

**Application of CE, HPLC and LC-MS-MS for the analysis and  
quality control of *Ginkgo biloba* dosage forms**

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## ABSTRACT

Natural products are complex mixtures of compounds with therapeutic effects which are often reported to be due to the synergistic action of multiple and sometimes unknown components. Consequently, standardization of these products is complex and a lack of effective quality control (QC) criteria in most countries has led to marketing of commercial products with questionable quality, safety and efficacy (QSE). The aim of this study was therefore to develop qualitative and quantitative analytical methods for use in the QC of *Ginkgo biloba* solid oral dosage forms.

Initially, a micellar electrokinetic chromatography (MEKC) method was developed for the identification of the flavonol glycosides, rutin and quercitrin as well as 3 flavonol aglycones, quercetin, kaempferol and isorhamnetin in crude extracts of 4 *Ginkgo biloba* solid oral dosage forms using ultraviolet (UV) detection. A reversed-flow cyclodextrin-modified MEKC method was subsequently developed for the simultaneous determination of the aforementioned flavonols as well as ginkgolide A, B, C, J and bilobalide (all positive markers) in *Ginkgo* commercial products. A non-aqueous capillary electrophoresis (CE) method was also developed for fingerprinting the presence of ginkgolic acids (negative markers) in *Ginkgo biloba* leaf extracts, which are purported to be associated with toxic properties. This method was also applied to 2 *Ginkgo biloba* commercial products.

Since the flavonols have strong UV absorbing chromophores, a reversed phase high-performance liquid chromatographic (RP-HPLC) method was developed and validated using photo-diode-array (PDA) detection which was then successfully applied to fingerprint commercially available *Ginkgo biloba* solid oral dosage forms as well as quantify the relevant flavonol markers present in these extracts. Sample preparation was simple, rapid and cost efficient with minimal clean-up and the employment of a minibore column which requires low mobile phase flow rates contributed to the economy of the method. Unlike the conventional QC approach, samples were not hydrolyzed and direct determination of 2 intact flavonol glycosides, together with the usual aglycone markers was facilitated which provided maximal content information for fingerprint comparisons. On the other hand, terpene trilactones possess poor chromophores and an alternative detection method to UV was required in order to obtain suitable sensitivity. RP-HPLC with evaporative light scattering detection (ELSD) was

selected for quantification of these non-volatile constituents in *Ginkgo* dosage forms and this method was deemed suitable for the routine QC analysis of these positive markers in commercial products.

Since approximately 33 flavonoids have been identified in *Ginkgo biloba* leaf extracts, baseline separation using UV/PDA detection normally requires complex gradient programs and long analysis times. In addition, unequivocal identification of the flavonoids with similar UV spectra and elution times cannot be guaranteed. A liquid chromatographic tandem mass spectrometric (LC-MS-MS) method was therefore developed and validated in order to ensure accurate quantification of the selected flavonol marker compounds in *Ginkgo* commercial products. LC-MS-MS analysis of *Ginkgo* extracts revealed, in addition to rutin, the possible presence of other quercetin analogues, quercetin-3-*O*-rhamnoside-7-*O*-glucoside or quercetin-3-*O*-glucoside-7-*O*-rhamnoside, previously unreported in *Ginkgo biloba* leaf extracts or dosage forms.

In terms of evaluating the most suitable analytical method for QC, CE shows exceptional potential in the future analysis of *Ginkgo biloba* dosage forms while HPLC-PDA and HPLC-ELSD are currently the most affordable and practical instruments for the routine analysis of the flavonols and terpenoids, respectively. LC-MS-MS proved to be pivotal for the accurate identification and quantification of the flavonols due to interference by other flavonoid compounds with similar retention times and UV spectra to the peaks of interest.

All quantitative and qualitative results revealed large discrepancies in the marker content between the products regardless of which batch was analysed and product labels disclosed little relevant information. Although currently not required by most regulatory agencies, some of the usual quality criteria applied to orthodox medicines was evaluated. In particular, dissolution analysis, disintegration, tablet hardness and weight uniformity were assessed and revealed similar inconsistencies. This thesis emphasises that implementation of effective QC criteria is long overdue and is essential to ensure consistent product QSE of commercially available *Ginkgo biloba* solid oral dosage forms.

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## CHAPTER 6

### QUALITY CONTROL

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## LIST OF ABBREVIATIONS AND UNITS

AD	Alzheimer's disease
ADAS-Cog	Alzheimer's Disease Assessment Scale for the measurement of performance-based cognitive dysfunction
ADR's	adverse drug reactions
ADME	absorption, distribution, metabolism and excretion
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
APP	amyloid precursor protein
ATM	African Traditional Medicine
ATP	lowered adenosine triphosphate
AUC	area under the curve
AUFS	absorbance units full scale
BB	bilobalide
BGE	background electrolyte
BP	British Pharmacopoeia
cAMP	cyclic adenosine monophosphate
CAMS	complementary and alternative medicines
CD	cyclodextrin
CE	capillary electrophoresis
CGE	capillary gel electrophoresis
CGIC	Clinical Global Impression of Change
CHMP	Committee for Herbal Medicinal Products
CIBI	Clinician Interview Based Impression
CID	collision induced dissociation
CIEF	capillary isoelectric focusing
CITP	capillary isotachopheresis
CM	complementary medicines
C <sub>max</sub>	maximum plasma concentration
CMC	Complementary Medicines Committee
CMEC	Complementary Medicines Evaluation Committee
CTAB	cetyltrimethylammonium bromide
CE	capillary electrophoresis
COX	cyclooxygenase
CZE	capillary zone electrophoresis
DPPH	1,1-diphenyl-2-picrylhydrazyl
DSHEA	Dietary Supplement Health and Education Act
DSVP	dietary supplement verification program
ECCMHS	Expert Committee on Complementary Medicines in the Health System



ED	electrochemical detection
ELSD	evaporative light scattering detection
EMA	European Agency for the Evaluation of Medicinal products
EOF	electroosmotic flow
ERP	Expedited Registration Procedure
ESCP	European Scientific Cooperative on Phytotherapy
ESI	electrospray ionization
EtOAc	ethyl acetate
EU	European Union
FAB	fast atom bombardment
FASI	field amplified sample injection
FDA	Food and Drug Administration
FIP	International Pharmaceutical Federation
FSDU	foods for special dietary uses
FST	forced swimming test
<i>g</i>	gravity
GA	ginkgolide A
GB	ginkgolide B
GBE	<i>Ginkgo biloba</i> extract
GC	ginkgolide C
GERRI	Geriatric Evaluation by a Relative's Rating Instrument
GIT	gastrointestinal tract
GJ	ginkgolide J
GK	ginkgolide K
GL	ginkgolide L
GM	ginkgolide M
GMP	good manufacturing practice
HCl	hydrochloric acid
HMP	herbal medicinal products
HPLC	high-performance liquid chromatography
5-HT	5-hydroxytryptamine
I	isorhamnetin
IS	internal standard
I Spray	ion spray
IT	ion trap
I/V	intravenous
K	kaempferol
LC	liquid chromatographic/liquid chromatography
LC-APCI-MS	liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry
LC-MS	liquid chromatography-mass spectrometry

LC-MS-MS	liquid chromatography-tandem mass spectrometry
LDL	low density lipoproteins
LOD	limit of detection
LP	Listing Procedure
MALDI	matrix-assisted laser desorption ionization
MAOA	mono-amine-oxidase A
MAOB	mono-amine-oxidase B
MCC	Medicine Control Council
MeCN	acetonitrile
MEKC	micellar electrokinetic chromatography
MeOH	methanol
MRM	multiple reaction monitoring
MRP 2	multidrug resistance-associated protein 2
MS	mass spectrometry
MW	molecular weight
N	normal
NaOH	sodium hydroxide
ng	nanogram
NHP	natural health product
NHPD	Natural Health Products Directorate
NHPR	Natural Health Products Regulations
NMDA	N-methyl-D-aspartate
N-MEKC	normal micellar electrokinetic chromatography
NMR	nuclear magnetic resonance
NP	normal phase
NSAIDS	non-steroidal anti-inflammatory drugs
OTC	over the counter
PAF	platelet activating factor
PD	plasma desorption
PDA	photodiode array
pH*	apparent pH
PMS	premenstrual syndrome
p.s.i.	pounds per square inch
PVDP	low protein binding durapore
Q	quercetin
QqQ	triple quadrupole
QSE	quality, safety and efficacy
Qtrin	quercitrin
R	rutin
RCT	randomized clinical trials

RDA	recommended daily allowance
RE	relative error
RF-MEKC	reduced flow micellar electrokinetic chromatography
RI	refractive index
RP	reversed phase
rpm	revolutions per minute
RSD	relative standard deviation
SA	South Africa
SC	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulphate
SIM	single ion monitoring
S/N	signal-to-noise
SRM	single reaction monitoring
SSI	sonic spray ionization
SSRIs	serotonin reuptake inhibitors
TFA	trifluoroacetic acid
TGA	Therapeutic Goods Administration
THF	tetrahydrofuran
TIC	total ion chromatogram
$T_{\max}$	time at $C_{\max}$
$T_{1/2}$	elimination half life
UK	United Kingdom
US	United States
USP	United States Pharmacopoeia
UV	ultraviolet
vs.	versus
WHO	World Health Organization

$\beta$	beta
$\lambda$	lambda
$\gamma$	gamma
$\Omega$	omega
d	capillary diameter
cm	centimeter
$R^2$	coefficient of determination
$^{\circ}\text{C}$	degrees Celsius
$\mu_{\text{eo}}$	electro-osmotic mobility
$\mu_{\text{eff}}$	electrophoretic mobility

g	gram
I.D.	inner diameter
C	ionic strength
kV	kilovolt
L	liter
$L$	total capillary length
$L_D$	length to detector
$L_T$	total length of capillary
$m/z$	mass-to-charge ratio
M	molar
$\lambda$	molar conductivity
$\mu\text{A}$	microampere
$\mu\text{g}$	microgram
$\mu\text{m}$	micrometer
$\mu\text{l}$	microlitre
mbar	millibar
mg	milligram
ml	millilitre
mm	millimeter
min	minute
MF	molecular formula
mM	millimolar
MM	molecular mass
nl	nanolitre
O.D.	outer diameter
$\text{pK}_a$	negative logarithm of the acid dissociation constant, $K_a$
sec	second
t	migration time of solute
$t_{e_0}$	migration time of neutral marker
V	applied voltage
W	rate of heat generated per unit volume

# CHAPTER 1

## 1. INTRODUCTION

Recent surveys on the universal use of complementary and alternative medicines (CAMS) indicate a burgeoning market in developed Western countries despite the availability of orthodox medicines. The sales of dietary supplements (often considered as CAMS) in the United States (US) ratify the popularity of CAMS with expenditures increasing from \$8.8 billion in 1994 to \$14.7 billion over the ensuing 5 years [1]. In 2001, sales of these so-called dietary supplements were estimated at \$17.8 billion of which herbal remedies accounted for \$4.2 billion [2]. Similarly, the CAMS industry in the United Kingdom (UK) has been reported to be expanding by approximately £1.6 billion annually [3]. The explanation for this unprecedented increase over the last decade is multifaceted and there are a number of unconfirmed speculations. Possible reasons include patient dissatisfaction with the outcomes of conventional treatment regimes, a desire for more holistic healing processes and increased patient participation in self-diagnosis and treatment [4, 5]. In addition, the general populace's initiative in taking proactive steps to ensure general well-being and the aversion of the onset of symptoms relating to chronic diseases are also dominant factors [5]. The dissemination of scientific information regarding the essential components in consumables as well as increased pressure to provide nutritious food to economically crippled nations has also emphasized the importance of the constitutional make-up of ingestible products [6].

As research has indicated, the emerging market of CAMS has not yet reached its full potential and many view this as the timely start of a so-called "herbal renaissance". In fact, CAMS is predicted to become a permanent facet of the health care system [4] and has also impacted other health sectors. Pharmacies have had to include natural products in their retail range of products [7] and it has been reported that the sales of herbal medicines are increasing at a rate that exceeds that of allopathic medicines [5]. Medical practitioners have also had to re-evaluate conventional treatment therapies and recognize the importance of a more holistic patient care approach [8] and more importantly at present, consider the impact of natural product use on the efficacy and safety of orthodox medicines [5]. In addition, some tertiary educational institutes have included certain fields of CAMS certifiable for degrees and diplomas [8]. It is therefore evident that CAMS is a ubiquitous phenomenon that requires definite recognition and attention. It is therefore logical that the increasing demand for CAMS is met with rising concern regarding the quality, safety and efficacy (QSE) of such products. Although the media has given much attention to the use of CAMS, very little scientific evidence regarding the QSE has been disseminated for a number of reasons which will be discussed in this thesis.

### 1.1 THE QUALITY OF CAMS

In order for a medicinal product to produce a consistent therapeutic response, it is essential that the final composition of the product is invariable and that the active ingredient/s is/are present in

appropriate, non-toxic amounts. This implies that if a product is standardized to ensure reproducible quality, the efficacy and safety of that product will be reasonably predictable and consistent [9]. Consistent quality is usually achievable when the active constituent/s has/have been unequivocally identified and isolated and the constitutional matrix is simple and controllable, as in the case of manufactured orthodox medicines [10].

Natural products on the other hand, are complex mixtures of compounds and their therapeutic activities are often reported to rely on the synergistic action of multiple and sometimes unknown components [9, 10]. In addition, various factors influence the levels of purported active constituents present in botanicals. These include geographical location, soil type, cultivation methods, natural biological variation, season and time of harvesting, harvesting, drying and processing techniques as well as storage conditions [2, 11]. In some cases, plant materials have been erroneously identified, processed and sold under the premise of another product, sometimes leading to disastrous ramifications [12]. Dried botanical products pose an additional difficulty in that characteristic macroscopic structures may be altered during dehydration processes and skilled, experienced personnel are required for correct herbal identification or confirmation of authenticity [12, 13]. The quality of the initial plant material used in botanical products has also been reported to be dramatically compromised by traders who have substituted costly herbs with more economical ones for their own financial benefit or in attempt to dilute the botanical material to comply with the increasing demands of manufacturers. The substitution of the herb *Stephania tetrandra* with *Aristolochia fragchi* in a slimming regime which also contained orthodox medicines epitomizes this phenomenon. A significant number of end stage renal failures and urothelial carcinomas were reported in Belgium in the early nineties and investigations concluded that the concurrent administration of the nephrotoxin, aristolochic acid and the orthodox drug acetazolamide was responsible for the potentially lethal effects [12].

Standardization of herbal products is attainable if the active ingredient/s has/have been identified with certainty and contributes exclusively to the therapeutic response. In such cases, the content of the active ingredient in a product may be altered by addition of the pure compound/s or excipients of interest to ensure appropriate concentration levels. If the pharmacological activity is linked to a constituent or groups of constituents which synergistically contribute to the desired effect of which the mechanism is largely unknown i.e. the active marker/s has/have been identified, then standardization can be achieved by blending batches of either raw botanical materials or herbal preparations of higher and lower quality. St. John's Wort and *Ginkgo biloba* can be classified in this category. In cases where herbal preparations have no documented evidence of identified active constituents, chemical markers which possibly do not contribute to any pharmacological effect may be selected as control measures for good manufacturing practice (GMP) purposes e.g. valerenic acid in preparations containing Valerian. In this way, although little may be known about the composition of a particular herbal preparation, a reasonable measure of quality control (QC) can still be attained [14].

## 1.2 THE SAFETY OF CAMS

### 1.2.1 Adverse effects

There is a general misconception that because herbal products are natural, they are also intrinsically safe and effective [11, 15]. Most patients do not regard natural products as a form of medication and are unlikely to document natural product use when questioned by health care professionals regarding their medication history [5, 11]. Natural products are indeed associated with some side effects as illustrated by the World Health Organization (WHO) which documented that in China, 9 854 cases of adverse events were reported in 2002 as opposed to 4 000 cases which marked the entire period from 1990 to 1999 [16]. The most frequently reported adverse events include gastrointestinal symptoms, headaches, allergic skin reactions and menstrual disturbances. Less common but more severe effects are related to central nervous system disorders, liver toxicity, renal failure, heart palpitations and respiratory complications [17]. Moreover, adverse effects associated with specific herbal product use are also documented. For example the anxiolytic, kava kava is a known hepatotoxin and serious cases of hepatitis, hepatic necrosis and even liver failure have been associated with its use. In 2002, in a follow-up study of 39 reported hepatotoxic effects related to the ingestion of various commercial kava preparations in Germany, nine patients developed fulminant hepatic failure and three deaths were recorded. The remaining patients spontaneously recovered after discontinuation of the kava products [18].

Some level of safety is usually considered justified when traditional herbal products have an inveterate history of use however it is difficult to determine cumulative toxic effects or even infrequent side effects in a traditional environment [6, 10]. In addition, the context of safety is an important consideration as herbal preparations prepared and administered in Western arenas might differ considerably from traditional use [10]. In general, an accurate reflection of the incidence of side effects caused by natural preparations is difficult to ascertain due to a lack of mandatory surveillance systems which are intact for orthodox medicines in most countries. In fact, a recent investigation concerning the adequacy of implemented surveillance systems in the US that 99% of all adverse events due to natural product use were probably not detected [19]. Furthermore, natural products may also indirectly affect patients' health and risk safety when pertinent conventional therapy is delayed or replaced with herbal preparations that have not demonstrated reasonable efficacy [20].

### 1.2.2 Adulteration and contamination of natural products

Botanical products have often been plagued by either intentional or unintentional adulteration of heavy metals such as lead, mercury, cadmium and arsenic [12] as well as by the inclusion of regulated proprietary medicines. Commonly undeclared drugs include ephedrine, chlorpheniramine, methyl testosterone and phenacetin but there have also been incidents where prescription medicines such as alprazolam, colchicine, fenfluramine, sildenafil [2] and steroids [20], amongst others have been

included in the manufacture of so-called “natural” products [2]. Chemical analysis of the popular “herbal” remedy, PC-SPES used in the treatment of prostate cancer, confirmed that it had been adulterated with various combinations of warfarin, indomethacin and diethylstilboestrol [2, 21]. In another incident, specific Asian natural products were linked to hyperthyroidism and hepatotoxicity which led to the hospitalization of some patients and three fatalities. Chemical profiling concluded that these products contained thyroid-stimulating agents, *N*-nitroso-fenfluramine and fenfluramine [2]. In fact, up to 25% of traditional Chinese medicines sold in Taiwan have been found to contain unspecified chemicals unrelated to the products’ originally prescribed indications [20].

Products containing abnormal levels of bioactive components which could potentially cause toxic reactions are also considered to be adulterated [11]. A recent publication on the analysis of *Ginkgo biloba* dosage forms suggested that one product was spiked with rutin in order to increase detected concentrations of flavonol glycosides [22] which are usually standardized to 22 - 26% [23]. In addition to adulteration, plant material may often be contaminated with insects, residual insecticides, aflatoxins and fungal and bacterial growth [9, 13]. Incidents such as these unnecessarily jeopardize the health of consumers and cast doubt on the integrity of the natural product industry in its entirety.

### 1.2.3 Interactions with orthodox medicines

Closely related to adverse effects is the rising concern of herb-herb interactions and herb-drug interactions [15]. A study in 1998 revealed that 20% of US adults on prescription medicines admitted co-medicating with herbal preparations with an overall estimation of 15 million people that were at risk for serious adverse complications or therapeutic failure due to herb-drug interactions [24]. A recent journal article has suggested that this phenomenon has not curtailed, especially in the elderly [1]. Countless examples of herb-drug interactions are documented, most of which can be detrimental and even lethal [15] with few references suggesting possible beneficial effects [12]. St John’s Wort is an extremely popular phytomedicine used to alleviate symptoms of mild to moderate depression [19]. It has been shown that this natural antidepressant is involved in the activation of the cytochrome P450 isoenzyme CYP3A4 as well as intestinal P-glycoprotein, thereby catalyzing the oxidative degradation of many therapeutic drugs which fall below their minimum effective plasma concentrations [2]. Allopathic medicines that are affected by this process include antiretrovirals, amitriptyline, warfarin, cyclosporine, theophylline and digoxin, to name a few [19]. On the other hand, grapefruit juice is an inhibitor of cytochrome P450 isoenzymes which would relate to elevated plasma levels of co-ingested orthodox medicines. This is of particular concern when conventional medicines exhibit narrow therapeutic indices [6].

A review on the impact of herbal remedies on surgical patients suggested that natural products could be responsible for physiological changes in perioperative patients and may interact with prescribed medications causing severe complications such as excessive bleeding, strokes, cardiac arrests, organ transplant rejections and alterations in anesthetic effects. The authors outlined possible perioperative



interactions with common herbals such as echinacea, garlic, *Ginkgo biloba* and St. John's Wort and provided a guideline on natural product discontinuation procedures since their investigations proved that 16% of a 2 186 patient cohort still used herbal medications in the month prior to major surgery [5]. Of notable significance however, is the fact that many patients are unaware of potential herb-drug interactions and/or adverse effects, posing questionable doubt on the circulating knowledge available to under-informed consumers [20].

### **1.3. THE EFFICACY OF CAMS**

To date, few herbal products have been subjected to rigorous randomized control studies which are routinely used to prove the efficacy of allopathic medicines. In most countries, natural products are not required to undergo the same testing procedures as conventional medicines and efficacy is usually credited to the fact that they have a well-established use or have been used traditionally over extended periods of time, sometimes even centuries [10]. The synergistic action of principal components present in herbals produce a "therapeutic response" that give the mechanism of action a degree of mystique and complexity. Moreover, CAMS use is generally associated with a promotion of overall health or healing and not direct measurable outcomes, as in the case of conventional treatment regimes. This view suggests that randomized control studies do not accommodate additional immeasurable parameters of unconventional treatment such as "spiritual enhancement, centeredness, harmony, unity, or healing" and only determine scientific outcomes, thereby invalidating the necessity to subject herbal preparations to clinical trials [25]. Although historical use does give natural products a degree of efficacious merit, it can be argued that more definitive data need to be accumulated to confirm appropriate levels of efficacy. For example, mistletoe has a history of anticarcinogenic activity but a randomized controlled clinical trial has shown that it did not significantly decrease morbidity or increase the quality of life of patients suffering from head and neck cancer [10].

Numerous clinical trials using herbal preparations have indeed been performed in recent years but multiple concerns have been highlighted regarding the applied methodology and overall quality of the presented data [10]. The difficulty lies in the complexity of natural products, including their multiple purported active constituents [10, 24]. Issues relating to product quality and safety are also problematic factors that cannot be disregarded [7]. Some herbal products have distinctive organoleptic properties which are difficult to mask when performing randomized or blind studies and most clinical trials have focused on the short term biological impact of herbals whilst cumulative effects have generally been neglected. The effects of concurrent administration of conventional and herbal medicines have also not been adequately addressed and comparative data of herbal preparations administered together with orthodox medicines of established efficacy is seriously lacking. Critical evaluations of the majority of trials have concluded that more evidence is required for unequivocal affirmation of current clinical outcomes [10]. Moreover, factually incorrect media reports result in misconceptions of the outcomes of research and contribute to the overall confusion relating to the efficacy of CAMS products [8]. Of the ten most popular herbal preparations on the US market, only three have consistently been found to be

clinically efficacious; St John's Wort, *Ginkgo biloba* and Saw Palmetto [10, 19]. It is obvious that there are currently limitations with regard to performing recognized clinical trials and protocols need to be re-evaluated and possibly standardized. Many of the limiting variables could be eradicated by addressing problems directly relating to the quality of herbal preparations.

#### **1.4. REGULATORY ASPECTS OF CAMS**

##### 1.4.1 Definitions

Universally congruent definitions for concepts involving non-conventional or natural substances used to improve health or treat diseases are currently not possible due to differing approaches countries have adopted in attempts to regulate these products [3]. Some definitions are used interchangeably – often incorrectly [26]. The following definitions are intended to differentiate terminology and are kept as neutral as possible however, it is important to note that CAMS is currently dynamic in nature and as perspectives are altered, the descriptive terminology may need to be modified.

**Food:** animal and/or botanical source of essential nutrients/substrates necessary for physiological metabolic processes which maintain vitality and enable growth. Food stuffs may include substances with little or no nutritional value such as flavourants and colourants [27].

**Functional foods:** food stuffs which are generally associated with a habitual diet but which also contain nutraceuticals i.e. they contribute to general health and may prevent disease states. In some instances, foods are modified in order to claim this effect [7].

**Foods for special dietary uses (FSDU)/medicinal foods:** functional foods that cater for specific dietetic needs e.g. sugar-free food stuffs for diabetics and products that contain cardio-protective antioxidants [7].

**Dietary/nutritional supplement:** nutrients such as essential vitamins and minerals which are usually administered independently from food stuffs in a liquid or oral dosage form and contribute to a balanced diet. However, the US has included botanical ingredients, amino acids and glandular extracts in this definition [7].

**Nutrients:** constituents inherently present in food that are essential for life and growth such as vitamins, minerals, trace elements and amino acids. Some nutrients have demonstrated reasonable evidence to be classified as nutraceuticals [7].

**Nutraceutical:** any compound present in a food or dosage form that provides health benefits to the consumer beyond contributing to basic physiological nutrition [7].

**Herbs:** crude botanical materials which encompass any plant matter in whole, fragmented or pulverized forms and may include stems, leaves, flowers, seeds, fruit, roots, bark, rhizomes etc. [28].

**Herbal preparations:** manufactured herbal products containing singular or combinations of complementary herbs in various forms of powdered materials, tinctures or extracts [19, 28].

**Phytomedicines/herbal medicines:** any botanical material that contains one/more inherent active constituent and are therefore theoretically capable of inducing a therapeutic response [28].

**Phytopharmaceuticals:** solid oral or liquid formulations originally derived from plant material and which are intended for therapeutic use [9, 26].

**Botanicals/botanical health product:** a preparation of plant origin that is not ingested as a nutritive source for metabolism but contains chemical components which are claimed to maintain or promote health [27].

**Natural Health Product (NHP):** virtually synonymous with herbal preparations but may also contain mineral and animal extracts [20].

**Naturopathy:** the use of non-toxic treatment methods adopted from various traditional healthcare systems for the prevention and cure of diseased states with accentuation on the individuals' health in its entirety [29].

**Conventional and orthodox medicines:** “any substance or mixtures of substances used or purporting to be suitable for use of manufacture or sold for use in:

- a) diagnosis, treatment, mitigation, modification, or prevention of a disease, or 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” [30].

**Complementary medicines:** unconventional drug treatment regimes to be used in conjunction with mainstream health care in order to achieve an appropriate therapeutic response with minimal or reduced side effects [29].

**Alternative medicines:** all health care practices that can be adopted instead of mainstream treatment regimes and are therefore mutually exclusive [29].

**Complementary and alternative medicines (CAMs):** “broad domain of healing resources that encompass all health systems, modalities, and practices and their accompanying theories and beliefs,

other than those intrinsic to the politically dominant health system of a particular society or culture in a given historical period” [29].

**Homeopathy:** a curative based approach involving administration of minute doses of substances that induce or potentiate diseased states at higher concentrations and which then stimulates the body’s own physiological healing mechanisms [7].

**Homotoxicology:** is based on the premise that disease is the body’s defense mechanism to combat the effect of accumulated toxins and uses a collaboration of medical science as well as homeopathy in the design of anti-homotoxic medication [31].

**Phytotherapy:** treatment that involves the medicinal use of plants or extracts from plants [7].

**Traditional phytotherapy:** phytopharmaceuticals whose evidence of QSE is drawn from traditional/historical use rather than scientific evidence and claim to aid in the prevention of diseases [26].

**Rational phytotherapy:** rational allopathic treatment regime using botanical dosage forms that have been subject to the same rigorous evidence-based procedures used to prove the QSE of orthodox medicines and are therefore considered to be of the same scientific stature as conventional treatment regimes. Rational phytotherapy is intended to cure ailments or diseases [26].

**Herbal medicinal products (HMP):** phytopharmaceuticals whose levels of QSE have been justified by rigorous scientifically approved pharmacological and clinical studies which are usually applicable to allopathic medicines [26, 27] i.e. are included in rational phytotherapy.

**Traditional herbal medicinal products:** phytopharmaceuticals which demonstrate appropriate standards of quality and safety, while efficacy is based on longstanding or historical use [31].

**Aromatherapy:** therapy involving the use of aromatic oils extracted from plants primarily by steam distillation or physical compression [31].

**Ayurveda:** ancient Indian treatment regime which focuses on the psycho-metabolic requirements of individuals and encompasses mineral, herbal and animal products as well as exercise and meditation in order to harmonize the inner self and promote a healthy, self-healing environment [31].

**Chinese medicine:** studies the human body’s energy patterns and aims to correct energy flow imbalances characterized by disease. Practitioners may employ various therapies to rectify the imbalance including herbal medicines, acupuncture, meditation, massage etc. [31]

**Flower Remedies/Essences:** uses spring water infused with flower essences to influence patients' emotional state and restore "happiness", thereby assisting the body to achieve wellness [31].

**Gemmotherapy/Embryophytotherapy:** a gentle, non-invasive treatment which effectively drains the body from toxins by using embryonic plant tissue harvested from seedlings [31].

**African Traditional Medicine (ATM):** similar to traditional phytotherapy however mineral and animal products may be included in the formulation and the treatment is of African origin [31].

**Unani Tibb (Unanai Sidda):** is a holistic treatment regime encompassing Greek, Arabic, Egyptian and Indian medicine. It is based on the theory that all matter comprises of 4 basic elements symbolized by water, earth, air and fire in different ratios which gives the object its own unique "Temperament". This "Temperament" is maintained by the bodily fluids which, in turn is influenced by food and liquids that are ingested. Disease is as a result of imbalance of the bodily fluids which are then corrected by natural medicines, diet, regimental and psychological therapies [31].

**Tibetan (Sowa Rigpa):** based on the tenet of so-called "humoural imbalances" and adopts Ayurveda, Bon shamanic, Chinese medicine and Unani Tibb practices in order to treat the physical and psychic "being". It is believed that certain disorders are caused by afflictive emotions and/or evil spirits and relies on karma to address or influence these diseases [32].

In order to avoid semantic problems with some terms used, terms such as herbal, CAMS and dietary supplements are used interchangeably throughout this thesis.

#### 1.4.2 Regulation of CAMS in Australia

In Australia, complementary medicines are regulated under the *Therapeutic Goods Act 1989* and include phytomedicines, homeopathic medicines, aromatherapy oils and nutritional supplements, amongst others. Under this act, the Therapeutic Goods Administration (TGA) is delegated to guarantee appropriate levels of product QSE. A therapeutic product is either classified as a low or high risk substance depending on the assessed safety of the product and is then referred to as either a Listed or Registered product, respectively. The overall safety of a product is evaluated in terms of:

- potential toxicity;
- indications for use i.e. whether the product claims overall health benefits and prevention of diseased states or intends to cure diseases or disorders;
- the possibility of the onset of significant side effects associated with its use and finally;
- the likelihood of adverse reactions after extended periods of administration [33].

Most complementary medicines are classified as Listed goods. Listed goods are required to comply with Australian legislation but the formulations/dosage forms are not evaluated in terms of product

safety, quality and efficacy. The ingredients used in the formulation of the product are however pre-evaluated for safety and quality but proof of efficacy is not required. All ingredients must be consistent with low risk substances as approved by the TGA. Listed products are limited to claims such as health maintenance and promotion, the relief of symptoms relating to ill-health and reduced risk of acquiring diseased states but may not claim to cure diseases. This information may appear on the label of the product but manufacturers are not required to obtain the approval of the TGA to make such structure/function claims. However, if the provided information is found to be deliberately misleading to the public or the product poses serious safety concerns after marketing, the TGA has the jurisdiction to request and evaluate evidence that suppliers have used to substantiate their indications. In addition the TGA, together with the Complementary Medicines Evaluation Committee (CMEC), have drafted guidelines for suppliers on appropriate interpretation of evidence used to substantiate therapeutic claims. The TGA is also concerned with the processing procedures and traditional use of herbal products since, if a product has been historically used for a particular ailment and prepared accordingly, the safety of that product can be presumed appropriate for Listing [33].

Registered complementary medicines, on the other hand are those products which profess therapeutic benefits beyond structure/function claims and may put consumers' health at risk by inappropriate self-medication or long term administration, thereby requiring that additional data be provided to substantiate use. These products are required to undergo pre-market evaluation of QSE and may include both over the counter (OTC) and prescription medicines. Both Listed and Registered products must bear the characteristic AUST L or AUST R numbers associated with their respective classification system [33].

The TGA is additionally responsible for ensuring that the manufacturers of therapeutic goods are licensed and comply with GMP. Imported phytomedicines are subject to the same GMP requirements before they can be marketed. The TGA employs a GMP task team that inspects industrial sites and evaluates adopted manufacturing procedures before issuing licenses. Thereafter, follow-up inspections are performed at regular intervals [33].

Post-marketing surveillance for Listed complementary medicines is mostly characterized by targeted, random audits for Listed products, chemical testing of commercial products or formulary ingredients and advertising and marketing surveillance. Australia also has an integrated system in place for reporting adverse reactions associated with complementary medicine use and has expedient recall procedures in place for warranted removal of products if they are found to compromise the health and safety of the Australian consumers [33].

Since the implementation of these systems, reports of substandard QC and the recalling of products due to complaints of severe adverse effects has indeed occurred and caused major damage to the Australian public's confidence in CAMS. Consequently, the government elected a new statutory committee, the Expert Committee on Complementary Medicines in the Health System (ECCMHS) to address the

shortcomings in the CAMS legislation [8]. The committee has proposed that the quality of complementary medicine ingredients should be enforced legally and the evidence used by manufacturers to make claims for therapeutic use and indications be subject to thorough scrutiny. The government has also agreed to disseminate accurate, interpretable information to the public to ensure that consumers make informed decisions regarding their choice of healthcare. Improvements in the efficiency of the adverse events surveillance system are also underway and the government has committed to support and promote CAMS research in Australia [34].

#### 1.4.3 Canadian Regulatory framework

NHPs comprise an eclectic array of compounds and may include “any product derived from a plant, animal or microorganism, vitamins, minerals, and homoeopathics that are used to diagnose, treat, prevent disease; restore or correct function, maintain or promote health”. However, items that are stipulated in the exclusion list are not permitted to be incorporated into NHP formulations such as biological blood products, marijuana, tobacco and intravenous (I/V) or subcutaneous formulations (SC) [35].

The Natural Health Products Directorate (NHPD) oversees NHP under official Natural Health Products Regulations (NHPR) which states that all products must be licensed and issued with identification numbers. NHP licenses are attainable if a monograph exists for the product in question [36] or if suitable evidence of safety and health benefits has been put forward to the Directorate [37]. The responsibility for safety of NHPs resides with the license holder of the product. Legitimate site licenses as well as individual importing, manufacturing, packaging and product labeling licenses are mandatory. GMP procedures are required for the processing, manufacturing, packaging, labeling and distribution of NHPs and include suitable standards of proficient personnel, premises, equipment, quality assurance, sanitation, stability and the maintenance of audited records, standard operating procedures etc. A site license holder is additionally responsible for adverse events that may occur with NHP use and QC reports which must be available for inspection by the NHPD upon request. The same QC standards are required for imported goods [36].

Licensed NHPs may lay claim to applicable benefits associated with product use as per definition. Package labels or inserts must contain indications for appropriate use, quantity, daily dosage, administration route, the recommended duration of the treatment, suitable storage conditions and also must include applicable warnings, precautions, side effects and possible drug interactions [37]. This promulgated information ensures the opportunity for cognitive, responsible decision-making on the consumers’ part, concluding autonomy of healthcare.

These Natural Health Products Regulations are currently being implemented incrementally over the next 6 years starting from 01/01/04 [37] and its success remains to be determined.

It is however important to conclude that although all NHPs fall under the national NHPD legislation, CAMS professions or practices are regulated individually by Canadian provinces and in some cases, not at all. For example, traditional Chinese medicine is regulated in only one province, naturopathic practitioners in four; whilst homoeopathy is not regulated in any. This essentially results in discrepancies regarding standards of training and competency of practitioners and may compromise public safety [35].

#### 1.4.4 US regulations

In the US, herbal products were included in the definition of dietary supplements under the Dietary Supplement Health and Education Act (DSHEA) in 1994. The DSHEA is an amendment of the Federal Food, Drug and Cosmetic Act governed by the Food and Drug Administration (FDA) [19]. Dietary supplements may comprise essential vitamins, minerals, glandular extracts, amino acids and herbal preparations and currently fall under the blanket cover of food [27]. The regulation of herbal preparations therefore differs drastically from orthodox medicines in that they are not required to undergo pre-market QSE testing. This regulatory framework was generated under the assumption that natural products are safe and that the consumer is entitled to affordable autonomous healthcare [19].

The safety as well as the scientific evidence used to substantiate therapeutic claims of marketed health products is the sole responsibility of the manufacturer and do not require the FDA's approval. Claims may describe the reputed structure/function benefits of the supplement [19] but may not make health claims. This has left a margin for ambiguous interpretation [38] and it has been reported that up to 55% of advertising over the internet make illegal health claims [19]. In addition, manufacturers that receive reports relating to adverse events are not obliged to relay that information to the FDA. As a result, the FDA's jurisdiction is demarcated to restriction measures or, in extreme cases, warranted recall of marketed products which have already been proven to jeopardize the health or safety of consumers. For example, ephedrine alkaloids which exhibit pharmacological effects comparable to that of methamphetamine are often used in natural slimming regimes. They have a potent stimulatory effect which is potentiated by caffeine and has led to lethal adverse effects such as cardiac arrhythmia, stroke, seizure, myocardial infarction and in a number of cases, death. Approximately 13 000 adverse incidences were reported to manufacturers, hundreds requiring medical assistance [2] and at least 12 deaths [39] directly associated with their use whereas the FDA apparently only received 1 200 complaints [2]. Whilst Canadian authorities have proscribed ephedra from their sales, the FDA has stipulated that clear warnings should be visible on all such products and depends on the US Congress to make any further interventions [40].

In the last decade, the US natural products market has been riddled with reports of substandard levels of QSE and there has been a thrust toward more stringent oversight of such products. As in the case of Australia, the public has become increasingly skeptical of dietary supplements in the US with good reason and in order for this industry to retain its position, the US government is compelled to address



these pertinent issues. As a result, appropriate standardized GMP procedures are under negotiation and may involve validation requirements and stability testing. Highly reputable third-party auditing will be available to companies who wish to certify the quality of their products and adopted GMP methods as well as to perform randomized testing for listed product ingredients. In addition, a dietary supplement verification program (DSVP) has been launched by the United States Pharmacopeia (USP) which verifies that actual content concurs with product label information and manufacturing procedures comply with USP standards. Companies who subscribe to such procedures may display DSVP logo seals on their manufactured products as a guarantee of quality. The USP has also committed to frequently updating official monographs on dietary supplements. Online analytical methods for popular dietary supplements are intended to be made available for routine product testing. The FDA commissioner has also issued perspicuous guidelines on the scientific evidence that is required to ratify health-related claims and has threatened legal action against companies that disseminate inept and misleading information [38].

#### 1.4.5 Regulations in the European Union (EU)

Countries within the EU differ substantially in regulating natural products due to historical and cultural heritages. Natural health products fall categorically between the realms of supplements and medicines, with subsequent diverse procurements required for pre-market authorization. Consequently, most natural products are regarded as either botanical health or herbal medicinal products and are regulated by food or pharmaceutical legislations, respectively. As a result, natural health products are limited to the state in which authorization was granted with minimal inter-state circulation especially of traditional herbal products [27].

Herbal medicinal products may obtain pre-market authorization under the European Council Directive 2001/83/EC. Registration requires adherence to official positive botanical monographs as compiled by WHO or European Scientific Cooperative on Phytotherapy (ESCOP) or by subscribing to the prerequisites required for traditional herbal medicinal products. Official monographs are established by the Committee for Herbal Medicinal Products (CHMP) which is involved in processing requisitions for registration and pre-market approval [41] and contain information pertaining to the definition (including the plant part used in preparation), identification (fingerprints), purity, radioactivity, adulterations and content of active constituents or chemical marker compounds of the product [9]. Traditional herbal medicines may undergo less onerous registration procedures if there is proof that the product involved has been traditionally used for a minimum of 30 years globally and 15 years within the EU. The intent is to make provision for those herbal medicines which are used for minor ailments, have minimal safety risks and proof of efficacy is too burdensome and expensive to pursue. The quality and safety of traditional medicines within the EU has to be verified by the country of origin and efficacy is based on historical use [27]. These products are required to contain the following inscription “traditional herbal medicinal product for use in specified indication(s) exclusively based upon longstanding use”. Traditional product exclusions occur if there are discrepancies in the declared and

actual content, the product poses significant risk of safety in its traditional context, the historical evidence of efficacy is unconvincing or the appropriate levels of pharmaceutical quality are not met. Products containing vitamins and minerals in conjunction with traditional herbal medicines may seek licenses under historical use if the pharmaceutical action of the herbal actives supersedes that of the supplementary ingredients [41].

In some EU countries, herbal medicinal products are exclusively found in pharmacies and in others (such as Germany) they may be sold in other retail sectors. In Germany, phytomedicines have shared equal status with orthodox medicines since 1978 regarding the requirement to submit full evidence of QSE prior to marketing. The German Commission E, a specialist committee under the German Federal Institute of Health, has published over 300 monographs on botanical products used in the assessment of herbal medicinal products and provides essential information on pharmacological-toxicological studies as well as the safety and clinical efficacy of popular herbal products [42]. This system is highly efficacious but has evolved over a considerable period of time and is also linked to exorbitant costs. Implementation of this system in other countries would cripple the natural products industry by effectively increasing costs and decreasing the availability of herbal products to consumers [12]. These regulations however serve as a framework for the current EU legislations with additional simplified provisions for traditional herbal medicines [27].

In some EU countries such as the Netherlands and Belgium, food supplements constitute substances that contribute to enhanced physiological effects and therefore inherently include botanical products. In such cases, information directly relating to the usual health claims permitted for herbal medicinal products is prevented by the EU food law and consumers remain unaware of potential health benefits. The EU has however no laws pertaining to the regulation of botanical products as food and some EU states have developed their own legislations, mostly using positive and/or negative lists [27].

#### 1.4.6 Regulations in the UK

In contrast to most other countries, the regulation of CAMS in the UK is focused on CAMS disciplines rather than the actual products used in phytotherapies. CAMS disciplines are divided into 3 categories namely professionally organized alternative therapies (acupuncture, chiropractic, herbal medicine, homoeopathy and osteopathy), complementary therapies (for example aromatherapy, hypnotherapy, Shiatsu, nutritional medicine and yoga) and finally, alternative disciplines which includes established and traditional healthcare systems (e.g. Ayurvedic, naturopathy, Chinese herbal medicine and traditional Chinese medicine) and other alternative disciplines (crystal therapy, dowsing, iridology, kinesiology and radionics). Of these, only two CAMS professions are regulated under an Act of Parliament - chiropractors and osteopaths which require qualified individuals to register with their respective Councils before legal permission is granted to practice under their professional titles [43].

With the increasing popularity of CAMS, authorities have recognized that effective regulation is necessary to ensure public safety and confidence in the CAMS industry. Since the statutory regulation of chiropractors and osteopaths has helped to maintain the integrity of those professions, there is a now general thrust towards the statutory self-regulation or the registering of united, single bodied organizations within all CAMS professions. The *Health Act 1999* facilitated this move by reducing the red tape usually required to become statutory professionals through an Act of Parliament and in addition, funding has been made available as award grants for organizations wishing to follow these recommended routes. Each profession will therefore have their own regulatory framework directly pertaining to that discipline with enlisted members whose practice will be monitored for suitable conduct [43]. In addition, professional bodies are responsible for ensuring adequate training and competency within their field of practice [44]. Statutory bodies will have the authority to enforce disciplinary action in cases of confirmed malpractice [43].

On the other hand, the quality and safety of herbal products themselves is also a contentious issue. Herbal preparations in the UK are mostly imported and enter the market as nutritional foods or are formulated in the country using imported dry herbal extracts. Consequently, a number of potentially serious QC issues could arise, ranging from incorrect plant identification to the accidental substitution of botanicals with similar nomenclature but differing pharmacology or toxicology [12]. In recognition of these problems, suppliers and manufacturers of these products are required to guarantee product quality, including absence of adulteration and contamination and have also been requested to provide information regarding the authenticity of raw materials, complete lists of product ingredients, harvesting dates and adopted processing techniques. Once again, however the onus is on the skilled CAMS practitioner to ensure that the herbal products available or issued to consumers are of the highest possible quality and safety [3]. This falls under the umbrella of appropriate and sufficient education and training in the respective CAMS disciplines [44].

The efficacy of CAMS is considered secondary to ensuring product quality and safety. However, products which make claims to treat specific diseases should have appropriate scientific evidence to prove efficacy “above and beyond the placebo effect” and is mainly directed to professionally organized alternative therapies in the first category. Those practices that fall into the second CAMS category and are generally used in conjunction with orthodox medicines are not required to have substantial evidence of efficacy but health related claims should be limited to currently available literature. The traditional healthcare systems and miscellaneous alternative therapies in the last group of CAMS disciplines are accepted with little or no scientific proof of efficacy. However, the general lack of CAMS evidence in all disciplines has prompted authorities to devote funding and attention to research, including the performance of appropriate Randomized Clinical Trials (RCT), with the intention of compiling a database comparable to that expected of conventional medicines [44, 45].

#### 1.4.7 Regulations in South Africa (SA)

Various approaches to regulate complementary and alternative medicines in South Africa have been attempted over recent years and although appropriate legislation is imminent, it is not without serious contention. South Africa has an ethnically diverse population and appropriate legislative criteria applicable to all CAMS practice has been a challenging task. The currently recognized CAMS in SA include amongst others, African Traditional Medicine, Anthroposophy, Aromatherapy, Ayurveda, Chinese Medicine, Energy Substances/Flower Essences, Nutritional Supplements, Homoeopathy, Tibetan Medicine (Sowa Rigpa), Unani-Tibb (Unani Sidda) and Western Herbal Medicine. In 1997, The Medicine Control Council (MCC) appointed a Complementary Medicines Committee (CMC) primarily to attend to issues relating to the QSE of available complementary medicines (CM) [46]. A separate committee was additionally established to focus exclusively on the regulation of ATM. It has been estimated that up to 70% of the South African population consult traditional healers and development of an optimal QSE framework is therefore imperative to ensure preservation of this cultural heritage [47].

Pending legislation requires the registration of products by compliance with either pre-market criteria defined for orthodox medicines or by a Listing Procedure (LP) also known as the Expedited Registration Procedure (ERP). Criteria for orthodox medicines refer to the universally accepted standardized methodology required to prove QSE and involves data requisition of pre-clinical and clinical trials. The LP procedure is less burdensome and more economically viable. It has been proposed that safety criteria should be drawn from regulating bodies of the relevant CM disciplines around the world. For example, expertise and dossiers regarding the legislation of herbal preparations could be obtained from the renown German Commission E and Australian TGA, that of Ayurveda from India's Department of Health and combined regulations from the Homoeopathic Pharmacopeia Committee of the US and German Commission C for homoeopathic drugs etc. In this way, the most appropriate systems can be selected to ensure optimal QSE whilst maintaining the authenticity or credibility of the CAMS discipline in question with simultaneous maintenance of international standards. Amendments based on scientific evidence or additional requirements will be considered and incorporated where appropriate to accommodate exceptions. Proof of appropriate levels of safety and use in the intended traditional context will be paramount for inclusion in the List. In addition, the following product information will essentially be required for each substance: the scientifically approved name and synonym, its common name, its traditionally understood name, which part of the plant is used, the appropriate safety levels, special warnings, side effects, contra-indications and also the proposed product grading i.e. if it is intended for OTC or prescription sale [46].

Valid indications of requisitioned products will be the responsibility of the applicant and a register of this information will be maintained by the CMC. The evidence used to substantiate claims must be acquired from approved references within the specified categories and label claims must identify the category in which the product is affiliated to. Furthermore, it is envisaged that provision will be made

for label claims that pertain to a specific discipline and will not be restricted to Westernized expressions. GMP related requirements will be specified according to the relevant CM discipline since some products such as herbal preparations will require comprehensive product assays while the potency of other products such as flower essences are not as crucial [46].

Whilst the regulations of herbal preparations have only recently been challenged, vitamins and minerals have been regulated since 1985 and 1981, respectively. Minerals were required to be registered with the MCC if the content in a product was higher than twice the recommended daily allowance (RDA) while exoneration of vitamin registration was granted if the concentration fell below the specified recommended level. Vitamins and minerals are now however classified as nutritional supplements in SA and will therefore be subject to the new regulations governing pre-market safety [46].

In addition, the implementation of the proposed Traditional Health Practitioner's Bill will facilitate the incorporation of ATM into the current health system, including the possibility of rebates by medical aid schemes. This will be actuated by the compulsory registration of the estimated 200 000 practicing traditional healers under the authority of a statutory council which will monitor ethical trade. Affiliated members will be issued with registration numbers which will make for conspicuous identification of approved traditional practitioners. Illegally operating healers are liable to twelve months imprisonment or a hefty fine. In this way, a more integrated health care system between traditional and Western practitioners is envisioned. The set criteria for the certification of traditional healers which are expected to include education/training and standards of practice have however not yet been specified [47].

## **1.5 CONCLUSIONS**

WHO has estimated that approximately 80% of Africa's population use traditional medicines originally derived from plants as a source of primary health care, 30 - 50% of China's population regularly use traditional Chinese medicine and in Western countries, 50% of the population admit to using CAMS, albeit in some cases only once [39]. Another literature source states that 80% of the entire World's population depend on botanical products for their health in either a direct or indirect capacity [26]. Either way, research consistently indicates that NHP use is deeply embedded in many cultures and is rapidly gaining unprecedented popularity in Western countries. This upsurge has resulted in the fiery, lucrative NHP market being riddled with substandard products and practices which have not only jeopardized the safety of consumers but unsolicited incidences have also sabotaged the integrity of the NHP industry in its entirety. Consequently, authorities in most countries have recognized the need for appropriate regulatory intervention and NHPs are now required to yield to long overdue legislations proposed to ensure product QSE.

As previously stated, the regulation of natural products is a contentious issue and currently the focus of a worldwide debate. Public misconception of the inherent safety of natural preparations has paved the way for minimal regulations, sometimes with disastrous ramifications. Until recently, it seemed that

most regulatory frameworks were constructed on a trial and error basis to the ultimate detriment of the consumer. The delicate balance remains between making natural health products available to consumers at an affordable price, appropriating autonomy of health care while simultaneously guaranteeing the highest possible levels of QSE.

Germany's phytopharmaceutical legislation is exemplary and is frequently used by other Western countries as a guideline to construct appropriate regulatory frameworks. It must be considered however that Germany's legislation has been in place for almost three decades and is inextricable from insurmountable costs. Rigorous QC testing which includes RCT demands high cost and time and impedes availability, especially in poorer nations. To make provision for this, the current approach is to award some level of safety to products which have been used historically over extended periods of time provided the product is processed and administered accordingly. In such cases, the priority of quality and safety supercedes that of efficacy.

Countries such as SA and the UK also intend to professionalize certain CAMS practices which gives such modalities a degree of credibility. Statutory regulation also ensures ethical trade, suitable standards of training and includes legal enforcement e.g. practitioners guilty of malpractice may ultimately face moratorium or disbarment. This approach does have its merits, but in the UK all facets of QSE are the sole responsibility of the professional which is extremely burdensome. It would seem more sensible to delegate responsibility to the various contributing sectors of the natural products industry and that the professionalization of CAMS practices adopts an ancillary role in terms of ensuring appropriate product quality.

In general, it can be concluded that more stringent criteria are required to ensure product quality and safety. Responsibilities within the natural products industry need to be explicitly and appropriately delegated and accountability needs to be enforced with legal action when necessary. More emphasis needs to be placed on post-marketing surveillance and efficient adverse events reporting to compensate for more lenient QSE criteria. False labeling and advertising claims have misinformed consumers resulting in inappropriate and often dangerous self-medication - it is therefore fitting that authorities pre-approve health claims and commit to promulgate accurate, scientific information to the public including special warnings, contraindications and possible interactions with orthodox medicines. Finally, continued interest and provision for research can only be considered an investment and benefit to those countries which rely on knowledge to provide more answers on this issue. The differences between CAMS regulations in the aforementioned countries is summarized in Table 1.1.

Table 1.1. Differences between regulatory approaches to CAMS in some Western countries

Criteria	Australia			Canada	Germany	South Africa		UK		US
	R ®	L	AR ®	a) Medicine/ b) Licensing Regulations	As for orthodox medicines	As for orthodox medicines	Proposed new regulations to be implemented	Regulatory scopes of practice	Proposed amended regulations	DSHEA
<b>Quality Control</b>										
GMP	✓	✓	✓	✓	✓	✓	✓	x	✓	voluntary
QC of Product Ingredients	✓	✓	✓	✓	✓	✓	✓	x	✓	voluntary
QC Final Product	✓	x	x	✓	✓	✓	✓	x	✓	voluntary
Standardization of Actives (if monograph exists*)	BP	BP	BP	Canadian NHPD	CHMP ESCOP WHO	BP/USP & Other Recognized Pharmacopeias	BP/USP & Other Recognized Pharmacopeias	x	BP & others?	USP & others?
<b>Efficacy &amp; Safety</b>										
Clinical data	✓	x	x	a) ✓ b) x	✓	✓	x	x	x	x
Historical Evidence	x	✓	✓	a) x b) ✓	?	?	✓	x	x	?
Adverse events reporting	✓	✓	✓ (improved system)	✓	✓	✓	✓	x	x	✓
Post-marketing surveillance	✓	✓	✓	✓	✓	✓	✓	x	x	x
All claims must be supported by acceptable sources	✓	✓	✓	✓	✓	✓	✓	x	x	✓
Legal pre-approval of claims	✓	x	x	a) ✓ b) ?	✓	✓	?	x	x	x
Legal enforcement/penalties	✓	?	✓	✓	✓	✓	✓	x	x	?

\*If a monograph for the product in question does not exist, appropriate proof of safety and efficacy is usually required; R ®, registered products; L, listed products; AR ®, amended regulations for registered products; BP, British Pharmacopeia; NHPD, Natural Health Product Directorate; CHMP, Committee for Herbal Medicinal Products; ESCOP, European Scientific Cooperative on Phytotherapy; WHO, World Health Organization; USP, United State Pharmacopeia ; DSHEA, Dietary Supplement Health and Education Act

## CHAPTER 2

### *GINKGO BILOBA*

#### 2.1 INTRODUCTION AND HISTORY

*Ginkgo biloba*, frequently referred to as a “living fossil”, is the oldest living tree species on earth and dates back more than 200 million years [48]. It is the only surviving member of the family Ginkgoaceae after the ice age and for centuries existed solely in China where it was cultivated for its ornamental beauty, mostly in Buddhist temples and gardens [49]. Western documentation was first recorded by a German, Engelbert Kaempfer during his travels with the Dutch East India Company more than three centuries ago. He named the tree “ginous” or “ginkgo” using the Japanese pronunciation of silver apricot “ginkyo” with reference to the appearance of the fruit. Later, Carolus Linnaeus supplemented the name with the Latin word “*biloba*” which describes the distinctive fan-shaped bilobed leaves of the tree. *Ginkgo biloba* was introduced in the US in 1794 by William Hamilton and today it can be found in most parts of the world, particularly in cities since its tolerance to adverse conditions make it an ideal decorative botanical. In fact, *Ginkgo biloba* may be regarded as a symbol of hope as it was reported that a Ginkgo tree was the first to sprout without genetic abnormalities after the Hiroshima atomic bomb blast during World War II. *Ginkgo biloba* is also known as “hill apricot, maidenhair tree, Oriental plum tree, Salisburia adiantifolia, silver apricot, silver fruit and silver plume” [50]. Figure 2.1 illustrates the appearance of a typical *Ginkgo biloba* tree and its leaves photographed in Dundee, South Africa in 2002.



Figure 2.1. Photograph of a young *Ginkgo biloba* tree during summer and a closer view of the leaves



*Ginkgo biloba* is a perennial dioecious tree and is columnar in shape with erratic, horizontal branching. It can live up to 1000 years old and may reach a height of 30 meters with diameter and circumference measurements of approximately 1 and 15 meters, respectively [48, 50]. After the age of 20, the male trees are capable of producing motile sperm, also characteristic of cycads, contained within inconspicuous flowers. The fruit borne on female trees resemble the shape and size of plums and are yellow in colour. They are infamous for their foul odour when decomposing and are therefore seldom planted in public areas [50]. The edible silver seed of the fruit is likened to the appearance of an almond and may be boiled or roasted and are often included in gourmet dishes in Japan and China or ingested as a digestive aid after meals [50, 51].

The medicinal use of Ginkgo leaves dates back to 2 800 BC where it was documented in the first Chinese Materia Medica that brewed leaf extracts were effective for the treatment of cardiovascular and bronchial diseases, circulatory complications, swelling of extremities, as an anthelmintic [50] and for the treatment of surface skin wounds and freckles [51]. It was also reported that Chinese royalty used leaf extracts to alleviate symptoms of senility. In the sixties, a German company known as Dr. Wilmar Schwabe GmbH & Co formulated a concentrated Ginkgo extract known as *Ginkgo biloba* extract (GBE) or EGb761® [50]. In this extract, the purported active constituents are enriched and standardized and the undesired components such as the ginkgolic acids are eliminated [52]. EGb761® is routinely used in many pharmacological and clinical trials and is endorsed by the German health authorities for the treatment of cerebral insufficiency, dementia, intermittent claudication, vertigo and tinnitus [50].

Today, Ginkgo formulations are produced by numerous manufacturers and consistently remain one of the top selling herbal preparations worldwide [53]. In order to meet the demands of this surging market,

large Ginkgo plantations have been cultivated in the US, France, Japan, South Korea and China exclusively for medicinal use [50]. In South Carolina (US), the Ginkgo trees are consistently pruned to shrub size to facilitate harvesting and are usually picked in the summer months [50, 54] when the concentrations of the purported actives are highest [52]. A typical processing procedure involves dehydrating and pulverizing the leaves followed by extraction under partial vacuum using a combination of acetone-water. The solvent is then evaporated and the extract is processed further in order to remove any undesired components followed by concentration and standardization of the active component [48, 55]. Currently however, no standardized protocol for extraction exists and the extraction process remains the sole prerogative of the manufacturer [53].

## 2.2 CHEMISTRY

### 2.2.1 Chemical composition of *Ginkgo biloba* leaves

Chemical analysis of Ginkgo leaf extracts has revealed a complex array of both polar and non-polar constituents which include terpenes, flavonoids, long-chain hydrocarbons, alicyclic acids and cyclic compounds, amongst others. From the review articles by van Beek [53, 56] and Sticher [57], Ginkgo constituents can be broadly categorized as follows:

1. *The terpenes*
  - Diterpenes: ginkgolides A, B, C, J, K, L, M
  - Sesquiterpene: bilobalide
  - Polyprenols
  - Steroids
  
2. *The flavonoids*
  - Flavonol glycosides
  - Flavone glycosides
  - Acylated flavonol glycosides
  - Biflavones (amentoflavone, bilobetin, ginkgetin, isoginkgetin, sciadopitysin, 5'-methoxybilobetin, sequojaflavone)
  - Flavan-3-ols (catechins)
  - Proanthocyanidins (procyanidin, prodelphinidin)
  
3. *Carboxylic acids*
  - Non-phenolic compounds (ascorbic acid, D-glucaric acid, quinic acid, shikimic acid)
  - Phenolic compounds (such as *p*-coumaric acid, 6-hydroxy-kynurenic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, isovanillic acid, *E*-ferulic acid, *Z*-ferulic acid)

4. *Alkylphenols*  
Ginkgolic acids (also known as 2-hydroxy-6-alkylbenzoic acids, 6-alkylsalicylic acids or anacardic acids), ginkgols
5. *Long-chain hydrocarbons and derivatives*  
Hydrocarbons, alcohols, aldehydes, ketones, acids
6. *Various other compounds* such as cytokinins,  $\beta$ -lectins, carotenoids, anti-vitamin B6 (4-*O*-methylpyridoxine), pinitol, sequoyitol, saccharose, high molecular polysaccharides

Of these groups, the terpene lactones and the flavonoids are purported to synergistically contribute to the beneficial pharmacological effects of Ginkgo leaf extracts and are frequently reported as the active components [53, 57]. Particular attention has been devoted to flavonol glycosides, the diterpenes and bilobalide. On the other hand, the alkylphenols are reported to cause allergic reactions and may possibly cause mutagenic, cytotoxic and even neurotoxic effects although more conclusive evidence is required to substantiate these postulations. Similarly, 4-*O*-methylpyridoxine, known to induce convulsions in high doses, has also been discovered albeit in sub-toxic quantities in Ginkgo leaves and commercial products. Methods for the quantitative analysis of 6-hydroxy-kynurenic acid in extracts has also more recently been developed since relatively high concentrations have been reported in leaf extracts (up to 1 - 2 mg/g) and it is speculated to have antagonistic N-methyl-D-aspartate (NMDA) receptor activity. Also, relatively little attention has been given to the polyprenols. They are apolar compounds which are predominantly extracted during processing of standardized extracts, probably during the same exclusion step designed to remove the ginkgolic acids and are therefore unlikely to significantly influence Ginkgo's therapeutic activity [53].

## 2.2.2 Chemical properties of the purported active components

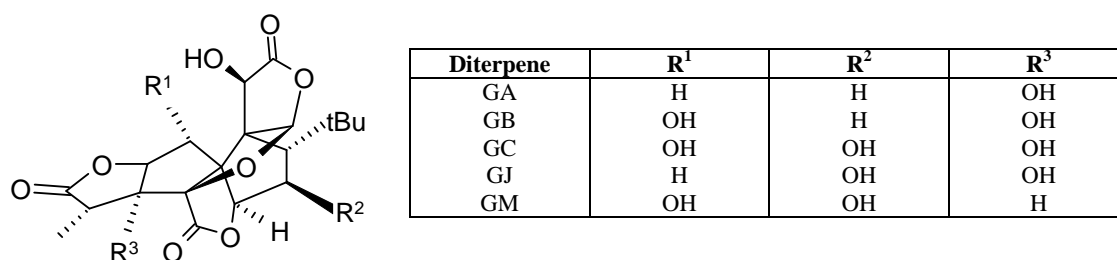
### 2.2.2.1 *Diterpenes and bilobalide (terpene trilactones)*

The terpene trilactones are unique chemical compounds found only in *Ginkgo biloba*. Their exclusivity may be attributed the presence of 3 lactone groups as well as a tertiary butyl group, a rare combination unprecedented in the plant kingdom [57]. The ginkgolides are 20 carbon diterpenes with cage-like structures consisting of six 5-membered rings. They can be differentiated by the positioning and number of hydroxyl functional groups at C-1, C-3 and C-7 of the spiro [4,4] nonane ring [58]. Seven ginkgolides have been identified in leaf extracts, ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC), ginkgolide J (GJ) [50], ginkgolide K (GK), ginkgolide L (GL) [56] and ginkgolide M (GM) [50]. GA, GB and GC are present in leaf extracts in significant amounts with small quantities of GJ being reported and rare mention of GM [59]. GK and GL were only identified and characterized as recently as 2001[56].

In addition to their pharmacological effects, the ginkgolides are thought to be responsible for the bitter taste of the leaves. Bilobalide (BB), a 15 carbon compound, is a related sesquiterpene which lacks the tetrahydrofuran nucleus and spirononane ring that the ginkgolides possess but comprises of 3  $\gamma$ -lactone rings and includes the butyl and lactone functional groups [50]. The leaves of the Ginkgo tree typically contain relatively small amounts of terpene trilactones, varying from 0.1 to 0.9% [51]. However, the German Pharmacopoeia specifies that standardized extracts should contain between 5.0 and 7.0% terpene lactones, with GA, GB and GC contributing to 2.8 - 3.4% and BB to 2.6 - 3.2% [23]. This, of course implies the need to ensure that commercial products contain concentrated extracts containing the recommended quantities of terpene trilactones. The chemical structures of the terpene trilactones are illustrated in Figure 2.2 and their chemical properties are further described in the section below.

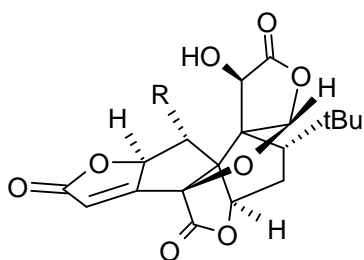
Figure 2.2. Chemical structures of terpene trilactones

A) Diterpenes GA, GB, GC, GJ and GM



The ginkgolides (A, B, C, J and M) have similar chemical formulae and properties. For example, GA [60] has a molecular formula (MF) of C<sub>20</sub>H<sub>24</sub>O<sub>9</sub> and molecular weight (MW) of 408.40 whereas GB [60], GJ (Chromadex™, Daimler St. Santa Ana, CA, USA) and GM's [56] MF are C<sub>20</sub>H<sub>24</sub>O<sub>10</sub> with the same MW of 424.40. GC's MF is C<sub>20</sub>H<sub>24</sub>O<sub>11</sub> with a MW of 440.40. Their corresponding melting points are, ~ 300°C (GA, GB and GC) [60] and 290°C (GJ), ~320-324°C (GM) [56]. GA, GB and GC form bitter crystals when dissolved in ethanol and their ultraviolet (UV) maxima are 219 nm (GA and GB), 220 nm (GC) in ethanol [60]. They are poorly soluble in water at room temperature as well as in apolar solvents, GB being the least polar [53]. They should be stored in a desiccator below 0°C away from direct sunlight (Sigma, St. Louis, MO, USA) whereas the suppliers of GJ (Chromadex™, Daimler St. Santa Ana, CA, USA) recommend storage at -20°C. Three pK<sub>a</sub> values have been reported for GB only, 7.14, 8.60 and 11.89 [56].

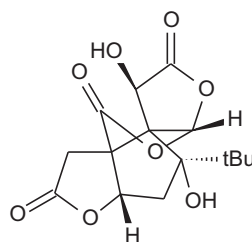
## B) Diterpenes GK and GL



Diterpene	R
GK	OH
GL	H

Ginkgolide K (GK), has a MF of  $C_{19}H_{19}O_9$  and MW of 391.36 compared to Ginkgolide L (GL) with a MF of  $C_{19}H_{19}O_8$  and MW of 375.36. Their melting points are given as  $>300^{\circ}C$  [56].

## C) Structure of the sesquiterpene, BB



BB has a MF of  $C_{15}H_{18}O_8$  and MW of 326.30 and should be stored in a desiccator below  $0^{\circ}C$ , away from direct sunlight. BB is unstable above pH 7 [53, 56] and is incompatible with strong oxidizing agents (Sigma, St. Louis, MO, USA). BB has a pKa value of  $11.80 \pm 0.20$  (<http://www.cas.org/SCIFINDER/SCHOLAR/index.html>, Registry number: 33570-04-6).

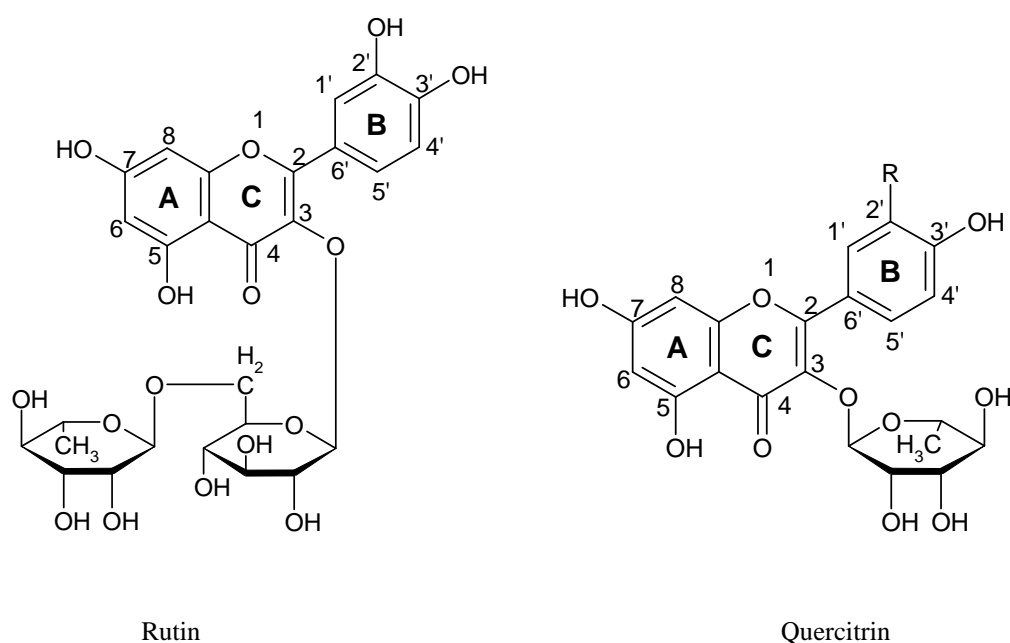
### 2.2.2.2. Flavonoids

Unlike the ginkgolides, the flavonoids comprise a large group of polyphenols that are ubiquitous in the plant kingdom [61] and occur in substantial proportions in *Ginkgo biloba* leaves [53]. Up to 33 flavonoids have been discovered [62], most of which occur as mono, di- or triglycosides of the major flavonol aglycones, quercetin and kaempferol. A small amount is also derived from isorhamnetin, the flavonols myricetin and 3'-methyl myricetin and also apigenin and luteolin, both of which have flavone skeletal structures [57, 61]. The stipulated flavonol glycoside content in standardized extracts is between 22 and 27% [23] however, the flavonol aglycones themselves occur only in small fractions in *Ginkgo* leaves, varying from 0.2 - 0.4% w/w. In addition, *p*-coumaric acid may be esterified with kaempferol or quercetin glycosides. According to Sticher *et al.* [57], these esters can be used as suitable QC markers.

The analysis of intact flavonol glycosides is a good indication of the source of raw material used in extracts [22, 53]. Quantification of the flavonol glycosides in leaf extracts and dosage forms is conventionally conducted by acid hydrolysis of the extracts followed by the high-performance liquid chromatographic (HPLC) determination of the resultant aglycones. The flavones glycoside concentration is usually approximated by back-calculation of the aglycone content using the average molecular weight of flavonol coumaroyl ester glycosides (MM 760) [52]. Although this procedure is relatively simple and reference materials for the aglycones are available, true flavonol glycoside content is exaggerated. This is due to the intrinsic aglycones already present in *Ginkgo biloba* and calculations based on the average glycoside mass [53]. In addition, glycosides are relatively stable compounds which hydrolyze only under rigorous extraction conditions and/or incorrect storage and their presence are therefore excellent QC indicators. An increase in the ratio of aglycones to glycosides in extracts suggests degradation [57].

The biflavones, whose content is not usually required to be standardized, have recently been incorporated into cosmetics with ginkgetin, isoginkgetin, bilobetin and sciadopitysin being the major components present in the leaves and extracts. The proanthocyanidins, on the other hand, are present in *Ginkgo* leaves to the extent of 4 - 12% and are usually standardized to 7% in extracts [53]. The proanthocyanidins and the flavonol glycosides are speculated to be responsible for pharmacological activity of the flavonoids [53, 57]. The flavonol glycosides are however by far the most dominant contributors to the flavonoid fraction and therefore are most frequently used as effective QC markers. The chemical properties of the flavonol glycosides, rutin and quercitrin (Figure 2.3) and the flavonol aglycones (Figure 2.4), quercetin, kaempferol and isorhamnetin which were selected as marker compounds for this research will be expounded upon further.

Figure 2.3. Chemical structures of the selected flavonol glycoside marker compounds



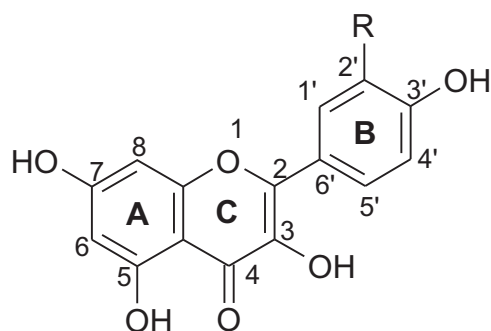
### Rutin

Rutin has a MF of  $C_{27}H_{30}O_{16}$ , a MW of 610.53 and forms pale yellow crystals when hydrated in water, which gradually darkens when exposed to light. The crystals are usually available in hydrous form and lose their water after 12 hours at  $110^{\circ}\text{C}$  and 10 mm Hg. Anhydrous rutin is hygroscopic and should be stored in a desiccator. One gram of rutin is soluble in approximately 8 litres of water, 200 ml boiling water and 7 ml of boiling methanol. It is soluble in aqueous alkaline solutions, formamide and pyridine, slightly soluble in acetone, alcohol and ethyl acetate and almost insoluble in carbon disulphide, chloroform, benzene, ether and petroleum solvents [60]. Rutin has UV maxima of 258 and 359 nm in methanol and a pKa of  $6.83 \pm 0.60$  [<http://www.cas.org/SCIFINDER/SCHOLAR/index.html>, Registry number: 153-18-4].

### Quercitrin

Quercitrin has a MF of  $C_{21}H_{20}O_{11}$  and a MW of 448.37. It forms yellow crystals from both methanol and ethanol and has a melting point of  $176 - 179^{\circ}\text{C}$  in water and  $167^{\circ}\text{C}$  in ethanol. It is soluble in alcohol and alkaline solutions, partially soluble in hot water and practically insoluble in cold water and ether. Quercitrin forms an intense yellow colour when dissolved in aqueous alkaline solutions which turns brown on exposure to air. It has UV maxima of 350 and 258 nm in ethanol [60].

Figure 2.4. Chemical structures of the selected flavonol aglycone marker compounds



Flavonol aglycone	R
Isorhamnetin	OCH <sub>3</sub>
Kaempferol	H
Quercetin	OH

### Quercetin

Quercetin has a MF of  $C_{15}H_{10}O_7$ , a MW of 302.23 and is the aglycone of both rutin and quercitrin. It exists in dihydrate form and becomes anhydrous at  $95 - 97^{\circ}\text{C}$ . It forms yellow needles from alcohol and 1 gram dissolves in 23 ml boiling alcohol, 290 ml absolute alcohol, in glacial acetic acid and in aqueous alkaline solutions. It is almost insoluble in water and alcoholic solutions are extremely bitter tasting. It has an LD<sub>50</sub> of 160 mg/kg in mice. Quercetin has UV absorbance maxima at 258 and 375 nm in alcohol [60] and a pKa of  $6.89 \pm 0.60$  [<http://www.cas.org/SCIFINDER/SCHOLAR/index.html>, Registry number: 117-39-5].

### Kaempferol

Kaempferol has a MF of  $C_{15}H_{10}O_6$ , a MW of 286.23 and is available as yellow needles. It has a melting point of  $276 - 278^{\circ}\text{C}$ , is soluble in hot alcohol, ether and alkaline solutions and is slightly

soluble in water. Kaempferol has UV absorbance maxima at 265 and 365 nm [60] and a pKa value of  $6.93 \pm 0.60$  [<http://www.cas.org/SCIFINDER/SCHOLAR/index.html>, Registry number: 520-18-3].

#### Isorhamnetin

Isorhamnetin has a MF of  $C_{16}H_{12}O_7$ , a MW of 316.26 and a melting point of 303 - 304 °C. It has a UV maximum of 365 nm in methanol and a pKa of  $6.90 \pm 0.60$

[<http://www.cas.org/SCIFINDER/SCHOLAR/index.html>, Registry number: 480-19-3].

### **2.3 PHARMACOLOGICAL ACTIVITY OF PURPORTED ACTIVE CONSTITUENTS OF *GINKGO BILOBA***

As with most herbal preparations, *Ginkgo biloba*'s pharmacological activity is attributed to the synergistic action of multiple chemical components [63, 64, 65] and it is generally accepted that both the flavonol glycosides and terpene trilactones are integrally responsible for its beneficial effects. However, it must be stated that the influences of other chemical entities present in extracts have not yet been determined as obsolete [14, 53] and some mechanisms of action of the extract in its entirety are unknown [57]. Ginkgo's proposed mechanism of action is fourfold and involves restoring i) haemodynamic imbalances, ii) abnormalities in Platelet Activating Factor (PAF) activity, iii) free radical formation resulting in cell damage and iv) the lowered adenosine triphosphate (ATP) levels associated with anoxia [66]. Although research regarding the pharmacological contributions of individual components is still seriously lacking, recently a number of papers have been published in this area indicating substantial progress in determining Ginkgo's precise active chemical constituents and mechanisms.

