic solvents primarily to alter separation selectivity, whereas techniques such as MEKC or CE with inclusion complexes use organic solvents primarily for adjusting hydrophobic interactions in solution. A wide range of effects on the physicochemical, electrochemical and chemical properties of both analyte and electrolyte have been observed with the addition of organic modifiers to the electrolyte solution. There are several theories relating to the mechanisms by which the organic solvents induce such changes, but the mechanisms are not yet fully understood. In general, addition of an organic solvent to an electrolyte in CE will cause an effect that will most likely be dependant upon the concentration of the modifier added. The . potential will be altered, as well as viscosity, EOF and selectivity. Organic solvents are known to reduce the EOF, which may result in better resolution, but at the expense of a longer analysis time [70,71,76,77].

In the case of MEKC and CE with inclusion complexes, presence of the organic modifier will affect hydrophobic interactions, and thereby affect separation. For example, the presence of an organic modifier in MEKC will disrupt the micellar structure and can even prevent micelle formation.

2.3.2.5 Electrolyte Replenishment

Buffer replenishment of anodic and cathodic solutions is an essential part of ensuring and maintaining high reproducibility in CE. In an aqueous electrolyte, electrolysis of solution occurs at the electrodes producing soluble protons at the anode and hydroxyl ions at the cathode. This phenomenon combined with ion migration is known as buffer depletion. Buffer depletion results in pH changes and buffer pH is probably the single parameter with the greatest influence on separation. Electrolyte buffer pH determines the electric charge on the analytes and their electrophoretic mobility as well as charge on the silanol groups of the capillary, and consequently EOF.

The extent of hydrolysis at the electrodes depends largely upon the current generated, the total run time and the buffer system capacity whereas the volume of the buffer reservoirs will determine the extent of pH change.

For the above reasons, it is recommended that the buffer reservoirs at both electrodes should be frequently replenished [71,74].

2.3.2.6 Electrolyte levelling

During sample injection and electrophoresis it is essential that the liquid levels in both the anodic and cathodic buffer reservoirs remain at the same horizontal level. If the height of liquid in the 2 reservoirs is not equal, a pressure difference will result and siphoning will occur. The influence of non-level liquid in reservoirs is dependent upon capillary dimensions and electrolyte viscosity. A higher liquid level in the inlet reservoir will result in a superposition of the laminar flow caused by siphoning onto the electrophoretic plug flow. This will decrease migration time and have deleterious effects on separation. The reverse also applies. Upon injection of sample, non-level liquid will either result in an excess of sample being loaded, or *vice versa.*

2.3.3 INJECTION CONDITIONS

The sample injection method in CE is required to reproducibly and efficiently deliver small volumes (nanolitres) of sample onto the capillary without introducing significant zone broadening. The 2 most common methods of injection are electrokinetic and hydrodynamic. Both of these injection methods inject sample directly onto the capillary, thereby eliminating zone broadening which occurs when sample injection valves are used.

2.3.3.1 Hydrodynamic Injection

Hydrodynamic injection, otherwise known as hydrostatic injection, can be accomplished by one of three methods. These include application of pressure at the injection end of the capillary, application of a vacuum at the exit end of the capillary, or by a siphoning action resulting from elevating the injection reservoir relative to the exit reservoir. The main advantage of hydrodynamic injection is that unlike electrokinetic injection, there is no inherent discrimination between different sample species upon injection. However, with pressure injection, difficulties arise in terms of controlling the pressure precisely and atmospheric conditions and elevation can affect the injection.

The volume of sample loaded onto the capillary is almost independent of sample matrix, and is dependent upon capillary diameter, the viscosity of the buffer, the applied pressure, and the time. The injection volume (V_C) can be calculated from the following Hagen-Poiseuille equation [70,71]:

$$V_C = \frac{\Delta P d^4 \pi t}{128 \eta L_T} \qquad (2.9)$$

ΔP = *pressure applied*
d = *internal diameter of capillary*
t = *injection time*
η = *buffer viscosity*
L_T = *total capillary length*

Typical injection pressures and times range from 25 to 100mbar and 0.5 to 5 seconds respectively.
For injection using the siphoning method, the pressure differential (ΔP) to be used in equation 2.9, is calculated from equation 2.10.

$$\Delta P = \rho g \Delta h \qquad (2.10)$$

ρ = *buffer density*
g = *gravitational acceleration*
Δh = *height difference of the reservoirs*

Siphoning is used in systems where there are no pressure injection capabilities. Typical siphoning injection parameters include raising the sample reservoir 5 to 10 cm relative to the exit reservoir for between 10 and 30 seconds.

Sample siphoning can cause poor peak area reproducibility and as well as sample overloading. In order to reduce unwanted sample siphoning, the liquid levels of the sample and exit buffer reservoirs should be equal. As well as this, the smallest injection volumes possible should be used where sensitivity is not limiting. Injection reproducibility frequently diminishes with shorter injection times however, and this tendency is exacerbated with short and/or wide bore capillaries and concentrated samples. A suitable compromise, therefore, has to be made.

2.3.3.2 Electrokinetic Injection

Electrokinetic injection is also known as electromigration injection. It tends to have a greater precision than that of the hydrodynamic techniques, although is not as reproducible. With this method, the electrode and capillary are inserted into the sample reservoir and a voltage applied for a brief period of time. A field strength of 3 to 5 times lower than that used for separation is usually used. The movement of sample onto the capillary is a result of the electrophoretic migration of charged species as well as the EOF of the sample solution. The quantity of sample loaded is therefore dependent upon the electrophoretic mobilities of the solutes, and discrimination between ions occurs since more mobile species will be loaded to a greater extent than those less mobile.

The quantity in moles (Q) of ions injected onto the capillary can be calculated according to equation 2.11.

$$Q = \frac{(\mu_{ep} + \mu_{EOF})\, V \pi r^2 C t}{L_T} \qquad (2.11)$$

μ_{ep} = *electrophoretic mobility of analyte*
μ_{EOF} = *EOF mobility*
V = *applied voltage*
r = *capillary radius*
C = *analyte concentration*
t = *injection time*
L_T = *total capillary length*

From equation 2.11, it can be seen that as well as being dependent upon sample electrophoretic mobilities and EOF, sample loading in electrokinetic injection also depends upon sample concentration, applied voltage, capillary radius and injection time.

2.3.3.3 Field Amplified Sample Injection (FASI)

Field amplified sample injection techniques are used to cause on-capillary enrichment of ionic analytes at the sample-buffer interface, thereby enhancing sensitivity and permitting the determination of low levels of sample. In FASI, sample is dissolved in water or buffer of lower ionic strength than the running buffer, and electrokinetically injected onto the capillary. Upon the application of reverse voltage, a proportionally greater electric field develops across the sample zone, causing sample ions to migrate rapidly to the sample-electrolyte interface, resulting in sample stacking. Stacking can also be induced by injection of a short plug of water before sample introduction [70,71].

2.4 PERFORMANCE CRITERIA

Performance criteria in CE are adapted from chromatography, and modified according to the concepts applicable to CE. The flat flow profile in CE allows extremely high efficiencies to be achievable along with good resolution, especially where selectivity is optimised for the separation.

2.4.1 SELECTIVITY

Selectivity (″) is the relative order of solute migration and is determined by the differences in electrophoretic velocities of the analytes. It is an important parameter in the optimisation of any CE separation. Selectivity can be calculated from equation 2.12.

$$\alpha = \frac{\Delta\mu}{\mu_{ave}} = \frac{\Delta t}{t_{mig(ave)}} = \frac{t_1 - t_{eo}}{t_2 - t_{eo}} \qquad (2.12)$$

μ_{ave} = *average migration velocity*
$\Delta\mu$ = *difference in velocity between 2 zones*
Δt = *difference in migration time*
$t_{mig(ave)}$ = *average migration time*
t_{eo} = *migration time of EOF marker*

Optimisation of selectivity is most readily achieved through alterations made to the electrolyte pH. The use of buffer additives and to a lesser extent the manipulation of ionic strength, are also methods of controlling selectivity.

2.4.2 EFFICIENCY

Efficiency is expressed by the number of theoretical plates (N) available by the capillary. Under ideal conditions, longitudinal diffusion is the sole contributor to solute-zone broadening. The following equation (equation 2.13) can be used to calculate efficiency under ideal conditions, and where there are no solute wall interactions, small injection plug length, and maintenance of plug flow through the whole length of the capillary:

$$N = \frac{:_{ep} V L_{eff}}{2DL_T} = \frac{:_{ep} E L_{eff}}{2D}$$

D = *Diffusion coefficient of solute*

(2.13)

The application of high electric field strength is beneficial in terms of reducing solute migration time, by reducing the time that the solute has to diffuse.

For Gaussian peaks, system efficiency can be determined experimentally from an electropherogram using the following equation:

$$N = 5.54\,(t/w_{1/2})^2$$

t = *migration time*
$w_{1/2}$ = *temporal peak width at half height*

(2.14)

The calculated efficiency (equation 2.13) is usually greater than the measured efficiency (equation 2.14), because equation 2.13 assumes that the only zone broadening is due to longitudinal diffusion. This is commonly not the case as other dispersive processes are often present.

Dispersion effects in CE contribute largely to longitudinal diffusion, thereby directly influencing system efficiency.

2.4.3 RESOLUTION

The resolution of sample analytes is the ultimate goal in any CE separation. Resolution (Rs) can be determined from an electropherogram as described in equation 2.15.

$$Rs = \frac{2(t_2 - t_1)}{w_1 - w_2}$$

t = *migration time*
w = *baseline peak width*

(2.15)

In CE separation is primarily driven by efficiency. Resolution can be expressed as a function of efficiency as follows:

$$Rs = (1/4)\, N^{1/2}\, (\,) : / :_{ave})$$

where time^{-1} *or velocity can be substituted for mobility*

$) : = :_2 - :_1$

$:_{ave} = (:_2 + :_1) / 2$

(2.16)

Resolution can also be described by an equation that does not require explicit calculation of efficiency, but rather uses the effects of EOF on resolution:

$$Rs = [1/ (4.2^{1/2})][\,) :][V / D (:_{ave} + :_{EOF})]^{1/2}$$

(2.17)

Efficiency increases linearly with increasing voltage. Resolution however is dependent upon the square root of the applied voltage. Due to this relationship, the voltage must be quadrupled in order to double the resolution. This principle applies only in theory as in practice the Joule heat generated by such an action is deleterious to the separation.

Infinite resolution can be obtained when the average electrophoretic mobility and the electroosmotic mobility are equal but opposite. However, the analysis time would also then approach infinity. Operational parameters should therefore be optimised and controlled in order to balance resolution and analysis time.

2.5 OPTIMIZATION OF EXPERIMENTAL VARIABLES

All procedures performed in the laboratory were carried out strictly according to Standard Operating Procedures which were developed in-house and are described in the/ Appendix at the back of the thesis.

2.5.1 INTRODUCTION

A range of variables and parameters can be optimised in CE to help achieve the desired selectivity, resolution and efficiency. These variables can be divided into three categories: [78]

- System variables: Capillary dimensions
  Temperature
  Applied voltage
  Injection system
  Method of detection
- Sample variables: Sample solution pH
  Solute concentration
- Electrolyte variables: pH
  Ionic strength
  Electrolyte composition
  Buffer additives

These variables simultaneously exert their influence on the separation. This means that optimisation of any method is challenging. A selection of these parameters were chosen and their effects on system performance and the separation of 5 flavonoid compounds (quercetin, quercitrin, isoquercitrin, hyperoside and kaempferol) were investigated.

2.5.2 INSTRUMENTATION

The capillary electrophoresis system consisted of:

- PrinCE (4 tray) Electrophoresis System, Model 0500-001 (Lauerlabs, Emmen, The Netherlands).
- Butler Buffer Replenishing Device, Model 0500-001 (Lauerlabs, Emmen, The Netherlands).

- Linear UVIS-206 Multiple Wavelength Detector, Model 0206-0000 (Linear Instruments Corporation, Reno, Nevada, USA).
- Polyimide fused-silica capillary I.D. 50ìm (Polymicro Technologies, Phoenix, Arizona, USA).

Additional equipment included:

- Perkin Elmer R100A Strip Chart Recorder, Model C0050005 (Perkin Elmer Corporation, Illinois, USA).
- Cole-Parmer Ultrasonic Bath, Model 8845-30 (Cole-Parmer Instrument Company, Chicago, Illinois, USA).
- Mettler Dual Range Electronic Balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland).
- Crison GLP21 pH Meter (Laboratory Equipment Company, Port Elizabeth, South Africa).
- Eppendorf Centrifuge, Model 54142770 (Ependorf Geratebau, West Germany)
- GBC UV/VIS 916 spectrometer, serial number V2105 (GBC Scientific Equipment Pty Ltd, Melbourne, Victoria, Australia).
- Water of HPLC grade was used, purified by reverse osmosis through a Milli-Q purification system (Millipore Corporation, Bedford, USA). Please refer to the appendices for the standard operating procedure (SOP) regarding the use of this system.

2.5.3 MATERIALS AND REAGENTS

All reagents used were of at least analytical grade quality

Quercetin [2]	Lot 109H0882
Quercitrin [1]	Lot 01072608
Rutin [2]	Lot 59H0388
Hyperoside [1]	Lot 01061516
Isoquercitrin [1]	Lot 01042326
Sodium dodecyl sulphate [2]	Lot 42H0019
Kaempferol [1]	Lot 01050219
Hypericin [1, 2, 7]	
Pseudohypericin [6, 7]	
Acetonitrile (HPLC grade) [4]	

Methanol (HPLC grade) [4]

Acetone (Batch 52724) [3]

Sodium tetraborate ($Na_2B_4O_7 .10H_2O$) [3]

Hydrochloric acid (Lot K20625547 405) [5]

[1] Indofine Chemical Company, Inc., Somerville, New Jersey, USA.

[2] Sigma Aldrich Co Ltd., Dorset, Poole, UK.

[3] Saarchem (Pty) Ltd., Johannesburg, South Africa

[4] Burdick and Jackson Division, Muskegon, Michigan, USA.

[5] Laboratory Supplies, Poole, England.

[6] Merck Laboratory Supplies , Half-Way House, Johannesburg, South Africa.

[7] PhytoLab GmbH & Co., KG, Wandaleng 24, D-20097 Hamburg, Germany.

2.5.4 METHODS

Standard operating procedures (SOPs) were followed for all procedures utilised. Refer to the appendix section for SOPs outlining the cleaning of glassware, the use of the balance, the use of the pH meter, the use of the Millipore Milli-Q System and the use of the PrinCE and related equipment.

The method chosen for initial use and optimization was adapted from a MEKC method reported by Pietta and Mauri [79] and used to separate flavonol-3-O-glyosides in *Ginkgo biloba* extract. The conditions used were as follows:

Capillary: 72cm fused silica (50.0ìm ID)
Applied voltage: 20kV
Buffer: 20mM sodium borate with 50mM SDS (pH 8.3)
Injection: 1 s aspiration
Detection: 260nm

Use of this method for separation of the above mentioned 5 flavonoid compounds was investigated and further developed. The total capillary length used was 82cm, with effective length of 53cm.

2.5.5 CHOICE OF WAVELENGTH

2.5.5.1 Procedure

Individual sample solutions of quercetin (Q), rutin (R), isoquercitrin (Iqr), hyperoside (Hd) and kaempferol (K) were made up in methanol. UV absorbance spectra for the compounds were determined. Unfortunately at the time of analysis, quercitrin (Qr) was not available and the UV absorbance for Qr was not determined.

2.5.5.2 Results and Discussion

The absorbance spectra of the flavonoid compounds are all very similar as can be seen in Figures 2.5 to 2.9. Quercetin and K are aglycones, the only difference between them being the positioning of an OH group, and K, Qr, Iqr and Hd are glycosides of Q. These four glycosides differ from each other according to the particular sugar molecule attached to the basic quercetin moiety. This accounts for the similarity of absorbance spectra for all these compounds.

Figure 2.5. UV absorbance spectrum of kaempferol in methanol.

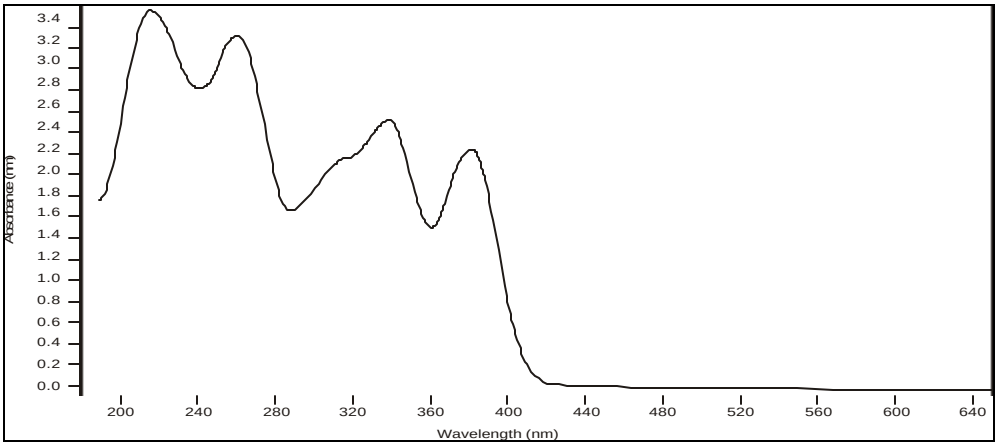


Figure 2.6. UV absorbance spectrum of hyperoside in methanol.

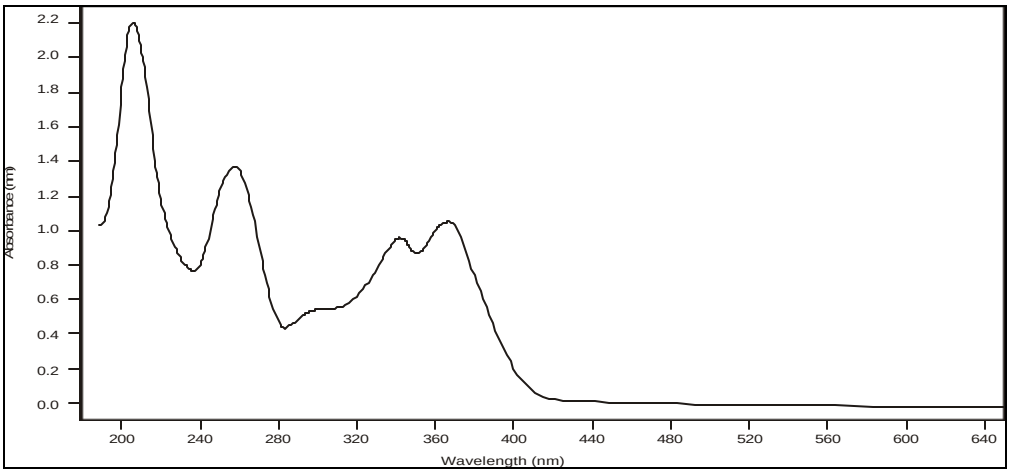


Figure 2.7. UV absorbance spectrum of isoquercitrin in methanol.

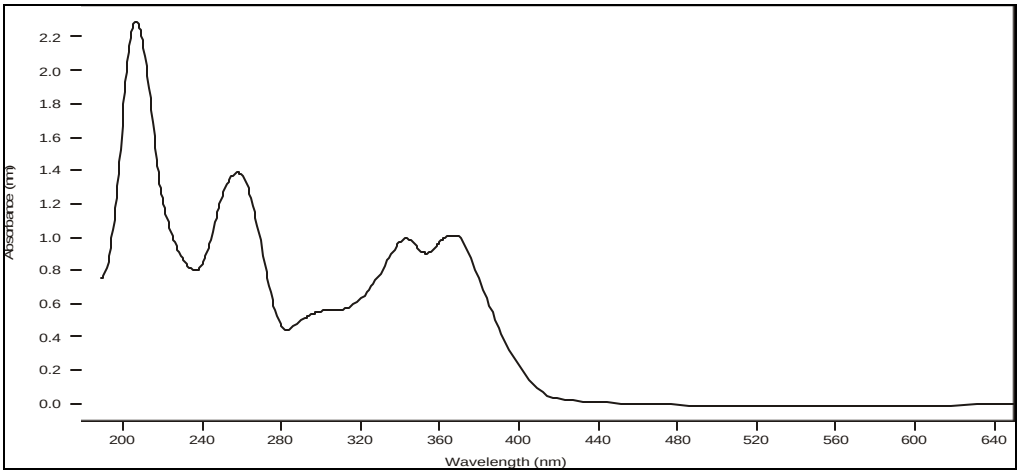


Figure 2.8. UV absorbance spectrum of quercetin in methanol.

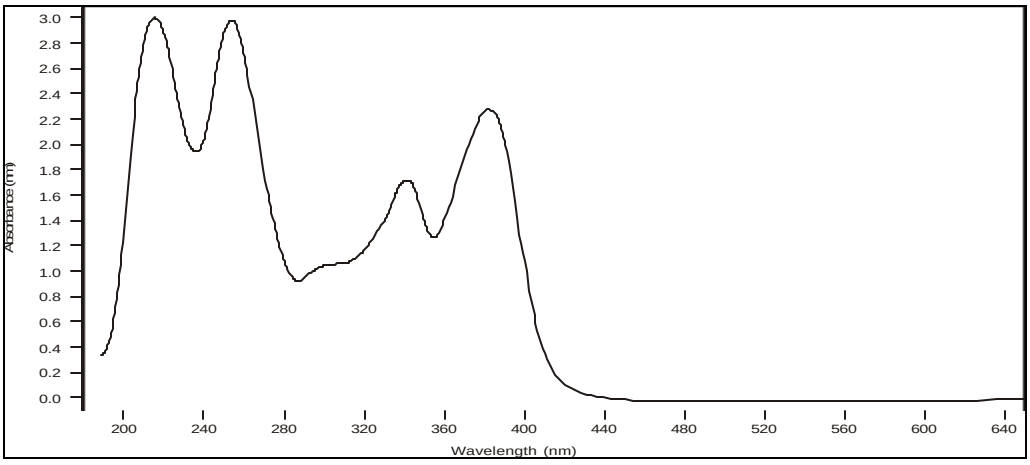
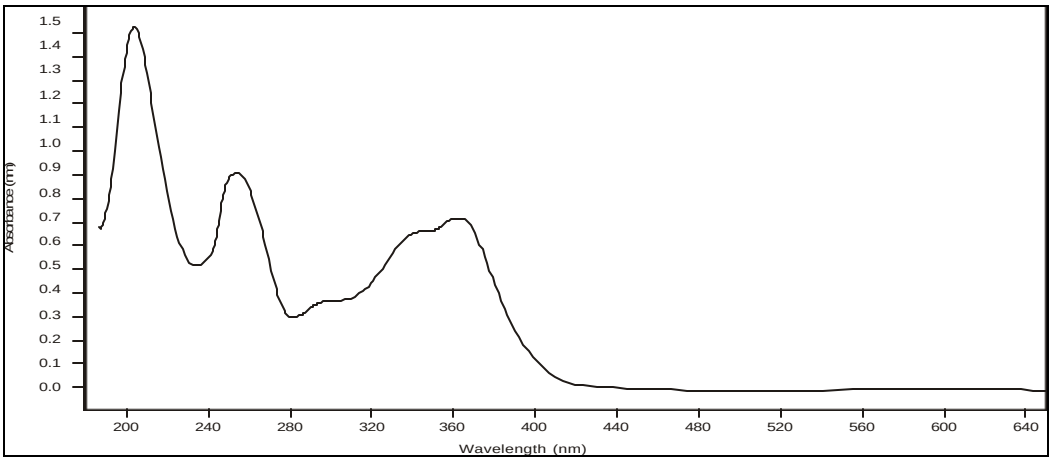


Figure 2.9. UV absorbance spectrum of rutin in methanol.



The literature reports quercitrin to have UV maxima at 258 and 350nm [40].

From the spectral information of these compounds, it can be seen that the absorbance maxima appear to be firstly around 207 or 216nm, followed by 254 to 260nm and finally around 340, 350 or 360 to 380nm

Many compounds absorb around 215nm which is unfortunately not very specific. The use of either 375 or 360nm was not possible as 375nm is on the edge of the visible lamp range (tungsten lamp in this case) and 360nm is on the outer limits of the deuterium lamp range. In both of these cases the noise that resulted at these wavelengths meant that the use of 375nm or 360nm would not be possible. In order to avoid unnecessary interference, 254nm was selected as the wavelength option of choice and 350nm was chosen as a secondary wavelength option where possible interferences at 254nm might necessitate its use.

2.5.6 OPTIMIZATION OF pH

2.5.6.1 Procedure

Three pH values between 8.3 and 10.0 were chosen to study the effect of pH on resolution and mobility. This pH range was chosen to fit the buffering capacity of sodium borate. One of the main considerations of this choice of pH range was also that the flavonoid compounds are soluble in alkaline aqueous solution. Polyimide coated capillaries can be used for separations between the pHs

of 2 and 12, but to ensure the maintenance of a reproducible internal surface of the capillary, a pH greater than 10 was not used.

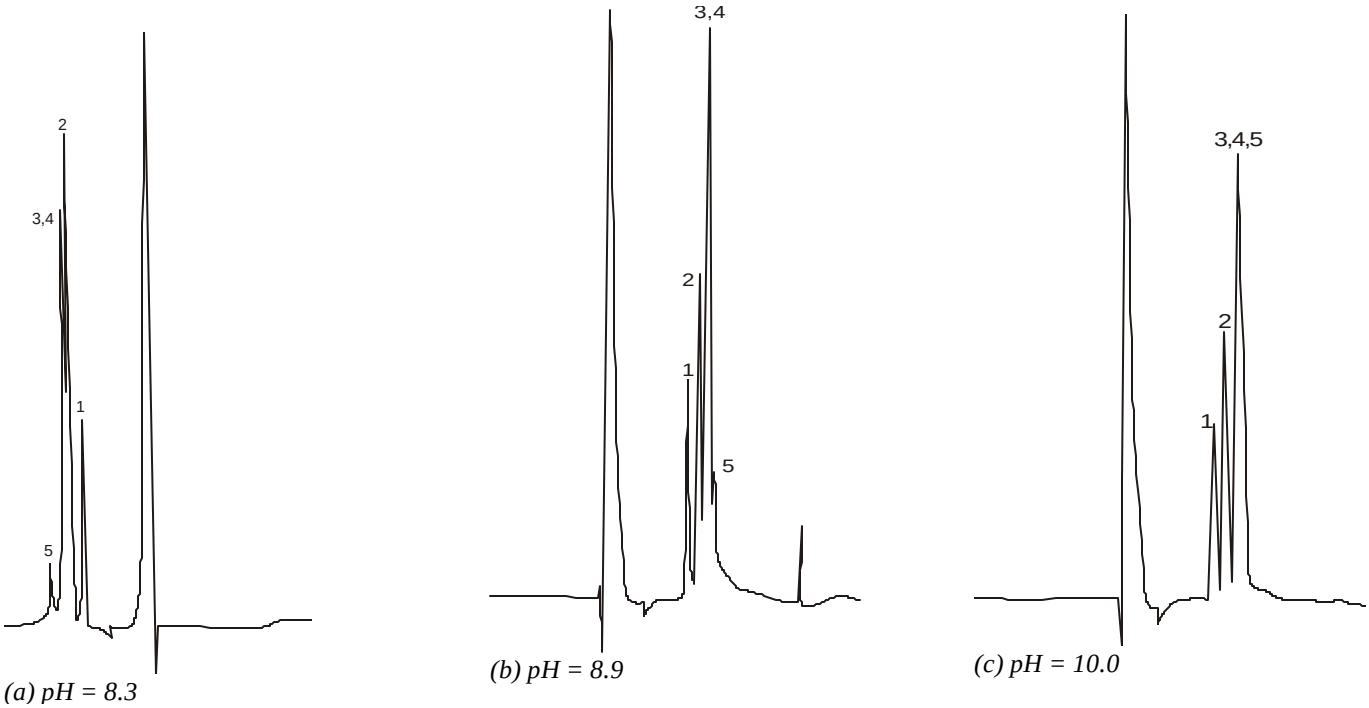
The analysis was performed with a 20mM sodium borate and 50mM sodium dodecyl sulphate buffer. The mode of injection was hydrodynamic; 200mbar for a duration of 0.1 minutes. A separation voltage of 20kV was used to effect the separation with the controlled voltage ramp programmed at 6kV/s to improve precision and accuracy of the migration time. The wavelength used was 254nm, with a rise time 0.5 seconds and an attenuation of 0.04 AUFS. The capillary used had a total length of 82.0cm and effective length of 53.0cm with internal diameter of 50.0ìm.

2.5.3.2 Results and Discussion

The electropherograms showing the separation of the flavonoids at pH's 8.3, 8.9 and 10.0 are shown in Figures 2.10a, 2.10b, and 2.10c respectively.

At pH 8.9 resolution was highest between all the flavonoids except Iqr and Hd which were still not resolved as illustrated in Figure 2.10b. With a further increase in pH to pH 10.0, resolution between all the flavonoids decreased with isoquercitrin, hyperoside and quercitrin all migrating together.

Figure 2.10. Electropherograms of a mixture of R, Iqr, Hd, Qr and Q at different pH's.

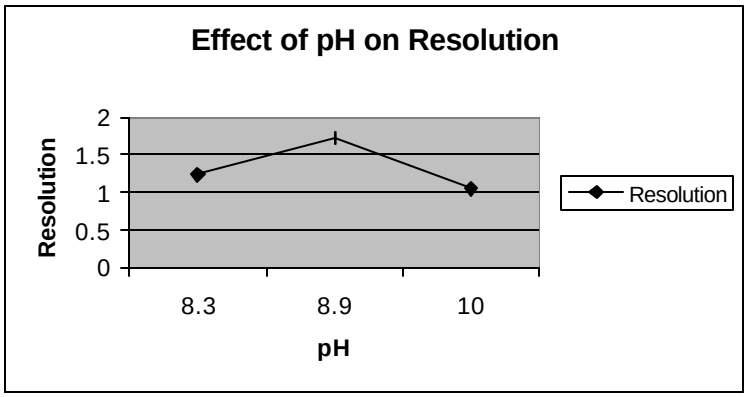


(a) pH = 8.3 *(b) pH = 8.9* *(c) pH = 10.0*

1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 4 - Quercitrin, 5 = Quercetin. Conditions: 20mM sodium borate with 50mM SDS buffer, Applied voltage: 20 kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm.

The effect of pH on resolution was determined from the electropherograms by applying equation 2.15. An illustration of the effect of pH on resolution between R and Iqr can be seen in Figure 2.11. This is an illustration of the trend of resolution between the flavonoids at the specific pHs chosen, except Hd and Iqr.

Figure 2.11. Variation of resolution between rutin and isoquercitrin with change in pH.



Conditions: 20mM sodium borate with 50mM SDS buffer, pH: 8.3 - 10.0, Applied voltage: 20 kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm.

The migration time for each component was used to calculate the electro-osmotic mobility ($:_{eo}$) and the electrophoretic mobility ($:_{eff}$) using equation 2.18 and 2.19 respectively.

$$:_{eo} = \frac{L_D L_T}{t_{eo} V} \qquad (2.18)$$

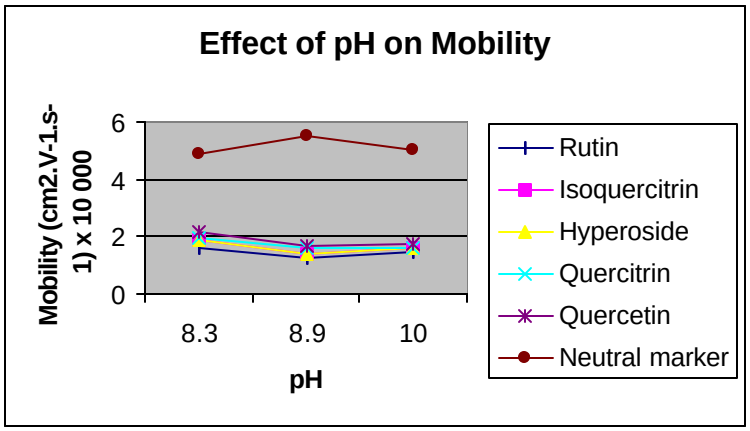
L_T = total length of capillary (cm)
L_D = length to detector (cm)
t_{eo} = migration time of neutral marker (s)

$$:_{eff} = \frac{L_D L_T}{V} \left[\frac{1}{t_{eo}} - \frac{1}{t} \right] \qquad (2.19)$$

t = migration time of solute (s)

These results can be seen in Figure 2.12 where the relationship between the electrolyte pH and electrophoretic mobility and electro-osmotic mobility is represented graphically.

Figure 2.12. Variation of electrophoretic and electro-osmotic mobility with pH.



Conditions: 20mM sodium borate with 50mM SDS buffer, pH: 8.3 - 10.0, Applied voltage: 20 kV, Injection: 200mbar, for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm.

The electrophoretic mobility of each component decreased between the pHs of 8.3 and 8.9, and then increased slightly at a pH of 10.0. The electro-osmotic mobility increased from pH 8.3 to 8.9, and then decreased slightly at a pH of 10.0.

As previously mentioned, optimization of selectivity is most readily achieved through alterations made to the electrolyte pH and selectivity of the separation is determined by the differences in electrophoretic mobility of the sample ions [73]. Changes in pH result in changes in sample ion dissociation and ionization and this can be utilised if the relationship between the electrophoretic mobility and pH is known for the sample ions.

In equation 2.20, the relationship between effective mobility of an ion to the effective charge of that ion is illustrated, emphasizing the importance of pH on effective mobility.

$$:_{eff} = \frac{Q_{eff}}{6B\,0\,R}$$

Q_{eff} = *effective charge of the ion (C)*
R = *total radius of ion (cm)*
$:_{eff}$ = *effective electrophoretic mobility* ($cm^2.s^{-1}.V^{-1}$)
0 = *Newtonian viscosity (Pa.s)*

(2.20)

In addition, the pH of the electrolyte influences the ionic state of the silica capillary surface by altering the charge density hence altering the zeta potential. The effective electrophoretic mobility is related to the zeta potential as described in equation 2.21.

$$:_{eff} = \frac{. \, ,}{6B\,0}$$

. = *zeta potential (V)*
, = *dielectric constant of the medium ($F.m^{-1}$)*
$:_{eff}$ = *effective electrophoretic mobility ($cm^2.s^{-1}.V^{-1}$)*
0 = *Newtonian viscosity (Pa.s)*

(2.21)

The general increase in EOF observed in Figure 2.12 with increasing alkalinity of the buffer may have resulted from the enhanced surface charge density of the capillary wall. With the increase in alkalinity, a relative increase in dissociation of silanol groups on the inner surface capillary wall may occur.

A pH of 8.9 was selected as the optimum pH for the separation and other parameters were subsequently investigated to attempt to effect separation of isoquercitrin and hyperoside.

2.5.7 OPTIMIZATION OF IONIC STRENGTH

Quercitrin was not available at the time of this study, and the flavonoids used for the study initially were R, Iqr, Hd and Q.

2.5.7.1 Procedure

(a) Optimization of Buffer Molarity

In order to optimise the separation of the flavonoid compounds, the effect of electrolyte ionic strength on separation was investigated for 20, 25 and 30mM sodium borate respectively, with a constant SDS concentration of 50mM, at a pH of 8.9.

The mode of injection was hydrodynamic; 200mbar for a duration of 0.1 minutes. A separation voltage of 15 kV was used to effect the separation with the controlled voltage ramp programmed at 6kV/s. The wavelength used was 254nm, with a rise time 0.5 seconds and an attenuation of 0.04 AUFS. The capillary used had a total length of 82.0cm,effective length 53.0cm and ID (internal diameter) of 50.0ìm.

(b) Optimization of Micellar Molarity

SDS has a critical micelle concentration (CMC) of 8mM. The effect of changing SDS concentration whilst maintaining a constant borate concentration was also investigated. Sodium borate (25mM) was

selected as the buffer molarity and the effects of using 25mM, 50mM and 75mM SDS were investigated.

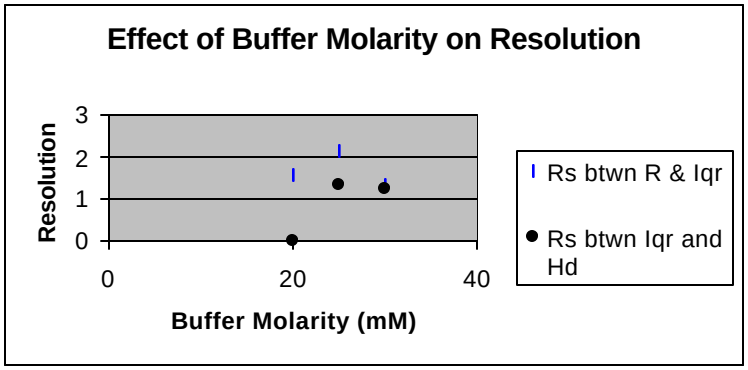
2.5.7.2 Results and Discussion

(a) Optimization of Buffer Molarity

The electrophoretic mobilities of the flavonoid compounds increased with increasing electrolyte concentration, whilst the electro-osmotic mobility decreased as electrolyte concentration increased. The migration times for each of the compounds increased with increasing buffer molarity, resulting in increased run times

With increase in electrolyte molarity to 25mM, an increase in resolution between all the flavonoids was exhibited and further increase to 30mM resulted in a decrease in resolution between all flavonoids. The effect of buffer molarity on the resolution of R, Iqr and Hd respectively is shown below in Figure 2.13. The resolution between Hd and Q followed the above-mentioned trend, but remained above 3.0.

Figure 2.13. Variation of resolution with electrolyte molarity.



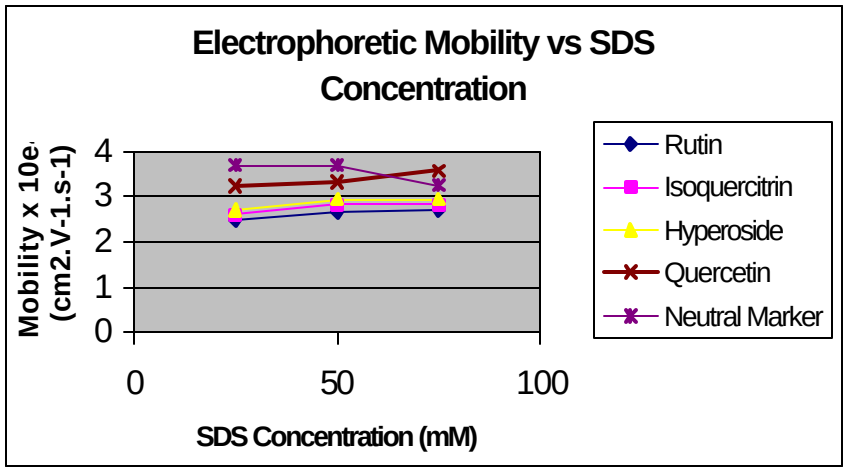
Conditions: 20, 25 and 30mM sodium borate with 50mM SDS buffer, pH 8.9, Applied voltage: 15 kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm.

As can be seen from Figure 2.13 optimum resolution was obtained at 25mM buffer molarity.

(b) Optimization of Micellar Molarity

Figure 2.14 illustrates the trend of mobility versus SDS for each of the flavonoids and the neutral marker. As can be seen there is a general increase in the mobilities of the flavonoids with increasing micellar molarity, and a general decrease in the mobility of the neutral marker.

Figure 2.14. Variation of electrophoretic and electro-osmotic mobility with micellar molarity.



Conditions: 25mM sodium borate with 25, 50 and 75mM SDS, pH: 8.9, Applied voltage: 15 kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm.

The increase in molarity of SDS did not have a large effect on the resolution of the glycosidic flavonoids. Resolution between R and Iqr remained approximately 1.4, and resolution between Iqr and Hd remained approximately 1.1. The SDS concentration seemed to have a large effect on the resolution of Hd and Q though, increasing from 5.4 to 9.5. This was a result of the relatively larger increase in migration time for quercetin than for Hd with the increase in SDS molarity.

The heat generated in CE is directly proportional to the electrolyte concentration and the square of the applied voltage, as expressed in Equation 2.7. In order to maintain high separation efficiency and resolution, electrolytes of moderate molarity and reasonable applied voltages should therefore be used. For the analysis of the flavonoids, a combination of 25mM borate buffer and 50mM SDS were found to yield the most satisfactory results.

2.5.8 OPTIMIZATION OF APPLIED VOLTAGE

The flavonoids used for this study were R, Iqr, Hd, Qr and Q.

2.5.8.1 Procedure

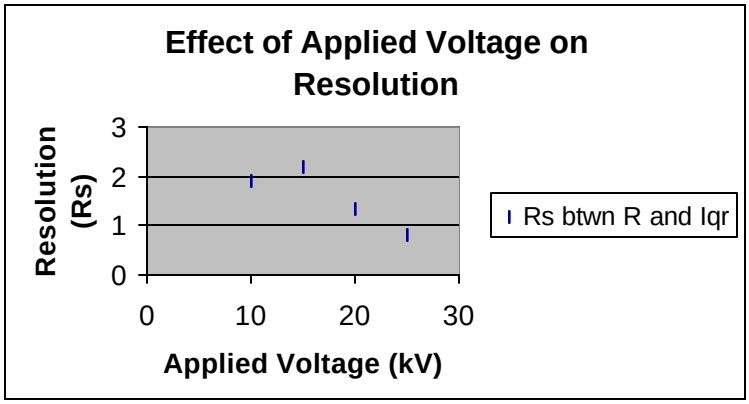
Four separation voltages of 10,15, 20 and 25 kV were investigated to affect the separation of the analytes. The controlled voltage ramp was programmed at 6kV/s. The mode of injection used was hydrodynamic; 200mbar for a duration of 0.1 minutes. The wavelength used was 254nm, with a rise time 0.5 seconds and an attenuation of 0.04 AUFS. The capillary used had a total length of 82.0cm,

effective length 53.0cm and ID (internal diameter) of 50.0ìm. Sodium borate buffer 25mM with SDS 50mM at pH 8.9 was used.

2.5.8.2 Results and Discussion

Increase in voltage resulted in increase in migration velocity and decrease in total run time. This is in accordance with equation 2.1, which states that the migration velocity is directly proportional to the applied voltage. At 10kV, the peaks were broad and the migration times were the longest. This followed a continuum to 25kV where the peaks were much sharper, and the migration times much shorter. Resolution between all the flavonoids was greatest at 15kV, followed by 10kV, then 20kV, and lastly 25kV. The resolution between rutin and isoquercitrin at each of the applied voltages is shown in Figure 2.15. This resolution illustrates the trend, which was observed between the other flavonoids.

Figure 2.15. Effect of applied voltage on resolution.



Conditions: 25mM sodium borate with 50 SDS, pH; 8.9, Applied voltages: 10, 15, 20 and 25 kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm.

The optimum applied voltage in terms of resolution, total run time and peak shape was found to be 15kV. This voltage was sufficiently low to avoid the generation of excessive joule heat. This relationship is expressed in equation 2.7.

2.5.9 EFFECT OF CAPILLARY LENGTH

2.5.9.1 Procedure

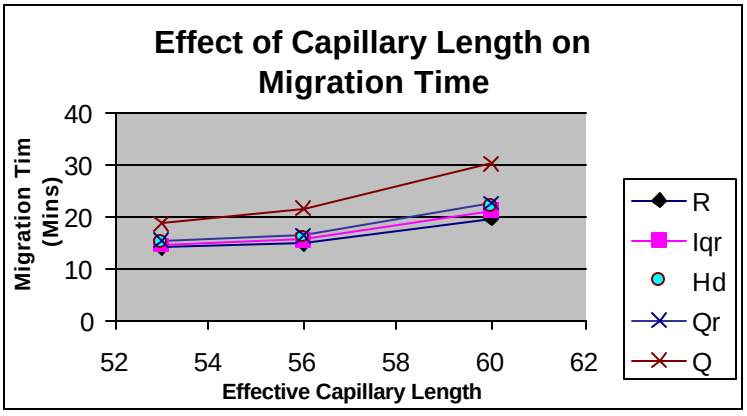
The effects of effective capillary length (length to the detector) were investigated within a range of 50.0 to 60.0cm using capillary of inner diameter 50.0ìm. The total lengths of capillary used were 82.0, 85.0 and 89.0cm, corresponding to effective lengths of 53.0, 56.0 and 60.0cm respectively. These lengths were selected to provide optimum separation time and still fit the instrumentation. The total length of the capillary could not be shorter than 82.0 cm, and longer capillaries resulted in much longer total run times.

A hydrodynamic injection technique was used; 200mbar pressure was applied for 0.1 minutes. A separation voltage of 15kV was applied to effect separation, with the controlled voltage ramp programmed at 6kV/s. The buffer used consisted of 25mM sodium borate with 50mM SDS at a pH of 8.9.

2.5.9.2 Results and Discussion

The results obtained from this investigation are illustrated in Figure 2.16 below. As can be seen, increase in capillary length resulted in a general increase in migration time for all the flavonoids.

Figure 2.16. Effect of capillary length on migration time.

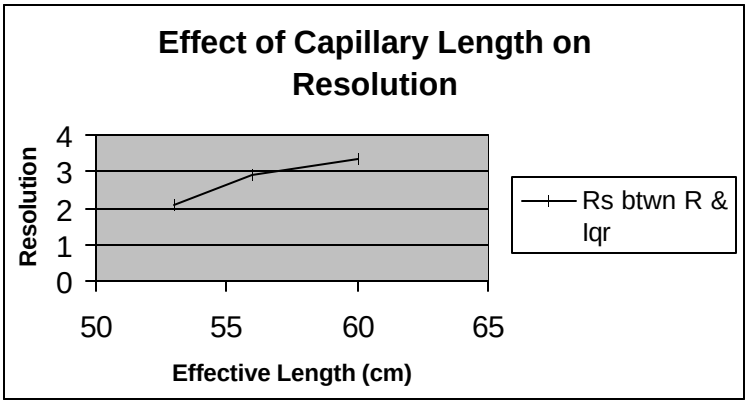


Conditions: 25mM sodium borate with 50 SDS, pH: 8.9, Applied voltage: 15kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total lengths = 82.0cm (effective length 53.0cm), 85.0cm (effective length 56.0cm) and 89.0cm (effective length 60.0cm).

The migration time for each flavonoid increased approximately 30% between those times obtained using the 82.0cm capillary and those obtained when using the 89.0cm capillary.

Each respective increase in capillary length resulted in a further increase in resolution between each of the flavonoids. The trend of increase in resolution is illustrated in Figure 2.17 where the resolution between rutin and isoquercitrin for each capillary length is indicated.

Figure 2.17. Effect of capillary length on resolution.



Conditions: 25mM sodium borate with 50 SDS, pH: 8.9, Applied voltage: 15kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm (effective length 53.0cm), 85.0cm (effective length 56.0cm) and 89.0cm (effective length 60.0cm).

The shortest capillary investigated (53.0cm effective length and 82.0m total length) offered the most desirable migration times of the flavonoids with the shortest total run time, and still offered sufficient resolution between the flavonoids.

2.5.10 EFFECT OF CAPILLARY DIAMETER

2.5.10.1 Procedure

To evaluate the effect of capillary diameter on separation, two capillaries of inner diameter 50.0 and 75.0µm were used. These were the only capillary diameters available. Sample solution consisting of the five flavonoids was injected onto each capillary separately. The hydrodynamic injection technique was used; 200mbar pressure was applied for 0.03 minutes. A separation voltage of 15kV was applied to effect separation, with the controlled voltage ramp programmed at 6kV/s. The buffer used consisted

of 25mM sodium borate with 50mM SDS at a pH of 8.9. The total length of the capillaries was 82.0cm, with effective length 53.0cm.

2.5.10.2 Results and Discussion

The increase in capillary diameter did not have much effect on migration times of the flavonoids, but increased the peak area and heights, and decreased resolution.

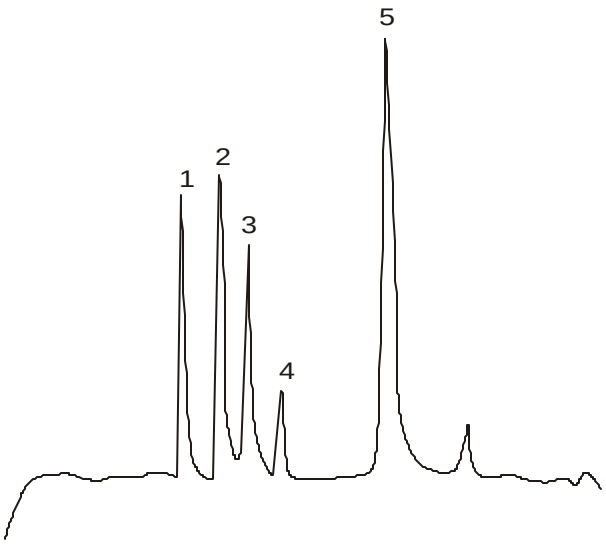
Narrow bore capillaries are more capable of effective heat dissipation than their wider bore counterparts. This is illustrated in the inverse relationship between the surface area to volume ratio and the inner diameter of the capillary. Increasing the capillary diameter increases the joule heat generated and thus decreases the efficiency of the separation. As well as this, the laminar flow that is often associated with wider bore capillaries results in peak broadening which may also be deleterious. Increase in capillary diameter however results in increased sensitivity of detection illustrated by the increase in peak areas and heights. A compromise between detection sensitivity and the level of joule heat generated is therefore required in selection of the capillary diameter for a separation.

The increased resolution and sharpness of the peaks indicated that the narrower bore capillary was most suitable for the separation of the flavonoids.

2.5.11 CONCLUSION

The optimized separation of R, Iqr, Hd, Qr and Q is shown in Figure 2.18. Parameters used were as follows: 25mM sodium borate buffer with 50mM SDS at a pH of 8.9. Hydrodynamic injection technique was used: 200mbar pressure was applied for 0.1 minutes. A separation voltage of 15kV was applied to effect separation, with the controlled voltage ramp programmed at 6kV/s. The total length of the capillary was 82.0cm, with effective length 53.0cm and ID of 50.0ìm. The capillary used was from Lot GFH03D. The rise time was set at 0.5s and AUFS were set at 0.004.

Figure 2.18. Optimized separation of the five flavonoids.



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 4 - Quercitrin, 5 - Quercetin. Conditions: 25mM sodium borate with 50 SDS, pH: 8.9, Applied voltage: 15kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm.