#### 2.3.1 Terpene trilactones

Until recently, the pharmacological effects of mainly GA and GB were studied [67, 68] however this scope has now been extended to GC and GJ as well as BB. In fact, a recent investigation has shown that GB is actually the most potent antagonist of PAF [69, 70] while GA, GC and GJ have demonstrated declining activities in comparison by exhibiting only 20%, 12% and 7% of GB's potency, respectively [71]. The more apolar the ginkgolide, the more anti-PAF activity it demonstrates [49].

PAF is produced by various cells (eg. basophils, neutrophils, endothelial cells and platelets) in response to certain stimuli such as hypersensitivity or oxidative stress and acts as an intra and inter-cellular messenger [71], triggering the conversion of cellular phospholipids to arachidonic acid with subsequent secretion of prostaglandins and leucotrienes which are potentiators of the blood clotting cascade and inflammation [68]. Inflammation can be characterized by an increase in microvascular permeability and oedema and the influx of intracellular  $Ca^{2+}$  causing smooth muscle contraction [71]. PAF may also be responsible for the excessive release of glutamate [72]. Ultimate physiological effects may include



bronchoconstriction, cardiac complications, anaphylaxis, hypotension and pulmonary hypertension, to name a few. As a result, the ginkgolides' anti-PAF activity is beneficial in the maintenance of an efficient haemostatic system as well as in the treatment of diseases and disorders primarily associated with abnormal levels of chronic inflammation [71, 73]. In addition, anti-PAF activity is associated with increased blood flow and therefore enhanced peripheral circulation [57].

The ginkgolides are also speculated to mitigate the neuronal excitotoxic effects of glutamate which is released during periods of ischemia and which may be responsible for augmenting PAF production [57]. One publication has also shown that the ginkgolides have high affinity for glycine receptors catalyzing research into the radiolabeling of GB for the future purposes of determining the neuromodulatory effects of this antagonistic interaction [58]. Recently, it was shown that the terpene trilactones were responsible for reducing inflammation possibly by inhibiting the macrophagal nitric oxide production at the site of infection after *Candida albicans* was injected into rat footpads which caused arthritic swelling [74].

BB on the other hand does not exhibit any anti-PAF activity but is regarded as a potent neuroprotectant which acts on neurons and astrocytes [67]. BB is responsible for aortic vasorelaxation by stimulating the influx of  $\text{Ca}^{2+}$  through non-selective cation channels in endothelial cells with resultant NO release. In the smooth muscle cell, cGMP is then activated by NO causing a decline in cytosolic  $\text{Ca}^{2+}$  and consequential vasodilation. In addition, the inhibition of both  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels and prostacyclin release may also contribute to the vasoregulatory effects. GA, GB and GC exhibited limited vasodilatory activity compared to BB in the same study and the authors subsequently suggested that BB may effectively be considered as the sole contributor to this pharmacological action due to its demonstrated concentration-dependent potency [66].

BB has exhibited electropharmacological actions by its negative chronotropic effect on the sino-atrial nodes in rats [66] and is capable of preventing hypoxic glycolysis by increasing the production of ATP, the mechanism of which is still unknown [73]. BB has also been tested for possible anti-bacterial effects in rats infected with *Pneumocystis carinii* where it was found that 10 mg/kg administration over 8 days proved as effective as the usual conventional treatment of trimethoxazole/sulphamethoxazole [50].

### 2.3.2 Flavonoids

The flavonoids, particularly the flavonol glycosides, biflavones, proanthocyanidins and phenolic acids are widely recognized for their broad-spectrum free radical scavenging effects [61, 75], their ability to terminate the propagation of free radical production and their affinity for metal ions involved in the production of these reactive species [76]. Superoxide anions, hydroxyl and lipid peroxide radicals are continuously generated as by-products of biological redox reactions and house unpaired electrons which make them highly reactive. These species are usually inactivated and destroyed by specialized

enzymes such as superoxide dismutase, catalase and peroxidase. However, in cases of excessive free radical production or decreased enzyme activities, these reactive species are capable of inducing oxidative stress and are associated with genetic mutations as well as chronic diseases such as cancer, rheumatism and hypertension [75].

The flavonoids help maintain the rheology of blood by preventing the free radical induced formation of lipid peroxides. Lipid peroxides inhibit the production of prostacyclin which is essential for vasodilation and for preventing platelet aggregation. A dynamic equilibrium exists between thromboxane A<sub>2</sub> and prostacyclin and the effects of thromboxane A<sub>2</sub> are therefore accentuated causing vasoconstriction and platelet aggregation. Thromboxane also inhibits adenylyl cyclase resulting in reduced intracellular cyclic adenosine monophosphate (cAMP) with consequential platelet aggregation. These effects result in decreased blood flow and in extreme cases could cause hypoxia or ischemia [57]. The flavonoids may also promote vasorelaxation by preventing the degradation of endothelium-derived relaxing factor by superoxide anion radicals [59]. The vasodilating effects of rutin and quercetin were compared using rat aorta ring strips. Both compounds exhibited positive concentration-dependent vasorelaxation effects, although quercetin was found to be significantly more potent, surpassing the effects of the terpene lactones [77].

The flavonoids are also responsible for reducing oedema by stabilizing cellular membranes, thereby promoting capillary integrity and are speculated to prevent the hemolysis of red blood cells [57]. In addition, the flavonoids may interfere with arachidonic acid metabolism by inhibiting cyclo-oxygenase and lipoxygenase involved in the production of thromboxane A<sub>2</sub> and leucotrienes, respectively. The leucotrienes are linked to the pathogenesis of inflammation [68].

In addition to stimulatory effects of EGb 761® on pancreatic β-cells, it has recently been shown that the beneficial pharmacological effects of the flavonoids described in the foregoing discussion may be applicable to type 2 diabetes mellitus patients. A recent study investigated the effect of Egb 761® ingested over a period of 3 months on blood platelets which were harvested from patients suffering from type 2 diabetes mellitus. It was found that the levels of malondialdehyde, a reactive metabolite from lipid peroxidation produced during activation of thromboxane, was significantly reduced. This action was attributed to the inhibition of cyclooxygenase (COX)-1, the rate-limiting isoenzyme in the synthesis of thromboxane and/or the reduction of available arachidonic acid with subsequent reduction in generation of prostanoids and free radicals formed as by-products of these biological reactions. Once again, the flavonoids were postulated to be responsible for these effects [78].

*Ginkgo biloba*'s free radical scavenging effects may extend to the inhibition of mono-amine-oxidase A and B (MAOA and MAOB). Inhibition of these enzymes prevents the oxidative deamination of dopamine and thereby limits the production of free radicals. MAOB is also involved in the catalysis of protoxins to toxins which cause neurotoxicity [79]. A study performed by Sloley *et al.* [80] showed that the inhibition of MAO in rat liver is predominantly due to the activity of kaempferol.

A study investigating the free radical activity of quercetin, rutin, *p*-coumaric acid, procatechuic acid, isogingetin and (+)-catechin using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method showed that quercetin was the most potent antioxidant. The presence of the *o*-dihydroxy functional groups on ring B of both quercetin and rutin were essential for radical scavenging activity [75, 76, 81] however, the addition of the rhamnose unit at position C<sub>3</sub> on ring C decreased the antioxidant capacity [76], probably due to steric hindrance [82]. The double bond present on ring C and the *m*-hydroxyl groups in positions C<sub>5</sub> and C<sub>7</sub> of ring A are also considered critical determinants of activity. Review Figure 2.3 for the structures of rutin and quercetin.

Pietta and co-workers [76] also investigated the *in vitro* antioxidant effects of flavonoid metabolites and other catechins and found that rutin exhibited more than twice the activity of vitamin E and was also more potent than the catechin metabolites. Although quercetin was included as a metabolite of rutin, its antioxidant activity was not reported.

In another study, the free radical scavenging efficiency of some flavonoids in cultivated human cells was investigated [83]. The results were somewhat contradictory to those reported by Ellnain-Woljtaszek [75] with catechins showing weak scavenging activity. The selected flavones however were collectively the most potent antioxidants although the flavonol aglycone, quercetin, had an antioxidant capacity second to that of the flavone apigenin which exhibited the highest overall activity. Once again, 3-*O*-glycosylation of quercetin with a rhamnose unit resulted in reduced activity. In addition, since human endothelial cells were used, the lipophilicities of the compounds were also considered a determining factor and may play a role in the effective penetrability of these antioxidants [83].

Although rutin's activity is considered subordinate to quercetin, a relatively recent publication which investigated the pharmacological effect of rutin on the antidepressant activity of St John's Wort found that rutin was required above threshold concentrations to decrease the immobility time of rats in the forced swimming test (FST). The authors hypothesized that rutin influenced the bioavailability of other chemical compounds present in the extract and advised that *Hypericum* extracts should routinely be analyzed for sufficient rutin content [84].

### 2.3.3 Other effects

The rate of lipid peroxidation of low density lipoproteins (LDL) has been reported to decrease in the presence of GBE [73]. Shah *et al.* [85] used an extract standardized to contain 9.4 mg of "ginkgoflavonglycosides" to demonstrate that GB is effective in adjusting abnormal levels of catecholamines (dopamine and adrenalin) and serotonin in the rat brain as well as plasma levels of corticosterone after using forced immobilization to induce stress in rats. Ginkgo may also increase levels of 5-hydroxytryptamine (5-HT) in the brain [80]. Although these mechanisms can presumably be extrapolated to the flavonoid fraction, these studies were all performed using *Ginkgo biloba* leaf extract in its entirety.

A study directed at determining the effect of EGb 761® on mice suffering from induced peripheral neuropathy showed that the extract was instrumental in reducing the symptoms in mice and also reduced damage to primary sensory neurons. The mechanism is however still unknown [86]. Studies have also postulated that *Ginkgo biloba* may act directly on the cholinergic system by preventing the uptake of the acetylcholine precursor, choline in pre-synaptic nerves and by upregulating cholinergic muscarinic receptors associated with long term ingestion of GBE. These effects may be partly responsible for improved cognitive function associated with GBE use [79].

Recently, the neuroprotective effects of EGb 761® have been ascribed to mitochondrial protection, thereby delaying ageing [87]. Another study has attributed GB's protection against rat brain dysfunction induced by chronic exposure to aluminum to the reduced production of amyloid precursor protein (APP) and caspase-3 in the hippocampus, ultimately reducing the concentration of  $\beta$ -amyloid [88]. Beta-amyloid plaques and vascular deposits are characteristically found in patients suffering from Alzheimer's disease (AD). In addition, GB helps to lower circulating cholesterol levels associated with the production of  $\beta$ -amyloid [89]. On the other hand, an investigation has shown that EGb 761® causes upregulation of caspase-3 activity thereby suppressing the proliferation of oral cavity cancer cells [90].

From these reports, it is obvious that *Ginkgo biloba*'s constitutional complexity makes elucidation of its precise mechanisms of action a difficult task. Overwhelming evidence does however support anti-PAF and free radical scavenging activity with the terpene trilactones and the flavonoids being the speculated dominant contributors, respectively. Although each chemical class has different and mostly multiple targets of action, their ultimate pharmacological effects complement each other, supporting the claim of synergism. This was also confirmed by the findings of Nishida and Satoh [77] where the vasorelaxing actions of the terpenoids and some critical flavonoids were compared. The authors noted that the sum of vasodilatory activity of the individual components was less than that observed from whole GBE extract and concluded that complex interactions of the chemical components present were responsible for the observed effect.

## **2.4 CLINICAL APPLICATIONS AND USE**

### **2.4.1 Cerebral insufficiency**

A decline in mental function is often the result of ischaemia where reduced blood supply to the brain results in insufficient oxygen delivery. Under these hypoxic conditions, free radicals are generated leading to the production of lipid peroxides with consequential cell damage and cerebral oedema. This process occurs gradually with the onset of symptoms of insufficiency and ultimately, AD [68]. The symptoms of cerebral insufficiency include "difficulty in concentration and loss of memory, absent-mindedness, confusion, lack of energy, tiredness, decreased physical performance, depression, anxiety, dizziness, tinnitus and headaches" which have all been linked to a decline in cerebral blood circulation and speculated to be the start of degenerative or multiple infarct dementia [59]. Another commonly

related condition is caused by the obstruction of the cerebral blood supply, invariably by thrombus formation and which is commonly known as a stroke [68].

GBE is available in Germany on prescription and OTC [50] and is primarily indicated for the treatment of cerebrovascular insufficiency [91], dementia [50] and postthrombotic syndrome [92] due to its vasoregulatory and stabilizing effects on arteries, capillaries and veins, antioxidant and anti-PAF activity, suppression of APP production, its effects on the cholinergic system and its regeneration of sensory neurons as described in section 2.3.

#### 2.4.2 Other central blood flow disorders

GBE is endorsed by the German Commission E for the treatment of vertigo and tinnitus [49, 50]. GBE has recently been postulated to improve cognitive and social skills in adults with Down syndrome [93]. Lay press frequently advertises the use of GBE for enhancing memory/cognitive function [72] and/or mental function and quality of life [94] in healthy adults.

#### 2.4.3 Peripheral blood flow disorders

Improved peripheral blood flow associated with GBE has been used to treat symptoms of peripheral vascular disease such as Raynaud's disease and arterial occlusive diseases [95], intermittent claudication, impotency [50], fluoxetine-induced sexual dysfunction [96], arteriosclerosis, varicose veins, haemorrhoids [81] and diabetes [50].

#### 2.4.4 Effect on Neurotransmitters

*Ginkgo biloba's* direct and indirect effect on neurotransmitters has resulted in GBE being used for the treatment of depression [97, 98], anxiety, stress [80] and mood swings [50].

#### 2.4.5 Anti-PAF effects

Due to Ginkgo's potent inhibition of PAF it has wide clinical application and its effect on inflammatory disorders such as shock, burns, ulcerations, anaphylaxis as well as skin conditions characterized by inflammation [64], are being researched on an ongoing basis [68].

#### 2.4.6 Free radical scavenging effects

In general, free radical scavenging is widely associated with anticarcinogenesis [97] and GBE's protection of mitochondria coupled with its free radical scavenging facilitates its application in creams and anti-ageing products [53]. In addition, GBE's free radical scavenging effects are suggested to

reduce the onset of many pathological states, including diabetes [78], rheumatism and hypertension [75].

In China *Ginkgo biloba* leaf extracts are commonly used for “asthma, cancer, impaired hearing, lung weakness and congestion, venereal disease, sexual energy and longevity” [50] and is prescribed by practitioners for the treatment of angina, atherosclerosis, dysentery, filariasis and hypercholesterolemia [91].

In the US, *Ginkgo biloba* dietary supplements are advocated for the treatment of “allergies, asthma, AD, brain trauma, circulatory disorders, depression, diabetes, eye diseases, headaches, hearing loss, impotency, memory impairment, multiple sclerosis, Raynaud’s disease, vertigo, tinnitus and varicose veins” [50].

## **2.5 CLINICAL EFFICACY**

### **2.5.1 Cerebral insufficiency**

Kleijnen and Knipschild [99] critically examined 40 clinical trials reviewing the efficacy of standardized extracts for the treatment of cerebral insufficiency in elderly patients. In 39 of the trials, positive outcomes were reported while one trial concluded that GBE was not effective in the treatment of symptoms related to dementia of vascular origin. Only 8 of the 40 evaluated trials were identified as having acceptable quality based on predefined criteria for meticulous methodology such as sufficient number of patients, comprehensive descriptions on randomization protocols and patient profiles, outcomes measurements and data acquisition and presentation. Positive results were observed in those 8 trials with no serious side effects. Daily doses of 120 - 160 mg were administered for at least 4 weeks and up to 3 months. The administered preparations included Tebonin®, Tanakan® and Rökan®, which are synonymous with EGb 761® and contain 24% flavonol glycosides and 6% terpene lactones and Kaveri®, standardized to contain 25% flavonol glycosides and also 6% terpenoids. The authors noted that a minimum of 4 - 6 weeks of GBE treatment was required before beneficial effects were observed. The evaluation of suitable rational clinical outcomes in trials also posed difficulty due to the fact that symptoms of cerebral insufficiency (section 2.4.1.) frequently vary between patients and seldom occur simultaneously. The authors concluded that, although there was justified merit to GBE’s beneficial effects, additional well designed studies needed to be performed to substantiate available evidence. These trials were also compared to 5 clinical trials using the orthodox drug, co-derocrine for dementia and its efficacy was rated of the same quality as GBE.

More recently a randomized double-blind, placebo-controlled, parallel group, multicenter study was performed over a 52-week period by Le Bars *et al.* [100] in order to determine the effect of EGb 761® in patients suffering from mild to moderate multi-infarct dementia and AD. Initially, 309 selected subjects were randomized however only 78 and 59 of the patients receiving EGb 761® (120 mg daily)

and placebo completed the study, respectively. Three primary outcome measures were used in the assessment of patients at set intervals during the course of the study, the Alzheimer's Disease Assessment Scale for the measurement of performance-based cognitive dysfunction (ADAS-Cog), the Geriatric Evaluation by a Relative's Rating Instrument (GERRI) which assessed the daily living as well as the social behaviour of the subject and the Clinical Global Impression of Change (CGIC) where the subject's general psychopathology was determined by a clinician. The authors stated that EGb 761® demonstrated a stabilizing function and in some cases even improved cognitive abilities as well as daily and social behaviour in subjects after a period of 6 months - 1 year while the CGIC score showed no improvement. The CGIC score has however been replaced by the Clinician Interview Based Impression (CIBI) rating scale which has been shown to be more sensitive but was not available when the initial study was designed. Adverse events were reported in both treatment groups in equal capacity with the exception of gastrointestinal tract (GIT) symptoms which were more frequent in the EGb 761® group.

In a separate randomized, placebo-controlled study, 18 subjects who were diagnosed with age-related memory dysfunction were given single doses of either 320 mg or 600 mg GBE an hour prior to testing their processing abilities in response to both verbal and visual information. Positive results on both accounts were reported [73]. According to Grant [11], GBE seems to be on the same efficacious level as acetylcholinesterase inhibitors for AD with the added advantage of being less toxic and should be considered as a replacement or ancillary preparation if conventional drug therapy fails.

### 2.5.2 Stroke

Fifty patients participated in a placebo-controlled clinical trial which demonstrated that 150 mg GBE given daily to patients who had suffered from aneurismal subarachnoid haemorrhages was successful in improving reaction times, accuracy and short-term memory compared to baseline and placebo recipient results [73].

### 2.5.3 Vertigo and tinnitus

At least 6 reportable trials for tinnitus have been performed, mostly using outcome measures based on subjective assessments with incongruous results [64, 101]. Daily dosages predominantly ranged from 16 - 120 mg and the largest clinical trial involved 1000 subjects where GBE was administered intravenously but the actual daily dose was not specified. Two open trials reported no significant improvements in tinnitus. One trial which used I/V GBE combined with laser treatment reported a 43% improvement in patients with tinnitus and 20% of the subjects reported that all tinnitus symptoms had disappeared. Two trials showed 60 and 16% improvements of tinnitus symptoms respectively, and 1 trial using the lowest dose of 16 mg daily reported significant improved effects of tinnitus intensity only. In all trials vital information was missing, the most obvious pertaining to patient information, dosage or duration of the trial which automatically indicates that additional comprehensive trials need

to be conducted to prove efficacy. In spite of the foregoing, according to Chavez and Chavez, GBE shows promising efficacy for the treatment of acute tinnitus [101].

Chavez and Chavez [101] have also reported the outcomes of 6 trials using GBE to alleviate symptoms of vertigo. Only 2 trials exhibited significant differences compared to the placebo.

#### 2.5.4 Down Syndrome

A preliminary study was recently conducted on 2 young subjects. One subject was 11 years old and received 80 mg GBE daily for 11 weeks while the other patient (17 years) received 120 mg every morning for 13 weeks. Both subjects were without any additional medical conditions or abnormalities and showed an 8.45% and 4.88% improvement in their Vineland scale score tests, respectively. In addition, marked improvements in social behaviour were observed in both patients and no side effects were reported [93].

#### 2.5.5 Memory in healthy individuals

*Ginkgo biloba* is frequently referred to as a “smart” drug in advertising campaigns aimed at healthy individuals. A few preliminary studies have been conducted using low, recommended and high dosing regimes on female volunteers to determine the acute cognitive effects of *Ginkgo biloba* on subjects showing no signs of dementia [102, 103, 104]. Moulton *et al.* [72] critically evaluated these trials and objected to the high dosage regime (240 - 600 mg GBE) required to achieve significant responses in memory scanning, picture recall and reaction speed to attention-related tasks since abnormally high doses would also implicate increased incidences of adverse effects and/or drug interactions. In addition, studies have demonstrated that memory variations may occur during different phases of the menstrual cycle and female subjects are therefore not a suitable choice for such trials.

As a result, Moulton *et al.* [72] designed a double-blind, placebo-controlled clinical study using 30 healthy male volunteers with a mean age of 20. Subjects were given the recommended daily dose of 120 mg of a standardized GBE for 5 days and then tested using the Sternberg Memory Scanning Test, a prose memory task and a reading span test. No significant enhancements of memory were observed in any of the assessments and the authors postulated that the inclusion of such young subjects may have excluded the possibility of even slight cognitive decline and therefore possible improvement in brain function [99]. Moreover, *Ginkgo biloba* was only administered over a period of 5 days whereas most trials have shown that a minimum of 4 - 6 weeks of administration is normally required before substantial beneficial effects are observed.

Recently, a 4-week, randomized, double-blind, placebo-controlled, parallel-group, monocentric study was performed involving 66 healthy subjects aged between 50 and 65 years to investigate the effect of EGb 761® (120 mg administered twice daily) on cognitive function. Primary and secondary outcome



measurements involved subjective assessments and tests based on neurobiological classification of functions, respectively. EGb 761® was found to positively influence the subjects' perceived mental health as well as quality of life. In addition, within 2 weeks of administration, EGb 761® had a positive impact on self-judged mood state and at the end of the trial an improvement in motor function was demonstrated by increased speed results in the Finger-tapping test in the EGb 761® recipients. Only minor adverse events were reported [94]. Although this trial shows promising effects of EGb 761® in mental functioning, it would be premature to conclude reasonable efficacy of standardized GBE for this indication.

#### 2.5.6 Peripheral Vascular diseases

Kleijnen and Knipschild [59] reviewed 15 clinical trials examining the efficiency of GBE for the treatment of intermittent claudication. All trials reported beneficial effects, however only 2 trials were deemed well performed. One trial demonstrated longer walking distances by 110 meters vs. 31 meters on placebo after 6 months and the other trial reported alleviation of leg pain during periods of rest. The authors considered these results as preliminary and once again recommended that further trials should be conducted for concrete evidence of efficacy for this indication.

One clinical trial has reported the effects of a 100 mg Ginkgo infusion administered twice a day on pain levels associated with Fontaine stage 3 chronic occlusive arterial disease. A visual-analogue scale was used to obtain results and subjects were requested to complete the McGill Pain Questionnaire compiled using qualitative and quantitative assessment criteria. Results showed a reduction in pain during rest compared to subjects receiving placebo and fewer patients requested the use of the analgesic pentazocine [95].

#### 2.5.7 Impotency

Two trials have shown significant improvements after ingesting GBE for impotency. An open trial which treated arterial erectile dysfunction in 60 patients resistant to papaverine injections showed improvements after 6 months of treatment with 60 mg GBE daily and increased blood flow after only 8 weeks. Another trial used GBE to alleviate impotency related to the administration of serotonin reuptake inhibitors (SSRIs). Of the 100 patients included in the study, only 16% did not report beneficial effects. The initial dose was 120 mg daily which was successfully increased in some cases up to 480 mg [101].

#### 2.5.8 Hypertension

A randomized, double-blind trial was performed to investigate the effect of either 40 mg or 80 mg GBE administered 3 times a day to 54 subjects with mild to moderate cognitive dysfunction over a period of 3 months. Arterial blood pressures were monitored at regular intervals when clinical and self-

assessments for cerebral function were performed. Clinically significant results were reported for both indications, with diastolic blood pressure measurements decreasing remarkably in the low dose (40 mg) GBE patient bracket [105].

#### 2.5.9 Anti-depressant activity

One study has been reported using GBE concurrently with antidepressants. The trial investigated the administration of 240 mg GBE for the treatment of patients with cerebral insufficiency also diagnosed with depression. The trial was placebo controlled and included 40 patients with statistically significant positive results reported after 8 weeks using the Hamilton Depression Scale and Short Test of General Intelligence as outcome measurements [73, 101].

#### 2.5.10 Anti-oxidant activity

Ginkgo's antioxidant activity was preliminarily tested in 30 patients due for coronary bypass surgery. The double-blind, placebo-controlled, randomized study showed GBE recipients had improved post ischemic cardiovascular damage using haemodynamic, myoglobin and thiobarbituric acid reactive agents in the evaluation. Administration of 320 mg EGb 761® daily before surgery showed that free radical generation was reduced [101].

#### 2.5.11 Asthma

Four studies have shown the effect of a standardized extract containing GA, GB and GC (in a preparation called BN52063) on asthma, atopic asthma and exercise-induced asthma. When tested, the terpenoids exhibited anti-PAF effects however expiratory flow rate in exercise-induced asthma did not improve [101].

#### 2.5.12 Premenstrual syndrome (PMS)

An open trial which was designed to determine the effect of GBE on cyclic oedema by I/V (200 - 300 mg) or oral (160 - 200 mg) administration in 11 women who reported complete restoration of PMS symptoms caused by sodium and water retention as well as hypoaldosteronism due to increased capillary permeability after 4 - 5 days of treatment [101].

In a separate double-blind, placebo-controlled study, 165 subjects were given 160 mg GBE daily mid-way through one cycle until the 5<sup>th</sup> day of the following cycle. Significant improvements were reported, most notably on breast tenderness with outcomes based on patient self-evaluation and physical assessment by a clinical physician [101].

### 2.5.13 Other

Isolated trials for hypovolemic shock, diabetic retinopathy, Raynaud's disease and chronic active hepatitis B have shown preliminary beneficial effects while other trials using GBE for hyperlipidemia and ulcerative colitis have shown it to be ineffective [101].

In summary, it is surprising that after 3 decades of clinical research on the efficacy of GBE for diseases related to its supposed beneficial pharmacological effects, authors have perpetually concluded that more extensive, empirical, well organized and documented trials need to be conducted to substantiate the available preliminary evidence [19]. A partial explanation may exist in that the establishment of suitable criteria for judging the quality of clinical trials has resulted in many trials performed in the 1970's and 1980's not having met the standards for good clinical practice and acceptable methodology [99]. In addition, the majority of clinical trials have focused on GBE's beneficial effects for cerebral insufficiency which is characterized by elusive symptoms. The employment of extensively different outcomes measurements has also added to the complexity of comparing and evaluating the results of these trials and some assessment methods in well-performed trials have been criticized for their lack of sensitivity [73]. Moreover, large multicenter trials are associated with exorbitant costs which impede the conduction of long overdue clinical studies. Nonetheless, the efficacy of GBE for cerebral insufficiency has not been disproved in approximately 3 decades since the documentation of clinical trials in 1977 [99] and the use of GBE for the treatment of symptoms relating to dementia and other diseases has not diminished [72, 106].

Ginkgo's synergistic pharmacological effects are both complex and extensive and random clinical trials therefore do serve a purpose in helping determine additional mechanisms of action in conjunction with providing new potential clinical indications. In addition, these trials do merit GBE with some level of safety since adverse events are routinely documented and reported and are determined under various conditions relating to the dosage, the pathological states of the subjects and duration of GBE administration.

## **2.6 DOSAGE**

*Ginkgo biloba* leaf extracts are marketed and sold as solid oral dosage forms such as tablets and gelatin coated capsules, concentrated liquids, tinctures, gels and sublingual sprays and also included as ingredients in some revitalizing drinks and vitamin preparations. Most clinical trials have been conducted using standardized GBE formulations or EGb 761® which contain 24% flavonol glycosides and 6% terpene trilactones. In some countries in Europe, a parenteral formulation is available which has been withdrawn from the market in Germany for safety reasons [107] and is not available in the US [95].

The German Commission E recommends the administration of 40 mg standardized GBE 3 times a day i.e. a daily dose of 120 mg daily [95]. However, clinical trials have typically used ranges within 80 - 300 mg daily [73] for most indications except for the enhancement of intellectual performance in healthy volunteers (600 mg) and for tinnitus where substantially lower doses were investigated [101]. Beneficial effects in positive trials were normally observed within 4 to 6 weeks [73, 101]. One publication advised infusing 500 ml of boiling water with 30 g of dried *Ginkgo biloba* leaves and drinking 125 ml of the infusion twice a day [64]. This may however not be advisable due to the presence of undesired chemical components such as the ginkgolic acids which are normally removed during extraction processes [53].

## 2.7 SIDE EFFECTS AND TOXICITY

Adverse events in clinical trials have been reported with doses starting from 80 mg per day with duration of treatment periods ranging from 1 week to a year [73]. The most common side effects were headaches and GIT symptoms [64, 73] such as stomach upset, diarrhoea, flatulence, nausea and vomiting [101]. Other side effects included dizziness, heart palpitations, allergic skin reactions [64], excessive bleeding [101], burning eyes and breathlessness [11]. Higher doses of 240 mg in 1 clinical trial showed increased incidences of dyspepsia, sleep disturbances and dizziness [105].

GBE administration is not advocated for pregnant women due to its effects on the arachidonic pathway nor to those that have previously displayed hypersensitivity to GBE [101]. Moreover, it is possible that GBE could precipitate cerebral blood vessel vasoconstriction if maximum vessel dilation exists prior to GBE ingestion [64]. Allergic reactions have frequently been attributed to the presence of excessive amounts of ginkgolic acids and German health authorities have restricted marketed preparations to contain  $\leq 5$  ppm [53]. *Ginkgo biloba* leaf extracts are however generally considered to be low risk preparations and adverse effects in most clinical trials were reported to occur with more-or-less the same frequency as placebo group recipients [99].

The LD<sub>50</sub> of oral GBE in mice and rats has been determined to be 7.73 mg/kg body weight [101] and chronic GBE administration over a 27 week period in rats with daily doses ranging from 100 to 1 600 mg/kg demonstrated no organ damage or alterations in hepatic or renal function [64]. Isolated incidences of excessive bleeding in humans have however occurred with chronic GBE use. A 33 year old woman with no history of head injuries spontaneously developed bilateral haematomas which required evacuation after ingesting 120 mg *Ginkgo biloba* daily for 2 years. Discontinuation of GBE reinstated the patient's recorded bleeding times to normal and the authors attributed GBE to be the possible cause for the incident [108]. A similar case was subsequently reported concerning a 72 year old woman who complained she was suffering from memory impairment and dizziness after ingesting 150 mg *Ginkgo biloba* daily for approximately 7 months. Scans showed that she had also developed a subdural haematoma [109]. The following year, an incident of subarachnoid haemorrhage was reported in a 61 year old man after taking 40 mg *Ginkgo biloba* three to four times daily [110].

Recently, bilateral haematomas were reported in 2 patients after rhytidoplasty and blepharoplasty surgeries. Extensive and diffuse bleeding was also noted during the surgical procedures and further investigations revealed that both patients self-medicated with 160 mg *Ginkgo biloba* leaf extract daily prior to surgery. The authors postulated that Ginkgo's anti-PAF activity was involved in the haemostatic disturbances [111]. These incidences support findings by Adusumilli *et al.* [5] described in section 1.2.3. These reports however cannot be regarded as conclusive evidence and in fact, Le Bars *et al.* [100] reported a placebo recipient developing a subdural haematoma during their study investigating the efficacy of *Ginkgo biloba* for dementia [100]. The normal incidences of haematomas in elderly patients suffering from dementia were not mentioned in any of the reports. Hyphema and retinal and retrobulbar haemorrhage have also been associated with *Ginkgo biloba* use [112].

With regards to toxicity, it has been postulated that some compounds may have beneficial effects under certain conditions and detrimental effects under others. Quercetin has been classified as both a carcinogen and anti-carcinogenic substance while others studies have demonstrated that its free radical scavenging ability renders it useful for the prevention of cardiovascular diseases. The toxicity of a chemical component may differ significantly when ingested as part of a matrix and studies on isolated entities may present unrealistic predictions for total extracts since the toxic effect of one component may be augmented, supplemented or neutralized by another [113]. As a result, toxic effects of *Ginkgo biloba* leaf extract in its entirety were mentioned in this section only.

## **2.8 DRUG INTERACTIONS**

Since pharmacovigilance and related surveillance systems for herbal side adverse drug reactions (ADR's) are either elementary or do not exist at this time in most countries, most herb-drug interactions are exclusively determined by isolated case reports resulting in a dearth of crucial information regarding the safety of such concurrent treatment therapies. Moreover, the causative nature of the reported adverse event is difficult to determine often resulting in ambiguous conclusions [19]. Until recently, it was perceived that *Ginkgo biloba* leaf extracts posed very few threats in terms of herb-drug interactions [59]. Some earlier publications merely suggested that concomitant administration with anticoagulants should not be recommended or should be closely monitored due to Ginkgo's anti-PAF activity and prevention of platelet aggregation [68].

GBE should not be co-ingested with MAO inhibitor drugs or anticoagulants [73] such as aspirin, warfarin [19] or ticlopidine [114]. Several cases of excessive bleeding have been reported in patients with histories of chronic use of GBE and blood thinning agents. A spontaneous hyphema developed in a 70 year old man who had been taking 80 mg GBE daily for 1 week and 325 mg aspirin daily for 3 years [95]. Recently, a case report of persistent postoperative bleeding in a 77 year old woman who received a hip replacement showed she had been taking both aspirin and 120 mg GBE daily. Discontinuation of the aspirin did not remedy the problem but after GBE self-medication was stopped,

gradual improvement of the surgical wound was observed [114]. Discontinuation of GBE 36 hours before surgery has been recommended by Adusumilli *et al.* [5]. Ginkgo may potentiate warfarin [5] and interact with clopidogrel and dipyridamole [115].

Additional case reports and studies performed on hepatic enzymes involved in drug metabolism as well as the biosynthesis of prostaglandins and steroids have however shed more light on this matter. A recent study found that GBE induced cytochrome P450 enzymes in rats, concluded by increased liver mass, cytochrome P450 liver content and glutathione S-transferase activity after feeding male Wistar rats with 0.5% GBE for 1 week. Discontinuation resulted in these observations being returned to normal baseline levels after 3 weeks. The dose was however 100 times higher per kilogram body weight than the recommended dose and the authors were undecided whether the same effects would occur in humans after ingestion of the recommended dosage [70].

Even more recently, a study has shown that EGb 761® is capable of suppressing the activities of CYP1A2, CYP2C9, CYP2E1 and CYP3A4 human hepatic enzymes with insignificant inhibition of CYP2D6. The terpene trilactones were responsible for substantial inhibition of CYP2C9 however the terpenoids constituted only 6% of the standardized GBE extract used in the study and their contribution to overall inhibition was considered to be minimal. The flavonol glycosides on the other hand, were found to suppress all but one of the selected P450 enzymes (CYP2D6) and since they were present in significant amounts (24%) in EGb 761®, the authors concluded that their potency warranted further studies. Decreased metabolism of diltiazem due to the inhibition of CYP3A by *Ginkgo biloba* leaf extracts in mice has also previously been reported [116]. It is obvious that the incongruous results of the effects of GBE on hepatic enzymes warrants further, intensive investigation since whether GBE has inducing or inhibiting activities on hepatic enzymes, plasma levels of co-administered drugs will either fall below minimum effective concentrations or may be toxic where conventional drugs exhibit narrow therapeutic indices. These effects may seriously compromise *Ginkgo biloba*'s safety as a complementary medicine.

GBE may increase blood pressure when taken concurrently with thiazide diuretics and augments trazodone sedation [19]. GBE should not be taken with excessive amounts of garlic [11] or vitamin E [115] and a recent study has shown that concomitant ingestion of GBE and calcium channel blockers should be avoided [117]. Non-steroidal anti-inflammatory drugs (NSAIDS) should also not be co-ingested with GBE [118].

## **2.9 PHARMACOKINETICS**

### **2.9.1 Terpene trilactones**

While a few studies have reported the bioavailability of GA, GB and BB [26], GC is apparently not bioavailable and no data on GJ and GM could be found [71]. The pharmacokinetics of GA, GB and BB

were determined by Mauri *et al.* [119] using liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS). Fifteen healthy male subjects were chosen for the study and divided into 2 groups for the administration of different formulations of GBE. One product was formulated as a phospholipid complex (Ginkgoselect Phytosome®) and the other was available in its uncomplexed form (Ginkgoselect®). Subjects were given 160 mg which corresponded to 9.6 mg of terpene trilactones/dosage form. The pharmacokinetic parameters of these formulations are given in Table 2.1 as provided by Mauri *et al.* [119].

Table 2.1. Pharmacokinetics of some terpene lactones

Parameters	Ginkgoselect®			Ginkgoselect Phytosome®		
	GA	GB	BB	GA	GB	BB
C <sub>max</sub> (ng/ml)	41.8 ± 14.0	5.6 ± 2.2	37.6 ± 14.2	108.1 ± 7.7	13.4 ± 2.2	60.3 ± 13.0
T <sub>max</sub> (h)	2	2	2	4	3	3
T <sub>1/2</sub> (h)	2.64 ± 0.45	2.35 ± 0.38	2.31 ± 0.24	1.89 ± 0.14	1.69 ± 0.31	3.17 ± 0.36

C<sub>max</sub>, maximum plasma concentration; T<sub>max</sub>, time at C<sub>max</sub>; T<sub>1/2</sub>, elimination half life

Although GC was included in both formulations, it was not detected in the plasma samples and the authors suggested that either minimal absorption or rapid biotransformation could have occurred. The limit of detection (LOD) was 3 ng/ml for spiked plasma samples [119]. The bioavailability of GA and GB in 2 other studies was determined to be between 80 and 100% and that of BB between 70 and 80% [71].

One single dose cross-over study comparatively evaluated the pharmacokinetic properties of the terpenoids in 12 subjects using 2 pharmaceutical brands available on the US market. Statistically significant results were reported in all parameters of the selected formulation in comparison to the reference product which contained EGb 761® extract. Each subject was given a 120 mg dose of either the test or reference product which was previously assayed in order to determine the GA, GB and BB content. Although the reference product was found to contain lower terpenoid amounts, AUC (area under the curve) values were 1.4, 3, and 1.1 times higher for GA, GB and BB compared to the test product, respectively with C<sub>max</sub> values exhibiting even more accentuated differences [120].

### 2.9.2 Flavonols

Although extensively studied due to their ubiquity in the plant kingdom, the absorption, metabolism and excretion of the flavonols remain a contentious issue. As previously mentioned, flavonoid compounds are mainly represented by the flavonol glycosides in *Ginkgo biloba* leaves with the aglycones occurring typically in insignificant quantities [57].

Initially it was thought that the flavonol glycosides could not penetrate the GIT wall and enter into the systematic circulation in its intact or hydrolyzed aglycone form since extensive degradation by intestinal flora would lead to the formation of phenolic acid fission products [121]. Later, it was generally accepted that the flavonol glycosides were hydrolyzed by resident intestinal flora in the colon to render the more hydrophobic aglycones available either for absorption or for further degradation by bacteria [26]. Consequently, further investigative studies mainly focused on the activities of the flavonol aglycones and in particular, quercetin. Initial studies performed midway through the last decade however yielded confounding results. Hollman and Katan [122] stated that the proposed mechanism of absorption of the aglycones through the intestinal wall required more intensive research after a study investigating the relative absorption of the aglycone, quercetin, the flavonol glycoside rutin and other quercetin glycosides from the human ileum showed that 24%, 17% and 52% of the administered amounts were absorbed, respectively. These results implied that the uptake of intact flavonol glycosides into the systematic circulation occurred more extensively and readily than their aglycone counterpart. The authors hypothesized that the absorption of the quercetin glycosides in the small intestine was mediated through sodium-dependant glucose transporter 1 situated in the membranes of enterocytes. This theory was supported by some studies [123, 124] however one study showed that flavonol absorption along this pathway was compromised by the subsequent efflux of some glycosides by the apical transporter multidrug resistance-associated protein 2 (MRP2). It has also been shown that the broad-specific enzyme,  $\beta$ -glucosidase located within enterocytes may hydrolyze flavonol glycosides if entry is indeed permitted [125].

Other investigations have demonstrated that it is possible that hydrolysis of some flavonol glycosides could indeed occur in the small intestine or even in the oral cavity indicating that absorption is not limited to the location of the colon or the glycosidic state of the flavonols. The enzyme, lactase phloridzin hydrolase present in the brush border of the small intestine has also been found to be capable of glycosidic cleavage [125].

In a stability study designed by Goh and Barlow [126], dried pulverized *Ginkgo biloba* leaves, EGb 761® and a *Ginkgo biloba* commercial product (purchased from a General Nutrition Center in the US) were exposed to simulated gastric and intestinal fluid (both prepared according to the USP National Formulary specifications) to determine the stability of the flavonoid components before absorption. Minimal evidence of hydrolysis was observed in the pulverized Ginkgo leaves, rather digestion seemed to facilitate the availability of the glycosides. EGb 761® showed marked conversion of quercetin glycosides to aglycones while the commercial products exhibited even lower glycoside and aglycone recoveries which indicated that extensive flavonol degradation had occurred. Based on the premise that the flavonol glycosides are the active components (as on label claims), the authors suggested that the raw leaves had potentially greater pharmacological benefits than commercial products. However, higher aglycone content was observed in commercial products as opposed to the leaves before digestion experiments were commenced. It was suggested that extraction processes could alter the flavonol content of commercial products. The authors stated that additional studies on the



bioavailability and preferential absorption of the glycosides *vs.* aglycones would be crucial in determining product superiority. In this study the authors also highlighted that the effects of matrices, brush border enzymes and resident colon micro flora were not considered.

A recent investigation studied the difference in absorption of quercetin-3-*O*-glucoside (isoquercitrin) and quercetin-3-*O*-galactoside (hyperoside). Isoquercitrin was detected in its mainly glucuronide form in the plasma however trace amounts of intact isoquercitrin was also seen which was confirmed using tandem mass spectrometry. Hyperoside was not detected in either its intact or quercetin state. Stability tests in buffers with pH 1.2 and 6.8 indicated that no chemical degradation occurred in the GIT tract however simulated GIT contents prepared from rat stomach, duodenum, jejunum, ileum and colon showed that intestinal bacteria were in most probability responsible for the hydrolysis of isoquercitrin. It was hypothesized that hyperoside was more resistant to hydrolysis and therefore could not be absorbed as quercetin or it was completely degraded by intestinal flora to phenolic acids. This study concluded that major differences in absorption and therefore bioavailability profiles may exist between different flavonol glycosides and that the sugar moiety was responsible for this observation [121].

This point is further illustrated by Manach *et al.* [127] where administration of separate doses of rutin and quercetin to rats was used to determine their respective bioavailability by quantification of plasma metabolites, quercetin, a 3'-*O*-methyl quercetin derivative known as isorhamnetin and a 4'-*O*-methyl derivative (tamarixetin) using HPLC with UV (HPLC-UV) detection. Quercetin was more rapidly absorbed than rutin shown by the presence of metabolites after 2 hours compared to the substantially longer lag time of 4 hours for rutin. Quercetin consistently showed greater plasma concentrations.

Erland [128] further investigated the bioavailability of rutin and quercetin in 16 healthy volunteers and these results are presented in Table 2.2. In both cases, quercetin glucuronides (detected as quercetin after conjugate hydrolysis) were used as the marker metabolites.

Table 2.2. Pharmacokinetic profiles of rutin and quercetin in healthy human volunteers [128]

Parameter	Rutin			Quercetin		
	8	20	50	8	20	50
Dose (mg)	8	20	50	8	20	50
C <sub>max</sub> (ng/ml)	24	48	90	41	66	86
T <sub>max</sub> (h)	6.5	7.4	7.5	1.9	2.7	4.9
AUC (ng.h/ml)	0.4	0.6	1.0	0.5	0.9	1.1
T <sub>1/2</sub> (h)	-	-	-	17.1	17.7	15.1

C<sub>max</sub>, maximum plasma concentration; T<sub>max</sub>, time at C<sub>max</sub>; T<sub>1/2</sub>, elimination half life; AUC, area under curve

These studies seem to favour the hydrolysis of quercetin glycosides to aglycones prior to absorption and it has generally been accepted that flavonol glycosides do not have access to the systematic circulation whether they are absorbed in their intact form and then hydrolyzed in intestinal enterocytes or hydrolyzed in the lumen of the gut prior to absorption in the aglycone form [125]. However, the

recent study by Chang *et al.* [121] which used both HPLC-UV and liquid chromatography-tandem mass spectrometry (LC-MS-MS) to confirm their findings, suggest that this conclusion may be premature since trace amounts of intact isoquercitrin were also found in plasma, albeit in subordinate quantities to glucuronidated quercetin. It seems that the exact mechanism of absorption remains elusive at this point and further studies are still required to determine that exact fate of various flavonol glycosides in the human body. What seems to be reiterated however is that fact that the sugar moieties influence the rate and extent of absorption and therefore influence relative bioavailability. This has also been confirmed by Hollman and Katan [122] and Bhattaram *et al.* [26].

Based on the postulation that most quercetin glycosides are hydrolyzed to their aglycone form by the time they reach systematic circulation, quercetin is then subject to glucuronidation, *O*-methylation and sulfation which occurs either in the liver or during the intestinal absorption process mediated by strategic enzymes [129]. The majority of the quercetin glucuronides (99.4%) are then hypothesized to be bound by plasma proteins. Due to the fact that most pharmacological studies have been performed using quercetin in its free aglycone form, appropriate questions are now directed at the activities of the quercetin metabolites. It has been hypothesized that conjugated quercetin may be a “prodrug” which is converted to its active form when required. This theory and related ones require further evidence to substantiate their claims [26].

It has been estimated that 4.5% of a flavonol dose administered as quercetin glycosides is excreted in the urine [26] as substituted benzoic acids [130] and renal clearance is estimated to be 0.7 L/min regardless of the original attached sugar moiety. It is further suspected that the remainder of the quercetin conjugates either undergo further biotransformation to unknown metabolites or are excreted through bile action. It is thought that carbon dioxide may be the dominant final metabolite of quercetin since a study showed that 23.0 - 81.1% of radiolabeled quercetin was exhaled through the lungs of healthy volunteers [26].

Kaempferol is a C-3' dehydroxylated derivative of quercetin and is also a component found in *Ginkgo biloba* leaf extracts in its aglycone and glycosidic form [52]. One study showed that kaempferol and quercetin are both glucuronidated by human UDP-glucuronosyltransferase UGT-1A9 and it was suggested that this enzyme plays a key role in the absorption and metabolism of these flavonols. It was determined that  $\beta$ -glucuronidase metabolized both quercetin and kaempferol extensively into 4 and 2 monoglucuronides, respectively. The presence of an identified quercetin glucuronide was found in the urine sample of a volunteer 4 hours after administration of 2 tablets of a *Ginkgo biloba* commercial product purported to contain 28.8 mg flavonol glycosides. Based on these results, the authors speculated that glucuronidation was the dominant metabolic pathway for quercetin, kaempferol and possibly other flavonoid compounds [131].

In summary, to-date, the pharmacokinetic profiles of both the terpene trilactones and the flavonols are lacking pertinent information. With regards to the terpene trilactones, the exact fate of GC is of

significance since it is well represented in most *Ginkgo biloba* leaf extracts [53]. GJ is typically present in lower concentrations and its pharmacological and pharmacokinetic parameters have not yet been extensively explored. Similarly, GM is rarely addressed in any capacity and further studies on these diterpenes are necessary to determine the absolute bioavailability and therapeutic windows of this class of posited active compounds.

The debate concerning the absorption mechanism of the flavonols remains a contentious issue. However, it seems to be unanimous that the sugar moiety attached to the flavonol aglycone influences the rate and extent of absorption. Few studies have been performed on the bioavailability of the various flavonoid glycosides that have been identified in *Ginkgo biloba* leaf extracts. In studies performed on the bioavailability of quercetin absorption following doses containing the aglycone alone, very little free aglycone content in plasma could be found since extensive conjugation of the aglycone occurs *in vivo* [26].

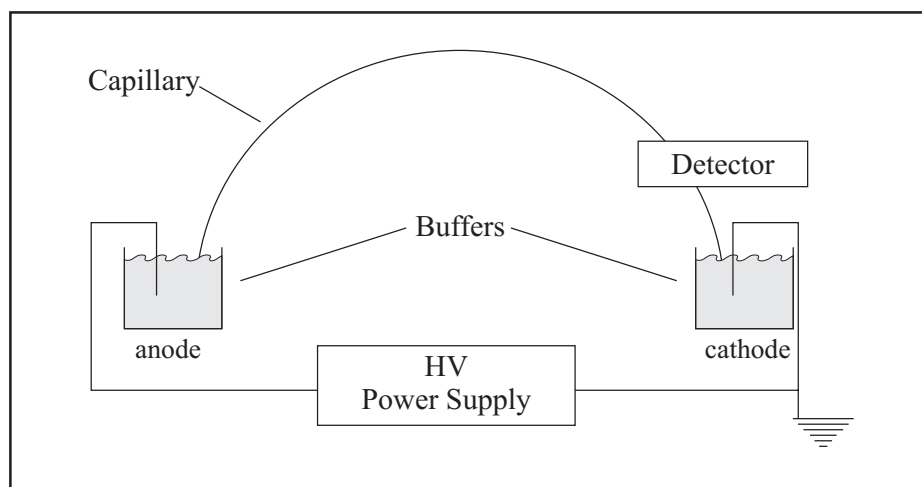
## CHAPTER 3

### CAPILLARY ELECTROPHORESIS

#### 3.1 PRINCIPLES OF CAPILLARY ELECTROPHORESIS (CE)

CE involves the separation of chemical components based on the differential migration of charged species under the influence of an applied electrical field. Typically small sample volumes are injected onto the anodic end of a narrow-bore capillary filled with buffer and are swept towards the cathode by the electroosmotic flow (EOF). The EOF is caused by the application of an electric field on the counter-ions (predominantly cations) accumulated on the negatively charged silanol groups of the capillary wall which form an electrical double layer [132]. These cations are solvated and carry the bulk of the buffer solution with them as they migrate toward the cathode [133]. Under these conditions, compounds are separated by their characteristic electrophoretic mobilities which are dependent on the solute's charge and size [132]. Small, positively charged species with high charge-to-mass ratios will reach the detector at the cathodic end first, followed by cations with lower ratios and then unresolved neutral compounds which migrate under the forced influence of the EOF. Next in the sequence are anions with small charge-to-mass ratios and finally, anionic species with large charge-to-mass ratios are dragged to the cathode by net bulk flow of the EOF and reach the detector last [132]. A schematic of a simplified CE system is presented in Figure 3.1.

Figure 3.1. Diagrammatic representation of a simple CE system [133]



CE is a relatively new technique which has gained popularity over the last 2 decades due to its high efficiency, short analysis times, wide application and low cost. It is particularly suited for content analysis of botanical products which may contain multiple chemical constituents and which usually require lengthy analysis times and complex separation methods [134]. Moreover, the problems associated with the adsorption of highly hydrophobic compounds or unknown natural product

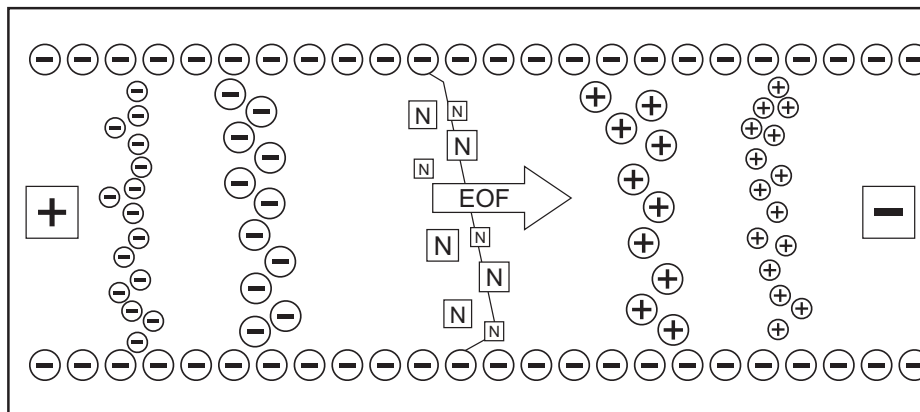
constituents to reversed phase high-performance liquid chromatographic (RP-HPLC) columns are eliminated by simple high pressure washing of the capillary with water between consecutive injections [135]. Draw backs of this micro-analysis method is the lack of sensitivity due to small volumes of sample injected onto the column (5-10 nl [136]) and the narrow optical path length through typical UV detector cells, requiring samples to be 2 to 5 times more concentrated to generate the requisite response achieved by UV detection using HPLC and which may pose problems when analyzing poorly soluble drugs [137, 138]. Recently, however various instrumental and operational adaptations have been attempted to overcome these limitations, including the use of bubble cells and Z-cells to increase the detector path length as well as various pre-concentration and sample stacking techniques [139].

CE's success is also partly due to various possible separation modes which have vastly different operative and separation characteristics and facilitates the analysis of distinctly different classes of chemical compounds using the same instrument. These include capillary zone electrophoresis, micellar electrokinetic chromatography, capillary gel electrophoresis, capillary isotachopheresis and capillary isoelectric focusing [133].

### 3.1.1 Capillary zone electrophoresis (CZE)

CZE may be described as an instrumental approach to CE. It is the most frequently used mode of CE due to its simplicity and versatility and has a wide application range which includes the analysis of peptides, amino acids and enantiomers. The electrolyte of this system consists of a simple buffer solution with a suitable known pH value and separation takes place in an uncoated polyimide fused-silica column with an internal diameter usually in the range of 25 - 75  $\mu\text{m}$ . Sample is introduced at the anodic end of the capillary and upon the application of an applied potential, charged analyte species separate into spatially discrete zones based on their electrophoretic mobilities. Neutral compounds are not capable of migrating in this environment and co-elute with the EOF [133]. Alteration of the buffer pH is the easiest way to manipulate the charge-to-mass ratios of ionizable compounds hence influencing their electrophoretic mobilities and subsequent separation [140]. This process is represented in Figure 3.2.

Figure 3.2. Diagrammatic representation of the CZE separation process



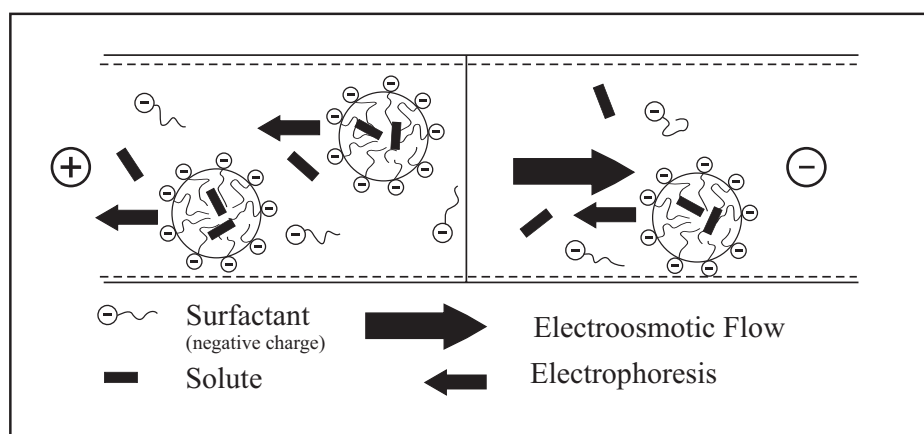
### 3.1.2 Micellar electrokinetic chromatography (MEKC)

MEKC is a hybrid of electrophoresis and chromatography and has the added advantage over CZE in that it is capable of separating both neutral and charged solutes within a single run. Here either an anionic or cationic surfactant is incorporated into the buffer solution in a concentration above the critical micelle concentration to form micelles. Micelles are spherical aggregates of surfactant molecules orientated with their polar heads towards the hydrophilic buffer phase and their hydrophobic tails closely associated within the micellar core [141]. The micellar phase in MEKC is often referred to as a pseudo-stationary phase and is likened to the stationary phase in conventional chromatography while the properties of the aqueous phase frequently corresponds to that of the mobile phase [139].

In an electrochromatographic system, the charged micelles migrate with or opposite to the EOF depending on their charge. Cationic micelles migrate with the EOF while anionic micelles gravitate towards the anode. Under neutral or alkaline buffer conditions, the magnitude of the EOF is however greater than the migration velocity of the anionic micelles and there is a net movement in the direction of the cathode [133]. Concurrently, complex interactions including hydrophobic, electrostatic and hydrogen bonding occur between the micelles and the analyte which facilitate separation [142].

In a system using a common anionic surfactant such as sodium dodecyl sulphate (SDS), charged analytes tend to migrate according to their electrophoretic mobilities in the aqueous phase while neutral analytes partition between the anionic micellar phase and aqueous phase, depending on their distribution coefficient. The more hydrophilic the analyte, the more it will partition into the aqueous phase and will be swept with the EOF towards the detection window while the hydrophobic compounds are more strongly associated with the negatively charged micelles and migrate at a slower rate. The MEKC separation process is depicted in Figure 3.3 [132].

Figure 3.3. Diagrammatic representation of the MEKC separation process [132]



Neutral analytes are compelled to migrate at a speed between the EOF and micellar velocity and this detection time frame is also known as the migration time window. It is possible to expand the migration time window by the addition of organic modifiers into the aqueous phase in concentrations of up to 20%. These organic solvents facilitate enhanced resolution or altered selectivity by reducing the EOF and by preventing the partitioning of highly hydrophobic molecules exclusively into the micellar core. The same effect on the migration window is observed by an increase in the micellar concentration. However higher conductivities may result in the production of joule heat which causes a parabolic temperature and (therefore) viscosity gradient in the running buffer from the wall to the core of the capillary. The ions in the centre of the capillary then migrate at a faster rate than those near the wall resulting in band broadening and micellar concentration ranges are thus typically constrained to less than 200 mM [132].

Although the focus of MEKC is on the separation of neutral analytes, enhanced selectivity of ionic species may also be achieved by the addition of a surfactant to the CZE buffer system. The negative charge on the surface of an SDS micelle results in electrostatic repulsion or attraction between the negatively or positively ionized analytes, respectively. Since the charge and hydrophobicity influences the distribution co-efficient of ionized species, these additional interactions facilitate enhanced separation. Consequently, cationic surfactants will exhibit markedly different selectivity in the separation of ionic analytes in comparison to anionic surfactants [132, 142]. In addition, the use of cationic surfactants causes a reversal in the direction of the EOF due to their electrostatic attraction to the negatively charged silanol groups, forming a bilayer of surfactant along the capillary wall. This bilayer is arranged by hydrophobic interactions between free and capillary bound cationic surfactant molecules with the cationic heads of the second layer facing the buffer solution [133].

In MEKC a variety of surfactants may be used and include anionic, cationic, non-ionic and bile salts. Bile salts such as sodium cholate, sodium deoxycholate and taurine conjugates of bile salts are suitable for the separation of highly hydrophobic compounds as well as enantiomeric separations. The selectivity in MEKC

can be manipulated further by the employment of surfactant mixtures and modification of the aqueous phase by the inclusion of cyclodextrins, ion-pairing reagents, urea and metal salts [132]. Constitutionally different micellar systems may have varying effects on selectivity due to alterations in the size, aggregation number and geometry of the micelles [142].

### 3.1.3 Capillary gel electrophoresis (CGE)

CGE involves the separation of macromolecules such as proteins and nucleic acids by electrophoresis of the compounds through a “molecular sieve” consisting of a suitable polymer network. Size-based separation occurs when the ionized compounds are hindered as they migrate through the gel-filled capillary under the influence of an applied potential with larger solutes experiencing more hindrance than smaller ones. This technique is suited for analytes which possess mass-to-charge ratios which change with an increase in size, as in the case of DNA and SDS-saturated proteins and which have similar mobilities in normal buffer systems [133].

Gel polymer networks are predominantly selected based on pore size. Covalently cross-linked polyacrylamide may be classified as a chemical gel network that has a small pore size and is therefore used for protein separations while agarose has larger mesh spacing and is employed for the separation of DNA. Cross-linked polyacrylamides are rigid in nature and therefore preclude the use of hydrodynamic sample injection. Linear polymers are characterized by greater flexibility and may be loaded onto the capillary hydrodynamically [133] however coated capillaries are necessary to eliminate the effects of the EOF [142]. Polyacrylamide gels are available in a range of concentrations and the amount required to produce an effective network is selected based on an inverse relationship with the size of the analyte [133].

CGE offers distinctive advantages over slab and tube gel electrophoresis in that the capillary itself offers anti-convective properties and higher voltages can be applied to facilitate separation. In addition, the automated CE system allows for on-line detection [133]. The selectivity of CGE may also be altered by the addition of chiral selectors, ion-pair reagents or complexing agents to the polymer network [142].

### 3.1.4 Capillary isotachopheresis (CITP)

CITP is often described as a “moving boundary” electrophoretic technique where 2 buffers are employed to create leading and terminating electrolytes with higher and lower mobilities than the analyte, respectively. The sample zone remains sandwiched between the electrolyte buffer systems and when the electric field is applied, the analytes separate into discrete zones and each band migrates at the same velocity as determined by the leading electrolyte. Although the electric field in each zone varies with the lowest field exhibiting



the greatest mobility, each field is self-adjusting and a steady-state velocity is maintained. If ions from one zone penetrate into an adjacent zone, the velocity difference causes it to immediately diffuse back into its own zone and in this way sharp boundaries are maintained. Additional zone sharpening or broadening may be achieved with the inclusion of lower or higher electrolyte concentrations than the leading or terminating zones in the sample. The leading zone dictates the constant concentrations observed in successive zones and since a constant ratio of concentration to mobility is maintained in each zone, a zone with a lower (or higher) concentration than the leading band results in zone sharpening (or broadening) in order to ensure an acceptable equilibrium [133, 142]. It is this principle that is used in pre-concentration or so-called “sweeping” techniques used in CZE, MEKC and CGE to enhance sensitivity [133].

CITP has limited application however since simultaneous determination of cations and anions is not possible and the selection of an appropriate discontinuous buffer system at the desired pH is often an arduous task [142].

### 3.1.5 Capillary isoelectric focusing (CIEF)

CIEF is a “high resolution” technique designed to separate proteins and peptides based on their isoelectric points or pI values. Ampholytes (zwitterions) are used to construct a pH gradient within the capillary with an acidic solution at the anode and a basic solution at the cathode. Once the capillary is filled with a mixture of ampholytes and solutes, the application of an electric field propels the charged ampholytes and amphoteric proteins to migrate until they reach a region where they are uncharged (at their respective pI values) and remain stationary or “focused”. High resolution is obtained since migration of a solute into a zone of differing pH would cause a disruption in its neutral charge and the solute rapidly migrates back to into its pI zone. The completion of amphoteric focusing is indicated by a break in current and mobilization is induced by application of pressure or by adding salt to either reservoir in order for the zones to migrate through the detector. As with CGE, this electrophoretic technique is well known and has been adapted for automated instrumental use [133].

## 3.2 CE METHODS USED FOR THE ANALYSIS OF FLAVONOIDS

The flavonols are predicted to be well suited for analysis by CE since they are negatively ionized at high pH [143] however, it has been shown that the use of a simple phosphate-borate running buffer does not sufficiently ionize the flavonol skeleton to allow for differential electrophoretic mobilities. Morin *et al.* [144] published a short communication in 1993 on the separation of flavonol-3-*O*-glycosides using a borate-complexing buffer. Under alkaline conditions, separation was facilitated by the *in-situ* complexation of borate with either the sugar moiety or the cis-1, 2-hydroxyl groups on the flavonol skeleton creating negatively charged borate complexes capable of migrating in the presence of an electric field. It was found

that an increase of borate buffer concentration resulted in retarded migration velocities due to enhanced borate-complex formation as well as suppression of the EOF and that complexation was dependent on the number of boration sites present on the saccharide moiety [144, 145].

Morin *et al.* [146] also reported the separation of flavonol-7-*O*-glycosides using MEKC with the addition of 50 mM SDS to a 20 mM Tris buffer (pH 7.1) which was attributed to hydrophobic interactions between the flavonols and the micellar core. It was concluded that the above CE methods were superior to published HPLC methods due to improved demonstration of efficiency, selectivity and speed. That same year, Bjerregaard *et al.* [147] reported a method to separate kaempferol and quercetin glycosides using a cationic surfactant, cetyltrimethylammonium bromide (CTAB) after sample isolation, purification and group separation procedures were performed.

In 1994, Pietta and co-workers [148] concentrated on optimizing the separation of flavonoids which differed in their degree of hydroxylation and found that the incorporation of organic solvents in the sample reduced migration times as well as resolution between the flavonoids. SDS concentrations positively effected separation at pH 8.3 but had no influence at higher pH values while beyond pH 10.5, separation was once again reliant on hydroxyl ionization and borate complexation of the sugar moieties.

There have subsequently been a number of CE methods developed for the analysis of flavonoid markers in various natural products which have one or more flavonoids which are also present in *Ginkgo biloba* extracts, the details of which are given in Table 3.1. [138, 149 - 158]. All reported analyses were performed under alkaline conditions except for the determination of kaempferol-3-*O*-rhamnoside in the aerial parts of *Epimedium brevicornum* [138] and only one paper described the determination of the content of the markers in a solid oral dosage form [157]. All analyses were performed using positive applied voltage.

Pietta and Mauri [149] published a promising MEKC method in 1991 for the analysis of 9 flavonoids commonly found in some natural medicinal products and applied this method for the determination of 5 flavonols as well as astragalin in a standardized *Ginkgo biloba* extract. Baseline resolution was reported although only one electropherogram of all selected flavonoid reference standards illustrated this observation, while another showed only the quercetin, kaempferol and isorhamnetin glycosides of which reference standards of hyperoside (not known to be present in *Ginkgo biloba*) and quercitrin were not fully resolved. The standardized Ginkgo extract showed incomplete resolution of rutin and isoquercitrin, both due to unknown interfering compounds however resolution was notably superior to that of HPLC using a C<sub>8</sub> Aquapore reversed phase column. This paper primarily focused on comparative separations of Ginkgo using MEKC and HPLC and commented on the improved resolution, speed, sensitivity and low solvent consumption using MEKC which would be beneficial for fingerprint analyses. It would be of interest however to perform this experiment using the more popular C<sub>18</sub> reversed phase column most frequently used in the HPLC analysis of Ginkgo preparations. This paper contradicts heavily documented findings of

decreased sensitivity using CE. Moreover, those authors used a standardized extract which dissolved completely in 30% aqueous methanol without having to use the usual laborious extraction procedures normally required for the analysis of both raw leaf extracts and formulated products. The reason as to why this method has not been subsequently used and developed for the QC of *Ginkgo biloba* extracts and formulations is not apparent. Perhaps the emphasis on the activities of the flavonol aglycones and the adopted industrial method of flavonol hydrolysis to aglycones prior to analysis has detracted from the value of analyzing intact Ginkgo extracts, even from a qualitative perspective.

This method was therefore used as a model to develop a qualitative method to fingerprint *Ginkgo biloba* solid oral dosage forms performed at high pH, particularly due to the fact that it was shown that it may be directly applied to the analysis of *Ginkgo biloba* preparations. Five flavonols were selected for this research namely 2 flavonol glycosides, rutin and quercitrin and the conventional aglycone markers, quercetin, kaempferol and isorhamnetin.

The following variables may be used in method development to optimize selectivity, resolution and efficiency and are categorized as follows [159]:

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

Since the following qualitative analysis was based on the method of Pietta and Mauri [149] as well as additional studies previously performed in our laboratories [140] using this same method to fingerprint *Hypericum perforatum* extracts containing similar flavonol markers, only minor method adjustments were necessary before analyzing *Ginkgo biloba* tablet extracts for the selected marker compounds. These

included choice of wavelength, pH and ionic strength. In addition, comparison of the extraction efficiencies of various solvents on the fingerprint profiles of *Ginkgo biloba* extracts was investigated.

Table 3.1. CE methods used for the analysis of flavonols

Matrix analyzed	Flavonoids present in <i>Ginkgo biloba</i> leaves and extracts	Running buffer composition	Buffer pH	Voltage	Detection wavelength (nm)	Run time (min)	Qualitative/quantitative Analysis	Reference
Standardized <i>Ginkgo biloba</i> extract	rutin, isoquercitrin, quercitrin, kaempferol-3- <i>O</i> -rutinoside, isorhamnetin-3- <i>O</i> -rutinoside, astragalgin	50 mM SDS, 20 mM sodium borate	8.3	+20 kV	260	14	Qualitative	149
Aerial parts of <i>Epimedium</i> species	quercetin, quercitrin, luteolin, kaempferol-3- <i>O</i> -rhamnoside	48 mM SDS, 20 mM sodium borate, 1 mM 1,3-diaminopropane	8.5	+20 kV	254	20	Qualitative	150
<i>Eucommia ulmoides</i> extract <sup>1</sup> , red wine <sup>2</sup>	rutin, quercitrin <sup>1</sup> , rutin, quercetin, myricetin <sup>2</sup>	30 mM disodium hydrogen phosphate, 30 mM sodium dihydrogen phosphate <sup>1</sup> ; 30 mM disodium hydrogen phosphate <sup>2</sup>	7.0 <sup>1</sup> , 8.9 <sup>2</sup>	+30 kV <sup>1</sup> +20 kV <sup>2</sup>	220 <sup>1</sup> , 220-380 <sup>2</sup>	21 <sup>1</sup> , 20 <sup>2</sup>	Qualitative	151
Enzymatically modified flavonoids	rutin, isoquercitrin	100 mM borate	10.0	+30 kV	254	7	Qualitative	152
<i>Rhododendron dauricum</i> L. leaves	quercetin	50-75 mM borax	8.7	+14 kV	Electro-chemical	16	Quantitative	153
<i>Hypericum perforatum</i> leaves or flowers	rutin, isoquercitrin, quercitrin, quercetin	50 mM Tris, 25 mM N-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid, 55 mM boric acid	8.8	Constant current (200 $\mu$ A)	254	35	Quantitative	154
<i>Eucommia ulmoides</i> bark and leaves	rutin, quercetin	50 mM boric acid, 50 mM SDS, 4% 1-butanol	9.5	+20 kV	214	16	Quantitative	155
Aerial parts <i>Achillea setacea</i>	rutin, apigenin	25 mM sodium borate, 20% methanol	9.3	+30 kV	270	19	Quantitative	156
Aerial parts of <i>Epimedium brevicornum</i>	kaempferol-3- <i>O</i> -rhamnoside	20 mM phosphoric acid, 100 mM SDS, 20% acetonitrile, 2% 2-propanol	2	+15 kV	254	23	Quantitative	138
<i>Hippophae rhamnoides</i> extract and tablet	quercetin, kaempferol, isorhamnetin	20 mM borate, 5 mg/ml $\beta$ -cyclodextrin	10.0	+15 kV	270	4.5	Quantitative	157
Grape wine	quercetin, kaempferol, myricetin, apigenin, luteolin	35 mM borax	8.9	+16.8 kV	270	16	Quantitative	158

### 3.3 CE ANALYSIS AT HIGH PH

#### 3.3.1 Choice of wavelength

##### 3.3.1.1 *CE Instrumentation*

The CE system comprising of a PrinCE (4 tray) Electrophoresis System, Model 0500-001 and Butler Buffer Replenishing Device, Model 0500-001 were both purchased from Lauerlabs, Emmen, The Netherlands. A Linear UV/VIS-206 Multiple Wavelength Detector, Model 0206-0000 (Linear Instruments Corporation, Reno, Nevada, USA) was used for UV detection. A Polyimide fused silica capillary (50 µm I.D. x 360 µm O.D.) was supplied by Polymicro Technologies (Phoenix, Arizona, USA).

##### 3.3.1.2 *Additional equipment*

A Perkin Elmer R100A Strip Chart Recorder, Model C0050005 (Perkin Elmer Corporation, Illinois, USA) was used to record the electropherograms. A Cole-Parmer Ultrasonic Bath, Model 8845-30 (Cole-Parmer Instrument Company, Chicago, Illinois, USA) was used in the sonication procedure for extraction purposes and a Mettler Dual Range Electronic Balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland) was used for weighing reagents and standards. A Crison GLP21 pH Meter (Crison, Barcelona, Spain) was used to measure and adjust pH values of relevant solutions while an Eppendorf Centrifuge, Model 54142770 (Eppendorf Geratebau, West Germany) was used to centrifuge tablet extracts. HPLC grade water was purified by reverse osmosis through a Milli-Q purification system (Millipore, Bedford, MA, USA).

##### 3.3.1.3 *Materials and reagents*

Methanol (HPLC grade), isopropyl alcohol, ethanol, acetone and ethyl acetate were obtained from Burdick and Jackson Division (Muskegon, Michigan, USA). Sodium tetraborate ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) was acquired from Saarchem (Pty) Ltd. (Johannesburg, South Africa). SDS, rutin (95%) and quercetin (95%) were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide pellets were purchased from BDH chemicals (Poole, UK) and quercitrin (85%), kaempferol (95%) and isorhamnetin (90%) from the Indofine Chemical Company (New Jersey, USA). The purity of these standards was assumed as provided by the certificates of analyses from the suppliers. A *Ginkgo biloba* product, Formule Naturelle was purchased from a local pharmacy in Grahamstown, South Africa.

##### 3.3.1.4 *Capillary conditioning*

New capillaries were conditioned using a constant pressure of 2 000 mbar to wash with 1 M sodium hydroxide for 30 minutes, 0.1 M sodium hydroxide for 30 minutes and water for 30 minutes. At the start of

each day, the capillary was flushed with 1 M sodium hydroxide for 10 minutes, 0.1 M sodium hydroxide for 10 minutes and water for 10 minutes and with 1 M sodium hydroxide for 2 minutes, 0.1 M sodium hydroxide for 2 minutes and with water for 2 minutes between consecutive injections to ensure optimal charge density on the capillary wall. To ensure reproducibility and prevent effects of pH changes in the background electrolyte (BGE) due to electrolysis, the buffer at both anodic and cathodic ends were replaced after each injection.

#### 3.3.1.5 Initial CE conditions

A capillary with a total length of 69.0 cm and effective length of 52.8 cm was used for the separation. The running buffer consisted of 20 mM sodium tetraborate and 50 mM SDS (pH 8.5). The applied voltage was +15 kV with a voltage ramp of +6 kV/s and samples were injected hydrodynamically at 200 mbar for 0.1 minutes. Various detection wavelengths were used, 254 nm, 270 nm and 350 nm, depending on the nature of the extracts. The detector rise time was 0.1 seconds and the sensitivity was set at 0.01 absorbance units full scale (AUFS).

#### 3.3.1.6 Preparation of reference standards

Unless stated otherwise, reference standards were prepared using 50:50 methanol-water as solvent as opposed to 30% aqueous methanol used by Pietta and Mauri [149]. The methanol fraction was increased to facilitate the solubility of the additional selected flavonol aglycones which are less hydrophilic than the glycosides and to decrease the migration time without compromising the separation, as investigated by Pietta *et al.* [148]. The negative methanol peak was used as the neutral marker and concentrations were typically in the order of 50 µg/ml.

#### 3.3.1.7 Results and discussion

From the UV absorption spectra of the flavonols in methanol-water (50:50) (Figure 3.4), the wavelengths for fingerprint analysis of the flavonols were selected at 270 nm and 254 nm. The flavonols form intense yellow solutions under alkaline conditions and are therefore well suited for analysis in the visible light range. It would probably be advantageous from a fingerprinting perspective to analyze extracts at less selective wavelengths for augmented comparisons of content, including that of the non-flavonoid fraction. Wavelengths lower than 254 nm were however not feasible due to excessive interference. Hence, most Ginkgo dosage forms were analyzed at 254 nm or 270 nm with 350 nm reserved for those extracts where interference precluded identification of the selected marker compounds. This point is illustrated in Figure 3.5 which shows the fingerprints of the same tablet extract analyzed at 254 nm, 270 nm and 350 nm with the identified marker compounds as numbered.

Figure 3.4. UV absorbance spectra of the flavonols in 50:50 methanol-water

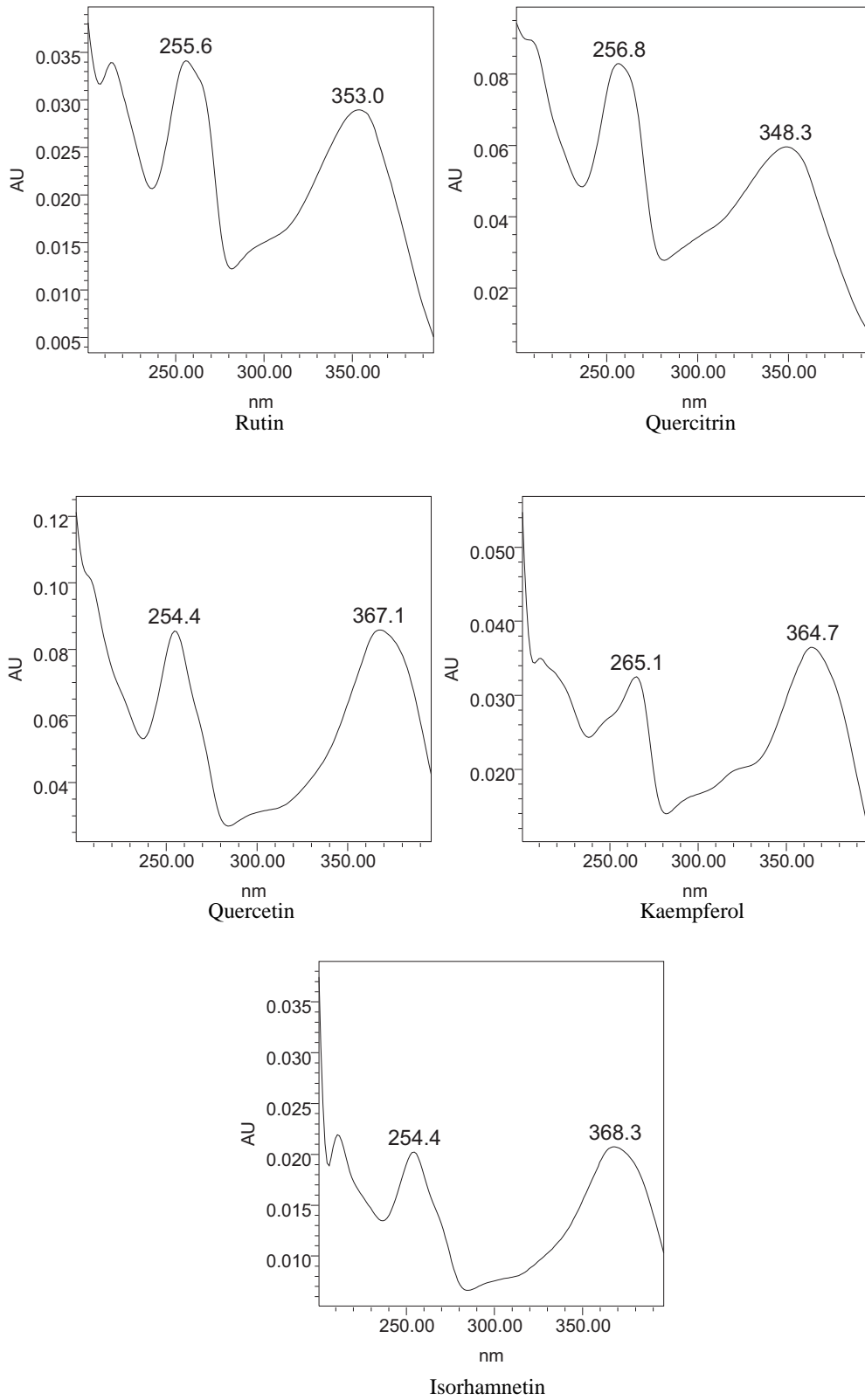
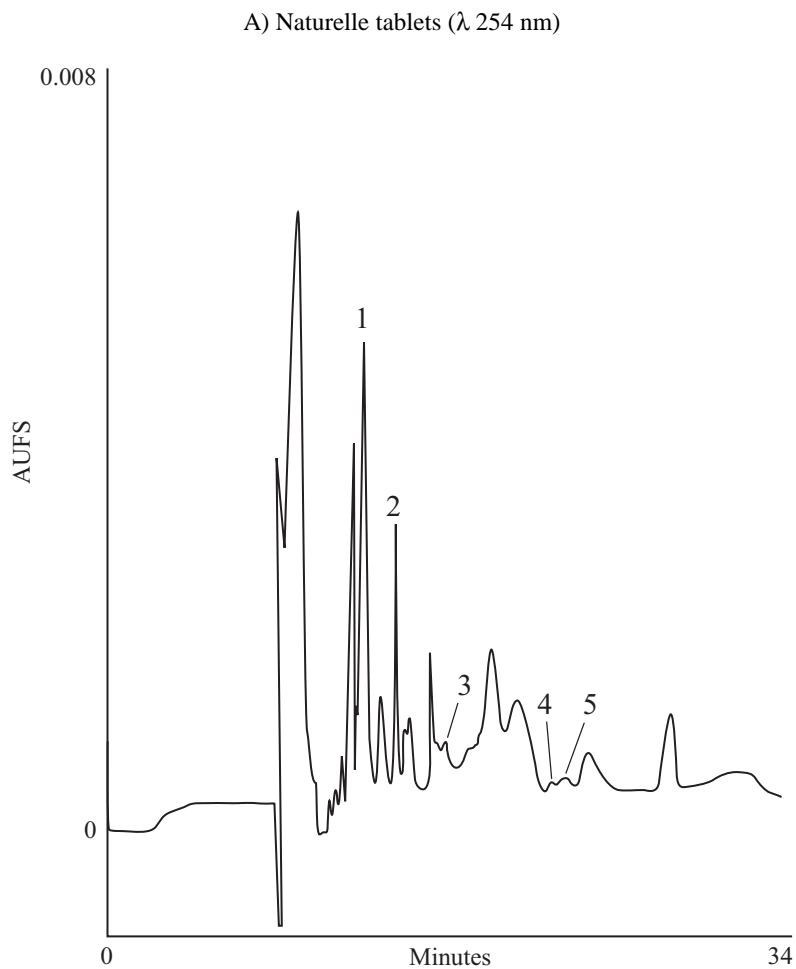


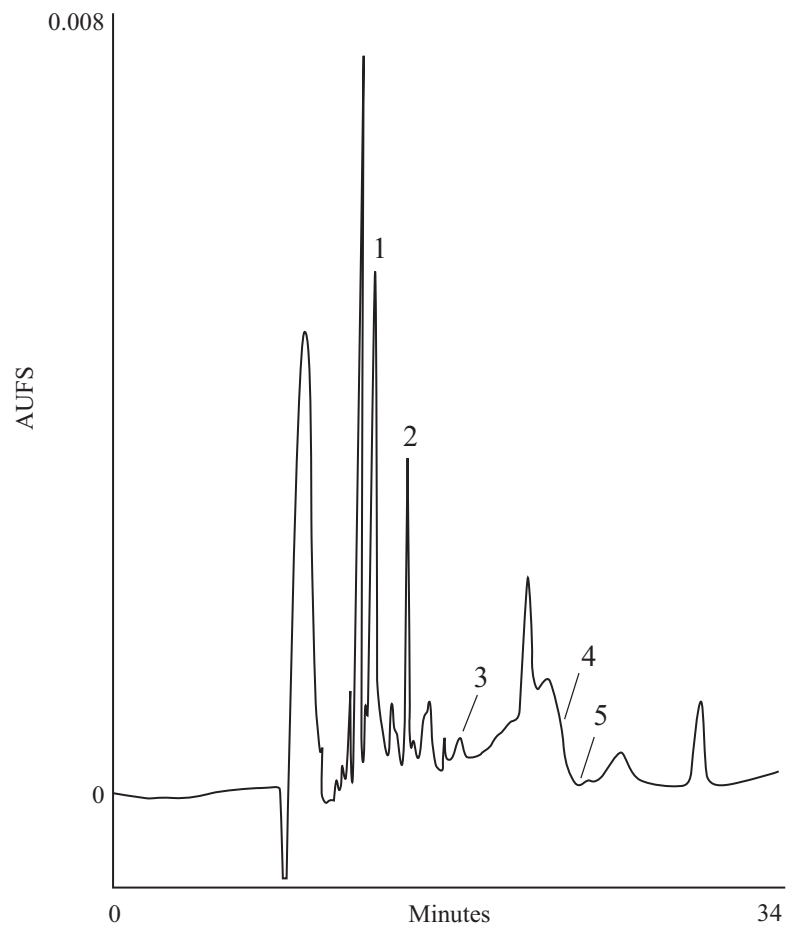


Figure 3.5. Fingerprint profiles of a tablet extract analyzed at different wavelengths



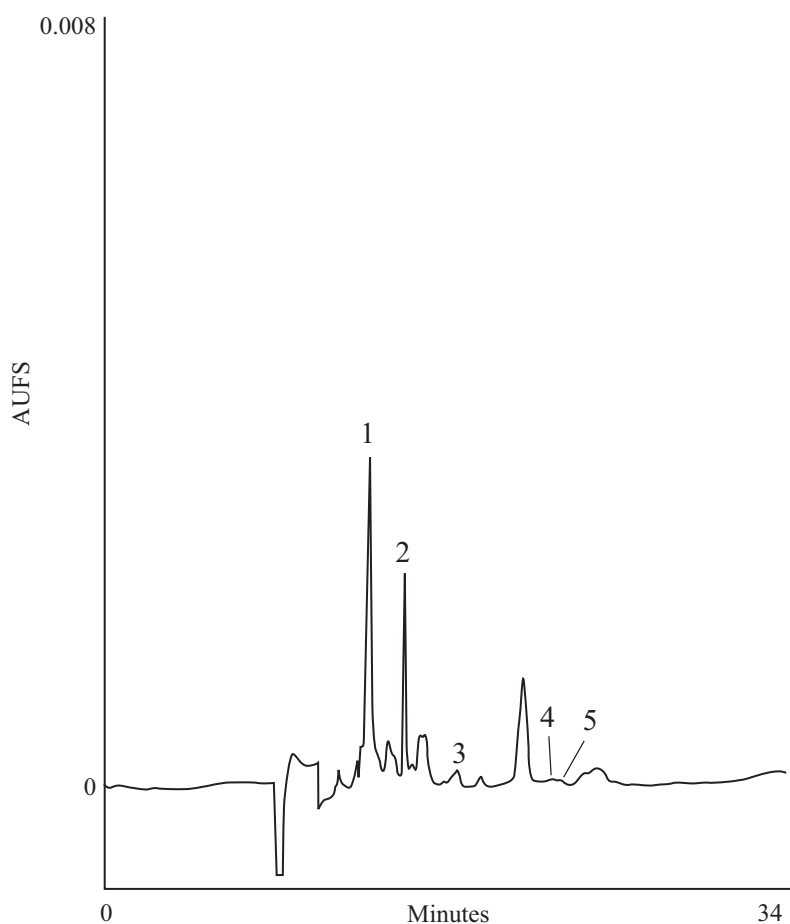
Conditions: Capillary: 50.0  $\mu$ m I.D., total length = 69.0 cm, effective length = 52.8 cm; Sample buffer: 50:50 methanol-water; Running buffer: 20 mM sodium borate, 50 mM SDS (pH 8.5); Hydrodynamic injection: 200 mbar for 0.1 minutes; Voltage: +15 kV; Labeled peaks: 1=rutin, 2=quercitrin, 3=quercetin, 4=kaempferol, 5=isorhamnetin.

B) Naturelle tablets ( $\lambda$  270 nm)



*Conditions: Capillary: 50.0  $\mu$ m I.D., total length = 69.0 cm, effective length = 52.8 cm; Sample buffer: 50:50 methanol-water; Running buffer: 20 mM sodium borate, 50 mM SDS (pH 8.5); Hydrodynamic injection: 200 mbar for 0.1 minutes; Voltage: +15 kV; Labeled peaks: 1=rutin, 2=quercitrin, 3=quercetin, 4=kaempferol, 5=isorhamnetin.*

### C) Naturelle tablets ( $\lambda$ 350 nm)



*Conditions: Capillary: 50.0  $\mu$ m I.D., total length = 69.0 cm, effective length = 52.8 cm; Sample buffer: 50:50 methanol-water; Running buffer: 20 mM sodium borate, 50 mM SDS (pH 8.5); Hydrodynamic injection: 200 mbar for 0.1 minutes; Voltage: +15 kV; Labeled peaks: 1=rutin, 2=quercitrin, 3=quercetin, 4=kaempferol, 5=isorhamnetin.*

### 3.3.2 Choice of pH

#### 3.3.2.1 *Experimental Procedure*

The optimal pH was selected based on the effects of the buffer at pH 8.3, 8.5, 9.3, 9.5, 9.7 and 9.9 on the resolution and mobility of the flavonol markers. Capillary conditioning, initial CE parameters and sample solutions were as described in section 3.3.1.4 - 3.3.1.6. The concentration of rutin, quercitrin, quercetin and kaempferol was 83.3  $\mu$ g/ml and that of isorhamnetin 41.7  $\mu$ g/ml. Detection was at  $\lambda$  254 nm. The pH adjustments were made with concentrated hydrochloric acid and a 1 M sodium hydroxide solution. The pH range was based on the buffering capacity of sodium borate and the fact that the flavonols are soluble in

aqueous solutions of high pH. A pH beyond 10.0 was avoided in order to maintain the integrity of the capillary, as recommended by the suppliers.

### 3.3.2.2 Results

The electropherograms displaying the effect of pH on the separation of the 5 flavonols are shown in Figure 3.6. Figure 3.7 shows the mobility of the flavonols at the investigated pH values. The electrophoretic mobility of each reference compound was calculated using the equations governing electro-osmotic mobility ( $\mu_{eo}$ ) [3.1] and electrophoretic mobility ( $\mu_{eff}$ ) [3.2] which are expressed below.

$$\mu_{eo} = \frac{L_D L_T}{t_{eo}} V \quad [3.1]$$

$L_T$  = total length of capillary (cm)

$V$  = separation voltage (kV)

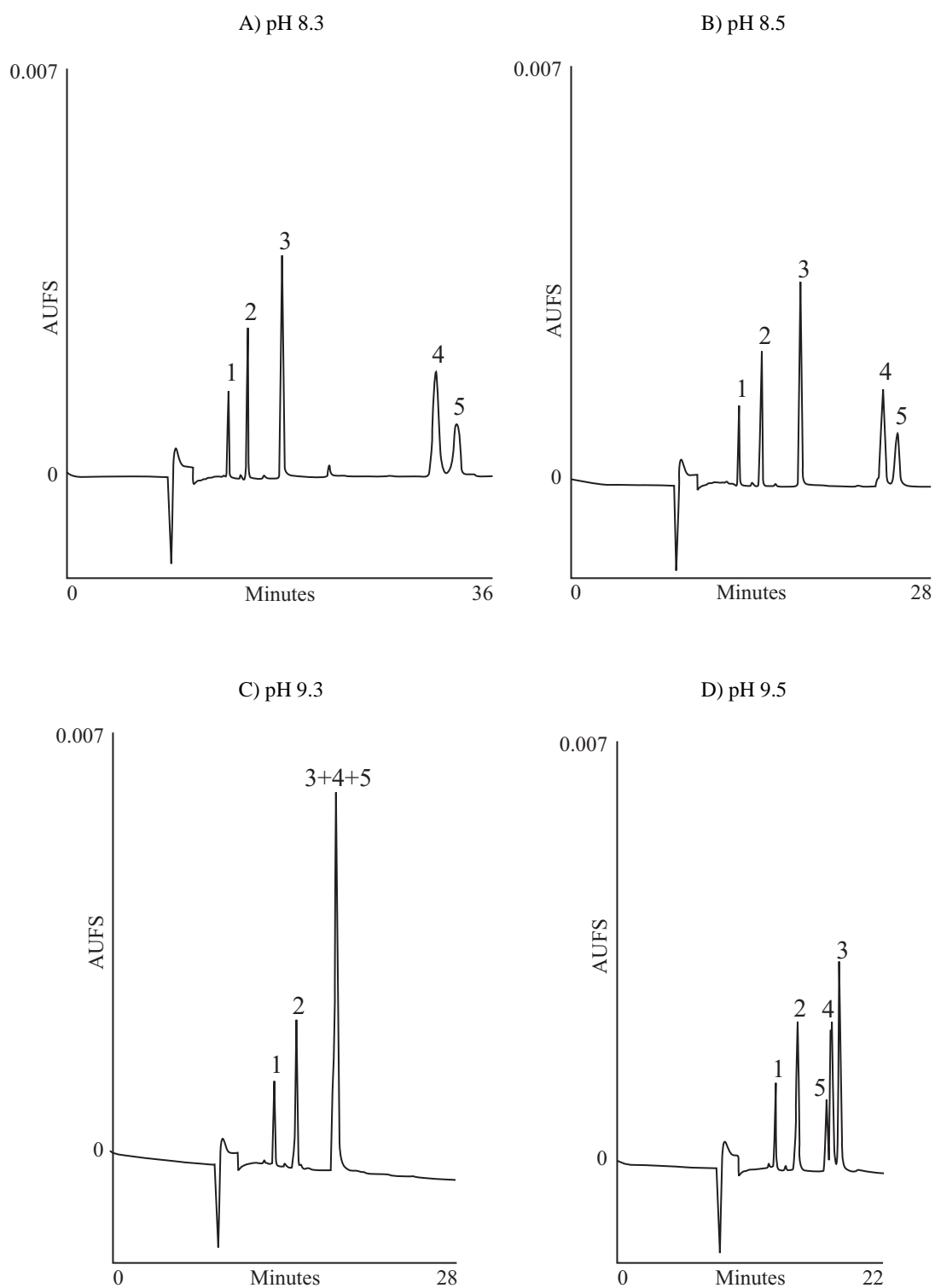
$L_D$  = length to detector (cm)

$$\mu_{eff} = \frac{L_D L_T}{V} [1/t_{eo} - 1/t] \quad [3.2]$$

$t_{eo}$  = migration time of neutral marker (sec)

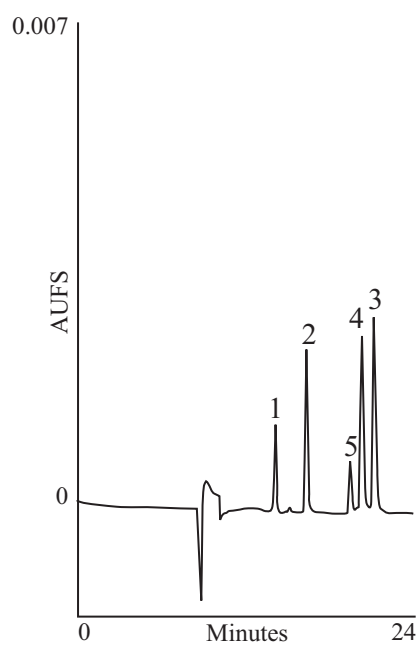
$t$  = migration time of solute (sec)

Figure 3.6. Effect of pH on the resolution of the flavonol marker compounds

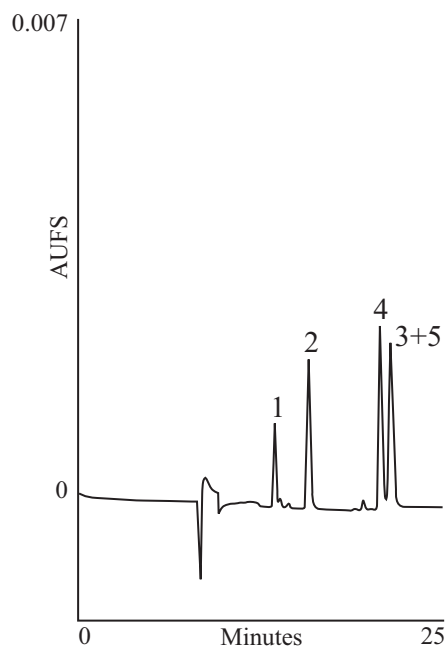


Conditions: Capillary: 50.0  $\mu\text{m}$  I.D., total length = 69.0 cm, effective length = 52.8 cm; Sample buffer: 50:50 methanol-water; Running buffer: 20 mM sodium borate, 50 mM SDS; Hydrodynamic injection: 200 mbar for 0.1 minutes; Voltage: +15 kV; Detection  $\lambda$ : 254 nm; Labeled peaks: 1=rutin, 2=quercitrin, 3=quercetin, 4=kaempferol, 5=isorhamnetin.

E) pH 9.7

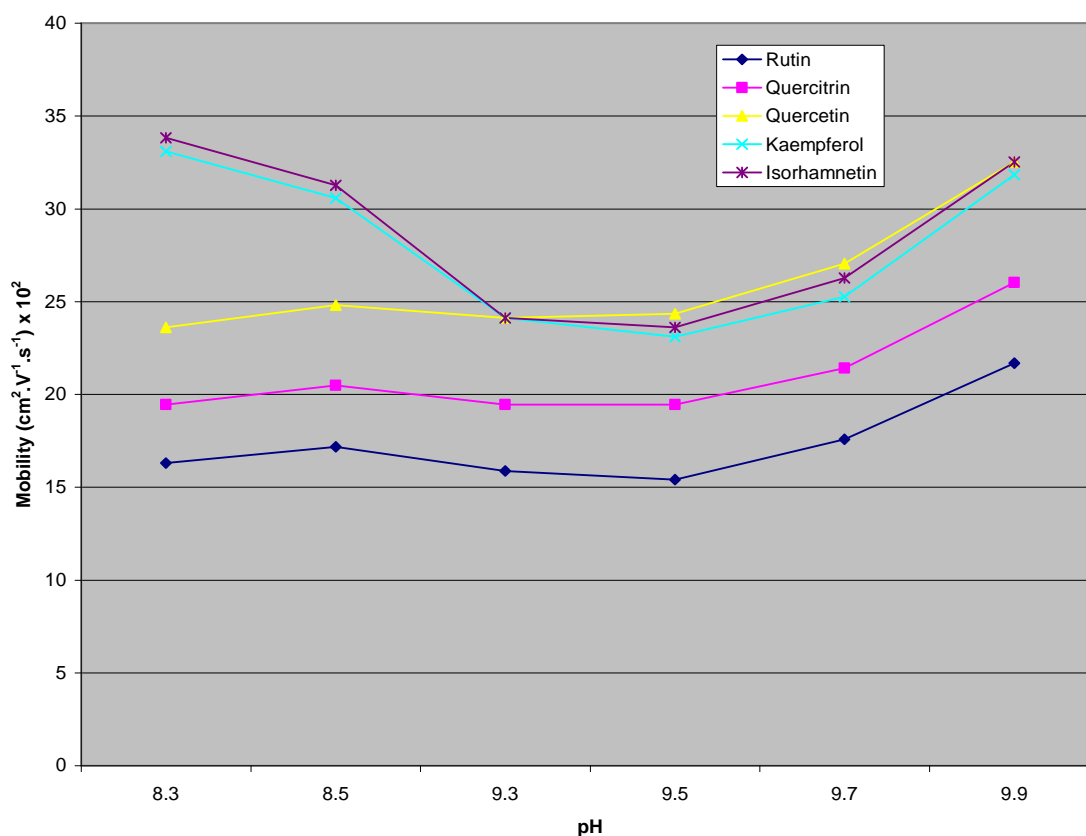


F) pH 9.9



Conditions: Capillary: 50.0  $\mu\text{m}$  I.D., total length = 69.0 cm, effective length = 52.8 cm; Sample buffer: 50:50 methanol-water; Running buffer: 20 mM sodium borate, 50 mM SDS; Hydrodynamic injection: 200 mbar for 0.1 minutes; Voltage: +15 kV; Detection  $\lambda$ : 254 nm; Labeled peaks: 1=rutin, 2=quercitrin, 3=quercetin, 4=kaempferol, 5=isorhamnetin.

Figure 3.7. Effect of pH on the electrophoretic mobility and resolution of the flavonols



### 3.3.2.3 Discussion

The pH of the buffer is the dominant parameter affecting the separation of flavonols at high pH which is clearly illustrated by the dramatically different results obtained with relatively small changes in pH. This is due to the fact that the flavonols are partially ionized at high pH and under these conditions any alteration of this parameter results in significant changes in the distribution co-efficients of the analytes between the micellar and aqueous phase [160]. From Figure 3.7 it can be seen that the electrophoretic mobility of isorhamnetin and kaempferol initially declined with an increase in pH until a pH of 9.3 after which a more gradual increase was observed. The resolution between the analytes at various pH values can also be deduced from this figure. For example, the graphs of compounds quercetin, kaempferol and isorhamnetin intersect at pH 9.3, indicating co-migration. Baseline resolution was obtained at pH 8.3, 8.5 and 9.7. At pH 9.5 and 9.7, the migration order of the flavonol aglycones was altered and kaempferol migrated before isorhamnetin, followed by quercetin. A similar migration order occurred at pH 9.9, however quercetin and isorhamnetin co-migrated.

The migration order of the flavonol glycosides, rutin and quercetin correspond to findings by Akiyama *et al.* [152] where the flavonol glycosides possessing larger sugar moieties migrated at a faster rate than those with shorter saccharide chains. Rutin and quercitrin are both 3-*O*-glycosides with glucose-rhamnose and

rhamnose units, respectively. When using borate alone in the running buffer, separation was dependent on borate complexation with the hydroxyl groups on ring B. Flavonol glycosides which have larger sugar moieties have smaller charge/size ratios resulting in shorter migration times. The inclusion of SDS into the running buffer allowed enhanced separation since the more hydrophilic marker compounds such as rutin and quercitrin predominantly partitioned into the aqueous phase with minimal hydrophobic interactions with the micelles and therefore migrated with the EOF.

Separation of the less hydrophilic flavonol aglycones using MEKC may be attributed to differences in partition co-efficients of the analytes with the micelles as described in section 3.1.2. In addition, a relationship between the substituents at positions C-2 of ring B (Figure 2.4) and the buffer pH must exist in order to explain the radically diverse migration orders and velocities of the flavonol aglycones. Quercetin has a hydroxyl group situated at C-2 of ring B and is the most hydrophilic aglycone, followed by kaempferol which has a hydrogen atom in the same position. Isorhamnetin is the most hydrophobic of the group due to a methoxy substituent in position C-2. At pH values between 8.3 and 9.3, minimal ionization of the flavonols results in a dominant interaction between the aglycones and the micelles with the distribution co-efficient of isorhamnetin favoring the strongest interaction with the micellar core which explains its substantially longer migration time in comparison to quercetin. Kaempferol in turn has greater hydrophobic interaction with the micelles than quercetin but spends less time in the micellar core than isorhamnetin and migrates between the other 2 aglycones. At higher pH values of 9.5 and 9.7, the aglycones were highly ionized which impeded hydrophobic micellar interactions and the aglycones are separated based on their respective negative charges and/or borate complex formation with the hydroxyl groups on ring B. Quercetin had the strongest borate complexation due to the presence of the dihydroxyl groups on C-1 and C-2, followed by isorhamnetin which has a methoxy and hydroxyl group in positions C-1 and C-2, respectively. Kaempferol has only one hydroxyl group in position C-2 of ring B available for borate complexation and was the least negatively charged under these conditions. The observed migration orders were thus altered and kaempferol migrated ahead of isorhamnetin while quercetin reached the detector at the cathodic end of the capillary last.

At a pH of 9.9, an increase in EOF was observed by differences in migration times of the methanol marker of 480 seconds as opposed to 528 seconds in all the other pH investigations. This dramatic increase explains the reduced selectivity and resolution of the flavonols and in particular, the separation of the aglycones. The increase in pH caused augmented surface charge density on the silanol capillary wall which created a higher zeta potential with subsequent generation of an enhanced EOF. The reverse is true at low pH values which will be further discussed in section 3.8 and 3.9. Hence, higher buffer pH values are generally associated with shorter run times usually at the expense of separation [142].

Following these investigations, a pH of 8.5 was selected due to enhanced selectivity and separation of both the flavonol glycosides and aglycones. Although a pH of 8.3 was shown to exhibit improved resolution



compared to 8.5, the excessively long run time of 34 minutes resulted in preferential selection of pH 8.5 where the run time was reduced to 28 minutes. Since more than 33 flavonoids have been identified in *Ginkgo biloba* leaf extracts, excluding other unknown compounds, it is paramount that adequate resolution and peak purity is achieved. Accurate, realistic results were therefore prioritized above the quicker analysis time of 22 minutes obtained at pH 9.7.

### 3.3.3 Ionic strength

#### 3.3.3.1 *Experimental procedure and results*

An increase in ionic strength from 20 mM to 38 mM was investigated. All conditions barring the buffer molarity were identical to those described in sections 3.3.1.4 - 3.3.1.6. As predicted, the increase in molarity resulted in slower migration of the flavonol compounds and an increase in run time from 28 minutes to 40 minutes. Although an increase in resolution was predicted with an increase in ionic strength, no significant improvement was observed. In addition, kaempferol and isorhamnetin had broad peak shapes.

#### 3.3.3.2 *Discussion*

According to Terabe [132], the buffer molarity should not be lower than 10 mM. Higher concentrations are normally associated with improved resolution due to slower migration times and therefore greater micellar and buffer interactions [155]. The increased buffer ionic strength supplies an abundance of counter-ions that effectively decreases the surface charge per unit area on the silica capillary surface. This results in the contraction of the electrical double layer and subsequent suppression of the EOF [142]. The wide peaks characteristic of slowest migrating compounds occurred due to zone broadening while the analytes diffused along the capillary length with consequential loss of efficiency [133]. An optimal ionic strength is therefore capable of facilitating separation within an acceptable migration time and with minimal generation of joule heat as described by equation 3.3 below. An ionic buffer strength of 20 mM was chosen for the analysis based on the paper by Pietta and Mauri [149] which gave a satisfactory result within the above mentioned constraints.

$$W = d^2 \pi \lambda C V^2 / 4L \quad [3.3]$$

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

The SDS concentration was also selected based on work performed by Pietta and Mauri [149] as well as previous investigations performed in our laboratories [140] where it was found that an SDS concentration of 50 mM adequately separated the flavonols in St. John's Wort with acceptable levels of joule heat production. Similarly, this concentration facilitated baseline resolution of all the selected flavonol marker compounds used in this study. Higher SDS concentrations are associated with greater partitioning of the analytes into the micellar pseudo stationary phase and therefore results in longer analyte migration times [155]. This effect is more pronounced with the less hydrophilic aglycones [140]. Moderate concentrations are usually recommended to minimize the production of joule heat [132].

#### 3.3.4 Separation voltage

The voltage selected was based on work previously performed on St. John's Wort in our laboratories [140]. It was found that the resolution between the flavonols rutin, isoquercitrin, hyperoside, quercitrin and quercetin using the same method was most efficient at +15 kV, followed by +10 kV, +20 kV and then +25 kV. An increase in the applied field strength generally results in an increase in analyte migration and therefore reduced run times however the resolution may be compromised. In addition, joule heating is a common phenomenon when separation voltages above +15 kV are applied [161], leading to disproportionate effects on the EOF and electrophoretic mobility of analytes with noticeable loss of efficiency [142]. The parameters which influence the temperature gradient within a capillary are presented in equation 3.3 in section 3.3.3.2. A suitable separation voltage is therefore one which enables baseline separation of compounds within a reasonable migration time with minimal production of joule heat.

#### 3.3.5 Effect of extraction solvents

##### 3.3.5.1 *Experimental Procedures*

The solvents used in the extraction process were methanol, ethyl acetate, isopropyl alcohol, ethanol and acetone. Conditions were identical to those described in section 3.3.1.4 - 3.3.1.6 except the total capillary length was 70.4 cm while the effective length remained the same at 52.8 cm. Detection was at  $\lambda$  254 nm.

##### 3.3.5.2 *Preparation of sample solutions*

A minimum of 10 tablets were individually weighed and then pulverized thoroughly using a mortar and pestle. For each selected extraction solvent, a mass of powder equivalent to the mass of a single tablet was then weighed and transferred to a 20 ml Kimax tube. Ten milliliters of each selected solvent were pipetted into the designated tubes and sonicated for 1 hour. The tubes were then centrifuged at 350 x g for 10 minutes. Equal aliquots of samples containing organic solvents were then blown down using nitrogen and

reconstituted in 50:50 methanol-water. Those samples which contained precipitate after reconstitution were centrifuged a second time before the supernatant was injected.

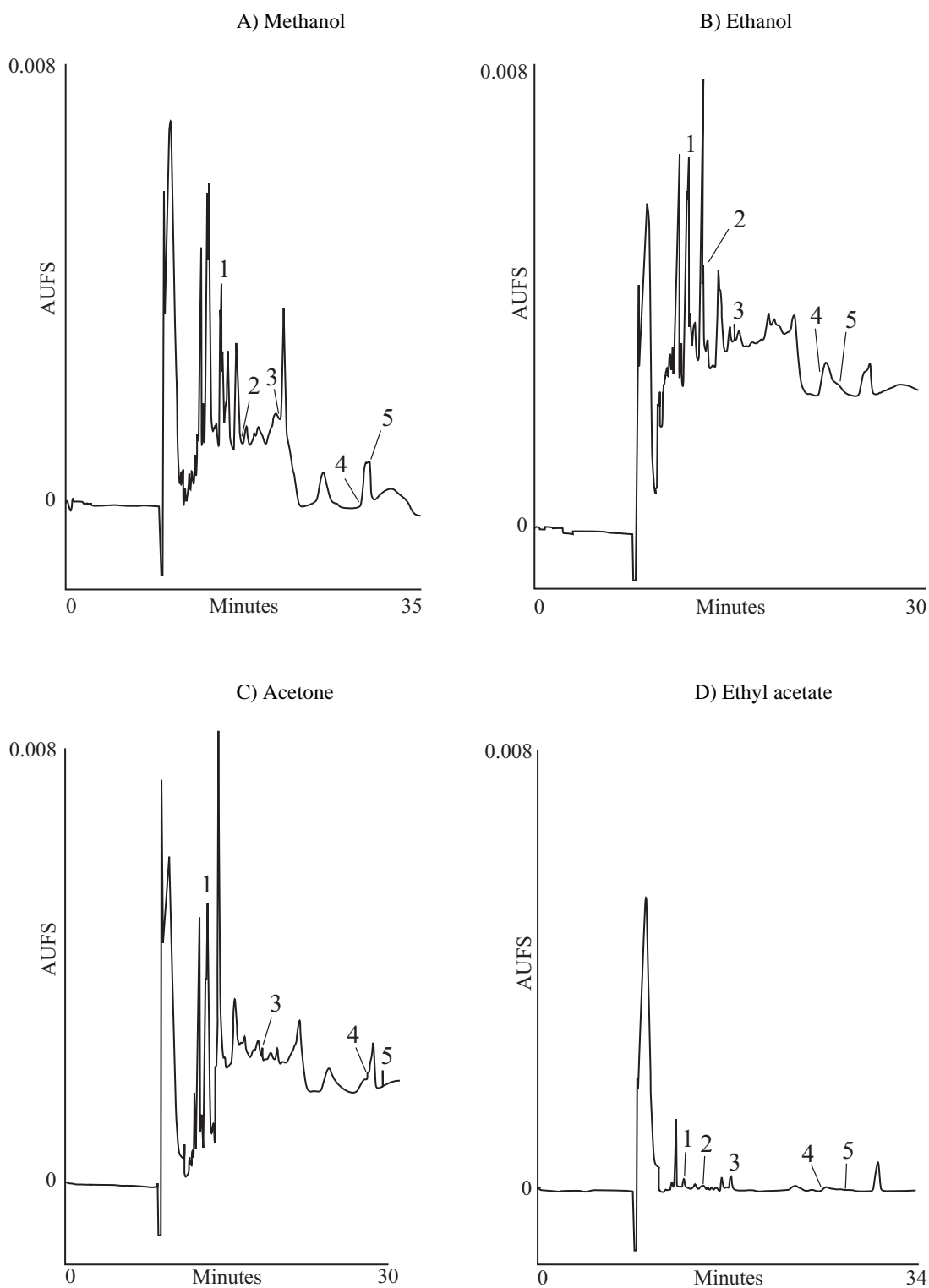
### 3.3.5.3 *Results*

Tablets extracted with methanol, ethanol and acetone gave similar profiles however baseline resolution of components was not achieved with any of these solvents. With critical examination of peak resolution and background absorbance, it could be deduced that ethanol was the least selective extraction solvent followed by acetone and then methanol. Unknown compounds co-migrated with the selected marker compounds in all 3 electropherograms shown in Figure 3.8 A, B, and C.

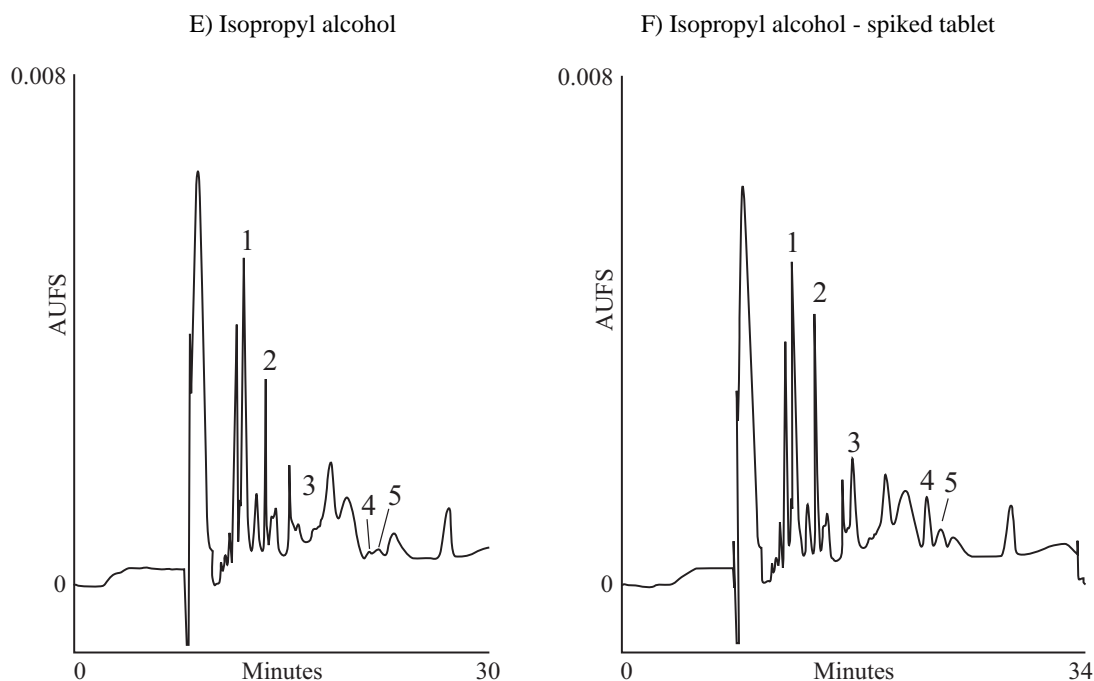
Extraction with ethyl acetate gave a simple profile with only 12 small peaks of which only a small rutin peak and larger quercetin peak were identified. An exhaustive extraction with ethyl acetate was then performed (3 x 10 ml), followed by a methanolic extraction of the residue. Both rutin and quercetin peaks were identified in the latter sample. Ethyl acetate seemed to preferentially extract the aglycones since only a few minor peaks were present in the flavonol glycoside window/zone in comparison to profiles extracted with more polar solvents. Moreover, there are generally more flavonol glycosides than aglycones present in unhydrolyzed extracts and a reverse ratio of rutin to quercetin substantiates selective extraction of the less polar aglycones. Figure 5.8 D shows the profile of a Naturelle dosage form extracted with ethyl acetate.

Isopropyl alcohol, on the other hand, gave a distinctive profile with 17 reproducible peaks. Baseline separation of all the peaks, except for quercetin was obtained. All 5 marker compounds were identifiable which was not achieved with any of the other solvents. The selected flavonol markers were identified in extracts by comparison of the migration times with reference standards prepared in the same solvent. The profile of an unspiked and spiked Naturelle tablet extracted with isopropyl alcohol is shown in Figures 3.8 E and F, respectively.

Figure 3.8. Effect of different extraction solvents on the profile of a *Ginkgo biloba* solid oral dosage form



Conditions: Capillary: 50.0  $\mu\text{m}$  I.D., total length = 70.4 cm, effective length = 52.8 cm; Running buffer: 20 mM sodium borate, 50 mM SDS (pH 8.5); Hydrodynamic injection: 200 mbar for 0.1 minutes; Voltage: +15 kV; Detection  $\lambda$ : 254 nm; Labeled peaks: 1=rutin, 2=quercitrin, 3=quercetin, 4=kaempferol, 5=isorhamnetin.



Conditions: Capillary: 50.0  $\mu\text{m}$  I.D., total length = 70.4 cm, effective length = 52.8 cm; Running buffer: 20 mM sodium borate, 50 mM SDS (pH 8.5); Hydrodynamic injection: 200 mbar for 0.1 minutes; Voltage: +15 kV; Detection  $\lambda$ : 254 nm; Labeled peaks: 1=rutin, 2=quercitrin, 3=quercetin, 4=kaempferol, 5=isorhamnetin.

#### 3.3.5.4 Discussion

The effect of solvents with differing polarity indices on the extraction of a Naturelle tablet was investigated in order to determine solvent selectivity. Of the products available during this investigation, the fingerprints of Naturelle were distinctive with a number of peaks that could be used in a comparative study. Vital was disregarded due to its formulation as a liquid in a gelatin coat which was more difficult to extract accurately and the remaining 2 products needed to be concentrated before distinctive profiles were observed.

The polarity indices of the organic solvents used in this extraction varied, with acetone and methanol having an index of 5.1, ethanol an index of 5.2, and ethyl acetate and isopropyl alcohol with indices of 4.4 and 3.9, respectively. Ethanol's high polarity index may explain its unselective extraction with high background absorbance. Although acetone and methanol have the same index value, peak resolution was marginally improved with the methanol extract as opposed to the acetone sample. Ethyl acetate has a higher polarity index than isopropyl alcohol however it extracted fewer compounds and was therefore not suitable for use as an extraction solvent for the fingerprinting Ginkgo extracts.

Selective extraction of the marker compounds used in this study posed difficulties since the flavonol glycosides are highly polar with the aglycones exhibiting less hydrophilic properties. Highly polar solvents used in extraction delivered electropherograms with unresolved peaks due to unknown interfering

compounds. Some measure of control was achieved by using higher detection wavelengths however higher flavonol absorbances and the inclusion of compounds that do not absorb in the visible range favored detection at lower, less selective wavelengths for fingerprinting purposes. In addition, a simple protocol of dilution to achieve separation is not possible due to varying amounts of compounds present in extracts as will be seen in section 3.4 where some extracts required concentration in order for the relevant peaks to be detected.

Isopropyl alcohol was therefore selected as the extraction solvent. Although its low polarity index is indicative of less efficient extraction of the more polar glycosides, a comparable profile was still possible. Moreover, the flavonol aglycones which migrate after the glycosides are ideally present in low concentrations in extracts and minimal interference is imperative for their detection.

### **3.4 QUALITATIVE ANALYSIS OF FLAVONOLS IN *GINKGO BILOBA* ORAL DOSAGE FORMS**

#### **3.4.1 Instrumentation and additional equipment**

The equipment used for this study has been described in section 3.3.1.1 and 3.3.1.2.

#### **3.4.2 Materials and reagents**

Isopropyl alcohol, methanol, sodium tetraborate, SDS and the reference standards used in this study were sourced as described in section 3.3.1.3. Four *Ginkgo biloba* products, Formule Naturelle, Ginkgoforce, Holotropic and Vital were purchased from a local pharmacy in Grahamstown, South Africa, the details of which are provided in Table 3.2.

Table 3.2. *Ginkgo biloba* dosage forms selected for qualitative analysis of flavonols

Manufacturer	Name of product	Dosage form	Label claim	Daily dose	Batch number	Extract
Formule Naturelle <sup>1</sup>	<i>Ginkgo biloba</i>	Tablet	Standardized to contain 24% Ginkgo Flavonglycosides	1 tablet tds*, ac <sup>5</sup>	69937	A
Bioforce <sup>2</sup>	Ginkgoforce	Tablet	Each 250mg tablet is equivalent to 10 drops of Ginkgoforce liquid	2 tablets tds*, ac <sup>5</sup>	109022E	B
Holotropic <sup>3</sup>	<i>Ginkgo biloba</i>	Tablet	Each tablet contains 400mg genuine imported <i>Ginkgo biloba</i>	2 tablets tds* prn <sup>6</sup>	0308	C
Vital <sup>4</sup>	<i>Ginkgo biloba</i>	Capsule	Each capsule contains <i>Ginkgo biloba</i> extract equivalent to 6 000mg of herb powder	1 capsule daily	1108A	D

<sup>1</sup>Formule Naturelle, Hout Bay, South Africa; <sup>2</sup>Bioforce S.A. (Pty) Ltd., Halfway House, South Africa; <sup>3</sup>Holotropic, Cape Town, South Africa; <sup>4</sup>Vital Health Foods (EDMS) BPK, Kuils River, Western Cape, South Africa; \*three times daily; <sup>5</sup>ac, before meals; <sup>6</sup>when required

### 3.4.3 Experimental Procedure

Operating conditions were as outlined in section 3.3.1.4 - 3.3.1.6 with exception of the total capillary length which was 70.4 cm with an effective length at 52.8 cm.

#### 3.4.3.1 *Preparation of sample solutions (Products A - C)*

Individual tablets were weighed and crushed with a mortar and pestle and the resulting powdered tablet was weighed and transferred into a 20 ml Kimax tube. Ten milliliters of isopropyl alcohol was pipetted into each tube and the mixture was sonicated for 30 minutes. The extracts were then manually agitated to facilitate re-dispersion of the contents before sonication was continued for a further 30 minutes. The extracts were centrifuged at 350 x g for 20 minutes and 3 ml of the supernatant was evaporated to dryness using nitrogen. The samples were reconstituted in 3 ml 50:50 methanol-water and centrifuged for 10 minutes before the clear supernatant was injected.

#### 3.4.3.2 *Sample preparation of Product D*

A single capsule was weighed before being sliced open with a blade and the contents squeezed into a 20 ml Kimax tube. The remaining residue on the gelatin coat was carefully washed into the tube with 10 ml isopropyl alcohol and the contents were extracted as described in section 3.4.2.1. Centrifugation was not

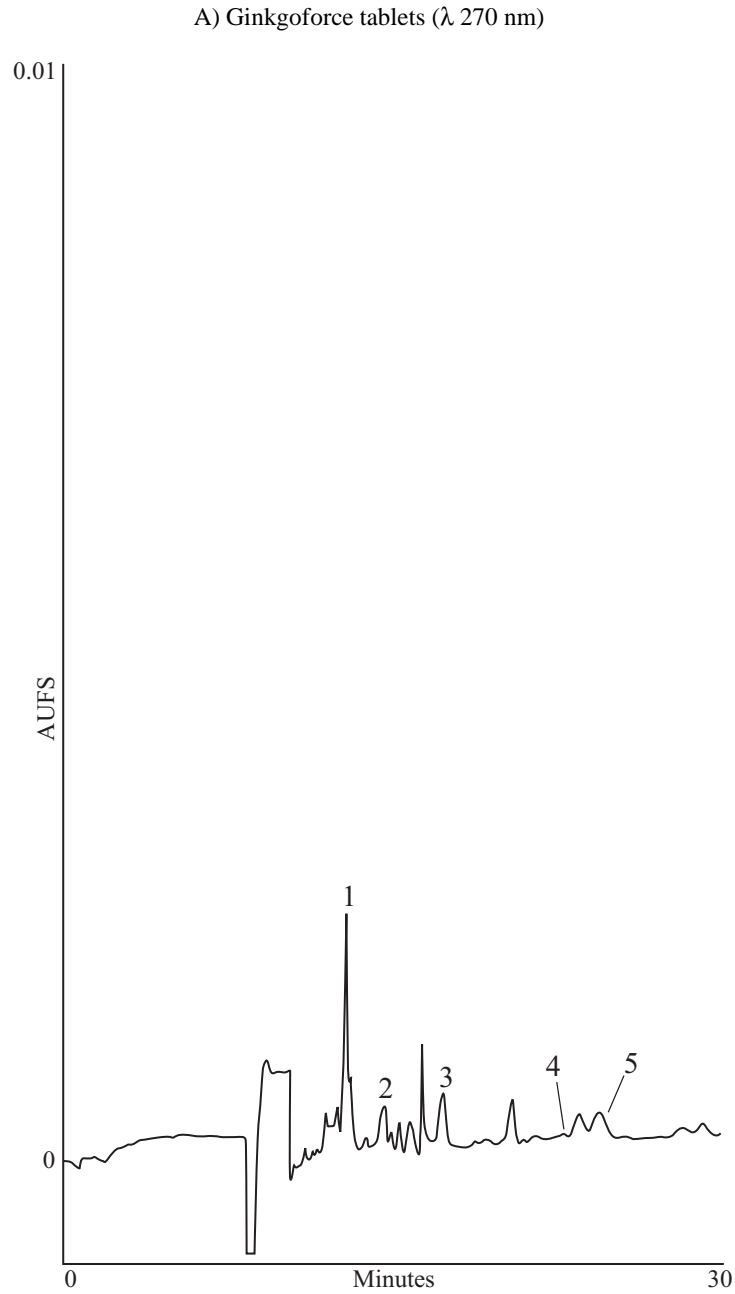
sufficient to remove undissolved components and filtration through a 0.22  $\mu\text{m}$  PVDF membrane (Millipore, Bedford, MA, USA) was required before injection.

#### 3.4.4 Results

The electropherograms with the identified marker compounds present in Product A ( $\lambda$  254 nm, 270 nm and 350 nm) were shown previously in Figure 3.5 A - C. Figures 3.9 A, B and C show Products B and C which were monitored at  $\lambda$  270 nm as well as Product D determined at  $\lambda$  350 nm, respectively. In view of the low concentrations of components in Products B and C, extracts were concentrated by using smaller volumes of solvent as well as changing detector sensitivity from 0.01 to 0.005 AUFS, in order to obtain a baseline fingerprint profile while Product D was monitored at  $\lambda$  350 nm in order to eliminate interferences.

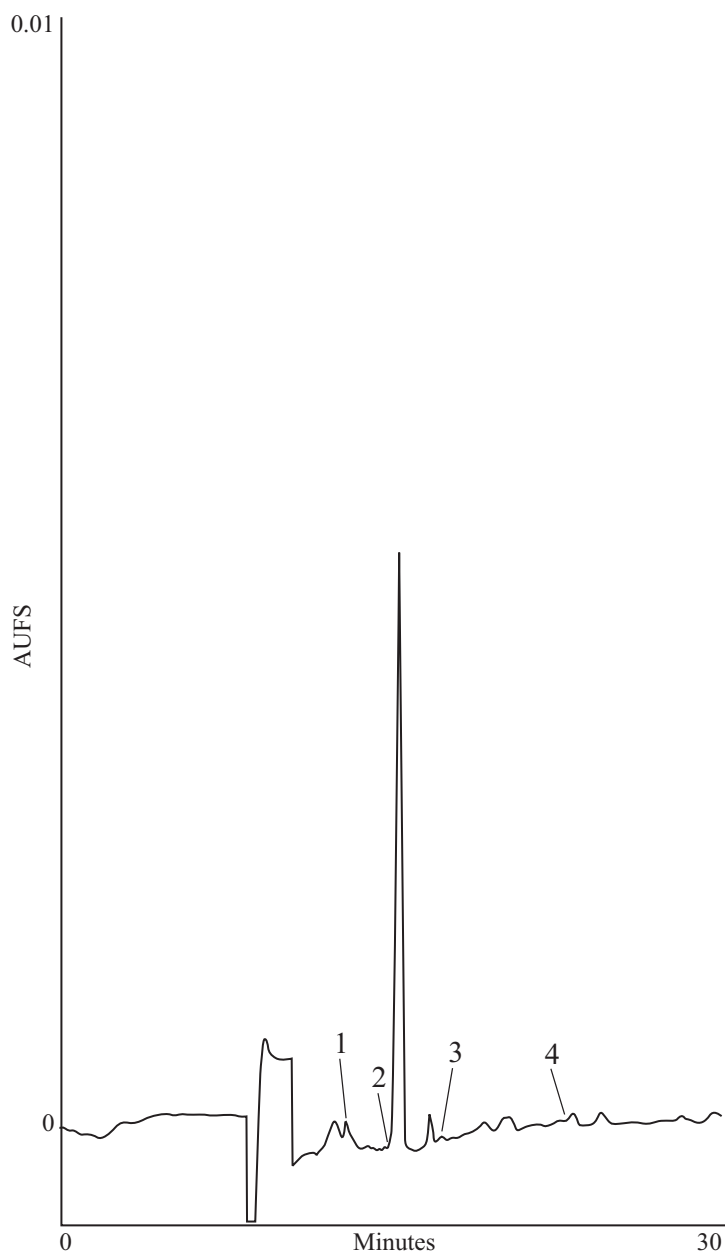


Figure 3.9. Fingerprint profiles of the *Ginkgo biloba* dosage forms



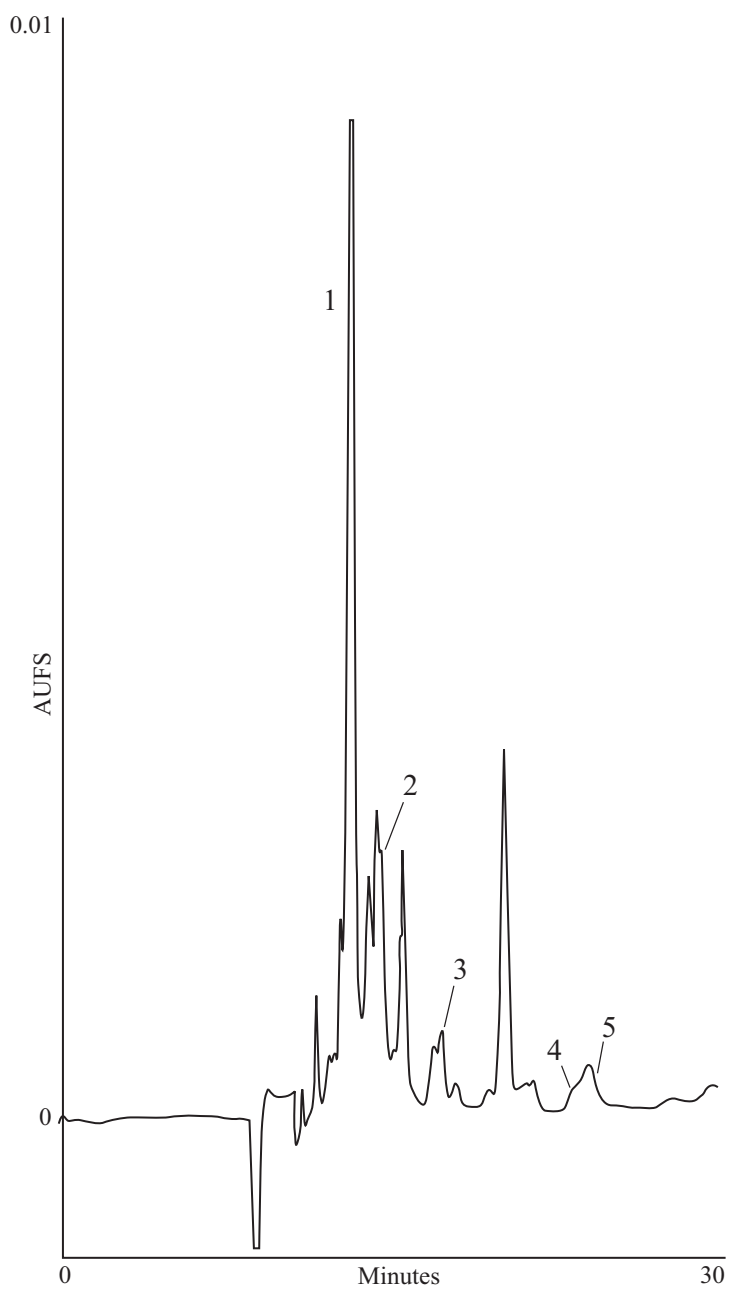
Conditions: Capillary: 50.0  $\mu\text{m}$  I.D., total length = 70.4 cm, effective length = 42.8 cm; Sample buffer: 50:50 methanol-water; Running buffer: 20 mM sodium borate, 50 mM SDS (pH 8.5); Hydrodynamic injection: 200 mbar for 0.1 minutes; Voltage: +15 kV; Detection  $\lambda$ : 350 nm; Labeled peaks: 1=rutin, 2=quercitrin, 3=quercetin, 4=kaempferol, 5=isorhamnetin.

B) Holotropic tablets ( $\lambda$  270 nm)



Conditions: Capillary: 50.0  $\mu\text{m}$  I.D., total length = 70.4 cm, effective length = 42.8 cm; Sample buffer: 50:50 methanol-water; Running buffer: 20 mM sodium borate, 50 mM SDS (pH 8.5); Hydrodynamic injection: 200 mbar for 0.1 minutes; Voltage: +15 kV; Detection  $\lambda$ : 350 nm; Labeled peaks: 1=rutin, 2=quercitrin, 3=quercetin, 4=kaempferol, 5=isorhamnetin.

C) Vital capsule ( $\lambda$  350 nm)



Conditions: Capillary: 50.0  $\mu$ m I.D., total length = 70.4 cm, effective length = 42.8 cm; Sample buffer: 50:50 methanol-water; Running buffer: 20 mM sodium borate, 50 mM SDS (pH 8.5); Hydrodynamic injection: 200 mbar for 0.1 minutes; Voltage: +15 kV; Detection  $\lambda$ : 350 nm; Labeled peaks: 1=rutin, 2=quercitrin, 3=quercetin, 4=kaempferol, 5=isorhamnetin.

### 3.4.5 Discussion

In order to ascertain the quality of the various products, fingerprint profiles are compared based on visual criteria of “integrity and fuzziness” or stated in another way, “sameness and differences” [162]. Fingerprinting provides a simple, effective and rapid means of determining the authenticity of the raw material used in the product i.e. it is possible to determine the integrity of the product based on its profile. Liang *et al.* [162] fingerprinted 17 *Ginkgo biloba* extracts purchased from pharmacies or pharmaceutical companies in China and compared them to the fingerprint of the standardized extract Egb 761®. From the results it could be ascertained that 3 products were adulterated with rutin in order to meet certain QC criteria. The fingerprinting technique could therefore provide a simple, rapid approach to identifying and eliminating false herbal preparations from the commercial market.

In this study, the fingerprints of Products A and D showed remarkable similarities in terms of peak densities with a large number of peaks present in the glycoside migration window which extended from approximately 12 - 16 minutes. After concentration and an increase in detector sensitivity, a comparable profile was observed for Product B while Product C bore little resemblance to any of the other products. The authenticity of Product C may therefore be questionable.

Although some parallels could be drawn from the profiles, major discrepancies in content were apparent in all 4 commercial dosage forms. Product D had the highest content, followed by Product A, then B and finally Product C. Daily dosages (Table 3.2) did not compensate entirely for these disparities. Both products B and C contained very few components in comparison to Products A and D, including the identified selected marker compounds. Product B contained the highest ratio of aglycones versus glycosides compared to all the other extracts and a substantial amount of quercetin was present. From a quality aspect, higher concentrations of glycosides compared to aglycones are preferred since this is indicative of suitable extraction and storage conditions which do not facilitate conversion of the glycosides to aglycones by hydrolysis [53].

Based on these results, it can be deduced that Products A and D are of comparable quality however, Product D was of superior quality to Product A in terms of flavonoid content. The extraction and/or storage conditions of Product B may require further evaluation due to the high aglycone to glycoside ratio. Based on the poor fingerprint profile of Product C, it can be deduced that this product contained very little flavonoid content and the lack of “sameness” compared to the fingerprints of the other products casts reasonable doubt on the authenticity of this product.

### 3.5 CONCLUSIONS

The method originally outlined by Pietta and Mauri [149] describing the separation of 9 flavonoids was used to determine mainly flavonol glycosides in *Ginkgo biloba* leaf extract. Their method was modified and fine tuned to fingerprint 4 *Ginkgo biloba* solid oral dosage forms using 2 flavonol glycosides and 3 flavonol aglycones as the selected marker compounds. In addition, various possible extraction solvents were tested for their selectivity in order to ensure profiles where marker compounds could be relatively easily identified at their UV maxima wavelengths.

Although this method was capable of ensuring high resolution of the peaks of interest, the run time of 28 minutes may be considered exceptionally long for this analysis technique. This may be attributed to the slower migration velocity of the flavonol aglycones which are more effectively solubilized by the SDS micelles than are hydrophilic glycosides. This also resulted in the wider aglycone peak shapes. In fact, in a recent publication [157] in which quercetin, kaempferol and isorhamnetin were analyzed in *Hippophae rhamnoides*, the authors opted for the use of cyclodextrin as modifier in CZE to facilitate separation instead of SDS due to this very reason. Pietta and Mauri's method is therefore particularly suited for the analysis of flavonol glycosides rather than for aglycones. Nevertheless, determination of aglycones together with the flavonol glycosides are very useful QC indicators since ratios of glycoside/aglycones have been shown to provide information of both the quality of the raw material as well as product quality. An increase in the ratio of aglycones to glycosides in extracts suggests degradation [57].

### 3.6 CE METHODS USED FOR THE ANALYSIS OF TERPENE LACTONES

To date, only 1 method has been published addressing the separation of the terpene lactones in *Ginkgo biloba* using CE. A little over a decade ago, Oehrle [163] briefly described an MEKC method for the separation of GA, GB and BB using 25 mM phosphate and 90 mM SDS. The author stated that the initial use of borate, boric acid and SDS buffers proved to be unsuccessful for the separation. Oehrle demonstrated that separation of the 3 terpenes could be achieved within 17 minutes using a capillary with dimensions 50.0  $\mu\text{m}$  I.D. x 360  $\mu\text{m}$  O.D. and a total length of 60.0 cm. UV detection was set at 185 nm and the oven temperature was maintained at 30°C. A stock solution (1 mg/ml) of the reference standards was prepared in methanol and further dilutions were made with running buffer in order to achieve concentrations of 1.12 mg/ml for all relevant compounds. A "terpene mix" was additionally analyzed which had a final concentration of 0.11 mg/ml. Sampling times were 5 seconds for the reference standards and increased to 15 seconds for the "mix". BB migrated ahead of GA and GB and reached the detector after approximately 10 minutes, followed by GA (16.4 minutes) and GB (16.6 minutes).

Oehrle's method however had a number of shortcomings. The resolution between GA and GB was not complete and three essential parameters were not stipulated, namely, the pH of the running buffer, the voltage and the injection mode. The pH of the running buffer is of notable significance for the optimization of resolution as illustrated in section 3.3.2. Of most critical importance however it the fact that BB degrades above pH 7 [53, 56]. To show its applicability, the method was used to qualitatively analyze a so-called "terpene mix" but no references were given regarding the source or the reasons for analyzing that "mix". No extraction procedures were described which implied that the "mix" was prepared by dissolving the Ginkgo extract in methanol with dilution using running buffer as described for the reference standards. This method was not validated and displayed no data pertaining to linearity, range, repeatability or accuracy.

Despite these obvious flaws, this method was used as a starting point for method development using GA, GB, GC and BB as markers. The difficulty and expense of procuring these reference compounds necessitated their economic use, at times excluding some of them during method development. GC was mostly used to investigate the effects of various parameters on the migration of the terpenoids since a sufficient amount of this reference standard was generously supplied by Dr. Egon Koch of Willmar Schwabe Pharmaceuticals. GJ was procured somewhat later when method development was almost complete.

### **3.7 METHOD DEVELOPMENT OF THE TERPENOIDS AT HIGH PH**

#### **3.7.1 Instrumentation**

##### *3.7.1.1 The CE system*

All experiments were performed using a PrinCE electrophoresis system Model 0500-002 (Lauerlabs, Emmen, The Netherlands) and a Linear UV/VIS 200 detector (Linear Instruments Corporation, Reno, NV, USA). A polyimide fused-silica capillary column (78  $\mu\text{m}$  I.D. x 360  $\mu\text{m}$  O.D.) was used for the separation which was supplied by Polymicro Technologies, Phoenix, AZ, USA.

##### *3.7.1.2 Additional equipment*

A Perkin Elmer R100A Strip Chart Recorder, Model C0050005 (Perkin Elmer Corporation, Illinois, USA) was used to record the electropherogram. A Mettler Dual Range Electronic Balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland) was used to weigh reagents and standards. A Crison GLP21 pH Meter (Crison, Barcelona, Spain) was used to measure and adjust the pH values of relevant solutions.

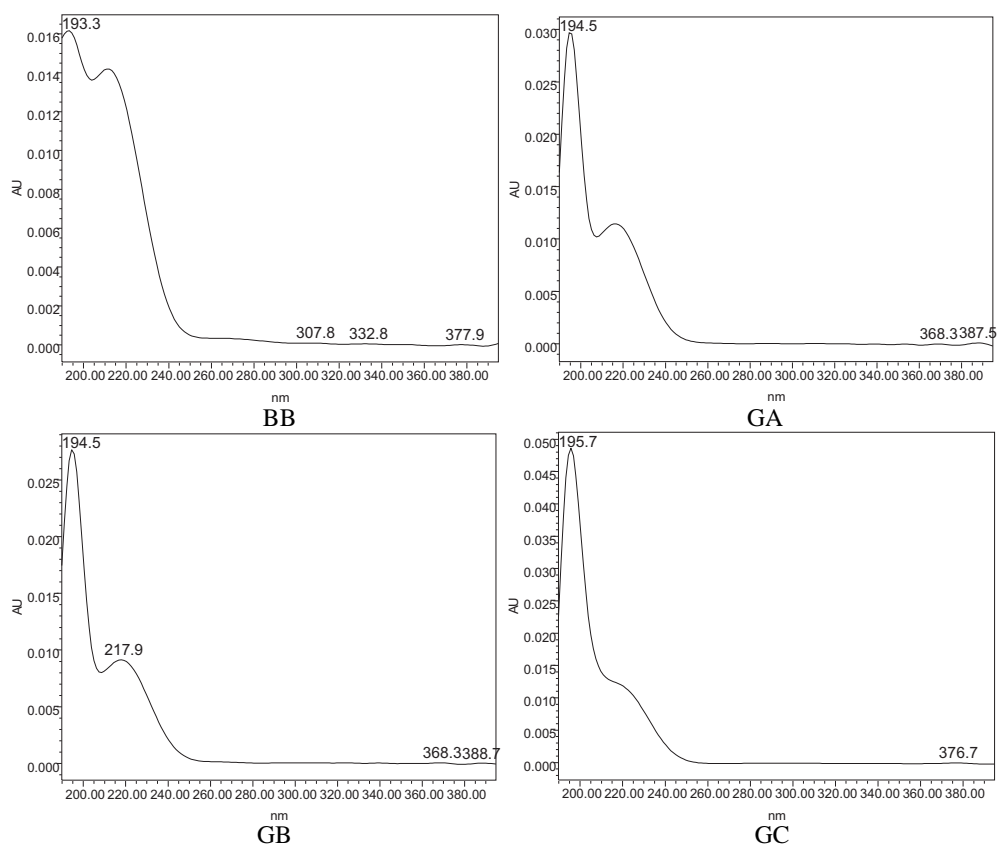
### 3.7.2 Materials and reagents

Methanol (HPLC grade) and sodium hydroxide pellets were purchased from BDH chemicals (Poole, UK), disodium hydrogen phosphate (analytical grade) from Roche Chemicals (Isando, Johannesburg, South Africa) and phosphoric acid (analytical grade) from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), GA (90%), GB (90%) and BB (95%) were purchased from Sigma (St. Louis, MO, USA). GC (90%) was a generous gift from Dr. Egon Koch of Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). The peak purity of the terpene trilactones used as reference standards were checked chromatographically and confirmed by NMR (Nuclear Magnetic Resonance) spectroscopy. Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA).

### 3.7.3 Initial experimental conditions and results

A capillary with a total length of 60.0 cm and effective length of 45.0 cm was used and conditioned as described in section 3.3.1.4. Our CE instrument was not able to adjust the operating temperatures however and experiments were therefore performed at ambient temperature (22°C). Since the pH of the buffer was not specified, a 25 mM solution using disodium hydrogen phosphate was prepared and the pH was measured at 9.3. Stock solutions of the reference standards were prepared in methanol and then diluted with running buffer to approximate concentrations of 0.1 mg/ml. Samples were injected hydrodynamically at 200 mbar for 0.03 minutes. The separation voltage was set at +15 kV with a voltage ramp of +6 kV/s. Detection was at  $\lambda$  190 nm, based on the UV spectra of the terpenoids shown in Figure 3.10.

Figure 3.10. UV spectra of BB, GA, GB, GC and BB



Initial experiments on the migration of GA, GB, GC and BB in the sample solution did not yield reproducible results and in an attempt to achieve a measure of consistency, numerous parameters were investigated. An increase in sample loading, field amplified sample injection (FASI) and lowered sample ionic strength was investigated to compensate for the low absorbance of the ginkgolides. Thereafter, the conditioning of the capillary was altered from the above mentioned procedure to washing with 10 minutes water and then 5 minutes buffer prior to injecting. A third experimental washing procedure involved conditioning the capillary with buffer only for 3 minutes in order to avoid a quasi-stable state caused by capillary conditioning with sodium hydroxide, a state which is augmented when using phosphate buffer due to its absorbing properties [142]. In order to ensure that excessively long run times were avoided, the voltage was increased incrementally to a maximum of +30 kV. Increased voltages resulted in lower efficiency however and aberrations in the baseline due to joule heat production resulted in the voltage of +15 kV being reinstated. The pH of the running buffer was also altered but kept in the high pH range of between 7 and 10. Lower pH values were attempted using positive applied voltage, however no peaks were obtained due to excessive suppression of the EOF.

In order to limit the use of reference materials and simplify peak identification, the reproducibility of GC only was investigated. The effects of ionic strength, SDS concentration, sample dilution and voltage on GC's migration time and peaks heights were determined. The current was monitored as an indicator of



joule heat production. Due to the noisy baseline in previous experiments, a smaller diameter capillary tubing (50  $\mu\text{m}$  I.D. x 360  $\mu\text{m}$  O.D.) with the same length was used in order to aid heat dissipation. Samples were dissolved in running buffer and were injected electrokinetically at +5 kV for 0.5 minutes. The capillary was conditioned between consecutive injections with 5 minutes of running buffer.

The optimal electrophoretic conditions for GC were determined and then also used for the analysis of GA, GB and BB. The running buffer consisted of 40 mM SDS and 25 mM disodium hydrogen phosphate (pH 9.2). Sample solutions (~1.0 mg/ml) were prepared in an electrolyte of lower ionic strength (10 mM disodium hydrogen phosphate and 40 mM SDS) and injected electrokinetically at +5 kV for 0.33 minutes. The applied voltage was +15 kV with a voltage ramp of +6kV/s. However, some degradation of the ginkgolides and BB occurred under these conditions. This has been confirmed by Van Beek [53] who cited the work of Fourtillin *et al.* (Thérapie 50 (1995) p. 137) that BB degrades above pH 7. This method at alkaline pH was therefore found to be unsuitable for the analysis of the terpene lactones and may explain initial difficulties in obtaining reproducible results. Further investigations at low pH were therefore required in order to determine the validity of the original method published by Oehrle [163].

### **3.8 METHOD DEVELOPMENT FOR TERPENOIDS AT LOW PH**

#### **3.8.1 Effect of sample loading on the resolution between GA and GB**

##### *3.8.1.1 Instrumentation and materials*

All experiments during development were performed using the equipment described in 3.7.1 and all materials and reagents were sourced as stated in section 3.7.2. A Vortexer model G-560E from Scientific Industries, Bohemia, N.Y., USA was used for sample mixing. HVLP filters were purchased from Millipore, Bedford, MA, USA.

##### *3.8.1.2 Capillary conditioning*

The washing procedures for unused capillaries and initial daily capillary conditioning were performed as described in section 3.3.1.4 and between consecutive injections, the capillary was conditioned with 15 minutes of buffer only. To ensure reproducibility, the buffer at the cathode was replaced after every injection while the anodic buffer was replenished after every third injection.

##### *3.8.1.3 Initial experimental conditions*

A capillary with a total length of 60.0 cm and effective length of 42.0 cm was used. The BGE comprised of 25 mM phosphoric acid and 40 mM SDS (pH 3) and was prepared by dispersing the appropriate amount of

phosphoric acid in HPLC grade water which was then filtered through a 0.45  $\mu\text{m}$  HVLP type filter. The surfactant was weighed and decanted into a volumetric flask and a sufficient volume of phosphoric acid solution was added for dissolution. The mixture was sonicated until the SDS dissolved completely. The solution was allowed to reach room temperature before filling it to volume with the same phosphoric acid solution. The buffer pH was altered using a 1 M sodium hydroxide solution. The separation voltage was -10 kV with a voltage ramp of -6 kV/s and samples were injected electrokinetically at -5 kV for 0.5 minutes. Detection was effected at  $\lambda$  220 nm with a rise time of 0.1 seconds and sensitivity of 0.01 AUFS.

#### *3.8.1.4 Preparation of reference standards*

Separate stock solutions of the reference standards were prepared by accurately weighing 1.15 mg of GA and 1.07 mg of GB into separate vials and then dissolving them in 2 ml of a 10 mM phosphoric acid and 40 mM SDS (pH 2.8) buffer solution. A mixture of the reference compounds was prepared by pipetting equal amounts of each stock solution into a vial and the sample vortexed for 30 seconds before injecting onto the capillary.

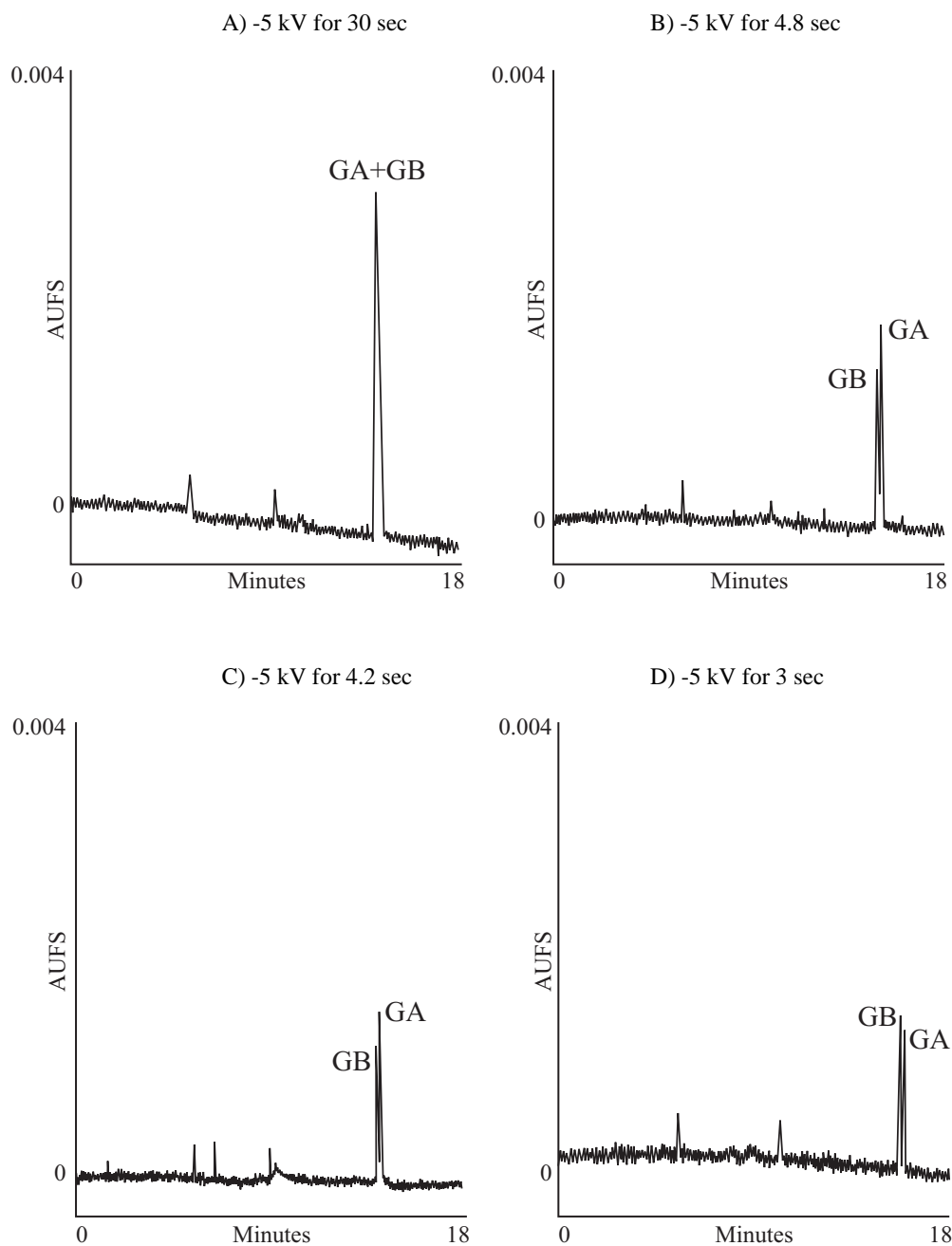
#### *3.8.1.5 Experimental Procedure*

The effect of sample loading on the resolution of GA and GB was investigated by systematically injecting sample solutions of the reference compounds at -5 kV for 30 seconds, 20 seconds, 4.8 seconds, 4.2 seconds and 3 seconds.