Numerous factors need to be taken into consideration when selecting optimal conditions for the separation. Many of these factors do not act individually, but have a combined effect. One of the main factors to be considered is the production of joule heat. Excess joule heat is detrimental to the separation and should be minimised. High molarity electrolytes, high applied voltages and wider bore capillaries can increase joule heat generated within the capillary independently. If two of these factors are increased simultaneously the increase in joule heat may be cumulative and impair separation significantly.

The impact of the selected experimental variables on separation was assessed and these variables were balanced against each other to provide optimal resolution, selectivity and efficiency within the most reasonable analysis time.

2.6 QUALITATIVE ANALYSIS

The method developed in Section 2.5 was utilised to qualitatively analyse two commercial tablet products and three commercial tinctures. The extracts and tinctures were spiked with the individual flavonoids to confirm the presence or absence of the respective flavonoids. Due to the lack of availability of Qr, the commercial products were not spiked with quercitrin.

2.6.1 INSTRUMENTATION

Refer to Section 2.5.2 for all instrumentation and additional equipment.

2.6.2 MATERIALS AND REAGENTS

The commercial samples chosen for use in the flavonoid analysis are listed below in Table 2.1. Two different brands of St John's Wort tablets, and three St John's Wort tinctures were analysed.

Table 2.1. St John's Wort products used for the qualitative analysis of the flavonoid compounds.

COMPANY	DOSAGE FORM	NAME OF PRODUCT	LABEL CLAIMS	BATCH	EXTRACT
Holotropic	Tablets	St John's Wort [9]	Each 500mg tablet contains 1000µg of Hypericin	0503	A
Vitality	Tablets	St John's Wort [11]	Each 500mg tablet contains 300mg *Hypericum perforatum* extract	1746	D
Burgess & Finch	Tincture	St John's Wort [12]	0.303g *Hypericum perforatum* per 1ml	F12916	F
Flora-Force	Tincture	Hypericum perforatum [10]	Contains homeopathic mother tincture of St John's Wort	PR5550266	G
Phyto-Force	Tincture	St John's Wort [13]	Contains purified water and ethanol	2324	H

Refer to Section 2.5.3 for all other materials and reagents.

[9] Holotropic, Cape Town, South Africa.

[10] Flora-Force Health Products, Rondebosch, South Africa.

[11] Vitality, Ridgeweg 534, Durban, South Africa

[12] Burgess and Finch Fine Natural Products, Natural Formulas Ltd., British Virgin Islands.

[13] Phyto-Force, Hillcrest, South Africa.

2.6.3 METHODS

Tablets from each product were ground into fine powder in a mortar and pestle. Three aliquots were accurately weighed out for each of the products and placed into individual Kimax tubes. Ten millilitres of methanol were pipetted into the Kimax tubes and the tubes stoppered.

The mixtures were vortexed for thirty seconds to ensure adequate dispersion of the powder throughout the methanol, and then sonicated for one hour at room temperature.

The respective extracts and tinctures were diluted with buffer, pipetted into vials and 0.2ml acetone added to each vial as the neutral marker. The dilutions were prepared on the day of analysis to avoid any unnecessary breakdown of the products. A standard solution containing R, Iqr, Hd and Q was run prior to injection of the samples for purposes of identification of components using relative migration times.

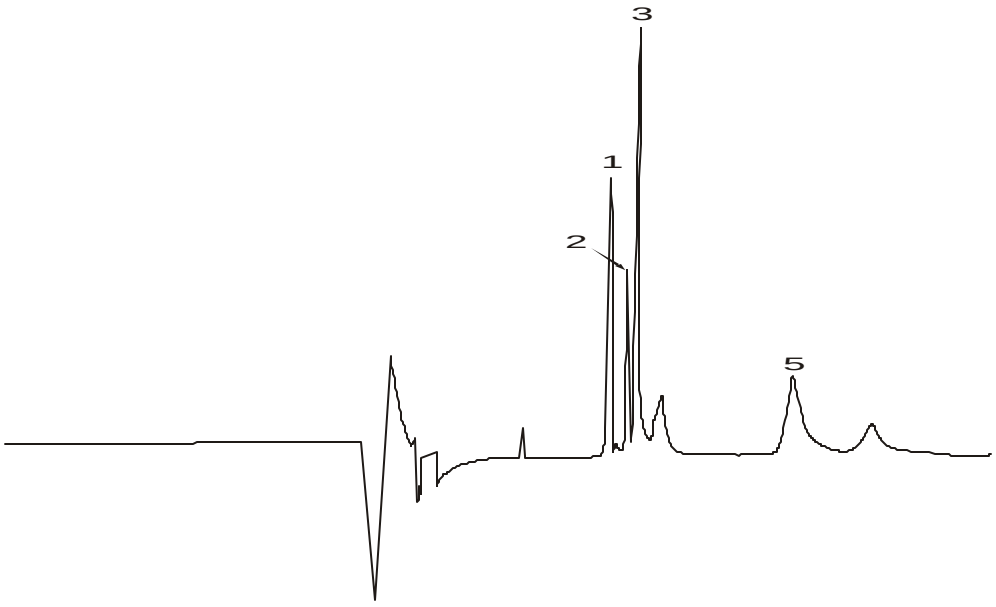
For each product, unspiked sample solutions were injected first and then the spiked samples. The sample solutions were spiked with 0.2ml of each individual flavonoid respectively, and then a separate sample was spiked with 0.2ml of a mixture of the flavonoid compounds.

The analysis was performed with a 25mM sodium dodecyl sulphate and 50mM sodium borate buffer at a pH of 8.9. The mode of injection was hydrodynamic; 200mbar for a duration of 0.1 minutes. A separation voltage of 15kV was used to effect the separation with the controlled voltage ramp programmed at 6kV/s to improve precision and accuracy of the migration time. The wavelength used was 254nm. The capillaries used had a total length of 82.0cm and effective length of 53.0cm with internal diameter of 51.0 : m.

2.6.4 RESULTS AND DISCUSSION

R, Hd, Iqr and Q were injected prior to analysis of A, D, F, G, and H of the commercial products. This separation is the one shown in Figure 2.19.

Figure 2.19. Separation of rutin, isoquercitrin, hyperoside and quercetin.



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 5 - Quercetin. Conditions: 25mM sodium borate with 50 mM SDS, pH: 8.9, Applied voltage: 15kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm.

The separation of R, Iqr, Hd, Qr and Q shown in Figure 2.18 was performed on a relatively old capillary. The separation of R, Iqr, Hd and Q shown in Figure 2.19 was performed on a relatively new capillary. The sample solution used in Figure 2.18 was more concentrated than that used in Figure 2.19. Other than this, all other conditions remained the same. As can be seen there is a decrease in resolution between the flavonoids with the use of the newer capillary, but the flavonoids were still separated. In Figure 2.18, the relative migration times for the five compounds varied from 1.63 to 2.34 and for the compounds in Figure 2.19 the relative migration times varied from 1.49 to 1.93, from R to Q respectively.

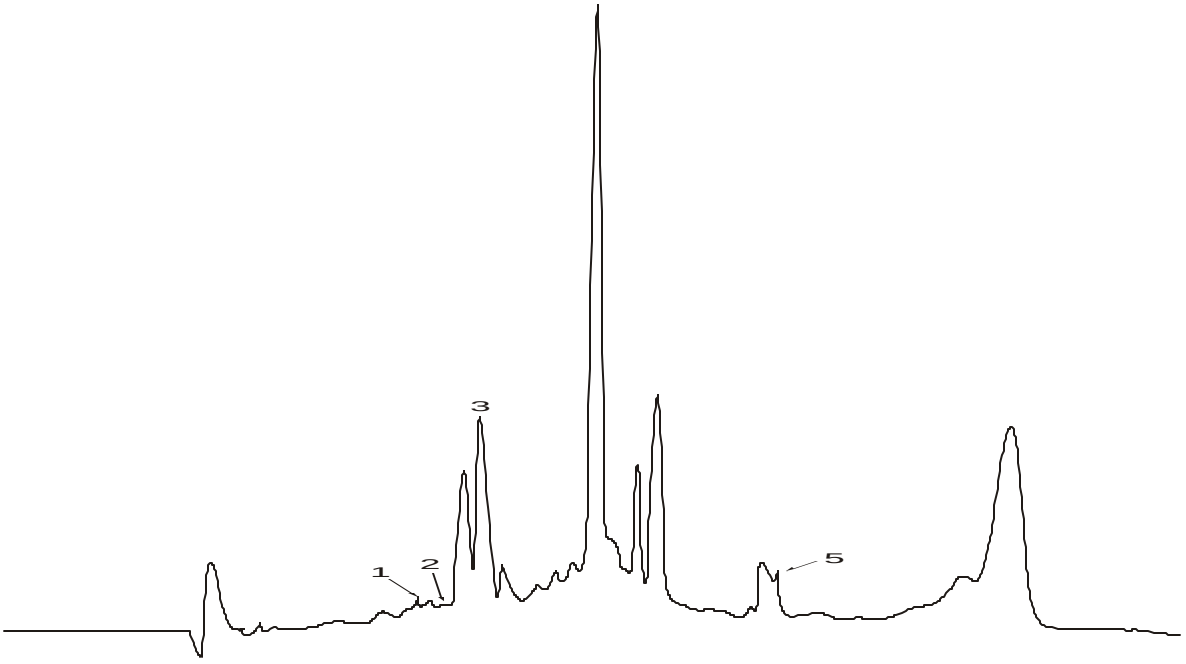
The separation obtained on any capillary was very sensitive to slight changes in the age of the capillary and slight changes to the charge on the capillary walls. As a result of this is, spiking of samples was performed in order to identify and confirm the presence or absence of each of the flavonoid components in the sample solution as opposed to simply using relative migration times. It was found that with new capillaries, resolution between the compounds tended to decrease as did migration times. As the capillary aged with use, the resolution tended to improve with an increase in migration time.

The spiked and unspiked electropherograms for each of the commercial products analysed are shown. In some cases the spiked electropherogram peaks appear smaller than those of the unspiked extracts. The extract samples were spiked in their respective sample vials and in spiking some dilution of sample occurred. The size of the spiked peaks relative to the other peaks present in the spiked electropherograms compared to the relative sizes of the same peaks in the unspiked electropherograms gives a more accurate indication of the relative increase in size of the spiked peaks.

2.6.4.1 Product A: Holotropic® Tablets

Figure 2.20 illustrates an unspiked electropherogram of an extract of Holotropic ® tablets. The positions of the respective flavonoids are indicated.

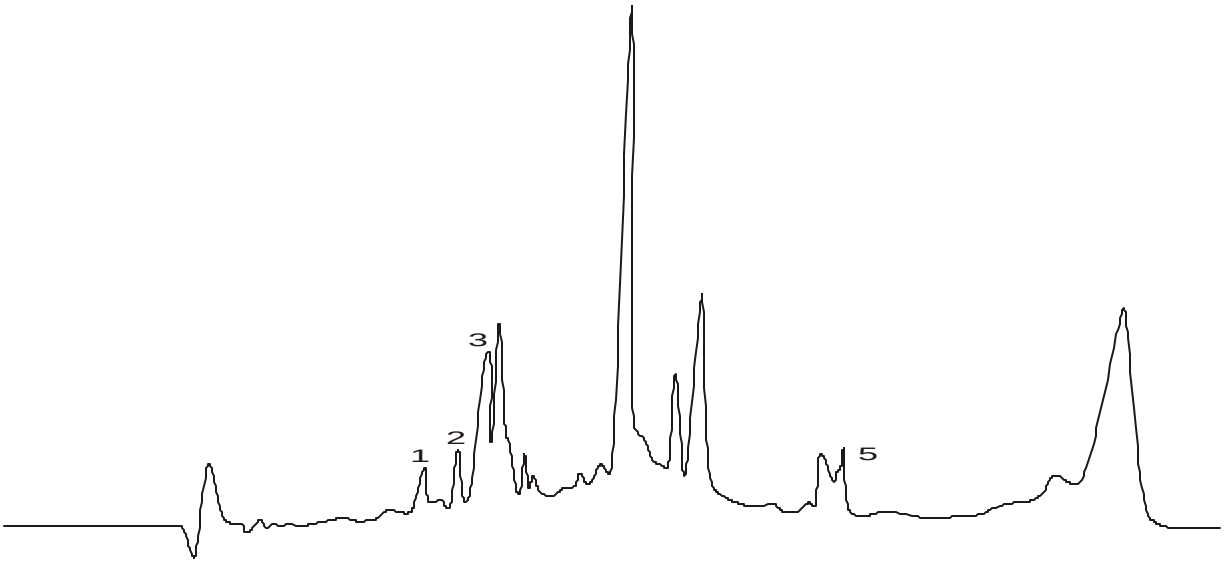
Figure 2.20. Unspiked Holotropic® extract.



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 5 - Quercetin. Conditions: 25mM sodium borate with 50 mM SDS, pH; 8.9, Applied voltage: 15kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm,

Figure 2.21 is an electropherogram illustrating a spiked Holotropic® extract.

Figure 2.21. Spiked Holotropic® extract.



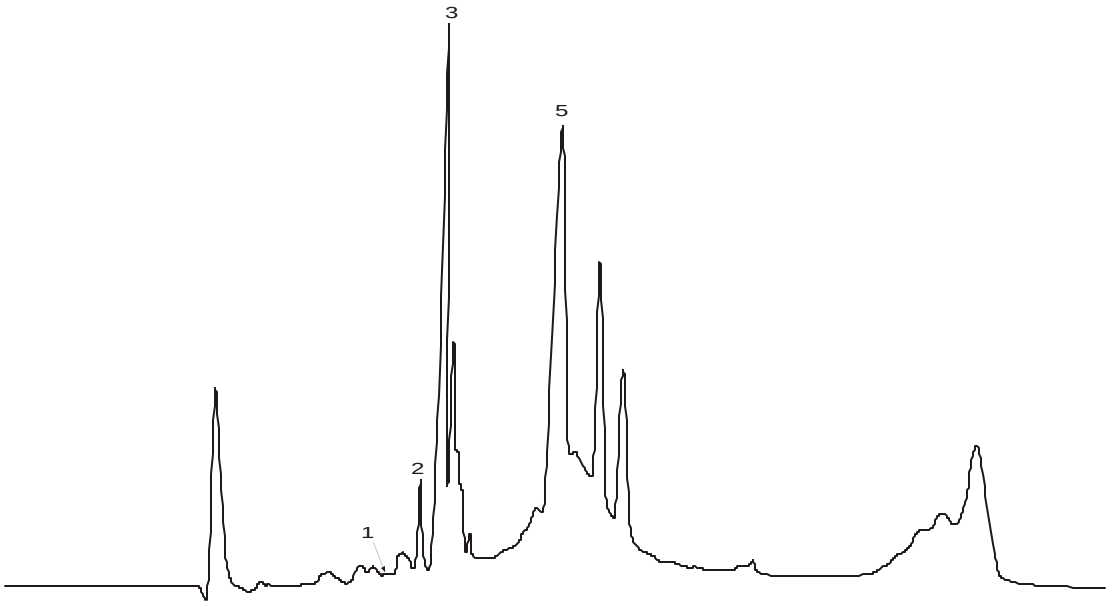
Conditions: As for Figure 2.20.

Figure 2.21 clearly shows the positioning of the four respective flavonoids. From Figure 2.20 it can be seen that this extract contained no significant amount of R, Iqr or Hd. The fingerprint does show some Q, although still not a significant amount. The results obtained from the quantitative analysis in Section 4.7 confirmed that there was no significant amount of R or Hd present in these tablets, although approximately 0.8mg of quercetin per 0.5g tablet was found. Therefore the results obtained here correlate with the results obtained from the HPLC assay of the same tablets.

2.6.4.2 Product F: Burgess and Finch® Herbal Tincture

Figure 2.22 is an electropherogram illustrating the separation of components in the Burgess and Finch® herbal tincture.

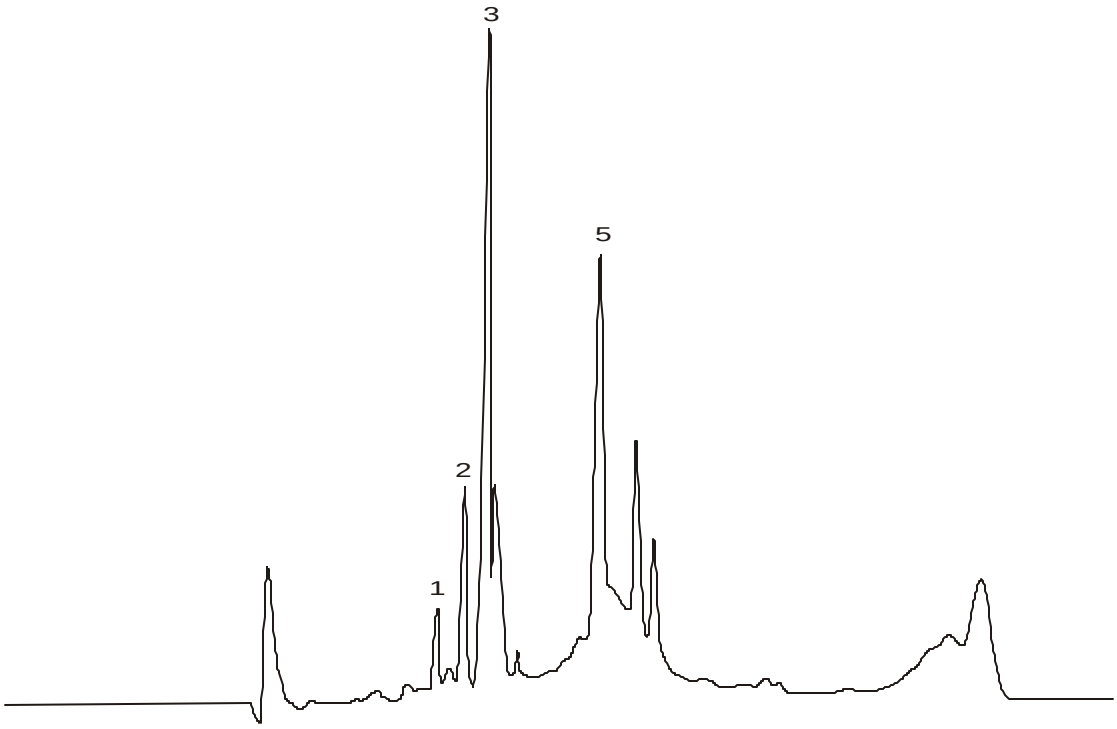
Figure 2.22. Unspiked Burgess and Finch® tincture.



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 5 - Quercetin. Conditions: 25mM sodium borate with 50 mM SDS, pH: 8.9, Applied voltage: 15kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm .

Figure 2.23 illustrates a spiked sample of the Burgess and Finch® tincture.

Figure 2.23. Spiked Burgess and Finch® tincture.



Conditions: As for Figure 2.22

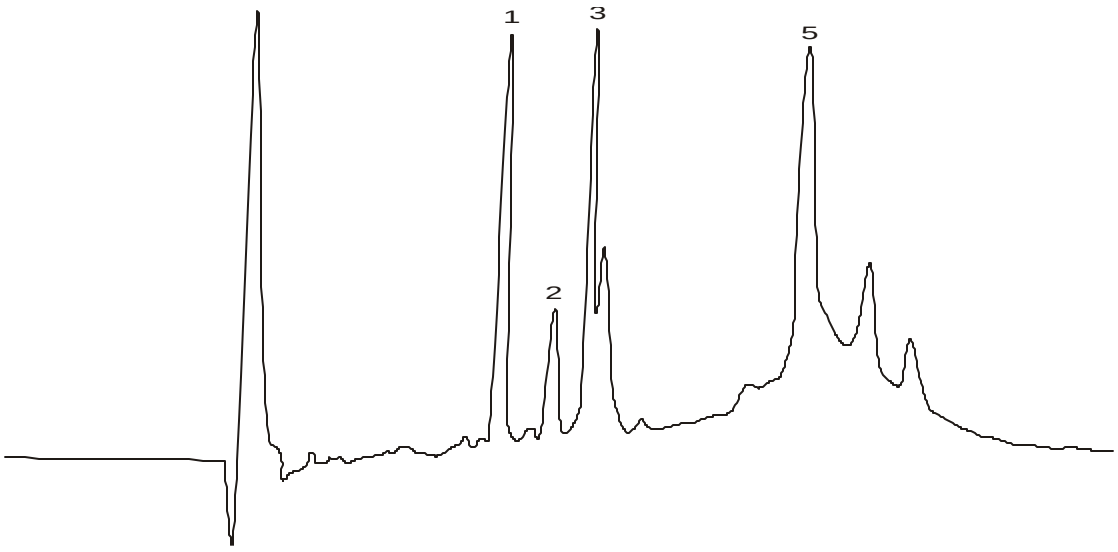
Figures 2.22 and 2.23 illustrate that Iqr, Hd and Q are present in the tincture in significant amounts. Figure 2.22 also illustrates the lack of R present in this tincture.

The peak labelled "5" is observed to be in a different position in Figures 2.22 and 2.23, than in Figure 2.21. This change in migration time could have been a mere effect of matrix interferences. This made fingerprinting of the extracts quite difficult and confirmation of the presence of certain compounds by spiking was necessary.

2.6.4.3 Product G: Flora-Force® Herbal Tincture

Figure 2.24 is an electropherogram illustrating the separation of components in an unspiked sample of the Flora-Force® tincture.

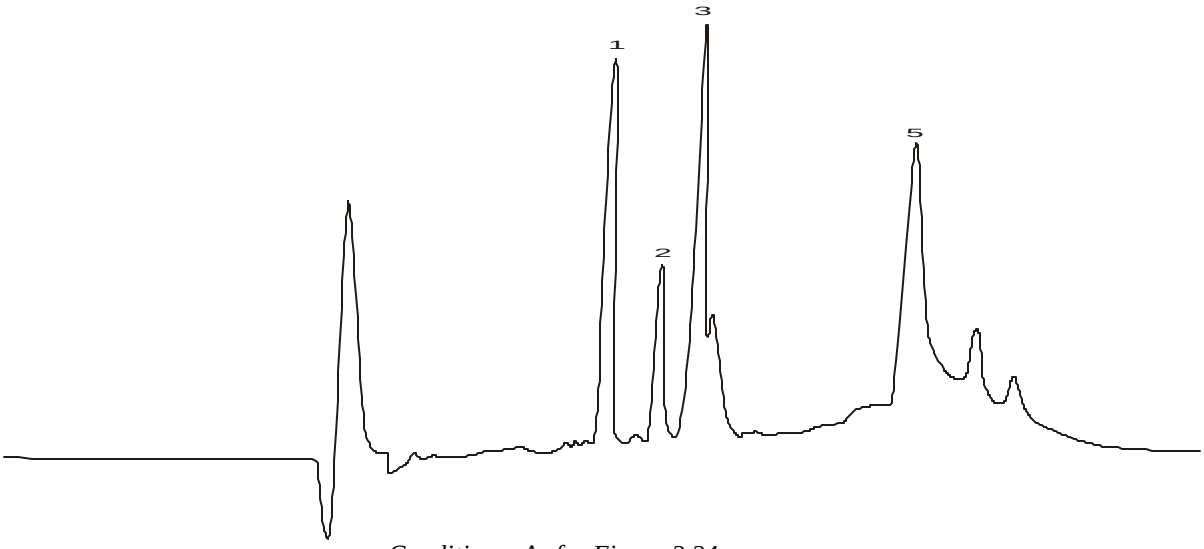
Figure 2.24. Unspiked Flora-Force® tincture.



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 5 - Quercetin. Conditions: 25mM sodium borate with 50mM SDS, pH: 8.9, Applied voltage: 15kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm.

Figure 2.25 is the electropherogram for a spiked sample of Flora-Force® tincture.

Figure 2.25. Spiked Flora-Force® tincture.



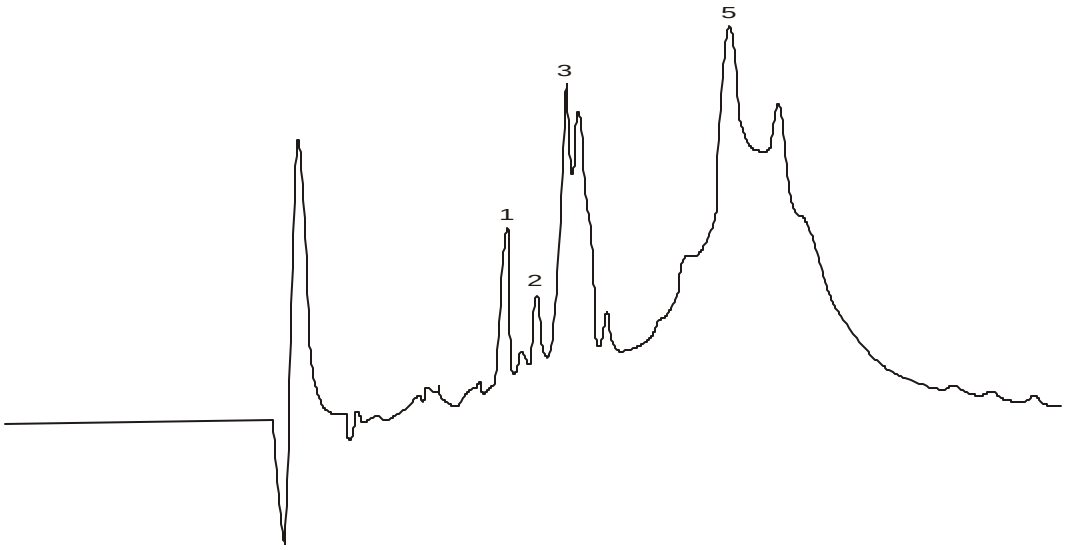
Conditions: As for Figure 2.24

As can be seen from these Figures 2.24 and 2.25, all four flavonoids are present in this tincture in significant amounts.

2.6.4.3 Product H: Phyto-Force® Tincture

Figure 2.26 is an electropherogram illustrating the separation of components in the Phyto-Force® tincture.

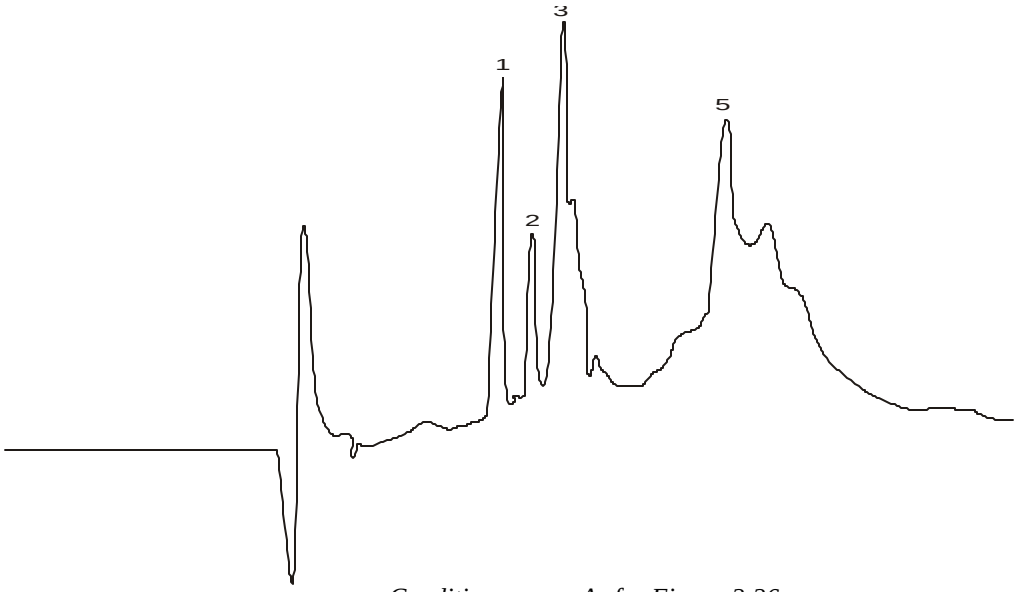
Figure 2.26. Unspiked Phyto-Force® tincture.



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 5 - Quercetin. Conditions: 25mM sodium borate with 50 mM SDS, pH; 8.9, Applied voltage: 15kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm.

The electropherogram of a spiked sample of Phyto-Force tincture is depicted in Figure 2.27.

Figure 2.27. Spiked Phyto-Force® tincture.



Conditions: As for Figure 2.26.

It can be seen that the Phyto-Force® tincture contains all four flavonoids in significant amounts.

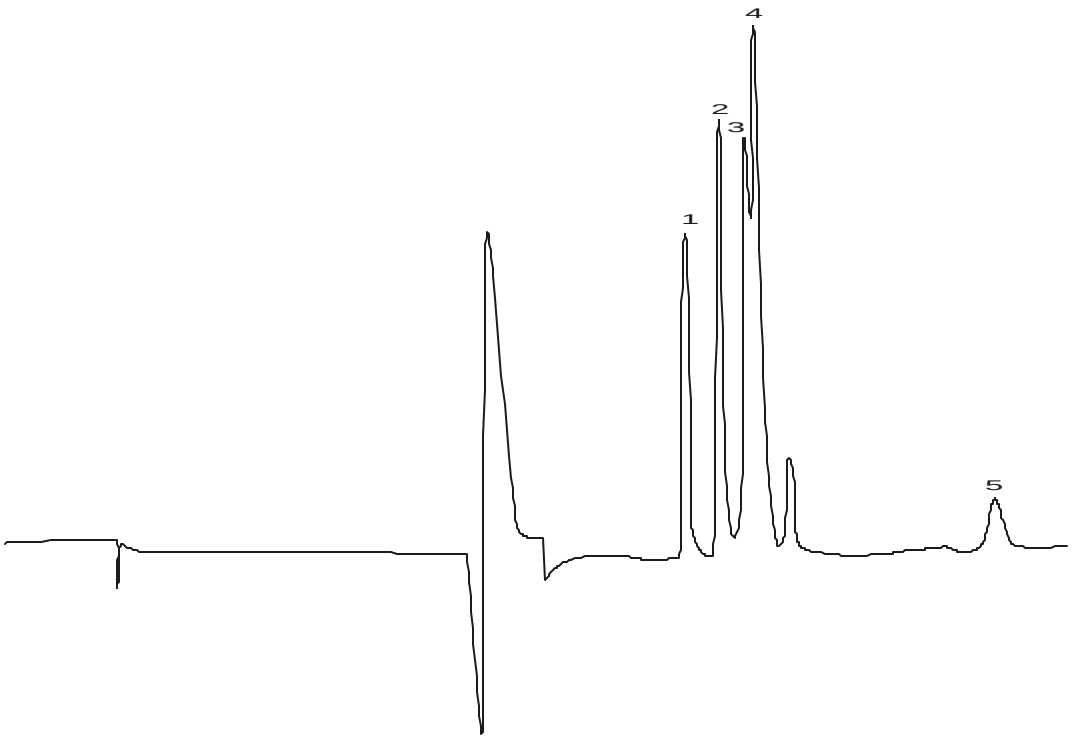
2.6.4.4 Product D: Vitality® Tablets

Product D was the last preparation to be investigated. An SSI UV/VIS detector was used for this analysis, as the Linear UV/VIS Detector that was used for all prior investigations became dysfunctional.

All capillaries used up to this point had been cut from lot GFH03D but the capillary needed replacing and a new capillary was cut from a new batch JGR07B.

An injection of the five flavonoids was performed with these two new features. At this point Qr was procured. This electropherogram is shown in Figure 2.28.

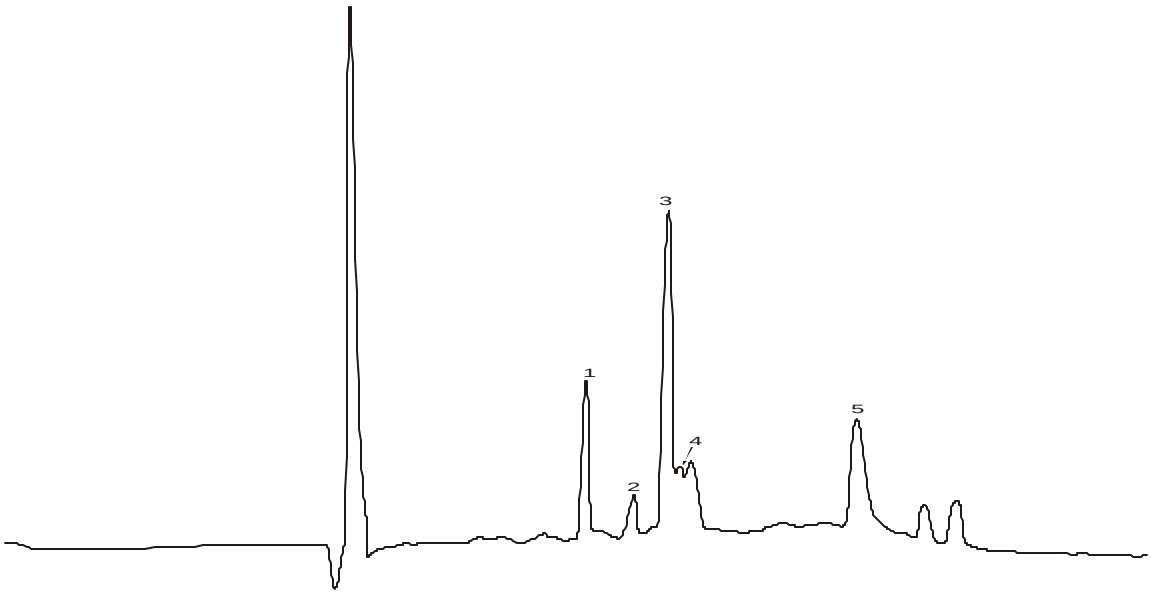
Figure 2.28. Separation of rutin, isoquercitrin, hyperoside, quercitrin and quercetin.



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 5 - Quercetin. Conditions: 25mM sodium borate with 50 mM SDS, pH: 8.9, Applied voltage: 15kV, Injection: 200mbar for 0.1 minutes, Capillary: 50.0ìm ID; total length = 82.0cm; effective length = 53.0cm.

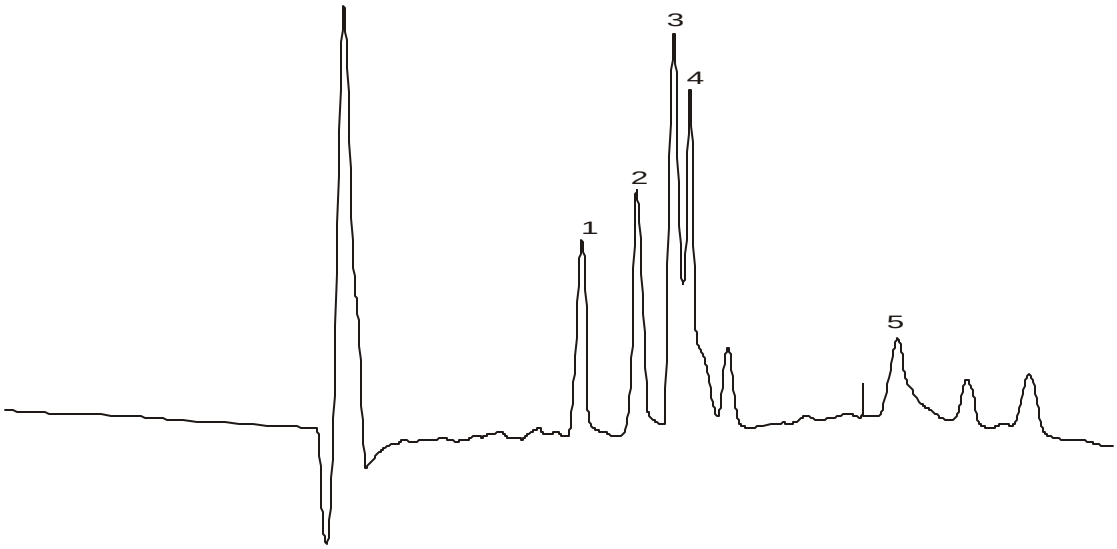
The unspiked and spiked Vitality® extracts are shown in Figures 2.29 and 2.30 respectively.

Figure 2.29. Unspiked Vitality® extract.



Conditions: *As for Figure 2.28.*

Figure 2.30. Spiked Vitality® extract.



Conditions: *As for Figure 2.28.*

The results shown in Figures 2.29 and 2.30 indicate that all five flavonoids were present in the Vitality® tablets used in this investigation. Figure 2.29 shows that this product contains a relatively large amount of Hd, R and Q, with less isoquercitrin and quercitrin. This is in agreement with the

quantitative results obtained in Section 4.7, where these tablets were found to contain approximately 1mg of rutin, 1.4mg hyperoside, 1mg of quercetin and 0.5mg quercitrin per 0.5g tablet.

Peak 5 appears to be smaller in the spiked sample than in the unspiked sample. As discussed at the beginning of this section, this frequently occurred as a result of relative dilution of extract sample upon spiking. The size of the spiked peaks relative to the other peaks present in the spiked electropherograms compared to the relative sizes of the same peaks in the unspiked electropherograms gives a more accurate indication of the relative increase in size of the spiked peaks. In this case however, it appears that the peak shape changed in the unspiked and spiked samples, and the use of peak height to compare spiked and unspiked samples does not appear to give an accurate reflection of the amount of Q present. In this case, judging from the peak area one can see that the spiked sample did in fact have a greater area than the unspiked sample.

2.6.5 CONCLUSIONS

The method developed was found to be suitable for the qualitative analysis and fingerprinting of various commercial St. John's Wort products. This method is advantageous in that it is simple, requires minimal sample preparation and utilises very small quantities of sample. It is cost effective in comparison to HPLC, as very small amounts of organic solvents are used and capillaries are less expensive than HPLC columns. In addition to these advantages, high separation efficiencies and the resolution of a large number of peaks are attainable in a reasonable analysis time.

However, there are drawbacks in that detection limits in CE tend to be an order of magnitude lower than those obtained with HPLC. This is due to the very low volumes of sample that are injected onto the capillary and the short light path through the capillary.

The results obtained in this section were in conformity with the quantitative assay findings of the tablet products, and served to qualitatively illustrate the inter-product variability in terms of content and quality of St John's Wort products on the market.

CHAPTER THREE

METHOD DEVELOPMENT FOR THE SEPARATION OF RELEVANT FLAVONOID COMPOUNDS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Due to the nature and number of different compounds chosen as markers for the analysis of St John's Wort products, development of separate chromatographic and extraction methods for the various groups of compounds was necessary. The flavonoids were the first group of compounds analysed and in this case included quercetin (Q), quercitrin (Qr), kaempferol (K), rutin (R), hyperoside (Hd) and isoquercitrin (Iqr).

3.1 INTRODUCTION

High performance liquid chromatography (HPLC) is an analytical procedure where components of a complex mixture can be separated, identified and quantitatively determined. The separation of sample constituents is facilitated by differences in the partition coefficients of solutes between a stationary and a mobile phase. HPLC has been widely applied to an enormous variety of compounds and has the capability to effect separation with great speed, sensitivity and precision [80,81].

In HPLC, the sample is injected onto the column (stationary phase) whilst mobile phase is permeating through the column. The average rate at which a solute migrates depends upon the average time it spends in the mobile phase. This rate will be less for solutes with partition ratios that favour retention on the stationary phase, and *vice versa* for solutes with partition ratios that favour partition in the mobile phase. Ideally, the differences in affinity of solutes between stationary and mobile phases cause the components in a sample mixture to separate into separate bands located along the length of the column, which then move along and out of the column with the mobile phase.

The object of HPLC is thus to separate sample components within a reasonable time. This separation depends on three main factors: column efficiency, column selectivity and retention.

3.1.1 SEPARATION EFFICIENCY

The original theory of chromatography, called the plate theory, was able to describe migration rates in quantitative terms. This theory envisages the column to be composed of a series of narrow discrete theoretical plates, the movement of the solvent and solute then being viewed as a series of stepwise transfers from each step to the next. At each step, the equilibration of the sample between the mobile and stationary phase is assumed to take place. The efficiency of a chromatographic column as a separation device can then be said to improve as the number of equilibrations or "steps" increase. Thus, the number of theoretical plates, N, is used as a measure of column efficiency. A second term, the height equivalent of a theoretical plate, H, also serves this purpose. The relationship between these two parameters is described by equation 3.1.

$$N = \frac{L}{H} \qquad (3.1)$$

L = Length of column packing (cm)
N = Number of theoretical plates
H = Height equivalent of a theoretical plate (cm)

The use of the plate theory was limited as it failed to describe the effects of numerous variables responsible for zone broadening. This theory was then replaced by the kinetic or rate theory, which is capable of accounting for these variables, however N and H are still used as criteria for the description of column efficiency.

N can be experimentally evaluated from a chromatogram by the substitution into equation 3.2 of the various parameters depicted in Figure 3.1.

$$N = \left[\frac{t_R}{W}\right]^2$$

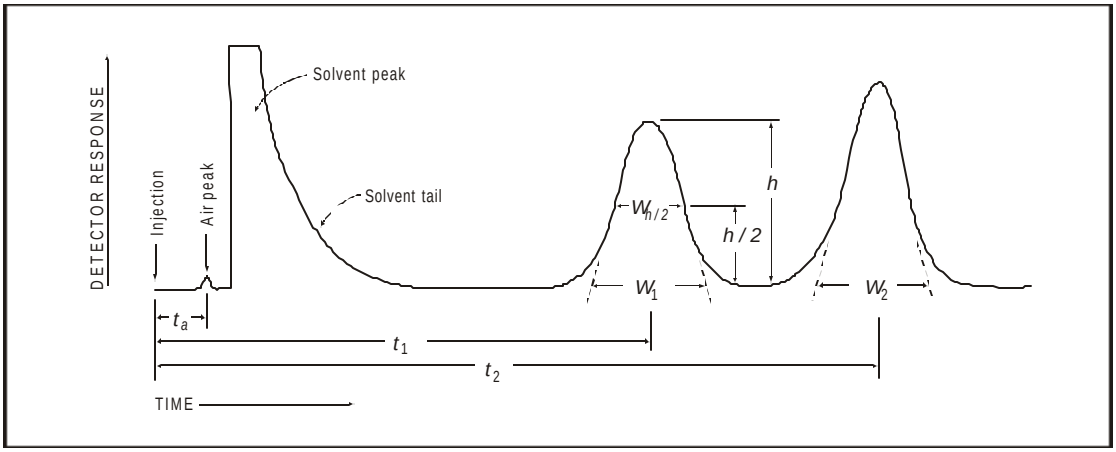
t_R = Retention time of solute
W = Width of peak at base

Or

$$N = 5.54 \left[\frac{t_R}{W_{h/2}}\right] \qquad (3.2)$$

t_R = Retention time of solute
$W_{h/2}$ = Peak width at half-height as shown in Figure 3.1

Figure 3.1. Chromatographic separation of two substances. [18]



The theoretical plate number (N) should ideally be greater than 2000.

3.1.2 CAPACITY FACTOR

The capacity factor (k') is an important constant that is related to the migration rate of the solute. The capacity factor simply describes where the solute peak of interest elutes relative to the solvent front or void volume. Equation 3.3 illustrates how k' can be derived from a chromatogram. t_R and t_M can be obtained from a chromatogram as illustrated in Figure 3.1.

$$k' = \frac{t_R - t_M}{t_M} \qquad (3.3)$$

t_R = Retention time of solute (s)
t_M = Retention time of unretained solute (s)
k' = Capacity factor

The peak of interest should be well resolved from other peaks and the void volume, and thus the capacity factor should be greater than two [82].

3.1.3 SELECTIVITY

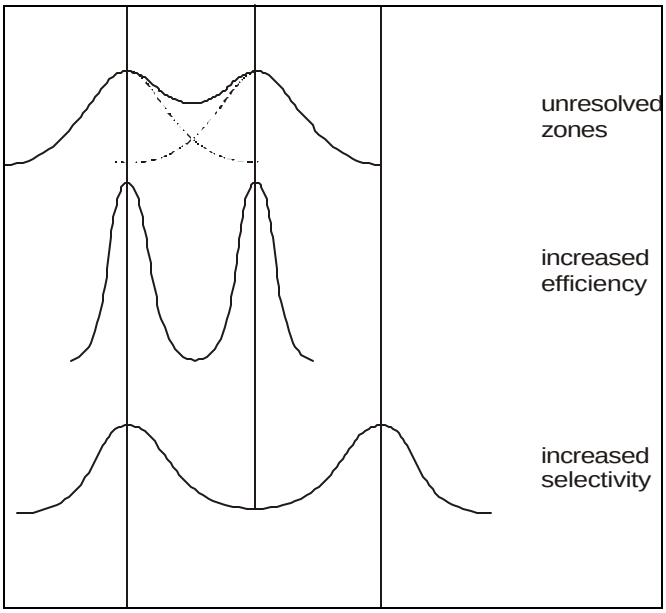
The selectivity factor (α), of a column for 2 solutes is defined as:

$$\alpha = \frac{k'_1}{k'_2} \qquad (3.4)$$

k'_1 = capacity factor for the solute more readily retained on the column
k'_2 = capacity factor for the more rapidly eluting solute

There are two primary ways to improve resolution. These are illustrated in Figure 3.2. The first is by decreasing peak width (zone width) whilst maintaining the zone centre constant, and the second by increasing the distance between the zone centers between the two peaks, whilst holding the peak width constant. The first method involves the efficiency of the column, and the second, the selectivity.

Figure 3.2. Illustration of the difference between increasing efficiency and increasing selectivity.



3.1.4 RESOLUTION

The ability of a column to resolve any two peaks of interest is of primary importance in HPLC. As seen in Section 3.1.3, resolution (R_s) is dependent upon both selectivity and efficiency. Resolution can be calculated from equation 3.5.

$$R_s = \left[\frac{\sqrt{N}}{4}\right]\left[\frac{(\alpha - 1)}{\alpha}\right]\frac{k'_2}{(1 + k'_2)} \qquad (3.5)$$

When two peaks are just resolved to the baseline, this corresponds to a resolution of 1.5 [81].

3.1.5 BACKGROUND OF METHODS USED

Summaries of published HPLC methods used for quantitative analysis of the relevant flavonoids are listed in Table 3.1 below. The column, method of detection, mode of operation, concentration ranges and retention times of the respective compounds are outlined. All of these are methods that have been used to separate flavonoids and/or naphthodianthrones and /or phloroglucinol components in various preparations including St. John's Wort extracts and products.

Table 3.1. Methods to date for the chromatographic separation of the flavonoids.

Reference	Column	Mode	Detection	Concentration Range of each Compound (: g/ml)	Retention times (mins) for each of the Flavonoid Compounds					
					1	2	3	4	5	6
Ganzera, *et al*., 2002 [85]	Synergi MAX-RP C_{12} 4: m 150 x 4.6 mm i.d.	GRADIENT	Photodiode Array 8 = 270nm	2-500	12.0	13.4	12.7	16.5	18.5	-
Li, *et al*., 2001. [86]	Waters YMC ODS-AQ C_{18} 5: m 250 x 4.6 mm i.d.	GRADIENT	Photodiode Array and UV 8 = 270nm	20-200	13.9	14.3	14.1	-	24.0	-
Pietta, *et al*., 2001. [87]	Symmetry C_{18} 5: m 250 x 4.6 mm i.d.	GRADIENT	UV 8 = 280nm	(1): 1mg/ml - injection volume varied	15.0	16.0	16.0	20.0	25.0	-
Brolis *et al*., 1998. [88]	201 TP 54 Vydac C_{18} 5: m 250 x 4.6 mm i.d.	GRADIENT	UV 8 = 270nm	(1): 4.5-100	16.2	16.9	16.5	19.7	28.2	-
Liu, *et al*., 2000. [89]	Phenomenex C_{18} 3: m 250 x 4.0 mm i.d.	GRADIENT	Photodiode Array 8 = 284nm	(1): 500-4000 (2): 250-2000 (3): 300-2400 (4): 200-1000 (5): 200-1000	42.0	42.8	42.5	46.5	50.0	-
AHP, 1997. [25]	Prosep C_{18} 5: m 150 x 4.0 mm i.d.	GRADIENT	UV 8 = 284nm	10-300	11.0	12.0	11.6	13.5	15.5	-

1 - rutin, 2 - isoquercitrin, 3 - hyperoside, 4 - quercitrin, 5 - quercetin, 6 - kaempferol.

The above methods have been used to separate flavonoid compounds along with naphthodianthrones and phloroglucinols, and which generally resulted in run times longer than twenty minutes. As well as this, the separations between rutin, isoquercitrin and hyperoside in all of the methods were not fully resolved as can be seen from their respective retention times in Table 3.1. Importantly, kaempferol was not determined using any of these methods. In the light of these observations, a rapid, accurate and precise HPLC method for the satisfactory separation of the relevant flavonoids, including kaempferol, was developed.

3.2. INSTRUMENTATION

The HPLC system consisted of:

- Waters 2690 Separation Module (Waters, Milford, Massachusetts, USA).
- Waters 2487 Dual Wavelength Absorbance Detector (Waters, Milford, Massachusetts, USA).
- LC-22 Temperature Controller, Model LC-22-240 (Bioanalytical Systems Inc., USA).
- Luna 5:m C18 (2) 150 x 2.00mm column, Part 00F- 4252 - B0 (Phenomenex, Torrance, CA, USA).
- Luna C18 Security Guard column, Cat No. AJO-4286 (Phenomenex, Torrance, CA, USA).

Additional Equipment included:

- Cole-Parmar Ultrasonic Bath, Model 8845-30 (Cole-Parmar Instrument Company, Chicago, Illinois, USA).
- Mettler Dual Range Electronic Balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland).
- Crison GLP21 pH Meter (Laboratory Equipment Company, Port Elizabeth, South Africa).
- Hettich D72 Universal II Centrifuge (Tuttlingen, Germany).

3.3 MATERIALS AND REAGENTS

Orthophosphoric acid (85%) was obtained from Elabtec, Port Elizabeth, South Africa [6].

Refer to Section 2.5.3 for all other materials and reagents

3.4 METHODS

Standard operating procedures (SOP's) were followed for all procedures. The appendix section at the back of this thesis outlines the SOP's for the cleaning of glassware, the use of the balance, the use of the pH meter, the use of the Millipore Milli-Q System and the use of the HPLC system.

The method used by Hasler [83] to separate the flavonoid aglycones and biflavones in *Gingko biloba* (including quercetin, kaempferol, and other compounds not found in St John's Wort) was adapted for the separation of the six relevant flavonoids. The separation of these flavonoid aglycones and biflavones was performed using gradient elution with 3 solvents, namely methanol, tetrahydrofuran and 0.5% phosphoric acid. Hasler used a Nucleosil 100-C_{18}, 3:m (100 x 4mm i.d.) column, a flow rate of 1.0ml/min, a temperature of 30°C and achieved separation of these compounds in twenty-five minutes. Studies in our laboratory illustrated that due to the higher eluting power of acetonitrile, methanol can be replaced by acetonitrile, and the tetrahydrofuran omitted. This modified method is illustrated in Table 3.2.

Table 3.2. Gradient 1. Modified gradient for the initial separation of flavonoids in St John's Wort.

Solvent A: Acetonitrile
Solvent B: 0.5% Phosphoric acid pH = 1.5

Gradient:	Time (minutes)	% A	% B
	0	20	80
	3.00	20	80
	3.01	40	60
	8.00	30	70
	20.00	20	80

Conditions: Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm; Detection l : 254nm; Temperature: 48°C; Injection volume: 5 : l.

The effects of altering the chromatography by varying different parameters and variables were investigated systematically in an attempt to improve the separation of the six flavonoids.

3.4.1. SELECTION OF ANALYTICAL COLUMN

Due to the relatively low concentrations of marker compounds, and high likelihood of interference in tinctures and extracts, a 2.00mm i.d. narrow bore column was selected primarily to increase resolution and also possibly selectivity and sensitivity of the method.

Over the past two decades, the most widely used analytical HPLC column has been the 4.6mm i.d. (internal diameter) column. The use of this column has been dictated primarily by the limitations of the HPLC instrumentation. With the vastly improved "state of the art" instrumentation, particularly in the area of miniaturisation, it is now possible to utilize reduced column bores and still maintain efficiency.

The use of narrow bore columns has several advantages over the more commonly used wider bore (4.6mm i.d.) column. The low flow rates (200 -300 : l/minute) associated with narrow bore columns result in a significant decrease in amount of solvent used with a consequent decrease in cost. Narrow bore columns also provide a potential gain in efficiency together with high-resolution capabilities, which is often associated with improvement in sensitivity. An additional important advantage is the reduced consumption of relatively toxic organic solvents which has environmental implications.

It has been noted that narrow bore HPLC columns can and will play an important role in analysis of samples where the amount of sample available is limited. This is due to the relatively greater sensitivity and smaller injection volumes or concentration of sample necessary for analysis [86].

3.4.2 CHOICE OF WAVELENGTH

The choice of wavelength was 254nm as reported in Section 2.5.5. A secondary wavelength chosen was 350nm in the event of any interferences which may occur with tablet extracts when monitoring at 254nm.

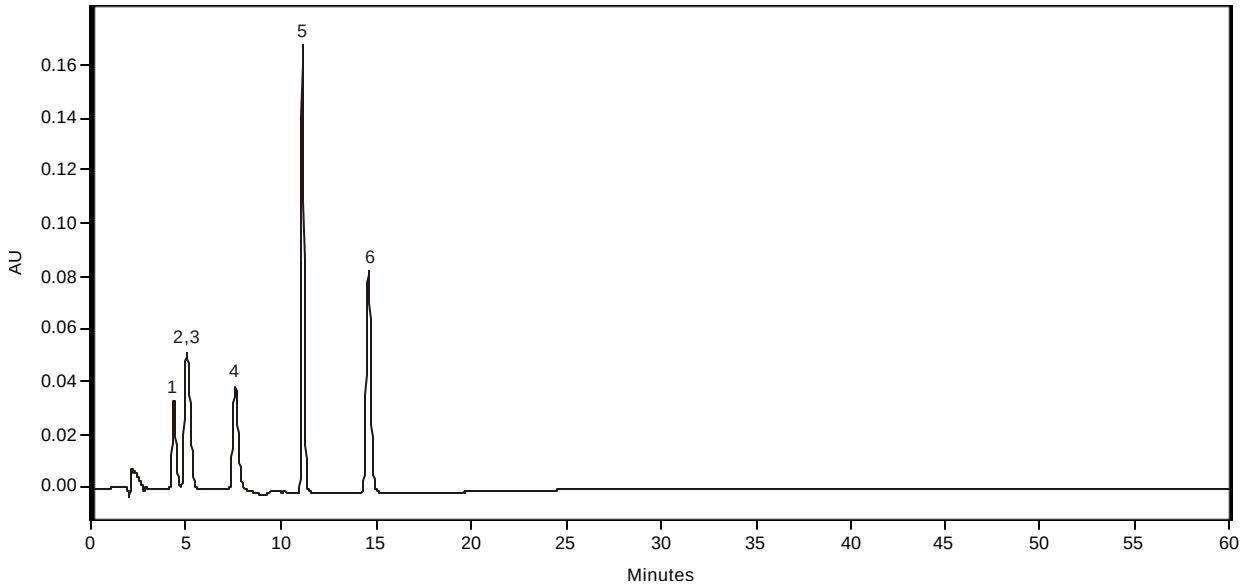
3.4.3 MOBILE PHASE SELECTION

The mobile phase selected consisted of acetonitrile and 0.5% phosphoric acid (pH = 1.5). A gradient was used to affect separation, as seen in Table 3.2.

3.5 RESULTS AND DISCUSSION

Figure 3.3 illustrates the separation of the flavonoids using the gradient system described in Table 3.2.

Figure 3.3. Chromatogram illustrating the flavonoid separation using the gradient profile illustrated in Table 3.2.



Conditions: Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm; Detection 8: 254nm; Temperature: 48°C; Injection volume: 5 : l.

In Figure 3.3 it can be seen that Iqr (2) and Hd (3) are unresolved. Resolution between R (1) and Iqr (2) is 1.393. An increase in resolution between the first three peaks (1, 2 and 3) was necessary to improve the separation. The k' of R (1) was 0.95, and the k' of Iqr (2) was 1.25 hence improved resolution was also required. The separations between Qr (4), Q (5) and K (6), however, were found to be acceptable.

Since separation of Qr, Q and K was acceptable with the initial gradient and remained so with all the gradient changes mentioned below, optimization of the gradient was primarily intended to improve the separation of R, Iqr and Hd, whilst maintaining the separation of the other three flavonoids. A summary of the significant investigations and the course that was followed is included as follows.

The initial changes made to Gradient 1 involved altering the time for the acetonitrile percentage to increase from 20 to 40 percent from 3 minutes into the gradient. The effect of increasing the percentage of acetonitrile was also investigated - taking the percentage up to 45 %. None of these

changes resulted in an improvement of chromatographic separation of Iqr and Hd. In fact the separation did not change very much at all.

The initial concentration of acetonitrile was then increased to 30% whilst maintaining the rest of the gradient as above. This did not improve resolution between isoquercitrin and hyperoside, and retention times decreased substantially causing a decrease in k' of R (1) and Iqr (2) to less than those obtained with Gradient 1.

After beginning the investigation with 20% acetonitrile at the start of the gradient, and increasing this to 30%, the next step was to decrease this percentage to 10%. This gradient, Gradient 2, is illustrated in Table 3.3.

Table 3.3. Gradient 2. Conditions: As per Table 3.2.

Time (minutes)	% A	% B
0	10	90
3	10	90
5	40	60
8	30	70
20	20	80

This change resulted in a value of 0.116 for the k' for R (1) and non-resolution of Iqr (2) and Hd (3).

Hd (3) and Iqr (2) remained unresolved with the use of this gradient, although slight separation was beginning to take place. An improvement occurred from the initial separation in the resolution between R (1) and the second merged peak (peaks 2 and 3 were merged), the resolution here was 2.56. The capacity factor, k' of rutin was 0.11 and k' of the second merged peak (peaks 2 and 3) was 0.53,- neither improving from the time before, and neither being acceptable.

The time of gradient change shown in Table 3.3 was sequentially altered between 3 and 20 minutes, maintaining the initial percentages as shown in Table 3.2. These changes decreased resolution between the first 3 peaks (1, 2 and 3) from that shown in Figure 3.5. The initial percentages were then changed to 5% solvent A and 95 % solvent B, with the gradient described in Table 3.3. No improvement was observed.

The effect of an increase in initial percentage of acetonitrile was then investigated, increasing the percentage to 15% as shown in Table 3.4.

Table 3.4. Gradient 3. Conditions: As per Table 3.2.

Time (minutes)	% A	% B
0	15	85
3	15	85
5	45	55
8	30	70
20	20	80

In this case, Hd (3) and Iqr (2) were unresolved, and the resolution between R (1) and the second merged peak (2 and 3) decreased from 2.56 in Gradient 2 to 1.45. The capacity factor k', of R increased to 0.98, and k' of the second peak increased to 1.29.

The initial isocratic portion of the gradient was lengthened from 3 minutes to 10minutes in an attempt to increase the retention time of the first three peaks, R (1), Iqr (2) and Hd (3), in order to increase k' to more acceptable values and improve resolution. The total run time for the gradient was also increased.

Table 3.5. Gradient 4. Conditions: As per Table 3.2.

Time (minutes)	% A	% B
0	16	84
10	16	84
12	45	55
15	30	70
30	20	80

Gradient 4 caused an increase in k' of R (1) and Iqr (2) to 2.55 and 2.96 respectively which was acceptable. This gradient resulted in the partial separation of Iqr (2) and Hd (3), but still not sufficiently adequate. The resolution between R (1) and the second merged peak (2 and 3) improved from Gradient 3 to 1.44. The increase in the isocratic portion of the gradient was retained for subsequent investigations, along with the overall increase in gradient run time, and changes in initial percentage of acetonitrile.

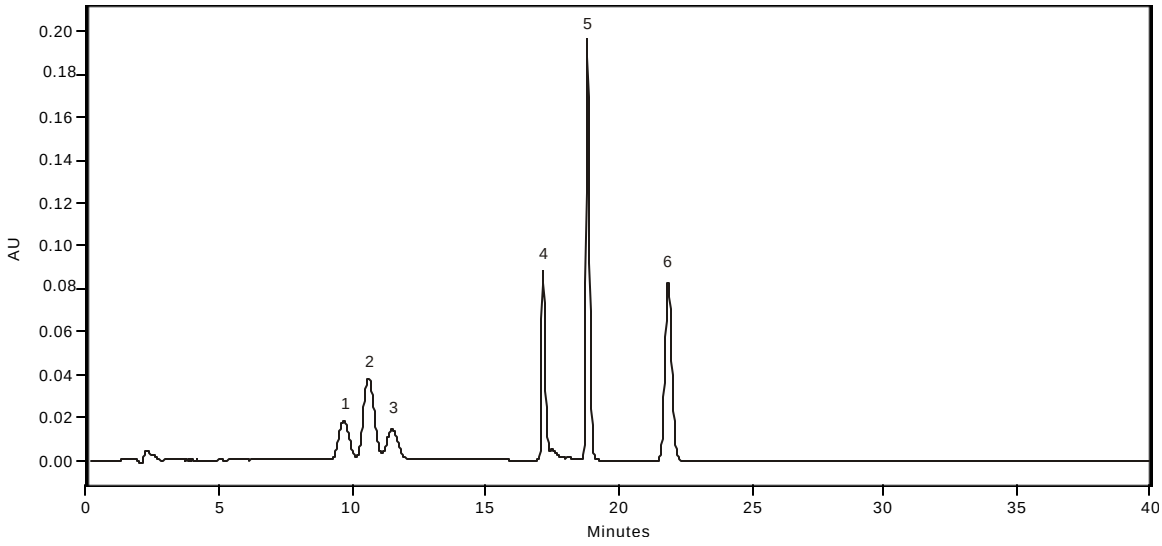
Gradient 5 involved a slight decrease in the initial percentage of acetonitrile, with an improvement in separation of the first three flavonoids compared to Gradient 4. Resolution between the last three peaks was excellent with Gradient 4, and the time for the last peak to elute was less than 23 minutes, hence gradient 5 involved a decrease in total run time from 30 to 25 minutes.

Table 3.6. Gradient 5. Conditions: As per Table 3.2.

Time (minutes)	% A	% B
0	15	85
10	15	85
12	45	55
15	30	70
25	20	80

Figure 3.4 illustrates the resultant chromatogram obtained with Gradient 5. From Figure 3.4 it can be seen that resolution between Hd (3) and Iqr (2) improved, being 1.12 and resolution between R (1) and Iqr (2) decreased to 1.26. The capacity factor, k' of both R (1) and Iqr (2) were acceptable, at 3.26 and 3.67 respectively. The decrease in overall run time had little affect on the separation of Qr (4), Q (5) and K (6), although Qr's (4) retention time increased slightly, bringing Qr (4) and Q (5) closer together.

Figure 3.4. Separation of the flavonoids with Gradient 5.



1 - rutin, 2 - isoquercitrin, 3 - hyperoside, 4 - quercitrin, 5 - quercetin, 6 - kaempferol. Conditions - Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection: 254nm; Temperature: 48°C; Injection volume: 5 : l.

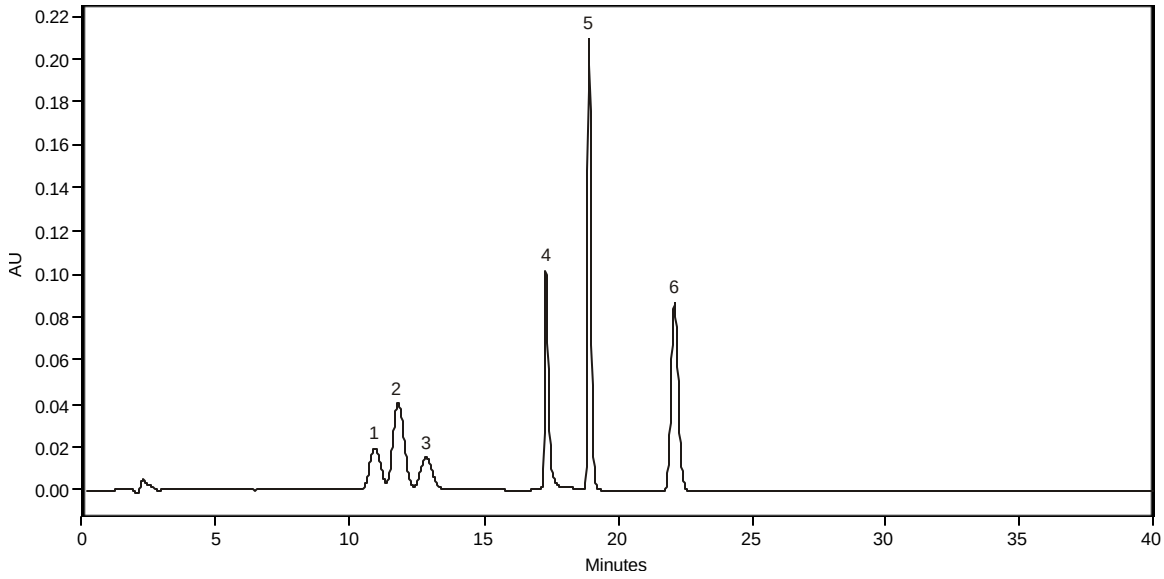
Gradient 6 involved a slight decrease in initial acetonitrile percentage, with the initial acetonitrile percentage set at 14.5%.

Table 3.7. Gradient 6. Conditions: As per Table 3.2.

Time (minutes)	% A	% B
0	14.5	85.5
10	14.5	85.5
12	45	55
15	30	70
25	20	80

As illustrated in Figure 3.5, resolution between R (1), Iqr (2) and Hd (3) was the most acceptable thus far, resolution between R and Iqr being 1.44, and between Iqr and Hd being 1.28. The capacity factors k' for R and Iqr were also acceptable - 3.80 and 4.19, respectively. The total run time was less than 23 minutes and the separation between Qr (4), Q (5) and K (6) was very good.

Figure 3.5. Separation of the flavonoids with Gradient 6.



1 - rutin, 2 - isoquercitrin, 3 - hyperoside, 4 - quercitrin, 5 - quercetin, 6 - kaempferol. Conditions - Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection l : 254nm; Temperature: 48°C; Injection volume: 5 : l.

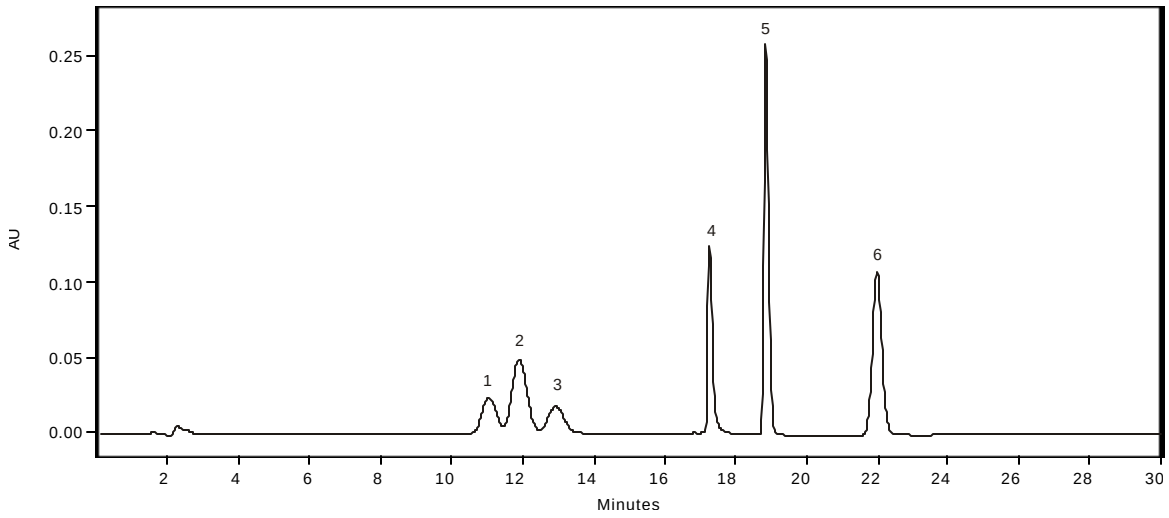
Using Gradient 7, R (1), Iqr (2) and Hd (3) eluted between 11 and 13 minutes. In an attempt to increase resolution between these three compounds, the gradient between 10 and 12 minutes in Gradient 6 (refer to Table 3.7) was increased between 10 and 15 minutes.

Table 3.8. Gradient 7. Conditions: As per Table 3.2.

Time (minutes)	% A	% B
0	14.5	85.5
10	14.5	85.5
15	45	55
25	20	80

This gradient produced the best separation thus far, although there was no improvement in the resolution between R (1) and Iqr (2), nor between Iqr (2) and Hd (3), compared to Gradient 6. As illustrated in Figure 3.6, separation of the 6 compounds was similar to that obtained using Gradient 6, resolution being 1.12 and 1.26 respectively for the first 3 compounds, R (1), Iqr (2) and Hd (3). As expected, the k's for the first 2 peaks, R (1) and Iqr (2) were acceptable and also remained similar to those obtained using Gradient 6.

Figure 3.6. Separation of the flavonoids with Gradient 7.



1 - rutin, 2 - isoquercitrin, 3 - hyperoside, 4 - quercitrin, 5 - quercetin, 6 - kaempferol. Conditions - Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection: 254nm; Temperature: 48°C; Injection volume: 5 : l.

As can be seen from Gradients 4, 5 and 6, the respective decreases in initial acetonitrile concentration increased the resolution between R (1), Iqr (2) and Hd (3). Using Gradient 8, a further decrease in initial acetonitrile concentration was investigated, whilst retaining other remaining aspects of Gradient 7.

Table 3.9. Gradient 8. Conditions: As per Table 3.2.

Time (minutes)	% A	% B
0	14	86
10	14	86
15	45	55
25	20	80

Gradient 8 resulted in a slight decrease in resolution between the first 3 peaks, resolution between R (1) and Iqr (2) being 1.06 and between Iqr (2) and Hd (3) being 1.25. The capacity factor, k', for R (1) and Iqr (2) increased marginally when compared with the results obtained from Gradient 7.

Gradient 9 involved further decreasing the initial percentage of acetonitrile.

Table 3.10. Gradient 9. Conditions: As per Table 3.2.

Time (minutes)	% A	% B
0	13	87
10	13	87
15	45	55
25	20	80

Rutin (1) and Iqr (2) were unresolved in this case, with the first peak (a merged peak consisting of R and Iqr) being incompletely resolved from Hd (3). The retention time of both peaks were sufficient to result in acceptable k' values. However, although the k's were acceptable, the peaks were unresolved.

Since Gradient 6 had resulted in the best separation, the respective percentages of Solvent A and B in Gradient 6 were retained, and an investigation into the effects of changing the times of solvent mixing (Gradient 10) was performed.

Table 3.11. Gradient 10. Conditions: As per Table 3.2.

Time (minutes)	% A	% B
0	14.5	85.5
6	14.5	85.5
15	45	55
25	20	80

As can be seen from Table 3.11, the respective times for gradient changes were altered. The resulting resolution between the first 3 peaks (R, Iqr and Hd) was not as good as that for Gradient 6, being 1.26 and 1.15 respectively, but were comparable to those obtained with Gradient 7. The k' values for R (1) and Iqr (2) were still acceptable, although slightly less than those using Gradient 6 and 7 (3.74 and 4.12 respectively).

Gradient 11 involved a minor alteration in the time of gradient change and resulted in similar resolution between the first 3 peaks (R, Iqr and Hd) as Gradient 10. The resolution between R (1) and Iqr (2) was 1.13, and the resolution between Iqr (2) and Hd (3) was 1.25.

Table 3.12. Gradient 11. Conditions: As per Table 3.2.

Time (minutes)	% A	% B
0	14.5	85.5
8	14.5	85.5
16	45	55
25	20	80

The capacity factors, k' of both R (1) and Iqr (2) remained acceptable.

Table 3.13. Gradient 12. Conditions: As per Table 3.2.

Time (minutes)	% A	% B
0	14.5	85.5
12	14.5	85.5
16	45	55
25	20	80

A further minor adjustment to produce Gradient 12 involved a small change in time (see Table 3.13) and did not make any significant difference to the resolution and k' of the respective peaks of interest, R (1), Iqr (2) and Hd (3).

Gradients 6 and 7 were subsequently selected as the most promising potential gradients for further optimization and development because the resolution and k' factors for the relevant flavonoid compounds were deemed satisfactory. Subsequent investigations involved the effect of addition of various percentages of methanol to the acetonitrile, or Solvent A.

3.5.1 EFFECT OF ADDITION OF METHANOL TO THE GRADIENT SYSTEM

Although the resultant resolution with Gradient 6 was slightly better than that obtained with Gradient 7, the latter gradient was selected for continuation of method development because the gradient itself was simpler than Gradient 6, and therefore possibly more stable in terms of reproducibility.
As shown in Table 3.14, Gradient 7 was selected, with an addition of 5% methanol to Solvent A. Flow rate was maintained at 0.2ml/min and the temperature maintained at 48°C.

Table 3.14. Gradient 7 with 5% methanol in Solvent A. Conditions: as per Table 3.2.

Time (minutes)	% A	% B
0	14.5	85.5
10	14.5	85.5
15	45	55
25	20	80

There was an overall decrease in resolution and k' of R (1), Iqr (2) and Hd (3) in comparison to Gradient 7. In order to confirm the effect of further addition of methanol, an addition of 10% methanol to Solvent A was subsequently investigated.

All conditions were maintained as for Gradient 7, except for the addition of 10% methanol to Solvent A.

The addition of 10% methanol caused a significant decrease in resolution between the first 3 peaks of the chromatogram (R, Iqr and Hd), and resolution was incomplete between these three peaks.

Although the overall run-time for all 6 peaks remained as for Gradient 13 (22.5 minutes), the first 3 peaks (R, Iqr, Hd) eluted 2 minutes later with Gradient 14 than Gradient 13. This resulted in an increase in capacity factors for R (1) and Iqr (2), compared with those obtained with Gradient 13.

Since an increase in percentage methanol from 5 to 10% did not improve resolution, a smaller percentage of methanol (3%) was added to Solvent A.

Addition of 3% methanol to Solvent A resulted in acceptable k's (4.71 for the merged peak comprising R and Iqr, and 5.02 for Hd). Improved resolution resulted compared to the addition of 5% or 10% methanol, however, overall, the resolution was not as good as that obtained previously with Gradients 6 or 7.

The effect of increasing flow rate to 0.5ml/minute with 3% and 10% methanol was then investigated. Although the peaks were much sharper with the methanol addition, they eluted more rapidly than with 0.2ml/minute flow rate, and resolution was not improved for the first 3 peaks (R, Iqr, Hd).

3.5.2 OPTIMIZATION OF FLOW RATE

Once again, Gradient 7 was selected as the starting point for this investigation.

All parameters were the same as for Gradient 7, except the flow rate was increased to 0.3ml/minute to produce Gradient 16.

As expected, there was a decrease in overall run time and retention times for all 6 compounds, and a decrease in k' values. The peaks were sharper than with 0.2ml/minute flow rate, although resolution between R (1), Iqr (2) and Hd (3) was not as good as that obtained with Gradient 6 and 7. Capacity factors of R (1) and Iqr (2) were 3.75 and 4.14 respectively.

Using this Gradient, R (1), Iqr (2) and Hd (3) all eluted before the gradient change at 10 minutes, under isocratic conditions.

Maintaining the flow rate at 0.3ml/minute, and all other parameters as for Gradient 7, R (1), Iqr (2) and Hd (3) were injected 3 times and run isocratically, each time changing the percentages of Solvent A and B in the proportions 14:86, 13:87 and 14.5:85.5. This was in order to see whether a flow rate of 0.3ml/minute was optimal when the initial ratio of Solvent A to Solvent B was 14.5:85.5. It was found that this latter ratio of solvents A and B was optimal. Resolution between R (1) and Iqr (2) was 1.17 and resolution between Iqr and Hd (3), 1.37

Gradient 17 consisted of the same conditions as Gradient 16 except the flow rate was set to 0.4ml/minute.

The resultant peaks were sharper than with any of the previous Gradients and the retention times for all peaks were greatly reduced. Resolution was no better than that obtained with Gradient 6, 7, or even Gradient 16, and there was a large amount of baseline drift between 14 and 25 minutes with this gradient. Resolution between R (1) and Iqr (2) was 1.16, and resolution between Iqr (2) and Hd (3) was 1.33.

An investigation into altering the initial time of change of gradient was conducted (Gradient 18), using 0.3ml/minute, and all other parameters remaining as for Gradient 6. The gradient was changed as illustrated in Table 3.15.

Table 3.15. Gradient 18. Conditions: As per Table 3.2, with flow rate: 0.3ml/min.

Time (minutes)	% A	% B
0	14.5	85.5
10	14.5	85.5
12	45	55
25	20	80

The capacity factors from this gradient were acceptable, with resolution being comparable to Gradient 7 and slightly less than that obtained with Gradient 6. Resolution between R (1) and Iqr (2) was 1.17, and resolution between Iqr (2) and Hd(3) was 1.38.

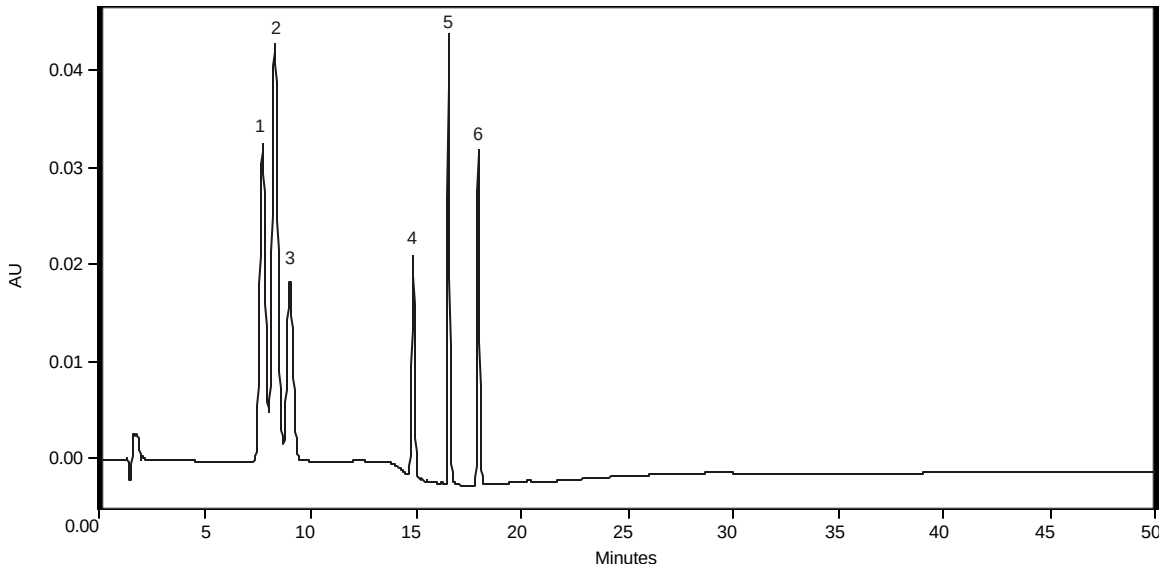
Gradient 19 involved a change in gradient as depicted in Table 3.16 with all other parameters maintained as for Gradient 18.

Table 3.16. Gradient 19. Conditions: As per Table 3.2, with flow rate: 0.3ml/min.

Time (minutes)	% A	% B
0	14.5	85.5
10	14.5	85.5
12	40	60
25	30	70

The resolution of R (1) and Iqr (2), and Iqr (2) and Hd (3) in Gradient 19 were comparable to that obtained with Gradient 6, being 1.19 and 1.36 respectively. As can be seen from Figure 3.7 below, the total run time for Gradient 19 was just over 18 minutes, whereas with Gradient 6, total run time was 23 minutes, and the peaks with Gradient 19 were much better compared to those obtained with Gradient 6.

Figure 3.7. Separation of 6 flavonoids with Gradient 19.



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 4 - Quercitrin, 5 - Quercetin, 6 - Kaempferol. Conditions: Flow rate: 0.3ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection: 254nm; Temperature: 48°C; Injection volume: 5 : l.

Gradient 19 was consequently chosen as the Gradient of choice for further development.

3.5.3 EFFECT OF pH

The pH of Solvent B up to this point had been maintained at 1.5. The pH of the 0.5% phosphoric acid was increased to 3.0 in order to investigate if a change in pH would improve the separation. All other conditions for Gradient 20 were the same as for Gradient 19.

The resultant resolution between R (1) and Iqr (2) was 1.14, and resolution between Iqr (2) and Hd (3) was 1.40. As can be seen, increase in pH did not have a great effect upon resolution. However, total run time increased slightly.

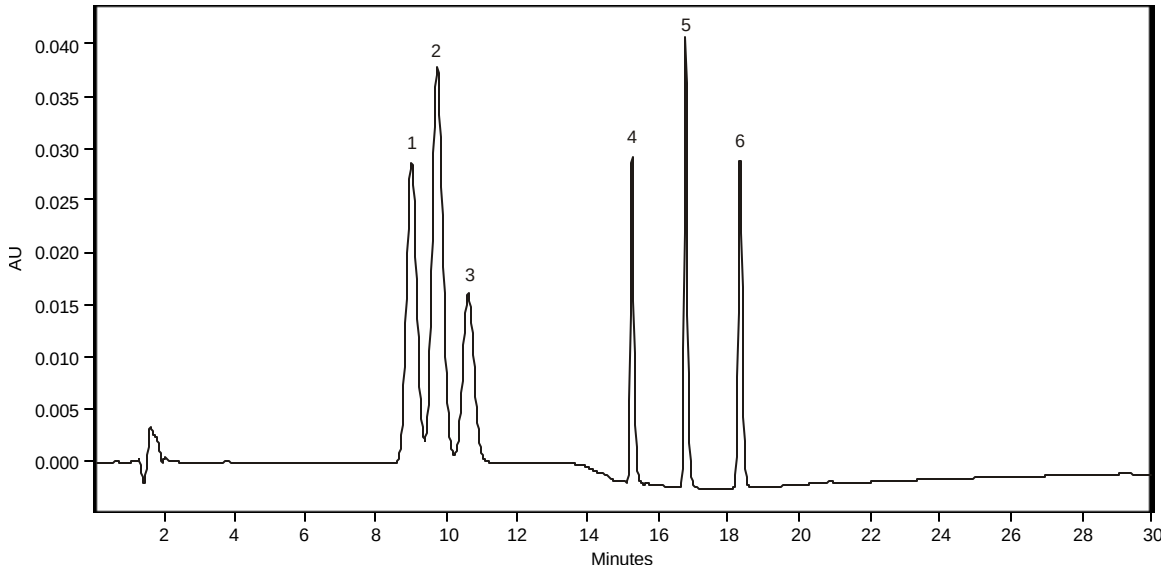
3.5.4 EFFECT OF TEMPERATURE

All investigations previously used a temperature of 48°C. The temperature was reduced first to 42°C, then further reduced to 30°C, and then finally allowed to equilibrate at room temperature (22°C).

Gradient 21 involved retaining all the conditions as for Gradient 19, except that the column temperature was maintained at 42°C.

As can be seen from the chromatogram below (Figure 3.8), the resolution between R (1), Iqr (2) and Hd (3) improved with the temperature held at 42°C. The resolution between the first two peaks (R and Iqr) was 1.28 and resolution between Iqr (2) and Hd (3) was 1.54. Total run time increased very slightly to 18.5 minutes.

Figure 3.8. Separation of 6 flavonoids with Gradient 21.

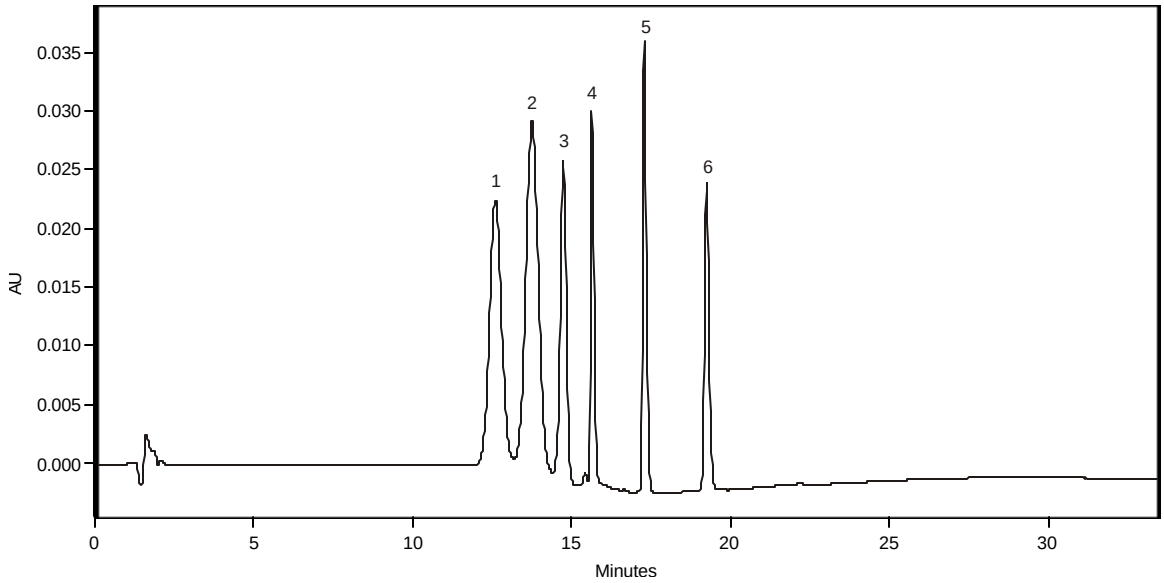


1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 4 - Quercitrin, 5 - Quercetin, 6 - Kaempferol. Conditions: Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection: 254nm; Temperature: 42℃; Injection volume: 5 : l.

Gradient 22 involved maintaining all the conditions above with a temperature of 30°C.

The results obtained indicated an improvement in resolution between the first 3 compounds compared to the previous gradient. Resolution between R (1) and Iqr (2) was 1.66, and between Iqr (2) and Hd (3) was 1.92. The first 3 peaks (R, Iqr and Hd) elute after the isocratic portion of the gradient, causing all 6 peaks to elute relatively close to one another as can be seen in Figure 3.9.

Figure 3.9. Separation of 6 flavonoids with Gradient 22.



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 4 - Quercitrin, 5 - Quercetin, 6 - Kaempferol. Conditions: Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection: 254nm; Temperature: 30°C; Injection volume: 5 : l.

A further increase in resolution by causing the first 3 compounds to elute more rapidly and therefore within the isocratic portion of the gradient was attempted (Gradient 23) by increasing the flow rate to 0.4ml/min whilst maintaining all other parameters as for Gradient 22.

The first 3 peaks did elute within the isocratic portion of the gradient, but the resolution obtained was similar to that using Gradient 22 (R_s between R and Iqr was 1.44 and R_s between Iqr and Hd was 1.72).

A further minor change, involving the reduction to room temperature (22°C) was attempted (Gradient 24).

Although the resolution between R (1) and Iqr (2) was very good (1.82), resolution between Iqr (2) and Hd (3) was 1.27 and not as good as that obtained with Gradient 22.

From the above results it was thus concluded that Gradient 22 was the method of choice for the separation of the 6 flavonoid marker compounds for St John's Wort products.

3.6 CONCLUSIONS

An HPLC method used by Hasler [83] to separate the flavonoid aglycones and biflavones in *Gingko biloba* (including Q, K, and other compounds not found in St John's Wort) was adapted and modified for the separation of the relevant six flavonoids in St John's Wort (including R, Iqr, Hd, Qr, Q and K). The effects of altering the chromatography by varying different parameters and variables were investigated systematically in an attempt to improve the separation of the six flavonoids. This investigation included varying the gradient, optimizing the ratios of solvents in the isocratic portion of the gradient, investigating the effects of addition of methanol to the mobile phase, optimizing the flow rate, investigating the effects of pH and the effects of temperature.

A rapid, reliable and accurate method for the quantitative analysis of 6 relevant flavonoids in St. John's Wort was developed. Details of this gradient elution system are depicted in Table 3.17 below.

Table 3.17. Gradient 22.

Time (minutes)	% A	% B
0	14.5	85.5
10	14.5	85.5
12	40	60
25	30	70

Using this method, the six flavonoids were satisfactorily separated within 20 minutes and the separation is illustrated in Figure 3.9.

The method developed is currently the only known method that separates all six relevant flavonoids in a reasonable run time (less than 20 minutes). It is also one of the few methods that has sufficient separation between R, Iqr and Hd. R, Iqr and Hd are not fully resolved in any of the methods listed in Table 3.1, and of those methods in that table, only 2 result in run times of less than 20 minutes. In addition, the method developed utilised a narrow bore column (2.00 mm, i.d.), which is advantageous in terms of cost, sensitivity, resolution and where only small sample volumes are available. Furthermore, it has the benefit of reducing the volume of toxic organic solvents.

Flavonoid compounds are common to many plants and plant products. When flavonoid glycosides (for example R, Iqr, Hd and Qr) are exposed to extreme conditions of harvesting, drying, storage, extraction or processing, they are hydrolyzed to their respective aglycone(s) (in this case, Q). This method can therefore be used as a qualitative tool in fingerprinting St John's Wort and herbal products which contain similar flavonoid components. It may also be used for quantitative determination of these flavonoid compounds in herbal products.

CHAPTER FOUR

METHOD VALIDATION AND QUANTITATION OF FLAVONOID COMPONENTS

4.1 INTRODUCTION

According to the United States Pharmacopoeia (USP) [18], method validation is performed to ensure that an analytical method is accurate, specific, reproducible and rugged for the specified range over which an analyte will be analysed. The USP specifies "eight steps of method validation", each step defined by an analytical performance parameter. However, in order to create a platform for universal understanding of terminology and definitions, the ICH (International Convention on Harmonization) developed a guideline defining terminology to describe validation parameters in analytical method validation. These ICH parameters are included below [90].

4.1.1 ACCURACY

Accuracy is the closeness of agreement between the value found by the test method and that which is the true or reference value. It is the measure of exactness of an analytical method. To document accuracy the ICH guideline on methodology recommends collecting data from a minimum of nine determinations over a minimum of three concentration levels, which cover the specified range.

4.1.2 PRECISION

Precision is a measure of the closeness of agreement of the data values from multiple sampling of the same homogenous sample under prescribed conditions. According to the ICH, precision should be performed at three different levels: repeatability, reproducibility and intermediate precision.

Repeatability reflects fluctuations in replicate procedures and is performed by multiple injections of a homogenous sample, within a short time period under the same operational conditions and within the

same operational run. It should be determined from a minimum of nine determinations covering the specified range of the procedure.

Reproducibility, as defined by the ICH, expresses the precision of inter-laboratory analysis. It is not normally expected that this parameter be cited, if data on intermediate precision is provided, as multiple laboratory analyses are not always feasible.

Intermediate precision expresses intra-laboratory variations. The analysis is either performed on different days, or by different operators, on different instruments or using reagents provided by an alternate supplier. Generally, the separation is repeated on a minimum of three separate days, and the overall percent relative standard deviation (% RSD) calculated. The % RSD should ideally be less than 3 %.

4.1.3 SPECIFICITY

Specificity is the ability of a method to unequivocally assess the analyte of interest in the presence of other compounds which may be expected to be present in the same sample. It is a measure of the degree of interference from such things as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due only to a single component and that no co-elutions exist. Specificity, therefore, is a measure of a method's sensitivity to potential sample-related interferents and reflects the ability of the system to resolve these compounds from the peak of interest.

Specificity and selectivity are often used interchangeably, but are distinguishable. A selective method provides accurate results for all analytes of interest, while a specific method provides accurate results for one analyte but the other analytes may interfere with each other. Specificity is measured and documented in a separation by the resolution, plate count (efficiency) and tailing factor [91].

4.1.4 LIMIT OF DETECTION AND LIMIT OF QUANTITATION

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, though not necessarily quantitated. The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and

accuracy under the stated operational conditions of the method. LOQ, like LOD is expressed as a concentration.

Recent articles [92] have included much discussion regarding the determination of LOD and LOQ values of an HPLC method. Included below are 3 techniques, which may be used to determine these parameters.

The first technique is a traditional technique where the signal-to-noise ratio is assessed and used. Baseline noise in the region that corresponds to a given peak is measured in a blank injection. The LOD concentration is equal to three times this value (i.e. a 3:1 signal-to-noise ratio). The LOQ concentration corresponds to 10 times the signal-to-noise ratio (i.e. a 10:1 signal-to-noise ratio).

The second technique is a relatively new convention, and is currently being promoted by the ICH. This is a graphical technique, which consists of plotting the standard deviation (in units of area) versus the concentration of successively more dilute samples as the concentrations approach the LOD value. The y-intercept of this plot is a representation of the virtual standard deviation at zero concentration. The LOD value is then defined as the concentration that corresponds to an area equal to three times the value of the y-intercept. Accordingly, the LOQ value is defined as the concentration that corresponds to an area of 10 times the value of the y-intercept [92].

A third technique that can be used involves choosing the LOQ as the lowest concentration for which the RSD of multiple injections is less than 5%. By convention, the LOD value is taken as 0.3 x LOQ [92].

4.1.5 LINEARITY AND RANGE

The linearity of a method is its ability to elicit results that are directly proportional to analyte concentration within a given range. Least-square linear regression is used to mathematically define the calibration line, if it is linear, and linearity is generally reported as the variance of the slope of the regression line.

The range of a method is derived from its linearity and is dependent upon the application of the procedure. Range is the inclusive interval between the upper and lower levels of analyte, that have been demonstrated to be determined with precision, accuracy, and linearity, using the prescribed method. The ICH guidelines specify a minimum of five concentration levels.

4.1.6 ROBUSTNESS

Robustness of method is its capacity to remain unaffected by small deliberate variations in method parameters. According to the ICH guidelines, robustness should be considered early in the development of the method.

4.1.7 SYSTEM SUITABILITY

System suitability tests are a fundamental component of many analytical procedures. These tests are designed to highlight any fluctuation in operating parameters, or any deterioration of the system, whilst providing information that is related to the origin of the problem. System suitability testing can be performed before or during the analysis. Parameters such as plate count, tailing factors, resolution and reproducibility (%RSD, retention time and area for repetitive injections) are determined and compared against the specifications set for the method.

4.2 VALIDATION PROCEDURES

All instruments and additional equipment, including materials and reagents have previously been described in Chapter 3, Sections 3.2 & 3.3. The method of choice for the validation was Gradient 22 with all the corresponding conditions as previously described.

4.2.1 CALIBRATION OF AUTOPIPETTES

Two adjustable autopipettes, one 1000 : l and one 100 : l were used in most of the analyses. They were calibrated by obtaining the mass of 20 weighings of a relevant volume of HPLC grade water from each autopipette. The electronic balance used is listed in Section 3.2, and the HPLC water was obtained from a Millipore Milli-Q System as listed in Section 2.5.2.

The results obtained are depicted below in Table 4.1.

Table 4.1. Table showing mean, standard deviation and % RSD from results of autopipette calibration.

	Mean Volume Delivered (mls)	Standard Deviation	% RSD
1.0ml pipette	0.9997	0.0051	0.51
0.1ml pipette	0.0988	0.0013	1.35

4.2.2 INTERNAL STANDARD SELECTION

The inclusion of an internal standard to standardize the fluctuations in injection volume can be beneficial. Various compounds, primarily based on availability, were randomly selected and injected, together with the 6 flavonoids, using Gradient 22. These compounds were all soluble in methanol.

Table 4.2. Retention times of potential internal standards using Gradient 22.

	Compound	Retention Time (min)
1.	Prednisone	16.8
2.	Toluene	19.0
3.	Propranolol	15.7
4.	Paracetamol	17.0
5.	Dimethylphthalate	18.4
6.	Cortisone acetate	24.1

The first 5 compounds in Table 4.2 displayed retention times between 15.0 and 20.0 minutes. This was unfavourable as quercitrin, quercetin and kaempferol elute within this window with Gradient 22. On the other hand, cortisone acetate did not interfere with the separation of the 6 flavonoids, but eluted soon after the last eluting flavonoid.

Multiple consecutive injections of the system suitability test mixture were performed in order to evaluate the precision and repeatability of injections. It was found that the variation in peak area for any one of the five compounds in the mixture was below 1.2%, and the variation in retention time for the same was less than 0.2%. Since injection volume error was thus improbable, it was concluded that the use of cortisone acetate as an internal standard was unnecessary.

4.3 METHODS

Two separate stock solutions were made up - one consisting of quercitrin (Qr), quercetin (Q) and kaempferol (K) and the other of rutin (R), isoquercitrin (Iqr) and hyperoside (Hd). Five dilutions in methanol were made from these respective solutions. The concentrations of each component in the stock solutions and in the dilutions are shown in Table 4.3.

Table 4.3. Concentration ranges of flavonoid solutions.

	Concentration (mg/ml)					
Flavonoid	Level 1	Level 2	Level 3	Level 4	Level 5	Stock Solution
Solution 1						
Rutin	5	10	20	40	80	100
Isoquercitrin	2	4	8	16	32	40
Hyperoside	2	4	8	16	32	40
Solution 2						
Quercitrin	2	4	8	16	32	40
Quercetin	5	10	20	40	80	100
Kaempferol	2	4	8	16	32	40

Repeatability was assessed by performing three replicate injections at each of five different concentrations (i.e. each dilution injected in triplicate) of both standard solutions, solution 1 containing R, Iqr and Hd and solution 2 containing Qr, Q and K. This procedure was repeated on three different days to assess intermediate precision. A calibration curve for each of the flavonoids was constructed by plotting the flavonoid peak area versus the respective flavonoid concentration. A straight-line fit of the data was made by least-squares linear regression analysis.

LOD and LOQ values were estimated by assessing the signal-to-noise ratio in the region that corresponds to each respective peak. The LOD concentration was taken as three times this value and the LOQ concentration taken as ten times this value.

System suitability was performed prior to, during and after the validation procedure. The column test method was used as the system suitability method. This test mixture consisted of toluene, uracil, naphthalene, uracil and benzene, and was injected six times at room temperature using a mobile phase of 65:35 acetonitrile:water. The specific parameters that were monitored include plate count, tailing factor, resolution, retention times and reproducibility.

4.4 RESULTS AND DISCUSSION

All system suitability tests resulted in acceptable column efficiency, k factor, and retention time.

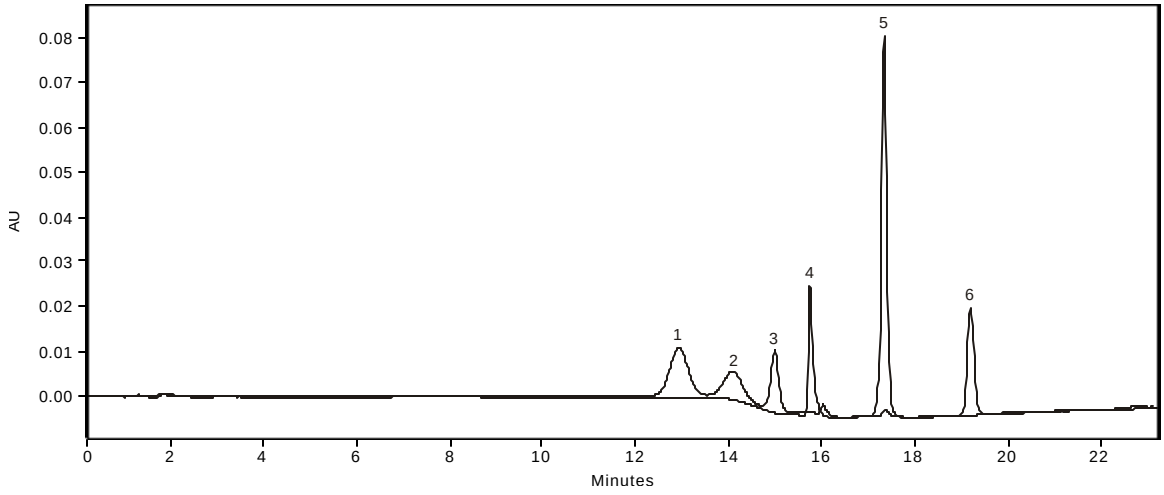
Table 4.4. Calibration data for all six flavonoids on three separate days.