#### *3.8.1.6 Results*

Since separation between GA and GB proved to be problematic, initial method development at low pH was performed using these 2 reference compounds only. In contrast to the results observed under alkaline conditions, injection of the individual stock solutions at low pH resulted in a single peak observed for GA and GB. Initially the sample solutions were injected at -5 kV for 30 seconds since this was the injection time used to obtain an appropriate response at high pH. When the mixture was injected, only one peak which was slightly broader compared to the individually injected peaks was observed which indicated co-migration and smaller sample loads were therefore investigated. Injection of the sample for 20 seconds only caused a decrease in peak height with no improvement in resolution. The injection times were then drastically reduced to 4.8, 4.2 and 3 seconds. Sample loading for 4.8 and 4.2 seconds resulted in decreasing peak heights with smaller sample injections but also improved separation. Baseline resolution was achieved when the sample was injected at -5 kV for 3 seconds and a further decrease in sampling time was therefore not attempted. Separation is shown in Figure 3.11. GB migrated ahead of GA which is the reverse order reported by Oehrle [163].

Figure 3.11. Effect of sample loading on the separation of GA and GB



Conditions: Capillary: 78  $\mu\text{m}$  I.D., total length = 60.0 cm, effective length = 42.0 cm; Sample buffer: 10 mM phosphoric acid, 40 mM SDS (pH 2.8); Running buffer: 25 mM phosphoric acid, 40 mM SDS (pH 3); Electrokinetic injection: Voltage: -10 kV; Detection  $\lambda$ : 220 nm; GA=ginkgolide A, GB=ginkgolide B.

### 3.8.1.7 Discussion

Sample loading contributed significantly to the resolution between GA and GB. GB only differs from GA in the positioning of a hydroxyl group at R<sup>1</sup> (Figure 2.2) and their structural similarity posed difficulties in achieving both chromatographic and electrophoretic separation. The ginkgolides lack strong chromophores and hence require extracts to be concentrated and/or higher sample loads to be injected to achieve an appropriate response. In CE however, only small sample volumes can be injected in order to ensure optimal efficiency [136] and a sample load at -5 kV for only 3 seconds facilitated optimal resolution and acceptable sensitivity of these 2 compounds.

### 3.8.2 Effect of capillary washing on the separation between GA and GB

#### 3.8.2.1 Experimental Procedure

The effect of capillary conditioning between consecutive injections on the resolution of GA and GB was investigated. Initial conditions including capillary washing procedures were the same as outlined in section 3.8.1.2 and 3.8.1.3. The same sample mixture of GA and GB that was prepared in section 3.8.1.4 was injected at -5kV for 3 seconds. The experimental washing programmes between consecutive injections are shown in Table 3.3. The flushing pressure was set at 2 000 mbar for all the steps involved in the washing sequences.

Table 3.3. Investigated washing procedures on the resolution of GA and GB

Method Number	Washing procedure
1	5 min BGE
2	15 min BGE
3	2 min 1 M NaOH, 2 min 0.1 M NaOH, 2 min distilled water, 5 min BGE
4	1 min distilled water, 1 min 1 M NaOH, 1 min methanol, 2 min 0.1 M NaOH, 2 min distilled water, 3 minutes BGE
5	10 min 0.1 M NaOH, 5 min distilled water, 5 min BGE
6	10 min 1 M NaOH, 5 min distilled water, 5 min BGE

### 3.8.2.2 *Results and Discussion*

Following the investigation to determine the effect of buffer pH on the resolution between GA and GB which produced irreproducible results, the capillary was replaced with freshly cut, conditioned new tubing since it was evident that the charge density on the capillary surface was no longer sufficient to facilitate separation. This however did not resolve the inconsistency and a further investigation into the washing procedures between consecutive injections was necessary since capillary cleaning is a major determinant of precision and accuracy [132].

Conditioning with buffer only between consecutive injections is normally recommended when using phosphate buffer [142] however it was found that methods 1 and 2 were both incapable of producing reproducible capillary surface conditions and results. Similarly, GA and GB co-migrated when method 3 was used. Method 5 delivered reproducible results with improved separation while consistent baseline resolution was achieved when method 6 was employed. Seven consecutive injections were performed using methods 5 and 6 washing procedures in order to ensure reproducibility. Although method 6 produced the most consistent results, this procedure was rather time consuming. The lengthy equilibration time was necessary due to the fact that conditioning of negatively charged silanol groups on the capillary wall with sodium hydroxide while working at low pH may cause hysteresis and moreover, phosphate buffer is known to adsorb to capillary surfaces. These two factors therefore necessitated a more extensive washing procedure [133]. Conditioning with an organic solvent (method 4) which was used by Wang *et al.* [164] while working at low pH was therefore attempted. This method did not produce satisfactory results however and GA and GB once again co-migrated. Method 6 was therefore selected as the optimal washing protocol and used in further experiments.

Capillaries coated with polyacrylamide are often used in protein analysis or separations involving high molecular mass compounds to prevent adsorption onto the capillary wall. This leads to improved reproducibility and precision [165, 166]. Janini *et al.* [167] and Jandera *et al.* [166] used polyacrylamide coated capillaries for their studies in a reduced flow environment at low pH. The use of coated capillaries could possibly decrease equilibration times between consecutive injections compared to using uncoated fused polyimide silica capillaries. Only the latter type capillaries were however available and were used throughout the course of this research.

### 3.8.3 Electropherograms of the terpene trilactones at low pH

#### 3.8.3.1 *Instrumentation and materials*

All experiments were performed using the equipment described in 3.7.1 and 3.8.1.1 and all materials and reagents were sourced as previously stated in section 3.7.2.

### 3.8.3.2 *Capillary conditioning*

The washing procedures for newly cut capillaries and initial daily capillary conditioning were performed as described in section 3.3.1.4 and between consecutive injections, the capillary was conditioned with method 6 described in Table 3.3 (Section 3.8.2.1). To ensure reproducibility, the buffer at the cathode was replaced after every injection while the anodic buffer was replenished after every third injection.

### 3.8.3.3 *Initial experimental conditions*

A capillary with a total length of 60.0 cm and effective length of 45.0 cm was used for the separation. The BGE comprised of 25 mM phosphoric acid and 40 mM SDS (pH 2.8). The separation voltage was increased to -15 kV with a voltage ramp of -6 kV/s to decrease migration time and samples were injected electrokinetically at -5 kV for 3 seconds. The detection wavelength was 190 nm with a rise time of 0.1 seconds and sensitivity of 0.01 AUFS.

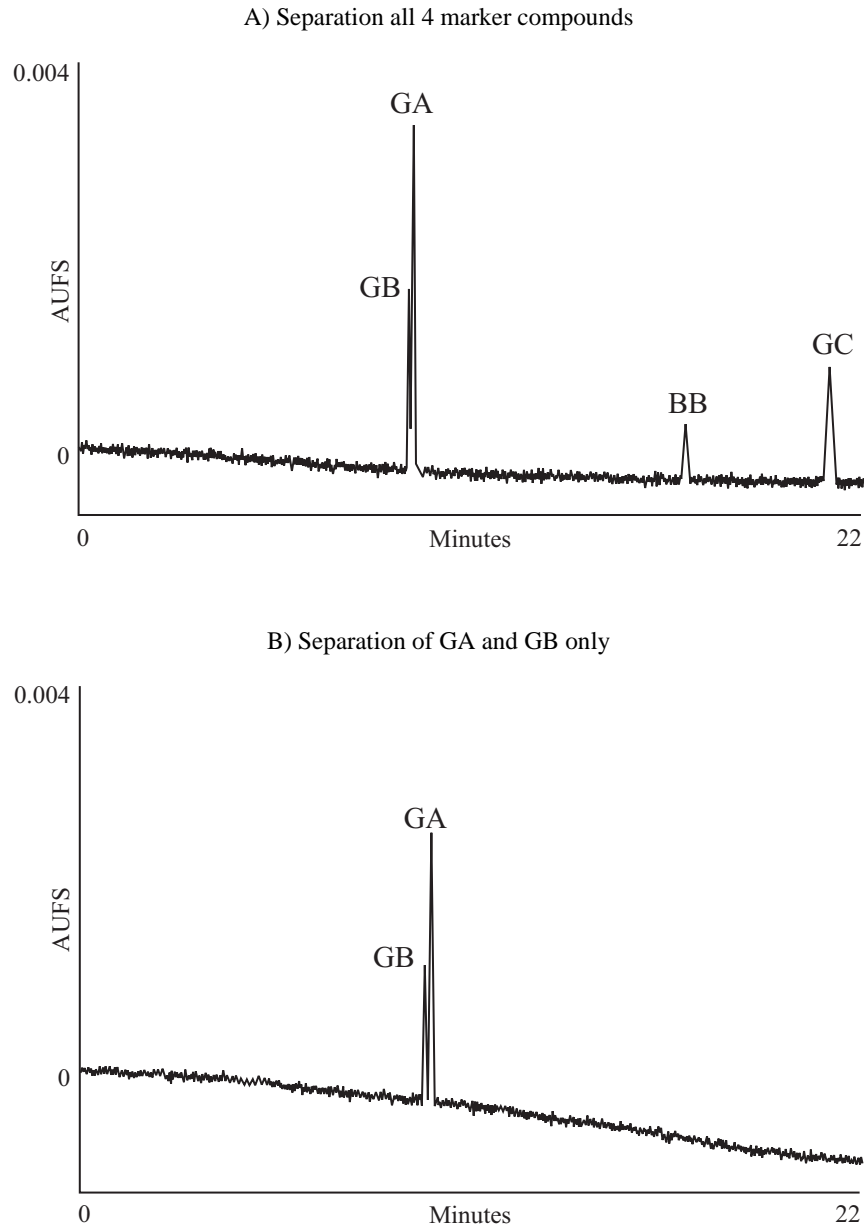
### 3.8.3.4 *Preparation of reference standards*

After individual injections of GA, GB, GC and BB were performed in order to determine the migration order, a mixture containing all 4 reference compounds was made by dissolving 1.50 mg, 1.12 mg, 1.10 mg and 1.05 mg of the respective compounds in 2 ml buffer consisting of 10 mM phosphoric acid and 40 mM SDS (pH 2.8).

### 3.8.3.5 *Results*

Figure 3.12 A shows the separation of all 4 marker compounds and Figure 3.12 B shows the separation when only GA and GB were injected. The run time was 21 minutes and the migration order was GB, GA, BB and then GC. During this method development, the reference compound GJ was not available.

Figure 3.12. Electropherogram of GA, GB, GC and BB at pH 2.8



Conditions: Capillary: 78  $\mu\text{m}$  I.D., total length = 60.0 cm, effective length = 42.0 cm; Sample buffer: 10 mM phosphoric acid, 40 mM SDS (pH 2.8); Running buffer: 25 mM phosphoric acid, 40 mM SDS (pH 3); Electrokinetic injection: -5 kV for 3 sec.; Voltage: -15 kV; Detection  $\lambda$ : 190 nm; Labeled peaks: GA= ginkgolide A, GB=ginkgolide B, GC=ginkgolide C; BB=bilobalide.

### 3.8.3.6 Discussion and conclusions

Baseline separation between GA, GB, GC and BB was achieved and no degradation was evident at low pH. Migration orders were the reverse of those describe by Oehrle [163] with GB migrating ahead of GA, followed by BB and then GC.

Suppression of the EOF under acidic conditions necessitated the application of a negative separation voltage (change of polarity) at the injection end of the capillary. This facilitated repulsion of the anionic SDS micelles from the cathode and migration of the negatively charged analytes towards the positive electrode at the detector end of the capillary at a velocity which superseded that of the non-ionic components. Compounds with greater distribution co-efficients towards the hydrophobic micellar core therefore migrated more rapidly than the more hydrophilic compounds. MEKC performed under acidic conditions where the EOF is suppressed is referred to as reduced flow micellar electrokinetic chromatography (RF-MEKC). Migration orders are therefore typically the reverse of those observed under alkaline conditions where the EOF allows for rapid migration of hydrophilic compounds. This conventional MEKC mode is known as normal micellar electrokinetic chromatography or N-MEKC [166].

It is apparent that Oehrle [163] used N-MEKC due to the migration order of the reference compounds where BB migrated ahead of GA and GB migrated last. N-MEKC is however not suitable for the ginkgolides since this technique has to be performed under alkaline conditions and BB is unstable above pH of 7. Oehrle's method could not be repeated in our laboratory even after numerous time-consuming attempts were made. Based on these facts, it was surmised that that his method is probably unreliable and thus inappropriate for the analysis of terpenoids.

Since RF-MEKC is suited for the analysis of hydrophobic compounds [166] and the flavonol aglycones have hydrophobic properties, this method was further developed for the simultaneous analysis of both the selected flavonol and terpenoid marker compounds.

## **3.9 DEVELOPMENT OF A SIMULTANEOUS METHOD FOR THE DETERMINATION OF FLAVONOLS AND TERPENOID**

### 3.9.1 Effect of organic solvents on the separation of the flavonols and terpenoids

#### 3.9.1.1 Instrumentation and materials

All experiments were performed using the equipment and some materials described in section 3.7.1 and 3.7.2, respectively. Acetonitrile and 1-propanol (all HPLC grade) were purchased from BDH chemicals



(Poole, UK) Quinine hydrochloride,  $\beta$ -cyclodextrin, rutin (85%), quercetin (85%), GA (90%), GB (90%) and BB (95%) were purchased from Sigma (St. Louis, MO, USA). Quercitrin (99%) was supplied by Phytolab (Hamburg, Germany) and kaempferol (95%) and isorhamnetin (90%) from Indofine Chemical Company (New Jersey, USA). GJ (95%) was purchased from Chromadex (Santa Ana, CA, USA). GC (90%) was a generous gift from Dr. Egon Koch of Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). The purity of the flavonol reference standards were assumed as provided by the suppliers. The peak purity of the terpene trilactones used as reference standards were checked chromatographically and confirmed by NMR spectroscopy. Water was purified in a Milli-Q system and 0.45  $\mu$ m HVLP filters (both supplied by Millipore, Bedford, MA, USA).

#### *3.9.1.2 Capillary conditioning*

Capillary conditioning was the same as previously described as in section 3.8.3.2.

#### *3.9.1.3 Initial CE conditions*

CE conditions were as stated in section 3.8.3.3. The flavonols were monitored at  $\lambda$  365 nm and the terpenoids at  $\lambda$  190 nm.

#### *3.9.1.4 Preparation of reference standards*

Since the flavonols are soluble in aqueous alkaline solutions, individual stock solutions of the flavonols were initially prepared in methanol and then diluted with the usual buffer solution consisting of 10 mM phosphoric acid and 40 mM SDS (pH 2.8). Solutions of rutin, quercitrin, quercetin and kaempferol were prepared by dissolving 0.5 mg in 200  $\mu$ l methanol and then adding 3 ml of buffer with a micropipette to give concentrations of 156.3  $\mu$ g/ml. Isorhamnetin was less soluble and 0.16 mg was dissolved in 3 ml 50% methanol-buffer to give a concentration of 53.3  $\mu$ g/ml. A mixture of all 5 reference compounds was prepared by the addition of 200  $\mu$ l of the rutin (62.5  $\mu$ g/ml) and isorhamnetin (21.3  $\mu$ g/ml) stock solutions and 100  $\mu$ l of the quercitrin, quercetin and kaempferol (31.3  $\mu$ g/ml each) stock solutions into a vial which was then vortexed for 30 seconds to ensure thorough mixing before injecting.

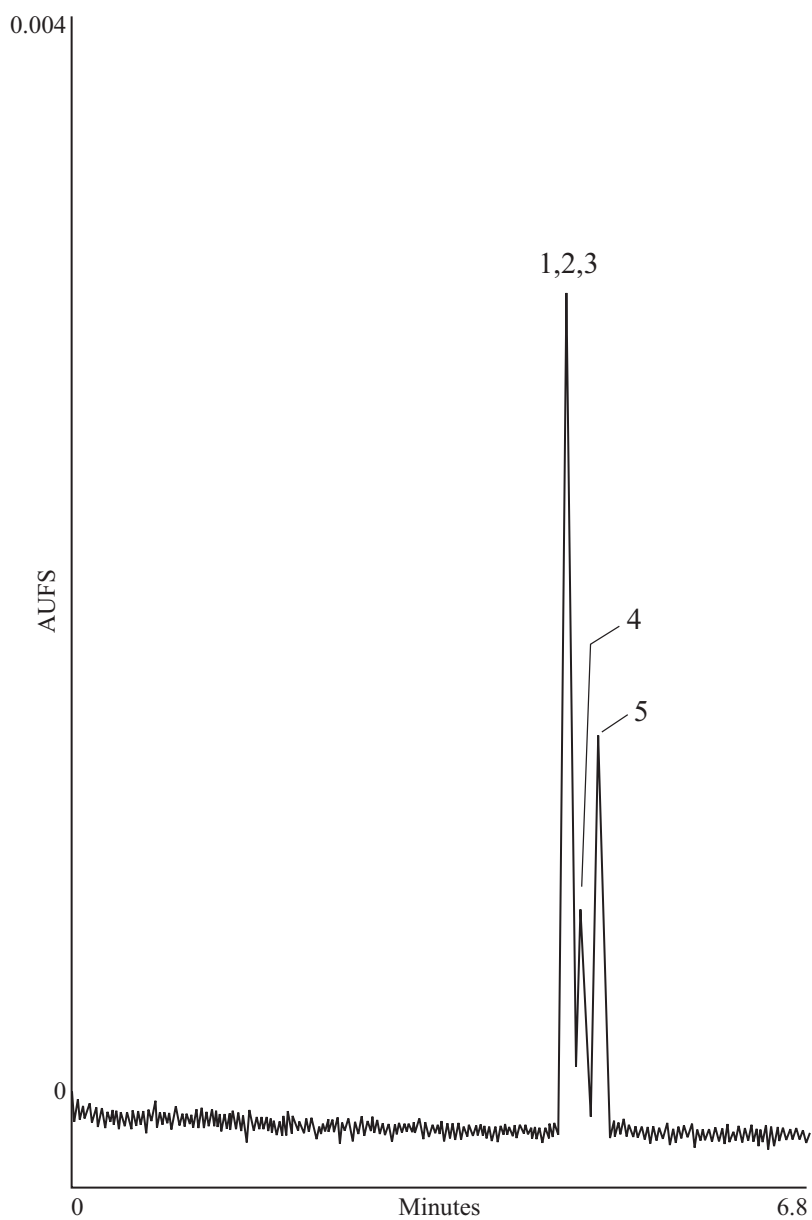
#### *3.9.1.5 Experimental Procedure*

The effect of the inclusion of various percentages of methanol (2, 5, 6, 7, 10, 20 and 30), 1-propanol (2, 5, 7, and 10) and acetonitrile (2, 7, 10, and 20) in the BGE on the separation of the flavonols was investigated.

#### 3.9.1.6 Results

Figure 3.13 shows the electropherogram of the flavonols injected using the identical conditions which separated the terpenoids. Isorhamnetin, kaempferol and quercetin migrated at the same velocity and only one peak was detected for these compounds. Thereafter quercitrin reached the detection window, followed by rutin. The voltage was then reduced to -10 kV and the pH was investigated over a range of 1.4 to 3.6 in an attempt to increase resolution however no pronounced effect on the separation was observed. Buffer additives in the BGE were then explored.

Figure 3.13. Electropherogram of the 5 selected flavonol marker compounds at pH 2.8



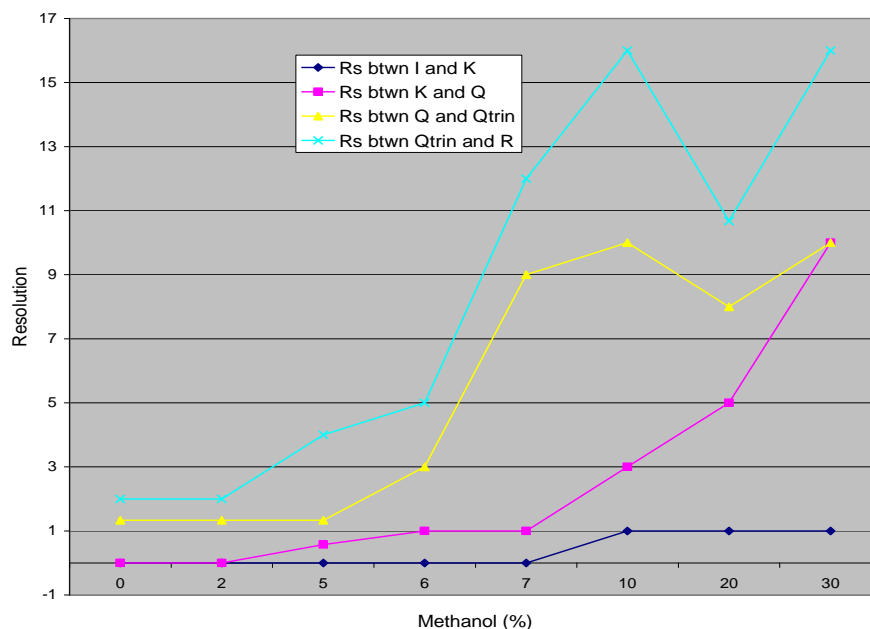
Conditions: Capillary: 78  $\mu\text{m}$  I.D., total length = 60.0 cm, effective length = 42.0 cm; Sample buffer: 10 mM phosphoric acid, 40 mM SDS (pH 2.8); Running buffer: 25 mM phosphoric acid, 40 mM SDS (pH 3); Electrokinetic injection: -5 kV for 3 sec.; Voltage: -10 kV; Detection  $\lambda$ : 365 nm; Labeled peaks: 1=rutin, 2=quercitrin, 3=quercetin, 4=kaempferol, 5=isorhamnetin.

The effect of methanol on the resolution of the flavonols is shown graphically in Figure 3.14. Resolution was calculated according to the following equation:

$$Rs = 2 (t_2 - t_1) / (w_1 + w_2) \quad [3.4] \quad t = \text{migration time (sec)}$$

$$w = \text{temporal peak width at baseline (sec)}$$

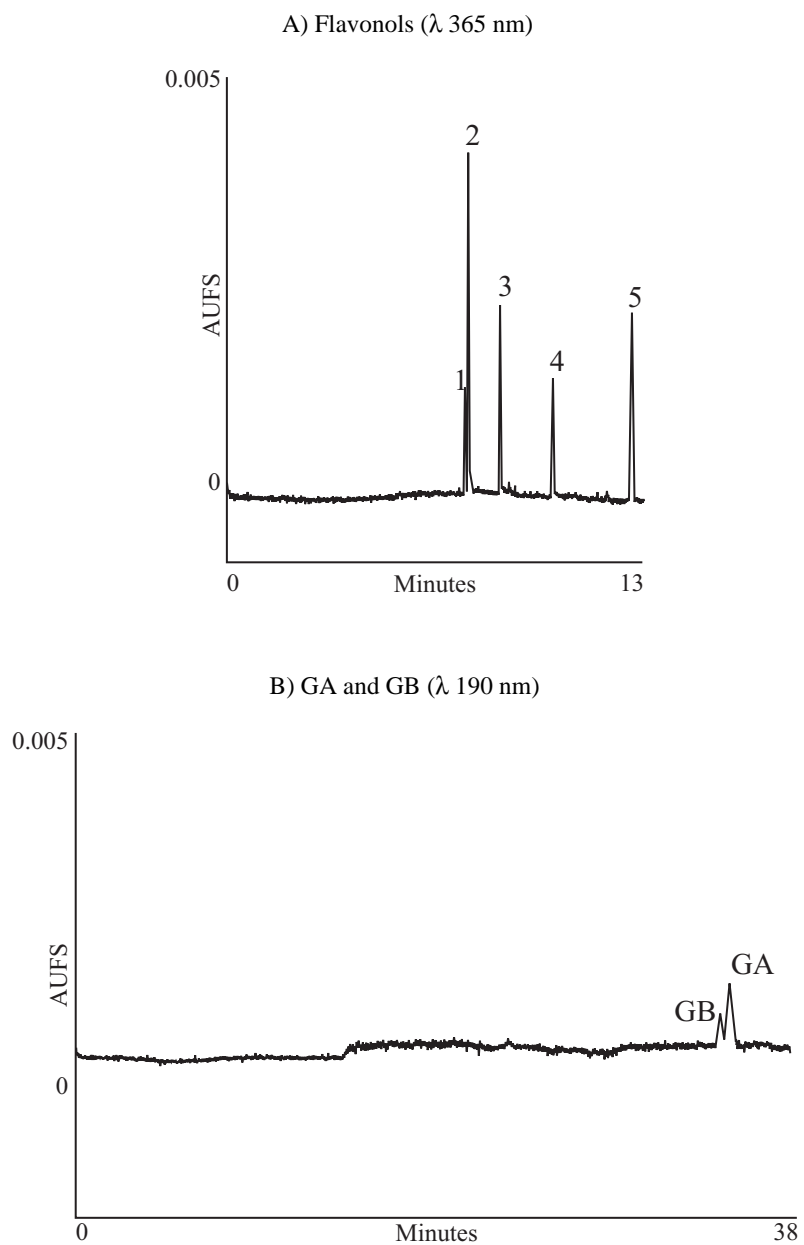
Figure 3.14. Effect of methanol on the resolution of the flavonols



Labels: I=isorhamnetin, K=kaempferol, Q=quercetin, Qtrin=quercitrin, R=rutin

With increasing methanol concentrations, the migration times increased but so did the resolution. Baseline resolution within 13 minutes was obtained using 30% methanol (Figure 3.15 A). When a sample solution of the terpenoids was injected under these conditions however, GA and GB reached the detection window after 35 minutes and the peaks were broad and not well separated as shown in Figure 3.15 B.

Figure 3.15. Electropherograms showing the effect of 30% methanol in the running buffer

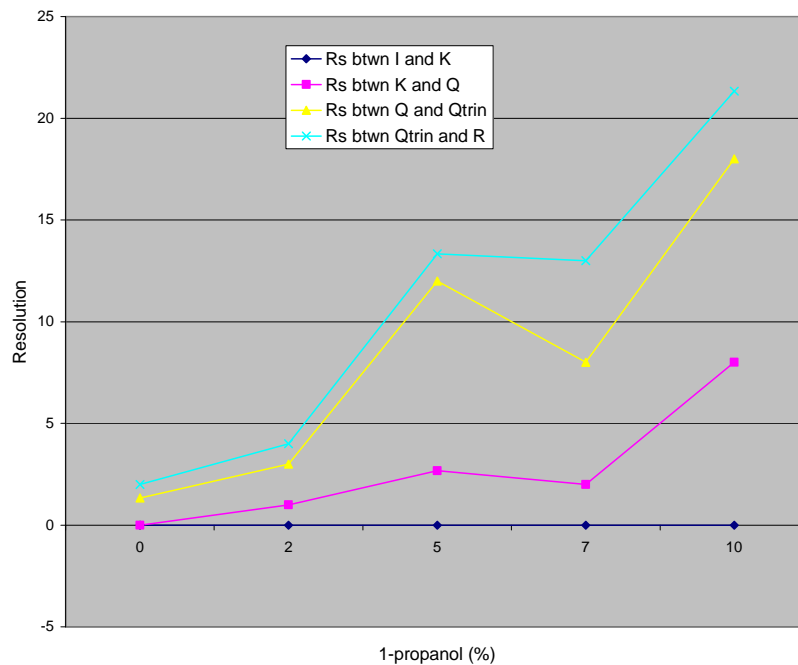


Conditions: Capillary: 78  $\mu$ m I.D., total length = 60.0 cm, effective length = 42.0 cm; Sample buffer: 10 mM phosphoric acid, 40 mM SDS (pH 2.8); Running buffer: 25 mM phosphoric acid, 40 mM SDS, 30% MeOH; Electrokinetic injection: -5 kV for 3 sec.; Voltage: -10 kV; Labeled peaks: 1=isorhamnetin, 2=kaempferol, 3=quercetin, 4=quercitrin, 5=rutin, GB=ginkgolide B, GA=ginkgolide A.

The effect of 1-propanol on the resolution of the flavonols is shown graphically in Figure 3.16. As with methanol, the addition of 1-propanol increased the migration time of all the flavonols. Resolution between

isorhamnetin and kaempferol was not achieved and the decrease in resolution at the inclusion of 7% 1-propanol was due to broader peak widths.

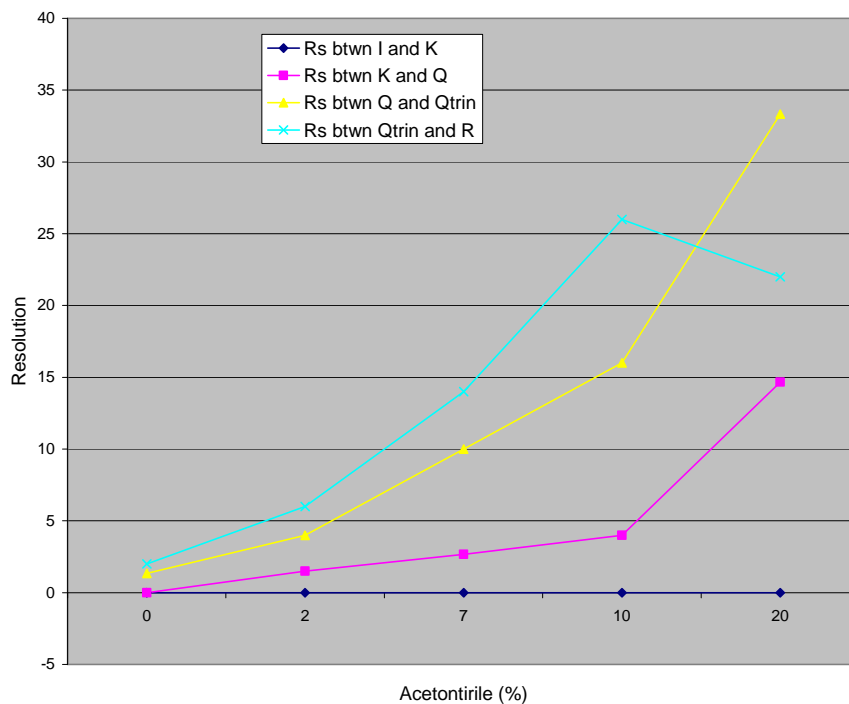
Figure 3.16. Effect of 1-propanol on the resolution of the flavonols



Labels: I=isorhamnetin, K=kaempferol, Q=quercetin, Qtrin=quercitrin, R=rutin

The effect of acetonitrile on the resolution of the flavonols is shown in Figure 3.17. As previously discussed, the organic solvent effects with an increase in acetonitrile percentages in the BGE resulted in longer migration times and improved resolution. Acetonitrile did not however facilitate separation between isorhamnetin and kaempferol. The marked decrease in resolution between quercitrin and rutin with the inclusion of 20% acetonitrile was due to the broader rutin peak caused by the excessive migration time of 29 minutes with consequential sample zone broadening.

Figure 3.17. Effect of acetonitrile on the resolution of the selected flavonols



Labels: I=isorhamnetin, K=kaempferol, Q=quercetin, Qtrin=quercitrin, R=rutin

### 3.9.1.7 Discussion

The flavonols are acidic compounds with pKa values of approximately 6.8 (Section 2.2.2). A change of buffer pH while working under acidic conditions did not affect the ionization of these compounds and no change in the resolution of the selected markers were observed.

The addition of organic solvents into the BGE generally results in an increase in buffer viscosity and limits the interaction of the analytes with the micelle by rendering the aqueous phase more amenable to hydrophobic analytes. This results in an increase in migration time of these compounds but also improved selectivity [167]. Migration times were the longest using acetonitrile which has a polarity index 6.2, followed by methanol (polarity index of 5.1) and then 1-propanol (polarity index of 4.3). Acetonitrile was therefore the most hospitable towards solubilization of the flavonols. Methanol was the most selective of the 3 solvents however and baseline resolution was obtained between isorhamnetin and kaempferol which was not observed with either acetonitrile or 1-propanol. On the other hand, the inclusion of methanol in the running buffer had a negative impact on both the migration time and resolution of GA and GB and therefore it was not suitable for the simultaneous analysis of the flavonols and terpenoids.

### 3.9.2 Selection of a suitable micellar marker and possible internal standard (IS)

RF-MEKC differs from N-MEKC in that the EOF is immobilized and migration is dependent on electrophoresis only [132]. Moreover, the migration window is considered infinite as the micellar marker is exclusively incorporated into the micellar core and reaches the detector first, followed by the remaining analytes in order of decreasing hydrophobicity. An aqueous marker used to indicate the migration time of the EOF in N-MEKC will therefore never appear and the micellar marker in RF-MEKC hence represents the “dead-time” marker as in conventional elution chromatography [167].

A micellar marker must be capable of partitioning exclusively into the micelle and numerous compounds were tested as potential suitable markers. These included: carbamazapine, sudan III, stigmasterol, benzyl alcohol, cyclohexane, xylene, toluene, carbon tetrachloride, ethyl acetate, cyclopentane, benzene, salicylic acid, paracetamol, chlorocresol, methyl hydroxybenzoate, cholesterol, estrone,  $\beta$ -estradiol and quinine hydrochloride. Terabe [132] recommends the use of trimepidium bromide or quinine hydrochloride and sudan III was used in an example of a micellar marker in a paper published by Janini [167]. Quinine hydrochloride was the most appropriate micellar marker and its inclusion into the SDS micelle was evident by the fact that it eluted ahead of all the reference compounds, including all the other chemical compounds present in extracts.

In terms of finding a suitable IS for potential quantitative analysis, a chemical compound that migrated within the zone between the flavonols and ginkgolides was desired and potential standards included chlorocresol, protryline, ibuprofen, verapamil, carbamazapine, propanolol hydrochloride, resorcinol, chlorbutol, betamethazone, paracetamol, cytosine, codeine, mometasone, fluorocinolone acetate, allopurinol, salicylic acid, methyl hydroxybenzoate, propyl hydroxybenzoate and phenacetin. Chlorocresol migrated directly after rutin and is a suitable IS for quantification of all reference compounds. Phenacetin migrated at 8 minutes with no apparent interferences with either reference compounds or tablet extracts. Although salicylic acid co-migrated with GA, it was considered a suitable IS for the quantitative determination of the flavonols since GA does not absorb UV light at the wavelength ( $\lambda$  250 nm) used to monitor the salicylic acid.

### 3.9.3 Effect of gamma cyclodextrin ( $\gamma$ -CD) and beta cyclodextrin ( $\beta$ -CD) on the resolution of the flavonols and terpenoids

#### 3.9.3.1 *Instrumentation and materials*

See sections 3.7.1 and 3.9.1.1.



### 3.9.3.2 *Capillary conditioning*

To increase reproducibility, the capillary was washed at the start of each day by flushing for 15 minutes with 1 M sodium hydroxide, 15 minutes with 0.1 M sodium hydroxide, 15 minutes with distilled water and then 40 minutes with 0.1 M sodium hydroxide. Capillary flushing between consecutive injections was kept as method 6, previously stipulated in section 3.8.2.1 but washes were applied using external pressure. To ensure constant pH at both the anode and cathode, the buffer at the cathode was replaced after every injection while the anodic buffer was replenished after every third injection.

### 3.9.3.3 *Initial experimental conditions*

In order to increase the path length and therefore sensitivity, a square capillary with dimensions 78  $\mu\text{m}$  I.D. x 360  $\mu\text{m}$  O.D. with a total length of 60.0 cm and an effective length of 45.0 cm was used. The buffer consisted of 40 mM SDS and 25 mM phosphoric acid (pH 2.2). The separation voltage for the sample containing both the flavonols and the terpenoids was set at -17.5 kV for the first 11 minutes and then increased to -20 kV to decrease the run time to 18 minutes. The separation voltage for the flavonol sample was applied at -17.5 kV. The voltage ramp was -6 kV/s and samples were injected at -5 kV for 0.05 minutes. Detection was at  $\lambda$  190 nm for both the terpenoids and flavonols and the rise time was 0.1 seconds with a sensitivity of 0.01 AUFS.

### 3.9.3.4 *Preparation of reference standards*

Samples were prepared in 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS (pH 2.2)). The increased methanolic fraction was essential to facilitate solubility of the reference compounds, particularly the aglycones. Individual stock solutions of the flavonols and terpenoids were prepared. The terpenoid stock solution had a concentration of approximately 0.50 mg/ml for GA, GB, GC, GJ and BB while the flavonol stock solution contained 0.13 mg/ml of all the flavonols except for isorhamnetin (0.06 mg/ml). The flavonol and terpene sample mix contained 200  $\mu\text{l}$  of each solution and 100  $\mu\text{l}$  each of a 1 mg/ml quinine hydrochloride solution and 1 mg/ml chlorocresol solution, both prepared in 50:50 methanol-buffer.

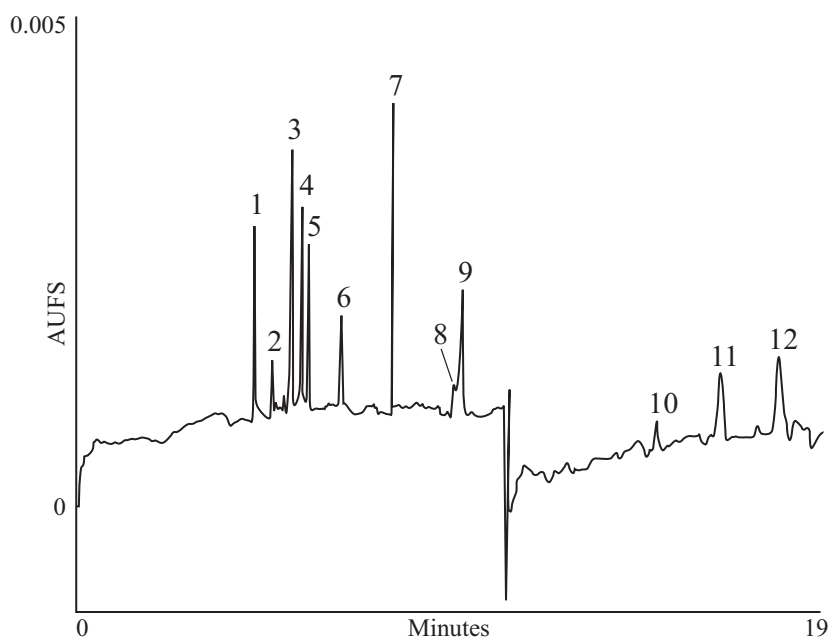
### 3.9.3.5 *Experimental Procedure*

The addition of  $\Upsilon$ -CD to the BGE on the resolution of the flavonols was investigated in concentrations of 10 mM and 20 mM. In addition, a 20 mM  $\Upsilon$ -CD and  $\beta$ -CD mixed buffer (10 mM each) was also prepared by their inclusion into the BGE. A final mixed cyclodextrin buffer containing 5 mM  $\Upsilon$ -CD, 15 mM  $\beta$ -CD, 40 mM SDS and 25 mM phosphoric acid was prepared and a sample solution containing both the flavonols and terpene lactones was injected.

### 3.9.3.6 Results

The BGE containing 10 mM  $\gamma$ -CD resulted in co-migration of isorhamnetin and kaempferol and also quercetin and quercitrin. The results were similar when the cyclodextrin concentration was increased to 20 mM however the joule heat production (current measurement of  $-104 \mu\text{A}$ ) caused aberrations in the baseline and a significant decrease in sensitivity was noticed, probably due to increased background absorbance. Peak broadening was also evident. The mixed buffer system comprised of 10 mM each  $\gamma$ -CD and  $\beta$ -CD showed a similar profile in terms of separation but the sensitivity was enhanced in comparison to the results when using the buffers containing  $\gamma$ -CD alone. The buffer containing only 5 mM  $\gamma$ -CD and 15 mM  $\beta$ -CD resulted in baseline separation of the flavonols however, GA and GB were not well resolved as shown in Figure 3.18.  $\beta$ -CD was therefore responsible for the improved separation of the flavonols and  $\gamma$ -CD did not contribute towards separation.

Figure 3.18. Effect of the addition of 5 mM  $\gamma$ -CD and 15 mM  $\beta$ -CD to the micellar buffer system



Conditions: 78  $\mu\text{m}$  I.D., total length = 60.0 cm, effective length = 45.0 cm; Sample buffer: 50:50 methanol-buffer (10mM phosphoric acid, 40mM SDS (pH 2.2)); Running buffer: 40 mM SDS, 25 mM phosphoric acid (pH 2.2), 5 mM  $\gamma$ -cyclodextrin, 15 mM  $\beta$ -cyclodextrin; Electrokinetic injection: -5 kV for 3 sec.; Voltage: -17.5 kV (0 - 11 minutes), -20 kV (11 - 19 minutes); Detection  $\lambda$ : 190 nm. Labeled peaks: 1=quinine hydrochloride, 2=isorhamnetin, 3=kaempferol, 4=quercetin, 5=quercitrin, 6=rutin, 7=chlorocresol; 8=ginkgolide B, 9=ginkgolide A, 10=bilobalide, 11=ginkgolide C, 12=ginkgolide J.

### 3.9.3.7 Discussion

Gamma and  $\beta$ -CD are oligosaccharides which have 8 and 7 glucose units respectively and are cylindrical in shape with hydrophilic surfaces and hydrophobic cavities. In addition, their cavity sizes differ with  $\gamma$ -CD having an internal cavity diameter of 0.75 - 0.83 nm and  $\beta$ -CD a measurement of 0.62 - 0.64 nm [132]. Separation using CD is based on forming stable complexes with the analytes which is dependent on hydrophobic and hydrogen bonding as well as the size and shape of the analyte [142]. It is therefore possible that the co-migration observed between kaempferol and isorhamnetin and quercetin and quercitrin was due to  $\gamma$ -CD's large cavity which enabled the simultaneous inclusion of more than one analyte.  $\beta$ -CD, on the other hand, has a smaller cavity and its selective interaction with the analytes resulted in improved separation. Although the mixed buffer system containing 5 mM  $\beta$ -CD facilitated baseline resolution of the flavonols, inclusion of GA and GB into the core of  $\gamma$ -CD still occurred which resulted in co-migration. Moreover, different types of cyclodextrins are also known to alter selectivity [132].

## 3.9.4 Effect of $\beta$ -cyclodextrin on the resolution of the flavonols and terpenoids

### 3.9.4.1 Instrumentation and materials

See sections 3.7.1 and 3.9.1.1.

### 3.9.4.2 Capillary conditioning

Capillary cleaning was performed as in section 3.9.3.2.

### 3.9.4.3 Initial experimental conditions

The separation was achieved on the same square capillary that was used in section 3.9.3.3 however due to buffer precipitation, both the anodic and cathodic ends were trimmed to give a total length of 59.4 cm with an effective length of 43.0 cm. The buffer consisted of 40 mM DS and 25 mM phosphoric acid (pH 2.2). The voltage was kept constant at -17.5 kV with a voltage ramp of -6 kV/s. The flavonols were monitored at a selective wavelength of 250 nm and after phenacetin reached the detection window, the wavelength was changed to 190 nm. The rise time was 0.1 seconds with the sensitivity set at 0.01 AUFS.

### 3.9.4.4 Preparation of the reference standards

The flavonol stock solution was prepared in 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS) with concentrations of 0.35 mg/ml, 0.31 mg/ml, 0.28 mg/ml, 0.22 mg/ml and 0.10 mg/ml of rutin, quercitrin, quercetin, kaempferol and isorhamnetin, respectively. A separate terpenoid stock solution was prepared with concentrations of 1.50 mg/ml, 1.39 mg/ml, 1.60 mg/ml, 1.40 mg/ml and 2.22 mg/ml of GA, GB, GC, GJ and BB, respectively. Quinine hydrochloride was used as the micellar marker and a stock

solution containing 1.35 mg/ml was prepared in methanol-buffer (50:50). Phenacetin was included as a potential IS and a stock solution of 1.13 mg/ml was prepared. The final sample solution consisted of 400  $\mu$ l of the flavonol stock, 700  $\mu$ l of the terpenoid stock and 100  $\mu$ l of both the phenacetin and quinine stock solutions. Samples were injected in triplicate at each concentration level.

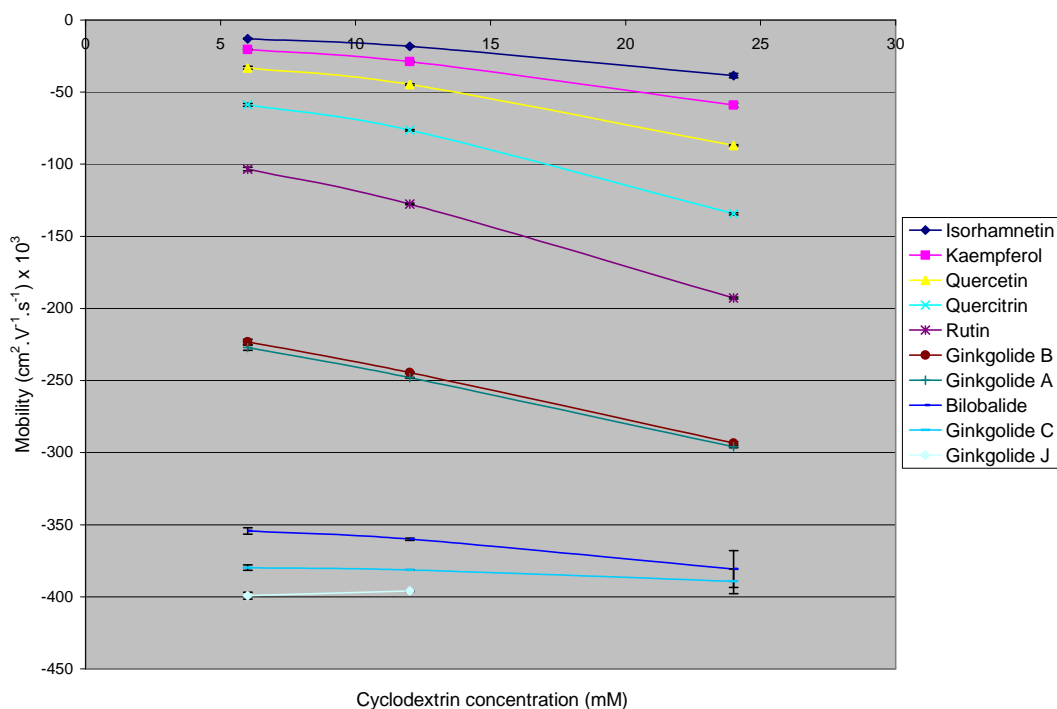
#### *3.9.4.5 Experimental procedure*

The effect of  $\beta$ -cyclodextrin ( $\beta$ -CD) on the separation of the flavonols and the terpenoids was investigated by the inclusion of 6 mM, 12 mM and 24 mM in the BGE.

#### *3.9.4.6 Results*

The effect of  $\beta$ -CD on the effective mobility and resolution of the flavonols is presented graphically in Figure 3.19 where the average effective mobility was plotted against the cyclodextrin concentration. The migration times lengthened with increasing  $\beta$ -CD concentrations. At a  $\beta$ -CD concentration of 24 mM, GC and BB were detected at a signal to noise (S/N) ratio of 3:1 while GJ could not be detected at all due to the baseline noise. Moreover, baseline aberrations also prevented separation between GA and GB. The inclusion of both 6 mM and 12 mM  $\beta$ -CD resulted in baseline resolution of the terpenoids however, the flavonols were better resolved at a concentration of 12 mM. The run times were 19 and 23 minutes, respectively.

Figure 3.19. Effect of  $\beta$ -CD on the mobility and resolution of the flavonols and terpene lactones



#### 3.9.4.7 Discussion

Initially the buffer consisted of phosphoric acid and SDS only and the flavonol aglycones, isorhamnetin, kaempferol and quercetin co-migrated. Increasing concentrations of  $\beta$ -cyclodextrin in the buffer (6 mM, 12 mM and 24 mM) improved the resolution between the flavonol aglycones present in the sample solution by changing the apparent distribution co-efficient of the more hydrophobic flavonol aglycones which prevented them from being completely incorporated into the core of the anionic SDS micelles [132]. Although the effective mobilities of all compounds decreased with increasing cyclodextrin concentrations, the resolution between GA and GB was unaffected. Resolution between BB and GC decreased slightly. However, ginkgolide J was not detected at a concentration of 24 mM  $\beta$ -cyclodextrin due to increased background noise which can be attributed to the production of joule heat, increased background absorbance and the lengthened analysis time. Beta-cyclodextrin concentration had minimal effect on the ginkgolides as these analytes are probably too large to be incorporated into the cavity of the  $\beta$ -cyclodextrin molecular structure. A  $\beta$ -cyclodextrin concentration of 12 mM was thus established as being optimal for the simultaneous separation of all the relevant compounds.

### 3.9.5 Effect of SDS on the resolution of the flavonols and terpenoids

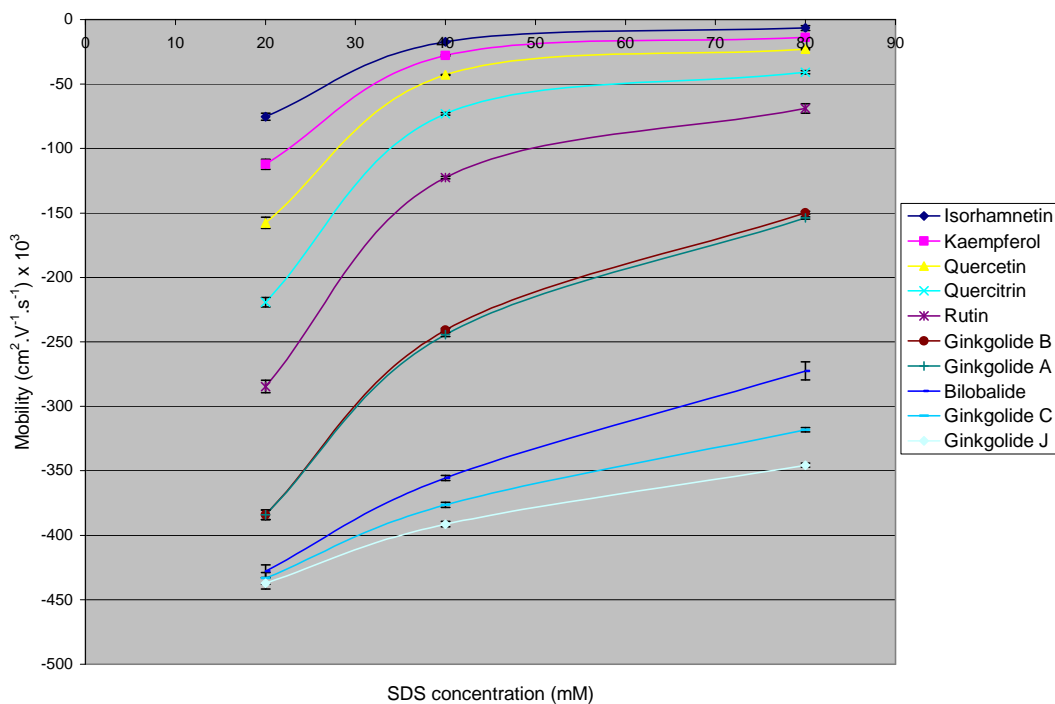
#### 3.9.5.1 *Experimental Procedure*

The effect of SDS on the effective mobility and resolution of the flavonols was investigated at 20 mM, 40 mM and 80 mM SDS concentrations. The phosphoric acid and  $\beta$ -CD concentrations remained fixed at 25 mM and 12 mM, respectively. The remaining conditions were as described in section 3.9.4.2 - 3.9.4.4.

#### 3.9.5.2 *Results*

Figure 3.20 shows the effect of SDS on the effective mobility and resolution. The mobilities of both the ginkgolides and flavonols increased with increasing concentrations of SDS. The migration time at 20 mM SDS was 33 minutes, at 40 mM it was 22 minutes and at 80 mM it was 14 minutes. At 20 mM SDS, the flavonols were well separated however, resolution between the terpenoids was compromised with co-migration of GA and GB. At 40 mM SDS, the flavonols were not as well resolved but baseline resolution of all the selected markers was obtained. At 80 mM SDS, both the separation of the flavonols and terpenoids was compromised, hence 40 mM SDS was selected as the optimal micellar concentration, shown in Figure 3.21 in section 3.10.4.

Figure 3.20. Effect of SDS concentration on the mobility and resolution of the flavonols and terpenoids



### 3.9.5.3 Discussion

Unlike the effect of  $\beta$ -cyclodextrin, the SDS concentration had a profound effect on the resolution of the terpenoids which indicates a substantial amount of micellar-analyte interactions, possibly because the ginkgolides are fairly neutral molecules and their electrophoresis is dependent on the charge of the anionic surfactant. Moderate concentrations of SDS provided optimal separation and prevented exclusive partitioning of GA and GB into the micellar core at 80 mM as well as minimal micellar-analyte interactions at 20 mM which also impeded separation. Increasing SDS concentrations resulted in heightened background absorbance and decreased sensitivity which was reflected in smaller peak areas. In RF-MEKC an inverse relationship exists between mobility and micellar concentration [167] which explains the decrease in run time (or increased mobility) with higher concentrations of SDS.

## 3.10 SIMULTANEOUS ANALYSIS OF FLAVONOLS AND TERPENE LACTONES IN *GINKGO BILOBA* SOLID ORAL DOSAGE FORMS

### 3.10.1 Instrumentation and additional equipment

Equipment that was used for this analysis has been described in section 3.7.1. However a polyimide fused-silica square capillary column (75  $\mu\text{m}$  I.D. x 360  $\mu\text{m}$  O.D.) was used for the separation. In addition, an ultrasonic bath, Model SC-211TH (Sonicator Instrument Corporation, Copiague, N.Y., USA) was used for extraction purposes and a centrifuge, Model HN/SII (Damon IEC Division, MA, USA) was used to centrifuge tablet extracts. A Vortexer, Model G-560E (Scientific Industries, Bohemia, N.Y., USA) was used for sample mixing. All extracts as well as the phosphate buffer were filtered through 0.45 $\mu\text{m}$  PVDF filters, purchased from Millipore, Bedford, MA, USA. HPLC grade water was purified by reverse osmosis through a Milli-Q purification system obtained from the same suppliers.

### 3.10.2 Materials and reagents

See Section 3.9.1.1 for details of materials and reagents used. Four solid oral *Ginkgo biloba* dosage forms (Products E - H) were bought from a local pharmacy in Grahamstown, South Africa (Table 3.4.). Three of the products were tablets and one product (Product H) contained pulverized leaf extract in a hard gelatin capsule.

Table 3.4. *Ginkgo biloba* dosage forms analyzed for flavonol and terpene lactone content

Manufacturer	Name of product	Dosage form	Label claim	Daily dose	Batch number	Extract
Formule Naturelle <sup>1</sup>	<i>Ginkgo biloba</i>	Tablet	Standardized to contain 24% <i>Ginkgo</i> Flavonglycosides <sup>6</sup>	1 tablet tds*, ac <sup>7</sup>	3011	E
Bioforce <sup>2</sup>	Ginkgoforce	Tablet	Each 250mg tablet is equivalent to 10 drops of Ginkgoforce liquid	2 tablets tds*, ac <sup>7</sup>	Unknown	F
Holotropic <sup>3</sup>	<i>Ginkgo biloba</i>	Tablet	Each 3 tablets provide: <i>Ginkgo biloba</i> botanical 300 mg, <i>Ginkgo biloba</i> extract equivalent to 900mg	1 tablet tds*	0319	G
Bioharmony <sup>4</sup>	<i>Ginkgo biloba</i>	Capsule	Each 350mg capsule contains 350mg <i>Ginkgo biloba</i> ( <i>Ginkgoaceae</i> )	2 capsules tds*	EP02.21	H

<sup>1</sup>Formule Naturelle, Hout Bay, South Africa; <sup>2</sup>Bioforce S.A. (Pty) Ltd., Halfway House, South Africa; <sup>3</sup>Holotropic, Cape Town, South Africa; <sup>4</sup>Vital Health Foods (EDMS) BPK, Kuils River, Western Cape, South Africa; <sup>5</sup>three times daily; <sup>6</sup>Additional information was provided, including 1.2 mg ginkgolides and 1.0 mg bilobalide per tablet; <sup>7</sup>ac, before meals

### 3.10.3. Experimental Procedure

The square capillary was cut to a total length of 60.0 cm and effective length of 45.0 cm. The BGE (pH 2.2) consisted of 25 mM phosphoric acid, 40 mM SDS and 12 mM  $\beta$ -cyclodextrin. The separation voltage was set at -17.5 kV for 11 minutes, thereafter the voltage was changed to -20 kV (the voltage ramp was -6kV/s) and samples were injected electrokinetically at -5 kV for 3 seconds. The measured current was ~ -80  $\mu$ A throughout the analysis. The flavonols were monitored at a wavelength of 250 nm and the ginkgolides at  $\lambda$  190 nm with a rise time of 0.1 seconds and sensitivity of 0.01 AUFS. The same conditioning and buffer replenishing protocols described in section 3.9.3.2 were used to wash the capillary.

#### 3.10.3.1 *Sample preparation (Products E - G)*

A minimum of 10 tablets of each solid oral dosage form (Products E - G) were weighed and powdered using a mortar and pestle. For Products E and G, a mass of powder equivalent to 1 tablet was weighed and for Product F, a mass of powder equivalent to 2 tablets was weighed and then transferred into a Kimax tube before dispersion with 20 ml of methanol. The mixture was sonicated for 30 minutes and then manually agitated to ensure re-dispersion before sonication was continued for a further 30 minutes. The extract was centrifuged at 350 x g for 10 minutes and the supernatant decanted into a 50 ml Kimax tube. Twenty milliliters of fresh methanol were then added to the remaining residue, the contents re-dispersed and sonicated for 30 minutes followed by centrifugation and decantation of the supernatant into the same 50 ml Kimax tube which was then evaporated to reduce the volume. The above procedure was then repeated and



the combined extracts were evaporated to dryness using nitrogen before reconstitution with appropriate volumes of 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS (pH 2.2)). All samples were filtered through 0.45µm PVDF filter membranes prior to analysis.

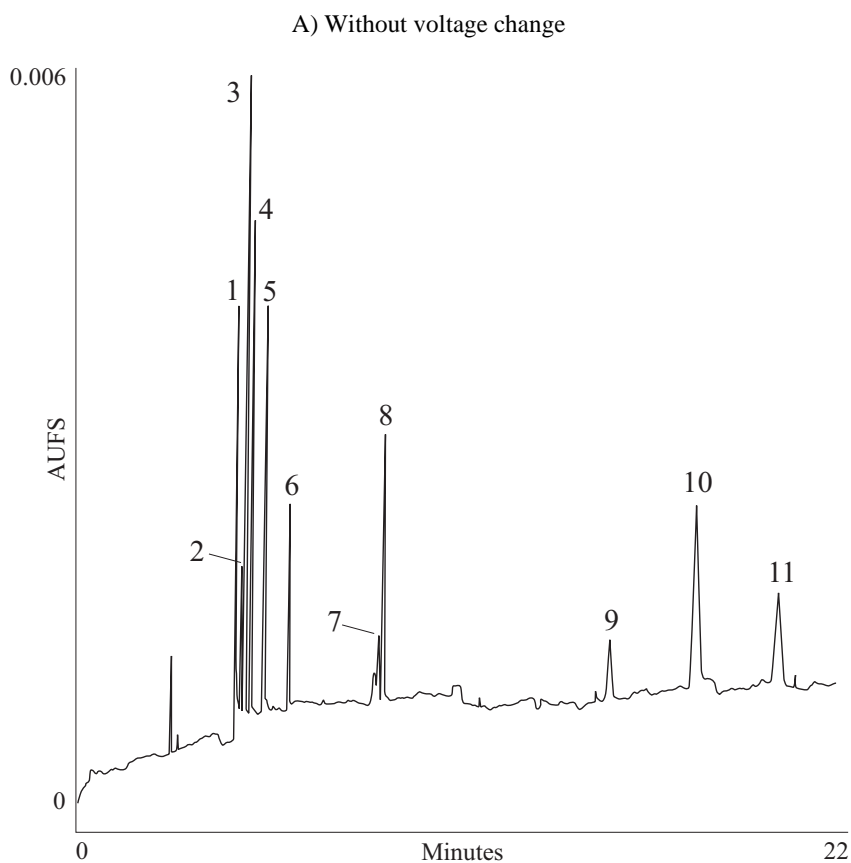
#### 3.10.3.2 *Sample preparation of Product H*

Ten capsules (Product H) were individually emptied and weighed and transferred to a mortar and pestle for mixing. A mass of powder equivalent to the contents of a single capsule was extracted according to the procedure described in Section 3.10.3.1 above.

#### 3.10.4 Results and discussion

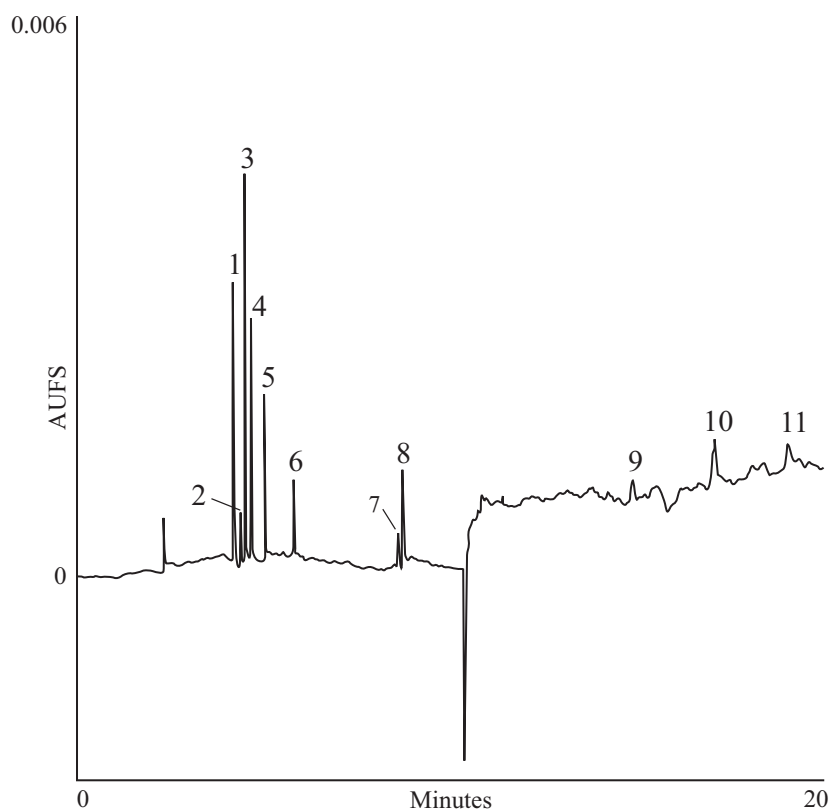
Figure 3.21 A shows the baseline separation of all 10 marker compounds within 22 minutes with a constant voltage of -17.5 kV. A typical electropherogram of the standards with the programmed voltage change is shown in Figure 3.21 B. Since a photodiode array (PDA) detector could not be configured to the CE instrument, the wavelength was manually changed from 250 nm to 190 nm after 11 minutes. The flavonols required detection at a more selective wavelength to ensure maximum absorbance and minimal interference.

Figure 3.21. Electropherogram of selected flavonol and terpene lactone reference standards



Conditions: 78  $\mu\text{m}$  I.D., total length = 59.4 cm, effective length = 43.0 cm; Sample buffer: 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS (pH 2.2)); Running buffer: 40 mM SDS and 25 mM phosphoric acid (pH 2.2), 12 mM  $\beta$ -cyclodextrin; Electrokinetic injection: -5 kV for 3 sec.; Voltage: -17.5 kV; Detection  $\lambda$ : 190 nm. Labeled peaks: 1=quinine hydrochloride, 2=isorhamnetin, 3=kaempferol, 4=quercetin, 5=quercitrin, 6=rutin, 7=ginkgolide B, 8=ginkgolide A, 9=bilobalide, 10=ginkgolide C, 11=ginkgolide J.

B) With voltage change programmed at 11 minutes



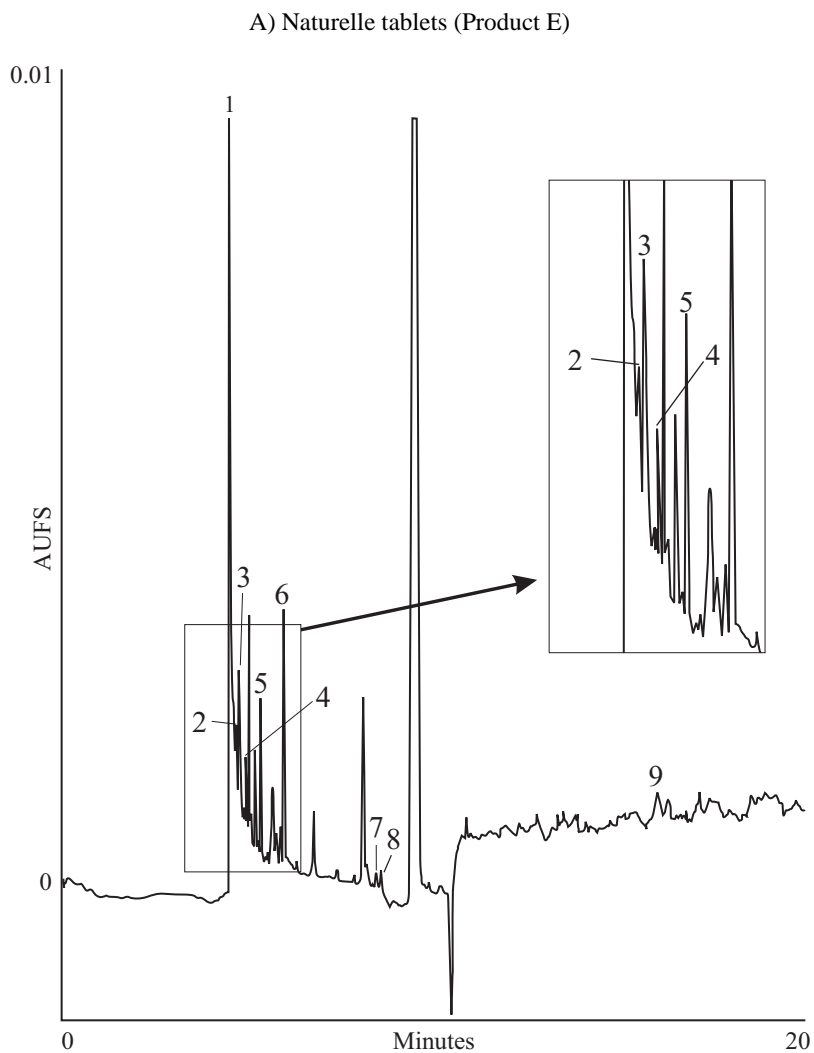
Conditions: 78  $\mu\text{m}$  I.D., total length = 60.0 cm, effective length = 45.0 cm; Sample buffer: 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS (pH 2.2)); Running buffer: 40 mM SDS and 25 mM phosphoric acid (pH 2.2), 12 mM  $\beta$ -cyclodextrin; Electrokinetic injection: -5 kV for 3 sec.; Voltage: -17.5 kV (0 - 11 minutes), -20 kV (11 - 19 minutes); Detection  $\lambda$ : 190 nm Labeled peaks: 1=quinine hydrochloride, 2=isorhamnetin, 3=kaempferol, 4=quercetin, 5=quercitrin, 6=rutin, 7=ginkgolide B, 8=ginkgolide A, 9=bilobalide, 10=ginkgolide C, 11=ginkgolide J.

The electrophoretic fingerprints of all 4 products are shown in Figure 3.22 and identification of the marker compounds was based on relative migration of the analytes compared to the micellar marker. All products had a greater number of peaks in the flavonoid migration window which extended from approximately 4.5 to 6.1 minutes (after the migration of rutin) than in the less selective terpenoid zone after 8.2 minutes when the wavelength was changed from 250 nm to 190 nm. Although this migration window is assumed, only a few peaks were observed between rutin and the time of wavelength change and since rutin is relatively hydrophilic compared to the other aglycones and glycosides, its migration is predicted to be one of the slowest of the flavonoids due to its dominant partitioning into the aqueous phase. Minimal interference in the terpenoid zone was crucial for the detection and identification of these markers using a very low UV non-selective wavelength - particularly since the ginkgolides have low absorbance.

The fingerprints of the commercial products indicated that large discrepancies occurred in both the flavonol and ginkgolide marker content. Products F and G exhibited similar profiles with less peaks present overall

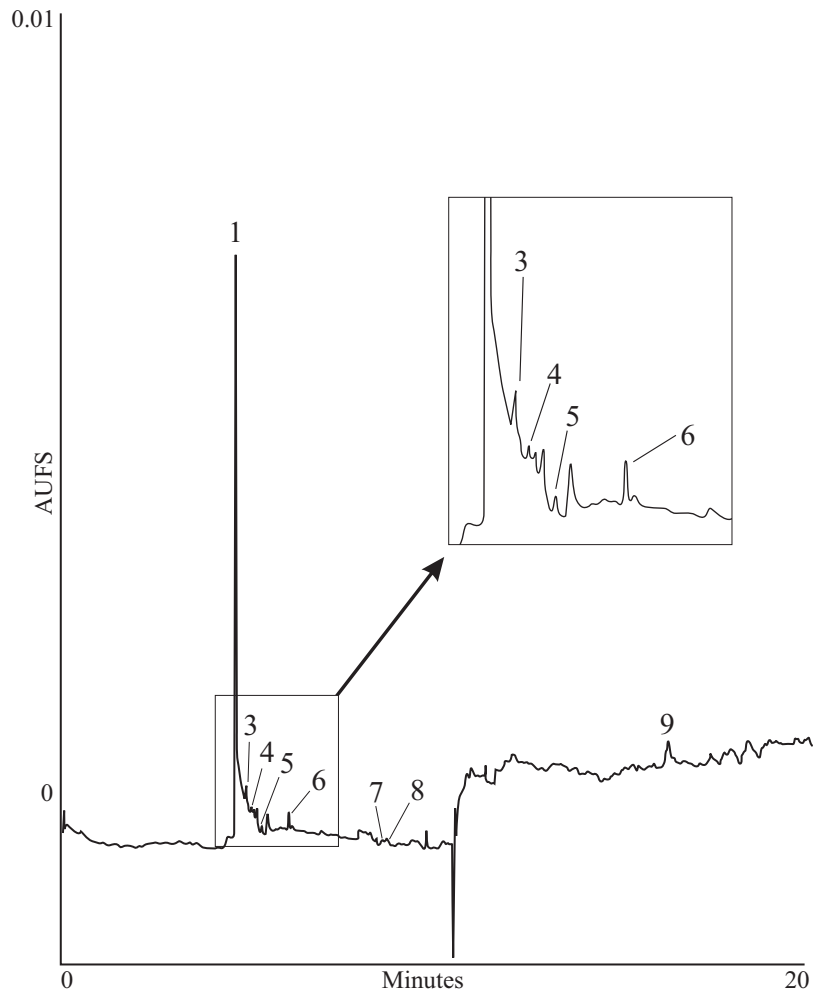
(identified and unidentified) in both the flavonol and ginkgolide migration windows compared to Products E and H. Product H revealed the presence of all selected marker compounds although baseline noise prevented unequivocal identification of the last marker compound, GJ. Product A seemed to have the highest content of flavonols but had less terpene trilactones than product H. Overall, based on peak intensity and height, Product E contained the most marker compounds, followed by Product H, then G and finally, Product F. Interestingly, Product E was the only product which claimed some level of standardization - albeit specified as “flavonglycoside” content only (Table 3.4).

Figure 3.22. Electrophoretic fingerprint of Products E-H



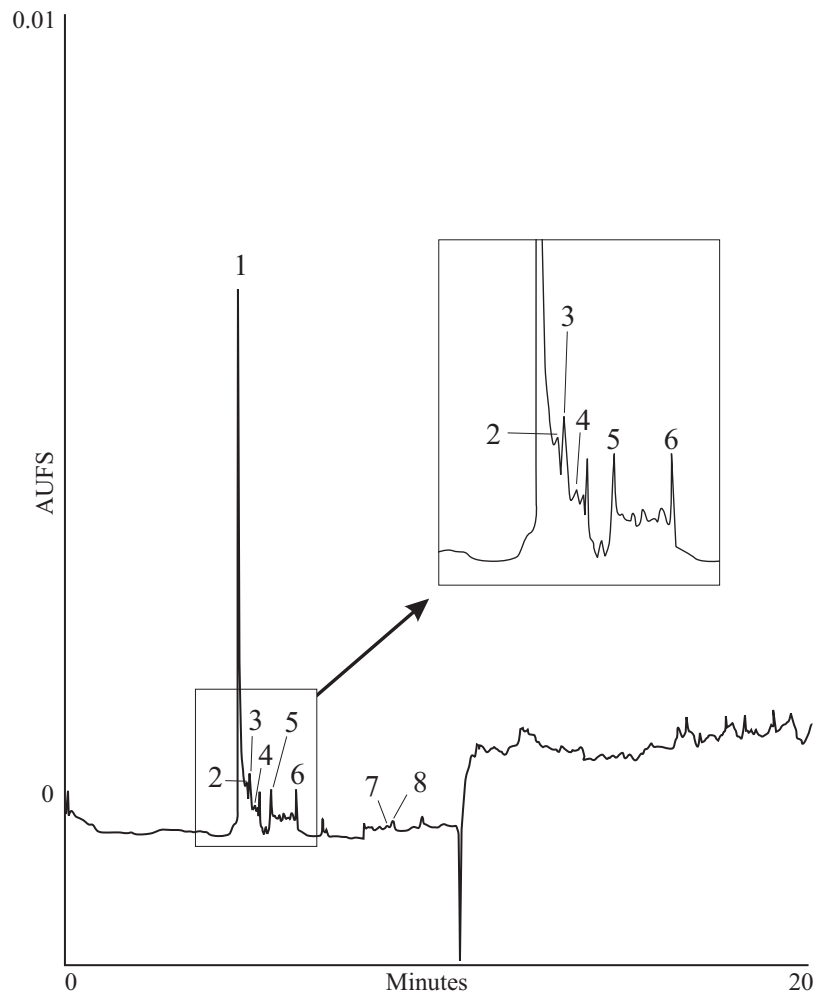
Conditions: 78  $\mu\text{m}$  I.D., total length=60.0 cm, effective length=45.0 cm; Sample buffer: 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS (pH 2.2)); Running buffer: 40 mM SDS and 25 mM phosphoric acid (pH 2.2), 12 mM  $\beta$ -cyclodextrin; Electrokinetic injection: -5 kV for 3 sec.; Voltage: -17.5 kV (0 - 11 minutes), -20 kV (11 - 19 minutes); Detection  $\lambda$ : 190 nm. Labeled peaks: 1=quinine hydrochloride, 2=isorhamnetin, 3=kaempferol, 4=quercetin, 5=quercitrin, 6=rutin, 7=ginkgolide B, 8=ginkgolide A, 9=bilobalide.