Name of compound	Concentration range (mg/ml)	Day	Slope	y-intercept	R^2
Rutin	5 -100	Day 1	$1.16e^{-2}$	$3.13e^{-2}$	0.999
		Day 2	$1.16e^{-2}$	$3.67e^{-2}$	0.999
		Day 3	$1.15e^{-2}$	$3.40e^{-2}$	0.999
Isoquercitrin	2 - 40	Day 1	$1.59e^{-2}$	$4.52e^{-2}$	0.998
		Day 2	$1.67e^{-2}$	$1.56e^{-3}$	0.999
		Day 3	$1.56e^{-2}$	$3.32e^{-2}$	0.998
Hyperoside	2 - 40	Day 1	$1.60e^{-2}$	$1.63e^{-3}$	0.999
		Day 2	$1.61e^{-2}$	$-2.40e^{-3}$	0.999
		Day 3	$1.57e^{-2}$	$1.91e^{-3}$	0.999
Quercitrin	2 - 40	Day 1	$1.77e^{-2}$	$1.06e^{-2}$	0.999
		Day 2	$1.78e^{-2}$	$2.11e^{-2}$	0.999
		Day 3	$1.80e^{-2}$	$4.83e^{-2}$	0.995
Quercetin	5 - 100	Day 1	$2.34e^{-2}$	$3.18e^{-2}$	0.999
		Day 2	$2.34e^{-2}$	$5.59e^{-2}$	0.998
		Day 3	$2.25e^{-2}$	$1.24e^{-1}$	0.999
Kaempferol	2 - 40	Day 1	$2.23e^{-2}$	$2.91e^{-3}$	0.999
		Day 2	$2.23e^{-2}$	$7.61e^{-3}$	0.999
		Day 3	$2.22e^{-2}$	$1.36e^{-2}$	0.999

The correlation coefficients for all six flavonoids fell within the range 0.995 to 1.000, as illustrated in Table 4.4 above confirming linearity over the concentration ranges studied.

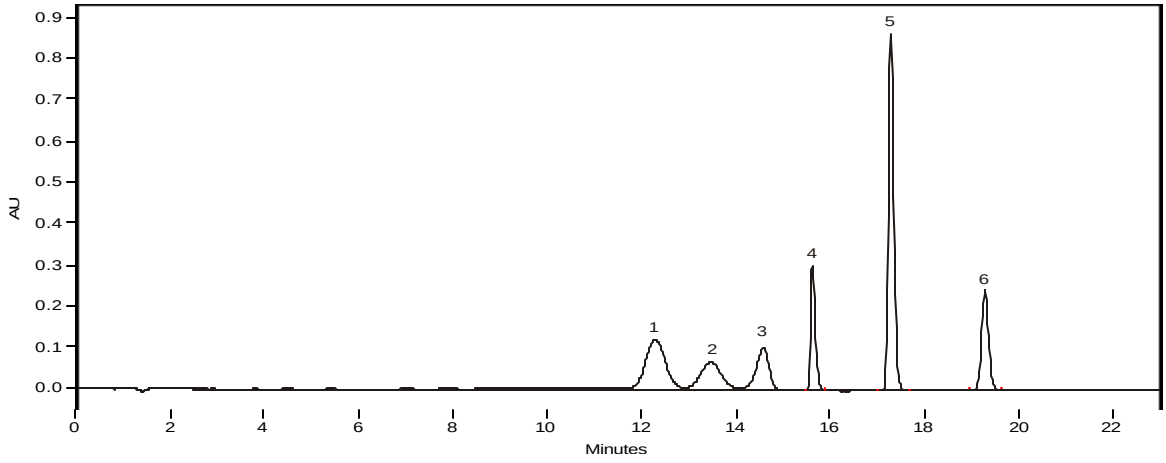
Figures 4.1a and 4.1b are illustrations of the chromatograms obtained for low and high calibrators respectively. The chromatograms for both sample solutions at each concentration are overlaid.

Figure 4.1a. Overlaid chromatograms of two low calibration solutions (AUFS 0-0.08).



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 4 - quercitrin, 5 - quercetin, 6 - kaempferol. Conditions: Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection: 254nm; Temperature: 30°C; Injection volume: 5ìl.

Figure 4.1b. Overlaid chromatograms of two high calibration solutions (AUFS 0-0.9).



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 4 - quercitrin, 5 - quercetin, 6 - kaempferol. Conditions: Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection: 254nm; Temperature: 30°C; Injection volume: 5ìl.

Figures 4.2a and 4.2b are examples of calibration curves obtained for each flavonoid from each solution.

Figure 4.2a. Calibration curve for rutin.

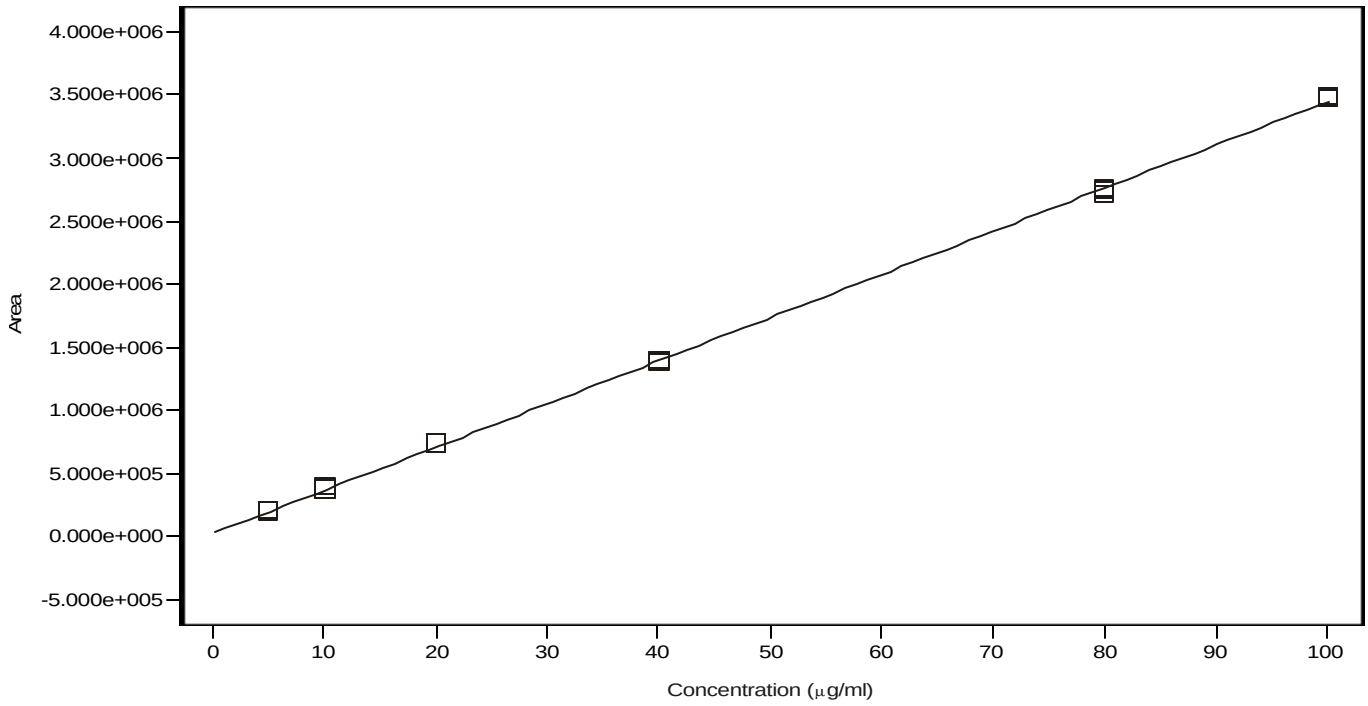
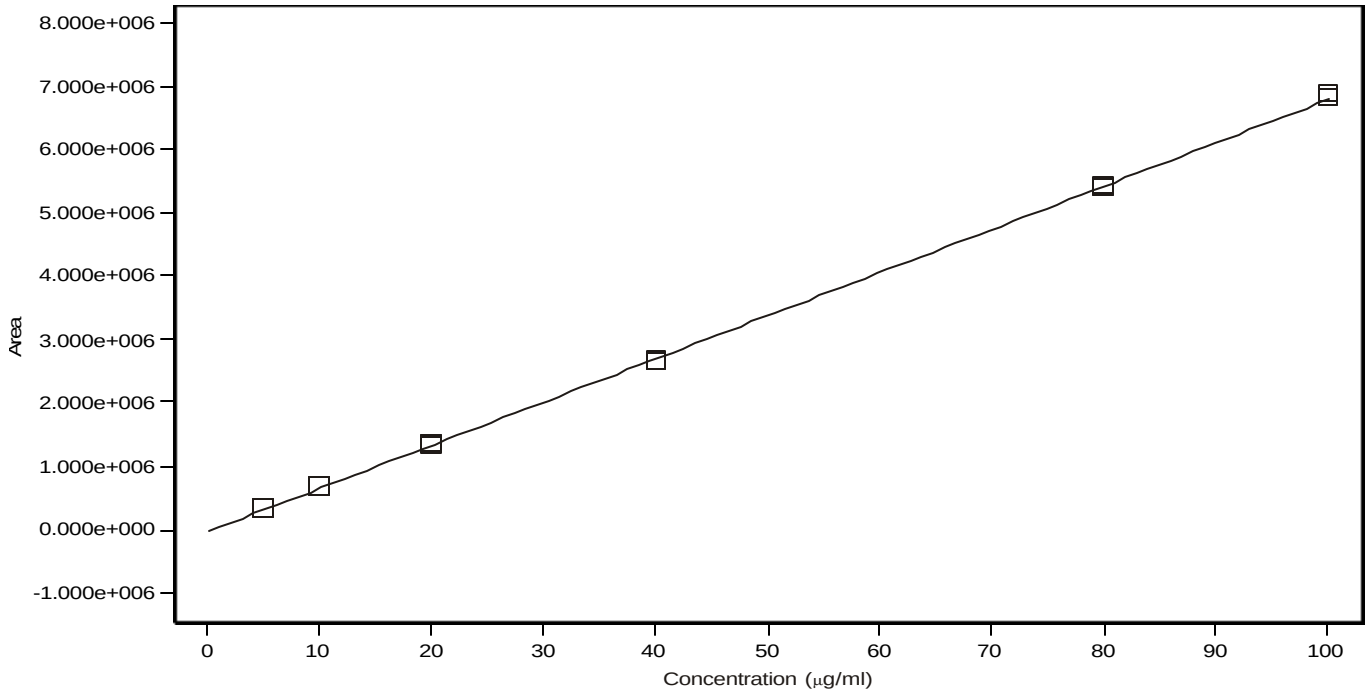


Figure 4.2b. Calibration curve for quercetin.



The precision and linearity of these calibration curves can be seen in Figures 4.2a and 4.2b. The intra- and inter-day day precision and accuracy results for R, Iqr and Hd are tabulated in Table 4.5.

Table 4.5. Intra- and inter-day precision and accuracy results for rutin, isoquercitrin and hyperoside.

Name of compound	Theoretical concentration (mg/ml)	Day	Measured concentration (: g/ml)	Precision CV (%)	Accuracy % Bias
Rutin	20	Day 1	21.16 ± 0.20	0.9	5.8
		Day 2	21.95 ± 0.15	0.7	9.7
		Day 3	21.27 ± 0.12	0.6	
	40	Day 1	39.88 ± 0.31	0.8	0.3
		Day 2	40.32 ± 0.07	0.2	0.8
		Day 3	40.84 ± 0.03	0.1	
	80	Day 1	79.34 ± 0.07	0.1	0.8
		Day 2	79.08 ± 0.53	0.7	1.1
		Day 3	79.52 ± 0.03	0.0	
Isoquercitrin	8	Day 1	8.57 ± 0.13	0.9	5.8
		Day 2	8.30 ± 0.51	0.7	9.7
		Day 3	8.94 ± 0.10	0.6	
	16	Day 1	15.32 ± 0.60	0.8	0.3
		Day 2	15.76 ± 0.41	2.6	1.5
		Day 3	16.19 ± 0.36	2.2	
	32	Day 1	31.56 ± 0.34	1.1	1.4
		Day 2	31.21 ± 0.53	1.7	2.5
		Day 3	32.28 ± 0.30	0.9	
Hyperoside	8	Day 1	8.47 ± 0.03	0.4	5.9
		Day 2	8.30 ± 0.51	6.1	3.8
		Day 3	8.58 ± 0.01	1.2	
	16	Day 1	15.68 ± 0.16	1.0	2.0
		Day 2	15.99 ± 0.18	1.1	0.1
		Day 3	16.27 ± 0.09	0.6	
	32	Day 1	31.54 ± 0.15	0.5	1.4
		Day 2	31.56 ± 0.24	0.8	1.4
		Day 3	31.93 ± 0.04	0.1	

The intra- and inter-day precision and accuracy results for Qr, Q and K are listed in Table 4.6.

Table 4.6. Intra- and inter-day precision and accuracy results for quercitrin, quercetin and kaempferol.

Name of compound	Theoretical concentration (mg/ml)	Day	Measured concentration (: g/ml)	Precision CV (%)	Accuracy % Bias
Quercitrin	8	Day 1	8.03 ± 0.01	0.1	0.3
		Day 2	8.19 ± 0.03	0.3	2.3
		Day 3	8.47 ± 0.02	0.2	
	16	Day 1	16.34 ± 0.01	0.1	2.1
		Day 2	16.58 ± 0.02	0.1	3.6
		Day 3	17.60 ± 0.88	5.0	
	32	Day 1	31.71 ± 0.15	0.1	0.9
		Day 2	31.60 ± 0.01	0.0	1.2
		Day 3	31.63 ± 1.08	3.4	
Quercetin	20	Day 1	20.35 ± 0.04	0.2	1.7
		Day 2	21.04 ± 0.04	0.2	5.2
		Day 3	20.51 ± 0.06	0.3	
	40	Day 1	41.04 ± 0.07	0.2	2.6
		Day 2	41.0 ± 0.10	0.2	2.5
		Day 3	39.76 ± 0.02	0.0	
	80	Day 1	79.10 ± 0.10	0.1	1.1
		Day 2	78.70 ± 0.07	0.1	1.2
		Day 3	79.22 ± 0.12	0.2	
Kaempferol	8	Day 1	7.96 ± 0.03	0.4	0.6
		Day 2	8.16 ± 0.18	2.2	2.0
		Day 3	8.41 ± 0.01	0.1	
	16	Day 1	16.44 ± 0.03	0.2	2.8
		Day 2	16.50 ± 0.01	0.1	3.2
		Day 3	16.52 ± 0.01	0.1	
	32	Day 1	31.63 ± 0.00	0.0	1.2
		Day 2	31.52 ± 0.04	0.1	1.5
		Day 3	31.73 ± 0.05	0.2	

The results obtained for Qr, Q and K were somewhat better than those obtained for the other three compounds, R, Iqr and Hd. The latter compounds eluted as the gradient was changing rapidly, and this tended to cause slight peak shape changes and retention time shifts. This did not occur with the last three compounds, Qr, Q and K. Although these three compounds eluted whilst the gradient was changing, there was a lack of baseline drift or retention time changes for these compounds since the change in mobile phase composition was less pronounced compared to the initial change. The slight peak shifting that occurred with R, Iqr and Hd on the rapidly changing gradient portion of the method was probably a result of the very low flow rate used (µl as compared with ml) coupled with the lack of appropriate micro- plumbing optimization in the HPLC system and the inability of the macro GPV valve (gradient proportioning valve) to cope with mixing such very low flow rates and volumes. So rapidly.

The calibrators used in Day 2 were used as "unknown" samples and assessed with the calibration curve from the first day and similarly the third day calibrators assessed with the calibration curve of Day 2, hence % bias was not determined for Day 3.

Table 4.7. Flavonoid LODs and LOQ.

: g/ml	Rutin	Hyperoside	Quercitrin	Quercetin	Kaempferol
LOD	0.0558	0.4661	0.2428	0.0500	0.2116
LOQ	0.186	1.5538	0.8093	0.1651	0.7053

The LODs and LOQs reported in Table 4.7 are similar to those reported by Ganzera *et al*. [85].

4.5 ASSAY OF DOSAGE FORMS

4.5.1 INSTRUMENTATION

Refer to Section 3.2 for all instrumentation used.

Additional equipment included:

- Meyer N-EVAP Analytical Evaporator, Model 112 (Organomation Inc. South Berlin, MA 01549, USA).

Refer to Section 3.2 for all other additional equipment.

4.5.2 MATERIALS AND REAGENTS

Holotropic® St John's Wort Genuine Imported Hypericum tablets, Batch 0503.
Refer to Section 2.5.3 for further details and other materials and reagents used.

4.5.3 METHODS

4.5.3.1 Tablet Extraction Efficiency Analysis

In their analysis of St John's Wort powder, Li and Fitzloff [86] found that they could extract most of the major constituents within four steps. Their extraction method consisted of weighing an amount of St John's Wort powder into a borosilicate glass tube and adding 10ml of methanol. This mixture was then sonicated for 30 minutes, centrifuged, and the supernatant poured off and made up to 10ml in a

volumetric flask with methanol. A further 10ml of methanol was added to the residue in the borosilicate glass tube, which was then sonicated for 30minutes, centrifuged, and the supernatant poured off and made up to 10ml in a volumetric flask with methanol. The residue in the glass tube was then extracted 2 more times in the same manner described above. According to their findings, within four steps (30 minutes each) of sonication, more than 99% of the major constituents could be extracted.

With this in mind, preliminary studies were performed and different sample sonication times for extraction were compared. In the first study, 30 minutes was used as the time for sample sonication, and the extraction repeated 3 times. In the second, third and fourth studies, 1hour, 1½ hours, and 2 hours were used as sample sonication times, respectively, and with each study the extraction was repeated 3 times. The results from these studies showed that 1 hour sample sonication yielded significantly more flavonoids than 30 minutes, and the same for 2 hour sample sonication versus 1½ hours. The final extraction efficiency analysis then compared the yield of flavonoids from 3 consecutive one hour sonications, (using the same residue, but decanting the supernatant and adding 10ml methanol to the residue after each hour), to 3 consecutive two hour sonications (performed in the same manner). Each of the triplicates was individually analyzed.

Ten Holotropic® tablets were accurately weighed and ground into fine powder with a mortar and pestle. Approximately half a gram of the powdered tablets was accurately weighed, and 6 replicate weighings were placed in 6 separate Kimax tubes. Ten millilitres of methanol was added to each Kimax tube, the tube stoppered and the mixture vortexed for thirty seconds to ensure that the powder was adequately dispersed in the methanol. These six tubes were then placed in a sonicator bath, three tubes being sonicated for one hour and the other for two hours. At the end of the respective sonication periods, the relevant tubes were removed from the sonicator and centrifuged for ten minutes until the residues had compacted at the bases of the Kimax tubes. The supernatants were poured into clean Kimax tubes, stoppered and kept for injection.

A further ten millilitres of methanol was then added to each residue, the tubes stoppered, vortexed for thirty seconds and sonicated again for the relevant time. The tubes were centrifuged for ten minutes and the supernatants decanted into clean Kimax tubes and kept for injection.

This procedure was repeated once more, both for the three one hour and the three two hour sonication samples.

A suitable aliquot (1.5ml) of each of the eighteen respective supernatants was pipetted into an HPLC vial. These vials were vortexed for thirty seconds, and placed in the HPLC system for injection.
The results were obtained by performing triplicate injections of each sample.

4.5.3.2 Recovery

A stock solution containing the concentrations of flavonoids listed in Table 4.8 was made up in methanol.

Table 4.8. Concentrations of flavonoids used in stock solution for spiking.

Flavonoid:	Rutin	Isoquercitrin	Hyperoside	Quercitrin	Quercetin	Kaempferol
Concentration (µg/ml)	120	120	120	120	300	120

Ten tablets were ground using a mortar and pestle. Six portions of approximately 0.5g of the tablet powder were accurately weighed out and placed in Kimax tubes. Three millilitres of the flavonoid stock solution was pipetted into each of 3 of those tubes containing the powdered tablets followed by the addition of 7 ml methanol to make the total volume up to ten millilitres per tube. Ten millilitres of methanol was pipetted into each of the other three Kimax tubes, containing powdered tablets.

The tubes were stoppered and the mixtures vortexed for thirty seconds to ensure that the powder was adequately dispersed throughout the methanol. The tubes were then sonicated for one hour and centrifuged for 10 minutes to ensure the residue was compacted at the base of the tubes. The resulting supernatants were poured off, vortexed for thirty seconds and an aliquot of each of the supernatants pipetted into a vial for injection into the HPLC system.

4.5.4 RESULTS AND DISCUSSION

4.5.4.1 Tablet Extraction Efficiency Analysis

Results from the two sets of samples are shown in Figures 4.3 and 4.4, and Tables 4.8 and 4.9 respectively. Hyperoside is not included in the figures and tables shown, as it was present in quantities

below the limit of quantitation (LOQ) in the Holotropic® tablets used for the extraction efficiency analysis. The Figures below depict the percentage flavonoid extracted after one, two, and three and after two, four and six hour sonication periods respectively.

Figure 4.3.

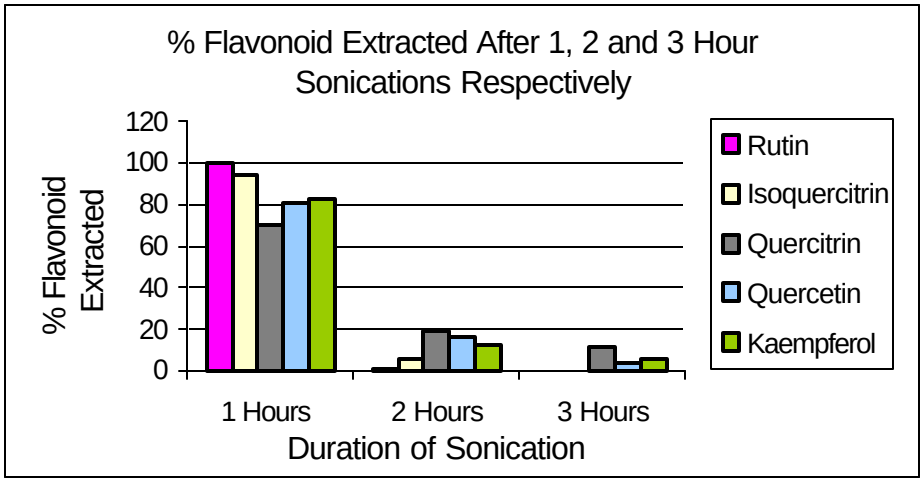


Figure 4.4.

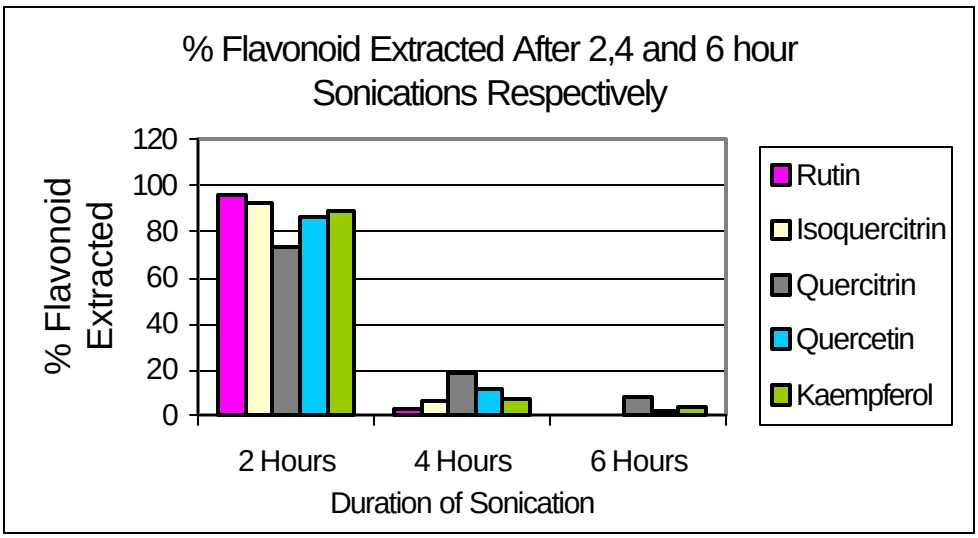


Table 4.9. Percentage flavonoid extracted after 1, 2 and 3 hours respectively.

	ONE HOUR SONICATIONS: PERCENTAGE FLAVONOIDS EXTRACTED					
FLAVONOID	**1 Hour**	% RSD	**2 Hours**	% RSD	**3 Hours**	% RSD
Rutin	**99.65**	0.6	**0.35**	0.6	**0**	0
Isoquercitrin	**93.93**	3	**6.07**	0	**0**	0
Quercitrin	**69.82**	2.96	**18.92**	2.78	**11.26**	1.12
Quercetin	**80.76**	2.35	**15.8**	2.42	**3.44**	0.55
Kaempferol	**82.78**	1.42	**12.11**	1.84	**5.11**	1.44

Table 4.10. Percentage flavonoid extracted after 2,4 and6 hours respectively.

	TWO HOUR SONICATIONS: PERCENTAGE FLAVONOIDS EXTRACTED					
FLAVONOID	**2 Hours**	% RSD	**4 Hours**	% RSD	**6 Hours**	% RSD
Rutin	**96.61**	2.75	**3.39**	2.75	**0**	0
Isoquercitrin	**92.92**	2.05	**7.08**	2.05	**0**	0
Quercitrin	**73.79**	3.33	**18.15**	0.35	**8.06**	3.62
Quercetin	**86.41**	1.06	**11.66**	0.79	**1.92**	0.28
Kaempferol	**88.67**	1.22	**7.58**	0.86	**3.75**	0.56

As illustrated in Table 4.9, sonication for one hour extracts eighty percent or more of the respective flavonoid, with the exception of Qr. The increase in % flavonoid extracted (0.4% - 19%) with doubling the sonication time was not seen to be so significant as to warrant 2 hour sonication.
In general, it can be seen that extraction using two hours as opposed to one hour resulted in a relatively slight increase in the percentage flavonoid extracted.

Tables 4.9 and 4.10 and Figures 4.3 and 4.4 show that the amount of flavonoid extracted using repeated sonications does not greatly increase the percentage flavonoid extracted. The exception to this appears to be quercitrin, where approximately 70% is extracted after one hour. The results thus indicate that a single one hour extraction suffices for extracting tablets for quantitation and was thus accordingly chosen for use in the tablet assay.

4.5.4.2 Recovery Analysis

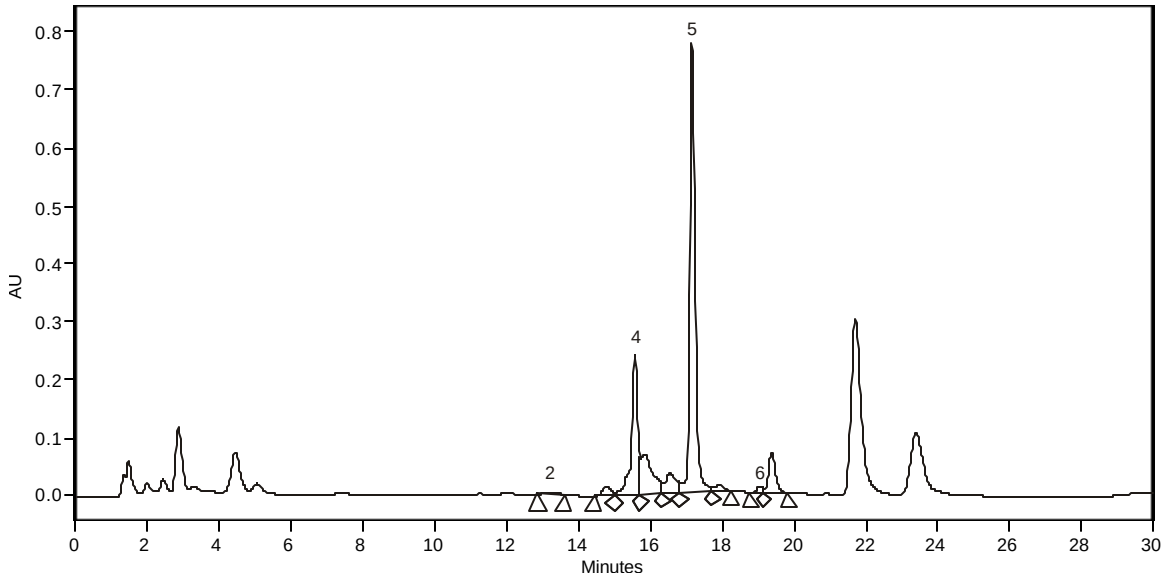
Table 4.11 below depicts the % recovery for each of the respective flavonoids. The % recoveries varied from 61 to 85%. With the exception of Qr and Q, the percentage RSDs obtained were 3% or less.

Table 4.11. Percentage recoveries of flavonoid compounds.

Flavonoid	% Recovery	% RSD
Rutin	77	1.2
Isoquercitrin	83	1.9
Hyperoside	64	3.0
Quercitrin	61	14.6
Quercetin	64	10.7
Kaempferol	85	1.3

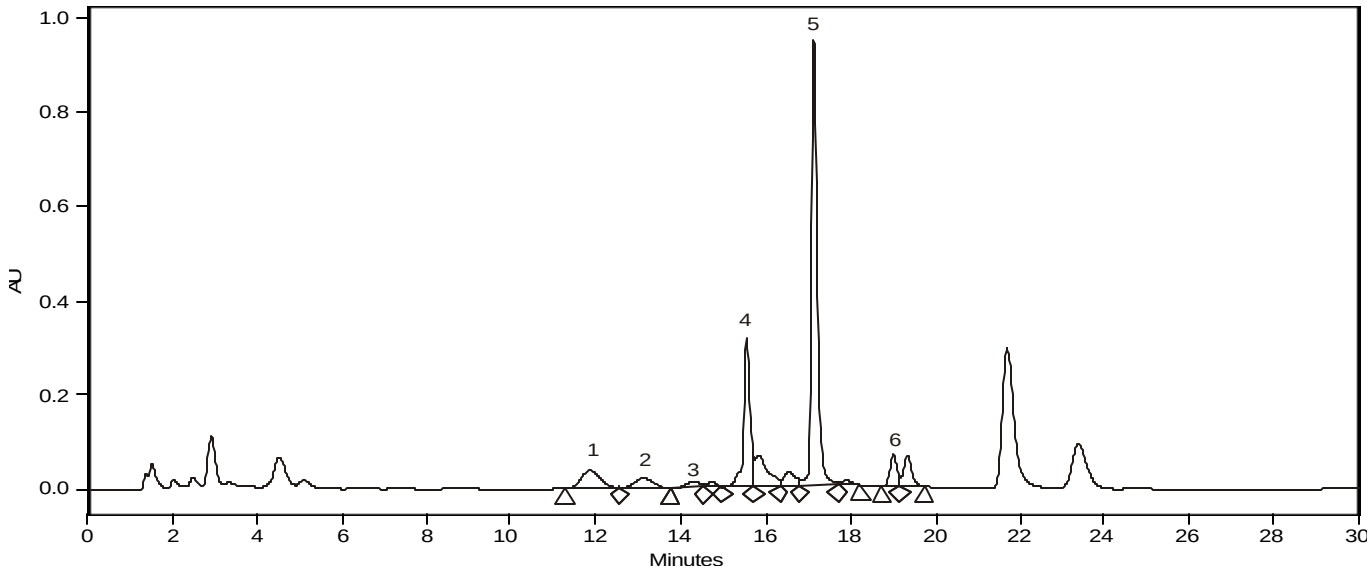
Eluent from the column was monitored at 254nm. It can be seen from the chromatograms in Figures 4.5a and 4.5b that there were interferences with the Qr (4) and Q (5) peaks making integration and quantitation difficult. It is thus quite possible that those may have contributed to the resultant higher percent RSDs for these two compounds.

Figure 4.5a. Unspiked tablet extract.



2 - Isoquercitrin, 4 - quercitrin, 5 - quercetin, 6 - kaempferol. Conditions: Flow rate: 0.3ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection: 254nm; Temperature: 30℃; Injection volume: 5 : l.

Figure 4.5b. Spiked tablet extract.



1 - Rutin, 2 - Isoquercitrin, 3 - Hyperoside, 4 - quercitrin, 5 - quercetin, 6 - kaempferol. Conditions: Flow rate: 0.3ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection: 254nm; Temperature: 30℃; Injection volume: 5 : l.

Figure 4.5a indicates that R (1) and Hd (3) were absent (below the LOD) in the unspiked extract, whereas small amounts of Iqr (2) and K (6) were present. The %RSDs for these four compounds are however, substantially lower than those obtained for Q and Qr. This is probably due to the slight interference at the bases of the latter 2 peaks. The spiked extract (Figure 4.5b) illustrates the increase in size of all six peaks due to spiking.

4.6 QUANTITATIVE ANALYSIS OF ST JOHN'S WORT TABLETS

The method developed in Section 3.5 was subsequently utilised to assay St John's Wort tablets to quantitatively determine the relevant flavonoid components, R, Hd, Qr, Q and K. At this point in time, a reference standard for isoquercitrin was not available and this compound could thus not be assayed.

Interferences from the tablet extract, resulted in difficulty with integration in the cases of hyperoside and quercitrin. These interferences were less pronounced at 350nm as opposed to 254nm. An unpublished validated LC/MS/MS method that was developed in our laboratory for the quantitation of flavonoids in *Ginkgo biloba* dosage forms (namely Q, isorhamnetin, R, Qr, and K) was utilised and further developed for the confirmatory quantitation by LC/MS/MS of Hd, Qr and Q. The LC/MS/MS

method was used as a tool for semi-quantitative purposes to check the integration and quantitation by HPLC with UV detection.

Another method that was used as a semi-quantitative tool to check the authenticity of the assay results obtained was the method that had been developed and used by Ganzera *et al*. [85]. Their method was successfully applied and used in this study to obtain semi-quantitative data from some of the previously assayed dosage forms containing St John's Wort, using the same extracts which had been stored at -20º C for 2 months.

4.6.1 INSTRUMENTATION

- Synergi 4ìm MAX - RP 80A, 150 x 4.60mm i.d. column, Part 00F- 4337 - E0 (Phenomenex, Torrance, CA, USA).
- Finnigan MAT, Model LCQ Mass Spectrometer System (355 Riveroaks Parkway, San Jose, CA 95134, USA)
- SpectraSystem binary gradient pump, Model P2000 (Thermoseparation Products, GmbH 63329 Egelsbach, Germany).

  Refer to Section 3.2 for all other instrumentation used.

Additional equipment included:

- Meyer N-EVAP Analytical Evaporator, Model 112 (Organomation Inc. South Berlin, MA 01549).

  Refer to Section 3.2 for all other additional equipment.

4.6.2 MATERIALS AND REAGENTS

The commercial samples chosen for use in the flavonoid analysis are listed in Table 4.12. Three batches of the same brand of St John's Wort tablet, and two other additional brands were used for the analysis.

Table 4.12. St John's Wort tablets used for the quantitation of the flavonoid compounds.

COMPANY	DOSAGE FORM	NAME OF PRODUCT	LABEL CLAIMS	BATCH	EXTRACT
Holotropic	Tablets	St John's Wort [9]	Each 500mg tablet contains 1000µg of Hypericin	0503	A
Holotropic	Tablets	St John's Wort [9]	Each 500mg tablet contains 1000µg of Hypericin	0502	B
Holotropic	Tablets	St John's Wort [9]	Each 500mg tablet contains 1000µg of Hypericin	0104	C
Vitality	Tablets	St John's Wort [11]	Each 500mg tablet contains 300mg Hypericum perforatum extract	1746	D
Alpha	Tablets	St John's Wort [14]	Each 500mg tablet contains 300mg Hypericum perforatum extract	4688	E

Formic acid [8] (Batch 12682/3078) was obtained from ACE.

[8] Associated Chemical Enterprises (Pty) Ltd. P O Box 379, Glenvista, 2058, South Africa

[9] Holotropic, Cape Town, South Africa.

[11] Vitality, Ridgeweg 534, Durban, South Africa.

[14] Alpha, Allied Drug Co. (Pty) Ltd., Durban, South Africa.

Refer to Section 2.5.3 for all other materials and reagents used.

4.6.3 METHODS

Standard operating procedures (SOPs) were followed for all procedures utilised. SOPs outlining the cleaning of glassware, the use of the balance, the use of the pH meter, the use of the Millipore Milli-Q System and the use of the HPLC system are described in the Appendix of this thesis.

Ten tablets from each product were ground into fine powder in a mortar and pestle. Three aliquots of approximately 0.5 grams each were accurately weighed out for each of the products and placed into individual Kimax tubes. Ten millilitres of methanol were pipetted into the Kimax tubes and the tubes stoppered.

The mixtures were vortexed for thirty seconds to ensure adequate dispersion of the powder throughout the methanol, and then sonicated for one hour at room temperature. Following sonication, the samples were centrifuged for five minutes, and the supernatants poured off and stored overnight at 4°C prior to injection.

4.6.3.1 HPLC Analysis with UV Detection

Supernatants were centrifuged and a suitable aliquot (1.5ml) of each supernatant transferred to a vial for injection. HPLC analysis using the method previously developed and validated with dual wavelength UV detection at 254 and 350nm was used for quantitative analysis. Details of the gradient are shown below in Table 4.12.

Table 4.13. Validated HPLC method (Gradient 22) used for flavonoid quantitation.

Solvent A: Acetonitrile
Solvent B: 0.5% Phosphoric acid pH = 1.5

Time (minutes)	% A	% B
0	14.5	85.5
10	14.5	85.5
12	45	55
25	20	80

The column used, as described previously, was a Luna C_{18} (2) 150 x 2.0mm narrow bore column. A flow rate of 0.3ml/min and a temperature of 30°C were used. Injections were performed in triplicate, and an equilibration time of 30 minutes was retained after each injection.

Where flavonoid concentrations fell outside the calibration range appropriate volumetric dilutions of the extracts were made.

4.6.3.2 Semi-Quantitative Confirmation with LC-MS-MS

The LC-MS-MS method that was developed in our laboratory for the quantitation of *Ginkgo biloba* flavonoids was *"fine-tuned"* for the confirmatory quantitation by LC-MS-MS of Hd, Qr and Q.

The original method used isocratic conditions together with a flow-rate of 0.3ml/min and temperature of 45°C. The mobile phase consisted of 0.3% formic acid in water: acetonitrile (75:25). The column used was the same as for the HPLC flavonoid method - a Luna C_{18} (2) 150 x 2.0mm narrow bore column. Using this method, the retention times of Hd, Qr and Q were 1.90, 2.74 and 7.82 minutes respectively. This method was optimized to increase the separation between Hd and Qr in order to `ensure that there would not be an apparent loss of Hd due to the narrow window for Hd coupled with any alteration in retention time. Various combinations of flow rates and temperature were used until the optimum combination of these two was found. Using 0.5ml/min and 30°C, the retention times for Hd, Qr and Q were 1.38, 2.09 and 7.17 minutes respectively. There was no interference with the Q peak using UV detection, and the inclusion of Q in this semi-quantitative confirmation was to act as a control when comparing integration results between LC/MS/MS and HPLC.

The LC-MS-MS tuning for these compounds was optimised to have sheath gas flow of 80 psi, spray voltage of 3.50 kV, capillary temperature of 200°C and capillary voltage of -2.70kV. The segments for each compound were as follows: Hd, from 0 to 1.8minutes, Qr from 1.8 to 5.0 minutes and Q from 5.0 minutes to 10.0 minutes. Optimum conditions for the three compounds were found to be: Hd, an isolation width of 2.0 and relative collision energy of 31%, Qr, an isolation width of 2.0 and relative collision energy of 30%, and Q, an isolation width of 2.0 and relative collision energy of 28%.

Fresh stock solution and appropriate dilutions of the flavonoid standards were made for the LC-MS-MS analysis. A three-point calibration curve was obtained at the start of each run. The same extracts that were assayed by the validated HPLC method were also used for assay by LC-MS-MS. Injections were performed in triplicate. This method was not validated, and was merely used to provide a semi-quantitative indication of the results from HPLC with UV detection.

4.6.3.3 Semi-Quantitative Confirmation Using the Ganzera Method

As well as the LC-MS-MS semi-quantitative check, the HPLC gradient method with UV detection developed and described by Ganzera *et al.* [85] was successfully applied as a semi-quantitative indication of the results obtained with the method developed in this thesis. Ganzera *et al*. reported baseline separation of nine of the major compounds in St John's Wort, with a total run time of less than thirty-five minutes. Their method was also applied to evaluate whether baseline resolution could in

fact be obtained with the compounds that were difficult to integrate using the currently developed method. The flavonoids which were separated by Ganzera *et al* included R, Hd, Iqr, Qr, Q and I3,II8-biapigenin. The aglycone K, however, was not included in their method.

The Ganzera method with its conditions, parameters, and column was set up on the instrumentation described in Section 4.6.1. A standard stock solution containing R, Hd, Q, Qr and the naphthodianthrones and hyperforin was made. Three dilutions were made of this. These four solutions were injected in triplicate and a four-point calibration curve constructed.

The extracts used for the chromatographic analysis of each of A, B, C, D, and E respectively were injected in triplicate immediately following the injections of the calibrator solutions.

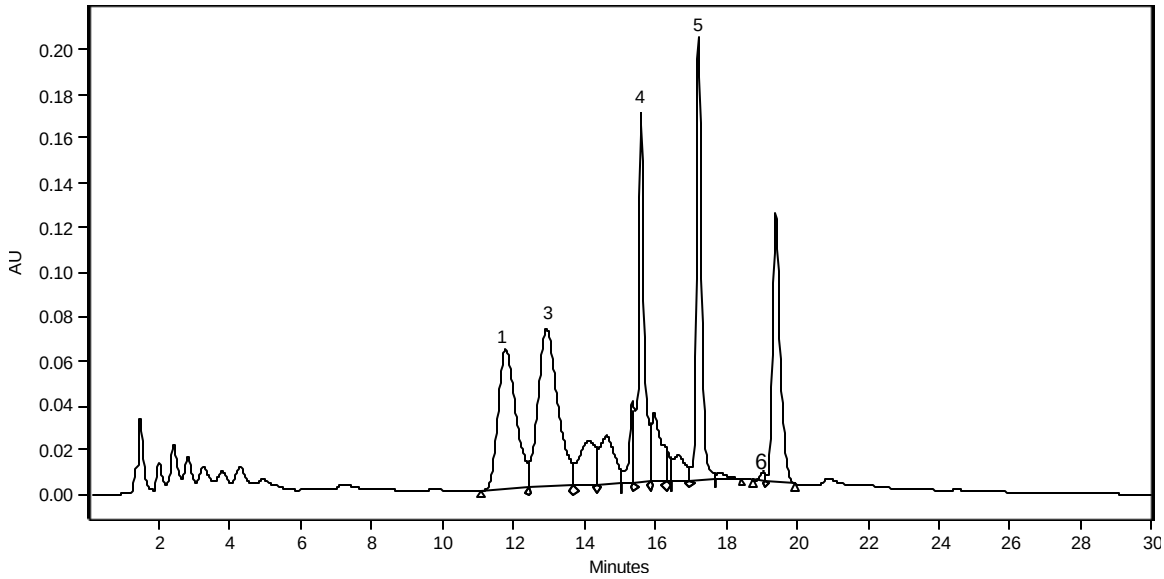
4.7 RESULTS AND DISCUSSION

4.7.1 HPLC ANALYSES

The HPLC analytical method developed in this study was validated over the concentration ranges used at a detection wavelength of 254nm. However, due to the nature of the tablets analysed, interferences with hyperoside and quercitrin were observed when the tablet extracts were injected and run at 254nm. Extract E is used as an example, and is shown in Figure 4.6, where there are interferences at the base of quercitrin (4).

Figures 4.1 and 4.5 show resolution of R, Iqr and Hd. Figure 4.6 implies that these components would not be resolved. If Iqr were present however, dilution of this extract to reduce the size of peaks 1 and 3 would allow Iqr to be identified and determined.

Figure 4.6. An example of a chromatogram from Extract E at 254nm.



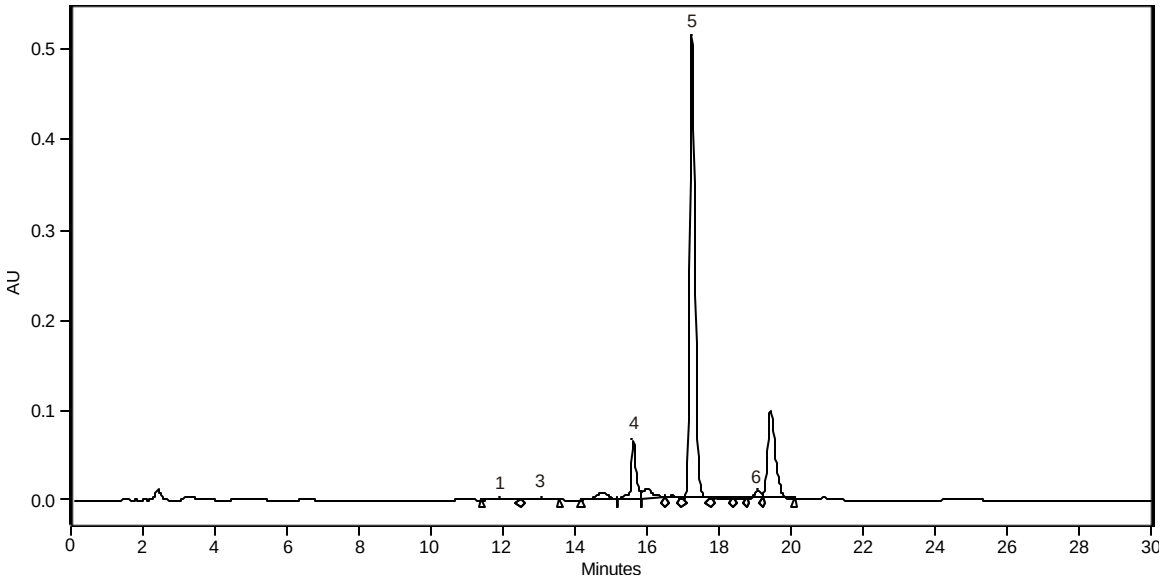
1 - Rutin, 3 - Hyperoside, 4 - quercitrin, 5 - quercetin, 6 - kaempferol. Conditions: Flow rate: 0.3ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm i.d. Detection: 254nm; Temperature: 30℃; Injection volume: 5 : l.

As a result of interferences with Hd and Qr in some of the extracts, the integration and ultimately, the quantitation of these chromatographic peaks was at times questionable. The interferences were slightly less prominent at 350nm and this is illustrated in Figure 4.9a, where the chromatogram of Extract E run at 350nm is shown. Calibration and quantitation were performed at 350nm in addition to 254nm. Further confirmation of the quantities of flavonoids in the tablets was obtained from LC/MS/MS and the Ganzera method.

Comparisons between the chromatography obtained using my method, and that obtained using the Ganzera method for Extracts A, D and E are shown in Figures 4.7, 4.8 and 4.9 respectively.

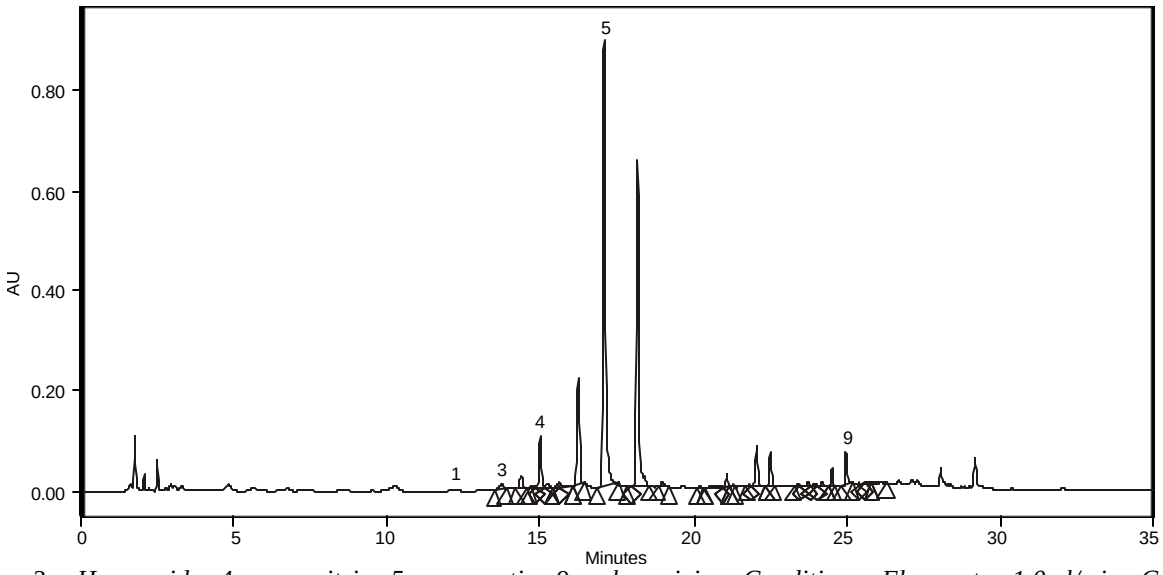
Figures 4.7a and b can be used to compare the results from each method used. The results from both methods illustrate that Extract A contains very little R and Hd, with the Iqr being below the LOD. Both chromatograms also illustrate that Extract A contained Qr, and a substantial amount of Q.

Figure 4.7a. Extract A: Example of Chromatogram using currently developed HPLC method at 350nm.



1 - Rutin, 3 - Hyperoside, 4 - quercitrin, 5 - quercetin, 6 - kaempferol. Conditions: Flow rate: 0.3ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm i.d. Detection: 350nm; Temperature: 30℃; Injection volume: 5 : l.

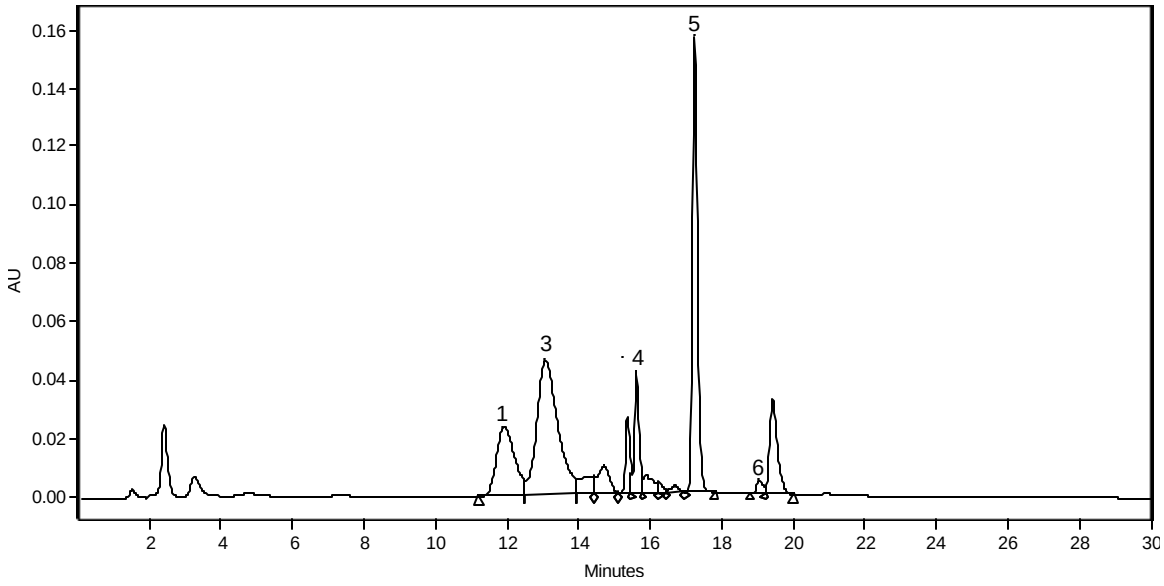
Figure 4.7b. Extract A: Example of Chromatogram using Ganzera et al. *HPLC method at 270 nm.*



1 - Rutin, 3 - Hyperoside, 4 - quercitrin, 5 - quercetin, 9 - hypericin. Conditions: Flow rate: 1.0ml/min; Column: Phenomenex Synergi MAX-RP 80A, 4 : m, 150 x 4.6 mm i.d. Detection: 270nm; Temperature: 40℃; Injection volume: 10 : l.

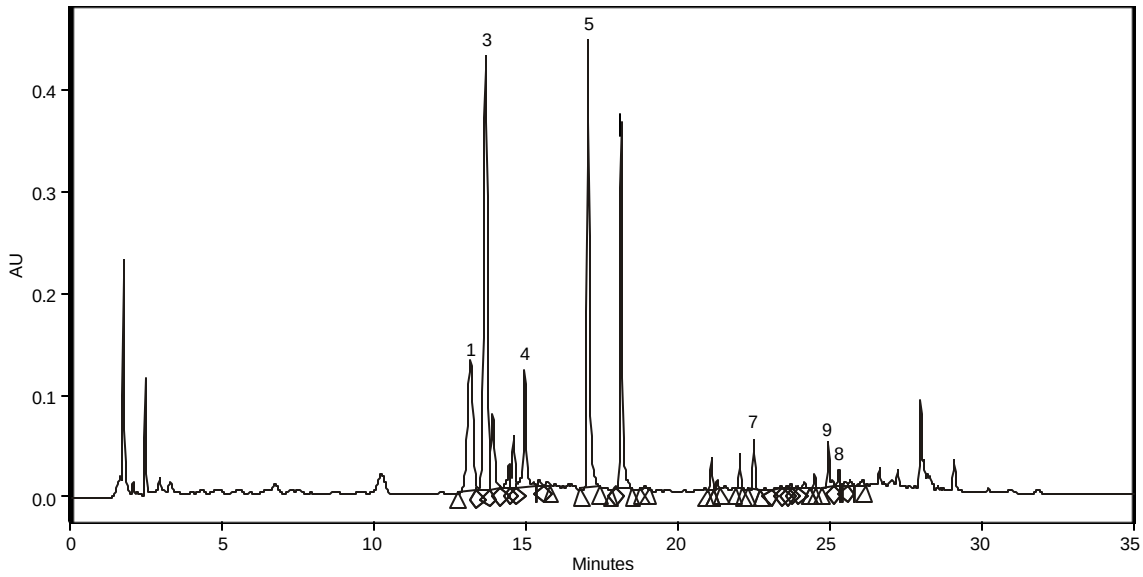
The chromatograms obtained from Extract D using both methods illustrate a similar situation to that occurring when Extract A was analyzed. It can be seen in Figures 4.8a and b that there was a substantial amount of R (1), Hd (3) and Q (5) in this extract. Qr (4) was present, with Iqr (2) again being below the LOD.

Figure 4.8a. Extract D: Chromatogram using currently developed method at 350nm.



1 - Rutin, 3- Hyperoside, 4 - quercitrin, 5 - quercetin, 6 - kaempferol. Conditions: Flow rate: 0.3ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm i.d. Detection: 350nm; Temperature: 30°C; Injection volume: 5 : l.

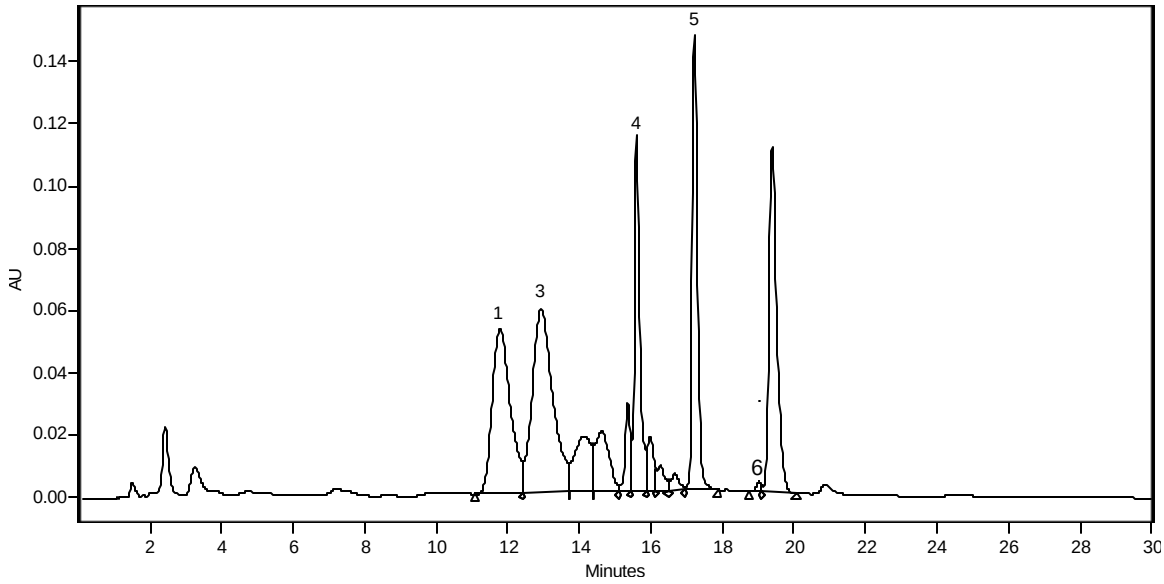
Figure 4.8b. Extract D: Chromatogram using the Ganzera method at 270nm.



1 - Rutin, 3 - Hyperoside, 4 - quercitrin, 5 - quercetin, 7 - pseudohypericin, 8 - hyperforin, 9 - hypericin. Conditions: Flow rate: 1.0ml/min; Column: Phenomenex Synergi MAX-RP 80A, 4 : m, 150 x 4.6 mm i.d. Detection: 270nm; Temperature: 40°C; Injection volume: 10 : l.

Figures 4.9a and b illustrate the chromatography obtained from Extract E. Extract E contained a substantial amount of R (1), Hd (3), Qr (4) and Q (5). Iqr (2) was below the LOD. Once again there is agreement between the results from both methods, and both chromatograms yield similar information about the product.

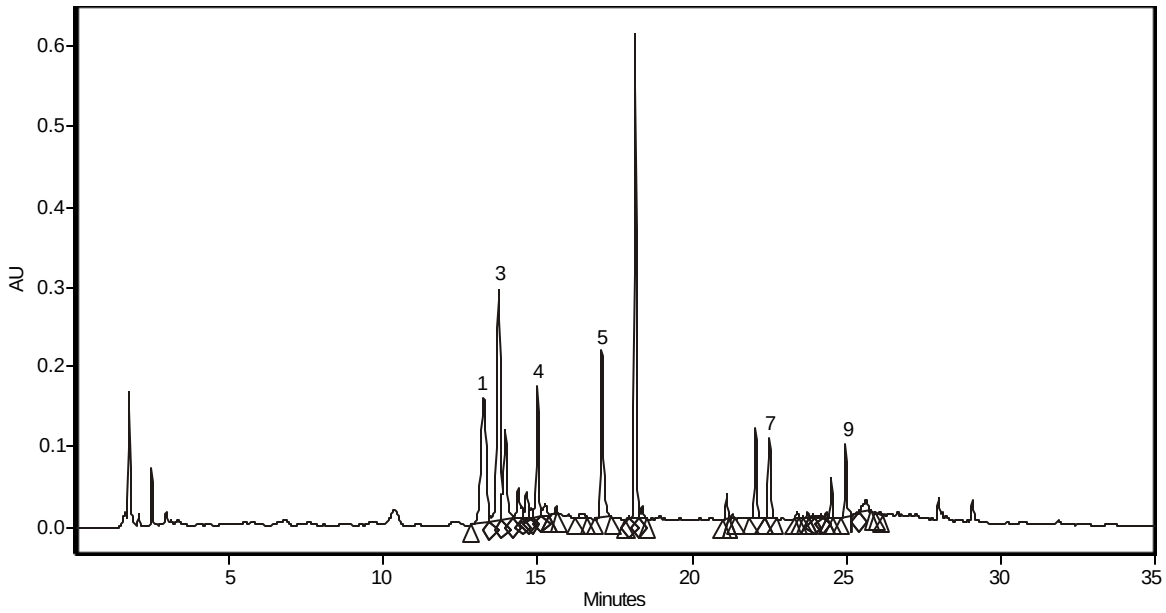
Figure 4.9a. Extract E: Chromatogram using currently developed method at 350nm.



1 - Rutin, 3 - Hyperoside, 4 - quercitrin, 5 - quercetin, 6 - kaempferol. Conditions: Flow rate: 0.3ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm i.d. Detection: 350nm; Temperature: 30°C; Injection volume: 5 : l.

As can be seen, Peak 3 in Figure 4.5b elutes a little after 14 minutes, whilst in Figures 4.6, 4.8a and 4.9a, Peak 3 elutes at approximately 13 minutes. The identification of this peak is correct. The peak shifting can be attributed to the lack of micro plumbing and inability of the GPV valve to cope with such low volumes, as well as the rapidly changing gradient at this point.

Figure 4.9b. Extract E: Chromatogram using the Ganzera method at 270nm.

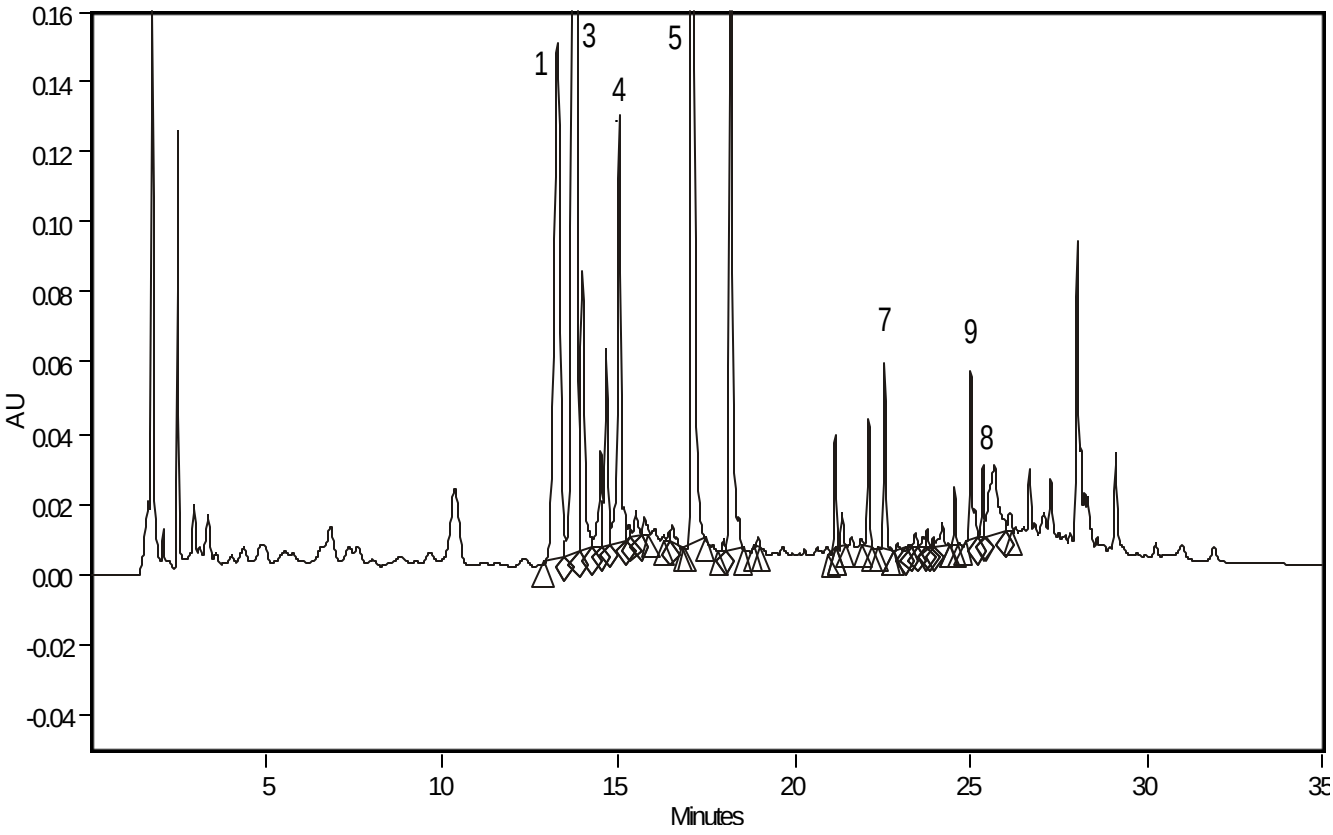


1 - Rutin, 3 - Hyperoside, 4 - quercitrin, 5 - quercetin, 7 - pseudohypericin, 9 - hypericin. Conditions: Flow rate: 1.0ml/min; Column: Phenomenex Synergi MAX-RP 80A, 4 : m, 150 x 4.6 mm i.d. Detection: 270nm; Temperature: 40°C; Injection volume: 10 : l.

Extracts A, B and C, were very similar, and Extract A reflects the chromatography (Figures 4.7a and b) of these extracts using both methods. As can be seen there was general agreement between the flavonoid peak sizes obtained using the currently developed method at 350nm and Ganzera's method at 270nm.

Ganzera *et al*. claimed baseline resolution of all the flavonoids with their method. Their method did not appear to have problems with interfering compounds (see Figures 4.7b, 4.8b and 4.9b). However, the calibrator and extract concentrations used by Ganzera and his coworkers were much greater than those used in this study. At first glance, it appears as if the Ganzera *et al* method results in baseline resolution of all compounds with no interference (see Figure 4.8b), however scaling that chromatogram to the same scale (0.16 AUFS) as that used for the currently developed method reveals some interference with Hd and Qr (see Figure 4.10). There was however, not as much interference using the Ganzera *et al* method at 270nm compared to detection at 254nm with the currently developed method. An example of the chromatography obtained from Extract D using the Ganzera *et al*. method is illustrated in Figure 4.8b. This chromatogram is adjusted to the same scale (0.16 AUFS) obtained from Extract D using the currently developed method and shown in Figure 4.10.

Figure 4.10. Extract D: Chromatogram obtained from Ganzera method at 270nm.



1 - Rutin, 3 - Hyperoside, 4 - quercitrin, 5 - quercetin, 7 - pseudohypericin, 8 - hyperforin, 9 - hypericin. Conditions: Flow rate: 1.0 ml/min; Column: Phenomenex Synergi MAX-RP 80A, 4 : m, 150 x 4.6 mm i.d. Detection: 270nm; Temperature: 40°C; Injection volume: 10 : l.

4.7.2 LC-MS-MS ANALYSIS

4.7.2.1 Introduction

Mass spectrometry is a powerful detection tool that is more selective and specific than other detection systems used with HPLC. Natural medicines usually constitute a multitude of constituents with many potentially interfering components. In this regard LC-MS-MS is a powerful tool to unequivocally identify target analytes regardless of the presence of interferences or complexity of matrix.

The theory behind mass spectrometry is that ions are produced which are then subsequently separated or filtered according to their mass-to-charge (m/z) ratio and subsequently detected. An essential and vital part of any mass spectrometric system is the interface. An LC-MS interface must accomplish nebulization and vapourization of the eluate, ionization of the analytes, removal of excess solvent

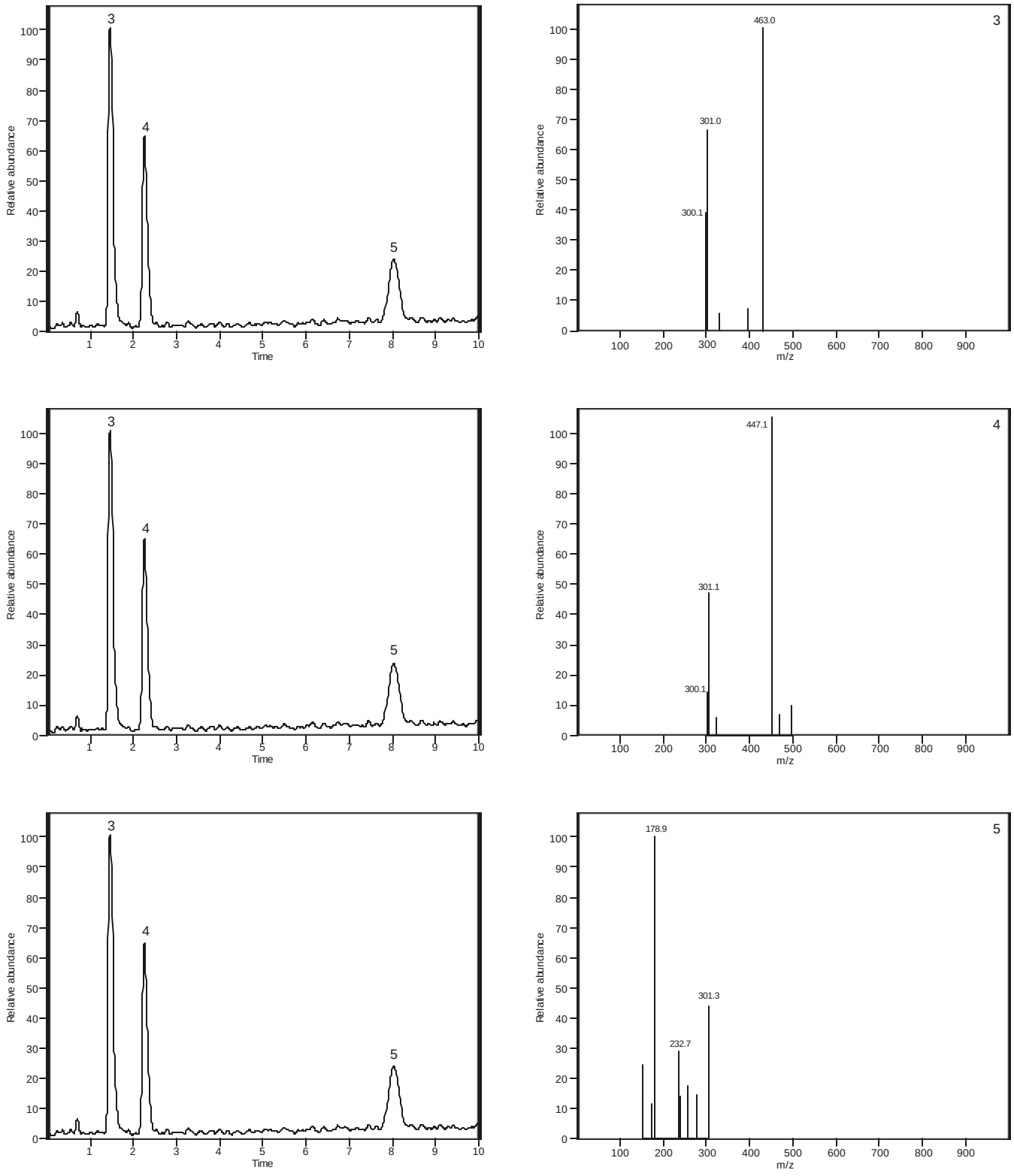
vapour and extraction of the ions into the mass detector. There are many different interfaces available, but the two most commonly used interfaces in the analysis of herbal extracts are electrospray (ESP) and thermospray (TSP) [104].

ESP or electric atomization occurs when a high potential is applied between a narrow bore capillary filled with a flowing liquid and a nearby plate, producing a mist of charged droplets. The ions observed in this case are mostly protonated molecules. The TSP interface on the other hand is based on the formation of a mist of vapour and small droplets from a heated vapourizer tube. It has been reported that with thermally labile ionic compounds, electrospray ionization (ESI) shows better performance than TSP, as ESI allows softer ionisation. Both ESP and TSP are, however, soft ionization techniques and most commonly do not produce many fragments. This is useful in qualitative and quantitative analyses [104,105].

ESI was used for the qualitative analysis of the content of Hd, Qr and Q in the five tablet products investigated in this thesis and ESI-MS-MS was performed on all the extracts from those respective tablets.

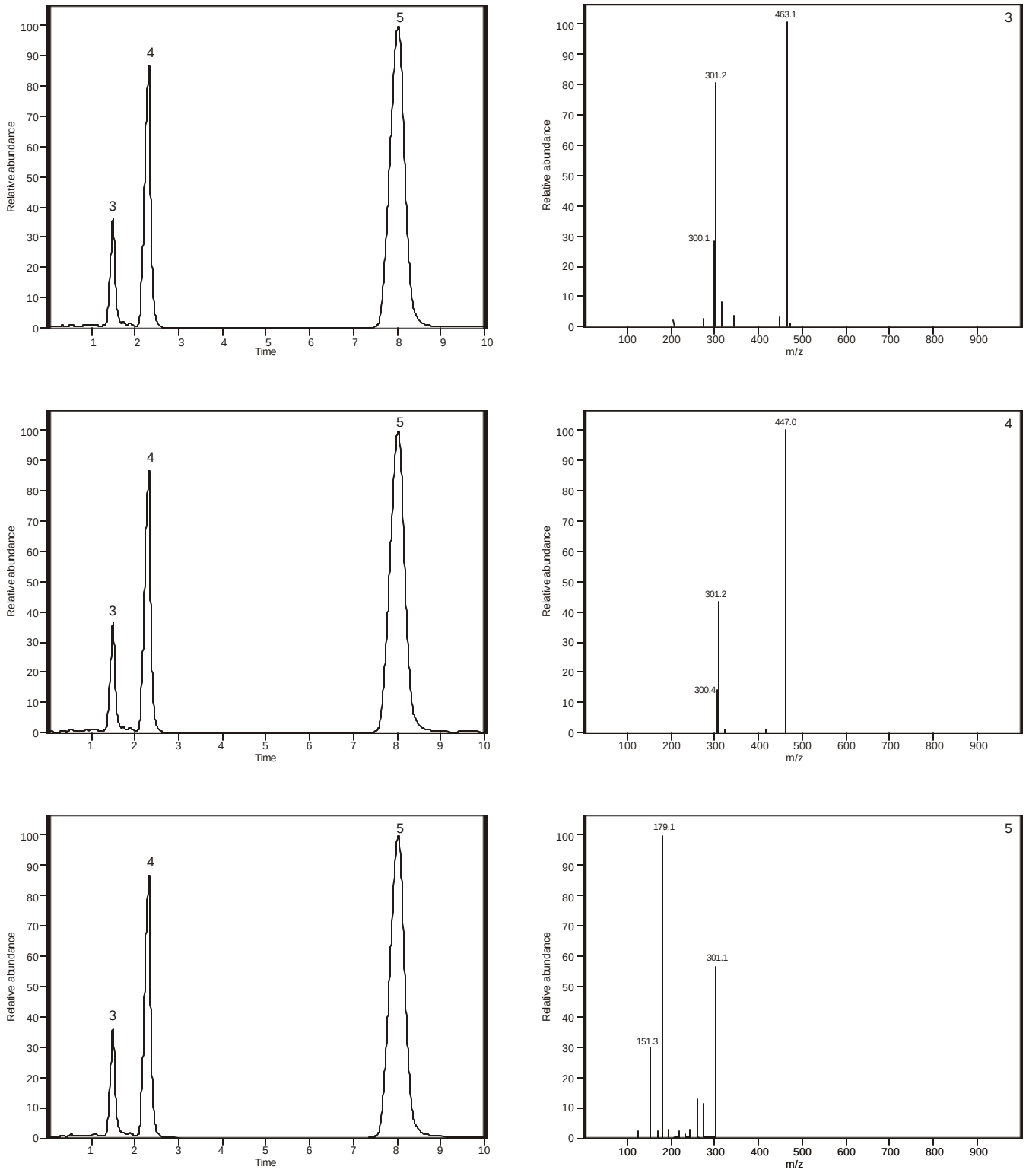
Figure 4.11 illustrates the total ion chromatogram and the mass fragmentation pattern for a calibrator that was used to construct calibration curves for the semi-quantitative analysis of all five products for Hd, Qr and Q. The left-hand block of each of the Figures illustrates the chromatography, whilst the right-hand block shows the mass fragmentation pattern of each of the respective flavonoids, numbered accordingly.

Figure 4.11. Low calibrator for LC/MS/MS.



3 - Hyperoside, 4 - Quercitrin, 5 - Quercetin. Conditions: Flow rate: 0.3ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm i.d. Temperature: 45℃; Injection volume: 20 : l.

Figure 4.12. Extract A.



3 - Hyperoside, 4 - Quercitrin, 5 - Quercetin. Conditions: Flow rate: 0.3ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm i.d. Temperature: 45℃; Injection volume: 20 : l.

As can be seen, the LC-MS-MS procedure was very effective in "filtering" out interferences.

The results from the 2 HPLC methods and LC/MS/MS are shown in Tables 4.14 to 4.18.

4.7.2.2 Rutin

The values obtained for R are shown in Table 4.14 below. Quantitation was performed at 254nm and 350nmn and semi-quantitative confirmation was obtained using the Ganzera method.

Table 4.14. Assay results for rutin.

Rutin: Amount (mg/0.5g tablet product)			
Product	254nm	350nm	Ganzera
A %RSD	0.026 5.2	0.028 7.5	*
B %RSD	0.023 14.4	0.021 12.0	*
C %RSD	0.016 53.9	0.012 21.1	*
D %RSD	0.940 4.34	0.967 4.1	1.150 3.2
E %RSD	1.077 2.0	1.090 1.7	1.180 2.0

** - Below LOQ*

The chromatography in Figure 4.7a illustrates that very small amounts of R were present in extracts A, B and C. The values obtained using the currently developed method at 254nm and 350nm for extracts A, B and C are seen to be consistent.

The most likely reason for the relatively high % RSD in the case of Extract C is that there was very little R present in Extracts A, B and C, and especially in the case of Product C the concentrations found were approaching the LOQ.

The chromatography obtained for the three products A, B and C using the method of Ganzera et al shows no R present (see Figure 4.7b). Although Ganzera *et al*. reported an LOQ for R of 0.16µg/ml, and the concentrations found in these three extracts using the currently developed were approximately an order of 10 higher than this, it was not possible to even detect the R using the Ganzera method. The

Ganzera method was not validated on the HPLC system used and the apparent absence of R may simply be that this method could not detect such low quantities of R.

The results from 254nm, 350nm and the Ganzera method for extracts D and E are all in agreement and serve to confirm the quantities of R present. The Ganzera method was not validated and served as a semi-quantitative tool.

In their analysis of nine products, Ganzera *et al.* [85] found between 0.08 and 0.705mg rutin per 0.5g product, and Liu *et al*. [89] found 3.35 to 10.95 mg R per 0.5g product. The current results indicate that the products evaluated contained a relatively low amount of R in comparison to other St John's Wort products on the market.

4.7.2.3 Hyperoside

The values obtained for Hd are shown Table 4.15 below. Quantitation was performed at 254 and 350nm, and semi-quantitative confirmation was obtained from LC/MS/MS and from applying the Ganzera method.

Table 4.15. Assay results for hyperoside.

Hyperoside: Amount (mg/0.5g tablet product)				
Product	254nm	350nm	LC-MS-MS	Ganzera
A %RSD	0.037 6.3	0.039 5.5	0.048 15.8	0.047 2.4
B %RSD	0.021 6.4	0.025 3.7	0.019 14.3	0.037 4.4
C %RSD	0.014 15.6	0.016 8.5	ND	0.026 12.0
D %RSD	1.34 4.6	1.409 4.2	2.672 1.5	1.458 27.0
E %RSD	0.728 0.8	0.791 1.6	1.807 9.9	1.063 2.7

ND - Not determinable

Extract A was found to contain approximately 0.04mg hyperoside per 0.5mg product. All four methods yielded similar results.

Product B was found to contain slightly less Hd than Product A, and the results using all four methods were similar. Similarly Product C was also found to contain very low amounts of Hd. It should be

noted that in spite of approaching the LOQ for Hd, the methods yielded relatively consistent results. Due to poor chromatographic results, with substantial peak shape changes, high %RSDs resulted, and realistic values were not obtained using LC/MS/MS. Any slight peak shape change would substantially change the area of the respective peak, and cause the high %RSDs.

The extracts from products D and E yielded much higher concentrations of Hd. The results from both extracts obtained from the three HPLC-UV methods were consistent, but the LC-MS-MS method yielded higher values, approximately twice as high. The reasons for this observation are difficult to explain.

Ganzera *et al* [85] and Liu *et al* [89] reported values of Hd to be in the ranges of 0.005 – 0.033mg and between 1.75 and 7.5 mg per 0.5mg product, respectively. The current results obtained from the products investigated are thus consistent with those previously reported.

4.7.2.4 Quercitrin

Quantitative analysis was performed at 254nm and 350nm, and semi-quantitative data were obtained from LC/MS/MS and from application of the Ganzera method.

Table 4.16. Assay results for quercitrin.

Quercitrin: Amount (mg/0.5g tablet product)				
Product	254nm	350nm	LC-MSMS	Ganzera
A %RSD	0.540 2.5	0.427 3.3	0.488 5.6	0.289 0.4
B %RSD	0.531 4.8	0.177 3.4	0.136 2.3	0.085 1.6
C %RSD	ND	0.177 5.4	ND	0.026 5.1
D %RSD	0.581 6.1	0.401 4.1	ND	0.363 2.9
E %RSD	0.728 0.8	0.644 2.3	1.643 3.1	0.465 3.1

ND - Not determinable

As previously mentioned, problems with interference were experienced. This can be seen in the chromatography in Figures 4.6.and 4.9a. The results obtained from calibration and integration at 254nm were higher than the actual quantities contained in the extract due to these interferences at the base of the peak. There was slightly less interference at the base of the peaks using a detection

wavelength of 350nm and hence the values obtained at this wavelength may only be a slight overestimate. In product C, no data could be obtained at 254nm due to interference. The LC-MS-MS values for C and D were omitted as the percentage RSDs were consistently high as a result of variable chromatography.

The results obtained for product A using the Ganzera method was slightly lower. This may be due to the fact that the extracts had been stored at -20°C for two months and then analyzed by the HPLC method of Ganzera *et al*.

With Extract E, the LC-MS-MS appeared to yield an overestimate of the results obtained by the other three methods. In LC-MS-MS, parent ions and interfering ions with the same mass-to-charge ratios are selected and hence full scan MS-MS helps determine from the mass spectral profile whether an interfering ion was present by observing the presence of ghost ions. There did not appear to be any ghost ions present, and it is unclear as to why the LC-MS-MS yielded an overestimate of the results. These same extracts were subsequently analyzed by the Ganzera *et al*. method.

Ganzera *et al*. [85] reported a range of 0.05 to 0.145mg Qr per 0.5g and Liu *et al*. [89] reported a range of between 0.03 to 1.45 per 0.5g product assayed. The current results for Qr obtained from the products analyzed in this thesis fall within those ranges.

4.7.2.5 Quercetin

The values obtained for quercetin are shown Table 4.17. Analysis was performed at both 254nm and 350nm, and semi-quantitative data were obtained from LC-MS-MS and from the application of the Ganzera method.

Table 4.17. Assay results for quercetin.

Quercetin: Amount (mg/0.5g tablet product)				
Product	254nm	350nm	LC-MS-MS	Ganzera
A	1.674	1.629	1.365	1.943
%RSD	2.4	2.1	9.2	1.3
B	0.783	0.730	0.865	0.913
%RSD	3.0	3.4	3.5	0.8
C	0.860	0.808	0.876	0.899
%RSD	3.8	3.7	10.2	5.3
D	0.867	0.851	1.356	1.096
%RSD	4.3	4.1	7.3	1.5
E	0.452	0.422	ND	0.503
%RSD	1.4	1.5		2.0

ND - Not determinable

Generally all four of the methods used for each extract yielded similar results in terms of quantities of quercetin present. The chromatography for Extract E using LC-MS-MS showed a high degree of variability and values for quercetin could thus not be determined.

4.7.2.6 Kaempferol

The values obtained for kaempferol are shown Table 4.17. Analysis was performed at both 254nm and 350nm.

Table 4.18. Assay results for kaempferol.

Kaempferol: Amount (mg/0.5g tablet product)		
Product	254nm	350nm
A	0.048	0.037
%RSD	5.2	2.6
B	0.016	0.016
%RSD	3.6	2.8
C	0.031	0.017
%RSD	16.5	4.9
D	0.029	0.031
%RSD	5.0	3.8
E	0.011	0.011
%RSD	1.7	1.2

The values obtained for K were very low for all the extracts. There was a peak that eluted directly after K, which resulted in a fused peak when K was present. This can be seen in Figure 4.8a. Integration was performed by dropping a vertical line where necessary. The values obtained for Extracts A and C differed slightly at the different wavelengths, but the concentrations of K were very

low (1.1 to 4.8 : g/ml) and close to the LOQ. There was effectively no significant amount of K present in any of the products assayed.

4.8 CONCLUSIONS

The nature of the flavonoids is such that under certain, usually extreme conditions, hydrolysis can occur resulting in the release of the aglycone moiety from its glycoside structure. Sticher reported [94] that the flavonoid glycosides and the biflavones are relatively stable, and that the ratios of these do not change when plant parts or extracts are stored properly. Thus an increase in aglycone content and a decrease in glycoside content indicate undesirable degradation processes. These processes may result from one or many external factors, such as, for example, poor storage which is a typical cause of degradation.

In this study each flavonoid investigated, except for kaempferol, was a quercetin derivative. Thus it can be seen that the lower the glycoside content and the higher the quercetin content, the poorer the quality of the product. Comparing flavonoid glycoside results obtained in this study with those reported from other studies, these results generally tended towards the lower end of the ranges reported, and in some instances were even lower than those reported elsewhere. The amount of quercetin found in the tablets assayed was relatively high when considered in terms of the ranges previously reported, and in some cases, even higher than those previously reported ranges. This implies that the products assayed in this study, especially A, B and C are of poor quality. However, although Product D had quite a high quercetin content, it also had a higher intact glycoside content than products A, B and C. In view of the fact Product E was found to contain the lowest amount of quercetin and in general, the highest amounts of in tact glycosides, this product can be considered to be most likely the best quality product assayed in this study. By virtue of it's content of intact glycosides, Product D is also a good quality product.

Interesting to note was the inter-batch variability between the amounts of flavonoids found in products A, B and C. Three batches of the same brand of tablets were found to possess largely differing flavonoid content. This was most pronounced with Product C, where the content of any specific flavonoid was often as much as 50% lower than the content in the other batches.

The LC-MS-MS method served as a semi-quantitative tool for checking purposes only. High percentage RSDs were not uncommon, and changes in peak shape and chromatography were also encountered. Whilst the LC-MS-MS method showed great promise for use as a quantitative tool for assay purposes, further investigations and optimisation are clearly necessary before it can be used as a routine quantitative method.

The semi-quantitative use of the Ganzera method also provided useful information. The method was reproducible in our laboratory without any further adjustments. However, when the chromatograms obtained from use of the Ganzera method were adjusted to the same scale as that used with the currently developed HPLC method, it could be seen that baseline resolution was not obtained when using the method developed by Ganzera *et al*. In addition, Ganzera *et al*. used higher concentrations for their calibrators, and their extracts were more concentrated than those used in the analyses reported in this thesis.

Most of the methods that have been used to separate flavonoid compounds in St John's Wort products include the naphthodianthrones and phloroglucinols in their separation, and as a result generally possess run times longer than twenty minutes. As well as this, the separations between rutin, isoquercitrin and hyperoside in all of the methods were not fully resolved. Importantly, kaempferol was not determined using any of these methods. The gradient method developed to quantitatively determine the various flavonoid components in St. John's Wort products and reported in this thesis was validated and had the necessary properties to effect a precise, reliable, accurate separation of relevant flavonoids found in St. John's Wort products (rutin, isoquercitrin, quercitrin, hyperoside, quercetin and kaempferol) in less than 20 minutes.

CHAPTER FIVE

QUANTITATIVE AND QUALITATIVE ANALYSIS OF NAPHTHODIANTHRONES AND HYPERFORIN

5.1 INTRODUCTION AND BACKGROUND

An HPLC method using a narrow bore column and UV detection was developed for the assay of hypericin (HY), pseudohypericin (PsHY) and hyperforin (Hypf) and validated to quantitatively determine these three compounds in some commercial St John's Wort products. In addition, an LC-MS-MS method was employed for semi-quantitative and qualitative analysis of those same St. John's Wort products.

Summaries of the most relevant and recent HPLC methods used for the quantitative analysis of the relevant naphthodianthrones and Hypf are listed in Tables 5.1 and 5.2. Some of the methods were designed for use to assay those components in pharmaceutical matrices (Table 5.1), and also, in some cases, in plasma (Table 5.2). The column, type of detection, mode of operation, concentration ranges and retention times of the respective compounds are outlined in this chapter.

The only method listed in Table 5.1 and 5.2 that separates all three compounds of interest in a relatively short run time is the method developed by De los Reyes and Koda [42]. The other methods reported in the Tables either focus on the determination of all the compounds of interest simultaneously in St John's Wort products, including the flavonoids, naphthodianthrones and Hypf, or the determination of either the naphthodianthrones and/or the phloroglucinols separately. In the first case, due to the wide range of polarity of all the compounds of interest, gradient methods were required to achieve full separation. This resulted in relatively long retention times for the naphthodianthrones and Hypf. The method developed by Ganzera *et al*. [85] separates all the compounds of interest in a relatively short total run time. This method was published subsequent to the initiation of the method development, validation, and quantitation procedures described in this thesis.

Table 5.1. Determination of compounds in extracts or pharmaceutical matrices.

Reference	Column	Mode	Detection	Concentration Range (µg/ml)			Retention times (mins)		
				HY	PsHY	Hypf	HY	PsHY	Hypf
De los Reyes, *et al*., 2001. [42]	Discovery C_{18} 5µm 150 x 4.6 mm i.d.	ISOCRATIC	Fluorescence (hypericins) and UV (hyperforin)	0.5-2.5	0.35-1.6	5-50	8	3	6
Draves, *et al*., 2000. [95]	Ultrasphere C_{18} 5 : m 250 x 4.6 mm i.d.	GRADIENT	Fluorescence and UV (hypericins)	0 - 14.6	-	-	16	12	-
Ganzera, *et al*., 2002. [85]	Synergi MAX-RP C_{12} 4 : m 150 x 4.6 mm i.d.	GRADIENT	Photodiode Array (Hypericins and hyperforin)*	2.1-500	2.1-500	2.1-500	31.5	26.6	32.3
Li, *et al*., 2001. [86]	Waters YMC ODS-AQ C_{18} 5 : m 250 x 4.6 mm i.d.	GRADIENT	Photodiode Array and UV (Hypericins and hyperforin)*	20-200	20-200	20-200	50	34	36
Sirvent, *et al*., 2000. [96]	Diazem-phenyl™ Metachem 5 : m 250 x 4.6 mm i.d.	ISOCRATIC	UV (hypericins)	0-1000	0-1000	-	5.3	8.7	-
Pietta, *et al*., 2001. [87]	Symmetry C_{18} 5 : m 250 x 4.6 mm i.d.	GRADIENT	UV (Hypericins and hyperforin)	0-1000	0-1000	0-1000	63	53	57
Brolis, *et al*, 1998. [88]	201 TP 54 Vydac C_{18} 5 : m 250 x 4.6 mm i.d.	GRADIENT	UV (Hypericins and hyperforins)*	Used relative response factor to external standard over range 4.5-100			43.4	40.9	46.1
Liu, *et al*., 2000. [89]	LiChrospher C_{18} 3 : m 250 x 4.0 mm i.d.	GRADIENT	Photodiode Array (Hypericins and hyperforin)*	20-100	40-200	200-1000	79	76	80
AHP, 1997. [25]	Prosep C_{18} 5 : m 150 x 4.0 mm i.d.	GRADIENT	UV (Hypericins and hyperforin)*	10-60	10-60	8-240	31	24	25

* Method also used for simultaneous determination of flavonoids and other components in product.

Table 5.2. Determination of compounds in plasma.

Reference	Column	Mode	Detection	Concentration Range (ng/ml)			Retention times (mins)		
				HY	PsHY	Hypf	HY	PsHY	Hypf
Chi, *et al*., 1999. [98]	Capital C_{18}/CN 5: m 150 x 4.6 mm i.d.	ISOCRATIC	UV (Hyperforin)	-	-	0.15-3.0	-	-	7
Chi, *et al*., 1999. [99]	Capital C_8 5: m 150 x 4.6 mm i.d.	ISOCRATIC	Fluorescence (Hypericin)	5-100	-	-	3.8	-	-
Bauer, *et al*., 2001. [100]	LiChrospher C_{18} 5: m 250 x 4.6 mm i.d.	ISOCRATIC	Fluorescence (Hypericins)	0.25-40	0.25-40	-	8.0	4.5	-
	Phenomenex Luna C_{18} 5: m 250 x 4.6 mm i.d.	ISOCRATIC	UV (Hyperforin)	-	-	0-300	-	-	6.7

5.2 METHOD DEVELOPMENT

An HPLC method to separate all three compounds of interest within twenty minutes was developed.

5.2.1 INSTRUMENTATION

Refer to Section 3.2 for all instrumentation and additional equipment used.

5.2.2. MATERIALS AND REAGENTS

- Hyperforin [7]
- Glacial acetic acid (Batch 40773) [3]
- Sodium hydroxide [3]
- Ammonium acetate (ACE - Batch 1483/767) [8]

[3] Saarchem (Pty) Ltd., Johannesburg, South Africa

[8] Associated Chemical Enterprises (Pty) Ltd., PO Box 379, Glenvista, 2058, South Africa

[7] PhytoLab GmbH & Co., KG, Wandaleng 24, D-20097 Hamburg/Germany.

Refer to Section 3.3 for all other materials and reagents used.

5.2.3 METHODS

5.2.3.1 Selection of Analytical Column

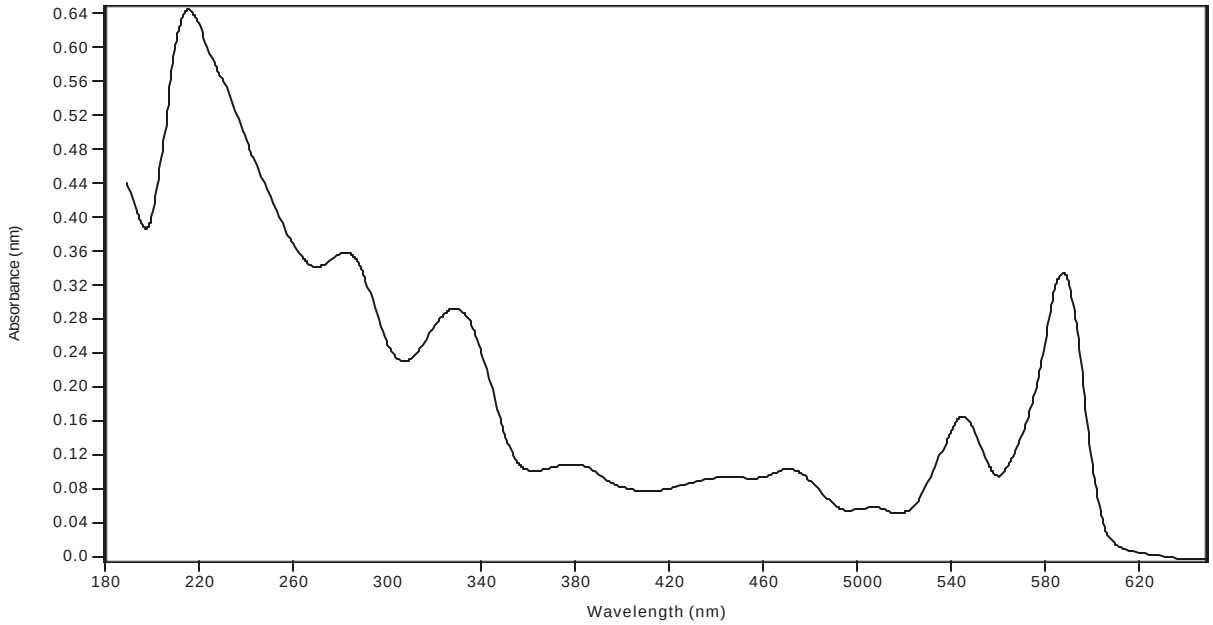
Due to the relatively low concentrations of these marker compounds, and high likelihood of interference in tinctures and extracts, a narrow bore column was selected in an effort to facilitate resolution, sensitivity and selectivity. The narrow bore column was the same as that used for the separation of flavonoids, described previously.

5.2.3.2 Choice of Wavelength

Due to the prohibitive cost of HY, PsHY and Hypf, and the small amounts of these compounds on hand, a UV/VIS spectrum was only determined for (PsHY) in methanol (Figure 5.1).

Peak maxima occur at 215nm, at 590nm and 282nm. The absorbance values show that PsHY does not have very good UV absorbance and this is true for HY too. The literature reports HY to have UV absorbance maxima at 590nm and 282nm [39]. Hypericin and PsHY have very similar structures (see Figure 1.2), the only difference being that PsHY has a hydroxyl-group at C in place of a hydrogen atom. Hence, the absorption spectra are similar and HY can also be expected to absorb around 215nm.

Figure 5.1. Absorbance spectrum for PsHY in methanol.



5.2.3.3 Mobile Phase Selection

Initially, attempts were made to reproduce the de los Reyes method [42] (acetonitrile: 0.3% ortho-phosphoric acid (v/v) 90:10), using a narrow bore (Luna, 2.0 mm i.d.) column which was not the same as the 4.6 mm i.d Discovery column used by those authors. However, under those conditions, both naphthodianthrones eluted in the void. The percentage of acetonitrile was systematically decreased and an acceptable separation of the naphthodianthrones was achieved using a ratio of 30:70 ortho-phosphoric acid (0.3%): acetonitrile and a flow rate of 0.2ml/min. This ratio resulted in HY eluting at 2.5 minutes and PsHY at 16 minutes. However, using this composition of mobile phase, Hypf was retained on the column for six hours. Further investigations involving modification of the mobile phase composition to an ultimate composition of 97:3 acetonitrile:acid using a flow rate of 0.2ml/min resulted in the elution of HY within ten minutes. This suggested that a gradient would probably be required to separate these three compounds using the mobile phase of 0.3% ortho-phosphoric acid and acetonitrile with the narrow bore column.

Subsequently, attempts were made, firstly, to develop a gradient method using 0.3% ortho-phosphoric acid: acetonitrile, and secondly to reproduce the method that Brolis *et al*. had developed [88]. (Brolis *et al*. developed a method to separate all the compounds of interest in St John's Wort extracts, using three solvents [A-water: ortho-phosphoric acid (99.7:0.3 v/v), B-acetonitrile, C-methanol]). Neither of

these approaches was successful. The wavelengths for analysis of these three compounds were chosen as 215nm and/or 270nm (590nm was an option for the naphthodianthrone compounds only). It was subsequently found that the phosphoric acid contained impurities that absorbed UV light, especially at lower wavelengths. These impurities adsorbed to the packing material of the reversed phase narrow bore columns. When a gradient was used, these interfering compounds only eluted with increasing solvent strength causing multiple peaks and interferences in the chromatography at 215nm and to a lesser extent at 270nm.

In light of the above observations the development of an isocratic method was undertaken for the separation of HY, PsHY and Hypf. The initial method used for the development was a method developed in-house at Phenomenex [97] for St. John's Wort that utilised acetonitrile: methanol: 0.1N ammonium acetate (pH 9.7) (50:30:20) and resulted in a total run time of 16 minutes for the separation of HY and PsHY. The column used was a Luna 5µm C18 (2), 150x4.6mm. The analysis was performed at 590nm at 22°C using a flow rate of 1.5ml/min. Hypf was not included in their separation. This method developed by Phenomenex was optimised for the separation of HY, PsHY and Hypf and successfully applied for the separation of all three compounds of interest.

5.2.3.4 Optimization of Chromatographic Method

Standard operating procedures (SOP's) which are outlined in the Appendix section of this thesis were followed.

In order to avoid possible degradation of the light-sensitive naphthodianthrones and Hypf, all procedures were performed in a dark room that was fitted with two special Philip's green light bulbs, which enabled low visibility without effecting the compounds' breakdown.

Many sample preparation techniques used for *Hypericum perforatum* involved intentional exposure of the extracts to a specified wavelength of light to facilitate complete conversion of the proto-forms of the hypericins to their conjugate analogues. This was not performed in this case, as light exposure may have been detrimental to Hypf [104].

All samples were stored at -20°C in amber glassware or covered with foil prior to analysis.

5.2.3.4.1 Effect of Buffer Molarity

The effects of buffer molarity on the retention time of HY were investigated. Due to the small amounts of sample present, and the cost of these compounds, initially only HY was used. PsHY has a similar structure to HY and was expected to respond to changes in a similar fashion as HY. The flow rate used was 0.3ml/min, wavelength, 215nm, and the analysis was performed at 22°C. The mobile phase consisted of ammonium acetate: acetonitrile: methanol in the ratio 20:50:30. Unadjusted ammonium acetate in solution was found to have a pH of around 6.8. This investigation was performed at pH values of both 9.7 (pH adjustment with sodium hydroxide) and 6.8.

Ammonium acetate solutions at pH values of 9.7 and 6.8 were made up to result in molarities of 0.1M, 0.05M and 0.01M, respectively. Using the method described above, these different buffer molarities were individually investigated to establish their effects on the chromatography of the relevant compounds. The change in buffer molarity did not significantly affect the retention time of HY. At a pH of 9.7, the retention time of HY was found to be approximately 12.4 minutes regardless of buffer molarity and at a pH of 6.8, the retention time of HY was approximately 10 minutes.