B) Ginkgoforce tablets (Product F)



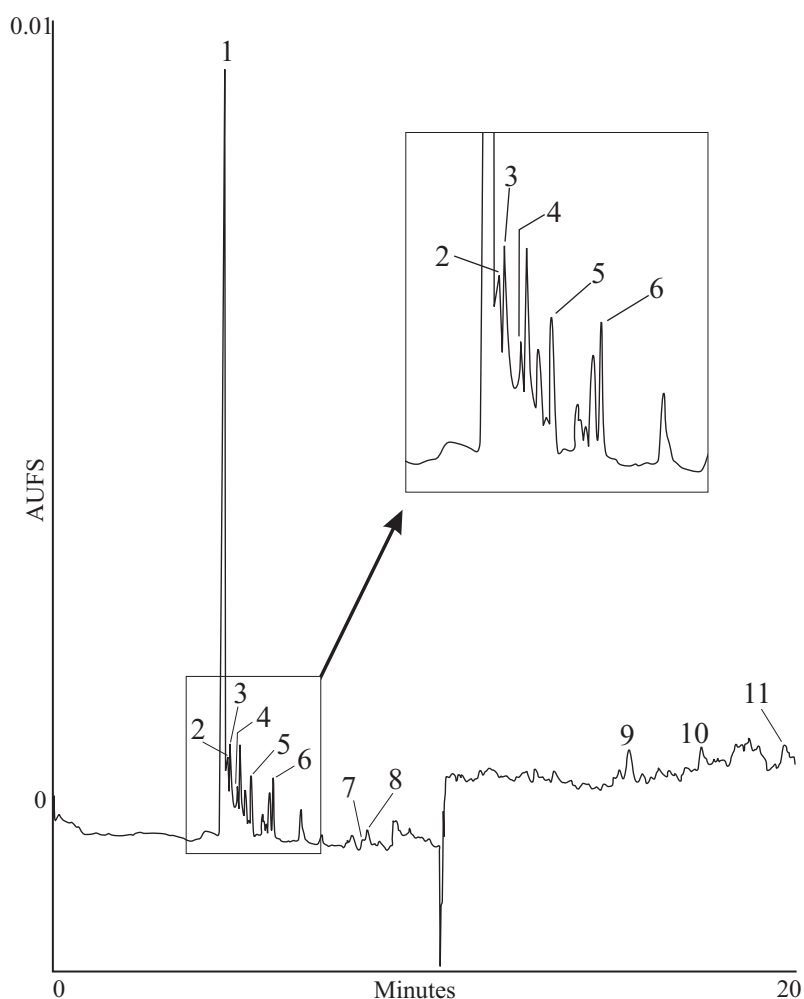
Conditions: 78  $\mu\text{m}$  I.D., total length=60.0 cm, effective length=45.0 cm; Sample buffer: 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS (pH 2.2)); Running buffer: 40 mM SDS and 25 mM phosphoric acid (pH 2.2), 12 mM  $\beta$ -cyclodextrin; Electrokinetic injection: -5 kV for 3 sec.; Voltage: -17.5 kV (0 - 11 minutes), -20 kV (11 - 19 minutes); Detection  $\lambda$ : 190 nm Labeled peaks: 1=quinine hydrochloride, 3=kaempferol, 4=quercetin, 5=quercitrin, 6=rutin, 7=ginkgolide B, 8=ginkgolide A, 9=bilobalide.

C) Holotropic tablets (Product G)



Conditions: 78  $\mu\text{m}$  I.D., total length=60.0 cm, effective length=45.0 cm; Sample buffer: 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS (pH 2.2)); Running buffer: 40 mM SDS and 25 mM phosphoric acid (pH 2.2), 12 mM  $\beta$ -cyclodextrin; Electrokinetic injection: -5 kV for 3 sec.; Voltage: -17.5 kV (0 - 11 minutes), -20 kV (11 - 19 minutes); Detection  $\lambda$ : 190 nm Labeled peaks: 1=quinine hydrochloride, 2=isorhamnetin, 3=kaempferol, 4=quercetin, 5=quercitrin, 6=rutin, 7=ginkgolide B, 8=ginkgolide A.

#### D) Bioharmony capsules (Product H)



Conditions: 78  $\mu\text{m}$  I.D., total length=60.0 cm, effective length=45.0 cm; Sample buffer: 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS (pH 2.2)); Running buffer: 40 mM SDS and 25 mM phosphoric acid (pH 2.2), 12 mM  $\beta$ -cyclodextrin; Electrokinetic injection: -5 kV for 3 sec.; Voltage: -17.5 kV (0 - 11 minutes), -20 kV (11 - 19 minutes); Detection  $\lambda$ : 190 nm. Labeled peaks: 1=quinine hydrochloride, 2=isorhamnetin, 3=kaempferol, 4=quercetin, 5=quercitrin, 6=rutin, 7=ginkgolide B, 8=ginkgolide A, 9=bilobalide, 10=ginkgolide C, 11=ginkgolide J.

#### 3.10.5 Conclusions

To our knowledge, no papers have been published which describe a method for the simultaneous determination of both ginkgolides and flavonols using CE. The novelty of this method therefore lies in the application of a RF-MEKC procedure for the simultaneous determination of a mixture of multicomponents, two flavonol glycosides, rutin and quercitrin, three flavonol aglycones, isorhamnetin, kaempferol and quercetin, four terpenic trilactones, GA, GB, GC and GJ and one sesquiterpene, BB in *Ginkgo biloba* solid oral dosage forms.



This RF-MEKC method was found to be particularly useful for the analysis of *Ginkgo biloba* solid oral dosage forms since this method was developed under acidic conditions in which both the flavonols and terpene trilactones are chemically stable. Importantly, RF-MEKC facilitated the UV-detection of the terpene trilactones (which possess poor chromophores) in extracts since slower migration of these hydrophilic components resulted in maximal separation and reduced interference which was facilitated by using an electrophoretic system which provided a reverse flow.

Also, by using RF-MEKC a novel QC approach was introduced by including the simultaneous determination of intact flavonol glycosides, rutin and quercitrin, together with the normal aglycones, quercetin, kaempferol and isorhamnetin in the assay in order to ascertain botanical authenticity and adulteration as well as to determine appropriate extraction and storage conditions.

### **3.11 METHOD VALIDATION AND THE QUANTITATIVE ANALYSIS OF RUTIN AND QUERCETIN IN *GINKGO BILOBA* COMMERCIAL PRODUCTS**

#### **3.11.1 Instrumentation**

All instruments and additional equipment described in section 3.10.1 was used for the validation.

#### **3.11.2 Materials and reagents**

See Section 3.9.1.1 for details of materials and reagents used. Salicylic acid was purchased from Sigma (St. Louis, MO, USA) and 2 commercial *Ginkgo biloba* products were bought from a local pharmacy in Grahamstown, South Africa (Table 3.5).

Table 3.5. *Ginkgo biloba* solid oral dosage forms used for the assay

Manufacturer	Name of product	Dosage form	Label claim	Daily dose	Batch number	Extract
Bioharmony <sup>1</sup>	<i>Ginkgo biloba</i>	Capsule	Each 350mg capsule contains 350mg <i>Ginkgo biloba</i> ( <i>Ginkgoaceae</i> )	2 capsules tds*	ER10.11	I
Nrf Herbal <sup>2</sup>	<i>Ginkgo biloba</i>	Tablet	Each tablet contains 350mg <i>Ginkgo biloba</i> , 50mg <i>Ginkgo biloba</i> extract 24% and 240mg FoodMatrix™	1 to 2 tablets or as directed by your healthcare practitioner	NTN042PW	J

<sup>1</sup>Bioharmony CC, Wynberg, South Africa; <sup>2</sup>Nrf Herbal, Centurion, South Africa; \*three times daily

### 3.11.3 Procedure

The capillary used was of the same dimensions as in 3.10.3 and the running buffer composition has been described in that same section. The separation voltage was -17.5 kV with a voltage ramp of -6 kV/s and samples were injected hydrodynamically at 20 mbar for 0.6 seconds. Detection was at  $\lambda$  250 nm at 0.01 AUFS with a rise time of 0.1 seconds. The capillary conditioning and buffer replenishment were performed as previously described in section 3.9.3.2.

#### 3.11.3.1 Preparation of standard solutions

On each of the 3 days, a 200  $\mu\text{g/ml}$  rutin and 100  $\mu\text{g/ml}$  quercetin stock solution with 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS (pH 2.2)) as the solvent was prepared and diluted to provide the calibrator ranges of 12 - 84  $\mu\text{g/ml}$  and 6 - 42  $\mu\text{g/ml}$ , respectively. Volumes were corrected for the addition of the IS and the micellar marker. A quinine hydrochloride stock solution (1.25 mg/ml) was prepared in 50:50 methanol-buffer which was used as the micellar marker and a 1 mg/ml salicylic acid stock solution was also prepared in the same solvent and was used as the IS for the validation. Each sample vial contained 250  $\mu\text{l}$  each of the quinine hydrochloride and IS solutions and 2 ml of the relevant calibration solutions.

### 3.10.3.2 Preparation of sample solutions

#### *Hard gelatin capsules (Product I)*

Forty capsules were individually emptied and weighed and transferred to a mortar and pestle for mixing. A mass of powder equivalent to the contents of a single capsule was extracted according to the procedure described in Section 3.10.3.1.

#### *Solid oral dosage forms (Product J)*

A minimum of 20 tablets of Product J was weighed and powdered using a mortar and pestle. A mass of powder equivalent to one tablet was accurately weighed and extracted according to the procedure in Section 3.10.3.1.

### 3.11.4 Method validation

#### *3.11.4.1 Calibration curves*

A calibration curve for each standard was constructed on each day of the validation by analyzing a mixture containing both rutin and quercetin at five different concentration levels on the first day and three concentration levels on consecutive days. The peak height of analyte/IS ratios were plotted against the concentration for each reference standard to obtain linear calibration responses.

#### *3.11.4.2 Precision and accuracy*

The precision and accuracy of the assay were assessed by spiking aliquots of powdered material equivalent to the content of one capsule of Product I chosen as the matrix for spiking, with low and high concentrations of rutin and quercetin. This process was performed in triplicate on each day of the validation. Product I contained a significant amount of rutin and therefore recovery of rutin at the lowest level was not assessed.

#### *3.11.4.3 Limits of detection (LOD's) and quantification (LOQ's)*

The LOD's and LOQ's were determined by serial dilutions of the lowest calibrator concentration and established at a S/N ratio of 3:1 and 10:1, respectively.

### 3.11.5 Results and discussion

#### 3.11.5.1 *Linearity and range*

Calibration curves for rutin and quercetin were constructed on each day of the validation. The response profile was linear for both compounds within the ranges of 12 - 84 µg/ml for rutin and 6 - 42 µg/ml for quercetin. The results of all 3 days are shown in Table 3.6.

Table 3.6. Linear ranges and co-efficients of determination of rutin and quercetin

<b>Name of compound</b>	<b>Concentration range (µg/ml)</b>	<b>Day</b>	<b>Slope</b>	<b>y-intercept</b>	<b>Co-efficients of determination (R<sup>2</sup>)</b>
Rutin	12.5 - 83.5	Day 1	13.6	-106.4	0.9943
		Day 2	12.8	-27.5	0.9977
		Day 3	11.4	-40.3	0.9989
Quercetin	6.3 - 41.9	Day 1	26.9	-82.5	0.9915
		Day 2	26.4	-58.0	0.9957
		Day 3	24.5	-77.1	0.9987

#### 3.11.5.2 *LOD's and LOQ's*

The LOD for rutin was found to be 3.13 µg/ml and for quercetin, 1.88 µg/ml. The LOQ for rutin and quercetin were 6.25 µg/ml and 3.75 µg/ml, respectively.

#### 3.11.5.3 *Accuracy and repeatability*

The accuracy and precision of the method was determined using QC samples as well as performing a recovery study. Two QC samples corresponding to low and high concentrations of each reference standard were injected in triplicate midway through the analysis on each day. The accuracy of the method can be gleaned from Table 3.7 where percentage relative error (% RE) values were less than 5% except for day 2 where the values for rutin QC 2 and quercetin QC 1 were +6.6 and -6.3, respectively. Intra-day percentage relative standard deviation (% RSD) values ranged from 0.9 to 8.5% for triplicate injections. Similarly, the inter-day results (Table 3.8) showed excellent accuracy and precision.

Table 3.7. Accuracy and precision of QC samples

Quality Controls	Day 1 (n=3)				Day 2 (n=3)				Day 3 (n=3)			
	Rutin		Quercetin		Rutin		Quercetin		Rutin		Quercetin	
	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2
Theoretical conc. ( $\mu\text{g/ml}$ )	33.57	58.74	16.97	29.69	33.97	59.44	16.85	29.49	33.37	58.39	16.83	29.46
Calculated conc. ( $\mu\text{g/ml}$ )	32.35	55.85	16.90	28.54	34.35	63.35	15.79	30.59	34.24	58.24	16.80	29.35
RSD (%)	8.5	3.1	4.7	2.4	1.6	4.4	2.6	0.9	8.4	3.8	2.7	2.3
RE (%)	-3.6	-4.9	-0.4	-3.9	+1.1	+6.6	-6.3	+3.7	+2.6	-0.3	-0.2	-0.4

Table 3.8. Inter-day accuracy of QC samples

Constituent	Average theoretical value ( $\mu\text{g/ml}$ )	Acquired average $\pm$ SD (n=9)	Accuracy (%) $\pm$ RSD	RE (%)
Rutin	33.64	33.67 $\pm$ 1.10	100.1 $\pm$ 3.3	+0.1
	58.86	59.13 $\pm$ 3.40	100.5 $\pm$ 5.8	+0.5
Quercetin	16.88	16.48 $\pm$ 0.57	97.6 $\pm$ 3.5	-2.4
	29.55	29.50 $\pm$ 1.21	99.8 $\pm$ 3.8	-0.2

In addition, one product was selected (Product I) and spiked with low and high concentrations of reference standards to assess the recovery of the method. On each of three days, Product I was assayed in triplicate to determine the contributions of rutin and quercetin to the overall recoveries. It was found that Product I contained a quantifiable amount of rutin near the lower recovery level and was therefore spiked with amounts which corresponded to medium and high concentrations of the calibration range. Table 3.9 depicts the results of this experiment and the precision is indicated by the intra-day and inter-day % RSD. Rutin had a higher overall recovery than quercetin which was expected since rutin has a sugar moiety present which enhances its polarity and thus aqueous solubility. Lower recoveries for quercetin at higher spiking levels have in fact, previously been documented in HPLC [168] and liquid chromatography-mass spectrometry (LC-MS) [169].

Table 3.9. Recovery of rutin and quercetin from Product I

Constituent	Mean spiking level ( $\mu\text{g}/350\text{mg}$ capsule)	Intra-day RSD (% , n=3)			Inter-day RSD (% , n=9)	Mean recovery (%) $\pm$ SD (n=9)
		Day 1	Day 2	Day 3		
Rutin	508	3.4	6.9	10.1	8.2	96.9 $\pm$ 8.0
	1015.8	6.5	5.2	3.6	5.7	91.7 $\pm$ 5.2
Quercetin	334.4	3.8	3.8	4.7	5.94	98.3 $\pm$ 5.8
	668.8	9.5	5.8	7.6	2.25	82.6 $\pm$ 1.9

#### 3.11.5.4 The specificity of the method

The specificity of the method was determined by comparing the relative migration times of rutin and quercetin to the incorporated micellar marker, quinine hydrochloride. In addition, on day 3 of the

validation, Products E and F were spiked with reference standards to confirm identification of the relevant peaks of interest since slight changes in elution times could possibly result in erroneous peak identification.

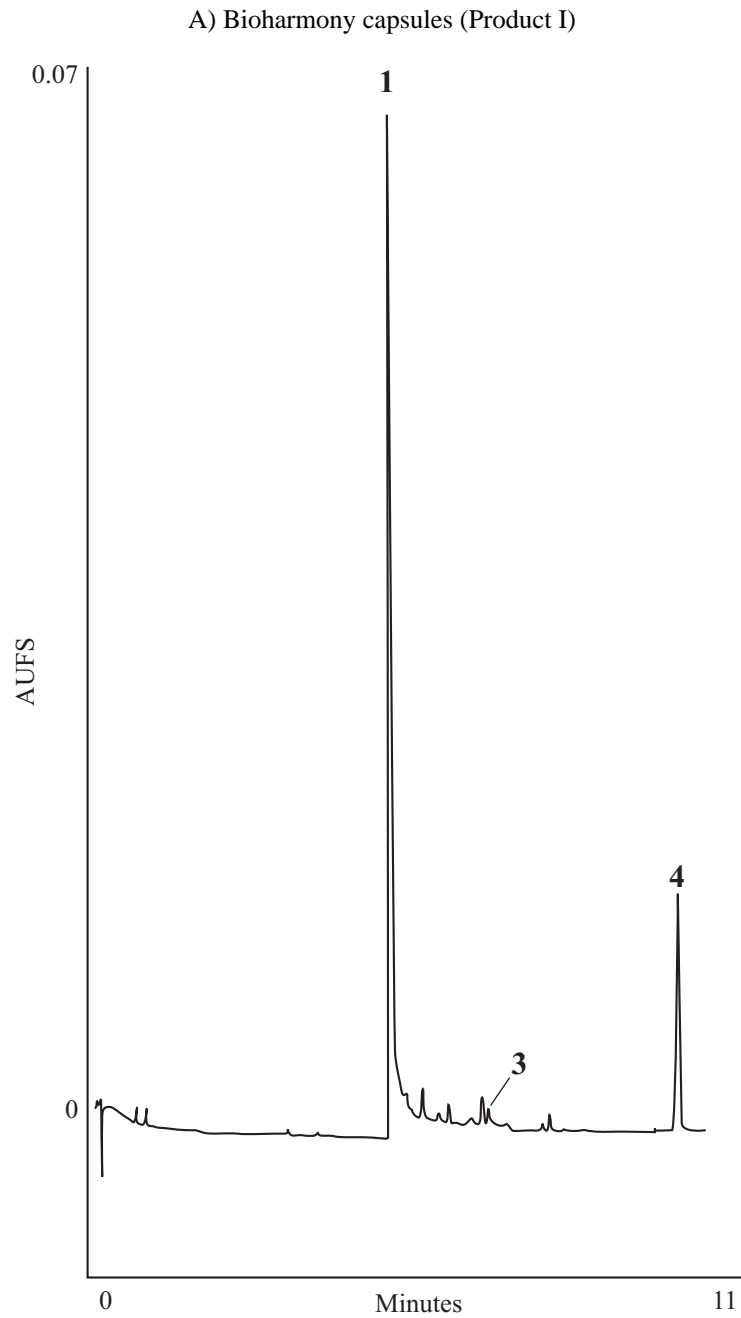
#### 3.11.5.5 Analysis of commercial products

Since 22 flavonol glycosides, 6 flavonol aglycones and 5 biflavones, amongst others, have been identified in *Ginkgo biloba* leaf extracts [57] and reference standards of many of these compounds are currently either unavailable or costly, quantification of the flavonol glycosides in leaf extracts and dosage forms is conventionally conducted by acid hydrolysis of the extracts followed by the HPLC determination of the resultant aglycones. Three main aglycones have been identified as quercetin, kaempferol and isorhamnetin of which quercetin and kaempferol are usually the main peaks of equal stature while isorhamnetin concentrations are typically 5 times lower. Three recent articles have suggested that manufacturers may adulterate Ginkgo extracts with rutin to claim a higher total flavonol content which is difficult to detect when using the acid hydrolysis method [22, 162, 170]. The analysis of intact flavonol glycosides is also a good indication of the source of raw material used in extracts [22, 53].

As a result, two flavonol markers were selected for quantification, the flavonol glycoside rutin and its corresponding aglycone, quercetin. Salicylic acid was chosen as the IS and migrated in a zone where there were no interferences and although it co-migrated with GA at lower, less selective wavelengths, the higher wavelength at 250 nm ensured unhindered determination of the IS. Products I and J were selected for quantification. Although the batch number of the Bioharmony product (I) was different to the lot used for the qualitative analysis in Section 3.10, it was predicted that it should contain sufficient rutin and quercetin to facilitate their determination. Similarly, the Nrf product (J) was also chosen for analysis since it too had previously shown consistently higher marker content than most other products (barring Vital), including Naturelle when using HPLC and LC-MS techniques of analysis (Chapters 4 and 5). Product I was also used to perform recovery studies since its moderate content facilitated the analysis of both lower and higher spiking levels.

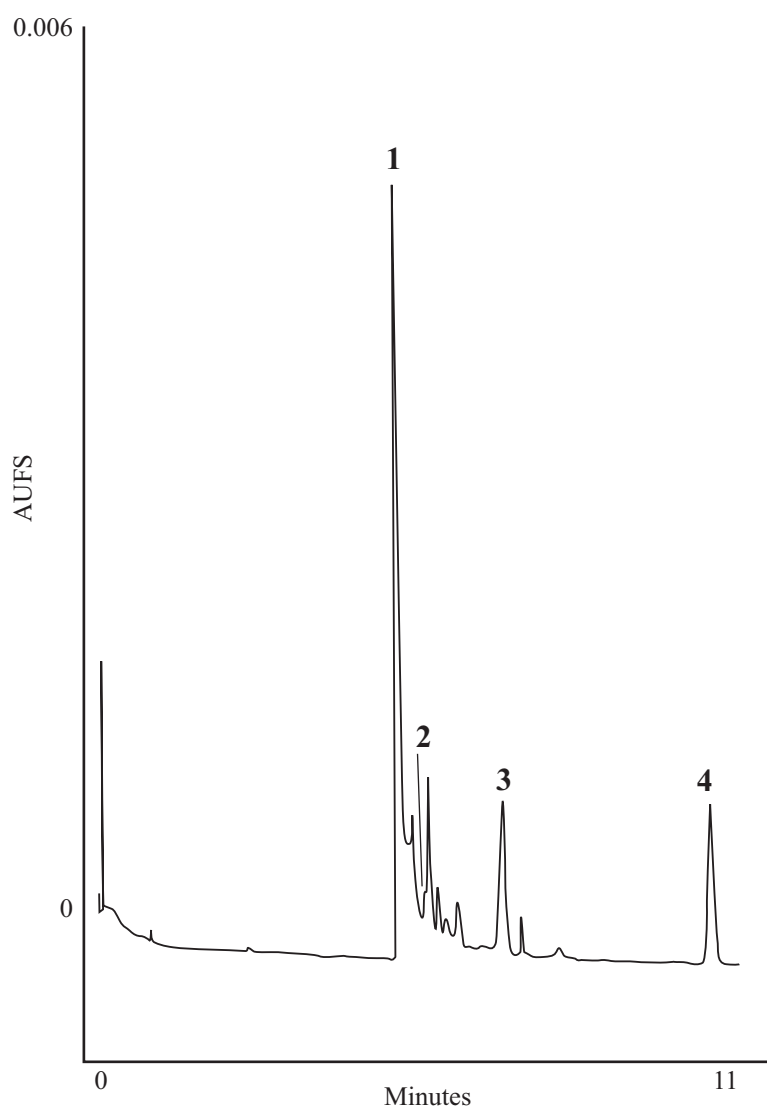
The electropherograms of products I and J are shown in Figure 3.23 and the results of the analyzed commercial products are given in Table 3.10. The total run time was ten minutes and the intra-day and inter-day RSDs were all less than 9%. There was an 11 fold difference in the amount of rutin present between the products. On the other hand, the high ratio of rutin to quercetin within products reflected appropriate storage conditions. From these results it can be concluded however that there are major disparities in the flavonol marker content of these two products and that Product J may be considered to be of superior quality when compared to Product I.

Figure 3.23. The fingerprinting profiles of Products I and J



Conditions: 78  $\mu\text{m}$  I.D., total length=60.0 cm, effective length=45.0 cm; Sample buffer: 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS (pH 2.2)); Running buffer: 40 mM SDS, 25 mM phosphoric acid (pH 2.2), 12 mM  $\beta$ -cyclodextrin; Hydrodynamic injection: 20 mbar for 0.6 sec.; Voltage: -17.5 kV; Detection  $\lambda$ : 250 nm. Labeled peaks: 1=quinine hydrochloride, 2=quercetin, 3=rutin, 4=salicylic acid.

B) Nrf tablets (Product J)



*Conditions: 78  $\mu\text{m}$  I.D., total length=60.0 cm, effective length=45.0 cm; Sample buffer: 50:50 methanol-buffer (10 mM phosphoric acid, 40 mM SDS (pH 2.2)); Running buffer: 40 mM SDS, 25 mM phosphoric acid (pH 2.2), 12 mM  $\beta$ -cyclodextrin; Hydrodynamic injection: 20 mbar for 0.6 sec.; Voltage: -17.5 kV; Detection  $\lambda$ : 250 nm. Labeled peaks: 1=quinine hydrochloride, 2=quercetin, 3=rutin, 4=salicylic acid.*



Table 3.10. Results of commercial products

Product	Constituent	Amount per dosage unit ( $\mu\text{g}$ )	Intra-day RSD (%) (n=3)			Inter-day RSD (%) (n=6)
			Day 1	Day 2	Day 3	
Product I	Rutin	$274.0 \pm 19.3$	6.2	4.7	6.6	7.0
	Quercetin	None	-	-	-	-
Product J	Rutin	$2395.3 \pm 160.0$	2.2	5.7	1.7	8.5
	Quercetin	$271.5 \pm 3.8$	3.9	3.4	2.8	1.4

### 3.11 CONCLUSIONS

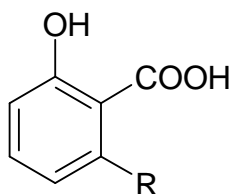
The RF-MEKC method designed for fingerprinting of both selected flavonols and terpene lactones was used for the quantitative analysis of the flavonol glycoside, rutin and flavonol aglycone, quercetin. This method proved to be suitable for the fingerprinting of these marker compounds and was also sufficiently accurate and reproducible for quantitative analysis of at least 2 of the markers, rutin and quercetin in commercially available *Ginkgo biloba* products. These 2 compounds were strategically selected QC markers since the ratio of rutin to quercetin relayed information regarding the authenticity of the raw material, the harshness/appropriateness of the manufacturing procedure and the conditions of storage. Although the profiles implied authenticity and suitable storage conditions, the quantification results indicated major disparities in the marker content in both products and re-iterated the fact that effective QC criteria need to be implemented to ensure consistent product quality and efficacy. When using CE-UV for the quantification of marker compounds, it is imperative that the assayed products are spiked in order to ensure accurate peak identification.

### 3.13 ANALYSIS OF GINKGOLIC ACIDS

As mentioned in chapter 2, the ginkgolic acids are chemically classified as alkylphenols and are speculated to cause allergic reactions and mutagenic, cytotoxic and even slight neurotoxic effects [53]. It should however be emphasized that these effects have not been adequately proven and there is some controversy whether these compounds should be removed from extracts. The ginkgolic acids are primarily found in high amounts in the Ginkgo fruits (which are commonly ingested as a digestive aid in Eastern countries) however more recently, the leaves have also been found to contain considerable quantities [53, 171]. Generally, standardized extracts are required to have total ginkgolic acid content limited to 5 - 10 ppm [53] although the German pharmacopoeia specifies that dry, standardized extracts may not contain more than 5 ppm [23]. Removal involves the inclusion of a simple partitioning step during the extraction process using an apolar solvent such as hexane or heptane [53]. Six ginkgolic acids have been identified and comprise a salicylic acid ring with a 6-alkyl side chain which varies in length (13, 15 or 17 carbons) as well as the presence (or absence) of double bonds (0, 1, 2 or 3) which

have a Z-configuration. The 2 main ginkgolic acids are C<sub>15:1</sub> and C<sub>17:1</sub> [53]. The structures of these alkylphenols are shown in Figure 3.24.

Figure 3.24. The structures of ginkgolic acids [53, 176]



R	Abbreviation
C <sub>13</sub> H <sub>27</sub>	C <sub>13:0</sub>
C <sub>15</sub> H <sub>31</sub>	C <sub>15:0</sub>
C <sub>15</sub> H <sub>29</sub>	C <sub>15:1</sub>
C <sub>17</sub> H <sub>33</sub>	C <sub>17:1</sub>
C <sub>17</sub> H <sub>31</sub>	C <sub>17:2</sub>
C <sub>17</sub> H <sub>29</sub>	C <sub>17:3</sub>

Due to the structural similarities between these compounds, separation using HPLC analysis has been difficult to achieve and is characterized by excessively long run times of over an hour [23], the use of complexation techniques [172] or dual column chromatography [171]. According to van Beek and Wintermans [171], separation between the pairs C<sub>13:0</sub> and C<sub>15:1</sub> as well as C<sub>15:0</sub> and C<sub>17:1</sub> was exceptionally difficult to achieve using reversed phase chromatography. However, when used in combination with a silver-loaded cation exchanger, HPLC separation was facilitated by complexation of the silver ions in the cation exchanger with the double bonds in the ginkgolic acid side chains and baseline resolution between all 5 ginkgolic acids was possible within 21 minutes using UV detection at 311 nm. This method was used to qualitatively examine a Ginkgo leaf extract where a sixth ginkgolic acid, C<sub>17:3</sub>, previously unreported, was discovered in trace amounts. This method was described by the authors as cheap and simple with potential application for routine phytochemical analysis of Ginkgo leaves and extracts. However, the procedure is relatively specialized and additional equipment and chemicals are required.

He and Xie [172] also used argentation chromatography with ESI-MS detection for the separation of 4 ginkgolic acids with similar results. Ginkgolic acids C<sub>13:0</sub> and C<sub>15:1</sub> co-eluted as well as C<sub>15:0</sub> and C<sub>17:1</sub> before addition of 0.03 mol/L solution of silver ions to the mobile phase consisting of (90:10) methanol: aqueous formic acid (0.5%). Baseline separation was achieved within 30 minutes and the authors stated that the use of low concentrations of silver nitrate did not cause corrosion or any other alterations in the stationary phase and column life was therefore not compromised. The analysis of various batches of Ginkgo leaves revealed differences in ginkgolic acid concentrations depending on geographical location and time of harvesting. He and Xie [172] showed that *Ginkgo biloba* leaves harvested in late autumn in China contained the lowest ginkgolic acid content (3 x) compared to those harvested in spring. On the other hand, Sticher [57] found that the green leaves from a female tree in Zürich showed the highest flavonol content during the month of May (spring). Based on these independent findings it would seem logical that since the ginkgolic acids can easily be removed using a

non-polar solvent such as hexane, the leaves should be harvested when all the purported active compounds, the flavonols and terpenes, are present in their highest possible concentrations.

Mass spectrometry has also been used for the quantitative determination of 2 ginkgolic acids, C<sub>15:1</sub> and C<sub>17:1</sub> in Ginkgo extracts using electrospray ionization in the negative ion mode. The low detection limits of 0.25 and 0.1 µg/ml, respectively and short run time of approximately 8 minutes using acetonitrile and acetic acid as the mobile phase makes this method highly suited for the analysis of Ginkgo preparations. Unfortunately the validation did not include the remaining ginkgolic acids, probably due to a lack of reference standards [173]. Jaggy and Koch [174] cited research by Verotta *et al.* (Phytochem. Anal (1993) p. 178) who used gas chromatography mass spectrometry (GC-MS) for the quantitative analysis of ginkgolic acids which were detected as trimethylsilyl derivatives. Although mass spectrometry (MS) is highly efficacious in unequivocally identifying relevant peaks with high sensitivity and compete liquid chromatographic (LC) resolution is not essential for quantification when using single ion monitoring (SIM), single reaction monitoring (SRM) or multiple reaction monitoring (MRM) modes, its expense and expertise can hardly justify its use for the analysis of these compounds classified as negative markers. Interest in these compounds is due to their potential negative pharmacological effects (although it has also been postulated that they may be anti-carcinogenic and have antimicrobial activity [174]) and their total content as opposed to individual quantification is currently sufficient in order to ensure product safety.

More recently, a relatively rapid HPLC-UV method was developed without resorting to the use of argentation, and validated for the determination of ginkgolic acids C<sub>13:0</sub>, C<sub>15:1</sub> and C<sub>17:1</sub> within 26 minutes which was then applied to analyze a *Ginkgo biloba* extract simply dissolved in methanol. The mobile phase consisted of (A) 0.1% aqueous formic acid and (B) 0.1% formic acid in acetonitrile programmed as a gradient. Only 3 ginkgolic acids were used for validation due to unavailability of pure reference standards and the tediousness of the isolation procedures [175]. This method appears to be simpler and more economical than the previously described HPLC methods and the fact that it was fully validated ensures suitable accuracy and repeatability for at least the main ginkgolic acids C<sub>15:1</sub> and C<sub>17:1</sub>, including C<sub>13:0</sub>.

Two studies have included the ginkgolic acids in methods used for the analysis of the terpene lactones and some flavonol components in *Ginkgo biloba* dosage forms [42, 170]. Kressmann *et al.* [42] quantified the ginkgolic acid content of 27 products found on the US market using a validated HPLC method. However, the individual ginkgolic acids were not specified and no chromatograms were shown to demonstrate the resolution of these compounds. Moreover, they stated that the ginkgolic acids elute at 80 - 90 minutes which is long after the more hydrophilic marker compounds have eluted. On the other hand, Liu *et al.* [170] used a rapid mass spectrometric method for the quantification of 2 ginkgolic acids, C<sub>13:0</sub> and C<sub>15:0</sub>, in addition to the qualitative analysis of some terpenoids and flavonol components. This method was however not validated and, as previously mentioned the use of a highly

sophisticated and expensive LC-MS technique for the quantification of these negative markers is currently unwarranted.

To our knowledge, no methods have been published using CE for the determination of the ginkgolic acids. This is surprising since CE is well known for its high resolution. A rapid, cost effective CE method was therefore developed for fingerprinting purposes to gauge the ginkgolic acid content in 2 commercial *Ginkgo biloba* products.

### **3.14 ANALYSIS OF GINKGOLIC ACIDS BY NON-AQUEOUS CE**

#### **3.14.1 The effect of apparent pH**

##### *3.14.1.1 Instrumentation*

See section 3.10.1 for the instrumentation and additional equipment used. A polyimide fused-silica capillary column (50  $\mu\text{m}$  I.D. x 360  $\mu\text{m}$  O.D.) was used for the separation which was supplied by Polymicro Technologies, Phoenix, AZ, USA.

##### *3.14.1.2 Materials and reagents*

Methanol, acetic acid and acetonitrile (all HPLC grade) were purchased from BDH chemicals (Poole, UK). Sodium acetate was procured from Roche Chemicals (Isando, Johannesburg, South Africa). A 100 mg ginkgolic acid mix (Bz04-274-08) was kindly donated by Dr. Egon Koch of Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany) and contained C<sub>13:0</sub> (3%), C<sub>15:0</sub> (3%) C<sub>15:1</sub> (47%), C<sub>17:1</sub> (43%) and C<sub>17:2</sub> (3%). The ginkgolic acid C<sub>17:1</sub> reference standard was obtained from Phytolab (Hamburg, Germany). Water was purified in a Milli-Q system and 0.45  $\mu\text{m}$  HVLP type filters were used to filter and degas the BGE (both from Millipore, Bedford, MA, USA).

##### *3.14.1.3 Capillary conditioning*

The capillary was washed at the start of each day for 30 minutes with 1M sodium hydroxide, 30 minutes with 0.1M sodium hydroxide and 30 minutes with distilled water. Between consecutive injections, the capillary was conditioned with buffer for 3 minutes. The BGE was replaced with fresh buffer at the cathode before each injection and the buffer at the anode was replenished after every third injection.

##### *3.14.1.4 Initial experimental conditions*

A mixture of the ginkgolic acids in methanol as well as methanol-buffer (50:50) was injected under the same conditions previously described in section 3.11. Although the ginkgolic acids are hydrophobic compounds and RF-MEKC seemed like a suitable CE mode, no peaks were observed after 60 minutes while monitoring at UV wavelength of 310 nm.

According to Altria [176], non-aqueous CE is suitable for compounds which are easily ionizable and insoluble in water. Organic solvents such as methanol and acetonitrile are popular BGEs due their low UV absorbance. The paper also gave an example of the separation of a number of acids, including benzoic acid using a buffer consisting of 50:50 acetonitrile-methanol with 10 mM sodium acetate and an applied voltage of +25 kV. These parameters were therefore used as the initial experimental conditions. The total capillary length was 60.0 cm with an effective length of 45.0 cm. The samples were injected hydrodynamically at 50 mbar for 3 seconds. The voltage ramp was +6 kV/s. Detection was at 310 nm with a rise time of 0.1 seconds and a sensitivity setting of 0.01 AUFS.

#### *3.14.1.5 Preparation of solutions of ginkgolic acid reference standards*

The ginkgolic acid mixture was transferred to a 20 ml volumetric flask and the remaining contents in the vial were dissolved in (3 x 2 ml) methanol and added to the same flask. The contents were dissolved in a suitable quantity of methanol before being filled to volume to yield a concentration of 5mg/ml of the mixture. Equal aliquots of the solution were evaporated to dryness using nitrogen and then stored at -5°C. When required, an aliquot of the ginkgolic acid mixture was reconstituted in methanol to give a total concentration of 2.5 mg/ml.

#### *3.14.1.6 Experimental Procedure*

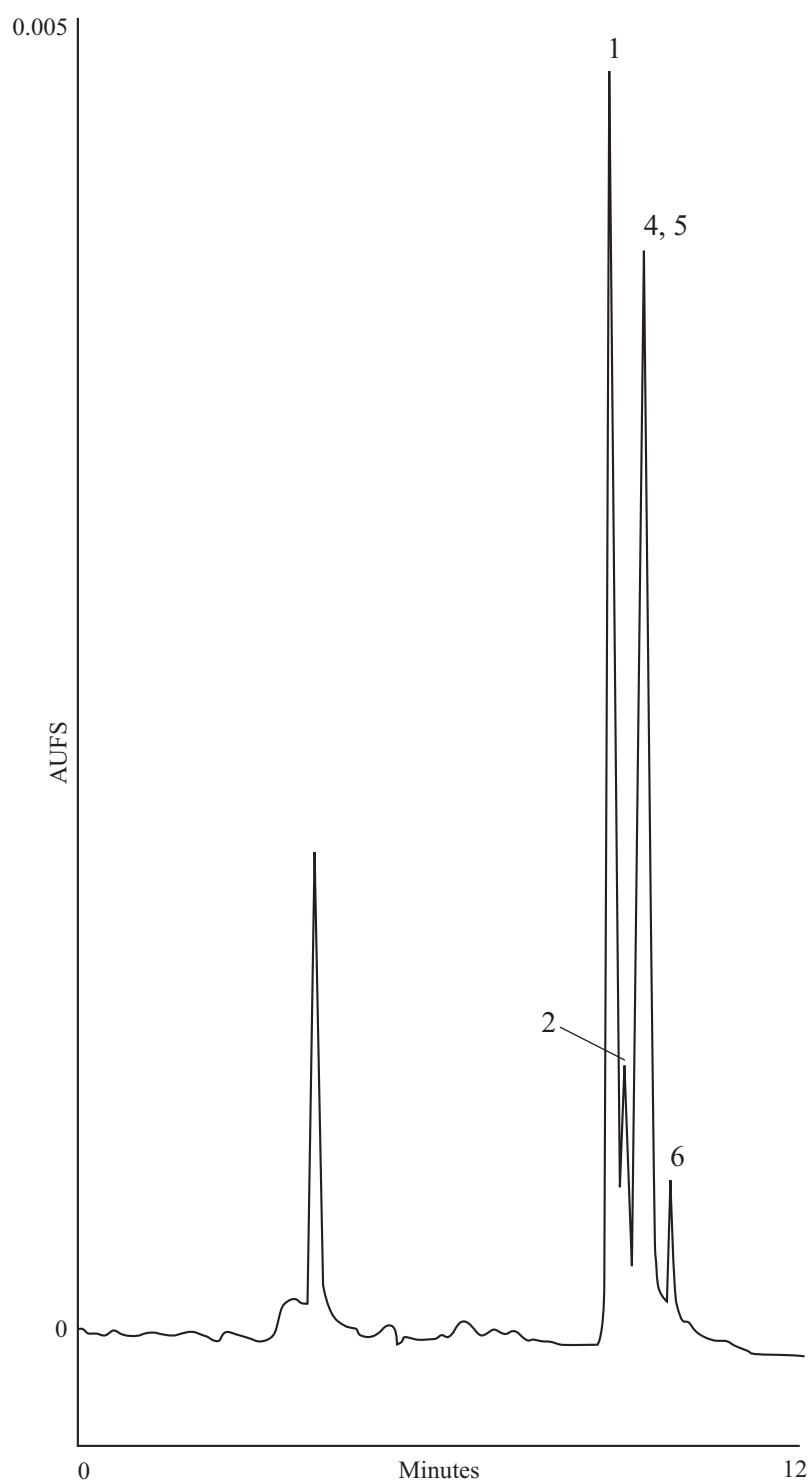
Technically pH cannot be extrapolated to non-aqueous systems. However, the addition of electrolytes such as ammonium or sodium acetate creates optimal acidic or basic conditions to facilitate analyte ionization and separation. This so-called apparent pH (pH\*) can therefore be measured [177].

The effect of the pH\* on the resolution of the ginkgolic acids was investigated. The BGE consisted of 50:50 acetonitrile-methanol with 10mM sodium acetate. The sodium acetate was weighed and transferred to a volumetric flask and dissolved by sonication in methanol. An equal quantity of acetonitrile was added and when the buffer reached room temperature, it was filtered through a 0.45 µm HVLP membrane and the pH\* was adjusted from 9.8 (the original pH\* value) to yield buffer solutions of pH\* 8.0, 7.0, 6.0 and 4.0, respectively, using acetic acid.

#### *3.14.1.7 Results*

Minimal effect on the separation was observed over the whole pH\* range. A slight increase in migration time was observed with a decrease in pH\* until a pH\* of 6.0 where the migration times were similar to those at pH\* of 8. At pH\* of 4.0, a single peak was observed. The pH\* was therefore kept at 9.8 and Figure 3.25 shows the separation of the ginkgolic acid mixture at this pH\* value. Since reference standards for each individual component of the ginkgolic acid mixture were not available, and on the basis that the mixture was purported to contain at least 5 ginkgolic acids, each peak was numerically labeled on the basis of its elution order, save for number 3 since a further peak was resolved when the separation was later optimized.

Figure 3.25. Separation of the ginkgolic acids at high pH\*



Conditions: 50  $\mu\text{m}$  I.D., total length = 60.0 cm, effective length = 45.0 cm; Sample solvent: methanol; Running buffer: methanol-acetonitrile (50:50), 10 mM sodium acetate; pH\* 9.8; Hydrodynamic injection: 50 mbar for 3 sec.; Voltage: +25 kV; Detection  $\lambda$ : 310 nm.

#### 3.14.1.8 Discussion

The ginkgolic acids are 2-hydroxy-6-alkyl benzoic acids and are therefore negatively ionized at high pH\* where the electrophoresis process based on charge and size is optimal. The EOF was enhanced under these conditions and the negatively charged compounds were swept to the cathode under its influence. Although a decrease in pH\* resulted in a slower moving EOF and hence slightly longer migration times, the separation did not improve. To conclude, the ginkgolic acids are optimally ionized at high pH\* and moderate changes in the pH\* of the BGE did not significantly influence resolution.

#### 3.14.2 The effect of ionic strength

##### 3.14.2.1 Instrumentation and materials

See section 3.14.1.1 and 3.14.1.2.

##### 3.14.2.2 Capillary conditioning

For the remaining experiments in this section, the initial usual daily capillary conditioning procedure was changed to washing with 1M sodium hydroxide for 15 minutes, 0.1M sodium hydroxide for 15 minutes, distilled water for 15 minutes and then with 0.1M sodium hydroxide for 40 minutes. The capillary was flushed before each injection with 5 minutes 1M sodium hydroxide, 5 minutes water and finally, 5 minutes BGE.

##### 3.14.2.3 Initial experimental procedure

The total capillary length (50  $\mu\text{m}$  I.D. x 360  $\mu\text{m}$  O.D.) was 60.0 cm with an effective length of 45.0 cm. The buffer consisted of 50:50 acetonitrile-methanol with 10 mM sodium acetate and the applied voltage was +25 kV with a voltage ramp of +6 kV/s. Injections were performed hydrodynamically at 50 mbar for 3 seconds. Detection took place at 310 nm with the rise time set at 0.1 seconds using a sensitivity setting of 0.005 AUFS.

##### 3.14.2.4 Preparation of reference standards

The ginkgolic acid mixture was prepared as previously described in section 3.14.1.5. Ginkgolic acid C<sub>17:1</sub> was prepared in methanol with a concentration of 0.25 mg/ml.

##### 3.14.2.5 Experimental Procedure

The effect of sodium acetate on the reference standard C<sub>17:1</sub> was investigated by changing the concentration of sodium acetate while keeping the ratio of acetonitrile and methanol constant at 50:50. The sodium acetate concentrations under investigation were 10 mM, 20 mM, 30 mM and 50 mM. Based on the results obtained, ionic concentration of 30 mM and 40 mM were chosen for further investigation of the ginkgolic acid mixture.

#### 3.14.2.6 Results

The effect of sodium acetate on the mobility of ginkgolic acid C<sub>17:1</sub> is shown in Figure 3.26 and an electropherogram at the 30 mM sodium acetate concentration is illustrated in Figure 3.27. The mobility decreases with increasing ionic strengths although higher peak heights were observed with increasing concentrations until 50 mM where the peaks were noticeably suppressed. From these results, 30 mM and 40 mM concentrations were selected to determine the effects on the ginkgolic acid mixture shown in Figure 3.28. From the graph it can be seen that although the migration of the analytes was slower at a concentration of 40 mM resolution was unaffected by the ionic concentration change. Once again, marked peak suppression was seen at a concentration of 40 mM sodium acetate. Although all the peaks had slightly different migration velocities (measurements were taken from the peak apex), baseline resolution between the peaks was not achieved and peak 3 and 4 were presented as a split peak. Ginkgolic acid C<sub>17:1</sub> was identified as the first ginkgolic acid in the mixture (peak 1) by calculation of the migration times relative to the EOF.



Figure 3.26. Effect of ionic strength on the mobility of ginkgolic acid C<sub>17:1</sub>

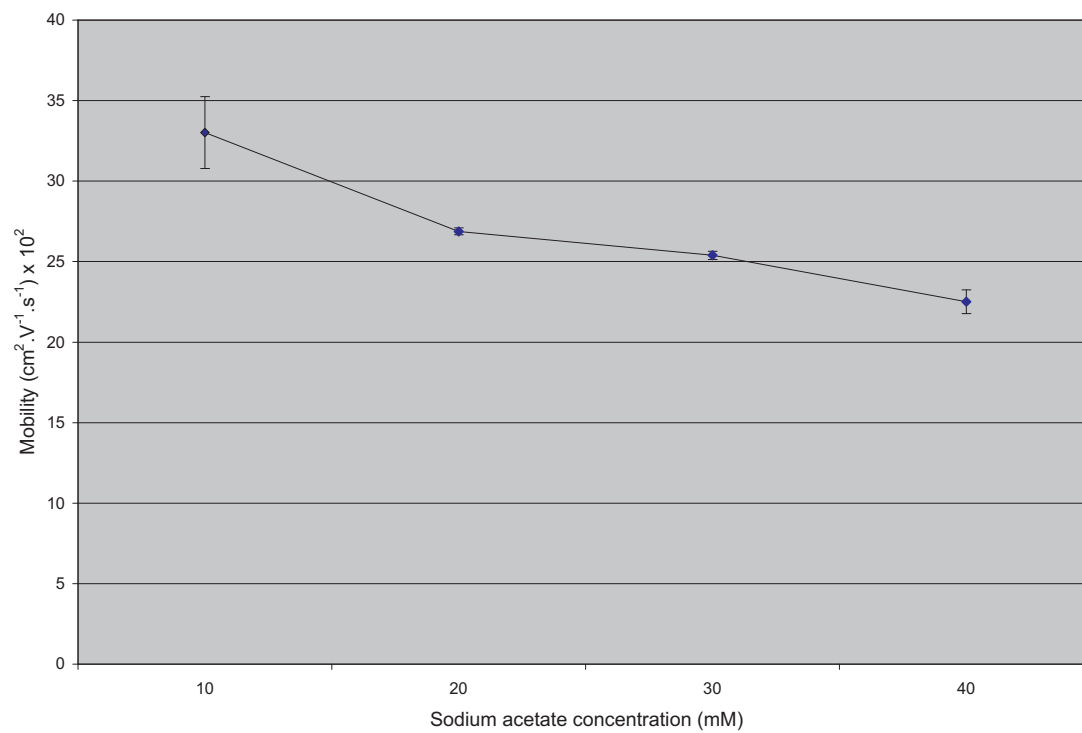
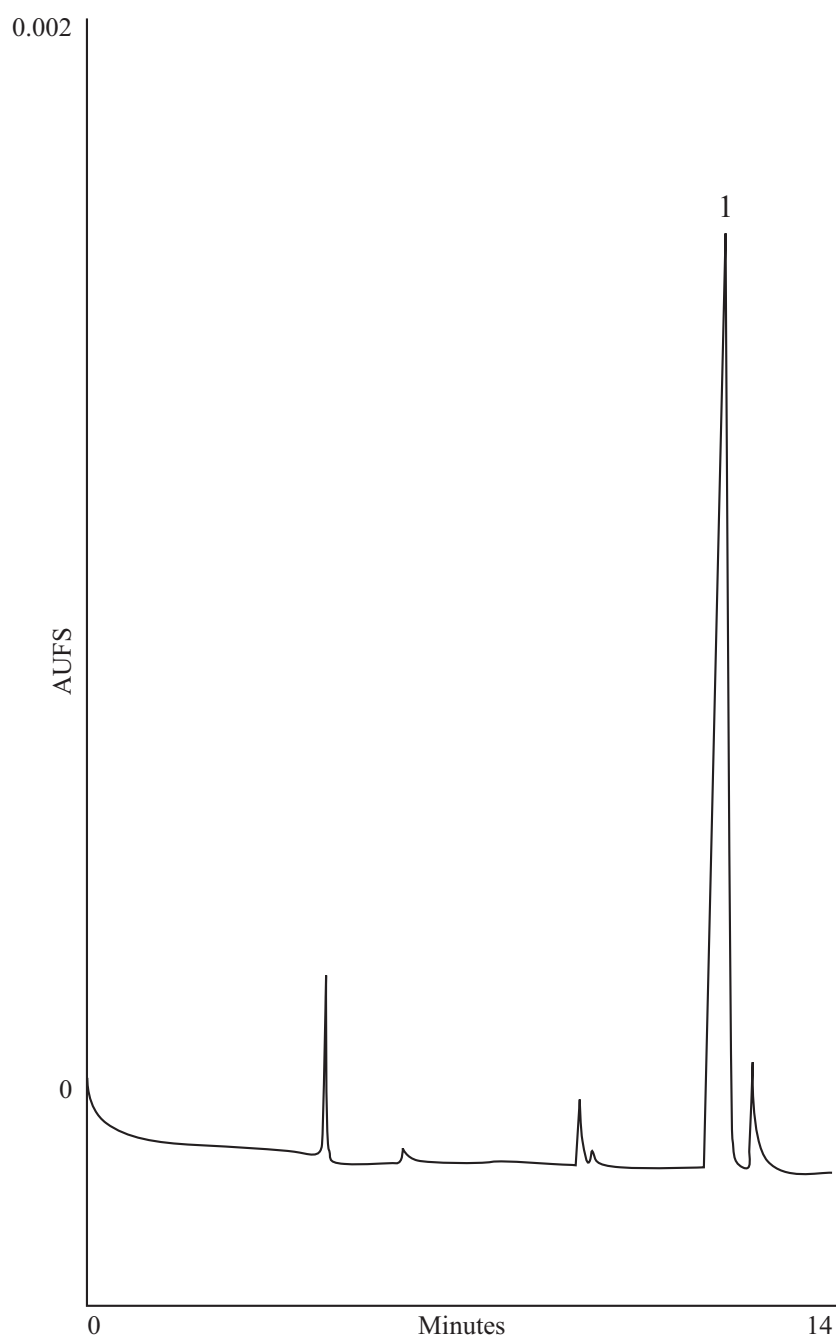
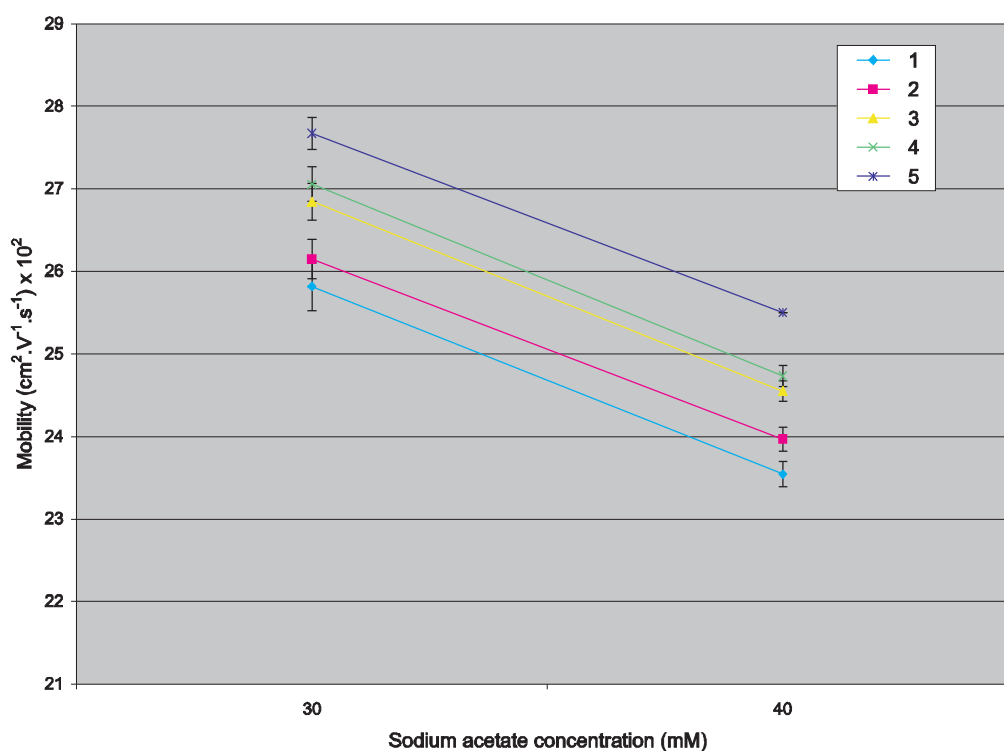


Figure 3.27. Ginkgolic acid C<sub>17:1</sub> using 30 mM sodium acetate



Conditions: 50  $\mu\text{m}$  I.D., total length = 60.0 cm, effective length = 45.0 cm; Sample solvent: methanol; Running buffer: methanol-acetonitrile (50:50), 30 mM sodium acetate; Hydrodynamic injection: 50 mbar for 3 sec.; Voltage: +25 kV; Detection  $\lambda$ : 310 nm; Labeled peak: ginkgolic acid C<sub>17:1</sub>.

Figure 3.28. Effect of ionic concentration on the mobility of the ginkgolic acid mixture



#### 3.14.2.7 Discussion

The increased migration times of the ginkgolic acids with increasing ionic strength was due to suppression of the EOF. Higher ionic strengths are normally also associated with broader peaks however, the reverse was observed. This can be explained by a process called “intrinsic sample stacking” which occurred when the BGE had a higher ionic strength than the sample zone [178]. The conductivity of the sample zone was initially lower than the running buffer and once the potential was applied, the analytes in this zone migrated rapidly under the influence of the greater field strength until they reached the boundary of the sample zone and BGE. The decrease in field strength at the boundary interface then caused the analytes to migrate slower. This process continued until all the analytes were concentrated at the boundary interface resulting in sample stacking which was observed by the sharper, higher peaks at 20 mM and 30 mM sodium acetate concentrations [133]. The decrease in peak heights at the 40 mM and 50 mM ionic strengths can be explained by an increase in background absorbance characterized by a decrease in sensitivity. The longer migration times also caused broadening of the sample zone with resultant wider peaks. As resolution was not significantly altered by changes in the ionic strength, the optimal sodium acetate concentration of 30 mM was selected, primarily based on the optimized peak shape and height of ginkgolic acid C<sub>17:1</sub> as well as the ginkgolic acid mixture.

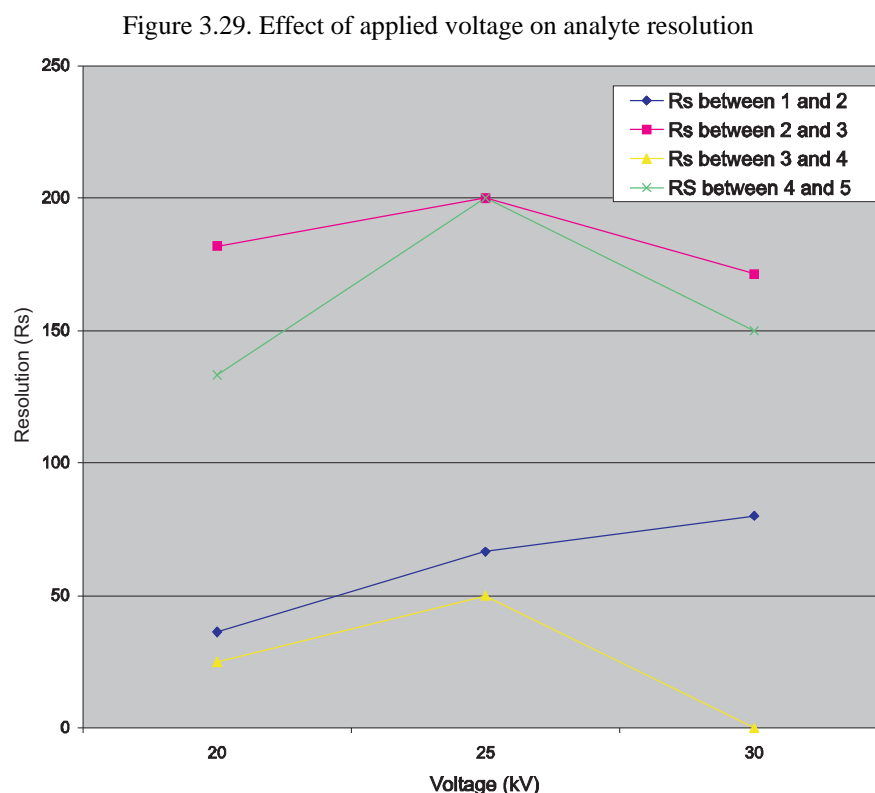
### 3.14.3 The effect of applied voltage

#### 3.14.3.1 *Experimental Procedure*

The effect of voltage on the resolution of the ginkgolic acids was investigated by changing the voltage settings to +20 kV, +25 kV and +30 kV with a constant voltage ramp of +6 kV/s. Conditions were the same as previously described in sections 3.14.2.1 to 3.14.2.4. The sodium acetate concentration used was 30 mM.

#### 3.14.3.2 *Results*

The results of this experiment are shown in Figure 3.29. Resolution was marginally improved at +25 kV. At +30 kV, peak 3 and peak 4 merged completely. With higher applied voltages, an increase in the mobility of all marker compounds was apparent and the run time decreased from 18 minutes at +20 kV to 14 minutes at +25 kV and then to 12 minutes at +30 kV. The peaks were sharper using higher applied voltages with the peaks at +20 kV being relatively broad. The applied voltage of +25 kV was considered optimal.



#### 3.14.3.3 *Discussion*

The use of non-aqueous CE had the advantage that there were minimal effects of joule heating and high voltages could be applied which ensured that the shortest possible run times were achieved with marginal loss of efficiency. At +30 kV, peak 3 and peak 4 co-migrated, probably due to the shortened

time spent in the capillary tubing however even at +20 kV and +25 kV, these peaks almost merged and baseline resolution was not achieved. The sharper peaks at +25 kV compared to +20 kV resulted in a minimal improvement in resolution and +25 kV was the most suitable separation voltage based on both resolution and total run time.

#### 3.14.4 The effect of the organic solvent in BGE

##### 3.14.4.1 *Experimental Procedure*

The ratio of acetonitrile to methanol on the resolution of the ginkgolic acids was investigated by changing the ratio from 50:50 to 25:75 and 75:25 while keeping the concentration of sodium acetate constant at 30 mM. The samples were injected once only. The remaining conditions were as stated in sections 3.14.2.1 - 3.14.2.4.

##### 3.14.4.2 *Results*

Figure 3.30 shows the effect of methanol and acetonitrile on the resolution of the ginkgolic acids and the corresponding electropherograms are shown in Figures 3.31 - 3.33. Higher fractions of methanol in the BGE resulted in improved resolution at the expense of an increased run time to 30 minutes. The separation between peak 3 and peak 4 was improved but baseline separation was still not achieved. An extra peak was present (peak 3) which can also be seen as a shoulder peak in the electropherogram where the BGE was 50:50 acetonitrile-methanol. Higher percentages of acetonitrile in the buffer resulted in sharper, higher peaks and a much shorter run time of 12 minutes, however resolution was compromised. Only 4 peaks were present and peak 3 and peak 4 co-migrated. The 50:50 acetonitrile-methanol composition was a compromise between the effects of the other 2 investigated buffers and was therefore chosen for further experiments.

Figure 3.30. Effect of acetonitrile composition on the resolution of the ginkgolic acids

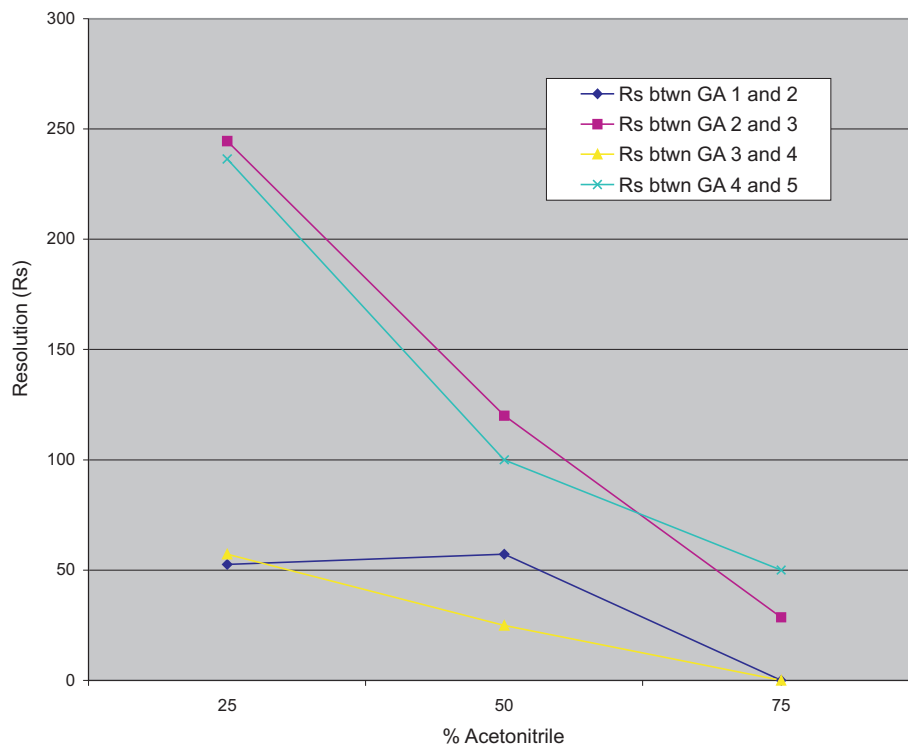
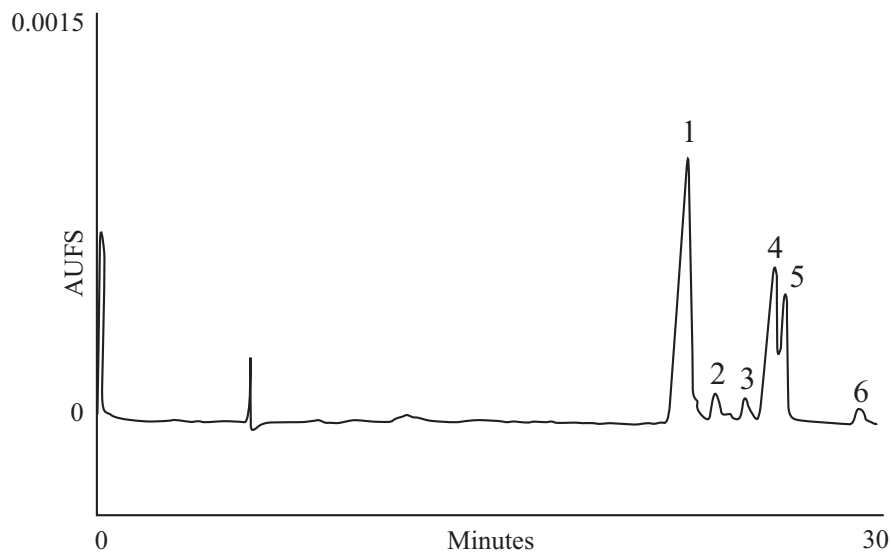
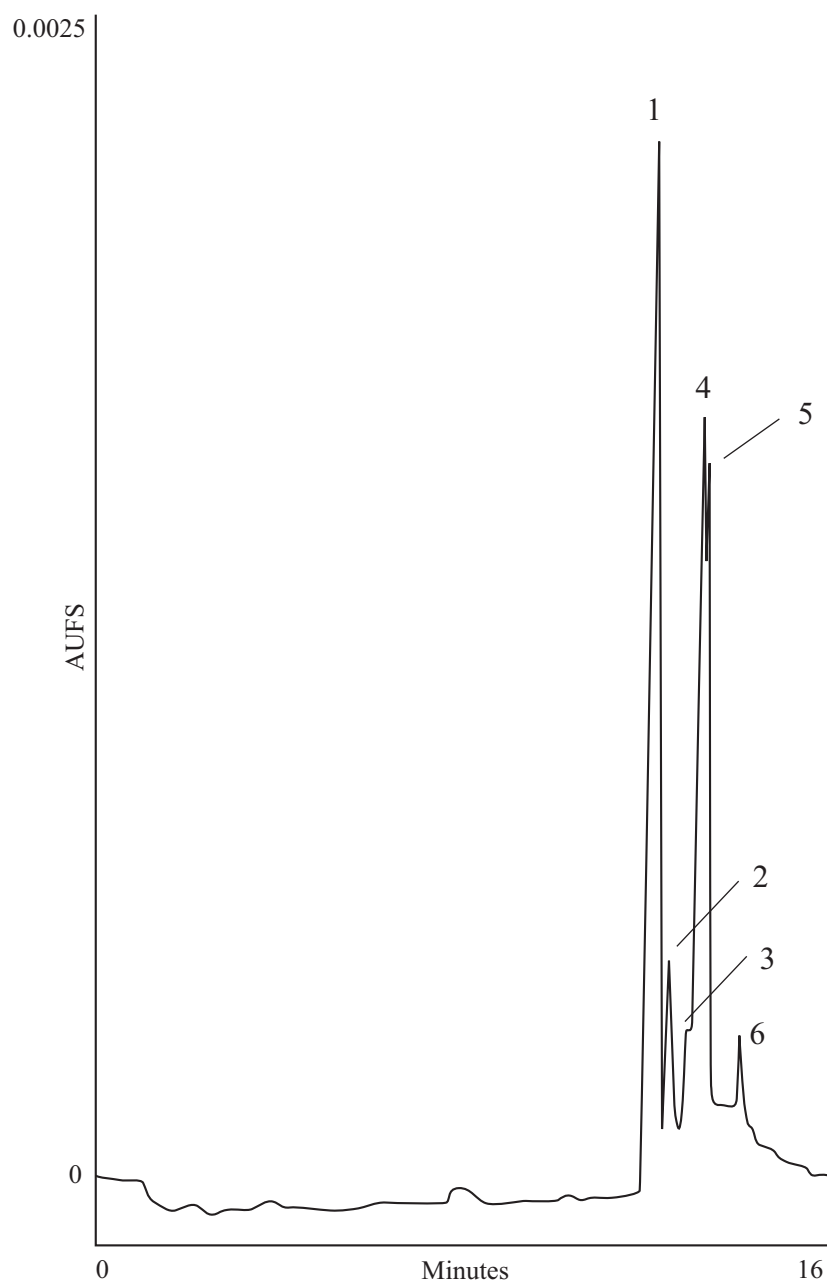


Figure 3.31. Electropherogram of ginkgolic acids with a running non-aqueous buffer of 25:75 acetonitrile-methanol



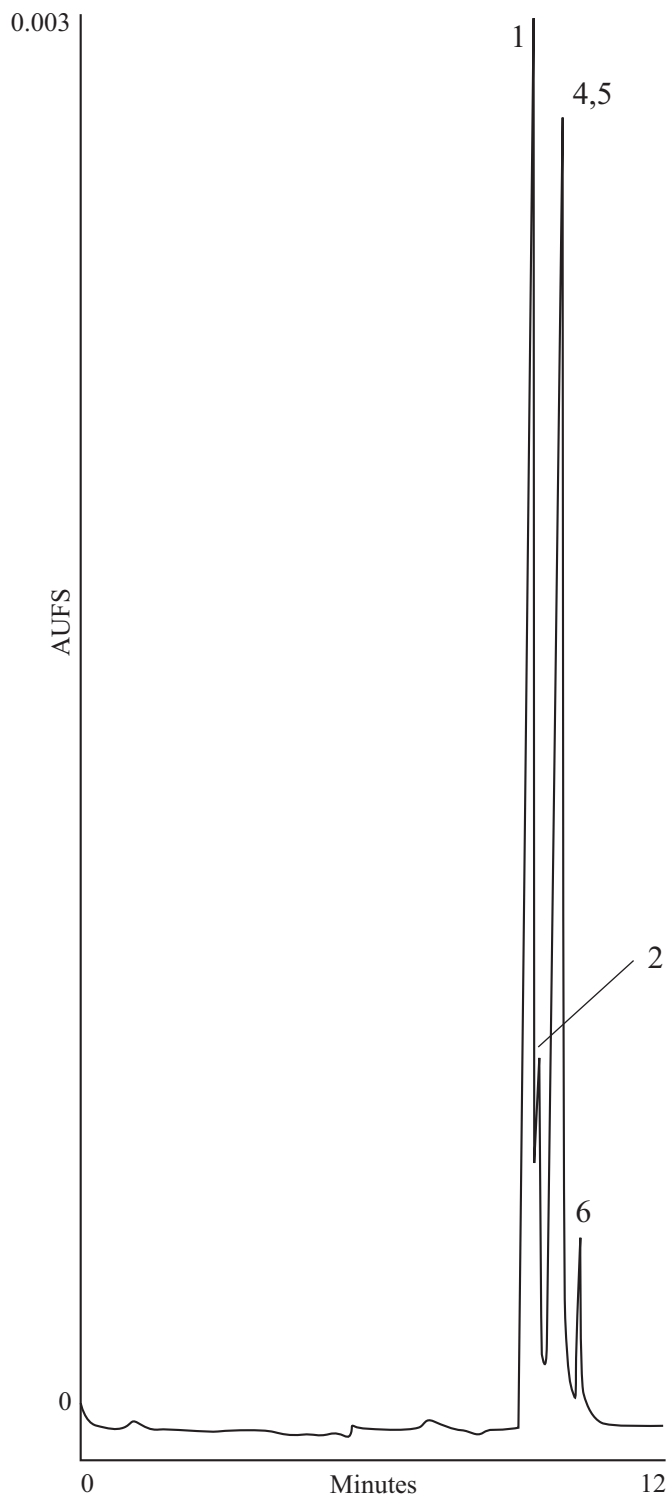
Conditions: 50  $\mu\text{m}$  I.D., total length = 60.0 cm, effective length = 45.0 cm; Sample solvent: methanol; Running buffer: methanol-acetonitrile (75:25), 30 mM sodium acetate; Hydrodynamic injection: 50 mbar for 3 sec.; Voltage: +25 kV; Detection  $\lambda$ : 310 nm.

Figure 3.32. Electropherogram of ginkgolic acids with a running buffer of 50:50 acetonitrile-methanol



Conditions: 50  $\mu\text{m}$  I.D., total length = 60.0 cm, effective length = 45.0 cm; Sample solvent: methanol; Running buffer: methanol-acetonitrile (50:50), 30 mM sodium acetate; Hydrodynamic injection: 50 mbar for 3 sec.; Voltage: +25 kV; Detection  $\lambda$ : 310 nm.

Figure 3.33. An electropherogram of ginkgolic acids with a running buffer of 75:25 acetonitrile-methanol



Conditions: 50  $\mu\text{m}$  I.D., total length = 60.0 cm, effective length = 45.0 cm; Sample solvent: methanol; Running buffer: methanol-acetonitrile (25:75), 30 mM sodium acetate; Hydrodynamic injection: 50 mbar for 3 sec.; Voltage: +25 kV; Detection  $\lambda$ : 310 nm.



### 3.14.4.3 Discussion

Although mixtures of acetonitrile and methanol are popular organic solvents for non-aqueous CE, their effect on the migration patterns of electrolytes is not yet fully understood. Separation has been broadly explained by ion-solvent interactions, ion-dipole interactions, ion-ion interactions and most importantly, electrostatic interactions between the ions and counter-ions present in the organic solvent mixture. Some solvent properties which play a role in these interactions are outlined in Table 3.11. [179].

Table 3.11. Properties of acetonitrile and methanol which facilitate separation in non-aqueous CE

Property	Methanol	Acetonitrile
Autoprotolysis at 25°C ( $pK_{\text{auto}}$ )	17.2	>33
Dielectric constant	32.63	36.01
Viscosity (centipoise)	0.544	0.345
Dipole moment (Debye units)	1.70	3.92

Methanol is popular in non-aqueous CE and is particularly useful for increasing the solubility of hydrophobic compounds. In these experiments, higher percentages of methanol resulted in slower migration, broader peaks and the most efficient resolution as seen in Figure 3.31. In the paper by Porras *et al.* [179], the same trend was seen. Methanol is an amphiprotic solvent and may alter the ionization of both acidic and basic compounds and therefore could possibly have affected the ionization of the ginkgolic acids at high pH with resultant lengthened migration times. The extra peak (peak 3) seen in Figure 3.31 was also seen as a shoulder peak in Figure 3.32. Whether this peak is an impurity or a ginkgolic acid is unknown. Six ginkgolic acids have in fact been identified in leaf extracts [171], however the mixture apparently contained only 5 namely,  $C_{13:0}$  (3%),  $C_{15:0}$  (3%),  $C_{15:1}$  (47%),  $C_{17:1}$  (43%) and  $C_{17:2}$  (3%) with their constituent proportions in parenthesis as provided by the supplier. Based on this, 3 smaller peaks (~3% of each) of approximately the same height/area and 2 larger peaks (47% and 43%) should have been present in the electropherogram. In the buffer containing 75% methanol and 25% acetonitrile (Figure 3.31), 3 smaller peaks and 3 larger peaks were present and in 50:50 acetonitrile-methanol (Figure 3.32), 2 smaller peaks (excluding the shoulder peak number 3) and 3 larger peaks were present. Only peak 1 could be identified as  $C_{17:1}$  by comparing its relative migration time to an authentic reference standard. Hence, either peak 4 or 5 could be an impurity unless the ginkgolic acid mixture actually contained 6 ginkgolic acids as opposed to the suggested 5.

Acetonitrile had a dramatically different effect on the ginkgolic acids compared to methanol. Increasing the acetonitrile content to 75% resulted in more rapid migration velocities although not as drastic compared to the change from 25% to 50%. Acetonitrile is less viscous and has a much larger dipole moment than both water (1.85) and methanol (1.70) and the former solvent is classified as an aprotic solvent [179]. These properties altered the buffer's selectivity and resolution and migration time decreased with an increase in peak height.

The BGE consisting of 50:50 acetonitrile-methanol was selected as optimal for fingerprinting purposes. Although resolution was greater with higher methanol content, the longer run time and lower sensitivity detracted from its benefits. Since the aim of the developed method was for qualitative purposes only, the same amount of information could be derived when equal quantities of organic solvents were used with the advantage of higher sensitivity and a shorter run time of 15 minutes.

### 3.15 QUALITATIVE ANALYSIS OF THE GINGKOLIC ACIDS IN *GINKGO BILOBA* ORAL DOSAGE FORMS

#### 3.15.1 Instrumentation

All instruments and additional equipment described in section 3.14.1.1 were used for the analysis.

#### 3.15.2 Materials and reagents

Refer to Section 3.14.1.2 for all materials used, including the relevant reference standards. Hexane was purchased from BDH chemicals (Poole, UK). Two commercial *Ginkgo biloba* products were bought from a local pharmacy in Grahamstown, South Africa (Table 3.12).