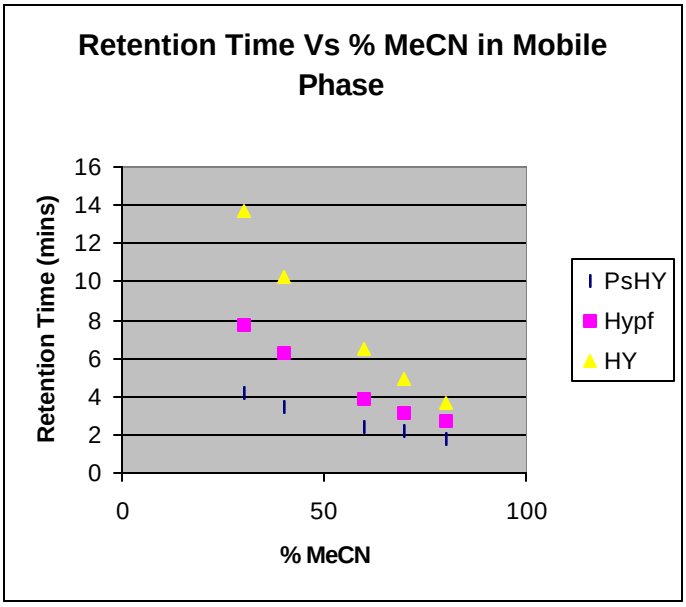
Ammonium acetate solution (0.01M) and a pH of 6.8 was subsequently chosen to continue with the investigations.

5.2.3.4.2 Effect of Mobile Phase Composition

In this investigation, the percentages of acetonitrile and methanol were systematically changed and the shift in retention times of HY, PsHY and Hypf was monitored. The % ammonium acetate solution (0.01M) was maintained at 20% throughout this at a pH 6.8 using a flow rate of 0.3ml/min and a temperature of 22°C.

Figure 5.2 below illustrates the variation in retention time with increasing percentage acetonitrile and relative decrease in percentage methanol in the mobile phase.

Figure 5.2. Effect of change in retention time with change in % acetonitrile.



Conditions: 20% 0.01M ammonium acetate (pH 6.8); Flow rate: 0.3ml/min; Temperature: 22°C.

As can be seen from Figure 5.2, decrease in retention times occurred for all three compounds with increase in the percentage of acetonitrile. This was expected, as acetonitrile has higher eluting power than methanol. The mobile phase composition of acetonitrile: 0.01M ammonium acetate (pH 6.8): methanol (30:20:50) was selected as the mobile phase for further development. This method had the lowest acetonitrile content of those investigated and resulted in the longest retention times for all three compounds.

A mixture of rutin, isoquercitrin, hyperoside, quercitrin, quercetin and kaempferol was injected using this method. This was in an attempt to see if these compounds could also be included in the method development for the separation using this isocratic method. As well as this it was useful in order to know where these compounds would elute when St John's Wort extracts were run. All the above-mentioned flavonoids were not retained on the column and eluted in the void volume. When St. John's Wort tablet extracts were injected using this method, a substantial amount of unknown potential interfering substances also eluted in the void volume during the first few minutes of the run. In order

to minimize potential interferences, a system had to be devised whereby the first eluting peak, PsHY, needed to be retained on the column for at least 4 minutes.

From Figure 5.2 it can be seen that PsHY, HY and Hypf have retention times of 4.2, 13.7 and 7.7 minutes respectively using this method.

5.2.3.4.3 Optimization of Flow Rate and Temperature

Using the method previously described in Section 5.2.3.4.2, the temperature was increased to 30°C whilst maintaining a flow rate of 0.3ml/min, and then the flow rate was decreased to 0.2ml/min whilst maintaining a temperature of 30°C. The resultant retention times are shown in Table 5.3.

Table 5.3. Effect of flow rate and temperature on retention times of the three compounds.

	Retention Time (minutes)		
Compound	0.3ml/min & 22°C	0.3ml/min & 30°C	0.2ml/min & 30°C
Pseudohypericin	4.2	3.6	5.4
Hyperforin	7.7	6.8	10.2
Hypericin	13.7	10.7	15.9

Conditions: MeCN: 0.01M ammonium acetate (pH 6.8): MeOH 30:20:50.

Table 5.3 depicts the decrease in retention time with increase in temperature, and increase in retention time with decrease in flow rate. This is also shown in Table 5.4 where the retention times decreased with increasing temperature.

In an attempt to increase the retention time of PsHY, a flow rate of 0.2ml/min and temperature of 30°C were maintained whilst the mobile phase composition was changed to acetonitrile: ammonium acetate 0.01M (pH 6.8): methanol 20:20:60. Using this mobile phase composition and a flow rate of 0.2ml/min, the effect of increasing the temperature to 35°C and then 40°C, temperatures of 35°C and 40°C were investigated. The results are shown in Table 5.4.

Table 5.4. Effect of temperature on the retention times of HY, PsHY and Hypf.

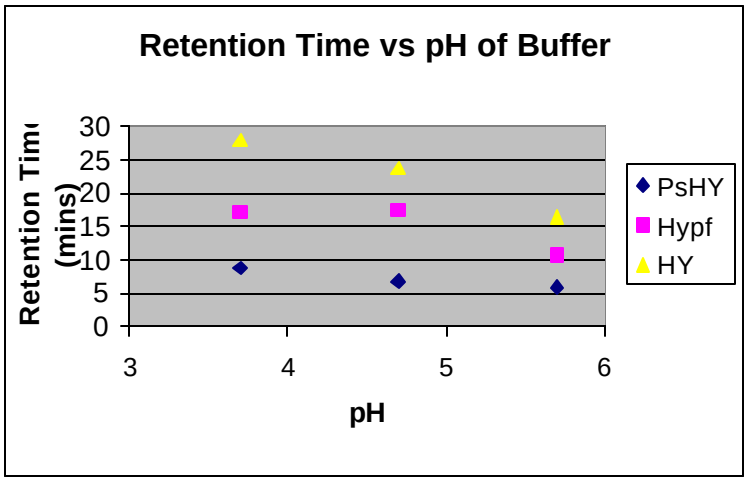
	Retention Time (minutes)		
Compound	0.2ml/min & 30°C	0.2ml/min & 35°C	0.2ml/min & 40°C
Pseudohypericin	5.9	5.3	4.9
Hyperforin	11.3	10.6	9.6
Hypericin	18.7	16.0	13.9

Conditions: MeCN: 0.01M ammonium acetate (pH 6.8): MeOH 20:20:60; Flow rate 0.2ml/min.

5.2.3.4.4 Effects of pH

Acetic acid has a reported pKa of 4.7. The pH investigation was designed so that the pHs values of the buffers used would be within the buffering capacity range of ammonium acetate. Using glacial acetic acid, the pH of the ammonium acetate solution was adjusted to obtain pH values of 3.7, 4.7 and 5.7, respectively for further investigations.

Figure 5.3. Effect of pHon retention times



Conditions: MeCN: 0.01M ammonium acetate (pH 6.8): MeOH 20:20:60; flow rate 0.2ml/min; Temperature: 35°C.

As can be seen in Figure 5.3 above, increasing the pH resulted in a reduction in retention times for all three compounds.

A pH of 5.7 was selected for final optimization of the separation conditions. Using a pH of 5.7, the total run time was kept within 20 minutes and permitted PsHY to elute without when injecting extracts.

5.2.3.4.5 Flow Rate, Temperature and Valve Investigations.

Injections were made using flow rates of 0.3ml/min or 0.2ml/min as well as on-line mixing. This was in order to investigate any effects of these conditions on retention time behaviour. In addition a mixture of HY, PsHY and Hypf was injected using a flow rate of 0.2ml/min at 40°C and the results are depicted in Table 5.5.

Table 5.5. Effect of temperature, flow rates and solvent mixing procedures on the retention times of HY, PsHY and Hypf.

	Retention Time (minutes)				
Compound	0.3ml/min & 35°C		0.2ml/min & 35°C		0.2ml/min & 40°C
	One line Single solution	Mixing on line	One line Single solution	Mixing on line	One line Single solution
Pseudohypericin	5.2	4.7	7.5	6.9	6.5
Hyperforin	10.7	9.3	16.0	13.7	13.3
Hypericin	17.1	15.1	25.3	22.1	19.9

Conditions: MeCN: 0.01M ammonium acetate (pH 6.8): MeOH 20:20:60.

A flow rate of 0.2ml/min and a temperature of 40°C resulted in the most acceptable retention times and provided good peak shapes for each of the compounds. This method was ultimately chosen for the separation and quantitative analysis of the naphthodianthrones and hyperforin.

Table 5.5 shows that using one line to deliver the pre-mixed mobile phase resulted in retention times compared with mixing the solvents on-line. Mixing solvents on-line resulted in variable retention times at the low flow rate used (0.2ml/min). The reasons for this have been briefly mentioned in Section 4.4. Most likely this was caused by the very low flow rate used (µl/min as compared with ml/min) coupled with the lack of appropriate micro-plumbing optimization of the HPLC system and the inability of the standard macro GPV valve to cope with mixing such low volumes and flow rates. Sixteen injections were made using the selected method using a single line to deliver the pre-mixed mobile phase. The percentage RSD for the retention times of PsHY, Hypf and HY were 0.9, 1.3 and 0.4% respectively. Subsequently only one line was used to deliver pre-mixed mobile phase.

5.3 METHOD VALIDATION

The method used for the validation involved the use of acetonitrile: 0.01M ammonium acetate (pH 5.7): methanol (20:20:60), at a flow rate of 0.2ml/min and a temperature of 40°C. The column used was a Luna 5µm ODS (2), 150 x 2.0mm i.d. with a Luna C18 SecurityGuard guard column.

De Los Reyes *et al*. [42] in their HPLC assay for the determination of hypericins and stabilized Hypf in commercial St John's Wort preparations described the extractability of the naphthodianthrones and Hypf and the stability of Hypf in various solvents. Their findings illustrated that Hypf was stable in aqueous acidified methanol and that this solvent had deleterious effects on the extraction or stability of the naphthodianthrones over the concentration range studied (0.35 to 1.6µg/ml and 0.5 to 2.5µg/ml for PsHY and HY respectively). From their data, the extraction method selected utilized acidified methanol. PsHY, HY and Hypf standards were made up in aqueous acidified methanol (methanol: water (80:20) adjusted to pH 2.5 with ortho-phosphoric acid).

Calibration curves were prepared with these standards over various ranges from 10-200µg/ml, to 1-20µg/ml. Hyperforin was linear over all the concentration ranges studied, but HY and PsHY revealed non-linear characteristics over all the ranges. The possibility that the presence of acid in the sample solution may have resulted in the non-linearity was considered and this is discussed in Section 5.3.5.

5.3.1 INSTRUMENTATION

- Meyer N-Evap Analytical Evaporator, model 112 (Organomation Associates Inc., South Berlin, MA 01549, USA).
- Durapore® membrane filters, Type: 0.45 : m HVLP (Millipore, Bedford, Massachusetts, USA).

Refer to Section 3.2 for all other instrumentation and additional equipment used.

5.3.2 MATERIALS AND REAGENTS

Refer to Section 5.2.2 for all materials and reagents used.

5.3.3 METHODS

Standard operating procedures (SOPs) were followed and these are outlined in the appendix section of this thesis.

The presence of acid in the sample solution was seen to be the cause of the non-linearity exhibited by the naphthodianthrones, when they were made up in acid solution. This is discussed in Section 5.3.5. From these investigations it was evident that although the method developed was suitable for the separation of Hypf and the naphthodianthrones, sample solutions would have to be made up separately, and extractions, recoveries and quantitative analysis of Hypf and the naphthodianthrones would have to be performed individually. Hyperforin samples were made up in aqueous acidified methanol, whereas, HY and PsHY were made up in methanol only.

All experimental procedures were performed with the exclusion of light in a dark room equipped with two Philips green dark room globes. Solutions and standards were stored at -20°C in amber glassware or covered with foil until analyzed.

5.3.3.1 Hyperforin

Hyperforin (10.4 mg) was obtained from PhytoLab (Wandaleng 24, D-20097 Hamburg/Germany). The sample vial containing hyperforin was weighed and the contents decanted into a 10ml volumetric flask. The sample vial was rinsed thoroughly with acidified aqueous methanol and the liquid was quantitatively transferred to the volumetric flask. Once all the Hypf had been rinsed out, the inside of the vial was dried using an N-Evap analytical evaporator, and the mass of the dried vial recorded. The volumetric flask was made up to volume and this stock solution was then divided into aliquots which were stored at -20°C.

Five Hypf calibrators were made up in concentrations of 2.5, 5.0, 10.0, 20.0 and 40.0µg/ml, respectively. The calibrators used for Hypf in constructing the calibration curves also contained the PsHY and HY. At the point when running the calibration for all three compounds it was assumed that HY and PsHY would posses linear characteristics in acidified solvent for this range, which, unfortunately, was not the case. Due to the prohibitive cost of Hypf and limited availability of standards, Hypf could not be run in isolation and thus was included with the naphthodianthrones in all calibrator solutions. All dilutions and samples were made with acidified aqueous methanol.

Quality control solutions were made up from the same stock solution (it was not possible to make two stock solutions from the small amount of viscous sample available), and comprised 6, 12 and 24µg/ml concentrations respectively.

Repeatability was assessed by performing triplicate injections at each of five different concentration levels of the calibration solutions. This procedure was repeated on three days to assess intermediate precision. A calibration curve for each of the compounds was constructed by plotting peak areas versus the respective compound concentration. A straight-line fit of the data was made by least-squares linear regression analysis and accuracy was assessed by triplicate injection of the three QC samples.

LOD and LOQ were determined by serial dilution of the lowest calibrator and five repeat injections made of each diluted solution. The LOQ was taken as the lowest concentration where the percent RSD of the areas of these peaks were less than 5% and the LOD was calculated as 0.3 x LOQ [94].

System suitability was performed prior to and after the validation procedure, as described in Section 4.3.

5.3.3.2 Hypericin and Pseudohypericin

Powder samples of HY and PsHY were obtained as solid material in 10.0mg vials. The material was transferred into a 50ml volumetric flask and accurately weighed. The flask was made up to volume with methanol and stored in aliquots for later dilution and use as calibrator solutions. The second stock solution, to be used for QC samples, was made up in a 10ml volumetric flask with methanol, and 4.1 and 4.7mg of HY and PsHY respectively were weighed into this flask. The concentrations of the calibrators are shown below in Table 5.6.

Table 5.6. Range of concentrations of naphthodianthrones used in the method validation.

Compound	Concentration (μg/ml)						
Hypericin	2.32	5.85	11.6	17.4	23.2	34.8	46.4
Pseudohypericin	2.24	5.6	11.2	16.8	22.4	33.6	44.8

Repeatability was assessed by performing triplicate injections at each of the seven concentrations shown in Table 5.6. This procedure was repeated on three days to assess intermediate precision. A calibration curve for each of the naphthodianthrones was constructed by plotting peak area versus the respective concentration. A straight-line fit of the data was made by least-squares linear regression analysis. Accuracy was assessed by triplicate injection of three QC samples following preparation of the calibration curves on each of the three days. The QC sample concentrations were 24.6, 12.3, 6.15 and 28.2, 14.1, 7.05 μg/ml for HY and PsHY respectively.

LOD and LOQ were determined by serial dilution of the lowest calibrator and five repeat injections made of each diluted solution. The LOQ was taken as the lowest concentration where the %RSD of the areas of these peaks were less than 5%. LOD was calculated as 0.3 x LOQ [96].

System suitability was performed prior to and after the validation procedure, as reported in Section 4.3.

5.3.4 RESULTS AND DISCUSSION

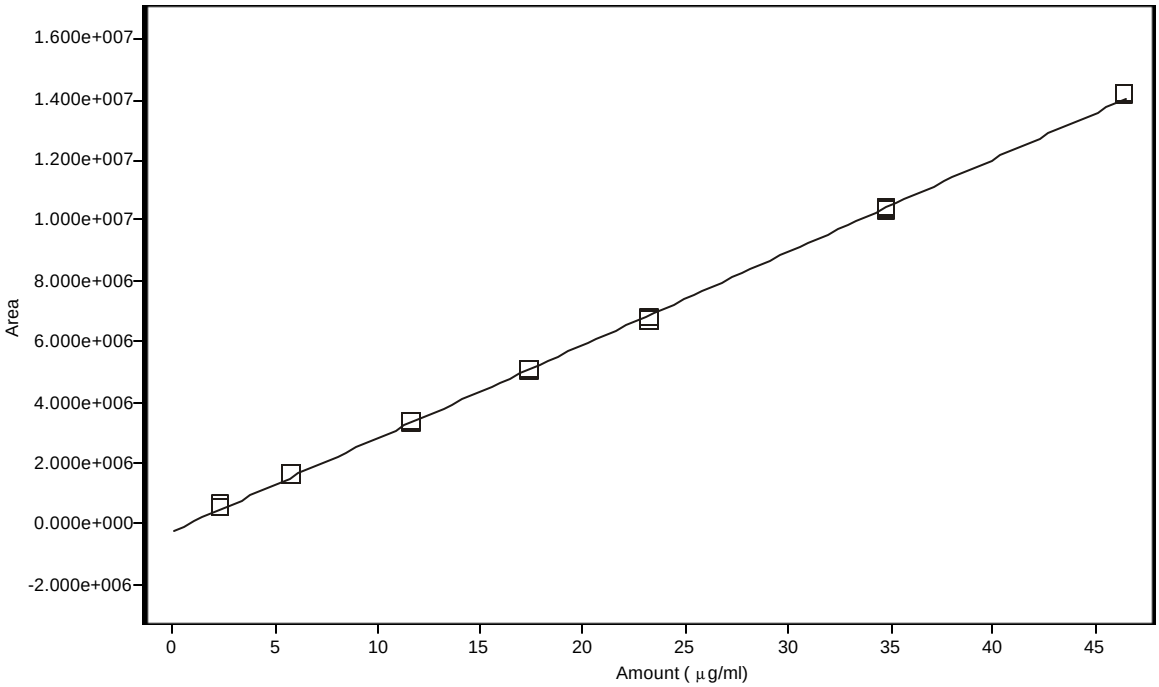
As can be seen (Table 5.7), all three compounds exhibited acceptable correlation coefficients.

Table 5.7. Calibration curve data for hypericin, pseudohypericin and hyperforin from three separate days.

Name of compound	**Concentration range (mg/ml)**	**Day**	**Slope**	**y-intercept**	**R^2**
Hypericin	2.32 - 46.4	Day 1	$3.07e^5$	$1.71e^5$	0.999
		Day 2	$2.98e^5$	$-5.69e^4$	0.999
		Day 3	$2.95e^5$	$-2.23e^4$	0.999
Pseudohypericin	2.24 - 44.8	Day 1	$1.65e^5$	$4.29e^4$	0.999
		Day 2	$1.60e^5$	$-5.35e^4$	0.998
		Day 3	$1.58e^5$	$1.02e^5$	0.999
Hyperforin	2.5 - 40	Day 1	$1.14e^4$	$2.30e^3$	0.999
		Day 2	$1.11e^4$	$4.71e^3$	0.999
		Day 3	$1.13e^4$	$3.85e^3$	0.999

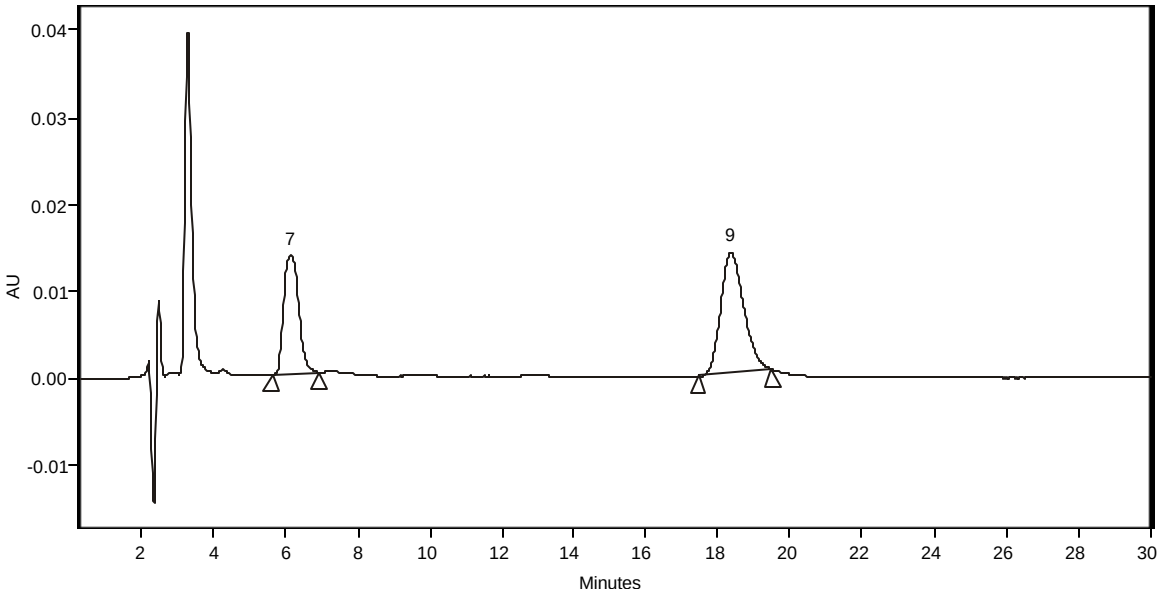
A typical example of the calibration curves for HY and PsHY is shown in Figure 5.4. The Hypf curve was linear as well. An example of a calibration curve obtained from Hypf is illustrated in Figure 5.7c, although in this case, the concentration range was 2.5-40 : g/ml and not 10-200 : g/ml.

Figure 5.4. Calibration curve for hypericin.



A typical chromatogram for pseudohypericin and hypericin is shown in Figure 5.5.

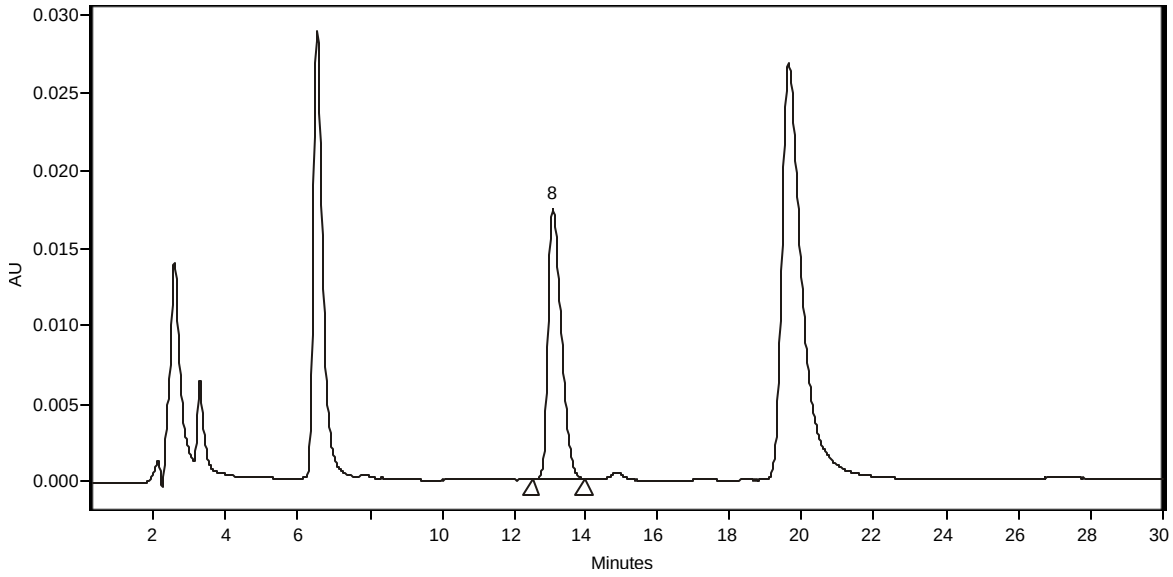
Figure 5.5. Chromatography of a calibrator containing PsHY and HY.



7 - pseudohypericin, 9 - hypericin. Conditions: Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection: 215nm; Temperature: 40°C; Injection volume: 5 : l.

Hypf was made up in acidified aqueous methanolic solution. For reasons discussed in detail in Section 5.3.5, it was discovered that it was necessary to make up methanolic calibration solutions containing HY and PsHY subsequent to running calibrators containing HY, PsHY and Hypf in acidified aqueous methanolic solution. A typical chromatogram of a calibrator for Hypf is shown in Figure 5.6. This chromatogram shows HY and PsHY as well as Hypf. The reasons for the presence of the naphthodianthrones in the Hypf calibrator solutions are as discussed previously and include the following. At the point when running the calibration for all three compounds it was assumed that HY and PsHY would posses linear characteristics in acidified solvent for this range, which, unfortunately, was not the case. Due to the prohibitive cost of Hypf and limited availability of standards, Hypf could not be run in isolation and thus was included with the naphthodianthrones in all calibrator solutions.

Figure 5.6. Chromatography of a calibrator for hyperforin.



8 - hyperforin. Conditions: As for Figure 5.5.

Inter and intra-day precision and accuracy results are shown in Table 5.8.

It can thus be seen that the precision of the method was extremely good with %RSD values, in most cases, being in the region of 1% or less, except for a few individual determinations. The accuracy of the method was also seen to be acceptable with %RSD values. In most cases being in the region of 5% or less, except for a few individual cases, only one of which just exceeded 10% (10.7%). The lower concentrations of samples tended to be associated with slightly higher percentage bias values, which was expected.

Table 5.8. Inter and intra-day precision and accuracy results for the naphthodianthrones and hyperforin.

Name of compound	Theoretical concentration (mg/ml)	Day	Measured concentration (mg/ml)	Precision % RSD	Accuracy % Bias
hypericin	6.15	Day 1	5.69 ± 0.02	0.3	7.4
		Day 2	5.75 ± 0.02	0.2	6.6
		Day 3	5.49 ± 0.01	0.2	10.7
	12.30	Day 1	11.65 ± 0.01	0.1	5.3
		Day 2	11.51 ± 0.05	0.7	6.4
		Day 3	11.19 ± 0.10	1.5	9.0
	24.60	Day 1	23.13 ± 0.09	1.3	6.0
		Day 2	23.27 ± 0.07	1.0	5.4
		Day 3	23.52 ± 0.07	1.0	4.4
pseudohypericin	7.05	Day 1	6.91 ± 0.02	0.3	1.9
		Day 2	7.48 ± 0.02	0.2	6.1
		Day 3	6.53 ± 0.03	0.4	7.4
	14.10	Day 1	14.76 ± 0.15	2.1	4.7
		Day 2	14.46 ± 0.12	1.7	2.6
		Day 3	14.24 ± 0.04	1.3	2.6
	28.20	Day 1	29.17 ± 0.07	1.0	3.4
		Day 2	26.97 ± 0.08	1.1	4.4
		Day 3	28.92 ± 0.09	1.3	2.6
hyperforin	5.00	Day 1	4.84 ± 0.06	1.2	3.2
		Day 2	4.64 ± 0.20	4.4	7.1
		Day 3	4.74 ± 0.07	1.6	5.2
	10.00	Day 1	10.39 ± 0.07	0.6	3.9
		Day 2	10.24 ± 0.33	3.2	2.4
		Day 3	10.35 ± 0.10	1.0	3.5
	20.00	Day 1	20.50 ± 0.22	1.1	2.5
		Day 2	20.92 ± 0.19	0.9	4.6
		Day 3	20.47 ± 0.21	1.0	2.3

Table 5.9. LOQs and LODs for pseudohypericin, hypericin and hyperforin.

Compound	LOQ (mg/ml)	% RSD	LOD (mg/ml)
Hypericin	0.1875	3.8	0.0563
Pseudohypericin	0.2425	2.7	0.0728
Hyperforin	0.2500	1.9	0.0750

The LOD and LOQ values for particular compounds will vary depending on the type of detection system used.

Table 5.10 contains LOQ values obtained by various researchers for HY, PsHY and Hypf using different detectors and/or wavelengths. It is interesting to note that Draves and Walker [95] obtained an LOQ for HY of 5.84 µg/ml using a wavelength of 236nm whereas a much lower LOQ of 0.25

μg/ml was found in this study using 215nm as the detection wavelength. This is not surprising since HY shows greater UV absorption at 215nm compared to 236nm. However, according to Draves and Walker the visible wavelength of 590nm resulted in a considerably lower value for the LOQ, of 0.088μg/ml. Fluorescence detection, however, provides a lower limit of detection [100]. The LOQ of 0.043μg/ml found for HY at 270nm using a photodiode detector [85] (Table 5.10) is somewhat surprisingly better than that obtained at 215nm.

Table 5.10 illustrates the range of values of LOQ that are published for the naphthodianthrones and Hypf, as well as the respective methods of detection used. Many methods do not quote these values.

Table 5.10. Published values of LOQ.

Author:	**Draves and Walker, 2000** [95]			**Bauer, *et al.*, 2001** [100]		**Ganzera *et al.*, 2002** [85]
	LOQ (μg/ml)			LOQ (μg/ml)		LOQ (μg/ml)
Compound	UV (236nm)	VISIBLE (592nm)	Fluorescence	UV (273nm)	Fluorescence	Photodiode array (270nm)
Hypericin	5.840	0.088	0.009	-	0.00025	0.043
Pseudohypericin	-	-	-	-	0.00025	0.030
Hyperforin	-	-	-	0.001	-	0.108

5.3.5. NON-LINEARITY EXHIBITED BY THE NAPHTHODIANTHRONES IN ACIDIC SOLUTION

The naphthodianthrones exhibited non-linearity in the concentration ranges 10-200 μg/ml and also in the range of 1-20 μg/ml when solutions of these compounds (HY and PsHY) were made up with aqueous acidified methanol. The non-linearity was more distinct over the higher range, i.e., 10-200μg/ml. Examples of this non-linearity are shown in Figures 5.7a and 5.7b. Hyperforin, on the other hand, was found to be linear over the same concentration range used for the calibration curve and its calibration curve can be seen in Figure 5.7c. Injections were made in triplicate at each concentration.

Figure 5.7a. Hypericin calibration curve. Range: 10-200 mg/ml.

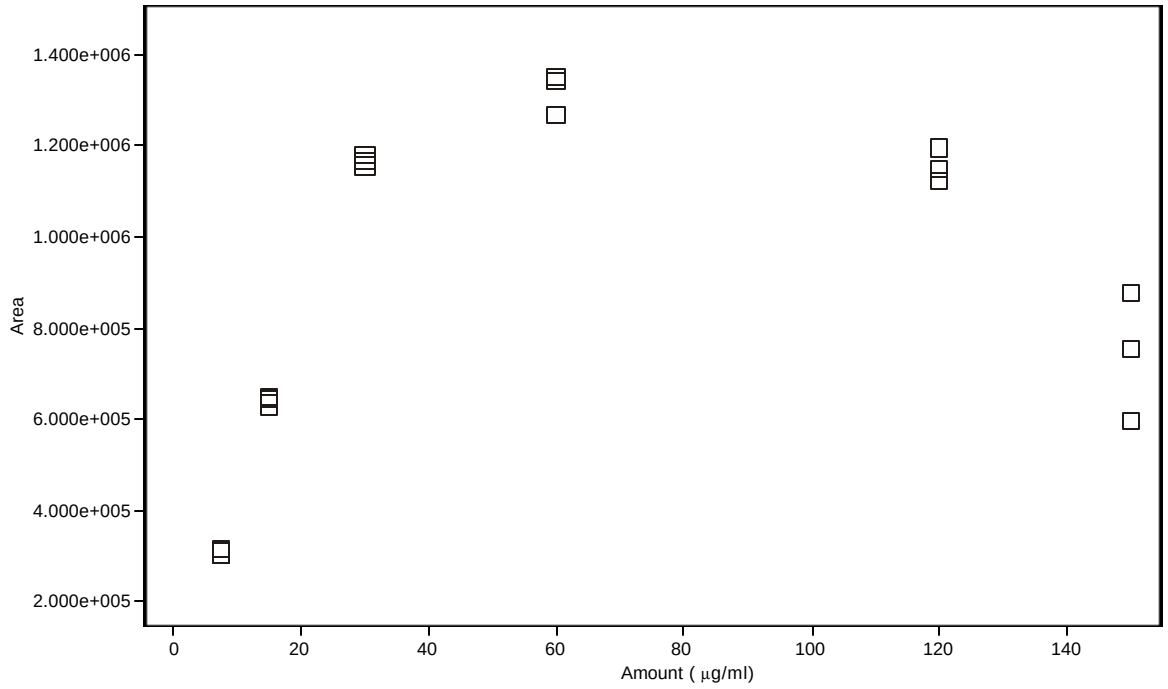


Figure 5.7b. Pseudohypericin calibration curve. Range: 10-200 µg/ml.

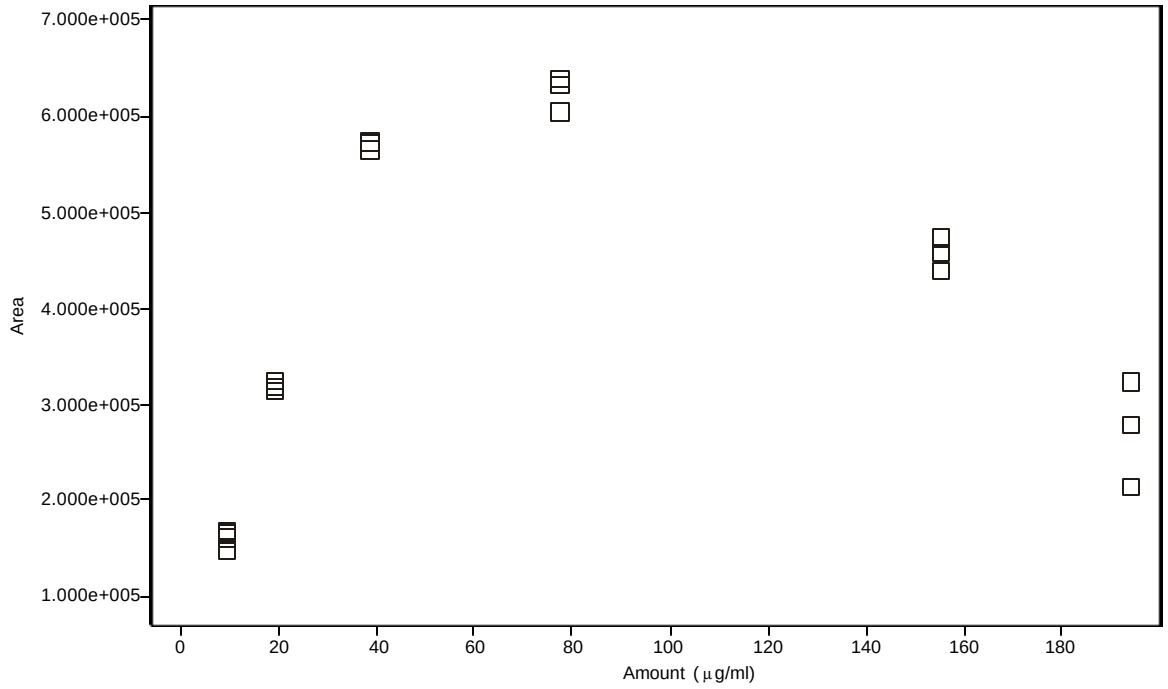
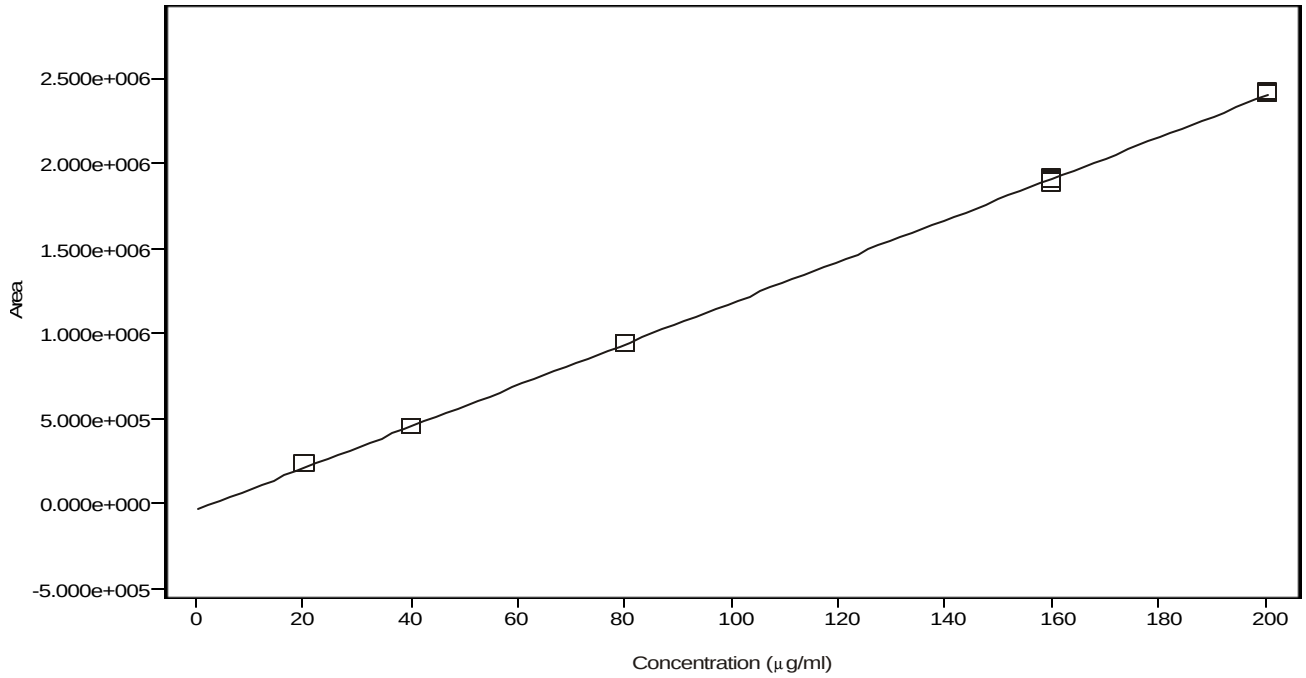


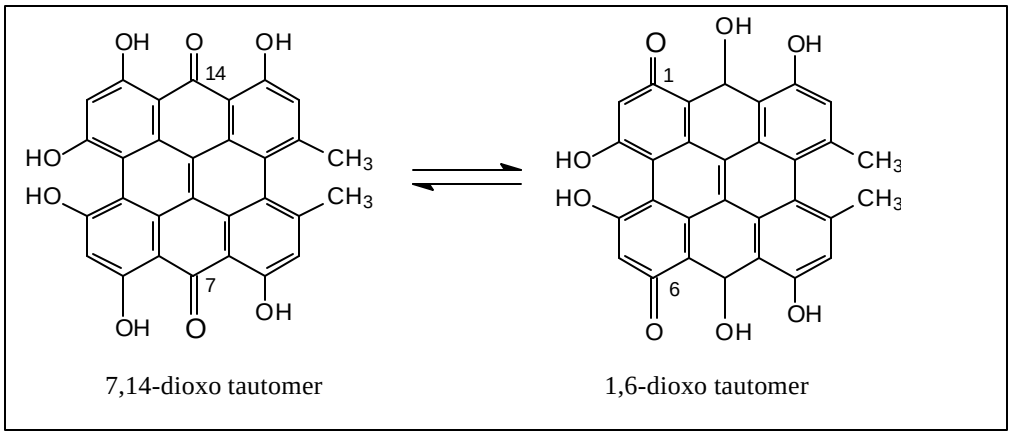
Figure 5.7c. Hyperforin calibration curve. Range: 10-200 : g/ml.



The results indicate that the non-linearity was characteristic of the naphthodianthrone compounds under those specific solution conditions, whereas Hypf was not affected. When the solutions were left to stand for two to three weeks, precipitation was observed in those solutions. When these samples were aggressively agitated by shaking or vortexing, the precipitate appeared to dissolve.

It has been reported in the literature that tautomerism of HY can occur under certain specific conditions (Figure 5.8). The literature, however, only cites HY in this regard and reports that the aggregation behaviour of PsHY has not been studied [39].

Figure 5.8. Tautomeric forms of HY.

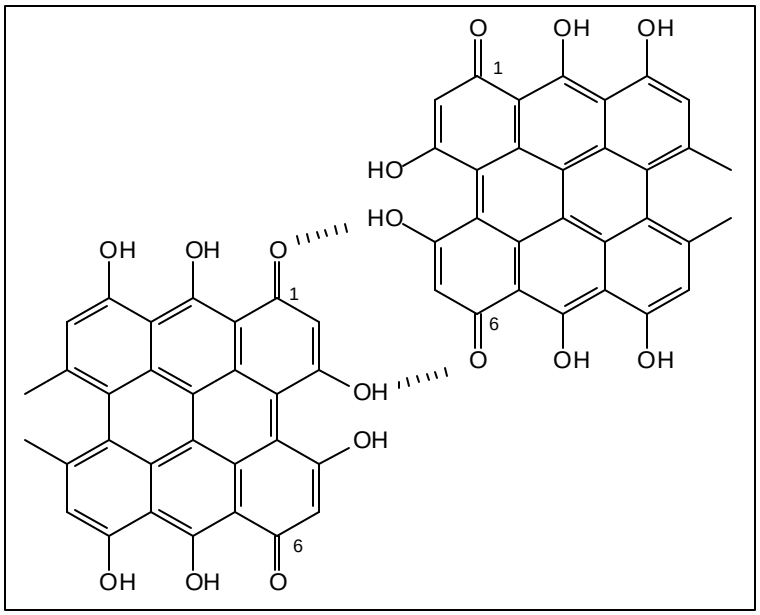


7,14-dioxo tautomer 1,6-dioxo tautomer

Kapinus and co-workers [103] observed that both tautomeric forms can occur in the solid state, and that transformation from one tautomer to the other only occurs in solution. They also observed that the 7,14 -dioxo tautomer is the most stable isomer, but that it can revert to the 1,6 tautomer by acidification. Dilution also can cause reversion of the 1,6-dioxo to the 7,14-dioxo tautomer.

It thus appears that the 1,6-dioxo tautomers form homo-associates, which are stabilized by efficient intermolecular hydrogen bonding, and it is this bonding that causes the 1,6-dioxo tautomeric form to be the most prevalent in saturated solutions and in the solid state. Figure 5.9 illustrates why the hydrogen bonding would only occur with the 1,6 tautomeric form.

Figure 5.9. Intermolecular hydrogen bonding of the 1,6-dioxo tautomer [39].



As can be seen, the 1,6 and not the 7,14 tautomer would possess this intermolecular hydrogen bonding, as the hydroxyl and carbonyl groups are optimally situated in the most beneficial location. It is thus reasonable to expect that PsHY would most likely also exhibit this property since the hydroxyl group added to one of the methyl substituents should not affect the tautomerism, or the intermolecular hydrogen bonding.

It has been noted that HY has poor solubility in most common solvents, and that hypericins have been seen to exhibit different solubility properties. It can thus be reasonably assumed that these different

solubility properties relate to different tautomeric forms. It has also been reported that the strong hydrogen bonds that are present with the 1,6-tautomer results in this tautomer possessing a lower solubility than the 7,14-tautomer [39].

From this evidence it is possible that the presence of acid (ortho-phosphoric acid) in the solvent used to stabilize HY and PsHY dissolution in the initial attempts to validate the method was probably responsible for the observed non-linear results. The presence of acid may have catalyzed the formation of the less soluble 1,6-dioxo tautomers, which then resulted in precipitation of the hypericins and non-linearity, especially at the higher concentrations.

5.4 METHODS

5.4.1 TABLET EXTRACTION EFFICIENCY ANALYSIS

De Los Reyes et al [42] performed studies on the extraction efficiencies of various solvents, including methanol, acidified (pH 2.5) methanol and acidified (pH 2.5) methanol:water (80:20 v/v). They reported poor extraction efficiency of Hypf using methanol, and showed that acidic pH and the addition of water to methanol improved Hypf extraction markedly, as well as its stability. They also stated that extraction of the hypericins by acidic methanol or by acidified aqueous methanol is comparable (95%) to that afforded by methanol, although slightly lower [42].

A comparison of the extraction efficiencies of methanol and acidified aqueous methanol was performed in order to ascertain whether one extraction method would suffice for the hypericins and Hypf or whether, contrary to what has been reported, 2 separate extraction systems would be required.

All procedures were performed in a dark room and all solutions were stored at -20°C and kept covered in foil or in amber glassware until analyzed. This was necessary to avoid possible degradation of the naphthodianthrones and Hypf. Many sample preparation techniques used for *Hypericum perforatum* require extract exposure to a specified wavelength of light to facilitate complete conversion of the proto-forms of the hypericins to their conjugate analogues. This was since light exposure may be detrimental to Hypf [102].

Sixteen tablets (Vitality® St. John's Wort) were ground in a mortar and pestle. Twelve approximate 0.5g portions of the powdered tablets were accurately weighed and placed in Kimax® tubes. Ten millilitres of methanol were added to each of six Kimax® tubes and ten millilitres of methanol: water (80:20 v/v) pH2.5 were added to the six other tubes. The tubes were then vortexed to ensure adequate dispersion of the tablet powder and sonicated for one hour. The temperature of the sonication bath was monitored and maintained below 10°C by addition of ice to the bath.

The samples were centrifuged and the supernatants decanted into Kimax® tubes for storage. A 1 in 10 dilution of each sample was made, and these diluted samples were injected onto the column. The supernatants in the Kimax® tubes were stored at -20°C in the dark for any further analysis.
Injections were performed in triplicate for each sample, and corrections made in all calculations for the respective masses weighed out (this was necessary as the masses weighed were not necessarily precisely 0.500g). In order to preserve the small amount of each standard on hand for use in the quantitative analysis, no calibrators were used for the tablet extraction efficiency analysis study. The results shown are based upon the area of the HY and PsHY peaks after integration.

5.4.2 RECOVERY ANALYSIS

All procedures performed were done with the exclusion of light to break down of the naphthodianthrones and Hypf. Recovery analyses for Hypf and the naphthodianthrones were performed separately. In the case of the naphthodianthrones, recovery was performed on filtered and unfiltered extracts.

5.4.2.1 Hyperforin

Fifteen Vitality® tablets were ground into fine powder with a mortar and pestle. Twelve 0.5g portions were accurately weighed and placed into separate Kimax® tubes. A low concentration, medium concentration and high concentration spike were chosen to ensure the final concentrations of Hypf in the spiked tablet extracts would fall within the concentration range of the calibration curve. Ten millilitres of aqueous acidified methanol was added to each of three tubes to represent the unspiked samples. One millilitre of low spike was added to each of three tubes, 1ml of medium spike to each of three tubes and 1 ml of high spike to each of three tubes. Nine millilitres of aqueous acidified methanol were added to these nine spiked samples, and the tubes stoppered. All twelve tubes were

vortexed and then sonicated for one hour below 10°C. The tubes were then placed in a centrifuge for 10 minutes, and the supernatants were poured off and vortexed prior to injection. All calibrators and samples were injected in triplicate.

5.4.2.2 The Naphthodianthrones

The procedure followed for Hypf was repeated for the naphthodianthrones. The only difference was that an investigation into the recoveries of filtered supernatants was performed simultaneously. Fifteen Vitality® tablets were ground into fine powder with a mortar and pestle and twelve 0.5g portions were accurately weighed, placed into separate Kimax® tubes and their masses recorded. Once again, low concentration, medium concentration and high concentration spikes were chosen. Ten milliliters of methanol was added to each of three tubes. One millilitre of low spike was added to each of three tubes, 1 ml of medium spike to each of three tubes, and 1 ml of high spike to each of three tubes. Nine millilitres of methanol was added to the spiked samples. The tubes were stoppered, vortexed, sonicated for one hour below 10°C, and then centrifuged for 10 minutes.

The approximate volume of each supernatant was 10ml from which approximately 2ml was removed and placed in a vial for injection, and the sample filtered through Durapore® membrane filters. The filtered supernatant was transferred into vials for injection.

The vials with supernatant contents were vortexed prior to injection. The calibrators run before the filtered supernatants were not filtered. All calibrators and samples were injected in triplicate.

5.5 RESULTS AND DISCUSSION

5.5.1 TABLET EXTRACTION EFFICIENCY ANALYSIS

The results were obtained from triplicate injections of each of four methanolic extracts and five acidified aqueous extracts.

5.5.1.1 Hyperforin

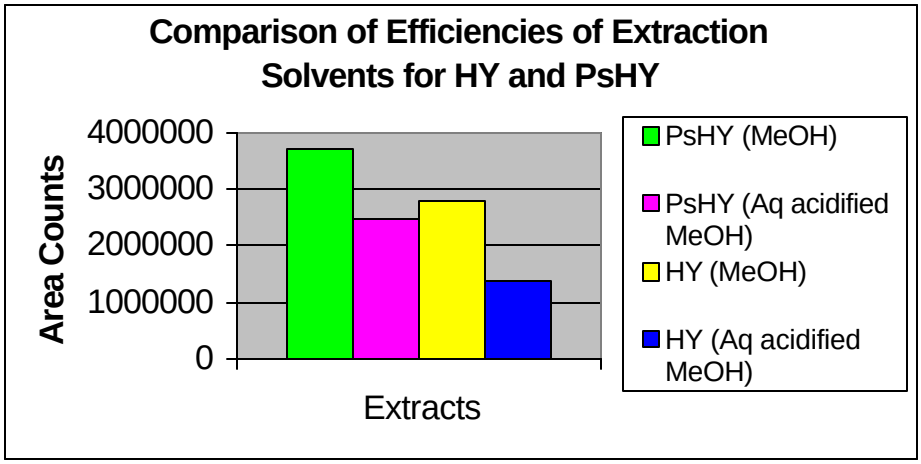
The product used for the extraction efficiency analysis did not have any quantifiable amount of Hypf present. This was not yet known at the time of the extraction efficiency analysis but was confirmed later in the assay. Out of the five commercial tablet products on hand, only two products were found to contain a quantifiable amount of Hypf. Only one of these contained a measurable amount of Hypf, but the presence of an interfering peak made the integration very difficult and not feasible.

De Los Reyes *et al* [42] reported that the most beneficial solvent in terms of extraction and stability of Hypf was acidified aqueous methanol. It was thus decided to use acidified methanol for the extraction and storage of Hypf.

5.5.1.2 The Naphthodianthrones

Thirty-three percent more PsHY was extracted with methanol than with acidified aqueous methanol, and 50% more HY was extracted with methanol than with acidified aqueous methanol. This demonstrates that there is in fact a substantial difference between the extractability of the hypericins in methanol compared to acidified aqueous methanol.

Figure 5.10. Extraction efficiencies of the two solvent systems for hypericin and pseudohypericin.



De Los Reyes et al [42] reported that the most beneficial solvent in terms of extraction and stability of Hypf was acidified aqueous methanol. Their findings also showed that extraction of the hypericins by acidic methanol or by acidified aqueous methanol is comparable (95%) to that afforded by methanol, although slightly lower. This is in contrast to the results of this study where it was found that there

was in fact a substantial difference between the extractability of the hypericins in methanol compared to acidified aqueous methanol.

De los Reyes *et al.* described the preparation of only one extract sample for each solvent extraction system as opposed to multiple samples in the case of this study. As well as this, their extractions were from crude powder extract and not powdered tablet dosage forms, as was the case in this study. These are important differences, and coupled with the fact that the preparations analysed by de Los Reyes *et al*. were different to those preparations used in this extraction efficiency analysis, the vastly different extraction efficiency results obtained in this study as opposed to their study can be readily explained.

The %RSDs for the results obtained in this study were all below 8.3% and the chromatography for the samples was very consistent. This, coupled with the knowledge that the hypericins are not very soluble in methanol, and any acid present tended to decrease solubility, affirms that the extraction efficiency results obtained are explainable and likely.

5.5.2 RECOVERY ANALYSIS

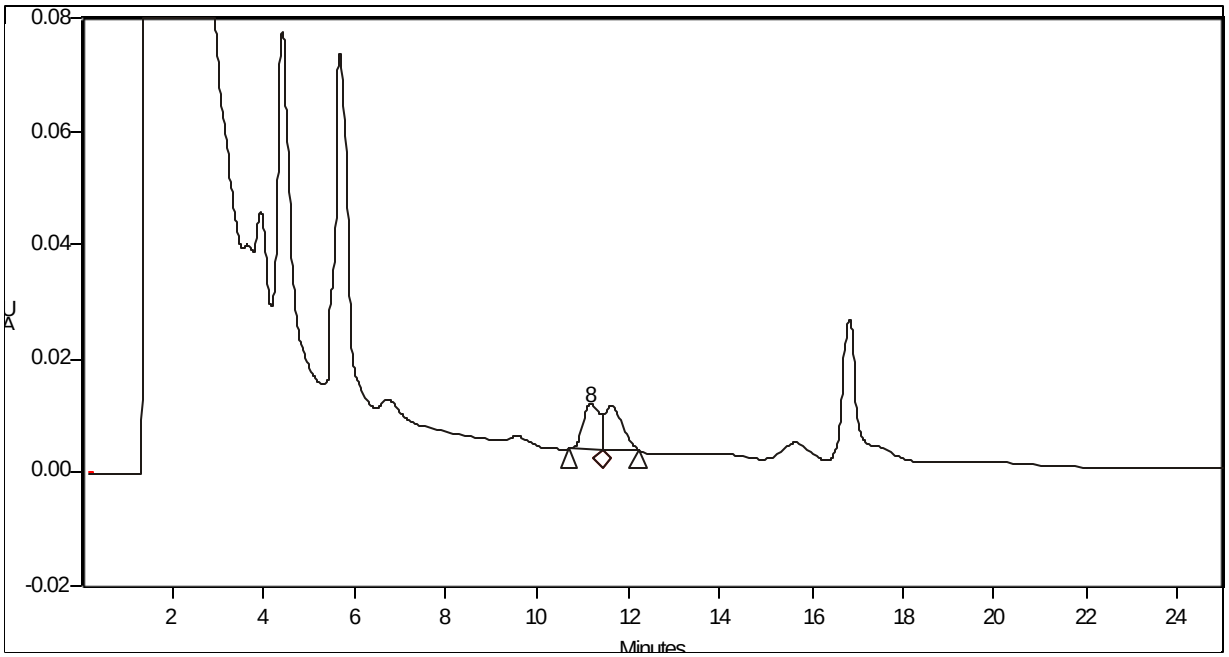
5.5.2.1 Hyperforin

Table 5.11. Hyperforin recoveries.

	Low Spike (50gm/ml)	**Medium Spike (100mg/ml)**	**High Spike (200mg/ml)**
% Recovery	104.4	87.8	85.8
% RSD	10.4	21.2	0.7

As can be seen, the recoveries were all above eighty percent. There was an interference that eluted after, but very close to Hypf, and resulted in a fused peak. This made accurate integration of the unresolved Hypf peak difficult, as can be seen in Figure 5.11 below.

Figure 5.11. Hyperforin recovery - high spike.



8 - hyperforin. Conditions: Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm. Detection: 215nm; Temperature: 40℃; Injection volume: 5 : l.

The high %RSD for the lowest spike can primarily be attributed to the inaccurate integration of this fused peak. In view of the extremely low amount of Hypf present, accurate quantitative determination of Hypf was not feasible. The situation was further aggravated in the case of the lowest spike which was near the LOQ and resulted in the largest %RSD.

The twelve extracts used for the recovery study were divided into four groups of three extracts each. The first group of extracts were unspiked. The second, third and fourth groups of extracts were spiked at low, medium and high concentrations respectively. The relatively higher %RSD that resulted from the medium spike extracts was caused specifically by the third extract in this group. The shape of the fused peak in this case was different to the shapes of the fused peaks from the other two extracts in this group. The anomalous shape of this fused peak caused the integration using a perpendicular drop to yield anomalous results. If the results from this anomalous extract are omitted, then the average of the other two medium spikes yields a recovery of 98.5% with an RSD of 2.8%.

5.5.2.2 The Naphthodianthrones

5.5.2.2.1 Recoveries from Unfiltered Supernatants

Table 5.12. Hypericin and pseudohypericin recoveries.

Hypericin			**Pseudohypericin**		
Spike	**% Recovery**	%RSD	Spike	**% Recovery**	% RSD
Low spike (56µg/ml)	97.5	4.4	Low spike (47µg/ml)	94.2	115.5
Medium spike (112µg/ml)	85.0	3.8	Medium spike (94µg/ml)	80.7	15.8
High spike (205µg/ml)	81.7	2.8	High spike (235µg/ml)	62.8	17.7

HY recoveries were all above 80%, as shown in Table 5.12. The lowest spike had the highest recovery, the medium spike less than that, and the highest spike, the lowest recovery. All of the %RSDs were less than 5 %.

The recoveries of PsHY followed a similar trend to those of HY, - decreasing recoveries with increasing spike concentration. However, the recoveries obtained from PsHY were substantially lower than those obtained from HY. This was most likely because the spiked extracts had to be diluted in order to fall within the calibration curve as a result of the PsHY concentration in the extracts already being relatively high in terms of the calibration curve. The % RSDs were also much higher than those of HY and this may also have been due to the dilution factor. In the calculations, correction for dilution generally magnifies any error. The chromatographic data relating to the lowest PsHY spike showed that the three injections of each of the three extracts were very precise.

5.5.2.2.2 Recoveries from Filtered Supernatants

The recoveries found for the filtered supernatants were in, general, very low, with high % RSDs. HY exhibited recoveries of between 45 and 60%, with % RSDs all above 7%. PsHY exhibited recoveries of 4 to 43% with % RSDs of above 19% for all filtered supernatants. The low % recoveries and high %RSDs for the filtered supernatants could be attributed to adsorption by the filters as the recoveries and %RSDs were much improved without filtration. It is interesting that Draves *et al* [95] reported using non-aqueous 0.5µm type filters with no naphthodianthrone loss. Li *et al* [86] reported using nylon, PP, Anotop, PTFE and PVDF filters, with the only high losses occurring with nylon filters. In this case the filter tended to adsorb a large proportion of the naphthodianthrones.

5.6 NAPHTHODIANTHRONE AND HYPERFORIN QUANTITATION

All procedures and storage of solutions and extracts were performed with the exclusion of light.

5.6.1 INSTRUMENTATION

- Meyer N-EVAP Analytical Evaporator, Model 112 (Organomation Inc. South Berlin, MA 01549, USA).
- Maxi mixer, Model M-1670-12 (Thermolyne Corporation, Subsidiary of Sybron Corporation, Dubuque, Iowa, USA)

  Refer to Section 3.2 for all additional equipment used.

5.6.2 MATERIALS AND REAGENTS

The commercial samples chosen for assay are listed in Table 4.12. Three batches of the same brand of St John's Wort tablets, (Holotropic) as well as two other brands of St John's Wort tablets (Vitality & Alpha) were analyzed.

Refer to Section 2.5.3 for other materials and reagents used.

5.6.3 METHODS

5.6.3.1 Hyperforin

Ten tablets of each product were ground into fine powder in a mortar in pestle. Three portions of approximately 0.5 grams were accurately weighed out for each of the products and transferred into individual Kimax® tubes. Ten millilitres of aqueous acidified methanol were pipetted into the t Kimax® tubes and the tubes were stoppered with teflon-lined screw caps.

The mixtures were vortexed for thirty seconds to ensure adequate dispersion of the powder throughout the methanol, and then sonicated for one hour with the temperature of the sonication bath maintained below 10°C. Following sonication, the samples were centrifuged for five minutes, the supernatant decanted and an aliquot of each supernatant transferred to a vial for injection. All sample solutions were covered with foil and stored at -20°C prior to injection where necessary.

HPLC analysis of products A to E for hyperforin using the validated method, with dual wavelength UV detection at 215 and 270nm was used. The column used was a Luna C_{18} (2) 150 x 2.0mm narrow bore column, with a Luna octadecyl guard column. A flow rate of 0.2ml/min and a temperature of 40°C were used and all injections were performed in triplicate.

5.6.3.2 Hypericin and Pseudohypericin

Ten tablets of each product were ground into fine powder in a mortar in pestle as previously described. Three portions of approximately 0.5 grams were accurately weighed out for each of the products, transferred into individual Kimax® tubes and the respective masses recorded. Ten millilitres of methanol were pipetted into the Kimax® tubes and stoppered with teflon-lined screw caps.

The mixtures were vortexed and dealt with as described above for Hypf.

Where the concentrations fell outside the calibration range, for some of the extracts, volumetric dilutions were made to bring the concentrations into the range, and allowance was provided for this in the calculations.

5.6.3.3 Semi-Quantitative Checks and Confirmation

This HPLC gradient method with UV detection developed and described by Ganzera *et al.* [85] was used as a semi-quantitative check for the results obtained for HY, PsHY and Hypf. Their method was applied to evaluate whether baseline resolution could in fact be obtained with Hypf, without interferences, as was the case with the current method developed and described in this thesis.

The Ganzera *et al*. method and all conditions and equipment has been previously described in Section 4.6.1. Four calibrators were injected in triplicate and a four-point calibration curve was constructed.

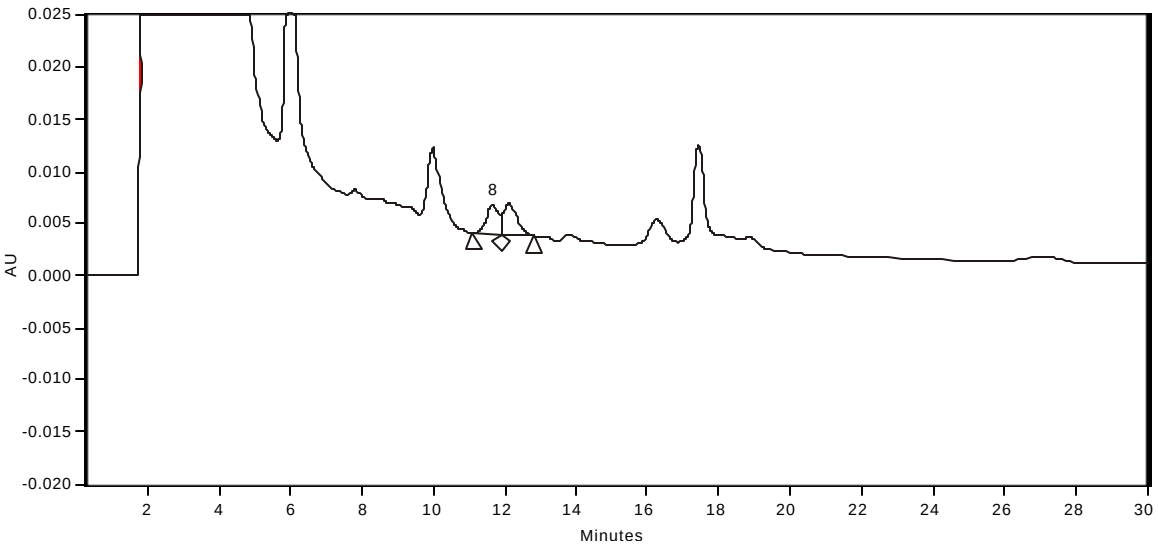
Each of the extracts from each of A, B, C, D, and E respectively were injected in triplicate directly after the calibration standards were chromatographed.

5.6.4 RESULTS AND DISCUSSION

5.6.4.1 Hyperforin

Product D was the only product found to contain a substantial amount of Hypf. Product C contained some evidence of Hypf, but in very small amounts. These amounts were very close to the LOQ using the current method hence the rather large %RSD found the hyperforin peak was incompletely resolved from an interfering peak that eluted immediately after Hypf. Integration of the Hypf peak was attempted using the “perpendicular drop” method. A typical example of the chromatography and integration of the Vitality® acidic aqueous methanolic extracts is shown in Figure 5.12.

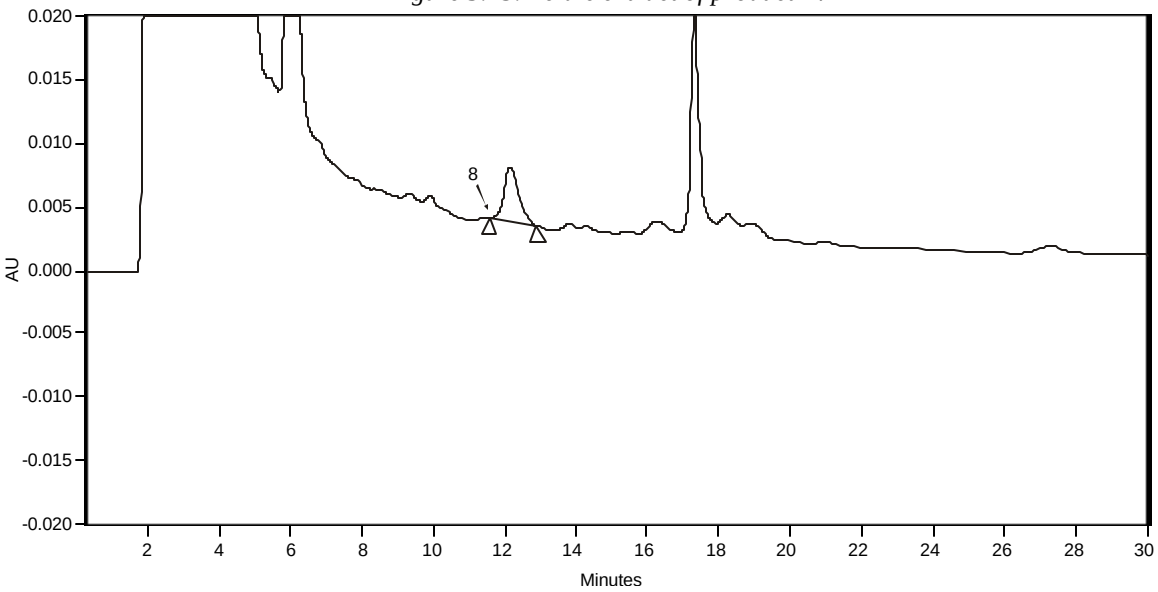
Figure 5.12. Acidic extract of product D.



8 - hyperforin. Conditions: Flow rate: 0.2ml/min; Column: Phenomenex Luna 5 : m C18 (2), 150 x 2.0 mm; Detection: 215nm; Temperature: 40°C; Injection volume: 5 : l.

The chromatography and integration shown in Figure 5.13 for product A is typical of that elicited by products B and E as well.

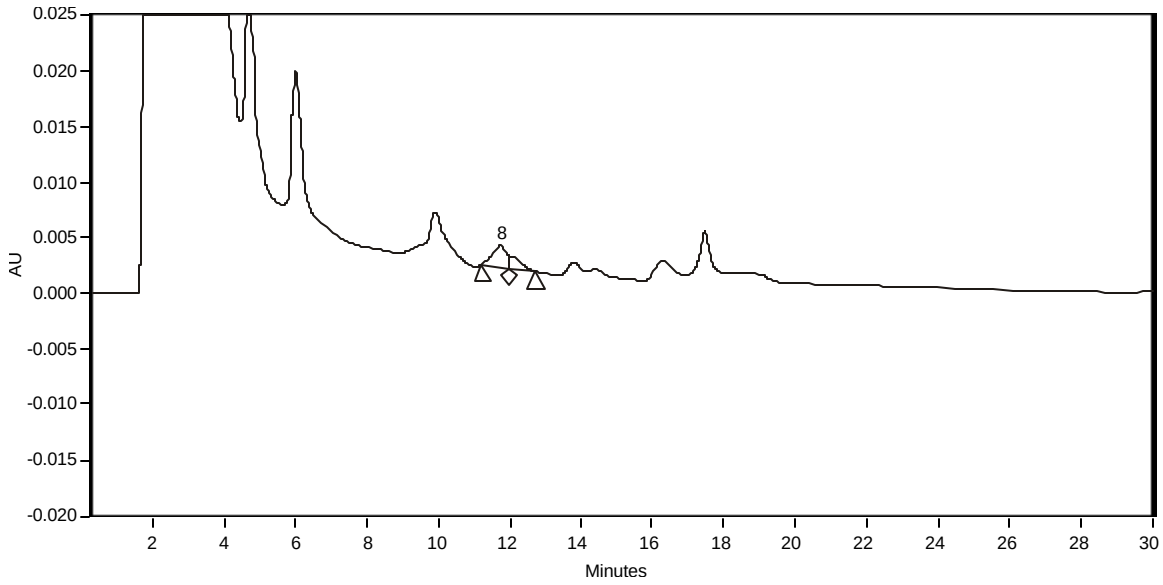
Figure 5.13. Acidic extract of product A.



8 - hyperforin. Conditions: As for Figure 5.12.

The chromatography and integration for product C are shown in Figure 5.14.

Figure 5.14. Acidic extract of product C.



8 - hyperforin. Conditions: As for Figure 5.12.

The assay results for Hypf are shown in Table 5.13.

Table 5.13. Hyperforin assay results using both the current HPLC method and the Ganzera et al method.

Hyperforin Assay Results Amount per 0.5g tablet (mg)		
Product	215nm	Ganzera
A %RSD	ND	ND
B %RSD	ND	ND
C %RSD	0.039 15.4	0.053 4.7
D %RSD	0.073 2.8	0.177 7.5
E %RSD	ND	ND

Both methods yielded similar results despite the very low amounts of Hypf present in the products.

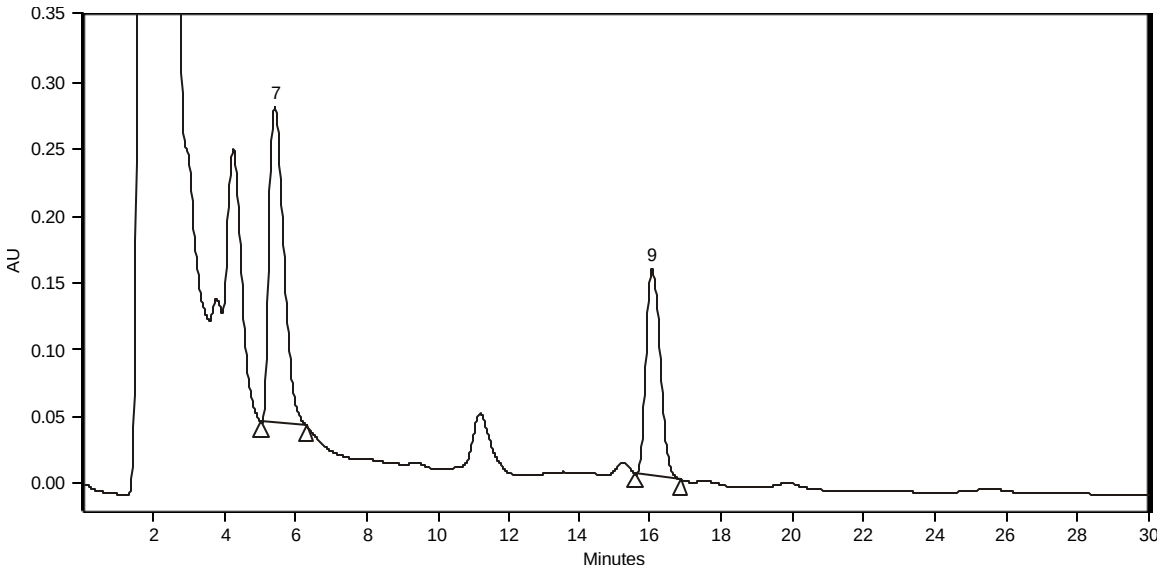
It is interesting to note that very little Hypf was present in the products investigated. This is of some concern especially as it is currently believed that Hypf contributes to the anti-depressant activity of *H. perforatum* [51]. Ganzera *et al.* assayed nine products in the USA for flavonoids, naphthodianthrones

and Hypf, and they found amounts of Hypf varying from 0.35 to 6.5mg of Hypf per 0.5g of product [85]. Liu *et al*. evaluated the quantities of major components in St John's Wort dietary supplements from five different sources and found the content of Hypf varying from 1.2mg Hypf per 0.5g product to 4.25mg Hypf per 0.5g product [89]. Hence, the Hypf results obtained from the assay of the various products investigated in this thesis indicate that these products are of substandard quality.

5.6.4.2 Hypericin and Pseudohypericin

The naphthodianthrones, HY and PsHY, were found to be present in all the tablet products assayed. The chromatography for all five tablet products was similar and a typical example of this is shown in Figure 5.15.

Figure 5.15. Chromatography of extract of product E



7 - pseudohypericin, 9 - hypericin. Conditions: As for Figure 5.12.

Table 5.14. Assay results for the naphthodianthrones

Hypericin and Pseudohypericin Assay Results Amount per 0.5g tablet (mg)		
Product	PsHY	HY
A	0.098	0.250
%RSD	6.8	5.5
B	0.049	0.102
%RSD	5.4	5.4
C	0.029	0.051
%RSD	4.3	6.2
D	0.068	0.175
%RSD	5.2	8.0
E	0.129	0.438
%RSD	3.2	5.3

The assay results for the naphthodianthrones are illustrated in Table 5.14. The content of PsHY varied between 0.029 and 0.129mg per 0.5g of product, - a four-fold difference, which is quite substantial. The inter-batch variability of PsHY was 3.4 fold. The content of HY was generally much higher than PsHY, and varied from 0.051mg to 0.438mg per 0.5g of product. There was a five-fold difference between the lowest and highest concentrations of PsHY in the different batches of Holotropic® tablets. HY exhibited a nine-fold difference in this case. This illustrates relatively high inter-batch variability for these compounds.

Ganzera *et al*. [85] reported the PsHY contents of nine products which varied from 0.05mg per 0.5g product, to 0.45mg per 0.5g product. The results from the tablets assayed with the method validated in this thesis are generally lower than this, but are in a similar range. Liu *et al*. reported a range of 0.15 to 0.85mg PsHY per 0.5g of product from the five products that were studied [89]. This range is higher than the range of PsHY in the products assayed in this thesis.

Ganzera *et al*. [85] also reported the content of HY in the products they assayed as ranging from 0.05mg to 0.45mg per 0.5mg product, whereas Liu [89] found the content of HY ranging from 0.1mg to 0.45mg of HY per 0.5g product. It can be seen that the range of content of HY in the products assayed in this thesis is consistent with similar data reported elsewhere.

5.7 HYPERFORIN STABILITY STUDY

Hyperforin is known to be a photolabile and oxygen sensitive compound [39]. As already discussed, the presence of acid in sample solutions is reported to have a stabilizing effect on Hypf. In this study, the stability of three samples of Hypf, each stored under different conditions of temperature and light were studied over two weeks using HPLC.

Refer to Sections 3.2 and 3.3 for all instrumentation, additional equipment, materials and reagents.

5.7.1 METHODS

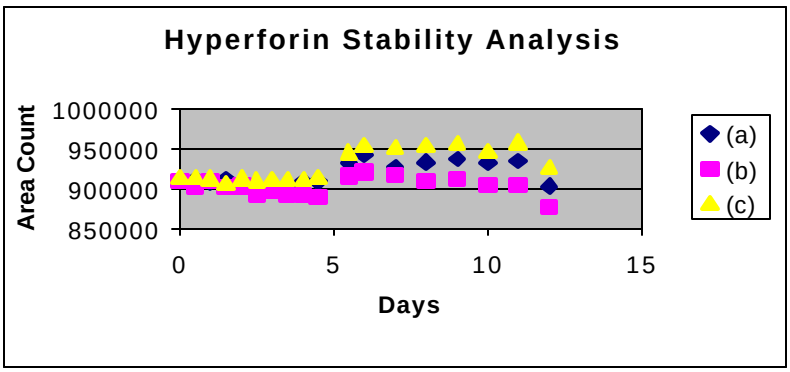
Three 40µg/ml calibrator solutions of Hypf were set aside for the study. The first calibrator solution was stored in the dark at -20°C, the second in the dark at room temperature, and the third at room temperature with exposure to light. The samples were injected every twelve hours for the first seven days, and then every 24 hours for the next seven days. Due to the shortage of Hypf standard and the cost thereof, calibrators could not be prepared hence a comparative study using relative peak areas was performed.

5.7.2 RESULTS AND DISCUSSION

The results from the fourteen-day stability study are illustrated in Figure 5.16.

(a) Represents sample stored in the dark at -20°C, (b) represents sample stored in the light at 20°C and (c) represents sample stored in the dark at 20°C.

Figure 5.16. Illustration of the stability of hyperforin over 14 days.



Due to various problems with the UPS (Uninterrupted Power Supply) and power cuts to the local power supply, the running of samples on days five and seven were interrupted and could not be included in this study. The HPLC system was therefore shut down on days five and seven, which may help to explain the slight shift in area values at these times. It can be seen however, that over the two week period studied there was no substantial break down or significant difference in the Hypf areas, regardless of storage conditions.

The phloroglucinols have been reputed to be unstable towards heat and light either on storage or in solution, and very susceptible to oxidation. It was thought that Hypf was chemically too unstable to play a role as a pharmacologically relevant constituent of SJW preparations. However, Erdelmeier [41] reports that if appropriately selected and dried SJW herb is used, both lipophilic and hydroalcoholic extracts may contain the relevant percentages of Hypf.

The stability of isolated Hypf in solution and solid form was investigated in several antioxidant systems. It was reported that Hypf showed greater stability in polar solvents than in non-polar solvents, and was much more stable in pH 2.0 methanolic solution than in pH 12 solution. It has also been reported that degradation under light exposure can be diminished in methanol by acidification (pH 2.0), and degradation under light exposure at pH 12.0 results in total decomposition within 30 days. However, when kept in the dark, hyperforin is stable in acid and alkaline methanolic solutions [39,42].

The results from this study show that there was no obvious or significant degradation or differences in the stability of hyperforin, whether stored in the dark and at -20°C, or in the light and at 20°C, or in the dark and at 20°C. This is contrary to what is reported in the literature [39,42].

5.8 QUALITATIVE ANALYSIS

5.8.1 INTRODUCTION

ESI was used for the qualitative analysis of the content of the naphthodianthrones and Hypf in the five tablet products investigated in this thesis and ESI-MS-MS was performed on all the extracts from those respective tablets.

LC-MS-MS analyses were performed in order to identify the constituents and to verify the specificity of the HPLC method developed during these investigations. All procedures were performed in a dark room fitted with two Philip's dark room green globes in order to avoid possible photodegradation of purportedly labile components. All extracts and dilutions thereof were stored at -20°C and in amber glassware or covered with foil prior to analysis.

5.8.2 INSTRUMENTATION

- Maxi mixer, Model M-1670-12 (Thermolyne Corporation, Dubuque, Iowa, USA)

Refer to Section 4.6.1 for all other instrumentation and additional equipment.

5.8.3 MATERIALS AND REAGENTS

Refer to Section 4.6.2 for materials and reagents used.

5.8.4 METHODS

The extracts prepared in Section 4.6.3 were removed from the freezer, allowed to thaw and equilibrate to room temperature prior to analysis.

5.8.4.1 Hyperforin

The acidic methanolic tablet extracts were diluted one in two with mobile phase. All samples were vortexed prior to injection. The method used for the LC-MS-MS analysis was the isocratic method validated in Section 5.3. The mobile phase consisted of 0.01N ammonium acetate (pH = 5.7): acetonitrile: methanol (20:20:60). The column used was a Luna C_{18} (2) 150 x 2.0mm narrow bore column. The flow rate was 0.2ml/min with a temperature of 40°C and an injection volume of 5µl.

The LC-MS-MS tune methods for Hypf, HY and PsHY were optimised using the following conditions: sheath gas flow = 80psi, spray voltage = 3.50 kV, capillary temperature = 40°C and capillary voltage of -4.00kV for Hypf and HY and –8.00kV for PsHY with isolation widths of 2.0 and relative collision energy of 50% for HY and PsHY and 40% for Hypf.

The experimental method was set up to qualitatively analyse the naphthodianthrones and Hypf. The segments for each compound were as follows: PsHY, from 0 to 10 minutes, Hypf from 10 to 17 minutes and HY from 17 to 23 minutes.

5.8.4.2 The Naphthodianthrones

Simple dilution of the methanolic extracts with mobile phase resulted in insufficient ionization of the naphthodianthrones. Two millilitres of each of the methanolic tablet extracts was evaporated to dryness using the N-Evap and made up in five times the original sample volume of mobile phase in order to elicit appropriate responses from the MS-MS detector. All samples were vortexed prior to injection. The method used for the LC-MS-MS analysis was the isocratic method used for Hypf (refer to Section 5.8.4.1).

5.8.5 RESULTS AND DISCUSSION

The naphthodianthrones have been reported to show little tendency to form $[M+H]^+$ quasi-molecular ions by addition of protons under acidic conditions. On the other hand, high yields of $[M-H]^-$ ions have been reported under basic and even neutral conditions. In this study, high yields of $[M-H]^-$ were obtained under slightly acidic conditions. The hypericins and related compounds are known to be very stable closed-shell anions, showing no fragmentation under mild electrospray conditions [105]. This could explain the need for the 50% collision energy required to obtain a daughter ion. Surprisingly, in spite of the reported instability of Hypf, 40% collision energy was required to obtain fragmentation.

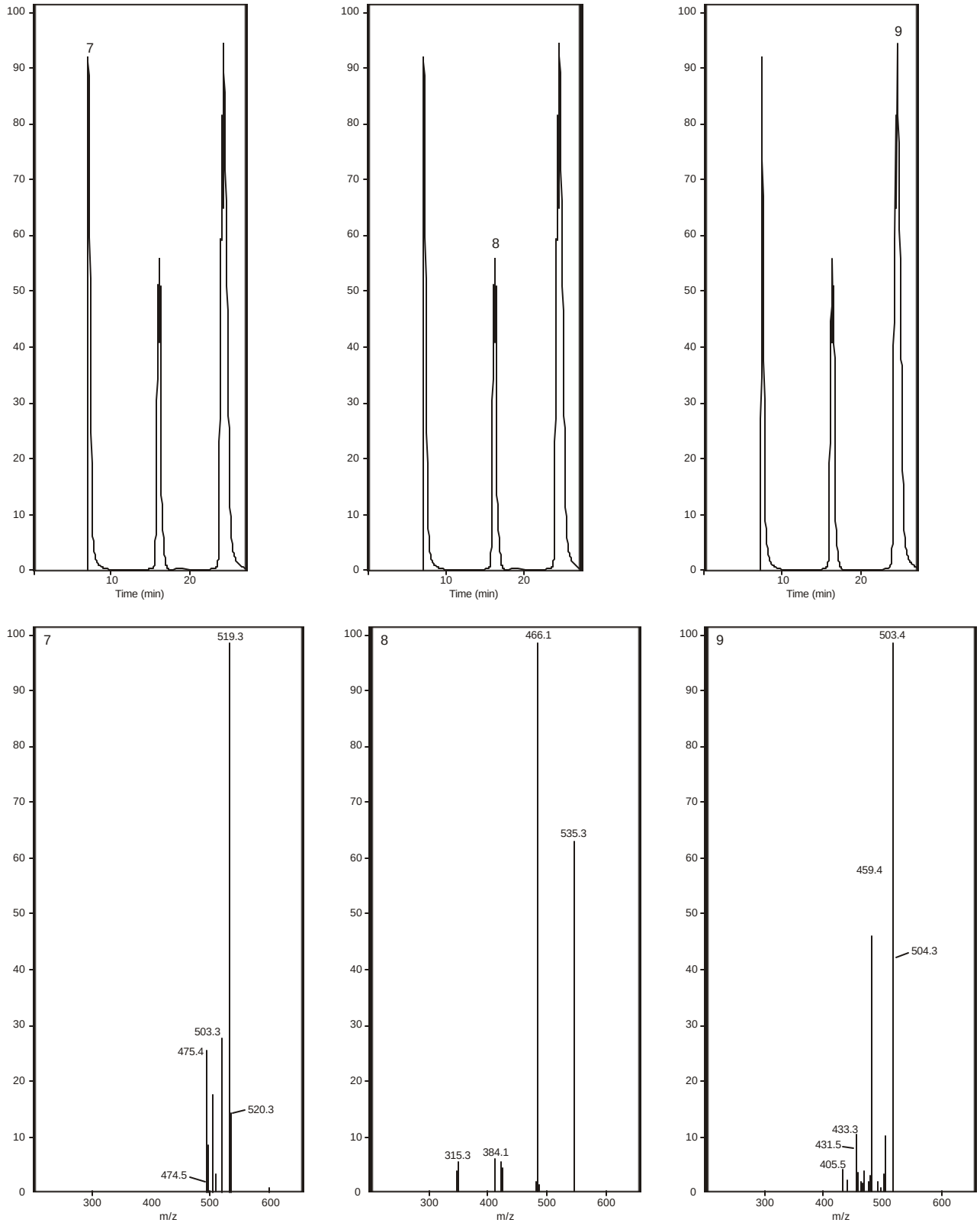
A full scan chromatogram with corresponding spectra and subsequently an MS/MS chromatogram with corresponding spectra were obtained for a mixture of PsHY, HY and Hypf. These were similar, and the MS/MS chromatogram with the corresponding spectra is illustrated in Figure 5.17. The molecular ions and daughter ions obtained in the negative ion mode for PsHY (7), Hypf (8) and HY (9) are shown in Table 5.15 and in Figure 5.17.

Table 5.15. Mass to charge ratios for pseudohypericin, hyperforin and hypericin

Constituent	Parent Ion (m/z)	Daughter Ion (m/z)
Pseudohypericin (7)	519.3	503.3
Hyperforin (8)	535.3	466.1
Hypericin (9)	503.4	459.4

Chromatograms in Figure 5.17 represent, from left to right, PsHY, Hypf and HY respectively with their corresponding spectra illustrated beneath

Figure 5.17. LC-MS-MS total ion chromatograms of PsHY, Hypf and HY with corresponding spectra.



7 = Pseudohypericin, 8 = Hyperforin, 9 = Hypericin

The results obtained from the qualitative analyses of the acidic methanolic and methanolic extracts are outlined in Sections 5.8.5.1 and 5.8.5.2 respectively.

5.8.5.1 Acidic Methanolic Extracts

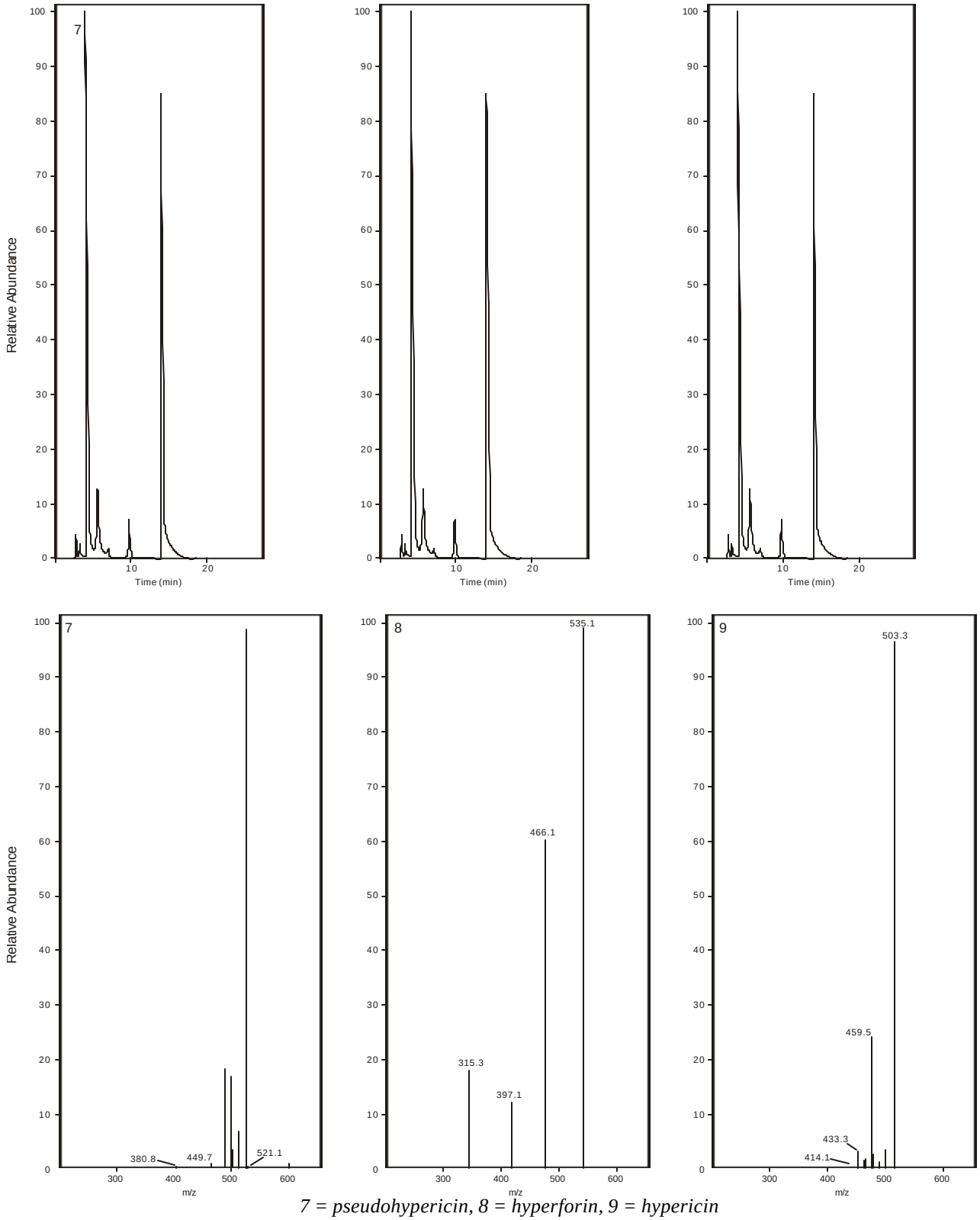
The total ion chromatograms and corresponding spectra for each of the diluted acidic aqueous methanolic extracts obtained from products A, B, C, D and E were obtained.

From the results it was evident the HY, PsHY and Hypf were present in all five extracts. Pseudohypericin content was present in the highest amount in all five extracts, followed by lesser amounts HY. The amount of Hypf was lowest in all five product extracts.

Extract A contained very little Hypf and the chromatogram and corresponding spectra for Extract A is illustrated in Figure 5.18. Extract E was similar to Extract A in that the size of the Hypf peak was very small in relation to the HY and PsHY peaks.

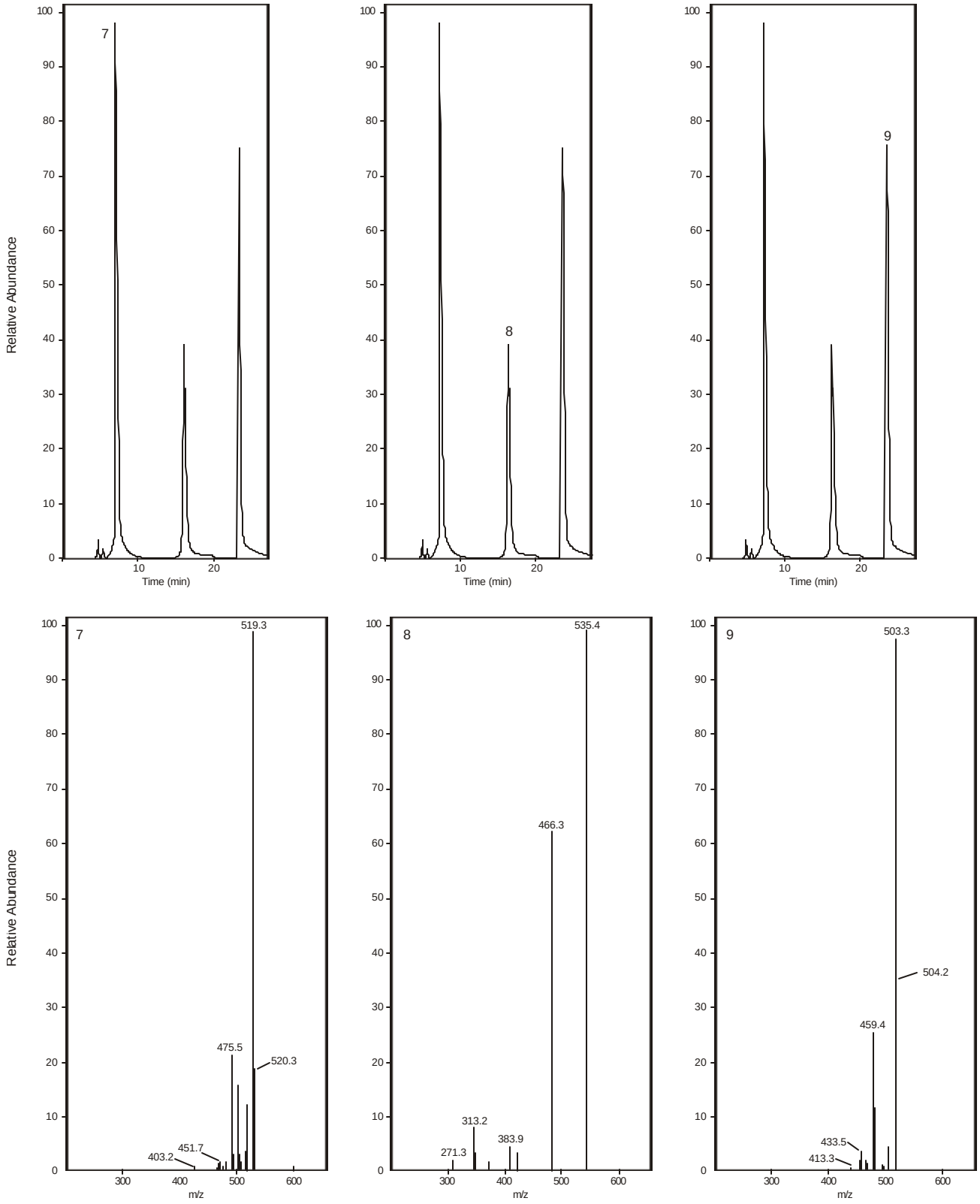
Figure 5.19 illustrates the chromatogram and corresponding spectra for Extract C. The chromatograms for Extracts C and D were very similar. As can be seen, there is a relatively greater amount of Hypf in this Extract C in relation to the naphthodianthrones than Extract A. Extract B contained relatively less Hypf than Extract C, but more than Extract A.

Figure 5.18. Extract A: LC-MS-MS chromatogram with corresponding spectra.



7 = pseudohypericin, 8 = hyperforin, 9 = hypericin

Figure 5.19. Extract C: LC-MS-MS chromatogram with corresponding spectra.



7 = pseudohypericin, 8 = hyperforin, 9 = hypericin

From these chromatograms and spectra it was evident that Hypf was present in all five of the extracts. It was also evident that the greatest quantities of Hypf occurred in extracts C and D. This is in agreement with the data obtained from the quantitative analysis of these products described in Section 5.6.4.1, where Hypf could only be determined in extracts from Products C and D. The amounts of Hypf in extracts from A, B and E were below the LOD.

Pseudohypericin and HY were present in all the acidic methanolic extracts. Tablet extraction efficiency comparisons of acidic aqueous methanolic and methanolic extracts of the naphthodianthrones (see Section 5.5.1) showed that the acidic aqueous methanolic extraction solvent was effective in extracting the naphthodianthrones. However, it was not as effective as methanol. The results from this qualitative analysis were in agreement with those findings.

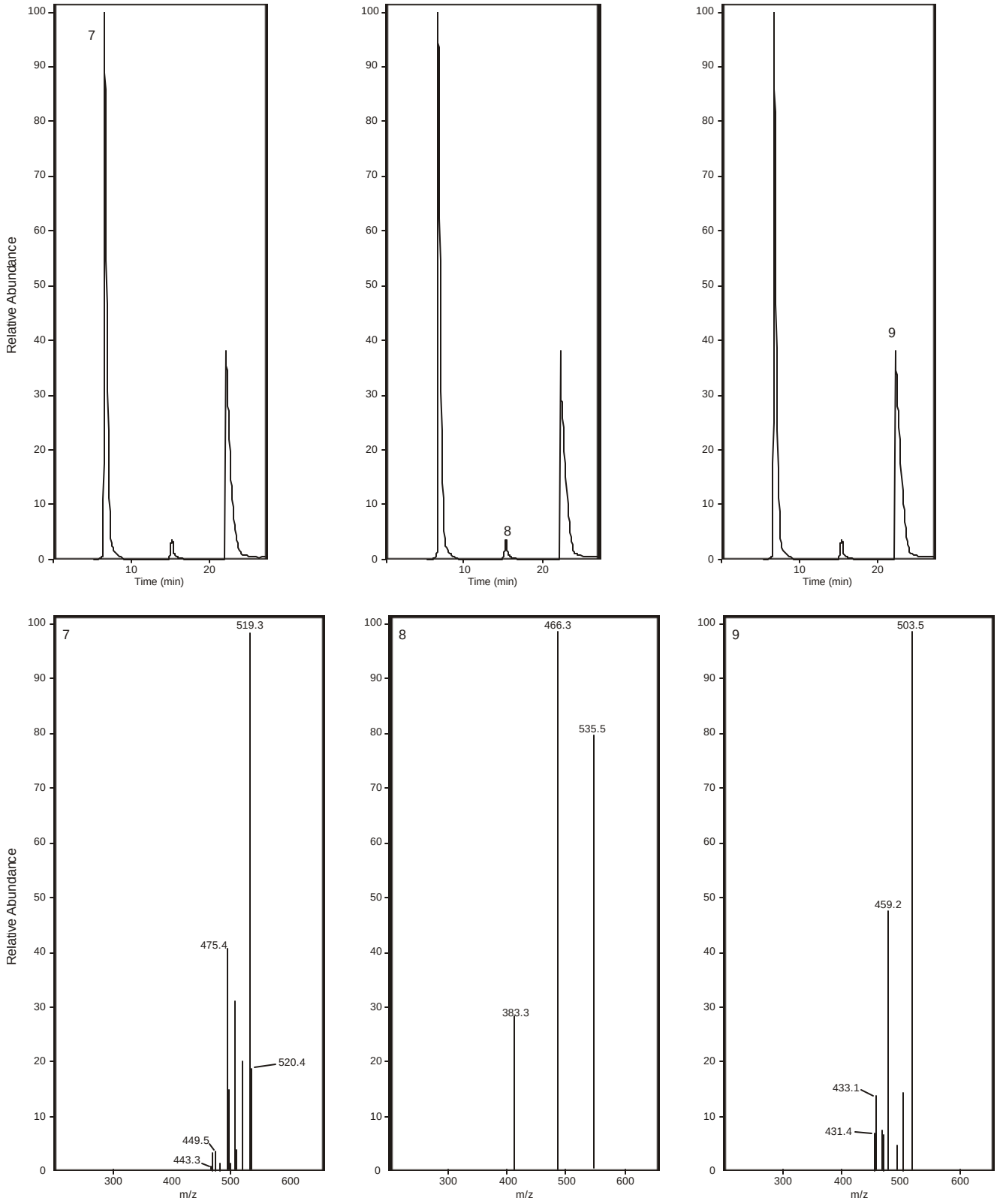
5.8.5.2 Methanolic extracts

The total ion chromatograms and corresponding spectra for each of the diluted methanolic Extracts A, B, C, D and E were obtained.

From the results it was evident that PsHY and HY were present in all five extracts, PsHY generally being present in greater quantities than HY. The total ion chromatograms and corresponding spectra are shown for Extracts C and E in Figures 5.20 and 5.21 respectively. Substantial amounts of PsHY and HY were present in all the product extracts.