Table 3.12. *Ginkgo biloba* dosage forms selected for qualitative analysis of ginkgolic acids

Manufacturer	Name of product	Dosage form	Label claim	Daily dose	Batch number	Extract
Bioforce <sup>1</sup>	Ginkgoforce	Tablet	Each 250mg tablet is equivalent to 10 drops of Ginkgoforce liquid	2 tablets tds*	406088E	K
Nrf Herbal <sup>2</sup>	<i>Ginkgo biloba</i>	Tablet	Each tablet contains 350mg <i>Ginkgo biloba</i> , 50mg <i>Ginkgo biloba</i> extract 24% and 240mg FoodMatrix™	1 to 2 tablets or as directed by your healthcare practitioner	2H2044PW	L

<sup>1</sup>Bioforce S.A. (Pty) Ltd., Midrand, South Africa; <sup>2</sup>Nrf Herbal, Centurion, South Africa; \*three times daily

### 3.15.3 Experimental Procedure

A polyimide fused-silica capillary column (50  $\mu\text{m}$  I.D. x 360  $\mu\text{m}$  O.D.) with a total length of 59.8 cm and effective length of 44.8 cm was used for the separation. The remaining conditions were as previously described in sections 3.14.2.2 - 3.14.2.4 except for the sodium acetate concentration which was 30 mM. The detector sensitivity was increased to 0.002 AUFS when analyzing the products (with the exception of Nrf extracted in methanol where the sensitivity was set at 0.005 AUFS).

#### 3.15.3.1 *Standard solutions*

Standard solutions were as prepared as previously described in sections 3.14.1.5 and 3.14.2.4.

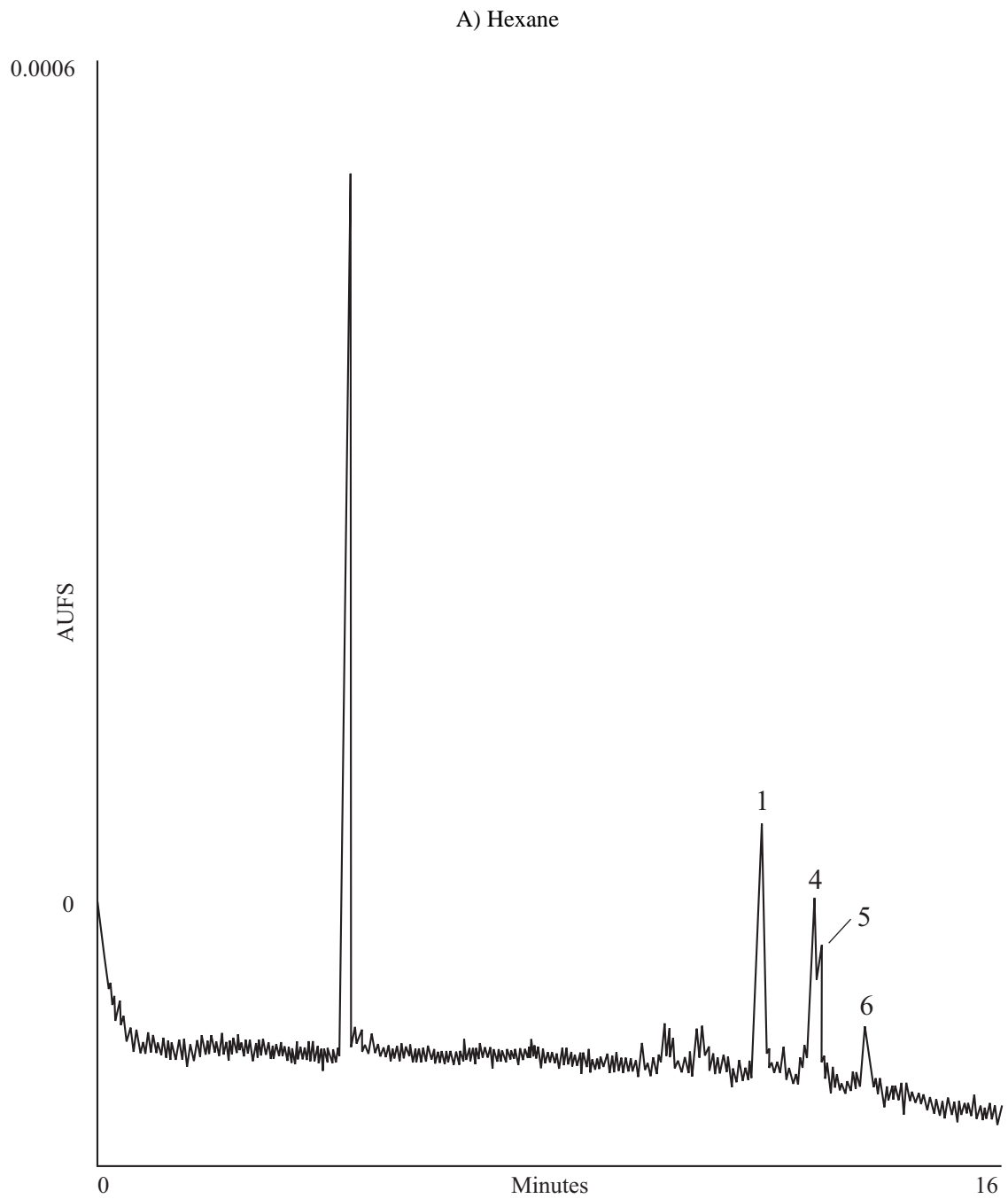
#### 3.15.3.2 *Sample solutions*

Twenty tablets from each dosage form were weighed and extracted according to the processes previously described (Section 3.10.3.1). An additional extraction using hexane instead of methanol was also performed according to the same procedure but the dry extracts were reconstituted in 5 ml methanol.

### 3.15.4 Results and discussion

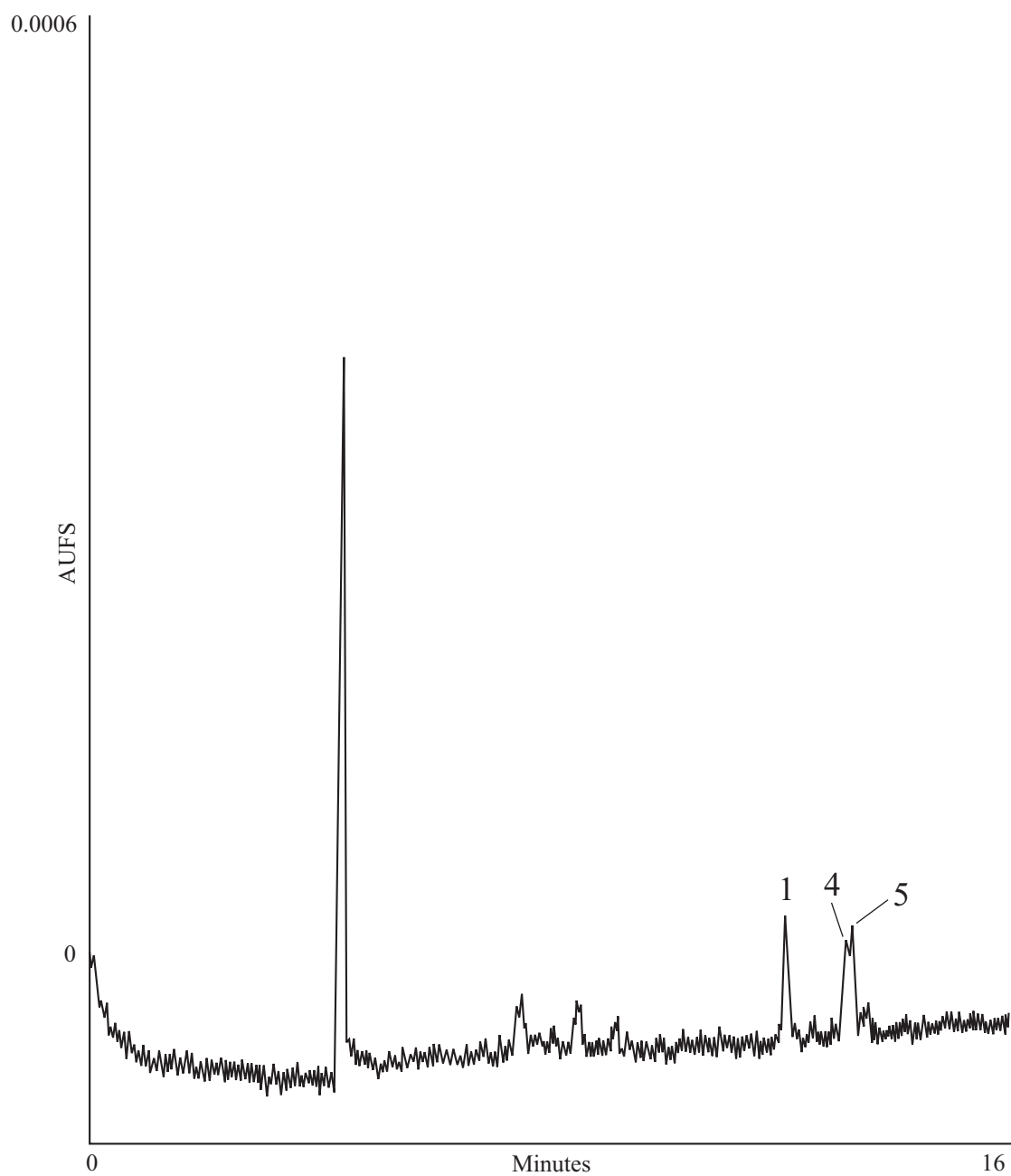
Methanol is considered a non-selective solvent and has been previously used for the extraction of ginkgolic acids [53], however these compounds are hydrophobic in nature and extraction using a solvent with a lower polarity index such as hexane was predicted to be more suitable. Extraction of the positive markers (the flavonols and terpenoids) with methanol was used for all other analysis techniques and, in order to simplify sample preparation procedures, it was presumed that the same samples used for the content analysis of the positive markers could also be used for the qualitative analysis of the negative ones. Both commercial products were therefore extracted with each solvent and the profiles were compared qualitatively for extraction efficiency and selectivity. The results are shown in Figures 3.34 and 3.35. Figure 3.36 shows the electropherogram of a blank injection of methanol only.

Figure 3.34. Effect of extraction solvent on the fingerprints of Products K



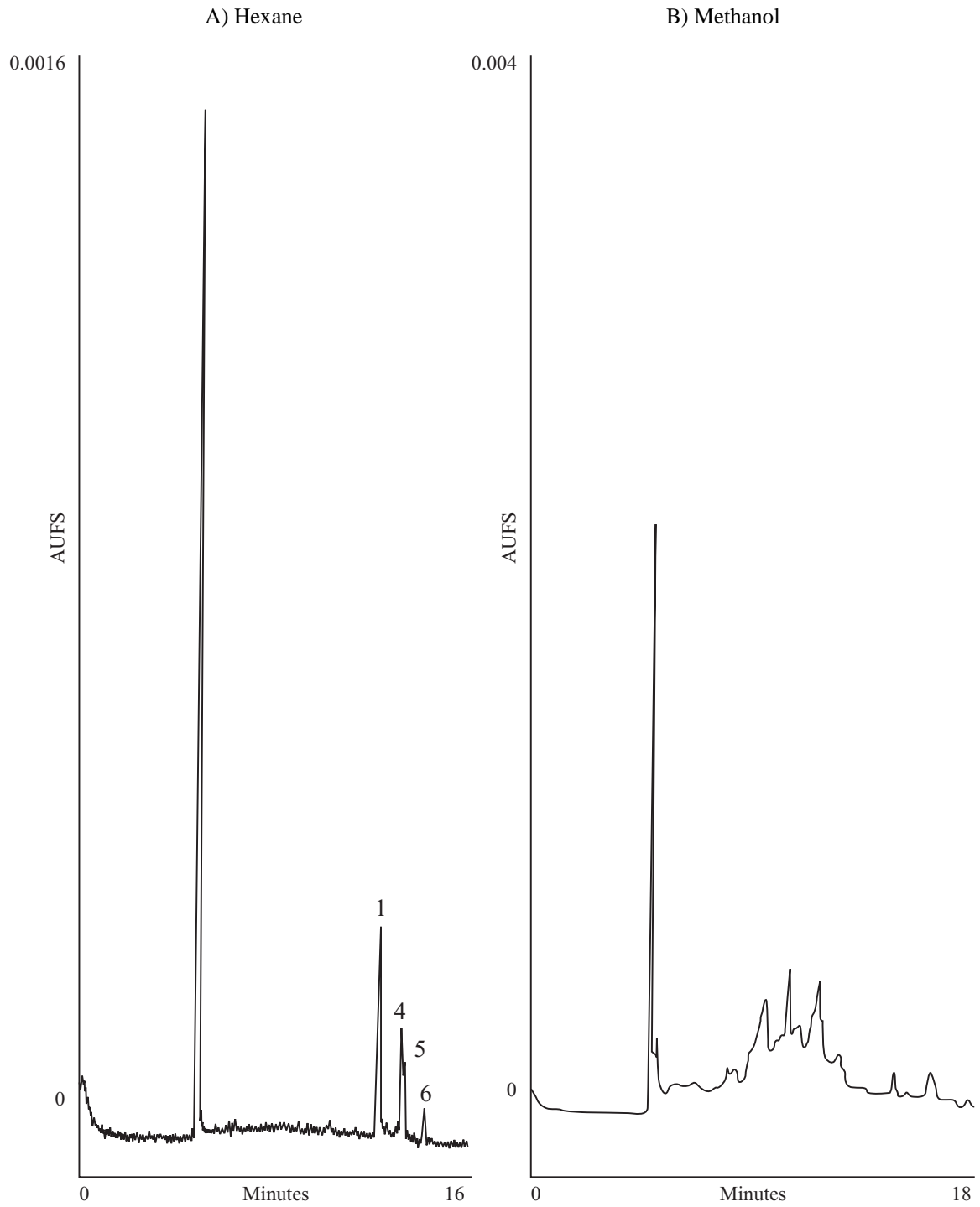
Conditions: 50  $\mu\text{m}$  I.D., total length = 59.8 cm, effective length = 44.8 cm; Sample solvent: methanol; Running buffer: methanol-acetonitrile (50:50), 30 mM sodium acetate; Hydrodynamic injection: 50 mbar for 3 sec.; Voltage: +25 kV; Detection  $\lambda$ : 310 nm.

## B) Methanol



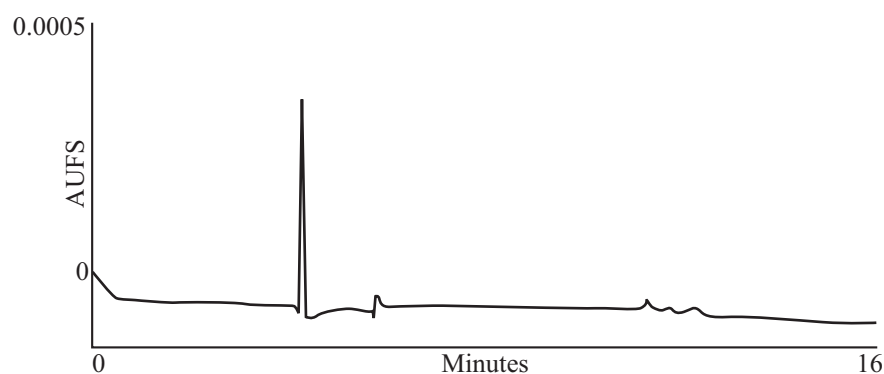
*Conditions: 50  $\mu\text{m}$  I.D., total length = 59.8 cm, effective length = 44.8 cm; Sample solvent: methanol; Running buffer: methanol-acetonitrile (50:50), 30 mM sodium acetate; Hydrodynamic injection: 50 mbar for 3 sec.; Voltage: +25 kV; Detection  $\lambda$ : 310 nm.*

Figure 3.35. Effect of extraction solvent on the fingerprints of Products L



Conditions: 50  $\mu\text{m}$  I.D., total length = 59.8 cm, effective length = 44.8 cm; Sample solvent: methanol; Running buffer: methanol-acetonitrile (50:50), 30 mM sodium acetate; Hydrodynamic injection: 50 mbar for 3 sec.; Voltage: +25 kV; Detection  $\lambda$ : 310 nm.

Figure 3.36. Electropherogram of a methanol blank



*Conditions: 50  $\mu$ m I.D., total length = 59.8 cm, effective length = 44.8 cm; Sample solvent: methanol; Running buffer: methanol-acetonitrile (50:50), 30 mM sodium acetate; Hydrodynamic injection: 50 mbar for 3 sec.; Voltage: +25 kV; Detection  $\lambda$ : 310 nm.*

Using hexane to extract Product K indicated that hexane had a higher extraction efficiency than methanol since peaks 1, 4, 5 and 6 could be detected in the hexane profile (Figure 3.34 A) while only peaks 1, 4 and 5 were seen in the methanolic extract (Figure 3.34 B). The methanolic extract of Product L (Figure 3.35 B) was less selective than the hexane extract profile (Figure 3.35 A). The high background absorbance prevented baseline resolution of the peaks and identification of the relevant markers was difficult. On the other hand, the hexane profile showed selective extraction of the ginkgolic acids only and peaks 1, 4, 5 and 6 could unambiguously be identified. The drastically different methanolic profiles of the 2 products (Figures 3.34 B and 3.35 B) is due to the fact that methanol is a less selective extraction solvent and Product L contained more extractable compounds than Product K.

Bioforce and Nrf were specifically chosen for fingerprinting the ginkgolic acids since Nrf contained consistently higher levels of positive markers when analyzed both qualitatively and quantitatively while Bioforce showed very low amounts i.e. Nrf was considered to be of better quality than Bioforce. It was therefore appropriate to choose these 2 products for fingerprinting of the negative markers to see if similar conclusions could be drawn. Nrf showed slightly higher levels of peaks 1, 4 and 5. Both products contained similar amounts of peak 6 and peaks 2 and 3 could not be detected in either product. Based on these results, toxic effects due to the presence of the ginkgolic acids would be more likely to occur with the ingestion of Nrf than Bioforce.

## 5.16 CONCLUSIONS

This non-aqueous CE method, although preliminary, has been shown to be superior to the published methods proposed to date in terms of simplicity, cost and speed. This method also demonstrated a measure of repeatability since preliminary studies were conducted one year prior to product analysis

and almost identical profiles of the reference standards were obtained. In addition, non-aqueous CE is ideal for analysis of these compounds since they are hydrophobic which normally results in excessively long run times using RP-HPLC and the inclusion of the methanolic fraction in the running buffer facilitates solubility of less polar analytes. Moreover, the problems associated with the adsorption of highly hydrophobic compounds to RP-HPLC columns were eliminated by simple high pressure washing of the capillary between consecutive injections. Extraction with hexane gave a clear fingerprint with no interferences and a blank methanol sample was injected which showed no peaks except for the EOF (Figure 3.36). He and Xie [172] also showed that hexane was highly efficient and selective in the extraction of the ginkgolic acids. The presence of C<sub>17:1</sub> in both the ginkgolic acid mixture and tablet extract was confirmed using a reference standard.

The ginkgolic acids are negative markers and although their speculated undesirable pharmacological effects have not been conclusively proven, their complete elimination from extracts is recommended [172] which is relatively simple to perform by the inclusion of a partitioning step using an apolar solvent such as hexane or heptane [53]. From a QC perspective, a simple fingerprinting method is therefore sufficient to determine if this step was included in the extraction and formulation process and unnecessary expenditure on analysis of these compounds can thus be circumvented. In addition, individual quantification at this point is tedious and expensive and to our knowledge, only C<sub>17:1</sub> is available commercially. Moreover, complex purification procedures have been a hindrance in the past [175]. Fingerprinting is the most affordable and practical technique and can provide sufficiently accurate information regarding the ginkgolic acid content. Although baseline separation between peak 4 and 5 was not obtained, both compounds could still be identified in both the ginkgolic acid reference mixture and in the commercial products. Product analysis revealed that substantial amounts of ginkgolic acids were present in both dosage forms analyzed which casts doubt as to whether any attempts were made to minimize their content.



## CHAPTER 4

### HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

#### 4.1 INTRODUCTION

Reversed phase high-performance liquid chromatography (RP-HPLC) is currently the most frequently used method for the analysis of herbal preparations [180, 162] since it is a well-established and relatively rudimentary analytical technique which is also economically viable for routine QC analyses. Furthermore, HPLC is versatile and is extremely useful for fingerprinting herbal medicines [181].

Although HPLC with UV detection is the most common technique used for the analysis of compounds with strong chromophores, assays using other detection modes are rapidly increasing due to technological advancement. PDA detection with its multiwavelength monitoring and scanning capabilities has the additional advantage of determining peak purity as well as identifying peaks with the aid of spectral libraries and is particularly useful when analyzing complex matrices such as plant extracts [180]. In fact, a recent article has promoted HPLC-PDA use for the fingerprinting of traditional South African herbal medicines [182].

Electrochemical detection (ED) also offers a measure of selectivity since substituents with differing electro activity may cause characteristic voltametric responses. HPLC with refractive index (RI) and evaporative light scattering detection (HPLC-ELSD) accommodates compounds which have poor UV absorbances [180]. Recent advances in MS instrumentation have paved the way for the incorporation of liquid chromatography tandem mass spectrometry (LC-MS-MS) assays for use in the pharmaceutical industry [183]. Other more complex hyphenated techniques have been explored more recently such as HPLC-PDA-MS, HPLC-NMR spectroscopy, HPLC-PDA-MS-NMR and HPLC-chemiluminescence [180].

#### 4.2 ANALYSIS OF FLAVONOIDS BY HPLC

Analysis of flavonoids with RP-HPLC was reviewed by Daigle and Conkerton [184] as early as 1988. UV detection was the most popular detection method due to the presence of strong flavonoid chromophores and availability of UV detection systems. Pietta and co-workers [185] developed a binary HPLC-PDA gradient system which was applied to *Ginkgo biloba* leaf extracts for the separation of naturally occurring flavonoids in 1991. Hasler *et al.* [62] separated 33 flavonoids found in *Ginkgo* leaves and extracts to obtain fingerprint profiles and also published a method for the quantification of the flavonol aglycones after acid hydrolysis [186]. In 1993, Sticher suggested such an approach to standardize phytomedicines containing flavonol components [52]. Table 4.1 summarizes the more recent HPLC methods [42, 187-191] used for the analysis of only flavonol aglycones which occur in *Ginkgo biloba* plant extracts, commercial products and human urine after consumption of a *Ginkgo*

*biloba* tablet using this QC approach. The exception is the method developed during this research [168], which also includes analysis of the glycosides, rutin and quercitrin. The importance and value of including the glycosides as positive markers has been discussed in Chapter 2.

Table 4.1. HPLC methods used in the analysis of selected flavonol markers common to *Ginkgo biloba*

Matrix analyzed	Sample extraction/ preparation	Compound	Mobile phase	Column	Qualitative/ quantitative	Detection	Ref
<i>Ginkgo biloba</i> extract and commercial products	80% MeOH followed by acid hydrolysis	quercetin, kaempferol, isorhamnetin	Isocratic: MeOH, phosphoric acid 0.3% (50:50)	Cosmosil 5C <sub>18</sub> -AR (150 x 4.6 mm I.D.)	Quantitative	UV (370 nm)	187
Commercial <i>Ginkgo biloba</i> products	MeOH-acid hydrolysis	quercetin, kaempferol, isorhamnetin	Isocratic: Isopropanol, MeCN, water (5:47:100 v/v/v) + citric acid	RP LiChrospher100 C <sub>18</sub> (125 x 4.0 mm I.D.)	Quantitative	UV (370 nm)	42
Commercial <i>Ginkgo biloba</i> products	80% MeOH followed by acid hydrolysis	quercetin, kaempferol, isorhamnetin	Gradient: A) Water-MeOH-TFA B) MeOH-TFA	Supelco Discovery C <sub>18</sub> (250 x 4.6 mm)	Quantitative	PDA (365 nm)	188
Single Commercial <i>Ginkgo biloba</i> product	MeOH, dry extract then extracted by liquid-liquid extraction using hot water and EtAc	quercetin, kaempferol, isorhamnetin	Gradient: A) Water-MeOH-TFA B) MeOH-TFA	Supelco Discovery C <sub>18</sub> (250 x 4.6 mm I.D.)	Quantitative	ELSD	189
Commercial <i>Ginkgo biloba</i> products	MeOH	rutin, quercitrin, quercetin, kaempferol, isorhamnetin	Gradient: MeCN, formic acid 0.3% (one step)	Phenomenex Luna C <sub>18</sub> (250 x 2.0 mm I.D.)	Quantitative	PDA (wavelength max for each analyzed compound)	168
Commercial <i>Ginkgo biloba</i> products	<b>PDA:</b> RP: 80% MeOH followed by acid hydrolysis  NP: 80% dioxane followed by acid hydrolysis  <b>ED:</b> MeOH	quercetin, kaempferol	<b>PDA:</b> RP: A) MeOH, MeCN B) THF in water  NP: dioxane in hexane  <b>ED:</b> RP: MeOH, MeCN, NaClO <sub>4</sub> NP: dioxane-hexane-MeOH	RP: Spherisorb ODS1 (150 x 4.6 mm I.D.)  NP: ODS (BAS™) (100 x 3.2 mm I.D.)	Quantitative	PDA (355nm), ED	190
Human urine after administration <i>Ginkgo biloba</i> tablet	acid hydrolysis	quercetin, kaempferol	Isocratic: Phosphate-THF-MeOH-isopropanol (70:15:10:20 v/v/v/v)	Platinum EPS C <sub>18</sub> (250 x 4.6 mm I.D.)	Quantitative	UV (380 nm)	191

MeOH, methanol; HCl, hydrochloric acid; TFA, trifluoroacetic acid; EtAc, ethyl acetate; MeCN, acetonitrile; THF, tetrahydrofuran; ED, electrochemical detection; RP, reversed phase; NP, normal phase

Lin *et al.* [187] focused on selecting the optimal C<sub>18</sub> column and mobile phase for the separation of aglycones only as well as determining the most efficient hydrolysis conditions. Nine different columns with dimensions 150 x 4.6 mm I.D. (5 columns), 250 x 4.6 mm I.D. (2 columns), 300 x 3.9 mm I.D. (1 column) and 125 x 4.0 mm I.D. (1 column) were supplied by Waters, Merck, GL Science and Phenomenex and 9 mobile phase compositions were tested by calculation of the retention times, resolution and selectivity. Table 4.1 depicts the specific mobile phases and related analytical columns chosen. The Cosmosil C<sub>18</sub> column resulted in optimal efficiency and resolution with a run time of 35 minutes. For the acid hydrolysis, 2 different hydrochloric acid (HCl) strengths (5.5% and 20%) were added to Ginkgo extract and hydrolyzed for 15, 30, 60 and 120 minutes, respectively. Complete hydrolysis was achieved by refluxing with 20% HCl for 15 minutes in a water bath (85°C) or alternatively with 5.5% HCl for 30 minutes. Precision and accuracy of the method was demonstrated and 12 *Ginkgo biloba* products formulated as film and sugar coated tablets, ordinary tablets, drops and oral solutions were assayed. The authors reported that there were no interferences with the peaks of interest however only one product chromatogram was shown.

Kressmann *et al.* [42] calculated total flavonol glycoside content from hydrolyzed flavonol aglycones to determine the pharmaceutical quality of 27 brands of *Ginkgo biloba* products available on the US market. The method used for the assays was adopted from an official WHO monograph and validation data were not provided.

Li and Fitzloff used HPLC with PDA detection to compare flavonol aglycone content in various pharmaceutical products [188] and also published a method to simultaneously determine the terpene lactones and flavonol aglycones using HPLC-ELSD [189]. In the latter method, a single commercial capsule was extracted using ethyl acetate without hydrolysis. This extraction procedure proved to be suitable for the analysis of both the terpenes and aglycones using a liquid-liquid extraction procedure of reference standards which showed 94 - 100% recovery. Although this method did not involve a hydrolysis step, the analysis would have been more informative for QC purposes if at least one flavonol glycoside such as rutin was included in the assay. Alternatively, hydrolysis with back calculation of the glycosides would have provided some indication of the glycoside content. Whether this extraction procedure would effectively extract the glycosides is not unknown however based on the relatively clean profile of the extract, either the product was of low quality or ethyl acetate failed to extract the more polar flavonol glycosides and this method cannot thus be sufficiently vindicated for fingerprinting of commercial products or extracts. Moreover UV detection is more sensitive for the analysis of the flavonoids as shown by the flavonol aglycone LOD values of 2 ng on-column load [188] compared to 20 - 30 ng for ELSD detection [189]. Since the flavonol aglycones are typically present in small quantities in leaf extracts, varying from 0.2 - 0.4% w/w [57] and quercetin, kaempferol and isorhamnetin contributed to only 0.07, 0.01 and 0.01% (w/w) of the *Ginkgo biloba* commercial product [189], these publications demonstrate that a more sensitive detection technique is paramount for accurate detection and quantification of the aglycone compounds, particularly if sample hydrolysis is not included in the assay.

In the last year, 2 methods have been published for the analysis of the flavonols in *Ginkgo biloba* commercial products, one of which compared both normal and reversed phase analysis of quercetin and kaempferol with either ED or PDA detection [190]. Results showed that peak tailing improved using normal phase HPLC (NP-HPLC) compared to RP-HPLC however when ED was used as the detection mode, RP-HPLC was more sensitive. ED demonstrated lower LOD levels than PDA detection. For the analysis of the commercial preparations, samples were hydrolyzed for PDA detection and the kaempferol and quercetin content of the commercial products were consistent with the label claims. The results using ED were largely inconsistent with the product label information and the authors suggested that this was due to the absence of the relevant compounds in extracts or interferences prevented accurate detection. It was thus concluded that the extraction procedure required further optimization to ensure successful application.

The other method was developed during this research and describes the quantitative analysis of selected marker compounds of both the flavonol glycosides and aglycones [168]. The following section describes the development and validation of a method for this simultaneous determination of the flavonol glycosides, rutin and quercitrin as well as their corresponding aglycones, quercetin, kaempferol and isorhamnetin.

### **4.3 METHOD DEVELOPMENT USING HPLC-PDA**

#### **4.3.1 Instrumentation**

A Waters 2690 Separations Module and Waters 2996 Photodiode Array equipped with an auto sampler, on-line degasser system and column heater was used for the HPLC analysis (Milford, MA, USA). A Mettler Dual Range Electronic Balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland) was used for the weighing of reagents and standards and a Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> column with dimensions 250 x 2.0 mm I.D. was used for the LC separation.

#### **4.3.2 Materials and reagents**

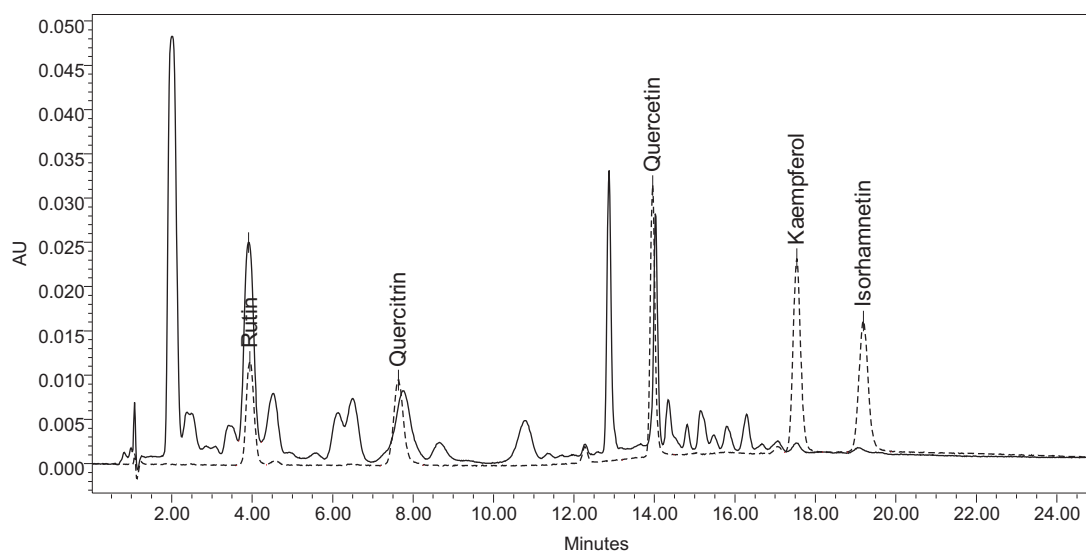
Formic acid (analytical grade) was purchased from Merck (Darmstadt, Germany) and acetonitrile (HPLC grade) was obtained from BDH chemicals (Poole, UK). Rutin (95%) and quercetin (95%) were purchased from Sigma (Missouri, USA) and quercitrin (85%), kaempferol (95%) and isorhamnetin (90%) from Indofine Chemical Company (New Jersey, USA). The purity of these flavonols was assumed as provided by the certificates of analyses from the suppliers. Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA) and HVLP filters were purchased from the same source.

#### 4.3.3 Method development

Initial LC conditions were based on the method used for LC-MS (section 5.3.4) using a Phenomenex Luna® C<sub>18</sub> 150 x 2.0 mm I.D. column maintained at 45°C. Although the short run time using isocratic conditions of 0.3% formic acid-acetonitrile (75:25) at 500 µl/min was achieved using the reference standards, exclusive determination of the peaks of interest was not possible when tablet extracts were analyzed and interfering peaks in the zone where quercitrin and quercetin eluted necessitated further method development for use with a PDA detector ( $\lambda$  200 – 400 nm) to confirm peak homogeneity. A Naturelle tablet extract which exhibited a fairly complex profile was prepared as described in section 4.4.4.1 and was used for further method development. A simple one step gradient method was then introduced which started with 0.3% formic acid-acetonitrile in a ratio of 85:15 and then changed to 75:25 at 5 minutes for the remainder of the analysis time. Although the run time was 17 minutes, detection of both the quercetin and quercitrin peaks was compromised by interferences.

The increase in percentage acetonitrile in the mobile phase was then delayed *seriatum* to 5, 6, 7, 8 and 8.5 minutes, respectively. With use of the higher portions of aqueous phase, resolution continued to improve at the expense of longer run times. Peak homogeneity testing was performed on the relevant peaks of interest as determined by their UV spectra relative to those of the pure reference standards. Interfering peaks still hindered the detection of quercetin however and the acetonitrile fraction was programmed to change linearly from 15% at 8 minutes to 25% at 11 minutes to further facilitate separation in this elution zone (Figure 4.1). The UV spectrum obtained at the peak at 14.0 minutes did not however correspond to that of quercetin.

Figure 4.1. Superimposed chromatogram ( $\lambda$  350 nm)



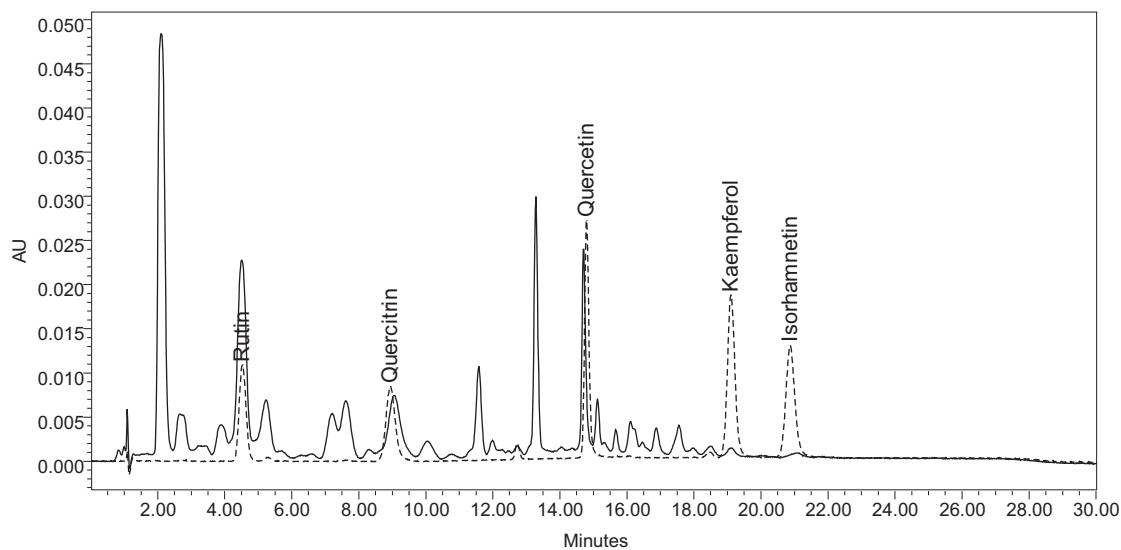
Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (150 x 2.0 mm I.D.); Column temperature: 45 °C; Mobile phase: MeCN-0.3% formic (gradient change 8-11 minutes); Flow rate: 500 $\mu$ l/min; Injection volume: 5  $\mu$ l; Detection  $\lambda$ : 350 nm; Solid line=chromatogram of tablet extract, dotted line=chromatogram of reference standards.

A final adjustment involved earlier introduction of the linear change from 15% acetonitrile at 7 minutes to 25% at 12 minutes to result in the gradient shown in Table 4.2 and although the peaks were better resolved and the peak shapes improved in the quercetin elution zone, quercetin could still not be unambiguously identified (Figure 4.2).

Table 4.2. Final mobile phase proportions used for further analyses

Time	Flow	Formic-water (%)	Acetonitrile (%)
0.01	0.50	85.0	15.0
7.00	0.50	85.0	15.0
12.00	0.50	75.0	25.0
35.00	0.50	75.0	25.0
35.01	0.50	85.0	15.0

Figure 4.2. Superimposed chromatogram using the gradient described in Table 4.2 above

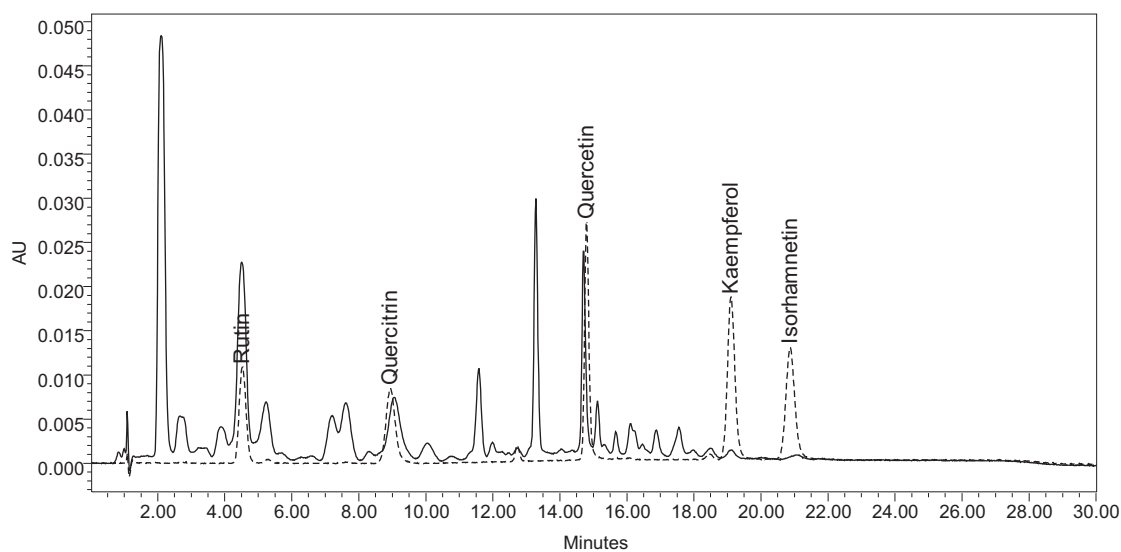


Conditions: Column: Phenomenex Luna (5  $\mu\text{m}$ )  $\text{C}_{18}$  (150 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 500 $\mu\text{l}/\text{min}$ ; Injection volume: 5  $\mu\text{l}$ ; Detection  $\lambda$ : 350 nm; Solid line=chromatogram of tablet extract, dotted line=chromatogram of reference standards.

A Phenomenex Luna (5  $\mu\text{m}$ )  $\text{C}_{18}$  column with dimensions 250 x 2.0 mm I.D. was then procured in an attempt to improve resolution and after performing column system suitability, the gradient in Table 4.2 was used again. Peak resolution was greatly improved and although quercetin and kaempferol were homogeneous (isorhamnetin was absent in the extract), the peak purity analysis of rutin and quercitrin suggested that those relevant peaks co-eluted with other unknown compounds present in the extract. (Figure 4.3).



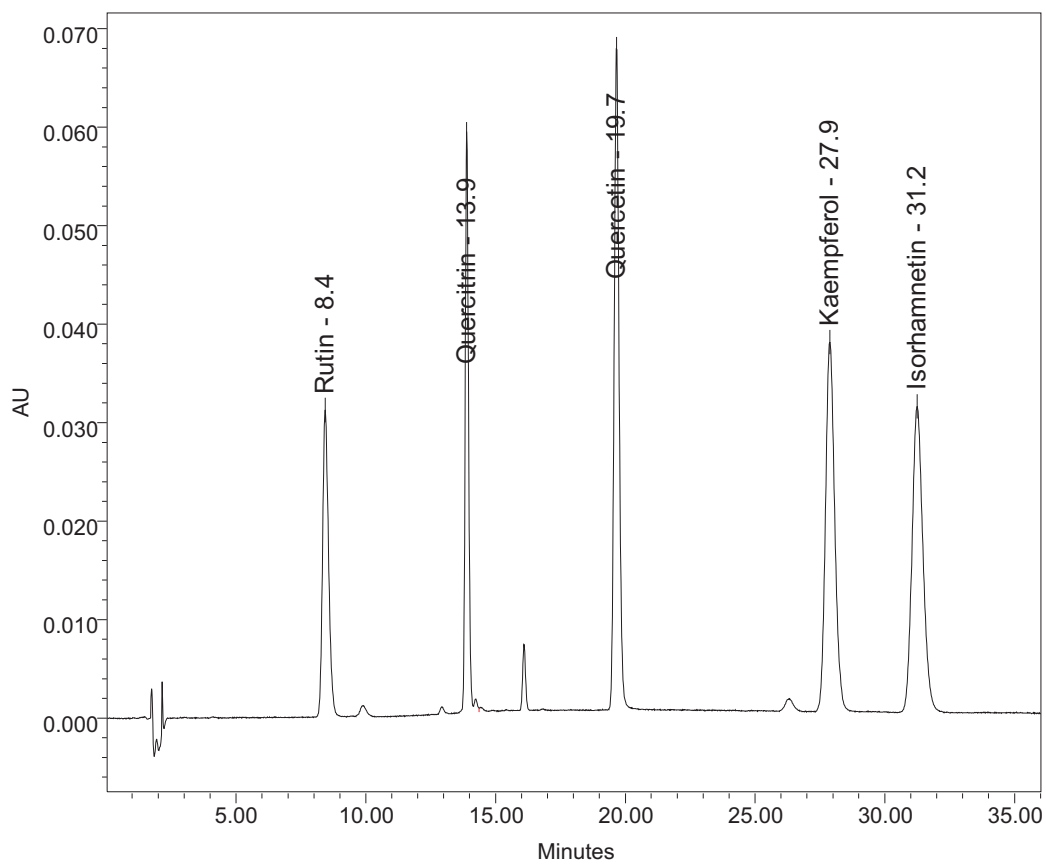
Figure 4.3. Superimposed chromatogram using the Phenomenex 250 x 2.0 mm I.D. column



Conditions: Column: Phenomenex Luna (5  $\mu\text{m}$ )  $\text{C}_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 500  $\mu\text{l}/\text{min}$ ; Injection volume: 5  $\mu\text{l}$ ; Detection  $\lambda$ : 350 nm; Solid line=chromatogram of tablet extract, dotted line=chromatogram of reference standards.

Thereafter, the effect of flow rate on the resolution was systematically investigated at 200  $\mu\text{l}/\text{min}$ , 300  $\mu\text{l}/\text{min}$ , 400  $\mu\text{l}/\text{min}$  and 500  $\mu\text{l}/\text{min}$ . Lower flow rates were generally preferred due to the lower back pressure and less solvent consumption however 400  $\mu\text{l}/\text{min}$  was the only flow rate whereby all 5 marker compounds were well resolved (save quercetin). Under these conditions, isorhamnetin eluted last at 31.2 minutes (Figure 4.4). Extracts of 4 other available dosage forms, Ginkgoforce, Bioharmony, Nrf and Vital were then prepared according to the procedures described in section 4.4.4 and injected under the same conditions as Naturelle (Figure 4.5). Peak homogeneity testing was performed on the relevant marker compounds present in the extracts. This method was then selected for validation as it was the only method in which the majority of the peaks were shown to be resolved. The exception was quercetin which was not quantified in Bioharmony due to interferences which were present even when monitoring at more selective wavelengths (350 - 370 nm).

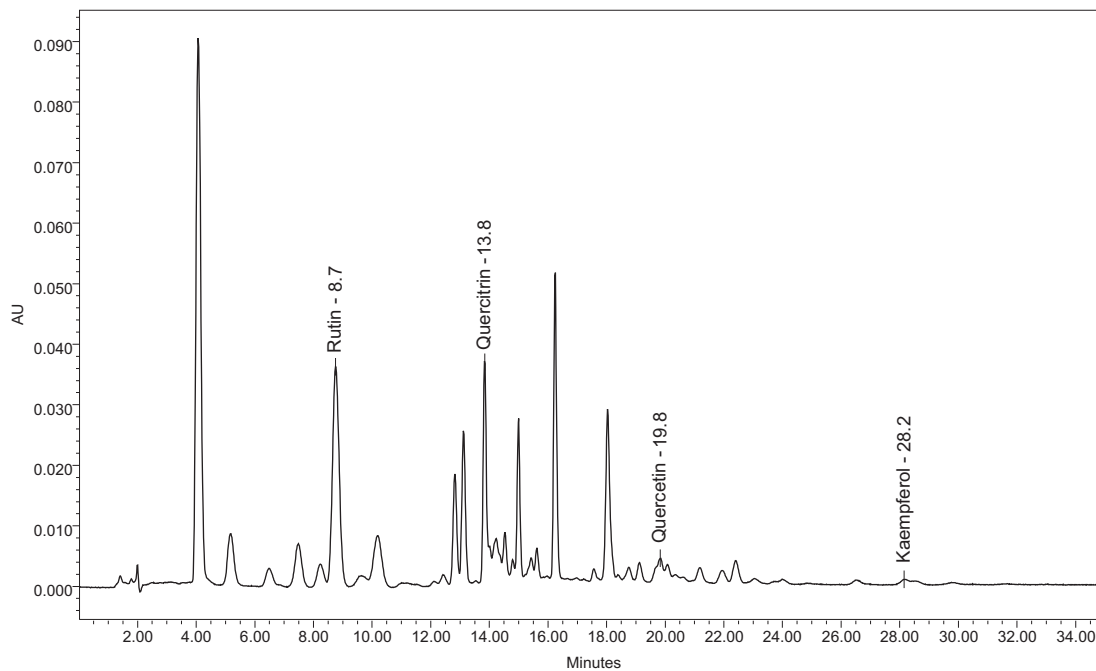
Figure 4.4. HPLC-UV chromatogram of the flavonol reference standards ( $\lambda$  350 nm)



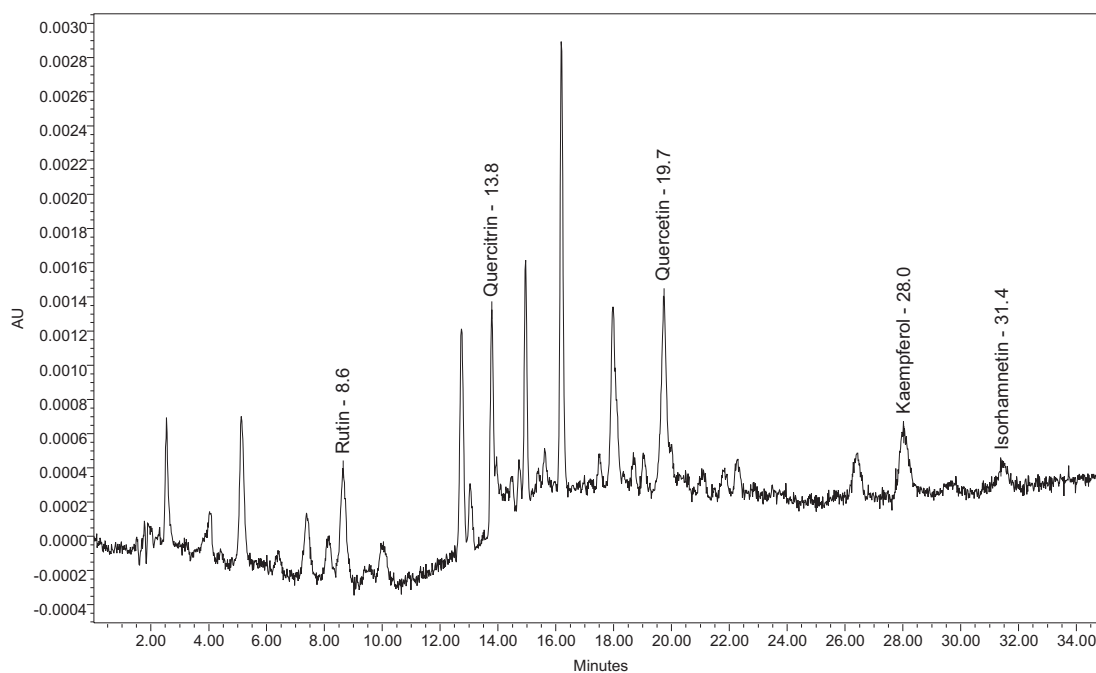
Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 400 $\mu$ l/min; Injection volume: 5  $\mu$ l; Detection  $\lambda$ : 350 nm.

Figure 4.5. HPLC-PDA profiles of 5 dosage forms

A) Naturelle tablets

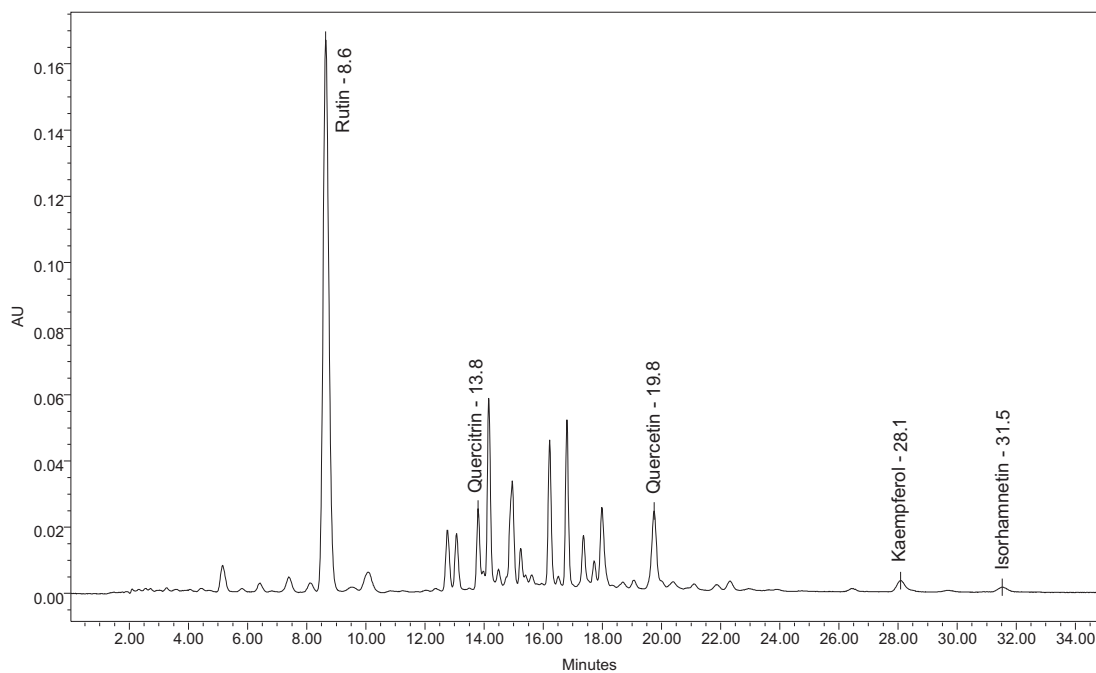


B) Ginkgoforce tablets

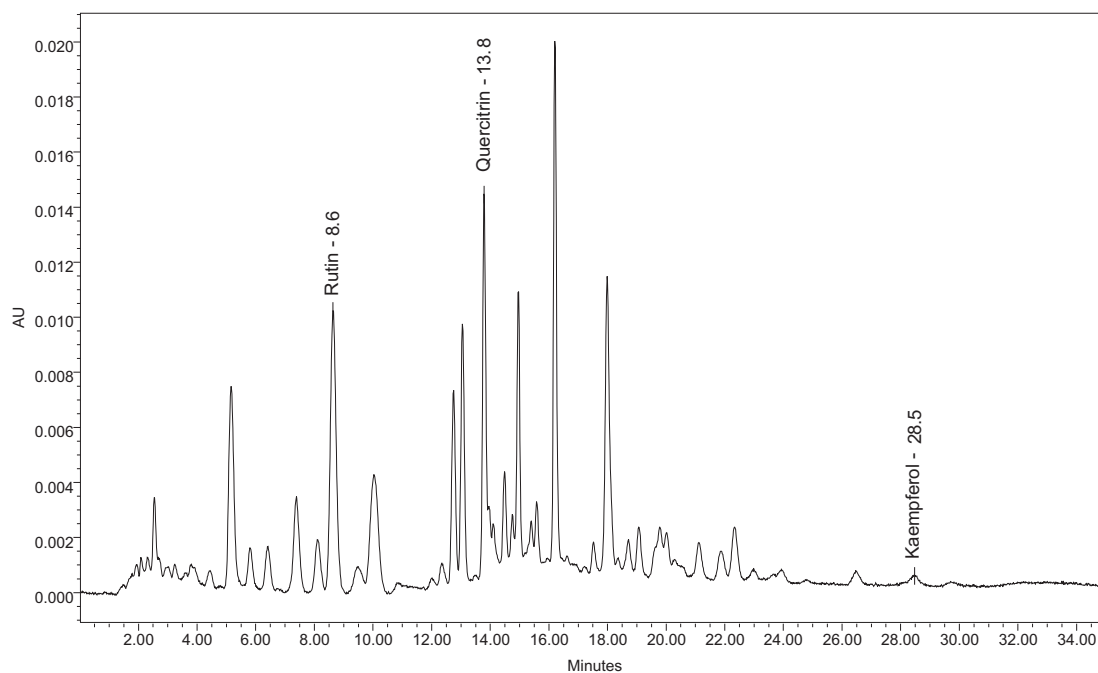


Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45 °C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 400  $\mu$ l/min; Injection volume: 5  $\mu$ l; Detection  $\lambda$ : 350 nm.

### C) Nrf tablets

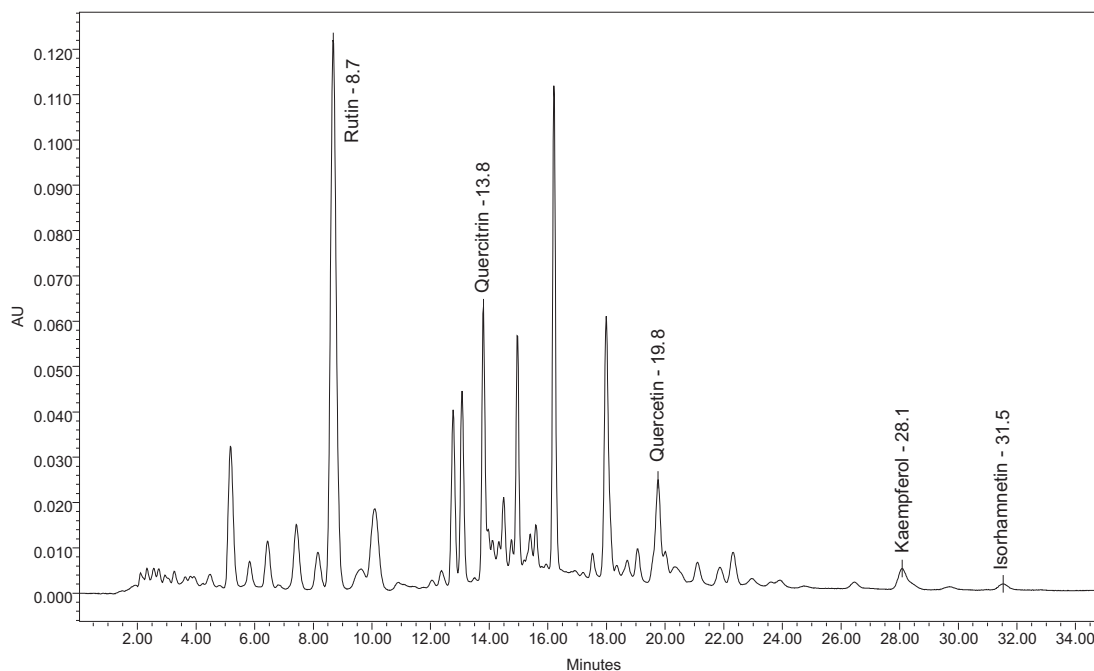


### D) Bioharmony capsules



Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45 °C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 400  $\mu$ l/min; Injection volume: 5  $\mu$ l; Detection  $\lambda$ : 350 nm.

### E) Vital capsules



Conditions: Column: Phenomenex Luna (5  $\mu$ m)  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45 °C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 400 $\mu$ l/min; Injection volume: 5  $\mu$ l; Detection  $\lambda$ : 350 nm.

#### 4.3.4 Tablet extraction efficiency

Tablet extraction efficiency was based on research involving St. John's Wort tablets [140]. The percentage of rutin, isoquercitrin, quercitrin, quercetin and kaempferol recovered from a Holotropic St. John's Wort tablet after exhaustive extraction with methanol (3 x 10 ml) was determined in that study. Two parallel studies were conducted in which 6 aliquots of powdered tablet material equivalent to the mass of a single tablet were weighed and decanted into separate Kimax tubes. Ten milliliters of methanol were added to each tube which was then vortexed to ensure even dispersion of the powder in the extraction solvent. Thereafter, 3 of the tubes were sonicated for 1 hour and the remaining 3 tubes for 2 hours. After the respective sonication times, the extracts were centrifuged, the supernatant was poured into clean Kimax tubes and fresh methanol was added. The sonication procedures were repeated twice more. The supernatants of each extraction procedure were then injected and analyzed by HPLC-PDA detection to calculate the percentage of the recovered marker compounds which were extracted for 1, 2 and 3 hours (the first 3 tubes) and for 2, 4 and 6 hours (the remaining 3 tubes), respectively.

From the results it was shown that a 1 hour sonication procedure extracted 99.7 %, 69.8%, 80.8% and 82.8% of the rutin, quercitrin, quercetin and kaempferol present in the tablet, respectively. After the following hour, more than 90% of the quercetin and kaempferol was extracted while 88.7% of the quercitrin was recovered. After the 3<sup>rd</sup> extraction, the remaining percentages were recovered. It was concluded that sonification for 1 hour was sufficient for the routine analysis of St. John's Wort dosage

forms and that the increase in the percentage flavonol extraction after 2 and 3 hours did not warrant the lengthy extraction times.

Regarding the 2 hour sonication intervals, a similar trend was seen with 96.6%, 73.8%, 86.4% and 88.6% of the rutin, quercitrin, quercetin and kaempferol being extracted after 2 hours, respectively. From these results it was concluded that the previously described protocol where methanol is replaced after 1 hour is more efficient than when the tablet mixture is sonicated continuously for 2 hours.

#### **4.4 METHOD VALIDATION AND QUANTITATIVE ANALYSIS OF *GINKGO BILOBA* SOLID ORAL DOSAGE FORMS**

##### **4.4.1 Instrumentation and additional equipment**

See section 4.3.1. In addition, a Cole-Parmer Ultrasonic Bath, Model 8845-30 (Cole-Parmer Instrument Company, Chicago, Illinois, USA) was used in the sonication procedure for extraction purposes and an Eppendorf Centrifuge, Model 54142770 (Eppendorf Geratebau, West Germany) was used to centrifuge tablet extracts.

##### **4.4.2 Materials and reagents**

Reagents were sourced as stated in section 4.3.2. PVDF filters were purchased from Millipore, Bedford, MA, USA. Six *Ginkgo biloba* products (A, B, C, D, E and F) were purchased from a local pharmacy in Grahamstown, South Africa. Four of the products were solid oral dosage forms and 2 preparations contained herbal extract in a gelatin capsule. Table 4.3 below depicts the product details.

Table 4.3. *Ginkgo biloba* dosage forms used for HPLC-PDA analysis of flavonols

Manufacturer	Name of product	Dosage form	Label claim	Daily dose	Batch number	Extract
Holotropic <sup>1</sup>	<i>Ginkgo biloba</i>	Tablet	Each 3 tablets provide: <i>Ginkgo biloba</i> botanical 300 mg, <i>Ginkgo biloba</i> extract equivalent to 900 mg	1 tablet tds <sup>7</sup>	0318	A
Formule Naturelle <sup>2</sup>	<i>Ginkgo biloba</i>	Tablet	Standardized to contain 24% <i>Ginkgo Flavonglycosides</i>	1 tablet tds <sup>7</sup> , ac <sup>8</sup>	82060	B
Bioforce <sup>3</sup>	Ginkgoforce	Tablet	Each 250mg tablet is equivalent to 10 drops of Ginkgoforce liquid	2 tablets tds <sup>7</sup> , ac <sup>8</sup>	210120E	C
Nrf Herbal <sup>4</sup>	<i>Ginkgo biloba</i>	Tablet	Each tablet contains 350mg <i>Ginkgo biloba</i> , 50mg <i>Ginkgo biloba</i> extract 24% and 240mg FoodMatrix™	1 - 2 tablets daily	200303047	D
Bioharmony <sup>5</sup>	<i>Ginkgo biloba</i>	Capsule	Each 350mg capsule contains 350mg <i>Ginkgo biloba</i> ( <i>Ginkgoaceae</i> )	2 capsules tds <sup>7</sup>	1EP02.21	E
Vital <sup>6</sup>	<i>Ginkgo biloba</i>	Capsule	Each capsule contains <i>Ginkgo biloba</i> extract equivalent to 6 000 mg of herb powder	1 capsule daily	0024B	F

<sup>1</sup>Holotropic, Cape Town, South Africa; <sup>2</sup>Formule Naturelle, Hout Bay, South Africa; <sup>3</sup>Bioforce S.A. (Pty) Ltd., Halfway House, South Africa; <sup>4</sup>Nrf Herbal, Centurion, South Africa; <sup>5</sup>Bioharmony CC, Wynberg, South Africa; <sup>6</sup>Vital Health Foods (EDMS) BPK, Kuils River, Western Cape, South Africa; <sup>7</sup>tds, three times daily; <sup>8</sup>ac, before meals

#### 4.4.3 Preparation of standard solutions

Separate stock solutions of the reference standards were made in methanol-water (50:50). A working solution of the combined standards was subsequently prepared in methanol-water (50:50) and diluted to provide a series of analytical standards ranging from 3 - 26 µg/ml for use in constructing calibration curves for each of the target analytes.

#### 4.4.4 Sample Preparation and Extraction

##### 4.4.4.1 *Solid oral dosage forms*

Twenty five tablets of each solid oral dosage form (Products A - D) were powdered in a mortar using a pestle. A mass of powder equivalent to 1 tablet was accurately weighed and dispersed in methanol (25

ml). The mixture was sonicated for 1 hour with manual agitation after 30 minutes and then centrifuged at 350 x g for 15 minutes. The samples were diluted with appropriate volumes of methanol and water in order to maintain a 50:50 methanol-water solvent ratio and filtered through 0.45 µm PVDF filters before injecting.

#### 4.4.4.2 *Hard gelatin capsules*

Twenty five capsules (Product E) were emptied, weighed and a mass of powder equivalent to the contents of a single capsule was extracted as described in section 4.4.4.1 above.

#### 4.4.4.3 *Soft gelatin capsules*

Individual capsules (Product F) were sliced longitudinally, the contents squeezed out and combined with the soft gel shell containing residual content. Methanol (25 ml) was added and the analytes were extracted by sonication for 1 hour with manual agitation after 30 minutes, followed by centrifugation at 350 x g for 15 minutes. The samples were diluted with appropriate volumes of methanol and water in order to maintain a 50:50 methanol-water solvent ratio and filtered through 0.45 µm PVDF filters before injecting.

#### 4.4.5 HPLC-PDA conditions

The flavonols were separated at 45°C on a minibore Phenomenex Luna 5µ C<sub>18</sub> (2) column with dimensions 250 x 2.0 mm using the gradient method described in Table 4.2. The total run time was 33 minutes at a flow rate of 400 µl/min. The PDA scanning range was set from λ 200 - 400 nm.

#### 4.4.6 Method validation

##### 4.4.6.1 *Calibration curves*

Linear calibration curves were constructed on 3 consecutive days by analysis of a mixture containing each of the flavonols at 4 concentration levels and plotting peak area against the concentration of each reference standard. Calibration curves were constructed at the relevant wavelength of maximum absorption of each reference compound. Specificity was determined by calculation of peak homogeneity facilitated by the PDA detector.

##### 4.4.6.2 *Precision and accuracy*

The repeatability of the assay was assessed by spiking tablet extracts (Product A) with high, medium and low concentrations of each reference standard. Each concentration level was prepared in triplicate over 3 days to determine both intra-assay and inter-assay precision.

Product A was chosen to perform recovery studies in triplicate and 3 blank extracts were prepared to determine the original concentration of the chosen marker compounds.



A mass of powder equivalent to the mass of 1 tablet was spiked with low, medium and high concentrations based on the suitable range for each reference standard except for the low spike for rutin, since a significant amount of rutin was found to be present in this product.

#### 4.4.6.3 LOD's and LOQ's

The LOD's and LOQ's were determined by means of serial dilution based on a S/N ratio of 3:1 for the former and 10:1 for the latter.

#### 4.4.7 Results and Discussion

##### 4.4.7.1 Linearity and range

Figure 4.4 shows a typical HPLC-UV ( $\lambda$  350 nm) chromatogram of 5 flavonols: rutin, quercitrin, quercetin, kaempferol and isorhamnetin with retention times 8.4, 13.9, 19.7, 27.9 and 31.3 minutes, respectively. Quantification was performed at  $\lambda$  350 nm for rutin and quercitrin, 375 nm for quercetin and 365 nm for isorhamnetin and kaempferol, respectively. Assessment of peak purity showed homogeneity (with the exclusion of quercetin in the Bioharmony capsules) thereby excluding the possibility of the presence of interfering components and rendering the method specific. Linear ranges and co-efficients of determination are depicted in Table 4.4.

Table 4.4. Linear ranges and coefficients of determination

Name of compound	Concentration range ( $\mu\text{g/ml}$ )	Day	Slope	y-intercept	Co-efficient of determination ( $R^2$ )
Rutin	3.1 - 25.1	Day 1	$1.94e^4$	$1.61e^3$	0.9997
		Day 2	$1.95e^4$	$1.90e^3$	0.9998
		Day 3	$1.96e^4$	$1.42e^3$	0.9996
Quercitrin	3.2 - 25.7	Day 1	$2.06e^4$	$4.59e^2$	0.9997
		Day 2	$2.06e^4$	$1.95e^3$	0.9998
		Day 3	$2.07e^4$	$1.20e^3$	0.9995
Quercetin	3.1 - 25.0	Day 1	$4.78e^4$	$1.20e^4$	0.9996
		Day 2	$4.78e^4$	$-7.92e^3$	0.9997
		Day 3	$4.78e^4$	$-1.83e^4$	0.9994
Kaempferol	3.3 - 26.4	Day 1	$4.32e^4$	$6.35e^3$	0.9998
		Day 2	$4.31e^4$	$5.83e^3$	0.9998
		Day 3	$4.34e^4$	$2.06e^3$	0.9996
Isorhamnetin	3.1 - 24.4	Day 1	$5.15e^4$	$-6.06e^3$	0.9997
		Day 2	$5.09e^4$	$-8.14e^3$	0.9998
		Day 3	$5.06e^4$	$-1.63e^4$	0.9997

##### 4.4.7.2 LOD's and LOQ's

Product D contained the highest quantities of marker compounds and was sequentially diluted to determine the LOD and LOQ in the tablet matrix. The LOD's were 1.36, 0.34, 0.87, 0.90 and 0.75  $\mu\text{g/ml}$  for rutin, quercitrin, quercetin, kaempferol and isorhamnetin, respectively and the LOQ's were found to be 2.76  $\mu\text{g/ml}$  for rutin, 0.77  $\mu\text{g/ml}$  for quercitrin, 1.11  $\mu\text{g/ml}$  for quercetin, 1.55  $\mu\text{g/ml}$  for kaempferol and 1.03  $\mu\text{g/ml}$  for isorhamnetin.

#### 4.4.7.3 Precision and accuracy

The intra-assay and inter-assay repeatability of the method for all 5 marker compounds are given in Table 4.5. The intra-assay precision represents data accumulated in triplicate in each day of the analysis. The RSDs ranged between 0.2 and 5.5%. The inter-day precision was calculated from nine determinations over 3 days for each concentration level and values were typically below 8.0% except for isorhamnetin at both low and medium concentrations which were 12.0% and 10.9%, respectively.

Table 4.5. Intra-day and inter-day repeatability of the HPLC analysis

Constituent	Mean (µg/ml)	Intra-day RSD (n=3) (%)			Inter-day RSD (n=9) (%)
		Day 1	Day 2	Day 3	
Rutin	4.3	1.6	0.9	5.4	4.8
	12.3	2.9	0.9	0.6	1.6
	17.6	1.7	0.4	0.4	1.7
Quercitrin	5.0	0.7	0.2	0.5	0.8
	11.8	1.2	0.9	0.7	1.2
	17.3	1.7	0.6	0.8	1.7
Quercetin	4.6	1.8	3.9	4.6	7.4
	9.4	1.9	1.9	1.0	4.3
	13.3	1.3	1.3	4.1	3.9
Kaempferol	6.4	5.5	1.7	2.5	5.4
	13.0	1.8	3.0	1.5	5.3
	18.6	1.7	1.4	1.3	2.0
Isorhamnetin	4.9	0.6	1.9	1.5	12.0
	9.1	2.5	1.5	0.5	10.9
	14.1	0.6	1.5	0.6	7.2

Recovery data obtained from Product A are depicted in Table 4.6. Isorhamnetin shows consistent but somewhat lower recoveries at all three spiking levels compared to the other flavonols. Isorhamnetin has a methoxy functional group at the R position (Figure 2.4) and exhibits lower polarity than the other marker compounds. Sample solutions were originally prepared in 0.3% formic-methanol (75:25) however decreases in peak areas with repetitive injections were observed for all of the flavonols, particularly the aglycones. Inspection of the samples showed precipitation of the standards which was particularly evident for isorhamnetin. Samples were subsequently prepared in methanol and diluted with water or a mixture of methanol-water in order to maintain a solvent ratio of 50:50. Low recoveries for isorhamnetin have been previously reported in gas chromatographic-mass spectrometric (GC-MS) [192], liquid chromatography mass spectrometry (LC-MS) [169] and HPLC-UV analysis [187].

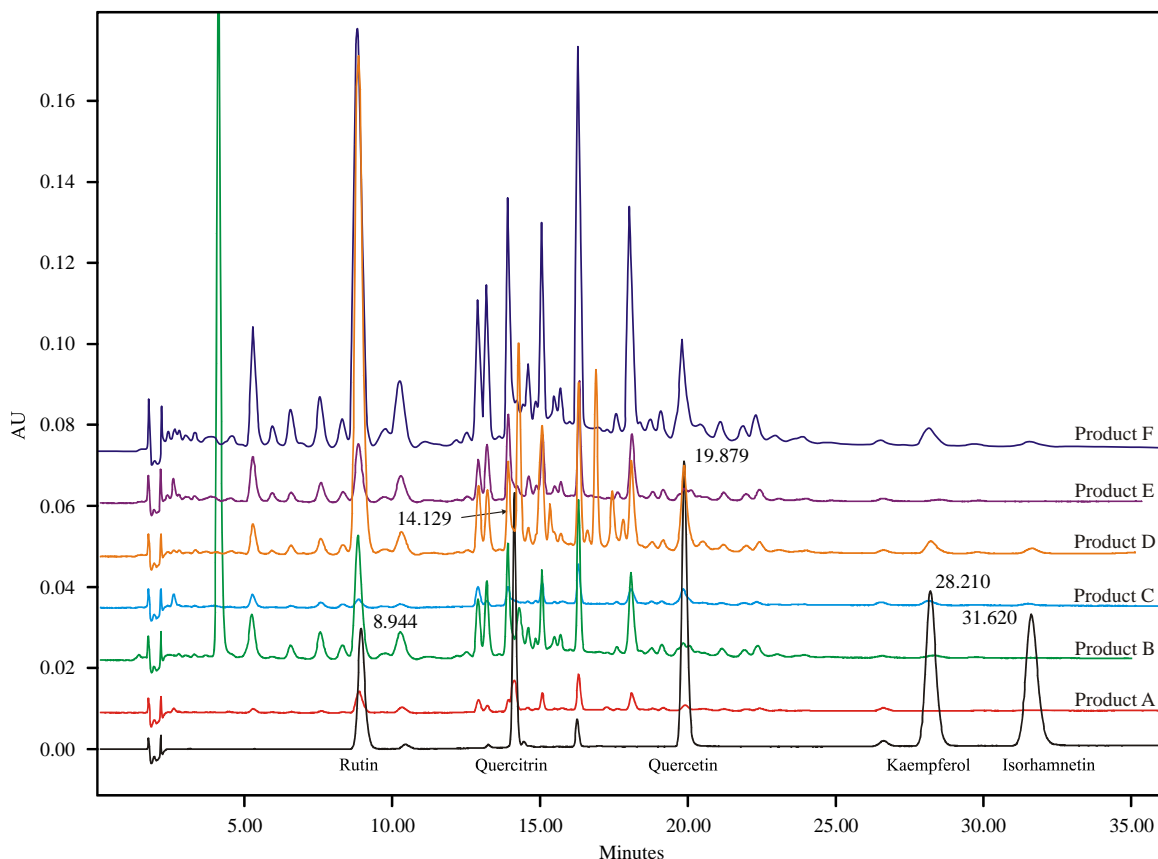
Table 4.6. Recoveries of flavonols from *Ginkgo biloba* extract (Product A)

Constituent	Amount added ( $\mu\text{g}/500\text{mg}$ tablet)	Mean recovery $\pm$ SD (%)
Rutin	-	-
	312.3	98.4 $\pm$ 2.9
	450.0	97.9 $\pm$ 1.7
Quercitrin	131.3	94.9 $\pm$ 0.7
	287.5	102.4 $\pm$ 1.2
	425.0	101.9 $\pm$ 1.7
Quercetin	143.8	89.6 $\pm$ 1.2
	312.5	81.1 $\pm$ 1.6
	437.5	79.5 $\pm$ 1.0
Kaempferol	165.0	96.7 $\pm$ 5.5
	330.0	98.9 $\pm$ 1.8
	475.3	97.9 $\pm$ 1.7
Isorhamnetin	157.3	78.5 $\pm$ 0.6
	314.5	72.5 $\pm$ 2.5
	453.0	77.9 $\pm$ 0.6

#### 4.4.7.4 Analysis of the commercial products

HPLC analysis of 5 *Ginkgo biloba* preparations gave remarkably similar fingerprint profiles (Figure 4.6) with more peaks present in the flavonol glycoside elution zone compared to the aglycone zone. This is in agreement with the literature where it was found that standardized leaf extracts contain only 0.4% (w/w) of kaempferol and 0.5% (w/w) quercetin [57]. Rutin was also present in substantial quantities in 5 of the 6 products - Product C (Ginkgoforce) was the exception. The exceptionally high ratio of rutin to other compounds present in Product D (Figure 4.5) suggests that this product may possibly be adulterated.

Figure 4.6. Superimposed HPLC-UV chromatogram of the *Ginkgo biloba* commercial products



Conditions: Column: Phenomenex Luna (5  $\mu\text{m}$ )  $\text{C}_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic; Flow rate: 400  $\mu\text{l}/\text{min}$ ; Injection volume: 5  $\mu\text{l}$ ; Detection  $\lambda$ : 350 nm.

Quantitative analysis of 6 *Ginkgo biloba* dosage forms is shown in Table 4.7 over the 3 consecutive days. Product F was formulated as a liquid in a soft gelatin capsule and some loss of material during the extraction procedure was evident by the higher % RSD values, hence only 2 of the 3 assays were included in the final assay results. Average marker content in each of the brands varied remarkably. A 160 fold difference in rutin content was found between products C and D. Product D was the only product where all 5 marker compounds were found to be present although as previously stated, the fingerprint profiles indicated that the rutin content may have been fortified. The quercetin content in Product E was not determined due to interferences. Label claims gave no indication of individual marker content. Product B gave detailed information regarding amounts of total flavonol glycosides, ginkgolides and BB, whereas the remaining products simply specified the contents based on raw material incorporated into each tablet/capsule (Table 4.3).

Table 4.7. Flavonol marker content (per tablet/capsule) in 5 *Ginkgo biloba* dosage forms

Product (n=9) <sup>1</sup>	Rutin (µg ± SD)	Quercitrin (µg ± SD)	Quercetin (µg ± SD)	Kaempferol (µg ± SD)	Isorhamnetin (µg ± SD)
A (Holotropic)	213.0 ± 7.4	38.4 ± 2.2	24.4 ± 4.2	-	-
B (Formule Naturelle)	1240.0 ± 5.2	508.0 ± 9.5	+	-	-
C (Bioforce)	35.4 ± 2.2	29.5 ± 1.4	26.35 ± 5.2	15.4 ± 2.1	+
D (Nrf)	5690.0 ± 5.1	318.2 ± 16.8	352.7 ± 7.7	95.2 ± 2.4	51.5 ± 2.6
E (Bioharmony)	293.2 ± 8.8	205.50 ± 6.2	ND	-	-
F (n=6) (Vital)	4200.0 ± 377.2	1074.2 ± 96.2	307.4 ± 11.0	137.0 ± 8.1	49.9 ± 8.7

<sup>1</sup>analysis performed over 3 different days and all samples were prepared in triplicate; <sup>2</sup>content expressed per 2 tablets; (+), indicates that the marker could be identified but was below the LOQ; (-), indicates that the marker was not detected; ND, the marker was not determined due to interferences

#### 4.5 CONCLUSIONS

A simple, precise, rapid and reproducible RP-HPLC-PDA method was developed to quantify 5 relevant flavonol marker compounds with successful application to *Ginkgo biloba* dosage forms. Sample preparation was simple and no tedious cleanup was necessary. In addition to the usual flavonol aglycones, 2 intact flavonol glycosides were determined in order to obtain comprehensive fingerprints which can be used to gauge the quality and authenticity of *Ginkgo biloba* extracts and dosage forms. This QC approach has recently been adopted by a few authors [22, 170] who have recognised the need for more stringent control on commercially available extracts and dosage forms. This is the first validated method however which has been published showing the quantitative determination of selected flavonols as well as their corresponding aglycones in *Ginkgo biloba* dosage forms [168].

Since up to 33 flavonoids [57] have been identified in *Ginkgo biloba* leaf extracts and these compounds have strong chromophores, HPLC-UV is seen to provide detailed fingerprints for product profiling at selective wavelengths. Complete separation of all the flavonoids was not possible using a simple gradient with a shorter analysis time. Moreover, since the analyzed dosage forms could contain differently extracted raw material as well as different excipients, complete resolution of all the peaks of interest using the same method was confounded by the presence of those varying and potentially interfering compounds. The PDA detector was extremely valuable for peak identification and homogeneity testing. This method can, nevertheless be readily modified to accommodate a specific formulation in order to determine marker compounds in those products where interferences may hinder detection.

Product analysis showed similar qualitative profiles however 1 of the products gave evidence of rutin fortification. The quantitative analysis of the dosage forms revealed major disparities in marker

compound composition. These results indicate that suitable QC measures need to be implemented to ensure the QSE of *Ginkgo biloba* products commercially available to consumers.

#### 4.6 ANALYSIS OF TERPENE LACTONES BY HPLC

The ginkgolides are non-volatile compounds and polar in nature and are therefore amenable to analysis by RP-HPLC. Early analytical papers used HPLC-UV/PDA [193] however, the terpene lactones possess poor chromophores with log  $\epsilon$  values of GA, GB and GC reported at 2.72, 2.37 and 2.30 [56] and they can therefore only be monitored at low, non-selective wavelengths (190 and 220 nm) [53]. UV detection of terpene trilactones is therefore difficult, especially for the analysis of crude Ginkgo extracts and extensive clean-up procedures are usually required prior to the analysis of leaf extracts and dosage forms [53, 189, 194].

RI detection has been used successfully [195, 196] but unstable baseline problems and low sensitivity [195] detract from its selectivity. In contrast, MS has proved to be a valuable detection method due to its sensitivity and LC-MS-MS facilitates exclusive analysis of selected peaks of interest with unequivocal peak identification which is particularly useful when analyzing crude extracts such as botanicals [197, 198]. Analysis of the ginkgolides using GC-MS has also been investigated [192, 199] but this method of analysis requires complex sample preparation as well as derivatization. In addition to these detection modes, van Beek *et al.* [200] obtained similar results to RI in terms of repeatability and selectivity using NMR for the successful quantification of GA, GB, GC, GJ and BB in Ginkgo leaves and phytopharmaceuticals. More recently,  $^1\text{H-NMR}$  has been used to quantitatively determine GA, GB, GC and BB in *Ginkgo biloba* leaves and commercial dosage forms. Advantages of this method included no sample clean-up or derivatization which contributed to the speed of the method [201]. However, NMR, GC and MS detectors require highly trained personnel for operation. Furthermore, the quite exorbitant costs, low availability and maintenance of such equipment makes these detection methods currently impractical for routine analyses [53, 198].

On the other hand, the relatively inexpensive evaporative light scattering detection (ELSD) has gained popularity for the analysis of non-volatile compounds that possess poor chromophores. Detection involves the nebulization of the LC effluent into a fine aerosol, followed by the evaporation of the more volatile solvent in a heated drift tube. A silicon photodiode then measures the scattering of light caused by the remaining solute particles as they pass from the drift tube into the light scattering cell [189]. The light scattering response is determined by the solute size and concentration [189, 202]. Recently, a number of papers have been published using HPLC with ELSD detection for the determination of terpene trilactones in complex matrices such as pharmaceutical dosage forms, beverage, snack and dietary supplement products [188, 189, 198, 203-205] and 1 paper has described the assay of a Ginkgo extract in an injectable formulation [202] (Table 4.8).

Table 4.8. HPLC methods used for the analysis of terpene lactones using RI and ELSD detection

Matrix analyzed	Sample purification/extraction procedure	Analyzed terpene lactones	Mobile phase	Column	Detection	Ref
<i>Ginkgo biloba</i> leaf extracts, phytopharmaceuticals	MeOH, dry extract then extracted by liquid-liquid extraction using hot H <sub>2</sub> O + EtOAc <sup>1</sup> ; MeOH <sup>2</sup>	GA, GB, GC, GJ, BB	Gradient: A) MeOH B) water	Nucleosil C <sub>18</sub> (250 x 4.0 mm I.D.) <sup>1</sup> Waters C <sub>18</sub> (150 x 3.9 mm I.D.) <sup>2</sup>	ELSD <sup>1</sup> , LC-MS <sup>2</sup>	198
<i>Ginkgo biloba</i> commercial products	MeOH	GA, GB, GC, GJ, BB	Gradient: A) AmAc B) MeOH-isobutanol	Synergy Max C <sub>18</sub> (150 x 4.6mm I.D.)	ELSD	203
<i>Ginkgo biloba</i> commercial products	EtOAc	GA, GB, GC, BB	Isocratic: THF, MeOH, water (10:20:75 v/v/v)	RP LiChrospher C <sub>8</sub> (250 x 4.0 mm I.D.)	RI	42
<i>Ginkgo biloba</i> commercial products	80% MeOH <sup>3</sup> MeOH <sup>4</sup> dry extract then extracted by liquid-liquid extraction using hot H <sub>2</sub> O + EtOAc	GA, GB, GC, GJ, BB	Gradient: A) water-MeOH-TFA B) MeOH-TFA	Supelco Discovery C <sub>18</sub> (250 x 4.6 mm I.D.)	ELSD	188 <sup>3</sup> 189 <sup>4</sup>
<i>Ginkgo biloba</i> injections	EtOAc, SPE	GA, GB, GC, BB	Isocratic: MeOH, water (33:67)	Diamonsil C <sub>18</sub> , 5 μm (250 x 4.0 mm I.D.), YWG C <sub>18</sub> , 10 μm (250 x 4.0 mm I.D.)	ELSD	202
<i>Ginkgo biloba</i> commercial products	MeOH	GA, GB, GC, GJ, BB	Gradient: A) water-TFA B) MeOH-TFA	Alltima C <sub>18</sub> , (100 x 4.6 mm I.D.)	ELSD	204
Beverage, snacks and commercial products containing <i>Ginkgo biloba</i>	Various: 5% KH <sub>2</sub> PO <sub>4</sub> , boiling water, EtOAc-THF, MeOH-acetic acid	GA, GB, GC, GJ, BB	Gradient: A) water B) MeOH-isobutanol	Phenomenex C <sub>18</sub> (250 x 4.6 mm I.D.)	ELSD	205

MeOH, methanol; GA, ginkgolide A; GB, ginkgolide B; GC, ginkgolide C; GJ, ginkgolide J; BB, bilobalide; SPE, solid phase extraction cartridge; DMF, N,N-dimethylformamide; EtOAc, ethyl acetate; AmAc, ammonium acetate; TFA, trifluoroacetic acid; Ref, reference

Ganzera *et al.* [203] quantified GA, GB, GC and GJ as well as the amount of BB present in 9 commercial products with a total analysis time of 25 minutes using a gradient program with a buffered mobile phase of 10 mM ammonium acetate, methanol and isobutanol. Validation of the method was only briefly described over a linear range of 31.2 - 500 μg/ml with an LOD of 20.3 μg/ml (203 ng on-column). LOQ values were not disclosed and although excellent recoveries were reported (99.31 - 100.10%), the experiment was performed once only at the lower end of the calibration range.

Kressmann *et al.* [42] assayed 27 US products for terpenoid content according to the official WHO and Pharmeuropa monograph. However, GJ was not included in the assay and no validation data were shown or referred to.

Li and Fitzloff [189] published a comprehensive method for the simultaneous determination of ginkgolides and only the flavonol aglycones, quercetin, kaempferol and isorhamnetin using a gradient method of methanol, water and trifluoroacetic acid (TFA). The final analysis time was 50 minutes, including an equilibration period however, all 4 ginkgolides as well as BB eluted within 16 minutes and this method has the potential for application to the analysis of terpene trilactones. The LOD was 20 - 30 ng on-column and the calibration range was 7 - 350 µg/ml for GJ with the remaining compounds demonstrating second-order polynomial calibrations within a slightly lower range of 4 - 200 µg/ml. The repeatability of the method as shown by the analysis of 3 sets of 3 controls over a 3 day period was relatively poor with a RE and RSD of 14.67% and 17.26%, respectively being recorded on the third day. Recovery studies were not reported. In addition, only a single product was analyzed resulting in no available comparative data to sufficiently assess the validation of their method.

Herring [204] recently published a method for the separation of 4 ginkgolides and BB within 14 minutes using a gradient consisting of water, methanol and TFA. The LOD was relatively high at 125 ng on-column. Four commercial products were analyzed and whilst the method showed acceptable repeatability, no recovery studies were performed which is a critical determinant of method accuracy.

Tang *et al.* [202] assayed the content of GA, GB and GC, together with BB in a Ginkgo injection for QC purposes. In addition, separation of the relevant peaks was compared using two columns, a Diamonsil and YWG (Table 4.8). Baseline separation was obtained on the Diamonsil column using a simple isocratic method of methanol and water but the run time was 41 minutes. The analysis time was halved using the YWG column however separation between BB and GC was compromised by an interfering peak which was speculated to be GJ but confirmation was circumvented due to a lack of GJ reference standard. Moreover, the calibration range was 75 - 800 µg/ml, the LOD and LOQ were not given and solid phase extraction was used for sample clean-up.

Lang *et al.* [205] published a method which focused on the extraction procedures required for accurate quantification of the terpene trilactones in beverages, snacks and capsules containing *Ginkgo biloba*. The 3 analyzed capsules were extracted with 5% KH<sub>2</sub>PO<sub>4</sub>, followed by a liquid-liquid extraction with sodium chloride and a mixture of ethyl acetate and THF. The terpenes were separated within 30 minutes on a Phenomenex C<sub>18</sub> (250 x 4.6 I.D.) column using a gradient of water and a mixture of methanol and isobutanol. Reference to a previous study [206] was provided concerning the recovery data of the capsules (95 - 102%) with RSDs of less than 7.4% performed at a single concentration level using GA, GB, GC and BB only. The LOD was 40 ng on-column load for all reference standards but the LOQ was not provided. Method repeatability was evaluated by both inter- and intra-day RSDs of ≤ 7.1%.



Although a number of proficient methods have already been published using HPLC-ELSD, its simplicity, easy operation and cost efficiency cannot be overrated and it was therefore a logical choice to attempt to use the same approach for the quantification of terpene trilactones in *Ginkgo biloba* solid oral dosage forms. As a result, an accurate, precise, rapid and reproducible RP-HPLC-ELSD method was developed and fully validated according to USP standards and applied to the quantitative determination of GA, GB, GC, GJ and BB using a simple gradient of methanol and water as mobile phase. Baseline separation of the reference standards was achieved within 14 minutes using a Phenomenex Luna (5 $\mu$ m) C<sub>18</sub> minibore column with dimensions 250 x 2.0 mm I.D. which normally requires lower solvent flow rates compared to the wider diameter conventional HPLC columns, thereby resulting in reduced analysis costs by decreasing the consumption of relatively expensive HPLC solvents. Sample clean-up involved an inexpensive liquid-liquid extraction procedure and this method was successfully applied to the quantitative determination of the selected marker compounds in various commercially available *Ginkgo biloba* oral dosage forms formulated as hard and soft gelatin capsules as well as tablets and is particularly suitable for the routine analysis and QC of such products.

#### **4.7 METHOD DEVELOPMENT**

##### **4.7.1 Instrumentation**

All experiments were performed using a Waters 2690 Separations Module and Waters 2996 Photodiode Array equipped with an auto sampler, on-line degasser system and column heater for the HPLC analysis (Milford, MA, USA). An Alltech ELSD 2000 detector system was supplied by the same source and Empower<sup>®</sup> Software was used for the processing and integration of data. A Mettler Dual Range Electronic Balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland) was used for weighing of reagents and standards. A Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> column with dimensions 250 x 2.0 mm I.D. was used to effect the LC separation.

##### **4.7.2 Materials and reagents**

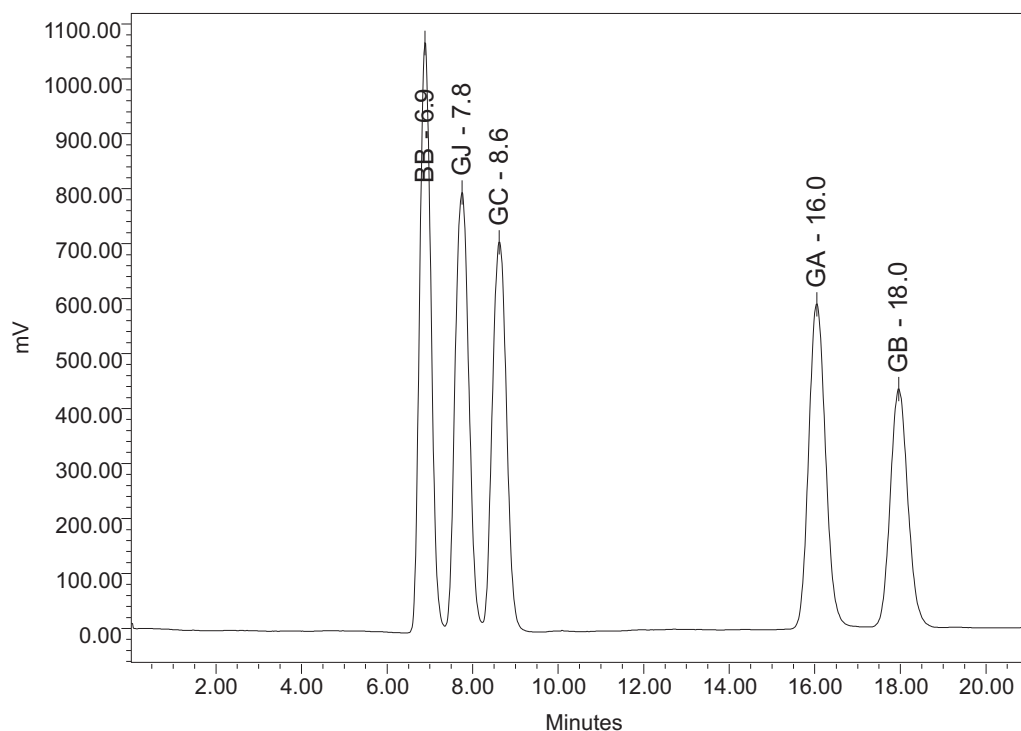
Methanol was supplied by Romil (The Source, Waterbeach, Cambridge, GB) and ethyl acetate by BDH chemicals (Poole, UK). GA (90%), GB (90%) and BB (95%) were purchased from Sigma (Missouri, USA) and GJ (95%) was obtained from Chromadex (Santa Ana, CA, USA). GC (90%) was a generous gift from Dr. Egon Koch from Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). The peak purity of the terpene trilactones used as reference standards were checked chromatographically and confirmed by NMR spectroscopy. Water was purified by reverse osmosis using a Milli-Q system (Millipore, Bedford, MA, USA) and HVLP filters were purchased from the same source.

#### 4.7.3 Optimization of separation of terpene trilactones

The mobile phase was selected based on a number of previously published methods where either methanol-water (~30:~70) or methanol-water-THF (~70:~20:~10) was used [53,188]. Although THF was reported to give better resolution [53], it was avoided as a mobile phase component due to its instability/explosive hazard properties [207]. The addition of acetonitrile, even in small quantities compromised the resolution between GA and GB which is consistent with the findings of Tang *et al.* [202]. The incorporation of phosphoric acid into the aqueous component of the mobile phase resulted in total peak retention on the column after 1 hour and methanol and water were subsequently selected as the mobile phase components.

All 4 ginkgolides and BB were well separated within 19 minutes using an isocratic method consisting of methanol-water (30:70) at a column temperature of 45°C and flow rate of 300 µl/min (Figure 4.7). However, when the proportions of the mobile phase were adjusted to 35:65 methanol-water, GC, GJ and BB co-eluted.

Figure 4.7. Isocratic HPLC-ELSD chromatogram of the terpene lactones



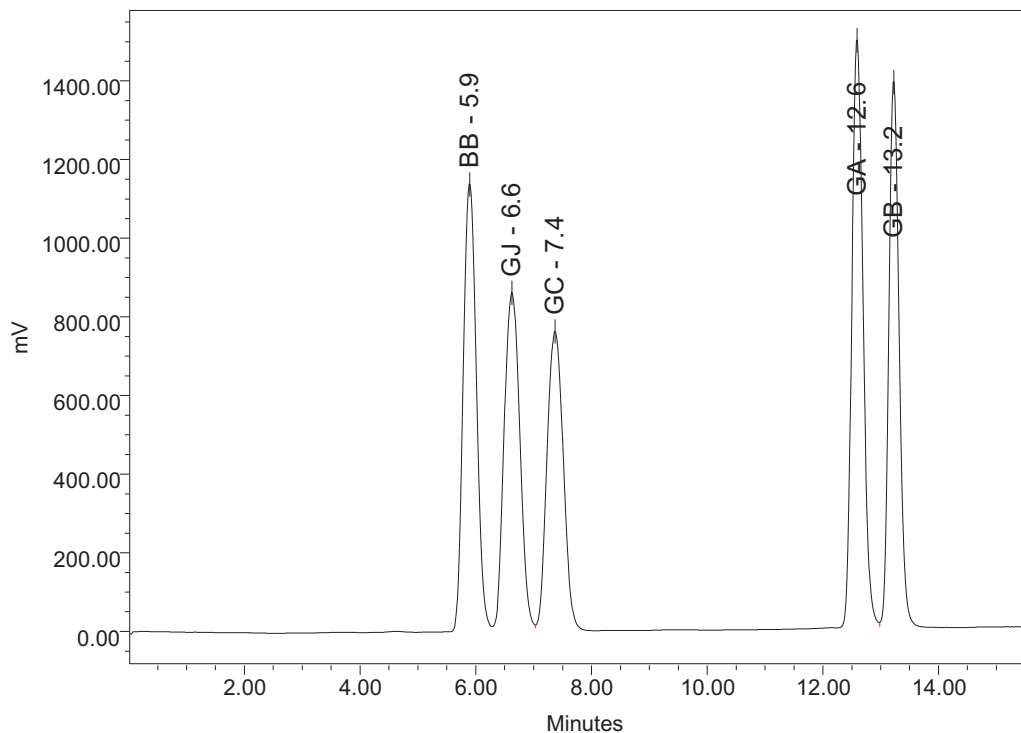
Conditions: Column: Phenomenex Luna (5  $\mu$ m)  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeOH:water (30:70); Flow rate: 300  $\mu$ l/min; Injection volume: 5  $\mu$ l; Drift tube temperature: 110°C; Gas flow rate: 2 L/min; Peak labels: BB= bilobalide, GJ=ginkgolide J, GC=ginkgolide C, GA=ginkgolide A, GB=ginkgolide B.

A gradient method was attempted to decrease the analysis time and also to improve sensitivity by changing the ratios of methanol-water from 30:70 after BB, GJ and GC had eluted. This was effected by linearly increasing the percentage methanol to 40:60 over the remaining 12 minutes of the 20 minute run time. The last peak eluted at 17 minutes and subsequently, the following gradient involved changing methanol-water from 30:70 at 7 minutes to a linear change of 50:50 over 20 minutes which decreased the run time by one minute only. Slight changes in the gradient profile ensued until changing the final gradient from 30:70 methanol-water at 6 minutes to 70:30 over the following 10 minutes resulted in an analysis time of less than 16 minutes. The flow rate was then increased slightly to 350  $\mu$ l/min and using the previous gradient all selected marker compounds eluted within 14 minutes (Table 4.9). Figure 4.8 shows the elution orders of the reference standards using this method. Both the isocratic and gradient method gave suitable chromatograms for analysis but the gradient method was selected due to the enhanced sensitivity and the improved peak shape of GA and GB caused by the higher organic composition of the mobile phase.

Table 4.9. Gradient profile used for analysis of the terpene lactones

Time (minutes)	Flow rate (ml/min)	Methanol (%)	Water (%)
0	0.35	30	70
6	0.35	30	70
16	0.35	70	30

Figure 4.8. Gradient HPLC-ELSD chromatogram of the reference standards



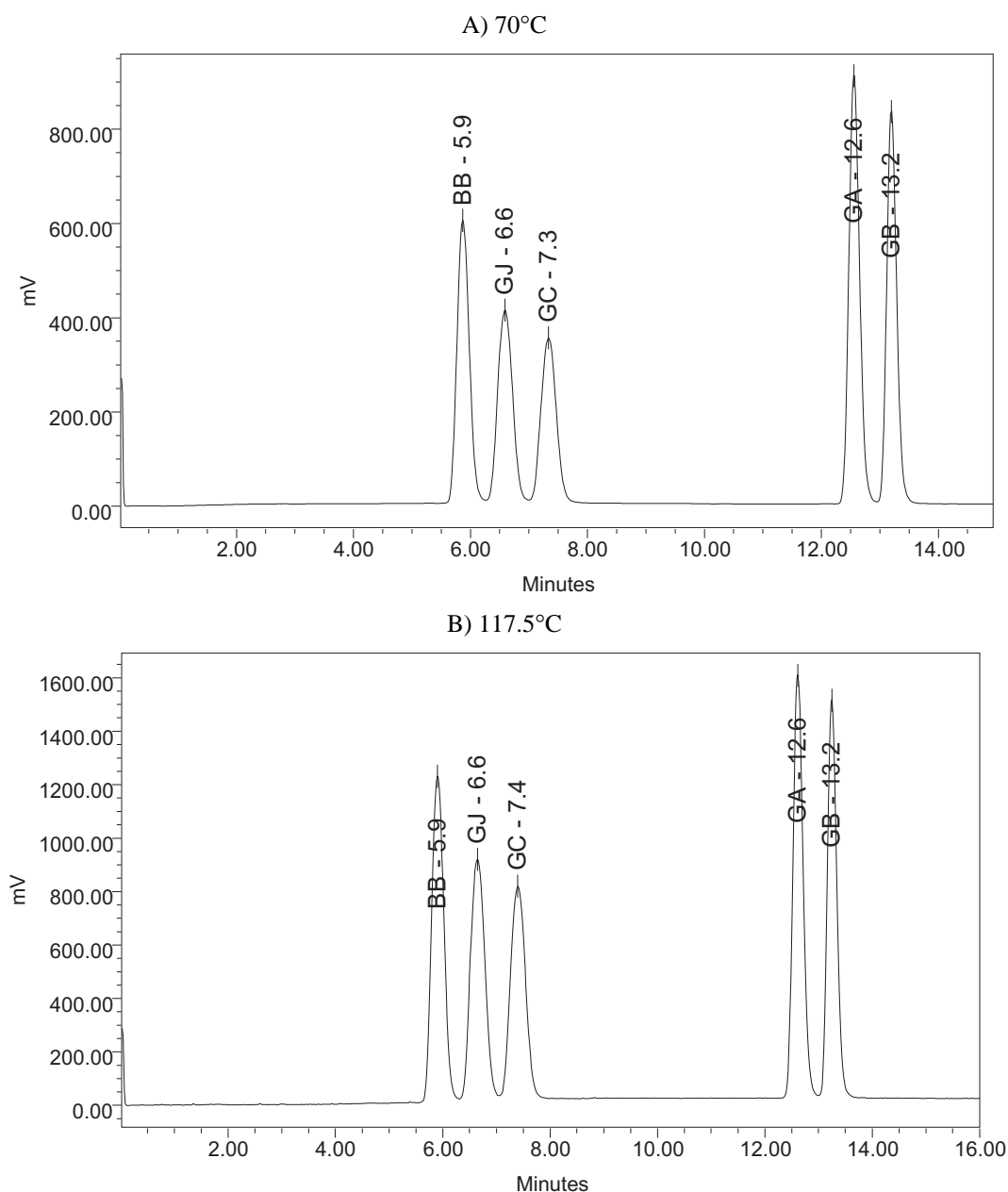
Conditions: Column: Phenomenex Luna (5  $\mu$ m)  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeOH-water (final gradient method); Flow rate: 350 $\mu$ l/min; Injection volume: 5  $\mu$ l; Drift tube temperature: 110°C; Gas flow rate: 2 L/min; Peak labels: Peak labels: BB= bilobalide, GJ=ginkgolide J, GC=ginkgolide C, GA=ginkgolide A, GB=ginkgolide B.

#### 4.7.4 ELSD parameter optimization

The most important parameters affecting the ELSD signal response is the drift tube temperature and nebulizer gas flow rate. The drift tube temperature facilitates the evaporation of the nebulized aerosol so that the light scattering response of the non-volatile solute can exclusively be determined. Mobile phases with a high aqueous content require higher drift tube temperatures for evaporation than those comprised predominantly of organic components. Similarly, optimal sensitivity of non-volatile solutes requires higher drift tube temperatures than semi-volatile solutes [208]. It was therefore predicted that a relatively high drift tube temperature would be required for adequate evaporation since the ginkgolides are non-volatile and the mobile phase consisted substantially of water. The effect of temperature on sensitivity was determined over the range of 70 to 120°C in increments of 10 degrees until 110°C and was thereafter increased by increments of 2.5°C. A drift tube temperature of 117.5°C was finally

selected based on the evaluation of the S/N ratio. Figure 4.9 shows the HPLC-ELSD chromatograms of the reference standards with drift tube temperatures of 70°C (A) and 117.5°C (B), respectively.

Figure 4.9. HPLC-ELSD chromatograms of the terpenes using various drift tube temperatures

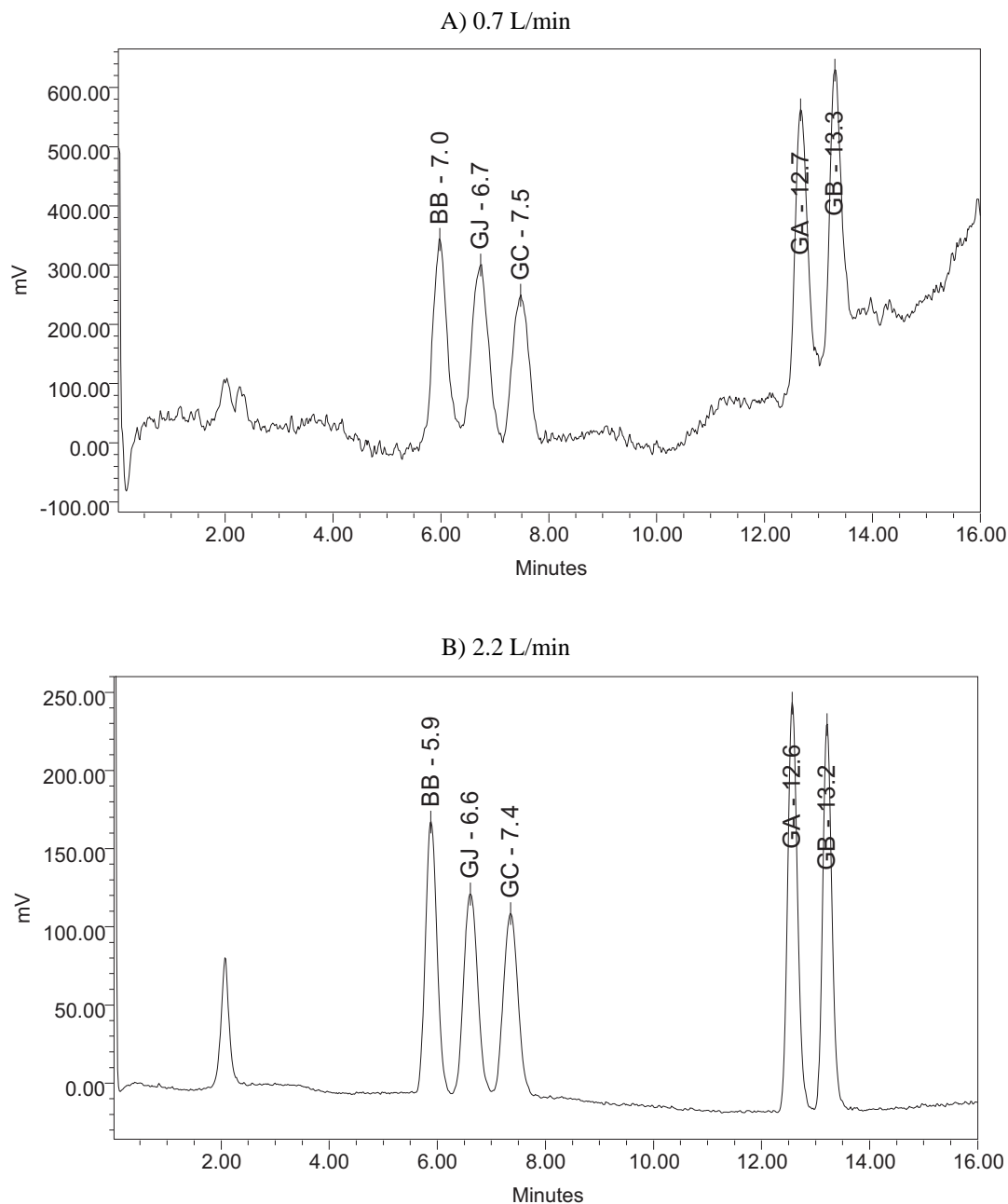


Conditions: Column: Phenomenex Luna (5  $\mu$ m)  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeOH-water (final gradient method); Flow rate: 350  $\mu$ l/min; Injection volume: 5  $\mu$ l; Gas flow rate: 2 L/min; Peak labels: BB= bilobalide, GJ=ginkgolide J, GC=ginkgolide C, GA=ginkgolide A, GB=ginkgolide B.

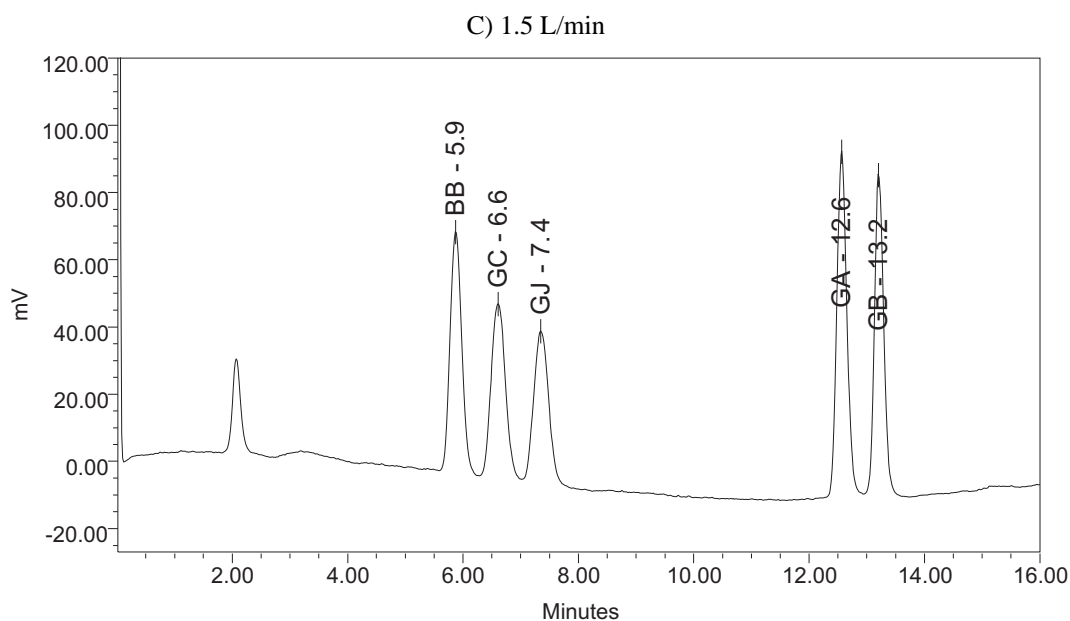
Gas flow rates influence droplet size of the column effluent before evaporation occurs. Higher flow rates result in the formation of smaller aerosol droplets and less scattering of light with subsequent lower sensitivity but a more stable baseline. On the other hand, lower gas flow rates are associated with larger droplet formation, augmented light scattering and therefore a higher response but the baseline is compromised. It is therefore imperative that this parameter is optimized to ensure that the highest S/N

is achieved [208]. The gas flow was therefore investigated over the range of 0.7 - 3.5 L/min in increments of 0.2 L/min. The sensitivity was highest at 0.7 L/min (Figure 4.10 A) as predicted and nebulizer gas flow rates higher than 2.2 L/min (Figure 4.10 B) obviously compromised the sensitivity. As a result, 1.5 L/min (Figure 4.10 C) was chosen since it exhibited the best S/N ratio.

Figure 4.10. HPLC-ELSD chromatograms of terpenes at various gas flow rates



Conditions: Column: Phenomenex Luna (5  $\mu$ m)  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeOH-water (final gradient method); Flow rate: 350  $\mu$ l/min; Injection volume: 5  $\mu$ l; Drift tube temperature: 117.5°C; Peak labels: BB= bilobalide, GJ=ginkgolide J, GC=ginkgolide C, GA=ginkgolide A, GB=ginkgolide B.

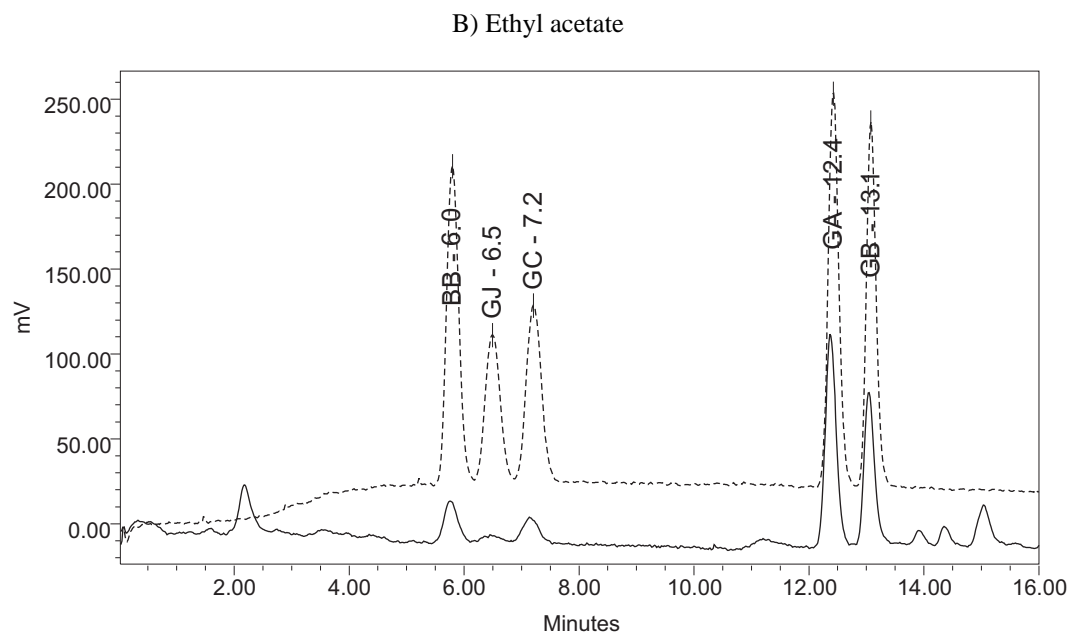
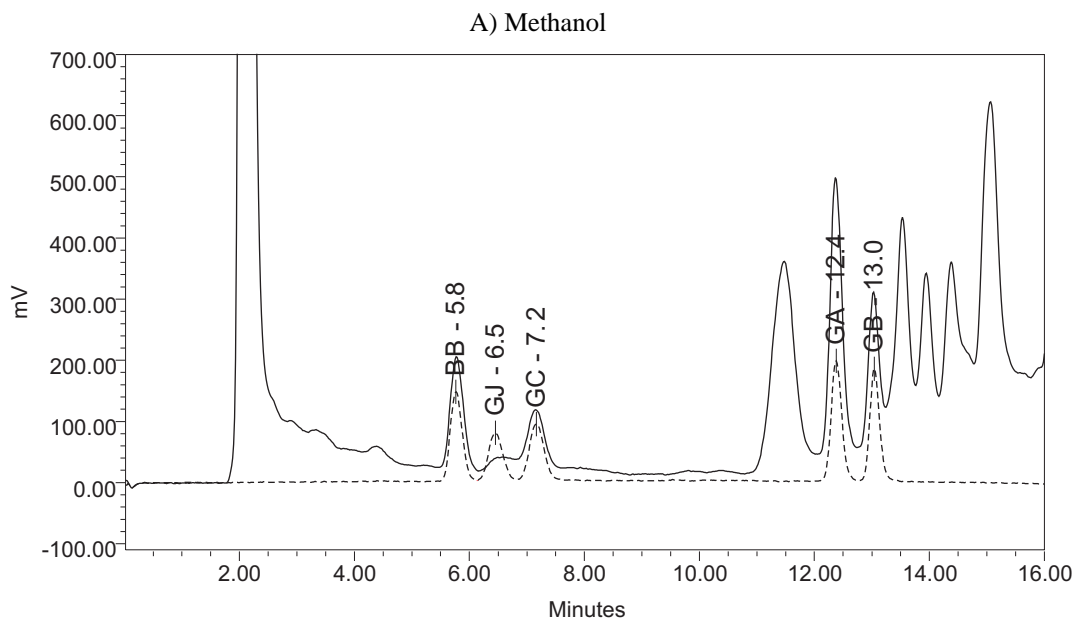


Conditions: Column: Phenomenex Luna (5  $\mu$ m)  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeOH-water (final gradient method); Flow rate: 350 $\mu$ l/min; Injection volume: 5  $\mu$ l; Drift tube temperature: 117.5°C; Peak labels: BB= bilobalide, GJ=ginkgolide J, GC=ginkgolide C, GA=ginkgolide A, GB=ginkgolide B.

#### 4.7.5 Tablet extraction efficiency

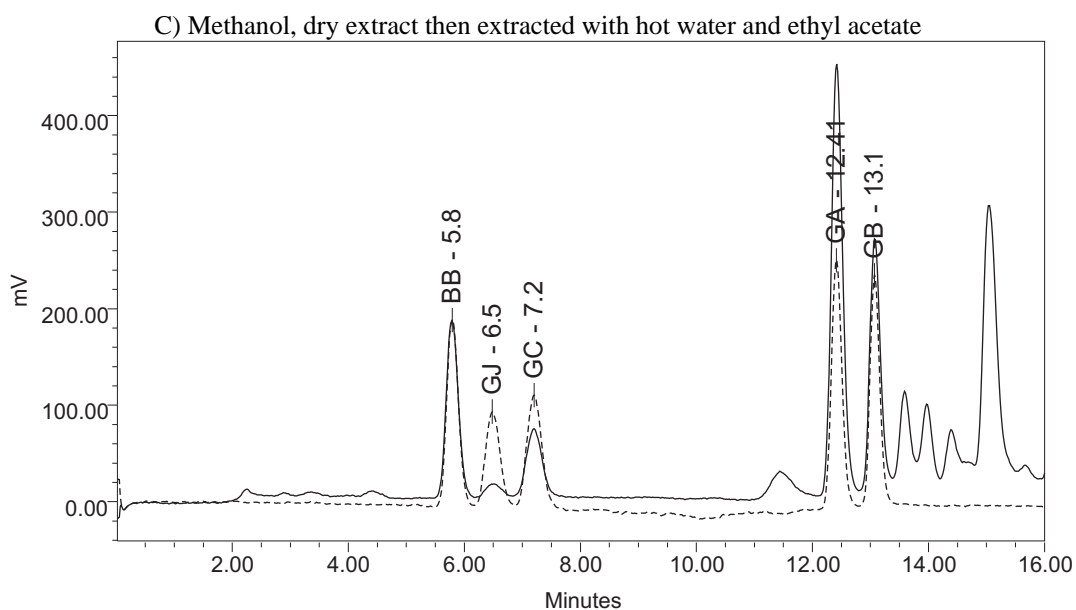
Tablet extraction efficiency was investigated using various solvents [53]. A mass of powder equivalent to the mass of a single tablet of Product C (Nrf) which contained sufficient quantities of the relevant marker compounds was extracted exclusively with methanol (3 x 20 ml) (Figure 4.11 A). This procedure is described in section 3.10.3.1. The same procedure was followed using ethyl acetate (3 x 20 ml) as extraction solvent (Figure 4.11 B) and finally the method of Li and Fitzloff [189] was investigated by using methanol as the primary extraction solvent followed by a liquid-liquid extraction using hot water and ethyl acetate as described in section 4.8.4.1 (Figure 4.11 C). All extract residues were reconstituted in 10 ml volumetric flasks. The various extraction procedures were also performed on the reference standards. For each extraction procedure, only one sample was prepared due to a shortage of reference standards. It was found that although methanol proved to be an excellent extraction solvent it was non-selective and interfering compounds co-eluted with the peaks of interest. When ethyl acetate was used as the primary extraction solvent, its lower polarity ensured little interference but the polar ginkgolides were not as effectively extracted as with methanol. It was concluded that the method described by Li and Fitzloff [189] was the most suitable and efficient extraction method since it extracted higher amounts of ginkgolides (based on peak area and height) with minimal interference. In addition, Li and Fitzloff reported extraction efficiencies of 94.7 - 99.2% when this method was used for the extraction of the terpene trilactone reference standards.

Figure 4.11. HPLC-ELSD chromatograms of reference standards superimposed with the dosage forms extracted with various solvents



Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeOH-water (final gradient method); Flow rate: 350  $\mu$ l/min; Injection volume: 5  $\mu$ l; Drift tube temperature: 117.5°C; Gas flow rate: 1.5 L/min; Peak labels: BB= bilobalide, GJ=ginkgolide J, GC=ginkgolide C, GA=ginkgolide A, GB=ginkgolide B; Solid line=chromatogram of tablet extract, dotted line=chromatogram of reference standards.





Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeOH-water (final gradient method); Flow rate: 350 $\mu$ l/min; Injection volume: 5  $\mu$ l; Drift tube temperature: 117.5°C; Gas flow rate: 1.5 L/min; Peak labels: BB= bilobalide, GJ=ginkgolide J, GC=ginkgolide C, GA=ginkgolide A, GB=ginkgolide; Solid line=chromatogram of tablet extract, dotted line=chromatogram of reference standards.

## 4.8 METHOD VALIDATION

### 4.8.1 Instrumentation and additional equipment

The instrumentation was the same as described in section 4.7.1. In addition, an ultrasonic bath, Model SC-211TH (Sonicator Instrument Corporation, Copiague, N.Y., USA) was used for extraction purposes and a centrifuge, Model HN/SII (Damon IEC Division, MA, USA) was used to centrifuge tablet extracts. A Vortexer, Model G-560E (Scientific Industries, Bohemia, N.Y., USA) was used for sample mixing.

### 4.8.2 Chemicals and materials

Chemicals and reagents were sourced as previously described in section 4.7.2. Five *Ginkgo biloba* commercial products (Products A - E) were purchased from a local pharmacy in Grahamstown, South Africa. Three of the products were formulated in tablet form, one product contained liquid Ginkgo extract in a soft gelatin capsule and one product contained pulverized Ginkgo leaf extract in a hard gelatin capsule (Table 4.10).

Table 4.10. *Ginkgo biloba* dosage forms used for HPLC-ELSD analysis of the terpenoids

Manufacturer	Name of product	Dosage form	Label claim	Daily dose	Batch number	Extract
Holotropic <sup>1</sup>	<i>Ginkgo biloba</i>	Tablet	Each 3 tablets provide: <i>Ginkgo biloba</i> botanical 300 mg, <i>Ginkgo biloba</i> extract equivalent to 900 mg	1 tablet tds <sup>6</sup>	0319	A
Formule Naturelle <sup>2</sup>	<i>Ginkgo biloba</i>	Tablet	Standardized to contain 24% Ginkgo Flavonglycosides, 1.2 mg ginkgolides, 1.0 mg bilobalide	1 tablet tds <sup>6</sup> , ac <sup>7</sup>	82060	B
Nrf Herbal <sup>3</sup>	<i>Ginkgo biloba</i>	Tablet	Each tablet contains 350mg <i>Ginkgo biloba</i> , 50mg <i>Ginkgo biloba</i> extract 24% and 240mg FoodMatrix™	1 - 2 tablets daily	MTM092PW	C
Vital <sup>4</sup>	<i>Ginkgo biloba</i>	Capsule	Each capsule contains <i>Ginkgo biloba</i> extract equivalent to 6 000 mg of herb powder	1 capsule daily	B0400209B	D
Bioharmony <sup>5</sup>	<i>Ginkgo biloba</i>	Capsule	Each 350mg capsule contains 350mg <i>Ginkgo biloba</i> ( <i>Ginkgoaceae</i> )	2 capsules tds <sup>6</sup>	1ER10.11	E

<sup>1</sup>Holotropic, Cape Town, South Africa; <sup>2</sup>Formule Naturelle, Hout Bay, South Africa; <sup>3</sup>Nrf Herbal, Centurion, South Africa; <sup>4</sup>Vital Health Foods (EDMS) BPK, Kuils River, Western Cape, South Africa; <sup>5</sup>Bioharmony CC, Wynberg, South Africa; <sup>6</sup>tds, three times daily; <sup>7</sup>ac, before meals

#### 4.8.3 Preparation of standard solutions

The stock solution for the calibration curve was prepared by accurately weighing ~5 mg of each reference standard into a 25 ml volumetric flask, dissolving the contents in methanol and then filling it to volume with the same solvent. In order to obtain the final concentration range of 12.5 - 100 µg/ml, appropriate concentrations of the stock solution were pipetted into 10 ml volumetric flasks and then filled to volume with methanol.

#### 4.8.4 Sample preparation and extraction

##### 4.8.4.1 *Solid oral dosage forms*

A minimum of twenty tablets of Products A - C were individually weighed and pulverized using a mortar and pestle. An aliquot of powder equivalent to the mass of a single tablet was then accurately weighed and carefully transferred to a 20 ml Kimax tube. Twenty milliliters of methanol were added

and the powder was extracted according to the procedure described in Section 3.10.3.1 but the dried extracts were not re-constituted in buffer as previously described. Instead, the dried extracts were re-suspended in 20 ml hot water and liquid-liquid extractions were then performed in triplicate (3 x 20 ml) with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness using nitrogen. Product A was reconstituted with 8 ml methanol, decanted into a 10 ml volumetric flask and made up to volume using the same solvent. Products B and C were reconstituted with 18 ml of methanol, decanted into 25 ml volumetric flasks and then filled to volume.

#### 4.8.4.2 *Soft gelatin capsule*

A single weighed capsule of Product D was sliced longitudinally and the contents were carefully squeezed out into a 20 ml Kimax tube. The remaining gelatin coat was then sliced into two halves and added to the contents. Twenty milliliters of methanol were added and the capsule was extracted as described in section 4.8.4.1 above. The extract was reconstituted with 25 ml methanol and then decanted into a 50 ml volumetric flask before filling it to volume.

#### 4.8.4.3 *Hard gelatin capsule*

A minimum of twenty capsules of Product E were individually emptied and weighed before ensuring thorough mixing using a mortar and pestle. Twenty milliliters of methanol was added to a mass of powder equivalent to the contents of one capsule and extracted as previously described in section 4.8.4.1. The extract was reconstituted with 8 ml methanol, decanted into a 10 ml volumetric flask and made up to volume using the same solvent. All samples were filtered through 0.45  $\mu\text{m}$  PVDF membranes before injecting 5  $\mu\text{l}$  onto the column.

#### 4.8.5 HPLC-ELSD conditions

The ginkgolides were separated on a Phenomenex Luna (5  $\mu\text{m}$ ) C<sub>18</sub> minibore column with dimensions 250 x 2.0 mm I.D. which was maintained at a temperature of 45°C. The mobile phase consisted of methanol-water in proportions of 30:70 for the first 6 minutes, after which the methanol was increased linearly to 70:30 for the remainder of the analysis time. The flow rate of the mobile phase was 350  $\mu\text{l}/\text{min}$ , the injection volume was 5  $\mu\text{l}$  and the total run time was 16 minutes. The ELSD gas flow and drift tube temperature were set at 1.5 L/min and 117.5°C, respectively.

#### 4.8.6 Method validation

##### 4.8.6.1 *Linearity and range*

Linear calibration curves were constructed using five concentration points on each of the 3 days and plotting the double logarithm of both  $x$  and  $y$ -axis values.

#### 4.8.6.2 Precision and accuracy

The precision and accuracy of the method was determined by spiking powdered tablet material from Product A with amounts corresponding to low, medium and high concentrations of each reference standard. Each concentration level was performed in triplicate and the recovery and % RSDs were used to determine the accuracy and precision of the assay, respectively. Since insufficient reference material was available, the recovery study for GJ was performed on the second day of the validation only.

#### 4.8.6.3 LOD's and LOQ's

The LOD's and LOQ's were determined by serial dilution of the stock solution containing all reference standards according to a S/N ratio of 3:1 and 10:1, respectively.

### 4.8.7 Results and discussion

#### 4.8.7.1 Linearity and range

The ranges and coefficients of determination are presented in Table 4.11. The RSDs over all 3 days ranged between 0.1 and 9.2% with the highest values recorded at the lower end of the concentration range. A typical HPLC-ELSD chromatogram of the reference standards (100 µg/ml) is shown in Figure 4.8.

Table 4.11. Linear ranges and co-efficients of determination

Name of compound	Concentration range (µg/ml)	Day	Slope	y-intercept	Coefficient of determination (R <sup>2</sup> )
GA	12.7 - 101.4	Day 1	1.45	3.52	0.9989
		Day 2	1.57	3.22	0.9918
		Day 3	1.48	3.47	0.9994
GB	12.7 - 101.4	Day 1	1.45	3.48	0.9986
		Day 2	1.66	3.00	0.9995
		Day 3	1.45	3.48	0.9999
GC	12.6 - 100.8	Day 1	1.38	3.47	0.9988
		Day 2	1.66	2.90	0.9996
		Day 3	1.41	3.45	0.9999
GJ	12.8 - 102.0	Day 1	1.34	3.52	0.9942
		Day 2	1.59	3.02	0.9984
		Day 3	1.40	3.47	0.9998
BB	12.9 - 103.4	Day 1	1.43	3.45	0.9974
		Day 2	1.56	3.14	0.9891
		Day 3	1.46	3.43	0.9999

#### 4.8.7.2 Precision and accuracy

The results of the recovery experiments are shown in Table 4.12. The precision is indicated by the intra-day and inter-day RSDs which were well below 5.0% except for the intra-day RSD for the medium recovery level of GC on the first day (5.4%). The accuracy of the method is represented by the % RE which ranged between +1.9 and -3.2 over all 3 days for GA, GB, GC and BB and +0.1 and -1.5 for GJ on the second day. These results reflect excellent method accuracy and precision. Moreover,

the repeatability of the method is better than the results obtained by Li and Fitzloff [189], Lang *et al.* [205], and Herring [204] while Ganzera *et al.* [203] did not report repeatability data.

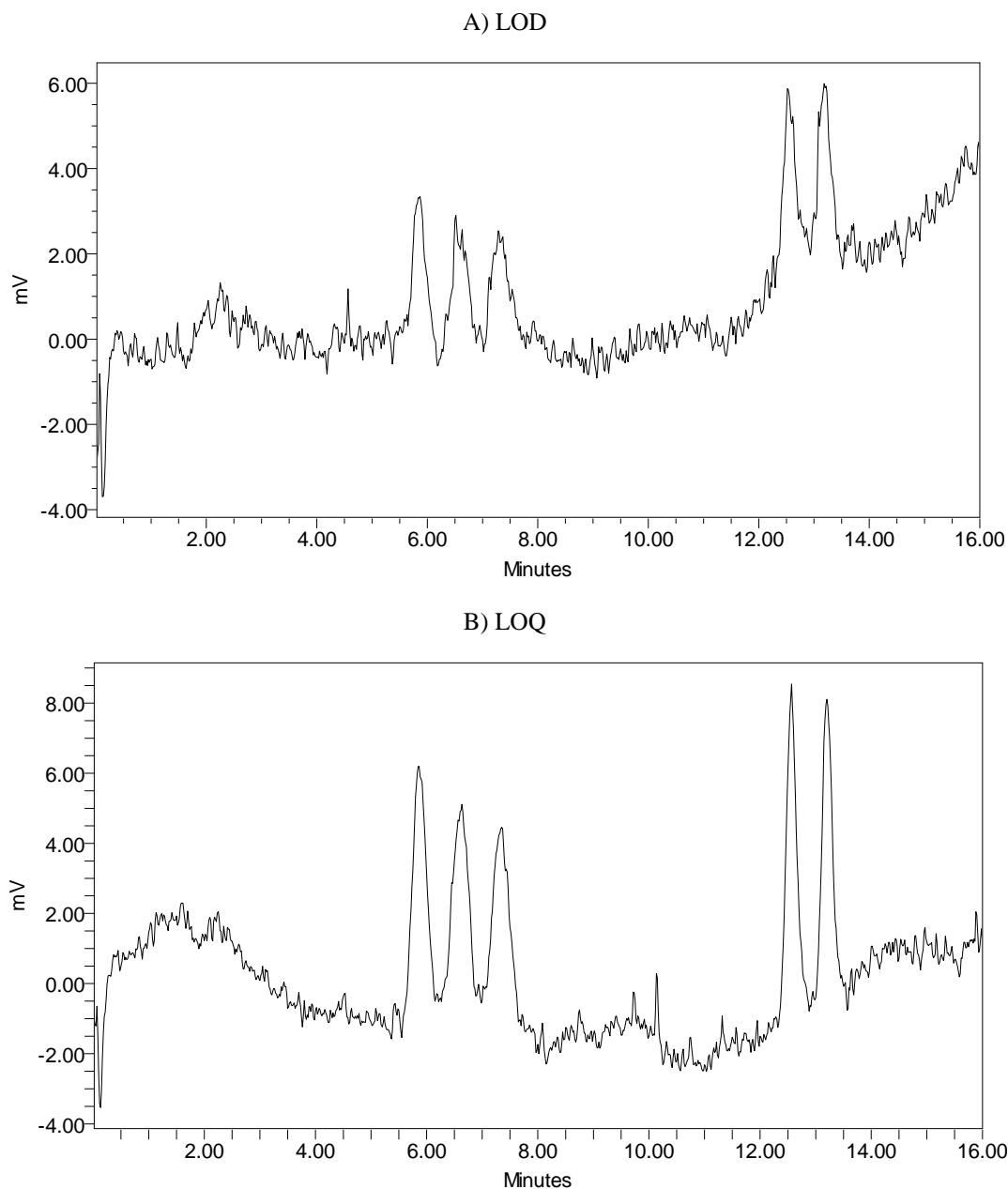
Table 4.12. Recovery of the ginkgolides and BB

Constituent	Mean Spiking level ( $\mu\text{g}/500\text{mg}$ tablet)	Intra-day RSD (% n=3)			Inter-day RSD (%, n=9)	Mean Recovery (%) $\pm$ SD (n=9)	% RE (%)
		Day 1	Day 2	Day 3			
BB	101.0	2.0	0.3	0.8	1.6	101.9 $\pm$ 1.6	+1.9
	403.8	3.6	1.8	0.9	1.1	99.4 $\pm$ 1.1	-0.6
	1245.4	0.5	1.0	1.5	3.0	100.4 $\pm$ 3.0	+0.4
GA	101.0	1.5	0.7	0.1	1.8	101.5 $\pm$ 1.8	+1.5
	403.6	4.8	1.9	0.3	1.3	100.2 $\pm$ 1.3	+0.2
	1245.0	0.6	1.5	1.2	2.3	101.0 $\pm$ 2.3	+1.0
GB	403.0	0.5	2.0	0.9	0.5	101.2 $\pm$ 0.5	+1.2
	806.2	4.2	1.3	0.3	1.2	99.6 $\pm$ 1.2	-0.4
	1612.2	0.9	0.9	1.9	0.9	100.1 $\pm$ 0.9	+0.1
GC	400.8	0.4	2.8	1.6	1.5	97.9 $\pm$ 1.4	-2.1
	801.6	5.4	2.0	1.7	0.5	96.8 $\pm$ 0.5	-3.2
	1603.2	0.7	1.4	1.3	4.0	98.3 $\pm$ 4.0	-1.7
GJ (Day 2)	288.4	-	1.3	-	-	100.1 $\pm$ 0.3 (n=3)	+0.1
	576.8	-	1.6	-	-	99.2 $\pm$ 0.6 (n=3)	-0.8
	1153.6	-	1.6	-	-	98.5 $\pm$ 1.0 (n=3)	-1.5

#### 4.8.7.3 LOD's and LOQ's

The LOD's of the ginkgolides were 31.3 ng (shown in Figure 4.12 A) and LOQ was 62.5 ng (Figure 4.12 B). The LOD's correlated with the range of LOD's given by Li and Fitzoff [189] for the ginkgolides and was better than those proposed by Herring [204], Ganzera *et al.* [203] and Lang *et al.* [205]. No LOQ values were given by the previously mentioned authors [189, 203, 204], and Tang *et al.* [202] reported neither LOD nor LOQ values.

Figure 4.12. The LOD's and LOQ's of the terpene lactones

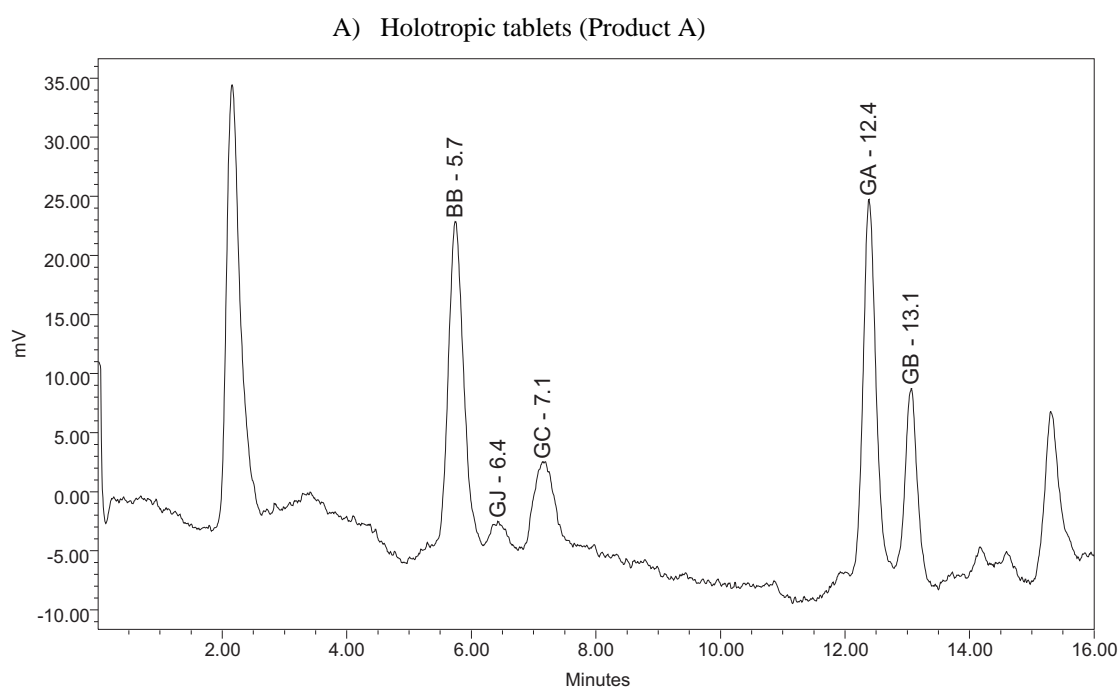


Conditions: Column: Phenomenex Luna ( $5\mu\text{m}$ )  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeOH-water (final gradient method); Flow rate: 350 $\mu\text{l}/\text{min}$ ; Injection volume: 5  $\mu\text{l}$ ; Drift tube temperature: 117.5°C; Gas flow rate: 1.5 L/min.

#### 4.8.7.4 Analysis of commercial *Ginkgo biloba* products

Figures 4.13 A - E show the HPLC-ELSD chromatograms of the five analyzed commercial *Ginkgo biloba* products and Table 4.13 indicates the content of the marker compounds. All five marker compounds were quantifiable within each product although inter-product content differed considerably. From these results it can be concluded that Product D is of superior quality to the other brands and since Product A contained consistently lower amounts of all the markers, this product may be considered of lower quality.

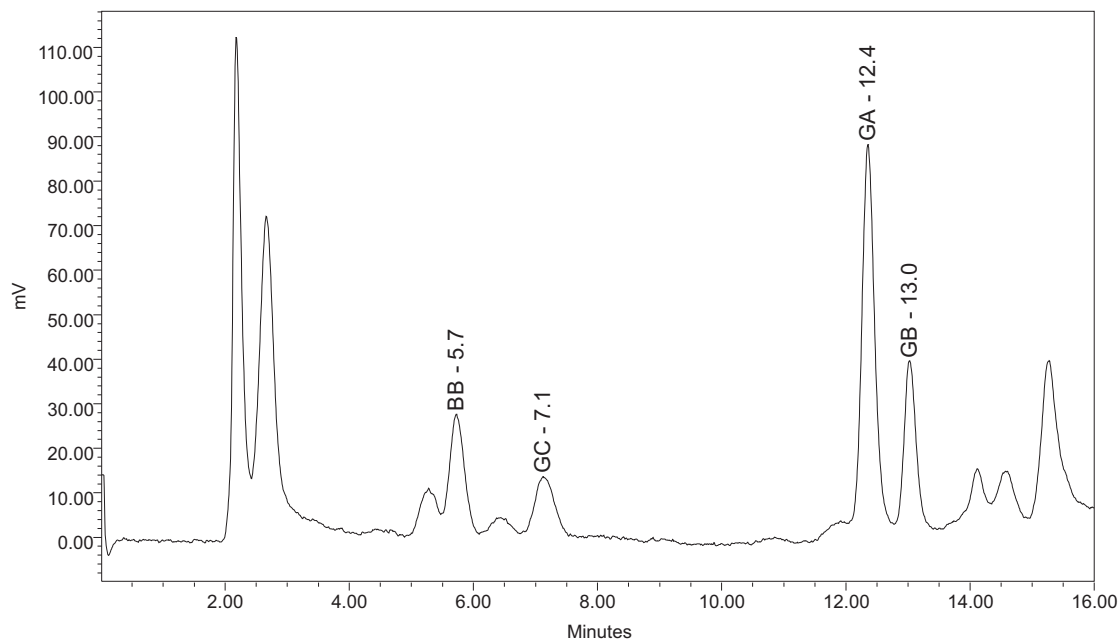
Figure 4.13. HPLC-ELSD chromatograms of the *Ginkgo biloba* commercial products



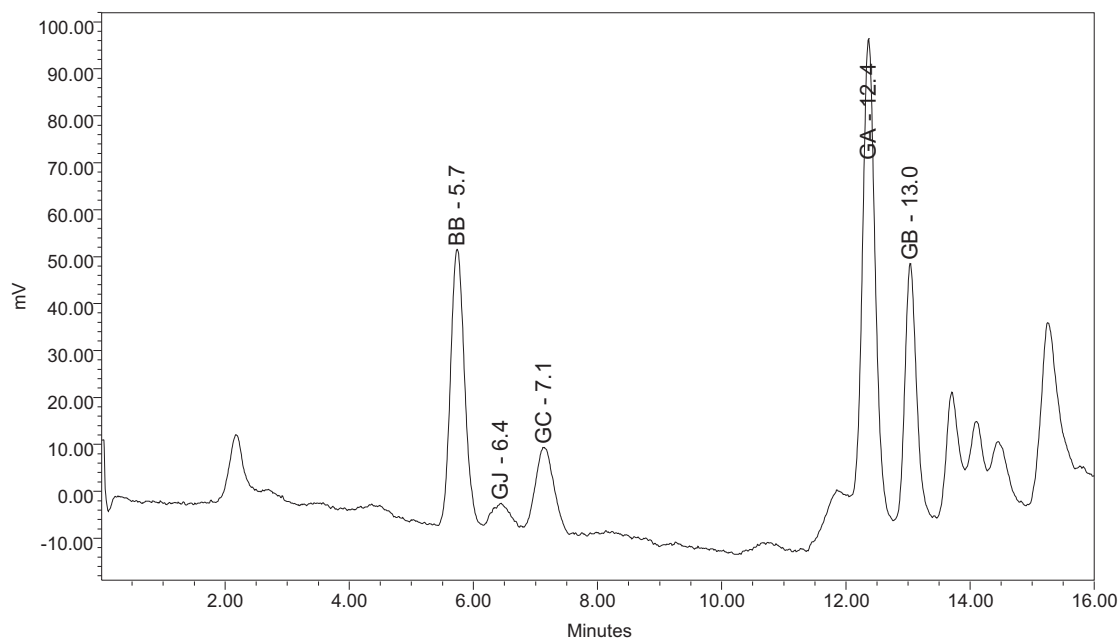
Conditions: Column: Phenomenex Luna ( $5\mu\text{m}$ )  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature:  $45^{\circ}\text{C}$ ; Mobile phase: MeOH-water (final gradient method); Flow rate:  $350\mu\text{l}/\text{min}$ ; Injection volume:  $5\mu\text{l}$ ; Drift tube temperature:  $117.5^{\circ}\text{C}$ ; Gas flow rate:  $1.5\text{ L}/\text{min}$ ; Peak labels: BB= bilobalide, GJ=ginkgolide J, GC=ginkgolide C, GA=ginkgolide A, GB=ginkgolide B.



B) Naturelle tablets (Product B)

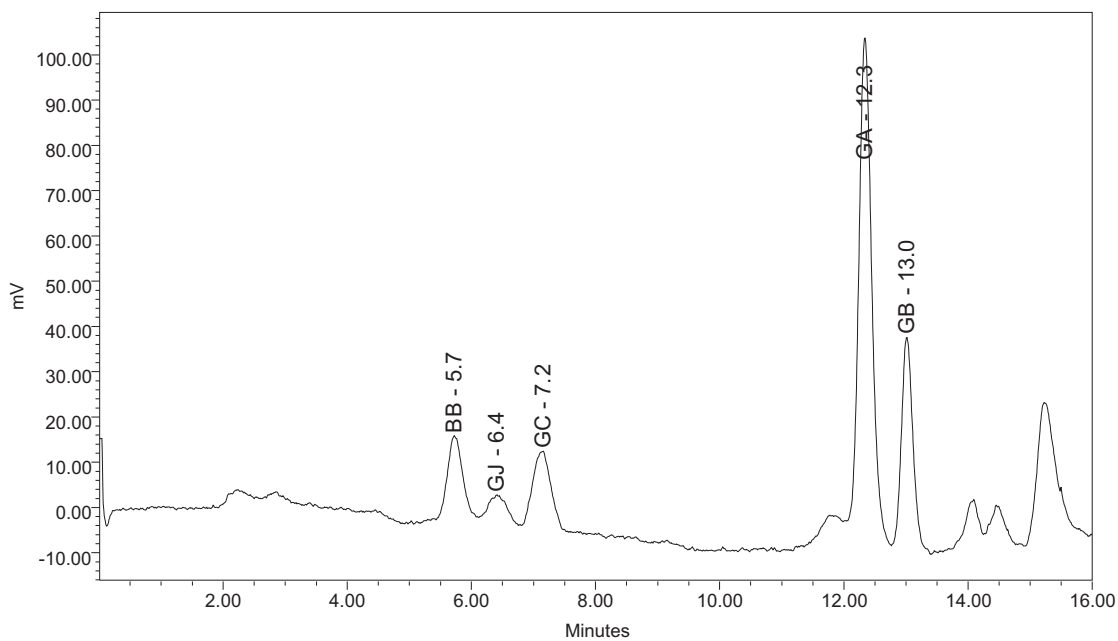


C) Nrf tablets (Product C)

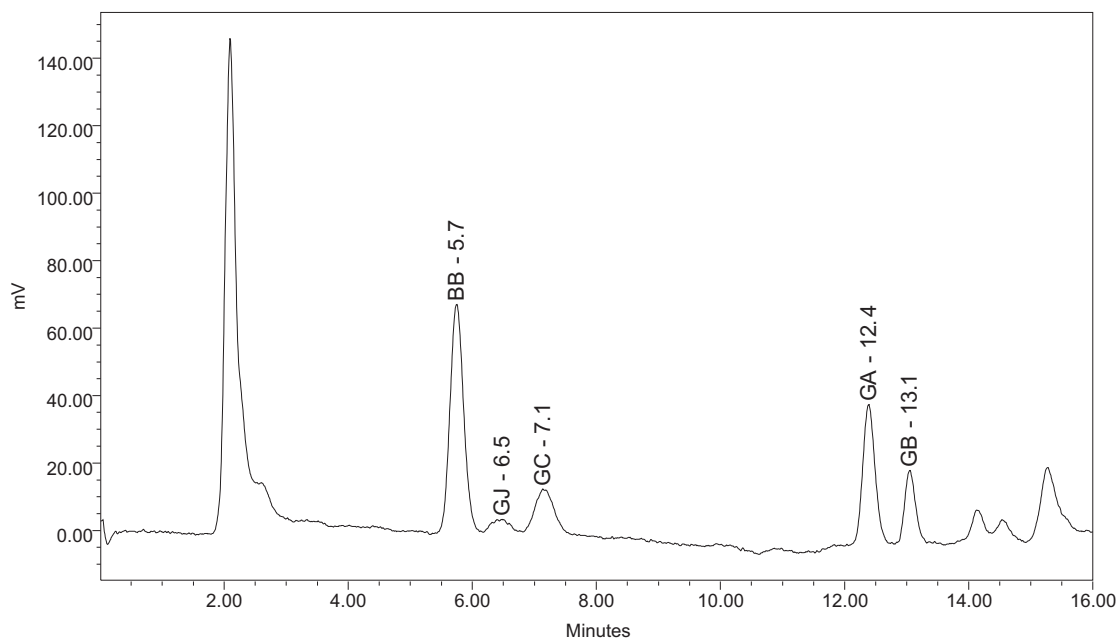


Conditions: Column: Phenomenex Luna ( $5\mu\text{m}$ )  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature:  $45^{\circ}\text{C}$ ; Mobile phase: MeOH-water (final gradient method); Flow rate:  $350\mu\text{l}/\text{min}$ ; Injection volume:  $5\mu\text{l}$ ; Drift tube temperature:  $117.5^{\circ}\text{C}$ ; Gas flow rate:  $1.5\text{ L}/\text{min}$ ; Peak labels: BB= bilobalide, GJ=ginkgolide J, GC=ginkgolide C, GA=ginkgolide A, GB=ginkgolide B.

D) Vital capsules (Product D)



E) Bioharmony capsules (Product E)



Conditions: Column: Phenomenex Luna ( $5\mu\text{m}$ )  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature:  $45^{\circ}\text{C}$ ; Mobile phase: MeOH-water (final gradient method); Flow rate:  $350\mu\text{l}/\text{min}$ ; Injection volume:  $5\mu\text{l}$ ; Drift tube temperature:  $117.5^{\circ}\text{C}$ ; Gas flow rate:  $1.5\text{ L}/\text{min}$ ; Peak labels: BB= bilobalide, GJ=ginkgolide J, GC=ginkgolide C, GA=ginkgolide A, GB=ginkgolide B.

Table 4.13. Terpene content in Ginkgo solid oral dosage forms ( $\mu\text{g}$  per tablet/capsule)

Product (n=3)	BB $\mu\text{g} \pm \text{SD}$	GA $\mu\text{g} \pm \text{SD}$	GB $\mu\text{g} \pm \text{SD}$	GC $\mu\text{g} \pm \text{SD}$	G J $\mu\text{g} \pm \text{SD}$
A (Holotropic)	314.7 $\pm$ 14.8	304.3 $\pm$ 2.5	176.0 $\pm$ 7.0	183.0 $\pm$ 2.7	110.34 $\pm$ 8.1
B (Formule Naturelle)	723.8 $\pm$ 15.2	1432.0 $\pm$ 32.1	809.5 $\pm$ 24.4	657.0 $\pm$ 25.9	298.8 $\pm$ 15.9
C (Nrf)	1358.3 $\pm$ 27.0	1590.8 $\pm$ 25.0	1062.0 $\pm$ 12.1	788.8 $\pm$ 14.5	292.0 $\pm$ 15.6
D (Vital)	1258.5 $\pm$ 30.6	3445.0 $\pm$ 82.6	1910.0 $\pm$ 74.0	1547.5 $\pm$ 68.0	641.5 $\pm$ 35.5
E (Bioharmony)	584.0 $\pm$ 7.0	353.3 $\pm$ 3.3	219.3 $\pm$ 5.3	252.0 $\pm$ 6.4	93.7 $\pm$ 2.1

According to the German Pharmacopoeia, extracts should be standardized to contain between 5.0 and 7.0% terpene lactones, with GA, GB and GC contributing to 2.8 to 3.4% and BB to 2.6 to 3.2% [23]. Table 4.14 shows the calculated BB and trilactone percentages based on the amount of *Ginkgo biloba* extract used per dosage unit as disclosed on the product labels. Since the content specifications are based on the percentage of the marker compounds present in *Ginkgo biloba* extract, Products B and D could not be compared since insufficient label information averted calculation. Product A and E did not meet the German Pharmacopoeia standards while product C marginally exceeded these specifications due to the incorporation of only 50 mg of standardized extract into each tablet.

Table 4.14. Terpene lactone content according to the label claim and German Pharmacopoeia standards

Product	Label claim	BB content (mg)	Total terpene content (mg)	BB (% of extract)	Total terpene (% of extract)	Meets GP <sup>2</sup> standard
A (Holotropic)	GB <sup>1</sup> extract equal to 900 mg	0.31	0.77	0.03	0.09	No
B (Formule Naturelle)	1 mg bilobalide, 1.2 mg ginkgolides	0.72	3.20	-	-	-
C (Nrf)	GB <sup>1</sup> extract 24% 50 mg	1.36	3.73	2.72	7.46	No
D (Vital)	GB <sup>1</sup> extract equivalent to 6000 mg of herb powder	1.26	7.54	-	-	-
E (Bioharmony)	350 mg GB <sup>1</sup>	0.58	0.92	0.16	0.26	No

GB<sup>1</sup>, *Ginkgo biloba*; GP<sup>2</sup>, German Pharmacopoeia; -, insufficient label information for calculation

## 4.9 CONCLUSIONS

The ginkgolides are unique chemical compounds found exclusively in *Ginkgo biloba* preparations and are therefore frequently used as marker compounds for QC purposes. For the analysis of these markers, ELSD detection provides advantages above using UV detection in terms of sensitivity and selectivity and is relatively inexpensive and easily operable compared to GC and MS equipment. While it is obvious that pertinent information relating to method validation is lacking in one or more areas of the RP-HPLC-ELSD methods published to date, a comprehensively validated method was developed for

the determination of GA, GB, GC and GJ as well as BB in commercially available *Ginkgo biloba* tablets and capsules. This method demonstrated the necessary speed, accuracy, precision, sensitivity and repeatability to be considered for routine QC analysis of such products. Furthermore, the inexpensive sample clean-up procedure, the use of methanol and water as the mobile phase and the lower solvent flow rate afforded by the employment of a minibore column offers definite economic advantages for high sample throughput.