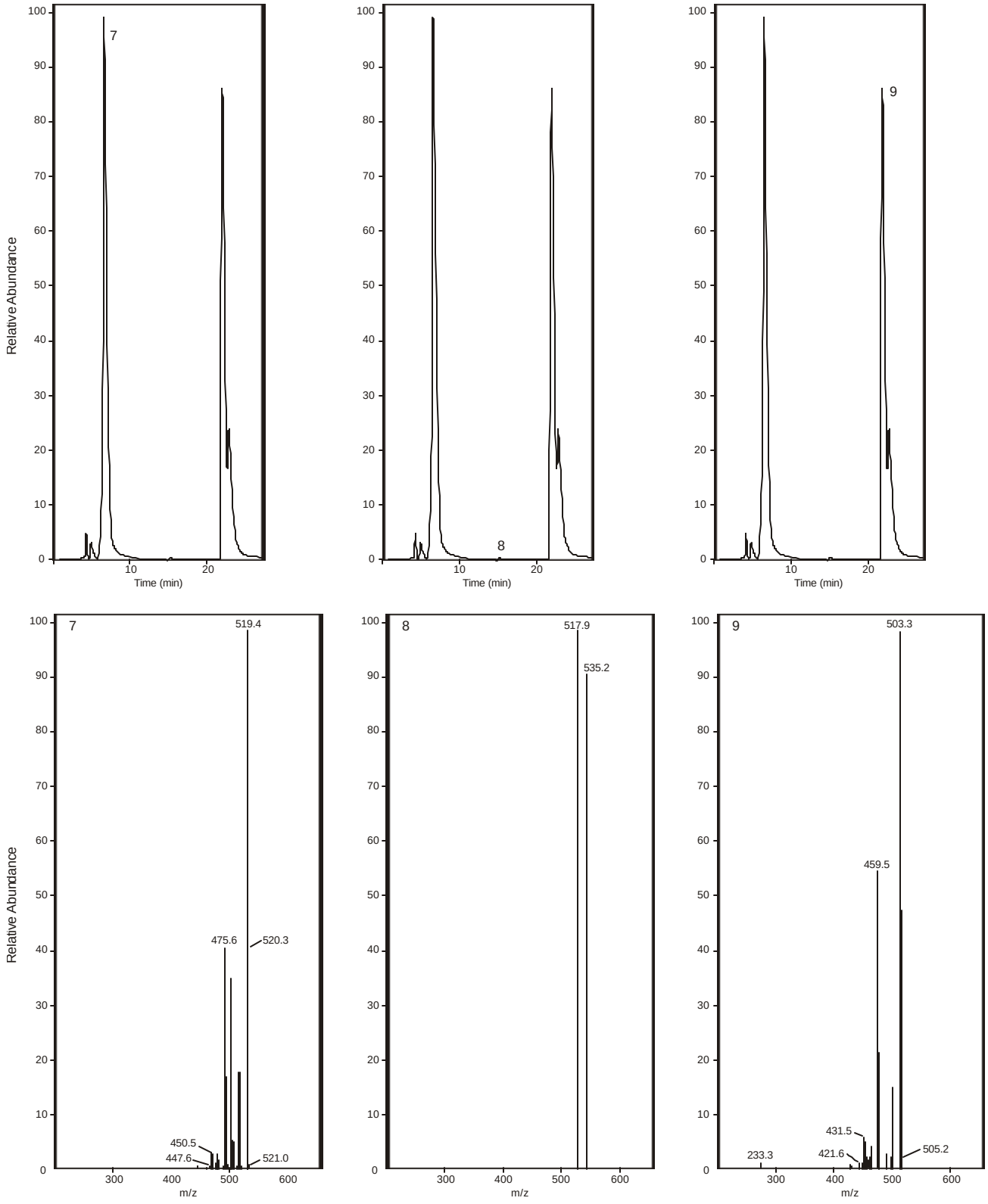
In terms of the amount of Hypf present in the methanolic extracts, the results from Extracts A and B were similar to Extract E, - the Hypf concentrations were below the LOQ. The results from Extract D were similar to those obtained from Extract C, - a very small amount of Hypf was present. These results confirm previous findings reported in Section 5.6.4.

Figure 5.20. Extract C: LC-MS-MS chromatogram with corresponding spectra.



7 = pseudohypericin, 8 = hyperforin, 9 = hypericin

Figure 5.21. Extract E: LC-MS-MS chromatogram with corresponding spectra.



7 = pseudohypericin, 8 = hyperforin, 9 = hypericin

As can be seen from Figures 5.20 and 5.21, both HY and PsHY were present in Extracts C and E. As already mentioned, this was also the case with Extracts A, B and D. This confirms the results previously obtained and reported in Section 5.6.4, where both naphthodianthrones were present in relatively substantial quantities in all five tablet product extracts. These extracts had to be diluted five times to obtain satisfactory LC-MS-MS results and to prevent detector overload, whereas the acidic extracts only had to be diluted by half. This shows the greater efficiency of methanol as extraction solvent for the naphthodianthrones as opposed to using an acidic aqueous methanolic solution.

From the spectra it can be seen that Hypf is detectable in Extracts C and D. The apparent absence of Hypf in products A, B and E could be due to two reasons: Hyperforin is purported to be more stable in acidic aqueous methanolic solutions than methanolic solutions, and may have degraded in these simple methanolic extracts. The methanolic extracts were 2.5 times more dilute than the acidic aqueous methanolic extracts and this dilution may have resulted in the inability to detect hyperforin in those extracts.

5.9 CONCLUSIONS

The isocratic method developed and reported in this thesis for the separation of HY, PsHY and Hypf had a total run time of less than 18 minutes. The method was validated and provided the necessary properties to enable successful quantitative analysis to be performed. Unfortunately, the extraction solvent that had to be used for the naphthodianthrones (methanol) was different to that which had to be used for hyperforin (acidified aqueous methanol). This implied that although the method was adequate for the separation of these three compounds in a single run, the methanolic extracts had to be run separately from the acidified aqueous methanolic extracts.

Hyperforin was only present in substantial quantities in Product D. This is both interesting and disconcerting since it is currently considered that Hypf contributes to the anti-depressant activity of *H. perforatum* [51]. From the assays performed on other St John's Wort products by Ganzera *et al*. [85] and Liu *et al*. [89] it can thus be reasonably assumed that the products investigated in this thesis were of dubious quality.

High inter-batch variability was observed for the naphthodianthrone compounds. There was a five-fold difference between the lowest and highest concentrations of PsHY in the different batches of Holotropic® tablets and a nine-fold difference for HY in this case. This illustrates the need for quality control of these products. The PsHY content in the tablets assayed in this study were generally lower than those reported elsewhere whilst the HY content in the products assayed in this was found to be consistent with HY content in St. John's Wort products reported elsewhere.

The HPLC method developed could be readily applied to LC-MS-MS and served as a useful qualitative tool to confirm results obtained from HPLC.

MAIN CONCLUSION

Two HPLC methods were developed, successfully validated, and utilised for the quantitation of the nine various active and marker compounds in St John's Wort products. Capillary electrophoresis and LC-ESI-MS-MS were also successfully used for qualitative analysis of St John's Wort products.

The gradient HPLC method developed for the separation of six of the relevant flavonoid compounds in St John's Wort was applied to assay selected commercially available St John's Wort products. This system provided the necessary accuracy, precision and reproducibility. In addition, the method developed utilised a narrow bore column (2.00 mm, i.d.), which is advantageous in terms of cost, sensitivity, resolution and where only small sample volumes are available. Furthermore, it has the benefit of reducing the volume of toxic organic solvents. The method developed is currently the only known method that separates all six relevant flavonoids in a reasonable run time (less than 20 minutes). It is also one of the few methods that has sufficient separation between rutin, isoquercitrin and hyperoside.

Flavonoid compounds are common to many plants and plant products. The nature of the flavonoids is such that hydrolysis can occur under certain, usually extreme, conditions resulting in the release of the aglycone moiety from its glycoside structure. Thus an increase in aglycone content and a decrease in glycoside content indicate undesirable degradation processes. These processes may result from one or many external factors, such as, for example, poor storage which is a typical cause of degradation. This method can therefore be used as a qualitative tool in fingerprinting St John's Wort and herbal products which contain similar flavonoid components. It may also be used for quantitative determination of these flavonoid compounds in herbal products.

In this study all of the glycosides investigated were quercetin derivatives. Thus it can be seen that the lower the glycoside content and the higher the quercetin content, the poorer the quality of the product. Comparing flavonoid glycoside results obtained in this study with those reported from other studies, the results tended towards the lower end of the ranges reported, and in some instances were even lower than those reported elsewhere. The amount of quercetin found in the tablets assayed was relatively high when considered in terms of the ranges previously reported, and in some cases, even higher than

those in previously reported ranges. Implications of this are that the products assayed in this study, especially A, B and C are of poor quality. By virtue of the fact that Product D and E possessed much higher glycosidic content and generally lower quercetin content than Products A, B and C, they can be considered to be the best quality products out of those analysed. Important to note is the high inter-product variability of flavonoid content, especially between the three batches of the same brand of tablets.

The qualitative MEKC method developed for the fingerprinting of the flavonoid components was successfully applied to qualitatively analyse various commercial St John's Wort products. This method was advantageous in that it was simple, required minimal sample preparation and utilised very small quantities of sample. It was cost effective in comparison to HPLC, as very small amounts of organic solvents were required and capillaries are less expensive than HPLC columns. The results obtained in this section were in conformity with the quantitative assay findings of the tablet products, and served to qualitatively illustrate the inter-product variability in terms of content and quality of St John's Wort products on the market.

The isocratic HPLC method developed for the separation of hypericin, pseudohypericin and hyperforin had a total run time of less than 18 minutes. The method was validated and provided the necessary properties to enable successful quantitative analysis to be performed, as well as the advantages associated with the use of a narrow bore column as opposed to the use of the more commonly used wider bore columns.

This method was subsequently applied to LC-MS-MS. ESI-MS-MS was used for the qualitative analysis of the content of the naphthodianthrones and hyperforin in the respective tablet products assayed with HPLC. These analyses identified the constituents, verified the specificity of the HPLC method and served as a useful qualitative tool to confirm results obtained from HPLC.

High inter-batch variability was observed for the naphthodianthrone compounds. There was a five-fold difference between the lowest and highest concentrations of PsHY in the different batches of Holotropic® tablets and a nine-fold difference for HY in this case. The content of PsHY in the tablets assayed in this study were generally lower than those reported elsewhere whilst the HY content in the products assayed was found to be consistent with HY content in St. John’s Wort products reported

elsewhere. In the case of hyperforin, although it is currently considered that Hypf contributes to the anti-depressant activity of *H. perforatum* [53], hyperforin was only found to be present in substantial quantities in Product D.

The results from these studies illustrate the large inter-product and inter-batch variability of active and marker constituents in various St. John's Wort products commercially available. This variability is not uncommon to herbal products and serves to highlight the necessity for quality control measures to be set in place in terms of the marketing and use of herbal products. This is especially important as there is a tremendous world-wide increase of interest and utilisation of these herbal medicines for preventative and therapeutic purposes.

APPENDICES

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STANDARD OPERATING PROCEDURE FOR LABORATORY

SOP No.: SOP/LAB/01	Revision No.: Original
Title: Procedure for the Operation of the Millipore Milli-Q System	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. Introduction

The Milli-Q system polishes/purifies deionised/distilled/RO water to HPLC quality water. The procedure below outlines the operation of the Milli-Q system.

2. References

a) Millipore Milli-Q Installation, Operation and Maintenance Manual
b) Related SOP's: SOP/LAB/19/01 - Procedure for the Maintenance of the Millipore Milli-Q System

3. Definitions

Milli-Q Function - The Milli-Q Water System polishes water that has been pre-treated (by deionization, distillation or reverse osmosis) to give the purest water commercially available (18 megΩ). Long life disposable cartridges remove dissolved inorganics by mixed bed deionization.

4. Responsibilities

It is the responsibility of all personnel that use the Milli-Q system to ensure that the correct operating instructions are followed.

5. Procedure

a) Switch on the power to the Milli-Q system at the wall plug.

b) Open the 3-way-valve (tap) so that the water can run to waste.

c) Allow the water to run to waste until the Meg-O-Meter reads 18 megohms.

d) Once, the meter reading has reached 18 megohms, then collect the required amount of Milli-Q water for use.

e) Log the amount of water taken in the logbook provided.

STANDARD OPERATING PROCEDURE FOR LABORATORY

SOP No.: SOP/LAB/02	Revision No.: Original
Title: Procedure for the Maintenance of the Millipore Milli-Q System	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. Introduction

The Milli-Q system polishes/purifies deionised/distilled/RO water to HPLC quality water. The procedure below outlines the maintenance of the Milli-Q system.

2. References

c) Milli-Q Installation, Operation and Maintenance Manual
d) Related SOP's: SOP/LAB/18/01 - Procedure for the Operation of the Millipore Milli-Q System

3. Definitions

Maintenance - This is a process whereby an item or instrument is cleaned and checked so as to ensure an extended life.

4. Responsibilities

It is the responsibility of all personnel that use the Milli-Q system to ensure that the maintenance is completed.

5. Procedure

5.1. Classification of the Milli-Q

Type: Milli-Q	Model: OMO 70	Serial No.: 17874

5.2. Milli-Q Expendables

a) Organex Cartridge (to remove organic contaminants)
b) Carbon Cartridge (to remove dissolved organic contaminants by absorption)
c) Ion Exchange Cartridges x 2 (to remove anions and cations)
d) Final filtration through a 0.22 µm filter (catalogue no. MPGL04SK2)

Note: A set of 4 Milli-Q cartridges (containing the cartridges described above) has a catalogue no. CFOF01205.

5.3. System Maintenance

5.3.1. Replacing Cartridge Elements

a) The frequency with which cartridge elements need replacing depends on the quality of the input water.

b) The Carbon and Ion Exchange cartridges must be replaced when the resistivity falls below 15 MΩ cm.

c) The outlet/stack filter should be replaced when the flow rate decreases to 0.5 litres per minute, or every 10 days when extremely low bacteria counts are required.

d) To replace the above cartridges and filter, first isolate the system by turning off the input water source and then stabilise the internal water pressure by opening the outlet ball valve. Disconnect the outlet/stack filter. Remove the cartridge housing and/or housing mount cover. Remove old/exhausted cartridge elements, by pulling straight down. Discard the exhausted cartridge elements and filter unit. Replace the cartridge elements and the filter unit in the proper sequence (as described in the manual in fig. 1-1).

5.3.2. Sanitisation/Cleaning

a) When cartridge elements are replaced and there is evidence residual slime deposits or dirt on the inner surface of the housing bowl, the system has become contaminated with bacteria and or dirt and must be sanitised with sodium hypochlorite solution (ordinary liquid bleach/ jik).

b) Remove all cartridge elements and set aside and remove the outlet/stack filter. Add 20 ml of the sodium hypochlorite solution to all each cartridge and replace. While the outlet-3-way valve is open, start the pump to fill the system with water from the attached source. Close the outlet-3-way valve when the water begins to flow. Turn off the pump and let it stand for 30 minutes. Remove all cartridge housing bowls, DISCARD contents and replace the bowls. Start the pump (with the outlet-3-way valve open) until the water begins to flow. Close the outlet-3-way valve and re-circulate again for 2 minutes. Open the outlet-3-way valve and flush the approximately 10 litres of water from the system to wash away any residual sodium hypochlorite solution.

c) Install cartridge elements and the outlet/stack filter and resume system operation.

5.3.3. **Prolonged Shutdown**

a) If a prolonged shutdown of more than 100 hours is anticipated, the system should be completely drained, cartridge elements removed and housing bowls cleaned.

b) When the system is full of water and has been inactive for longer than 100 hours, it should be completely drained before operating again. Remove and empty the cartridge housing bowls, and clean them if necessary, as directed above in 5.3.2. After reassembly and before starting to restore desired water quality, the residual degraded water in all the extra plumbing must be discarded with the initial flow of effluent from the system.

c) If the system is full of water and has been inactive for longer than 2 weeks, it must be sanitised as directed above in 5.3.2. and all the cartridge elements must be replaced.

SOP No.: SOP/LAB/03	Revision No.: Original
Title: Procedure for the Operation of the Crison GLP 21 pH Meter	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. **Introduction**

The procedure below outlines the operational procedure for the Crison pH Meter.

2. **References**

Crison Users Manual for pH Meters GLP 21-22

Related SOP: SOP/LAB/01/01

3. **Definitions**

The measurement of pH can be defined as the measurement of the activity of the hydrogen ions in a solution.

4. **Responsibilities**

It is the responsibility of all personnel that use the pH meter to ensure that the correct operating instructions are followed.

5. **Procedure**

i. Press the power button to switch on the pH meter.

ii. The main menu will display calibrate pH if it is the first pH measurement of the day. The display will notify you that the pH meter is out of calibration and it will not allow any pH measurement until the calibration has been done. The pH meter has to be calibrated once a day. Refer to SOP/LAB/01/01 for the procedure for the calibration of the pH meter.

iii. Once, the pH meter has been calibrated the main menu will display measure pH.

iv. Select “measure pH” by pressing the ENT button. The pH meter then displays the temperature and measures the pH. Once, the stabilised pH measurement has been obtained it will be displayed on the screen.

If the pH measurement has not stabilised within 30 seconds the flashing * will be replaced with the recorded value, which will be updated until stability is established.

SOP No.: SOP/LAB/04	Revision No.: Original
Title: Maintenance and Calibration Procedure for Crison pH Meter	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. Introduction

The procedure below details all the calibration and maintenance requirements for the classified pH meter.

2. References

The standard form that is relevant to this procedure: SF/LAB/01/01

3. Definitions

pH Meter - This is an instrument that determines the pH of an aqueous solution.
Calibration - This is a process whereby an instrument is set to read at a previously determined point.
Maintenance - This is a process whereby an item or instrument is cleaned and checked so as to ensure an extended life.

4. Responsibilities

It is the responsibility of the first person using the pH meter for the day, to calibrate it.

5. Procedure

5.1 Classification of the pH Meter

Type: Crison pH Meter	Model: GLP 21	Serial No.: 935027

5.2 Calibration Frequency

Calibration is to be done daily. (Note: After 1 day the pH meter will not allow any pH measurement until the pH calibration is done.)

5.3 Calibration Procedure

a) Pour 5 to 10 ml of the pH 7.0 and pH 4.0 buffer into separate beakers.

b) Go to pH calibration mode on the pH meter and press “ENT”. (Note: After 1 day the pH meter will not allow any further readings until a calibration is done; i.e. the pH meter will automatically be in calibration mode.) On the screen it will indicate that it is ready for the pH 7.0 buffer. Place the

electrode in the solution and press “ENT”. The screen will indicate that it is calibrating the pH 7.0 buffer.

c) When it has finished calibrating it will indicate that it is ready for the second buffer (pH 4.0). Remove the pH 7.0 buffer and rinse the electrode well. Then place the electrode in the pH 4.0 buffer and press “ENT”. Once the calibration has been completed, it will indicate that “calibration OK” or “calibration error”. (If “calibration error” is indicated refer to the operating manual page 9 for the different errors that can be displayed.)
d) If the “calibration OK” message is displayed on the screen, press “ENT” (within 2 seconds) and it will display the calibration data. When “ENT” is pressed again more calibration data can be obtained.
e) Record the information of the calibration on the standard form (SF/LAB/01/01) “Daily Calibration of the Crison pH Meter” and place it in the drawer below the pH meter.

5.4 Maintenance Procedure

a) Daily: Check that there is sufficient 3 M KCl in the protective sleeve.

b) Weekly: Replace the 3 M KCl in the protective sleeve with fresh 3 M KCl.

OP No.: SOP/LAB/05	Revision No.: Original
Title: Procedure for the Operation of the Mettler AE 163 Balance	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. Introduction

The procedure below outlines the operational procedure for the Mettler AE 163 Balance

2. References

Mettler AE 163 Operating Instructions

Related SOP: SOP/LAB/17/01

3. Definitions

None.

4. Responsibilities

It is the responsibility of all personnel that use the balance to ensure that the correct operating instructions are followed.

5. Procedure

i. Switch the balance on, by pressing the control bar once. All the display elements light up for several seconds; this permits a functional check of the display. Then zero is displayed.

ii. Place the container on the weighing pan.

iii. Press the control bar to tare; then zero is displayed.

iv. Weigh the desired target into the vessel. If different components must be weighed in, one after the other, tare can be pressed after each component is weighed in.

v. When weighing has been completed, remove the vessel from the weighing pan. Before leaving ensure that the weighing pan and the balance area is free of powder or any of the substances weighed.

vi. Switch off the balance by pressing the control bar once. By switching the balance off using the control bar, only the display is turned off. The electronic components are on as long as the power cable is connected. This allows the balance to be operational at all times and eliminates the need for warm-up time.

Note: During weighing the balance doors must remain closed.

SOP No.: SOP/LAB/06	Revision No.: Original
Title: Procedure for the Maintenance and Calibration of the Mettler AE 163 Balance	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. Introduction

The procedure below details all the calibration and maintenance requirements for the Mettler AE163 balance.

2. References

Operating Instructions for Mettler AE163

3. Definitions

Calibration - This is a process whereby an instrument is set to read at a previously determined point.
Maintenance - This is a process whereby an item or instrument is cleaned and checked so as to ensure an extended life.

4. Responsibilities

It is the responsibility of personnel using the balance to ensure that the maintenance and calibration is done.

5. Procedure

5.1 Classification of the Balance

Type: Mettler Balance	Model: AE163	Serial No.: F79058

5.2 Calibration Frequency

a) Weekly

The person using the balance must do a calibration on the balance using the built-in calibration weight, as well as checking the balance against 50 and 100 mg external calibration weights. This weekly calibration must be done on a Monday morning before any weighing has been performed.

b) Annually

Annually the supplier must calibrate the balance with certified calibration weights and supply a calibration certificate.

5.3 Calibration Procedure

a) Weekly Calibration

- Remove all objects from the pan and close the sliding doors.
- Set the balance to calibration mode by pressing the control bar until "CAL" is displayed. Then release the control bar and "CAL - - - -" will appear in the display.
- The calibration weight is placed in position by means of the calibration lever. As soon as "CAL 100" appears (the 100 blinks), slowly move the calibration lever to the rear, all the way until it stops moving.
- First, "CAL - - - -" appears, then "100.0000". When the display "CAL 0" appears (0 blinks), move the calibration lever back to the original position. Wait a few seconds and then the display will show "- - - -" followed by "0.0000".
- The balance is now calibrated with the built-in calibration weight at 100 g and 0 g.
- When the procedure above has been completed, place a 50 mg weight on the pan and record the result obtained in the calibration logbook provided. Do the same with the 100 mg weight.
- If the results obtained for the 50 and 100 mg weights exceed the limits tabulated below, then repeat the balance calibration using the built-in calibration weight. If the results of the 50 and 100 mg weight still exceed the limits then the supplier must be contacted, so that the balance can be serviced and calibrated.
- If the results obtained for the 50 and 100 mg weight are within the limits tabulated below the balance can be used.

Limits for 50 and 100 mg Weights	
50 mg weight	Result = 0.0500 g ± 1 digit
100 mg weight	Result = 0.1000 g ± 1 digit

b) Annual Calibration

Annually the supplier must calibrate the balance and a certificate of calibration must be supplied with the calibration.

5.4 Maintenance Procedure

a) Daily

Before using the balance ensure that the bubble in the spirit level is in the middle of the circle. If bubble is not in the middle of the circle adjust the two leveling screws so that it is in the middle of the circle.

After using the balance ensure that the pan, the balance and the area around the balance is clean.

b) Annually

The supplier must service the balance, before the annual calibration is done.

SOP No.: SOP/LAB/07	Revision No.: Original
Title: Procedure for the Operation of the Sartorius 2004 MP6 Balance	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. Introduction

The procedure below outlines the operational procedure for the Sartorius 2004 MP6 Balance.

2. References

Sartorius 2004 MP6 Instructions for Installation and Operation

Related SOP: SOP/LAB/21/01 - Procedure for the Maintenance and Calibration of the Sartorius 2004 MP6 Balance

3. Definitions

None.

4. Responsibilities

It is the responsibility of all personnel that use the balance to ensure that the correct operating instructions are followed.

5. Procedure

5.1. Switching on the Balance

a) Switch the balance at the power supply. All the display elements light up followed by the automatic taring.

b) Switch on the balance at least 30 minutes before operation.

c) Do not switch the balance off at the power supply, unless it is for a prolonged shutdown.

5.2. Weighing a Sample

a) Place the weighing vessel on the weighing pan.

b) Press the tare switch. Once, stability has been reached the “g” symbol lights up to show that zero has been reached.

c) Open the sliding door and weigh the desired target into the vessel. If different components must be weighed in, one after the other, tare can be pressed after each component is weighed in.

d) When weighing has been completed, remove the vessel from the weighing pan. Before leaving ensure that the weighing pan and the balance area is free of powder or any of the substances weighed.

Note: During weighing the balance doors must remain closed.

SOP No.: SOP/LAB/08	Revision No.: Original
Title: Procedure for the Maintenance and Calibration of the Sartorius 2004 MP6 Balance	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. Introduction

The procedure below details all the calibration and maintenance requirements for the Sartorius 2004 MP6 balance.

2. References

Sartorius 2004 MP6 Instruction for Installation and Operation

Related SOP: SOP/LAB/20/01 - Procedure for the Operation of the Sartorius 2004 MP6 Balance

3. Definitions

Calibration - This is a process whereby an instrument is set to read at a previously determined point.
Maintenance - This is a process whereby an item or instrument is cleaned and checked so as to ensure an extended life.

4. Responsibilities

It is the responsibility of personnel using the balance to ensure that the maintenance and calibration is done.

5. Procedure

5.1 Classification of the Balance

Type: Sartorius Balance	Model: 2004 MP6	Serial No.: 3010059

5.2 Calibration Frequency

a) Weekly

The person using the balance must do a calibration using a 15 g weight, as well as checking the balance against 50 and 100 mg external calibration weights. This weekly calibration must be done on a Monday morning before any weighing has been performed.

b) Annually

Annually the supplier/agent must calibrate the balance with certified calibration weights and supply a calibration certificate.

5.3 **Calibration Procedure**

b) Weekly Calibration

- Remove all objects from the pan and close the sliding doors.
- Set the motor stop switch to STOP.
- Once the balance has reached stability the "g" symbol will light up.
- Press the tare switch. The weight indication will display 0.00000g. (Permissible tolerance: ± 1 digit.)
- Open sliding doors and place 15 g test weight on balance pan.
- Close the sliding doors.
- Set the calibration switch to CAL. During the determination of the correction factor the weight indication is blanked out. (Decimal and stability indicator "g" will be flashing.)
- When the correction factor has been determined an acoustic signal sounds and the weight of the test weight flashes in the weight indication.
- Release the CAL switch used to call up the program.
- If a weight is displayed which deviates more than ± 1 digit from the test weight, repeat the operation.
- When the procedure above has been completed, place a 50 mg weight on the pan and record the result obtained in the calibration logbook provided. Do the same with the 100 mg weight.
- If the results obtained for the 50 and 100 mg weights exceed the limits tabulated below, then repeat the balance calibration using the 15 g test weight. If the results of the 50 and 100 mg weight still exceed the limits then the supplier must be contacted, so that the balance can be serviced and calibrated.
- If the results obtained for the 50 and 100 mg weight are within the limits tabulated below the balance can be used.

Limits for 50 and 100 mg Weights	
50 mg weight	Result = 0.05000 g ± 1 digit
100 mg weight	Result = 0.10000 g ± 1 digit

b) Annual Calibration

Annually the supplier must calibrate the balance and a certificate of calibration must be supplied with the calibration.

5.4 **Maintenance Procedure**

b) Daily

Before using the balance ensure that the bubble in the spirit level is in the middle of the circle. If bubble is not in the middle of the circle adjust the two leveling screws so that it is in the middle of the circle.

After using the balance ensure that the pan, the balance and the area around the balance is clean.

b) Annually

The supplier must service the balance, before the annual calibration is done.

SOP No.: SOP/LAB/09	Revision No.: Original
Title: Cleaning of Laboratory Glassware	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. **Introduction**

This procedure is a guide of the essential requirements of cleaning all laboratory glassware.

2. **References**

Vogel's Textbook of Quantitative Chemical Analysis; Fifth Edition; pages 79.

3. **Definitions and Abbreviations**

a) Open Vessels - Open vessels include most vessels where the diameter of the opening is similar in size to the maximum diameter of the vessel.

b) Semi-Closed Vessels - These are vessels that have a small opening compared to their maximum diameter.

4. **Responsibilities**

It is the responsibility of every individual that resides or does any work in the Laboratory to ensure that the glassware is kept clean.

5. **Procedure**

5.1 **General Cleaning Requirements**

a) It is important that all glassware and graduated/volumetric glassware is perfectly clean and free from grease, otherwise the results that are being generated may be unreliable. One test for cleanliness of glassware is that on being filled with distilled water and the water withdrawn, only an unbroken film of water remains. If the water collects in drops, the vessel is dirty and must be cleaned.

b) For the daily cleaning of glassware a mild detergent can be used (e.g. Teepol, Decon or an equivalent). A dilute solution of the detergent is used to wash the glassware. Graduated/volumetric glassware must not be washed in a glassware washer, as the washer could damage the glassware. Once, the glassware has been washed with the detergent solution, it must be rinsed well with tap water and then again with distilled water. All graduated/volumetric glassware must not be placed in an oven to dry, but must be left to drip dry. The reason is that graduated/volumetric glassware is calibrated to contain specified volumes at a definite temperature (usually 20°C) and when the glassware is exposed to higher or lower temperatures the glassware expands and contracts, respectively and may not necessarily hold the defined volume.

c) Each individual using graduated/volumetric glassware (pipettes and volumetric flasks) must clean the glassware used themselves and not place it with the general glassware (e.g. beakers, measuring cylinders, etc.) in the glassware-washer for washing.

d) If the daily cleaning procedure does not clean the glassware properly or the glassware needs degreasing, then the glassware can be soaked in a dilute nitric acid solution overnight. (NOTE: DO NOT USE CHROMIC ACID.)

5.2 Cleaning of Laboratory Glassware

The Gallay 910 Glassware Washer is designed for the washing of laboratory glassware.

For the purpose of glassware washing using the Gallay, the glassware is divided into three categories:

Category 1: Open vessels and totally closed items. (e.g. Beakers, autosampler vials, test tubes and stirring rods.)

Category 2: Semi-closed vessels. (e.g. Buchner/vaccuum flasks, conical flasks, Schott bottles and measuring cylinders.)

Category 3: Pipettes and long narrow items. (e.g. Pasteur pipettes, small burettes and glass tubing.)

5.2.1. Cleaning Category 1 Glassware

a) Place the glassware in the baskets provided. The autosampler vials and test tubes must first be soaked overnight in a dilute Decon solution and then grouped into groups of 7 and bound together with rubber bands. Place the test tubes and autosampler vials into small baskets.

b) Once the procedure described in a) above has been completed, the glassware can be placed in the washer. Attach the “Oscillating Spray Fitting” and place the soap powder in the soap container. (Ensure that the container is dry before adding the soap powder – use paper towel to dry the soap container.)

c) Start the wash cycle, which will take about an hour to complete (a brief rinse, a hot wash with soap and several rinses with de-ionised water). If it is necessary to add an item or soap after the wash cycle has started, open the door at any time and add the item or soap and the cycle will resume again after the door is closed. If it is found that soap

was not added and the cycle has advanced too far, stop the cycle using the abort button.

d) After completion of the wash, the machine may be opened and the glassware moved to the drying cabinet. (If there is soap left in the dispenser at the end of the wash cycle, the glassware should be considered contaminated and the wash should be repeated. See the note 1 at the end of the document for reasons for soap remaining in the dispenser.) The temperature of the drying cabinet is set to 70°C. Draining and warm air-drying facilities are available.

e) All the small dry items must be packed in styro-foam trays and sealed with cling film. All the large dry items must be sealed with cling film. Once the glassware is sealed, it is to be placed in the storage cupboards.

5.2.2. **Cleaning Category 2 Glassware**

a) Place the right-angle fitting in the washer. Place glassware on each of the individual water sprays of the individual-spray fitting. Small and light items should be secured using rubber bands.

b) Continue as described in 5.2.1. c) to e) above.

5.2.3. **Cleaning of Category 3 Glassware**

a) Place the right-angle fitting in the washer. The pipettes are then placed in the pipette washer and then placed in the glassware washer.

b) Continue as described in 5.2.1. c) to e) above.

Note 1: Reasons for soap remaining in the dispenser are as follows:

1) Dispenser not dried properly before soap was added.
2) Dispenser over-filled causing a leakage during the initial rinse.
3) Tall glassware items between the upper spray-head and the dispenser.

Note 2: It is possible to adjust the temperature of the washing water and to vary the number of rinses of the glassware washer. The temperature is set to 70°C and the number of de-ionised water rinses is set to maximum. It is preferable that these setting are NOT adjusted. If the settings must be for a cycle, then restore the "default" setting when the cycle is completed.

SOP No.: SOP/LAB/10	Revision No.: Original
Title: Procedure for the Operation of the Prince Capillary Electrophoresis Test System	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. Introduction

The procedure below outlines the operation of the PrinCE test system (including linked equipment).

2. References

Anatech PrinCE Capillary Zone Electrophoresis Instruction Manual

Related SOP's: SOP/LAB/13/01 - Procedure for the Maintenance and Calibration of PrinCE Capillary Electrophoresis Test System

3. Definitions

None.

4. Responsibilities

It is the responsibility of all personnel that use the PrinCE test system to ensure that the correct operating instructions are followed.

5 Procedure

5.1 Equipment Related to the Prince Test System

a) PrinCE - serves as an autosampler, pump and applies voltage across the capillary.

b) Butler - replenishes the liquid in the reservoir at the terminal end of the capillary, or the terminal electrode.

c) LINEAR UV Detector - detects the absorbance of the sample being analysed.

d) Perkin Elmer Chart Recorder - records the responses obtained from the LINEAR UV detector.

5.2 Operating Procedure

a) Measure a suitable length of capillary, marking the total desired length and effective length on the capillary. Burn a detection window, using a butane torch, about 1.5 cm long after the mark where the effective length was indicated. Gently rub the charred coating off with lens tissue.
The following standard capillary lengths are used:
Total length = ± 81cm
Desired length (to window) = ± 52 cm

b) Ensure that the detector is switched off and remove the detection window covering on the detector. Insert the capillary, from the cathodic/terminal electrode side of the detector, through the detector and then into the PrinCE. Ensure that the detection window of the capillary is in line with the detection window of the detector and then replace the detection window covering on the detector. Adjust the capillary length at the anode (inside the PrinCE), so that upon insertion of the electrode and capillary into the vial, the length of the capillary is 1 to 2 mm shorter than the electrode.

c) Switch on the PrinCE, the detector and the chart recorder.

d) Make up the respective buffers, solutions and samples, filter through HV 0.45 µm filter paper into the respective vials.

c) Choose the conditioning method required from the table below and program the PrinCE according to the method chosen.

Method for when capillary is used for the first time:		
1,0 M NaOH	2000 mbar	30 minutes
0,1 M NaOH	2000 mbar	30 minutes
Water	2000 mbar	30 minutes
If capillary is being reconditioned in the morning before a days use:		
1,0 M NaOH	2000 mbar	10 minutes
0,1 M NaOH	2000 mbar	10 minutes
Water	2000 mbar	10 minutes
For conditioning capillary between runs:		
0,1M NaOH	2000 mbar	2 minutes
Water	2000 mbar	2 minutes
Working buffer	2000 mbar	10 minutes

Note: The capillary must be conditioned before use everyday.

d) Set chart recorder speed and range to the required settings (normally, 5mm/min and 10 mV respectively).

e) Fill the Butler's flask with the working buffer and place it in position 1. Turn the clamp to allow the Butler to pump the buffer into the electrode reservoir and to pump the buffer through the Butler until there are no air-bubbles in the electrode solution reservoir. Place the capillary end into the reservoir.

f) Command the PrinCE to lift the vial and check that the level of the buffer in the vial is the same as the level of the buffer in the Butler/cathodic reservoir.

g) Start running the program and as the buffer flows through the capillary, before the first injection, zero the chart recorder.

h) Ensure that both buffer reservoirs (Butler's flask and PrinCE vial) are replenished regularly (i.e. after each run).

i) When finished using the system for the day, empty the Butler's flask of electrolyte and rinse with water. After rinsing, fill the Butler's flask with water. Pump the water through the Butler and the reservoir for one minute. Remove the capillary from the reservoir and flush with water for 20 minutes at 2000 mbar.

j) Switch off the PrinCE, LINEAR detector and chart recorder.

Date Prepared:

SOP No.: SOP/LAB/10	Revision No.: Original
Title: Procedure for the Maintenance and Calibration of the Prince Capillary Electrophoresis Test System	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. Introduction

The procedure below details all the calibration and maintenance requirements for the PrinCE Capillary Electrophoresis Test System (including linked equipment).

2. References

a) Anatech PrinCE Capillary Zone Electrophoresis Instruction Manual

b) Related SOP's: SOP/LAB/13/01 - Procedure for the Operation of PrinCE Capillary Electrophoresis Test System

3. Definitions

Calibration - This is a process whereby an instrument is set to read at a previously determined point.

Maintenance - This is a process whereby an item or instrument is cleaned and checked so as to ensure an extended life.

4. Responsibilities

It is the responsibility of personnel using the PrinCE test system to ensure that the maintenance and calibration is done.

5. Procedure

5.1 Classification of the PrinCE (including linked equipment)

Type: PrinCE	Model: 0500-001	Serial No.: 03 03 33
Type: Linear Detector 206PHD	Model: 0206-0000	Serial No.: 902689
Type: Butler	Model: 0550-001	Serial No.: 55-04-01-0-13

5.2 Calibration/System Suitability Frequency

The system suitability must be done monthly or when changes to the system have been made (e.g. changing capillary).

5.3 Calibration/System Suitability Procedure

a) Condition the capillary using the following method:
30 minutes 1M NaOH
30 minutes 0.1M NaOH
30 minutes H_2O

2 minutes 1M NaOH
2 minutes 0.1 NaOH

2 minutes H_2O

b) Make up the following solutions:
- 2M NaOH solution for adjusting the CAPS solution to pH 11
- 40 mM CAPS buffer solution and adjust to pH 11 with 2M NaOH
- Cytosine solution 100 ppm (made up in CAPS buffer)

c) Program the PrinCE using the following method:

CAPS	2000 mbar	1 minute
Cytosine	50 mbar	0.1 minute
CAPS	30kV	8 minutes
Water	2000 mbar	1 minute

Repeat the method above for as many injections as is required for your RSD. Run the following wash method between each repeat of the method above:
2 minutes 1M NaOH
2 minutes 0.1 NaOH
2 minutes H_2O

Wavelength: 254 nm
Rise time: 0.5 seconds
Sensitivity: 0.1

d) Run system suitability using SOP/LAB/13/01 for operating instructions.

e) To calculate the RSD for cytosine a minimum of 3 injections is required. The RSD calculated from the cytosine injections must be less than or equal to 5%.

5.4 Maintenance Procedure

a) Butler (Daily Maintenance)

Daily or when you have finished using the system, empty the Butler's flask of electrolyte and rinse with water. After rinsing, fill the Butler's flask with water. Pump the water through the Butler and the reservoir for one minute. Remove the capillary from the reservoir and flush with water for 20 minutes at 2000 mbar.

b) Capillary (Monthly Maintenance)

Monthly or when the migration time increases by more than 5 % the capillary must be replaced.

Measure a suitable length of capillary, marking the total desired length and effective length on the capillary. Burn a detection window, using a butane torch, about 1.5 cm long after the mark where the effective length was indicated. Gently rub the charred coating off with lens tissue.
The following standard capillary lengths are used:
Total length = ± 81cm
Desired length (to window) = ± 52 cm

Ensure that the detector is switched off and remove the detection window covering on the detector. Insert the capillary, from the cathodic/terminal electrode side of the detector, through the detector and then into the PrinCE. Ensure that the detection window of the capillary is in line with the detection window of the detector and then replace the detection window covering on the detector. Adjust the capillary length at the anode (inside the PrinCE), so that upon insertion of the electrode and capillary into the vial, the length of the capillary is 1 to 2 mm shorter than the electrode.

c) Inlet Electrode (Annual Maintenance)

Annually or when the electrode becomes corroded or when the electrode is not responding it must be replaced using the following method:

- Loosen the electrode connector screw by 2 turns (do not remove the screw) and pull down the electrode. Guide the electrode down through the inlet cone to remove it.
- Guide a new electrode in reverse direction upward through the inlet cone (a conical inlet will guide the electrode through its hole) and into the hole behind the electrode connector screw. Fasten the screw (gently) to secure the electrode.
- Check the length of the electrode – the tip of the electrode should remain just in the top of the cone when the inlet cone is its lowest position. (IF ELECTRODE PROTRUDES FROM THE FLAT SEAL, ALIGNMENT FAILURE WITH INSERTS MAY OCCUR.)
- Check the sealing device by using a vial filled with 80% fluid. At 2500 mbar (program in manual mode) there should be no leakage.

d) Outlet Electrode (Annual Maintenance)

Annually or when the electrode becomes corroded or when the electrode is not responding it must be replaced using the following method:

- Remove the outlet vial, loosen the electrode connector screw by 2 turns and pull down electrode.
- Push a new electrode upward in the hole (make sure that you put in enough length) and secure it in place by tightening the electrode screw (gently to avoid difficulties with a future replacement).

SOP No.: SOP/LAB/12	Revision No.: Original
Title: Procedure for the Operation of the Alliance HPLC using Millennium Software	

	Name/Position		Signature	Date
Prepared by:	Name:			
	Position:			
Authorised by:	Name:			
	Position:			
	Name:			
	Position:			

Date Prepared:	
Date Implemented:	
Date Removed:	

Table of Contents

1. Introduction

Samples are analysed using high performance liquid chromatography. The procedure below outlines the operational procedure for the Alliance (Waters 2690 Separations Module) HPLC.

2. References

a) Waters 2690 Separations Module – Quick Start Guide

b) Millennium^{32} Waters – Getting Started Guide

c) Related SOP: SOP/LAB/25/01 - Procedure for the Maintenance and Calibration of the Alliance HPLC

3. Definitions

a) Liquid Chromatography (LC):
Makes use of a stationary phase and mobile phase. Components of a mixture are carried through the stationary phase by the flow of the mobile phase; separations are based on the differences in migration rates of the sample components. These differences (or separations) can be detected using different techniques (ultraviolet, fluorescence, etc.), recorded and calculated.

b) High Performance Liquid Chromatography (HPLC):
HPLC is the modern version of LC. Columns with a high performance stationary phase (with particle sizes of 10μm and smaller) are used. The mobile phase is pumped at a high pressure through the column and the differences in migration (or separations) of the sample components are detected and processed electronically.

4. Responsibilities

It is the responsibility of all personnel that use the Waters Alliance to ensure that the correct operating instructions are followed.

5. Procedure

5.1 System Components

a) Waters 2690 Separations Module
b) Waters 2487 Dual Wavelength Detector
c) Computer Loaded with Millennium software

5.2 Start-up

a) Power up the separations module, detector and then the computer.
b) Prepare mobile phases and solvent washes and degas the mobile phases and solvent washes before use.

5.3 Preparation of Separations Module before the Start of a Run

5.3.1 Dry Prime

a) Set up the solvent reservoirs and insert the solvent tubing into the solvent reservoirs. Be sure the detector waste line and the sample loop waste line drain into an appropriate container.

b) Gently shake the filters in the reservoirs to remove any bubbles that may be trapped.

c) Attach and empty syringe to the prime/vent valve, then open the valve by turning it counterclockwise ½-turn.

d) Press the "Direct Function" screen key in the "Status" screen. The "Direct Functions" Menu appears.

e) Select "Dry Prime", then press "Enter". The "Dry Prime" dialog box appears. Press the screen key corresponding to the solvent line you want to prime.

f) Withdraw the syringe plunger to pull solvent through the tubing. You may need to exert force to pull the air and the solvent through the system. Continue until you pull the air through the solvent line into the syringe.

g) Repeat e) and f) above for each solvent line that needs priming and then close the prime/vent valve.

h) Press a screen key corresponding to the solvent line with which you want to prime the system. (It is recommended that you prime using the solvent with the lowest viscosity to help purge air from the lines, especially if an in-line vacuum degasser is installed).

i) In the "Enter a Duration" field, enter the length of time (in minutes) to prime the solvent management system. Start with a value of 5 minutes. Press "Continue".

The solvent management system begins to operate. At the end of the priming period, the solvent management system turns off and the Separations Module enters the "Idle" mode.

j) Now perform a wet prime as described below.

5.3.2 Wet Prime

To avoid damage to the face seals, perform a wet prime only when there is solvent in the solvent management system fluidic path.

Perform a wet prime when reservoirs or solvents are changed, or if the Separations Module has been idle for some time. The wet prime replaces solvent in the path from the reservoir to waste. If the solvent lines are dry, perform the dry prime procedure before continuing.

To avoid precipitating salts in the system, use an intermediate solvent such as water when you change from buffers to high-organic content solvents.

a) In the "Status" screen "Composition" field, enter "100%" for the first solvent you want to use for wet priming. It is recommended to start the wet prime using the solvent with the lowest viscosity to help purge air from the lines.

b) Press the "Direct Function" screen key in the "Status" screen. The "direct Function" menu appears.

c) Select the "Wet Prime" option, the press "Enter". The "Wet Prime" dialog box appears.

d) Gently shake the filters in the reservoir to remove any bubbles that may have formed.

e) Enter the flow rate and the duration of the prime, then press "OK". The solvent management system begins to operate.

At the end of the time period you specify, the solvent management system returns to previous conditions and the Separations Module enters the "Idle" mode.

f) Repeat the steps above for each solvent, as appropriate.

5.3.3 Purging the Sample Management System

Purge the sample management system whenever you:

- Prime the solvent management system.
- Change the solvents.
- See bubbles in the syringe.
- Start using the Separations Module at the beginning of each day.

To avoid precipitating salts, use an intermediate solvent such as water when you change from buffers to high-organic-content solvents.

a) In the "Main" screen, press the "Menu/Status" key to display the "Status" screen.

b) Enter the appropriate solvent composition in the "Composition" fields.

c) Press "Direct Function" to display the "Direct Functions" menu.

d) Select "Purge Injector", then press "Enter". The "Purge Injector" dialog box appears.

e) Enter the number of sample loop volumes in the "Sample Loop Volumes" field (use the default value of 6.0 volumes), then press "Enter". The solvent management system ramps up to a predetermined flow rate to flush the sample with the selected volume.

f) Leave the "Compression Check" box blank the first time you start up the system to prevent the compression test from occurring. The Separations Module does not allow a compression test before the seal pack is adjusted.

g) Press "OK". The purge cycle begins.

5.3.4 Priming the Needle-Wash Pump

The needle-wash pump flushes the needle in the sample management system, preventing carry-over of sample between injections. The needle wash also extends the life of the injector seals by removing buffered mobile phase and sample from the needle.

Selecting a Needle-Wash Solvent:

Select a needle-wash solvent based on the sample and mobile phase chemistries, making sure all solutions/buffers are miscible and soluble. The table below lists some needle-wash solvents recommended for use with certain mobile phase conditions. High sample concentrations may require other needle-wash solvents.

Chromatographic Condition	Needle-Wash Solution
Buffered aqueous, reverse phase	90% water, 10% MeOH
Non-aqueous, reverse phase	100 % MeOH
Normal phase	Mobile phase
GPC	Mobile phase
Ion exchange	H_2O

a) Be sure the needle-wash fluidic lines are properly installed.

b) Press the "Diag" screen in the "Main" screen. The "Diagnostics" screen appears.

c) Press the "Prime NdlWsh" screen key. The 30-second needle-wash procedure begins. If the solvent does not flow out of the waste tube within 30-seconds, press the "Start Again" key.

5.3.5 Adjusting the Seal Pack

Adjust the seal pack whenever:

- You start up the Separations Module for the first time. The Separations Module does not perform injections and compression checks until the seal pack has been adjusted.
- A "Compression Check Failed" error appears on the screen.
- A "Missing Restrictor" alarm appears during diagnostic procedure.
- You change the seal pack.
- You rebuild the seal pack.

Before you adjust the seal pack, purge the sample management system to ensure that there is no air in the syringe. Air in the system may cause "Alarm Seal Geometry" or "Alarm Missing Restrictor" dialog boxes to erroneously appear.

a) Press the "Diag" screen key in the "Main" screen to display the "Diagnostics" screen.

b) Press the "Adjust Seals" screen key. The adjustment procedure begins. When the test is complete, the results are shown in the dialog box that appears.

5.3.6 Loading Carousels

Loading carousels involves:

- Loading vials into the carousel.
- Loading the carousel into the sample compartment.

Loading Vials

Use 12x32 mm, 2 ml vials with screw caps.

Insert the prepared vials into the appropriate positions in the carousel(s). Be sure to place the vials in the numbered slots that correspond to the sample set that is being used.

Loading the Carousel

Carousels and their codes are listed in the table below.

Code	Colour	Vial Numbers
A	Blue	1 – 24
B	Yellow	25 – 48
C	Red	49 – 72
D	Green	73 – 96
E	White	97 – 120

a) Open the carousel door. The "Door is Open" dialog box appears on the screen.

b) Press the "Next" screen key (or select the desired carousel screen key) to position the carousel turntable for loading the appropriate carousel.

c) Load the carousel into the sample compartment.

d) Repeat steps b) and c) above until all the carousels are loaded.

e) Close the carousel door.

5.4 Start a Run Using Millennium Control

An automatic run is started from "QuickSet" in the Millennium software at the Millennium workstation.

a) Create a chromatographic system that includes the Waters 2690 Separations Module.

b) Create an instrument method that uses the system you created in a) above. (Refer to Millennium documentation for information on creating or modifying an instrument method).

Any Parameter values not set in the Millennium instrument method automatically takes on the values in the Default separation method in the Separations Module.

You can edit the Default separation method in the Separation Module to set parameter values for your application.

c) Create a method set using the instrument method you created in b) above. (Refer to the Millennium documentation for information on creating or modifying a method set.)

d) Run the samples in QuickSet (from Millennium) using the method set created in c) above. When entering QuickSet, use the HPLC system created in a) above. (Refer to the Millennium documentation for information on using QuickSet.)

e) Process and print out the data acquired from the chromatographic run. (Refer to the Millennium documentation on developing a processing method.)

5.5 Flushing System

Before the Separations Module is powered off, remove any buffered mobile phase present in the fluidic path.

a) Replace the buffered mobile phase with HPLC-quality water and prime the system for 10 minutes at 3 ml/min. (See section on “Wet Prime”.)

b) Perform 3 injector purge cycles to ensure that the sample loop is clean.

c) Wash the entire system with the HPLC-quality water for 10 minutes.

d) Replace the water mobile phase with methanol or another miscible solvent or mobile phase.

e) Prime the system as described in a) above.

f) Purge the injector as described in b) above.

g) Wash the entire system with the methanol or other mobile phase for 10 minutes.

h) Perform a needle-wash prime with a solvent that is miscible.

Note: Washes and purges can be programmed at the end of a run using Millennium software.

5.6 Powering Off

a) Ensure that the system has been flushed properly (as described in 5.5 above).

b) Press the power switch to the **O** (Off) position.

Note: A “Stop Method” can be programmed into the end of the Millennium run, which will stop the mobile phase flow, but will not power off the system.