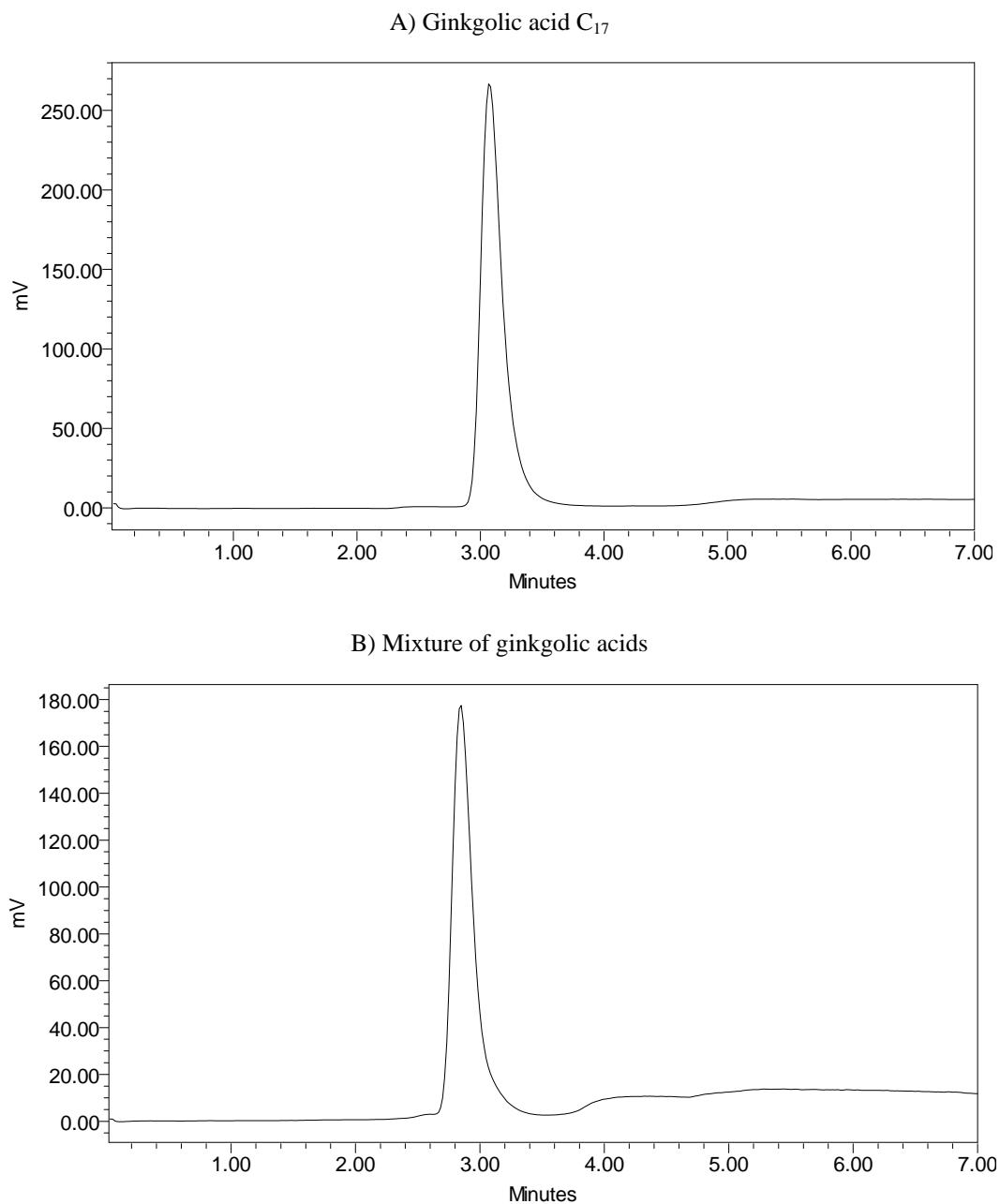
Although quantifiable amounts of all marker compounds were present in the Ginkgo commercial products analyzed, there were major disparities in the actual terpene content. In some cases, the information disclosed on the product labels provided little or no relevant information. None of the commercial products met the German Pharmacopoeia's specifications for terpene trilactone content although one product did exceed the standards. Whether these results are due to inappropriate or false label claims or incongruity between the actual content and the claims is unknown at this point and can only be determined once standardization of individual component content is disclosed on the product labels. For example, at times product content is described as % extract rather than in terms of the mass of individual components (Table 4.14). Furthermore, since clinical trials have used standardized extracts for dosing, the efficacy of these products is dubious and these results have effectively demonstrated that implementation of effective QC criteria is essential in ensuring consistent product QSE.

#### **4.10 HPLC-ELSD ANALYSIS OF THE GINKGOLIC ACIDS**

The methods used for the analysis of the ginkgolic acids are described in section 3.13. Separation of the ginkgolic acids using a simple HPLC method has not yet been achieved and attempts were therefore made to include these compounds in the method described above. Since the ginkgolic acids are lipophilic compounds [53], it was predicted that these compounds would elute after the terpenoids and method development was conducted using the ginkgolic acids alone. The methanolic component in the mobile phase was increased in order to prevent excessively long run times and a mobile phase of 95:5 methanol-water resulted in the C<sub>17</sub> ginkgolic acid (section 3.14.2.4) eluting at 3 minutes (Figure 4.15 A). The peak's identity was verified using the online PDA detector at  $\lambda$  310 nm. The ginkgolic acid mixture (section 3.14.1.5) was then injected using the same methanol-water ratio illustrated in Figure 4.14 B. A decrease in methanol content to less than 85% resulted in a run time in excess of 40 minutes and method development ensued using various ratios of methanol-water with the methanol content ranging between 87% and 95%. However no improvement in resolution of the ginkgolic acids was evident. Thereafter, the methanol content was maintained at 95% and the water fraction was incrementally substituted with 1%, 2% and 4% acetonitrile however, only 1 peak was still observed. The water fraction was then completely replaced with acetonitrile to give ratios of 85:15, 90:10 and 95:10 methanol-acetonitrile, respectively. The best separation was obtained using 95:5 methanol-acetonitrile shown in Figure 4.15. The resolution and peak shapes were extremely poor and it was

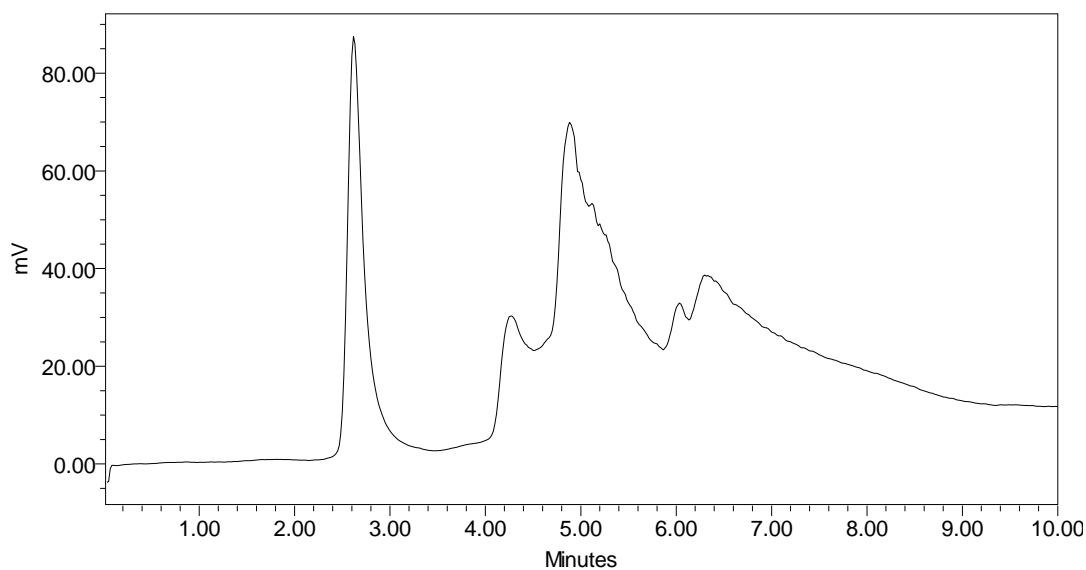
concluded that this method is unsuitable for the detection of the ginkgolic acids in *Ginkgo biloba* extracts and dosage forms.

Figure 4.14. HPLC-ELSD chromatograms using 95:5 methanol-water as mobile phase



Conditions: Column: Phenomenex Luna (5 $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeOH-water (95:5); Flow rate: 300 $\mu$ l/min; Injection volume: 5  $\mu$ l; Drift tube temperature: 110°C; Gas flow rate: 2 L/min.

Figure 4.15. HPLC-ELSD chromatogram of a mixture of ginkgolic acids with mobile phase methanol-acetonitrile (95:5)



Conditions: Column: Phenomenex Luna (5 $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeOH-acetonitrile (95:5); Flow rate: 300 $\mu$ l/min; Injection volume: 5  $\mu$ l; Drift tube temperature: 110°C; Gas flow rate: 2 L/min.

#### 4.11 SIMULTANEOUS DETERMINATION OF FLAVONOLS AND TERPENE TRILACTONES USING HPLC-PDA-ELSD

A method was published in 2002 [189] for the simultaneous determination of flavonol aglycones and terpene trilactones using HPLC with ELSD detection and has been discussed in section 4.2. The same authors also published a paper using HPLC-PDA for the analysis of flavonol aglycones and HPLC-ELSD for the analysis of terpenoids in the same year [188]. In that publication, sample preparation of the flavonols involved hydrolysis of the glycosides to aglycones, RP-HPLC separation and subsequent quantification at  $\lambda$  of 365 nm using PDA detection. The ginkgolides were extracted with methanol followed by a liquid-liquid extraction of the dry extract with hot water and ethyl acetate, evaporation to dryness and reconstitution with methanol. GA, GB, GC, GJ and BB were then determined using HPLC-ELSD. Nine commercial products were separately analyzed for their terpene and “flavonol glycoside” content.

During the period of this current research project and concurrently with the work of Li and Fitzloff [188,189], development of an HPLC-PDA-ELSD method was also underway. In this particular project, attempts were made to develop a method for the simultaneous determination of the flavonol glycosides, rutin and quercitrin in addition to the flavonol aglycones quercetin, kaempferol and isorhamnetin and the terpene trilactones GA, GB, GC and BB. GJ was not available at that time.

## **4.12 METHOD DEVELOPMENT**

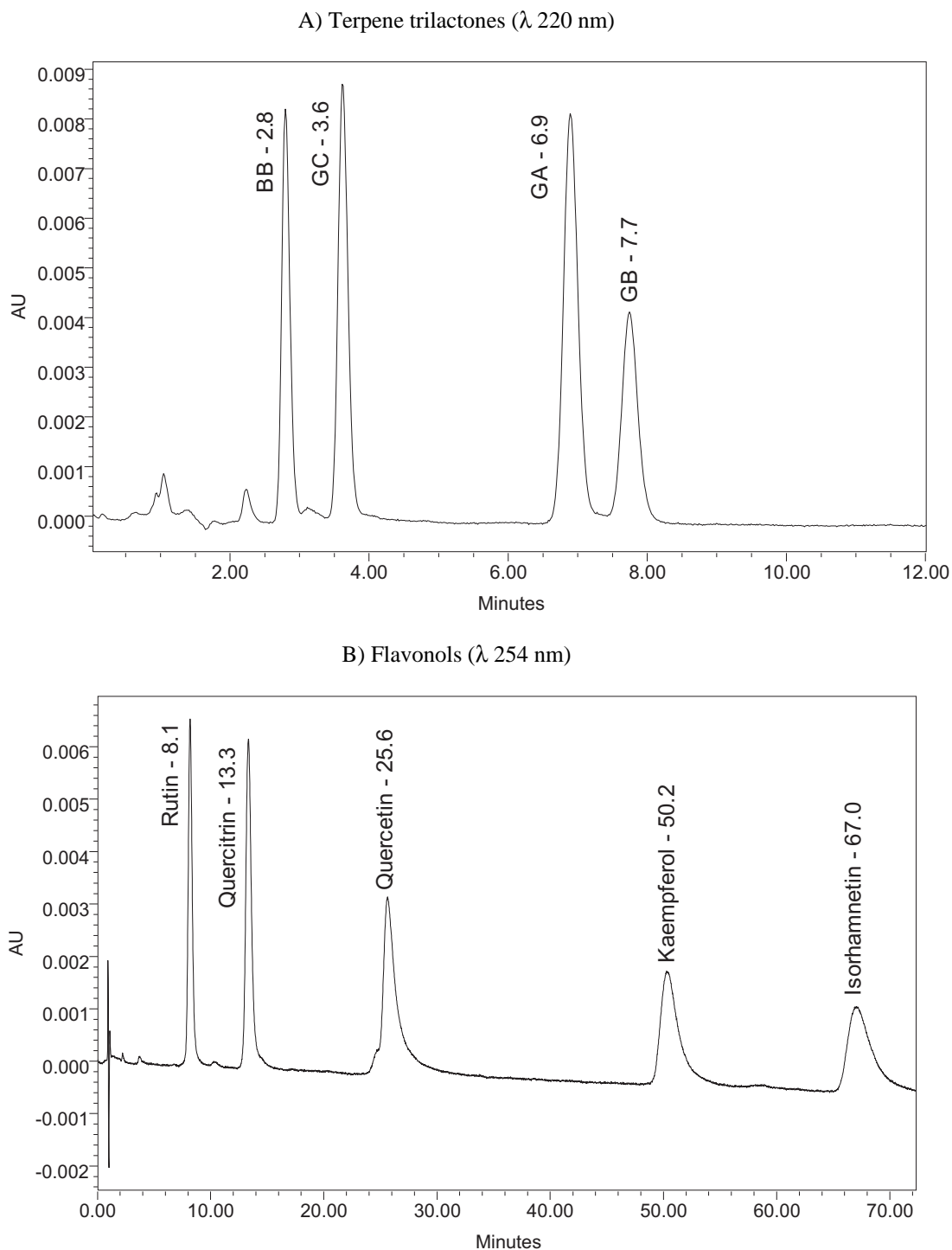
### **4.12.1 Instrumentation and materials**

The instrumentation used and sourcing of materials and reagents has previously been described in sections 4.7.1 and 4.7.2. As previously mentioned, GJ was not available during the early part of this study.

### **4.12.2 Initial conditions and LC optimization**

Since the ginkgolides are strongly retained on the C<sub>18</sub> column when phosphoric or formic acid are included in the mobile phase and the flavonols are readily eluted using a mobile phase of methanol-water without buffer, the latter solvent combination was selected for method development. GA, GB, GC and BB (1 mg/ml solution) were successfully separated within 9 minutes using an isocratic method of methanol-water (30:70) at a flow rate of 500 µl/min and column temperature of 40°C (Figure 4.16 A). A flavonol (100 µg/ml) solution containing the usual marker compounds was then injected under the same conditions and the peaks resolved within 70 minutes. Due to the long analysis time, peak tailing of the flavonol aglycones occurred (Figure 4.16 B).

Figure 4.16. HPLC-PDA chromatograms using an isocratic mixture of methanol-water (30:70)



Conditions: Column: Phenomenex Luna ( $5\mu\text{m}$ )  $C_{18}$  (150 x 2.0 mm I.D.); Column temperature: 40°C; Mobile phase: MeOH-water (30:70); Flow rate: 500 $\mu\text{l}/\text{min}$ ; Injection volume: 5  $\mu\text{l}$ ; Drift tube temperature: 110°C; Gas flow rate: 2 L/min.

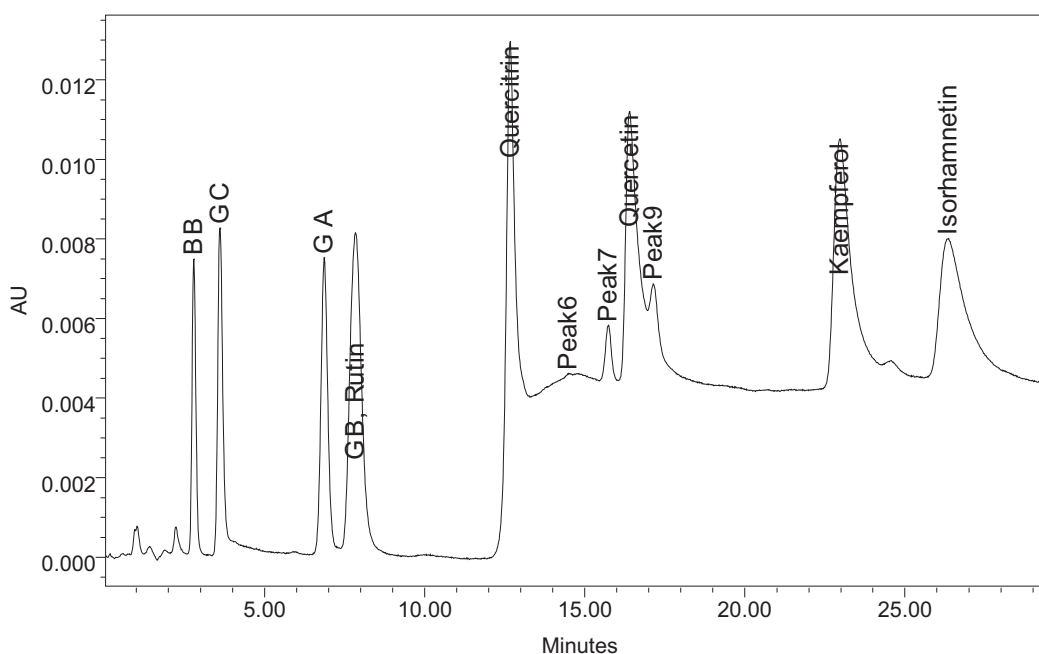


A mixture of the flavonols and terpene trilactones was then prepared and a simple one step gradient was introduced after the elution of the terpenoids according to the method in Table 4.15. The run time was 30 minutes and GB and rutin co-migrated (Figure 4.17).

Table 4.15. Gradient 1 used for the separation of the flavonols and terpenoids

Time (mins)	Flow rate (ml/min)	% Methanol	% Water
0.01	0.50	30.0	70.0
10.00	0.50	30.0	70.0
10.01	0.50	40.0	60.0
60.00	0.50	40.0	60.0

Figure 4.17. HPLC-PDA chromatogram of the flavonols and terpenoids using gradient 1 ( $\lambda$  220 nm)



Conditions: Column: Phenomenex Luna ( $5\mu\text{m}$ )  $C_{18}$  ( $150 \times 2.0$  mm I.D.); Column temperature:  $40^\circ\text{C}$ ; Mobile phase: MeOH-water (gradient: Table 4.15); Flow rate:  $500\mu\text{l}/\text{min}$ ; Sample solvent: methanol-water (40:60); Injection volume:  $5\mu\text{l}$ ; Detection  $\lambda$ : 220 nm; Drift tube temperature:  $110^\circ\text{C}$ ; Gas flow rate: 2 L/min; Peaks 6, 7, and 9 were unknown impurities.

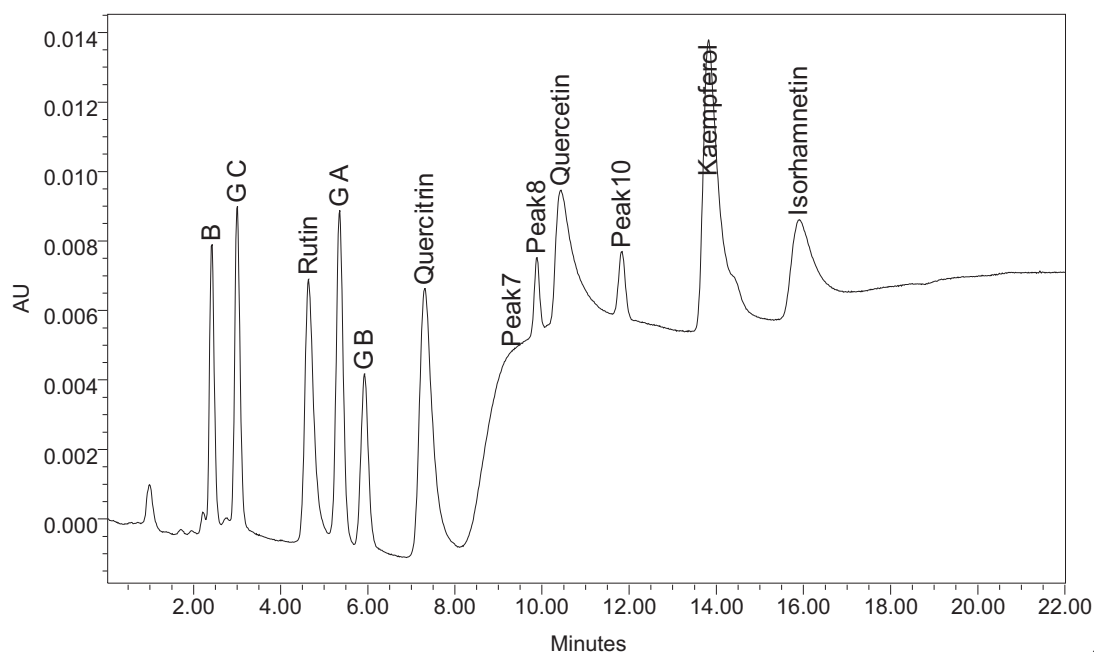
An increase in the methanol content was then introduced at 7 minutes in the gradient program (Table 4.16) in order to facilitate separation between GB and rutin and baseline separation of all the peaks was achieved (Figure 4.18). Peak tailing of the aglycones was still evident however and the one step change in mobile phase caused a drastic baseline shift when monitoring the eluent at  $\lambda$  220 nm due to the UV absorbance of methanol. This shift was not observed at more selective wavelengths of 254 nm and higher. Various attempts were made to minimize this shift by linear changes in the methanol content over longer periods of time from 8 to 10 minutes and 8 to 11 minutes however this shift was still

apparent. Moreover, quercetin eluted where the baseline drifted which would have complicated quantification. Less drastic changes in the methanol component were also attempted by changing the methanol percentage from 30% to 35% instead of 40% at the gradient step and although baseline resolution between all the compounds was still achieved, the lower methanol content had a negative impact on the peak shape of isorhamnetin. The addition of acetonitrile in the gradient, even in small percentages also had a negative impact on the peak shape of the aglycones. Optimal separation was therefore obtained using gradient 2 in Table 4.16.

Table 4.16. Gradient 2 used for the separation of the flavonols and terpene trilactones

Time (mins)	Flow (ml/min)	% Methanol	% Water
0.01	0.50	30.0	70.0
7.00	0.50	30.0	70.0
7.01	0.50	40.0	60.0
60.00	0.50	40.0	60.0

Figure 4.18. HPLC-PDA chromatogram of the flavonols and terpenoids using gradient 2 ( $\lambda$  220 nm)



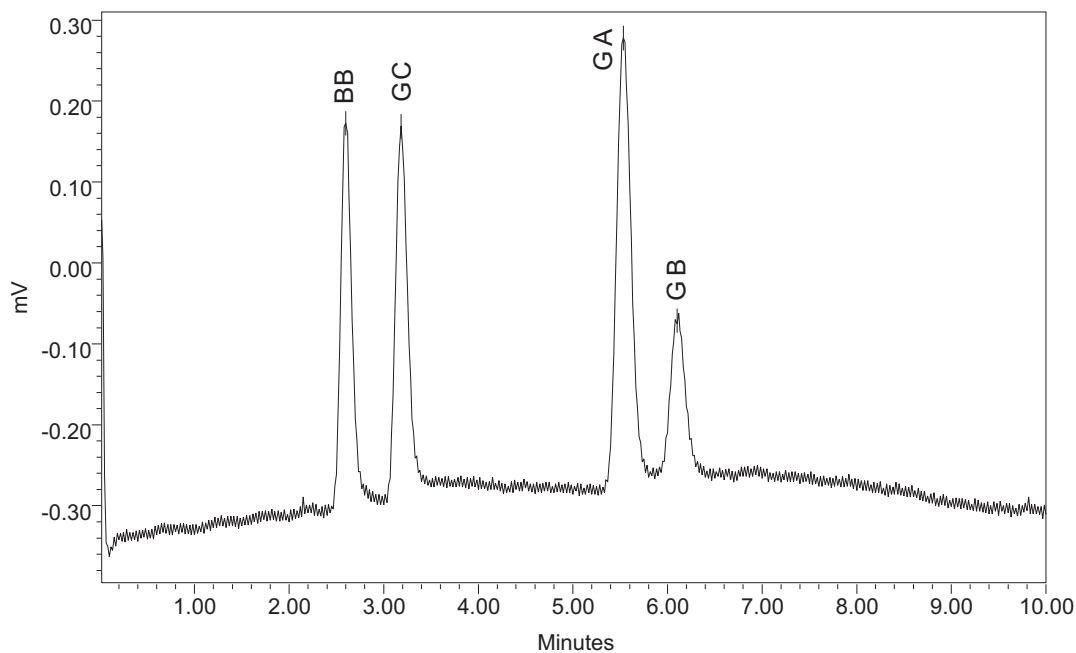
Conditions: Column: Phenomenex Luna ( $5\mu\text{m}$ )  $C_{18}$  ( $150 \times 2.0 \text{ mm I.D.}$ ); Column temperature:  $40^\circ\text{C}$ ; Mobile phase: MeOH-water (gradient 2); Flow rate:  $500\mu\text{l/min}$ ; Sample solvent: methanol-water (40:60); Injection volume:  $5 \mu\text{l}$ ; Detection  $\lambda$ :  $220 \text{ nm}$ ; Drift tube temperature:  $110^\circ\text{C}$ ; Gas flow rate:  $2 \text{ L/min}$ ; Peaks 7, 8 and 10 were unknown impurities.

#### 4.12.3 ELSD optimization

Since this method was preliminary, extensive optimization of the ELSD parameters was not undertaken. The original drift tube temperature was calculated according to the ELSD manual [208] in which the methanol to water ratio used in the mobile phase was used to determine the optimal temperature required to facilitate vaporization. The temperature was calculated to be  $98.5^\circ\text{C}$  for a

methanol-water ratio of 30:70 and flow rate of 500  $\mu\text{l}/\text{min}$ . This value was however increased slightly to 110°C, based on work performed by Thompson *et al.* [209] and greater sensitivity was obtained using this higher temperature. The gas flow remained set at 2.0 L/min throughout all experiments. Under these conditions, suitable sensitivity was obtained to detect the ginkgolides but the flavonols were not detected. Figure 4.19 shows the ELSD response of GA, GB, GC and BB.

Figure 4.19. HPLC-ELSD chromatogram of the terpene trilactones



Conditions: Column: Phenomenex Luna ( $5\mu\text{m}$ )  $C_{18}$  ( $150 \times 2.0 \text{ mm I.D.}$ ); Column temperature: 40°C; Mobile phase: MeOH-water (gradient change at 7 mins); Flow rate: 500 $\mu\text{l}/\text{min}$ ; Sample solvent: methanol-water (40:60); Injection volume: 5  $\mu\text{l}$ ; Detection  $\lambda$ : 220 nm; Drift tube temperature: 110°C; Gas flow rate: 2 L/min.

#### 4.13 DISCUSSION AND CONCLUSION

A preliminary method was attempted for the simultaneous determination of the terpene trilactones GA, GB, GC and BB, the flavonol glycosides, rutin and quercitrin and the flavonol aglycones, quercetin, kaempferol and isorhamnetin. Baseline resolution was obtained within 20 minutes which is faster than the method described by Li and Fitzloff [189] which took 35 minutes however GJ was not included in this method due to its unavailability at that time.

This method was developed under the premise that the flavonols could be detected and quantified using the PDA detector with the eluent subsequently directed to the ELSD for detection of the terpene trilactones. In fact, it was implied by Li and Fitzloff [188, 189] that greater sensitivity was obtained using UV than ELSD detection for the flavonols. In this way, optimal sensitivity for both chemical classes could be facilitated within a single run.

Detection of the flavonols using ELSD is clearly possible since the LOD levels obtained by Li and Fitzloff [189] were in the order of 20-30 ng under their conditions of operation of the ELSD. It is thus highly likely that optimization of the ELSD parameters or the use of higher concentrations of flavonol marker compounds would probably have resulted in detection of the flavonols.

However, based on subsequent work undertaken where the flavonols were successfully quantified using LC-MS-MS (Chapter 5), this ELSD method for the simultaneous analysis of flavonols and terpene trilactones was not pursued further but a rapid HPLC-ELSD method was developed for the exclusive quantification of the terpenoids only in *Ginkgo biloba* solid oral dosage forms and is described in sections 4.6 - 4.9 of this thesis.

## CHAPTER 5

### LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY OF FLAVONOLS

#### 5.1 INTRODUCTION

To date, approximately 4000 flavonoids have been identified in the plant kingdom [210], classified structurally by the presence of a chromane skeleton with a phenyl group on either position C2 or C3 of ring B (Figure 2.3). Although the flavonoids are produced by all vascular plants for protection against pathogens and herbivores [211], flavonoid composition is not identical in all plant species and various combinations of the polyphenols may facilitate species differentiation [212].

The flavonoids have strong chromophores resulting in RP-HPLC with UV detection being the most common method of analysis [213]. Although this method is economically viable, a major deterrent for its successful application is the lack of selectivity. Numerous flavonoids with similar retention times and UV spectra have been identified in crude plant extracts and unequivocal identification of the target analyte using UV or PDA detection alone cannot be guaranteed.

On the other hand, LC-MS facilitates component (peak) confirmation by mass detection as well as collision-induced dissociation (CID) from which intricate structural details such as the glycosylation position can be deduced [214]. Baseline resolution of natural compounds in complex mixtures is possible but often at the cost of long analysis times using complex gradient programs with UV/PDA detection. However, the use of SIM, SRM and MRM modes when using MS detection facilitates the detection and accurate quantification of co-eluting or unresolved peaks [197]. According to a recent review article published by Molnár-Perl and Füzfaí [180], LC-MS is the preferred method for qualitative analysis of flavonoids. Moreover, *C*-glycosidic flavonoids, where the sugar moiety is attached to the 6-*C* or 8-*C* carbon (Figure 2.3), are resistant to conventional hydrolysis protocols and tandem MS facilitates accurate structure elucidation [215].

Ionization techniques predominately applied for the analysis of polyphenolic compounds include ion-spray detection techniques such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and thermospray and then ion-desorption techniques which include fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI) and plasma desorption (PD). Of these, the most commonly used methods are ESI and APCI which are both atmospheric pressure ionization (API) techniques and can be used in either positive or negative ion modes [213, 214, 216].

LC-ESI involves nebulization of the LC effluent to form an aerosol by the application of an electric field on the metallic capillary of the ESI probe and depending on the voltage polarity, the aerosol droplets are then either positively or negatively charged. This nebulization process is aided by a supply of nitrogen which encapsulates the capillary. Desolvation then follows which results in an increase in the charge density on the surface of the droplets and as the droplets reduce further in size, repulsion forces eventually cause a coulombic explosion and the suspended ions are then transported into the vacuum of the mass analyzer [217].

The APCI interface, on the other hand, consists of a glass capillary surrounded by a high velocity nebulizing gas as well as a heater block which facilitates the rapid formation of a fine mist of vapor as the LC eluent passes through the capillary. Near the capillary tip, a corona discharge needle causes ionization and reacts chemically with the vaporized analyte molecules before transportation of these ions to the mass analyzer. Although both ESI and APCI techniques are classified as soft ionization methods, thermally labile compounds are generally not suitable for APCI detection [217].

## 5.2 METHODS USED FOR THE ANALYSIS OF FLAVONOLS USING LC-MS

A number of papers have been published in the past 5 years using LC-MS for the analysis of natural plant extracts containing 1 or more of the selected flavonol markers present in *Ginkgo biloba* leaf extracts, examples of which are shown in Table 5.1 [211, 218-224]. In addition, several papers have been published on the analysis of the flavonoids present in *Ginkgo biloba* leaf extracts [169,170, 214] and 1 paper has been published using GC-MS for the determination of the flavonol aglycones in human plasma after ingestion of a *Ginkgo biloba* dosage form [225].

Mauri and Pietta [214] fingerprinted a crude *Ginkgo biloba* leaf extract by means of direct infusion into an electrospray interface in positive ion mode which facilitated the simultaneous identification of GA, GB, GC and GJ as well as 6 flavonol glycosides of quercetin, kaempferol and isorhamnetin. The sample was dissolved in 50% methanol-water. Since the paper concentrated on the rapid ESI fingerprinting of at least 8 different medicinal plants, little experimental information and method development was divulged. The authors however managed to successfully demonstrate the diverse application of ESI-MS for the QC of medicinal plants.

Recently, 2 papers have focused on the analysis of intact flavonol glycosides [169, 170] as opposed to the more usual process of sample hydrolysis with conversion of the glycosides to their corresponding aglycones quercetin, kaempferol and isorhamnetin. One paper was based on the work described in this chapter [169] and the other paper was published less than a month later by Liu *et al.* [170] who developed a direct infusion nanoelectrospray ionization tandem quadrupole time-of-flight method (nanoES-MS) for the simultaneous qualitative detection of the terpene lactones GA, GB, GC and BB, the flavonols rutin, quercetin, kaempferol and isorhamnetin and also quantified ginkgolic acids “I” and “II” using the same method in negative ion mode. To our knowledge, this is the only method published

which is capable of analyzing some positive and negative markers in a single analysis. The results showed that 4 of the 14 analyzed marketed products were potentially fortified with rutin, quercetin, kaempferol or isorhamnetin as deduced by the abnormal ratios of glycosides to aglycones and also by the fact that the aglycones are known to be present in small quantities of 0.2 - 0.4% w/w in Ginkgo leaves [57]. These aglycones as well as rutin are commercially available in their pure form. The authors commented that this adulteration would not be detected if QC involved the usual method of glycoside hydrolysis and in addition, tedious sample hydrolysis procedures were circumvented. Moreover, two products had toxic levels of ginkgolic acids, one of which contained more than 60 000 times the upper limit of 5 µg/g material.

Although this direct infusion method has contributed significantly in terms of emphasizing the need for more effective QC criteria and provides a potentially proficient method for the fingerprinting of *Ginkgo biloba* extracts, it also had a few shortcomings, especially the lack of a chromatographic separation step. References concerning the flavonol and terpene content in the commercial *Ginkgo biloba* products were made without construction of calibration curves and although the method showed a measure of repeatability by the repeated analysis of a standardized Ginkgo extract over 18 days, it was not validated. Of the 6 ginkgolic acids identified in Ginkgo leaf extracts, only 2 ginkgolic acids were quantified, C<sub>13:0</sub> and C<sub>15:0</sub> while the concentration of C<sub>17:1</sub> was estimated using C<sub>13:0</sub> as the reference standard. According to van Beek [53], the main ginkgolic acids are C<sub>15:1</sub> and C<sub>17:1</sub>. The effect of various strengths of aqueous methanol (20 - 100% methanol) on the extraction of the Ginkgo compounds was also investigated by observation of the peak intensity and 100% methanol was shown to extract the highest levels of ginkgolic acids (which was predictable since water has a negative effect on extraction efficiency [53]). However, as illustrated in section 3.15.4, hexane is a superior extraction solvent compared to methanol which is an important consideration when these compounds are required to be quantified. The ginkgolic acids are considered as negative markers and the focus on their quantification may be considered as misguided since the toxicity of the ginkgolic acids have not yet been adequately proven. Hence, quantification of the purported bioactive components should therefore take precedence. Moreover, for quantitative determinations, it is advisable to include an LC separation step in order to avoid matrix effects with subsequent ion suppression [217]. Finally, although the presence of rutin, quercetin, kaempferol and isorhamnetin could be confirmed by spiking the extracts with reference standards, a number of other flavonols were also identified without explaining how these flavonol glycosides were structurally defined.

Table 5.1. LC-MS methods used for the analysis of flavonol markers in plant extracts

Matrix analyzed	Analyzed flavonols present in <i>Ginkgo biloba</i> leaf extracts	Mobile phase	API mode	Type of MS	Reference
St. John's Wort capsules	rutin, quercitrin, quercetin,	Gradient: A) water-0.5% TFA B) 0.5% TFA in MeOH-MeCN (13:7)	ESI (+)	IT	219
Onion, elderflower and lime blossom, St. John's Wort	quercetin, quercitrin, kaempferol	Gradient: water-MeCN-AA	ESI (+)	IT	220
Pulp mill effluent	quercetin, kaempferol	Gradient: A) water-0.5% AA B) MeOH	ESI ( $\pm$ )	QqQ	211
<i>Theobroma cacao</i>	rutin, quercitrin, quercetin, kaempferol, isorhamnetin	Gradient: A) water-0.1% FA B) MeCN-0.1% FA	I Spray (-)	QqQ	218
Artichoke waste	rutin, quercitrin, quercetin	Gradient: A) water-0.1% FA B) MeCN-0.1% FA	I Spray (-)	QqQ	221
Apple peel	rutin, quercitrin, quercetin	Gradient: A) MeOH B) water-0.5% AA	APCI (+)	QqQ	222
Strawberry fruit powder and fruit extract	rutin	Gradient: A) MeCN B) water-0.1% FA	ESI ( $\pm$ )	IT	223
<i>Adinandra nitidra</i> leaf extract	quercitrin	Gradient: A) water-0.1% FA B) MeCN-0.1% FA	ESI (-)	Q	224

MeOH, methanol; MeCN, acetonitrile; TFA, tetrahydrofuran; FA, formic acid; AA, acetic acid; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; IS, ion spray; IT, ion trap; TOF, time of flight; Q, single quadrupole; QqQ, triple quadrupole; (-), negative ion mode; (+), positive ion mode; ( $\pm$ ), positive and negative ion mode

### 5.3 LC-MS METHOD DEVELOPMENT OF FLAVONOLS

#### 5.3.1 Instrumentation

HPLC analyses were carried out using a SpectraSYSTEM P2000 pump equipped with an AS 1000 autosampler and UV 1000 variable-wavelength UV detector (all supplied by Thermo Separation Products, Riviera Beach, FL, USA). Separation was achieved on a Phenomenex Luna® C<sub>18</sub> 150 x 2.0 mm I.D. reversed-phase minibore column packed with 5  $\mu$ m ODS-2. Negative ion electrospray ionization mass spectrometry (ESI-MS) was performed using a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA).



### 5.3.2 Materials and reagents

Formic acid (analytical grade) was purchased from Merck (Darmstadt, Germany) and acetonitrile (HPLC grade) was obtained from BDH chemicals (Poole, UK). Rutin (95%) and quercetin (95%) were purchased from Sigma (St. Louis, MO, USA). Quercitrin (99%) was supplied by Phytolab (Hamburg, Germany) and kaempferol (95%) and isorhamnetin (90%) from Indofine Chemical Company (New Jersey, USA). The purity of the flavonol reference standards were assumed as provided by the certificates of analysis from suppliers. Water was purified by reverse osmosis in a Milli-Q system (Millipore, Bedford, MA, USA).

### 5.3.3 Preparation of reference standards

Due to the mobile phase composition of 0.3% formic acid-acetonitrile (75:25), samples were originally prepared in the same solvent, however inter-day repeatability was poor due to precipitation of the standards in solution, particularly the flavonol aglycones. Samples were subsequently prepared in methanol-water (50:50), which also was used as the sample solvent in the qualitative analysis performed by Mauri and Pietta [214].

### 5.3.4 LC optimization

LC conditions were originally developed independently using UV detection only. An isocratic method using methanol-0.5% phosphoric acid (50:50) published on the Institute for Nutraceutical Advancement's website [226] for the analysis of hydrolyzed Ginkgo extracts was further developed for the analysis of the 2 flavonol glycosides, rutin and quercitrin as well as the flavonol aglycones, quercetin, kaempferol and isorhamnetin. The original run time was 30 minutes at 35°C using a flow rate of 1.2 to 1.5 ml/min and a 250 x 4.6 mm I.D. Phenomenex Prodigy® ODS-3, 5 µm column.

A Phenomenex Luna® C<sub>18</sub> 150 x 2.0 mm I.D. minibore column was used in these experiments which demanded a relatively low flow rate, hence 500 µl/min was used with a column temperature of 45°C. A reference solution containing 10 µg/ml of the flavonols was used for method development and 5 µl was injected onto the column. The methanol component was replaced with acetonitrile to improve resolution and this fraction was further decreased to 25% to aid separation between the flavonol glycosides which eluted close to the void. Since phosphoric acid is too viscous for MS use, it was initially replaced with HPLC grade water. However, the peak shape was poor and formic acid was added with increasing percentages and optimized to a final concentration of 0.3%.

This choice of mobile phase composition was partially substantiated in a study performed by de Rijke *et al.* [213] where the use of various buffer-modifier combinations using acetonitrile, methanol, 10 mM ammonium formate and 10 mM ammonium acetate on the UV and MS responses of 15 known flavonoids was tested. Two of these flavonoids were common to those found in *Ginkgo biloba* leaf

extracts, namely rutin and kaempferol. Since higher responses are normally desired to maximize method sensitivity, it was generally concluded that for LC-UV, the methanol-formate combination gave the best overall results, however acetonitrile-formate was also capable of generating greater peak areas for some flavonoids. More specifically, rutin showed a double increase in peak height when acetonitrile-formate was used compared to methanol-formate while kaempferol exhibited a lower response with the acetonitrile-formate combination. Since acetonitrile is a powerful organic modifier which aids resolution, the peaks were sharper and run times were substantially shorter when it was employed as the organic modifier. The effect of the solvent combinations on the resolution of the flavonoids was however not discussed.

### 5.3.5 API source selection

According to Sánchez-Rabaneda *et al.* [218], soft ionization techniques are well suited for the detection of polar, non-volatile and relatively thermostable compounds such as the flavonoids. A soft ionization technique is essential to ensure that degradation of the glycosides to aglycones does not occur [214]. ESI is generally suitable for the detection of highly polar analytes which are ionized in solution while APCI is used for non-polar, non-ionic compounds. However this distinction is not always apparent [216] and selection of the most suitable technique is further complicated when analyzing complex mixtures containing both polar analytes such as glycosides and relatively non-polar compounds, for example the flavonol aglycones [213].

A comparative study of ESI versus APCI was investigated in both positive and negative modes by de Rijke *et al.* [213] using 15 flavonoids which included rutin and kaempferol. Although APCI was reported to give better results than ESI for the glycosides, the responses of both rutin and kaempferol were approximately the same with either source using an ion-trap MS instrument. The aglycone responses were comparable using either APCI or ESI in the negative mode. The S/N ratio of the peaks also improved with both ionization techniques when the negative ion mode was used. From Table 5.1 it can be seen that the majority of the reported analyses were performed using an ESI interface.

Since ESI is the softer technique and preservation of the glycosidic flavonols was crucial to this study, it was selected as the most appropriate API source and a comparative APCI study was not conducted.

### 5.3.6 MS optimization and tuning

The MS parameters for each flavonol were optimized by direct infusion of a 25 µg/ml mixture of the relevant flavonols at 3 µl/min into the source. The capillary temperature was maintained at 240°C and the sheath and auxiliary gas flow parameters were 80 and 20 arbitrary units, respectively. The spray voltage remained constant for all compounds at 4.5 kV. Both positive and negative ion modes were attempted, however the latter mode gave a more stable ion signal for all of the selected flavonol

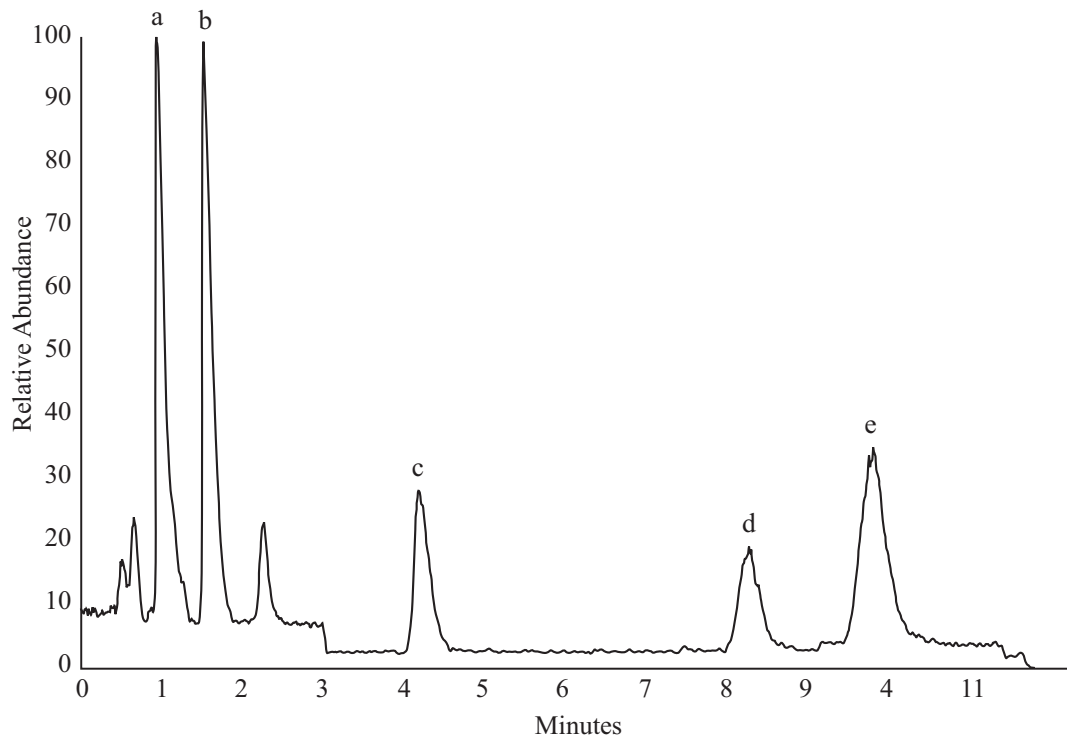
markers. The deprotonated molecular ions for rutin, quercitrin, quercetin, kaempferol and isorhamnetin were observed at  $m/z$  609, 447, 301, 285 and 315, respectively.

In a recently published review by Tsao and Deng [216], they reported that ESI in the negative ion mode is generally the first choice for the analysis of the flavonoids. This result is further supported by literature where it has consistently been shown that the best sensitivity for rutin is obtained in the negative ion mode when applying electrospray tandem mass spectrometry (ES-MS-MS) [227]. While the positive ion mode is considered as the most informative regarding structure elucidation, the negative ion mode demonstrates higher sensitivity [210, 227, 228]. This was also confirmed by Wolfender *et al.* [228] who found that the positive ion mode provided more structural information and was easier to interpret than the negative ion mode. Fabre *et al.* [210] used the negative ion mode due to its augmented sensitivity and specificity for analysis of some flavonoids commonly found in plant extracts. For the purposes of this study, maximum sensitivity was required for the detection of the flavonol aglycones which normally occur in very small quantities in Ginkgo extracts.

### 5.3.7 LC-MS in full scan mode

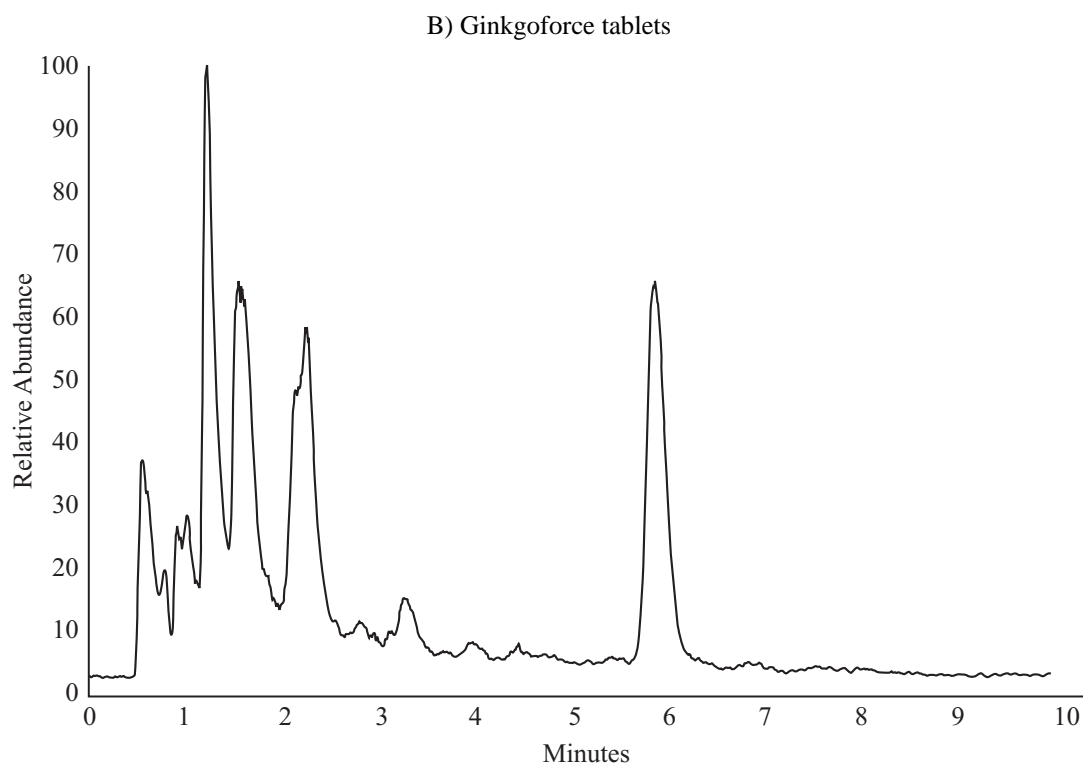
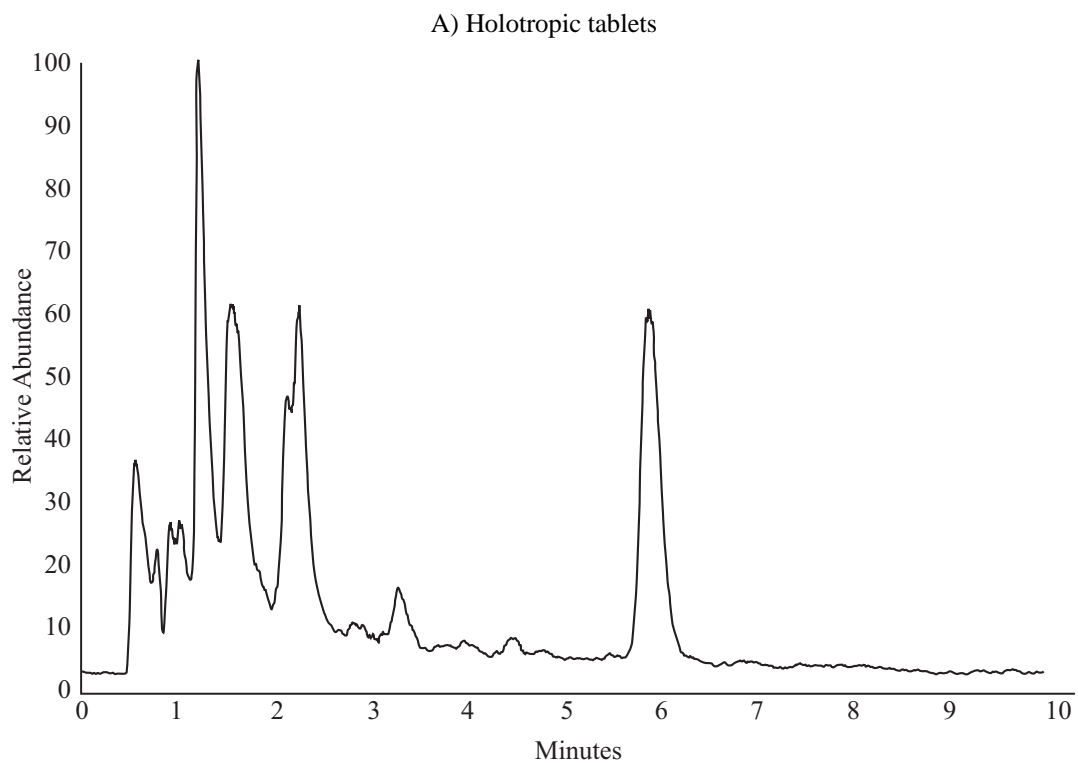
Preliminary studies were performed in another laboratory equipped with the same MS instrument models described in section 5.3.1. LC separation was achieved isocratically at 45°C on a 150 x 2.0 mm I.D. Luna C<sub>18</sub> reversed-phase minibore column packed with 5 µm ODS-2. The mobile phase consisted of 0.3% formic acid-acetonitrile (75:25) which was set at a flow rate of 700 µl/min in order to maintain a stable pump flow action and the injection volume was 20 µl. MS conditions were identical to those in section 5.3.6. Individual LC-MS full scans of the reference standards were conducted using the optimized conditions for each compound and the retention times for rutin, quercitrin, quercetin, kaempferol and isorhamnetin were 1.03 minutes, 1.61 minutes, 4.23 minutes, 8.33 minutes and 9.90 minutes, respectively (Figure 5.1). LC-MS full scans of 4 *Ginkgo biloba* commercial products, Holotropic (batch number: 0312), Ginkgoforce (batch number: 109022E), Naturelle (batch number: 69936) and Vital (batch number: 1108A) were fingerprinted as illustrated in Figure 5.2 using the optimised MS conditions. The fingerprints had remarkably similar profiles while interfering peaks prevented identification of the relevant marker compounds. LC-MS-MS was therefore shown to be the method of choice for further analyses.

Figure 5.1. Full scan mass chromatogram of the flavonol marker compounds



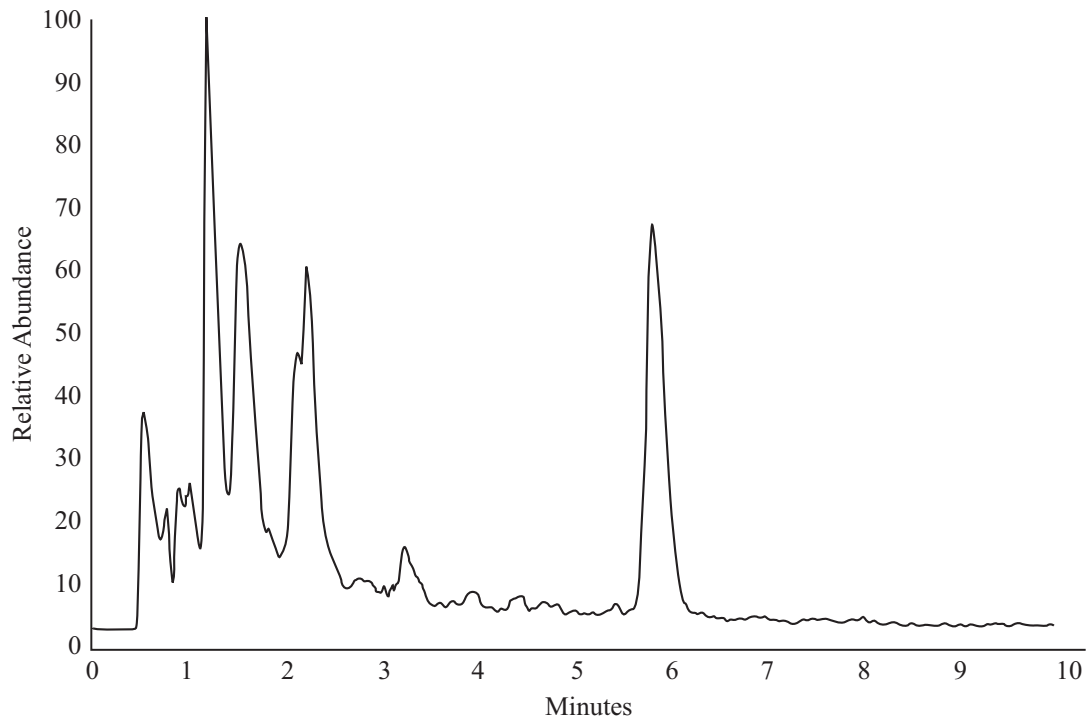
Conditions: Column: Phenomenex Luna (5  $\mu$ m)  $C_{18}$  (150 x 2.0 mm I.D.); Column temperature: 45  $^{\circ}$ C; Mobile phase: MeCN-0.3% formic acid (75:25); Flow rate: 700  $\mu$ l/min; Injection volume: 5  $\mu$ l; Peak labels: a=rutin, b-quercitrin, c=quercetin, d=kaempferol, e=isorhamnetin.

Figure 5.2. Full scan mass chromatograms of 4 commercial products using LC-MS

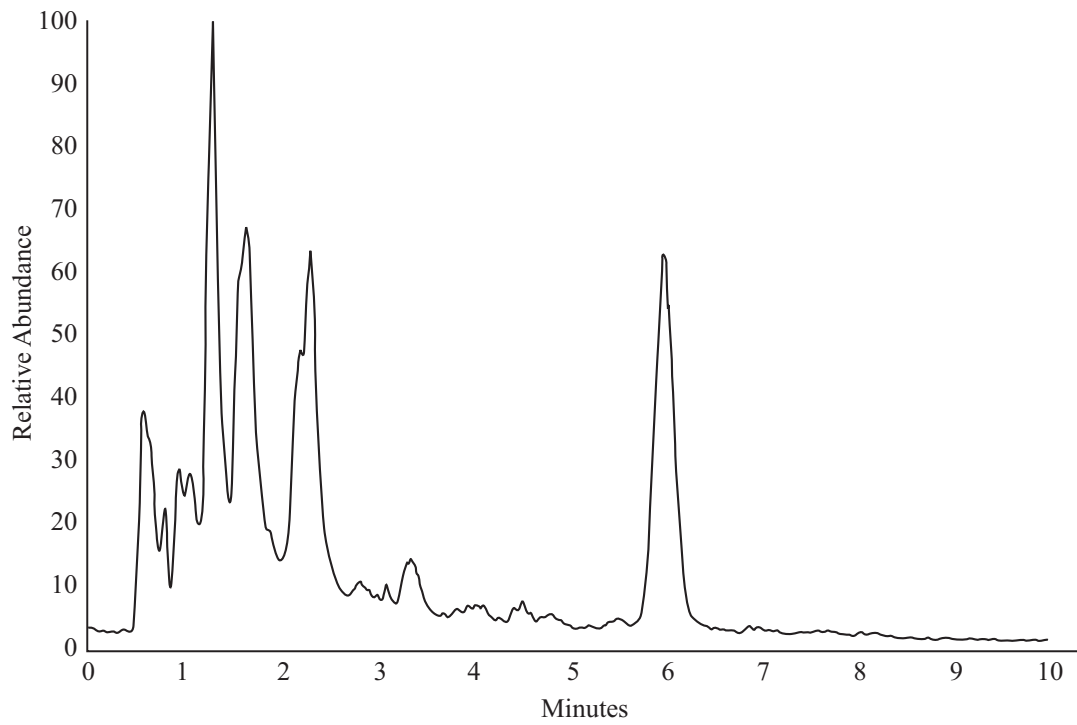


Conditions: Column: Phenomenex Luna (5  $\mu\text{m}$ )  $\text{C}_{18}$  (150 x 2.0 mm I.D.); Column temperature: 45  $^{\circ}\text{C}$ ; Mobile phase: MeCN-0.3% formic acid (75:25); Flow rate: 700  $\mu\text{l}/\text{min}$ ; Injection volume: 5  $\mu\text{l}$ .

C) Naturelle tablets



D) Vital capsules



Conditions: Column: Phenomenex Luna (5  $\mu\text{m}$ )  $\text{C}_{18}$  (150 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic acid (75:25); Flow rate: 700 $\mu\text{l}/\text{min}$ ; Injection volume: 5  $\mu\text{l}$ .

### 5.3.8 Collision induced dissociation (CID)

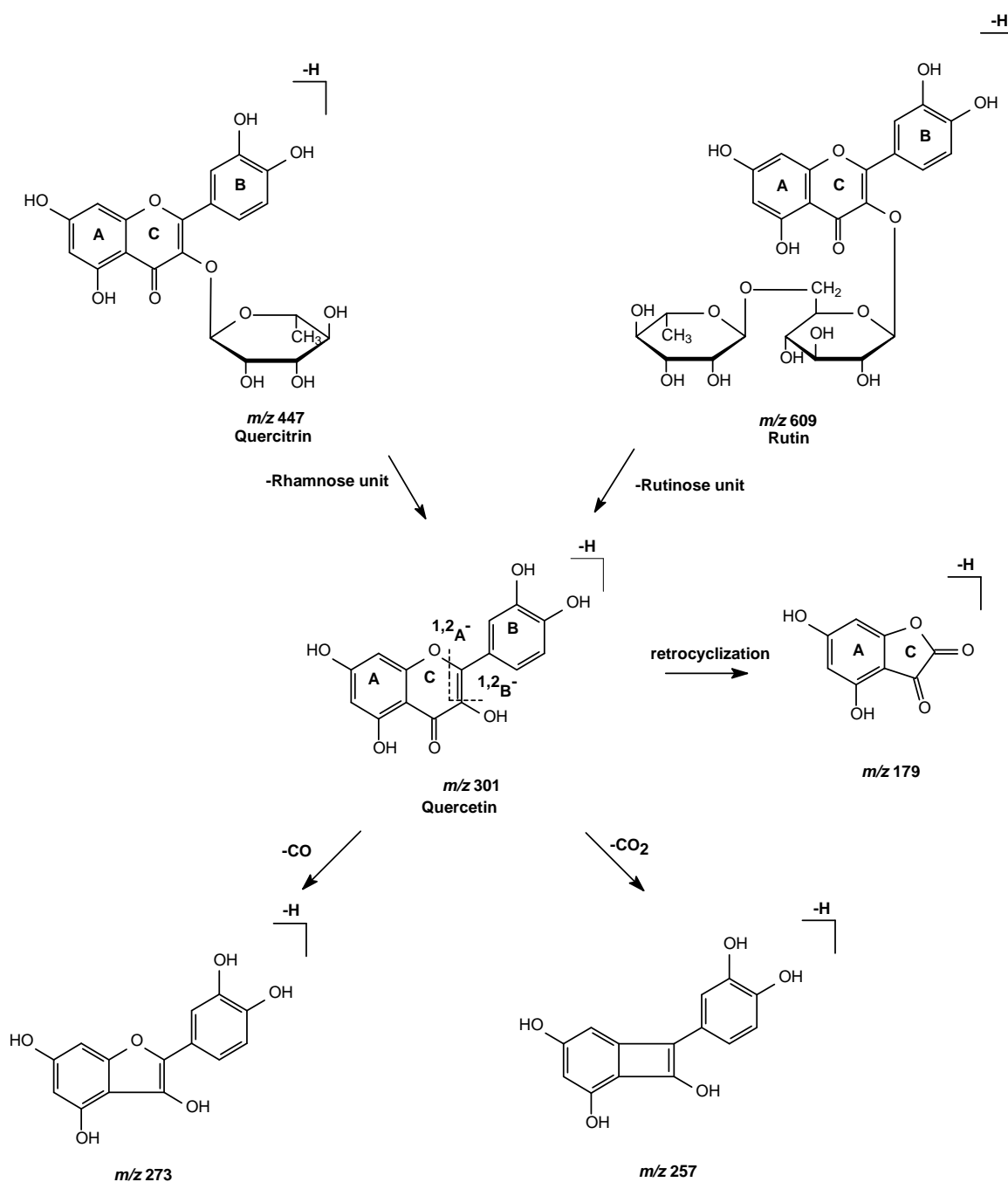
CID was undertaken in the MS-MS mode to yield diagnostic product ion mass spectra, which were characteristic of the structural moieties present in the analytes. While infusing the standard mixture, the collision energy was varied from 10% to 50% and the fragmentation profile investigated for each of the flavonols. The collision energies for rutin, quercitrin, quercetin, kaempferol and isorhamnetin were 32%, 29%, 35%, 47% and 37% respectively and were chosen so as to obtain product ion mass spectra without total loss of the deprotonated parent molecular ion. Partial integrity of the parent ion aided in the identification of flavonol glycosides which differ only in terms of the sugar moieties and therefore have similar fragmentation spectra (Figure 2.3). The scanning ranges for each flavonol and their characteristic fragments ions are shown in Table 5.2.

Table 5.2. Characteristic fragment ions of the flavonol standards

Flavonol standard	Scanning range	Target analyte m/z	m/z of fragment ions observed
Rutin	150.0 - 650.0	609	301 179
Quercitrin	150.0 - 650.0	447	301 179
Quercetin	120.0 - 350.0	301	273 257 179
Kaempferol	150.0 - 350.0	285	257 239 151
Isorhamnetin	150.0 - 350.0	315	300

The fragmentation of rutin gave rise to intense ions at  $m/z$  301 and  $m/z$  179 corresponding to the loss of the rutinoside unit and subsequent retrocyclization of the C-ring (between bonds 1 and 2) leading to the  $^{1,2}A^-$  fragment. Quercitrin was observed to lose its terminal rhamnose unit and also produce a product ion at  $m/z$  301 followed once again by the subsequent retrocyclization pathway to produce the product ion at  $m/z$  179. Quercetin, in addition to a  $m/z$  179 fragment, produced product ions resulting from the neutral losses of CO and CO<sub>2</sub> from the C-ring corresponding to the fragments  $[M-H-CO]^-$  and  $[M-H-CO_2]^-$  at  $m/z$  273 and  $m/z$  257, respectively. The ion at  $m/z$  179 also indicated a retrocyclization fragment, which was consistent with the fragmentation of the flavonols rutin and quercitrin (Figure 5.3).

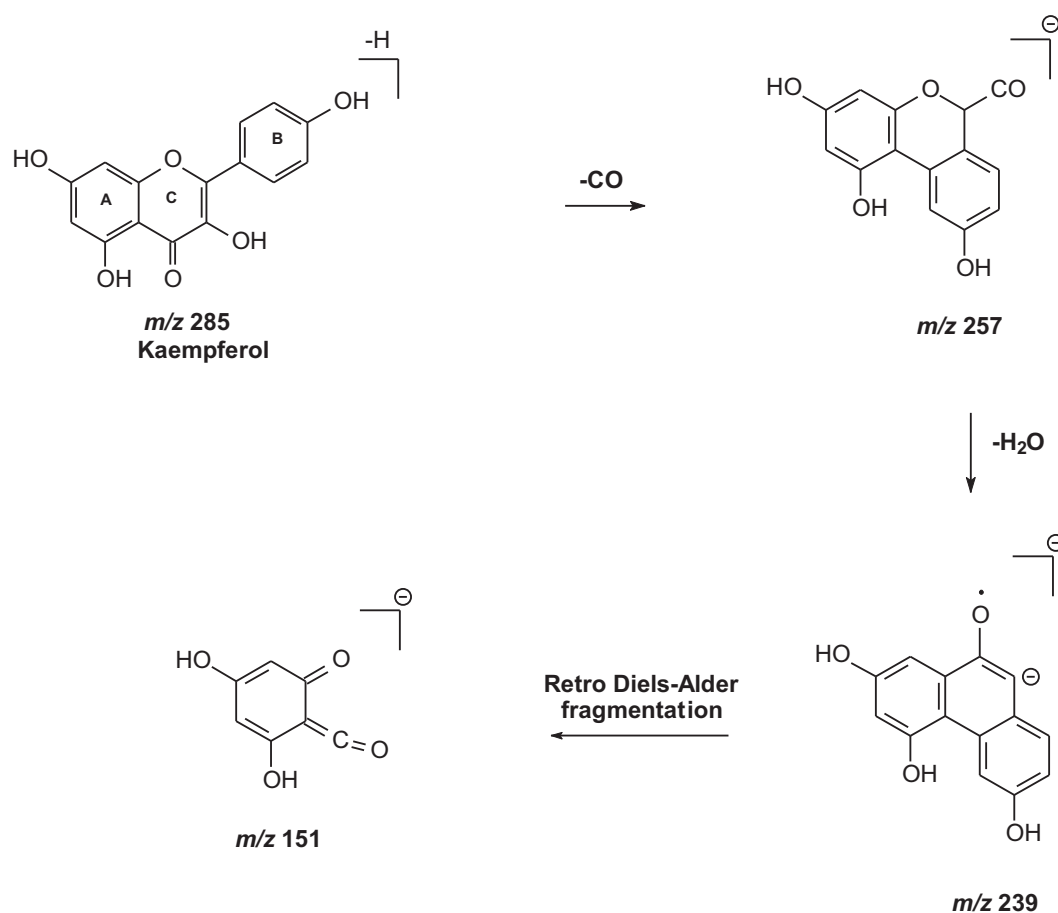
Figure 5.3. Fragmentation patterns of rutin, quercitrin and quercetin



The product ion mass spectrum of kaempferol contained ions at  $m/z$  257,  $m/z$  239 corresponding to the fragments  $[M-H-CO]^-$  and  $[M-H-CO-H_2O]^-$ , respectively. The ejection of CO is notably followed by B-ring rotation and bonding with the A-ring to form the fused ring structure of  $m/z$  257. The retro-Diels-Alder fragmentation product wherein bonds 1 and 3 undergo scission leading to the formation of the  $^{1,3}A^-$  ion at  $m/z$  151 was also evident. The fragmentation pathway of kaempferol, originally proposed by March and Miao [229] is presented in Figure 5.4.

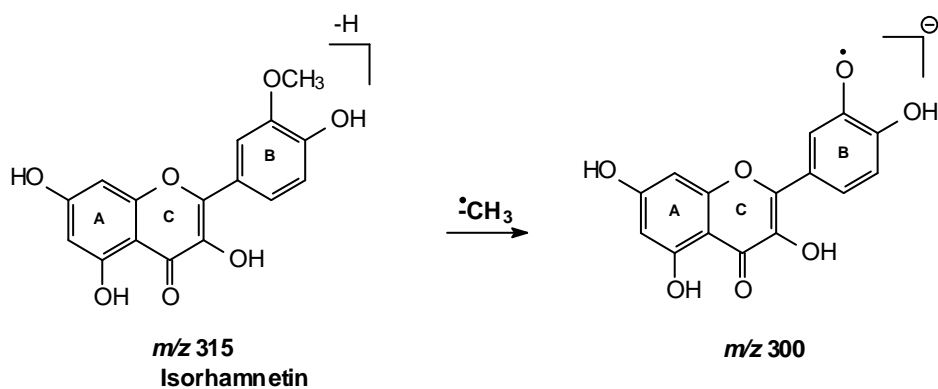


Figure 5.4. Proposed fragmentation pattern of kaempferol [229]



Isorhamnetin exhibited fragmentation with the loss of the  $\cdot\text{CH}_3$  radical from the deprotonated aglycone molecule ( $m/z$  315), thus producing the ion at  $m/z$  300 (Figure 5.5). These product ion mass spectra were similar to those observed in other mass spectrometry studies of flavonoids [210, 211, 229] and thus provided unequivocal identification of the relevant flavonols.

Figure 5.5. Fragmentation pattern of isorhamnetin



## **5.4 METHOD VALIDATION AND QUANTITATIVE ANALYSIS OF *GINKGO BILOBA* SOLID ORAL DOSAGE FORMS**

### **5.4.1 Instrumentation and additional equipment**

The same LC-MS system described in section 5.3.1 was used for the validation. In addition, A Cole-Parmer Ultrasonic Bath, Model 8845-30 (Cole-Parmer Instrument Company, Chicago, Illinois, USA) was used in the sonication procedure for extraction purposes and a Mettler Dual Range Electronic Balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland) was used for weighing the reagents and standards. An Eppendorf Centrifuge, Model 54142770 (Eppendorf Geratebau, West Germany) was used to centrifuge tablet extracts.

### **5.4.2 Materials and reagents**

Reagents were sourced as stated in section 5.3.2. PVDF filters were purchased from Millipore, Bedford, MA, USA. Six *Ginkgo biloba* products (I, II, III, IV, V and VI) were purchased from a local pharmacy in Grahamstown, South Africa. Four of the products were solid oral dosage forms and 2 preparations contained herbal extract in a gelatin capsule. See Table 5.3 for product details.

Table 5.3. *Ginkgo biloba* dosage forms selected for quantitative LC-MS-MS analysis of the flavonols

Manufacturer	Name of product	Dosage form	Label claim	Daily dose	Batch number	Extract
Holotropic <sup>1</sup>	<i>Ginkgo biloba</i>	Tablet	Each 3 tablets provide: <i>Ginkgo biloba</i> botanical 300 mg, <i>Ginkgo biloba</i> extract equivalent to 900 mg	1 tablet tds <sup>7</sup>	0318	I
Formule Naturelle <sup>2</sup>	<i>Ginkgo biloba</i>	Tablet	Standardized to contain 24% Ginkgo Flavonglycosides	1 tablet tds <sup>7</sup> , ac <sup>8</sup>	82060	II
Bioforce <sup>3</sup>	Ginkgoforce	Tablet	Each 250mg tablet is equivalent to 10 drops of Ginkgoforce liquid	2 tablets tds <sup>7</sup> , ac <sup>8</sup>	210120E	III
Nrf Herbal <sup>4</sup>	<i>Ginkgo biloba</i>	Tablet	Each tablet contains 350mg <i>Ginkgo biloba</i> , 50mg <i>Ginkgo biloba</i> extract 24% and 240mg FoodMatrix™	1 - 2 tablets daily	200303047	IV
Bioharmony <sup>5</sup>	<i>Ginkgo biloba</i>	Capsule	Each 350mg capsule contains 350mg <i>Ginkgo biloba</i> ( <i>Ginkgoaceae</i> )	2 capsules tds <sup>7</sup>	1EP02.21	V
Vital <sup>6</sup>	<i>Ginkgo biloba</i>	Capsule	Each capsule contains <i>Ginkgo biloba</i> extract equivalent to 6 000 mg of herb powder	1 capsule daily	0024B	VI

<sup>1</sup>Holotropic, Cape Town, South Africa; <sup>2</sup>Formule Naturelle, Hout Bay, South Africa; <sup>3</sup>Bioforce S.A. (Pty) Ltd., Halfway House, South Africa; <sup>4</sup>Nrf Herbal, Centurion, South Africa; <sup>5</sup>Bioharmony CC, Wynberg, South Africa; <sup>6</sup>Vital Health Foods (EDMS) BPK, Kuils River, Western Cape, South Africa; <sup>7</sup>tds, three times daily; <sup>8</sup>ac, before meals

#### 5.4.3 Preparation of standard solutions

Separate stock solutions of the reference standards were made in methanol-water (50:50). A working solution of the combined standards was subsequently prepared in methanol-water (50:50) and diluted to provide a series of analytical standards ranging from 3 - 26 µg/ml for use in constructing calibration curves for each of the target analytes.

#### 5.4.4 Sample preparation and extraction

##### 5.4.4.1 *Solid oral dosage forms*

Twenty five tablets of each solid oral dosage form (products I to IV) were powdered in a mortar using a pestle. A mass of powder equivalent to 1 tablet was accurately weighed and dispersed in methanol (25

ml). The mixture was sonicated for 1 hour, with manual agitation after 30 minutes and then centrifuged at 350 x g for 15 minutes. The samples were diluted with appropriate volumes of methanol and water in order to maintain a 50:50 methanol-water solvent ratio; and filtered through 0.45 µm PVDF filters before injecting.

#### 5.4.4.2 *Hard gelatin capsules*

Twenty five capsules (product V) were emptied, weighed and a mass of powder equivalent to the contents of a single capsule was extracted as described in section 5.4.4.1 above.

#### 5.4.4.3 *Soft gelatin capsules*

Individual capsules (product VI) were sliced longitudinally, the contents squeezed out and combined with the soft gel shell containing residual content. Methanol (25 ml) was added and the analytes were extracted by sonication for 1 hour with manual agitation after 30 minutes, followed by centrifugation at 350 x g for 15 minutes. The samples were diluted with appropriate volumes of methanol and water in order to maintain a 50:50 methanol-water solvent ratio; and filtered through 0.45 µm PVDF filters before injecting.

#### 5.4.5 LC conditions

LC separation was achieved isocratically at 45°C on a 150 x 2.0 mm I.D. Luna C<sub>18</sub> reversed-phase minibore column packed with 5 µm ODS-2. The mobile phase consisted of 0.3% formic acid-acetonitrile (75:25) which was pumped at a flow rate of 500 µl/min. The injection volume was 5 µl and the column eluate was monitored on-line with UV detection at λ 350 nm prior to MS detection.

#### 5.4.6 MS conditions

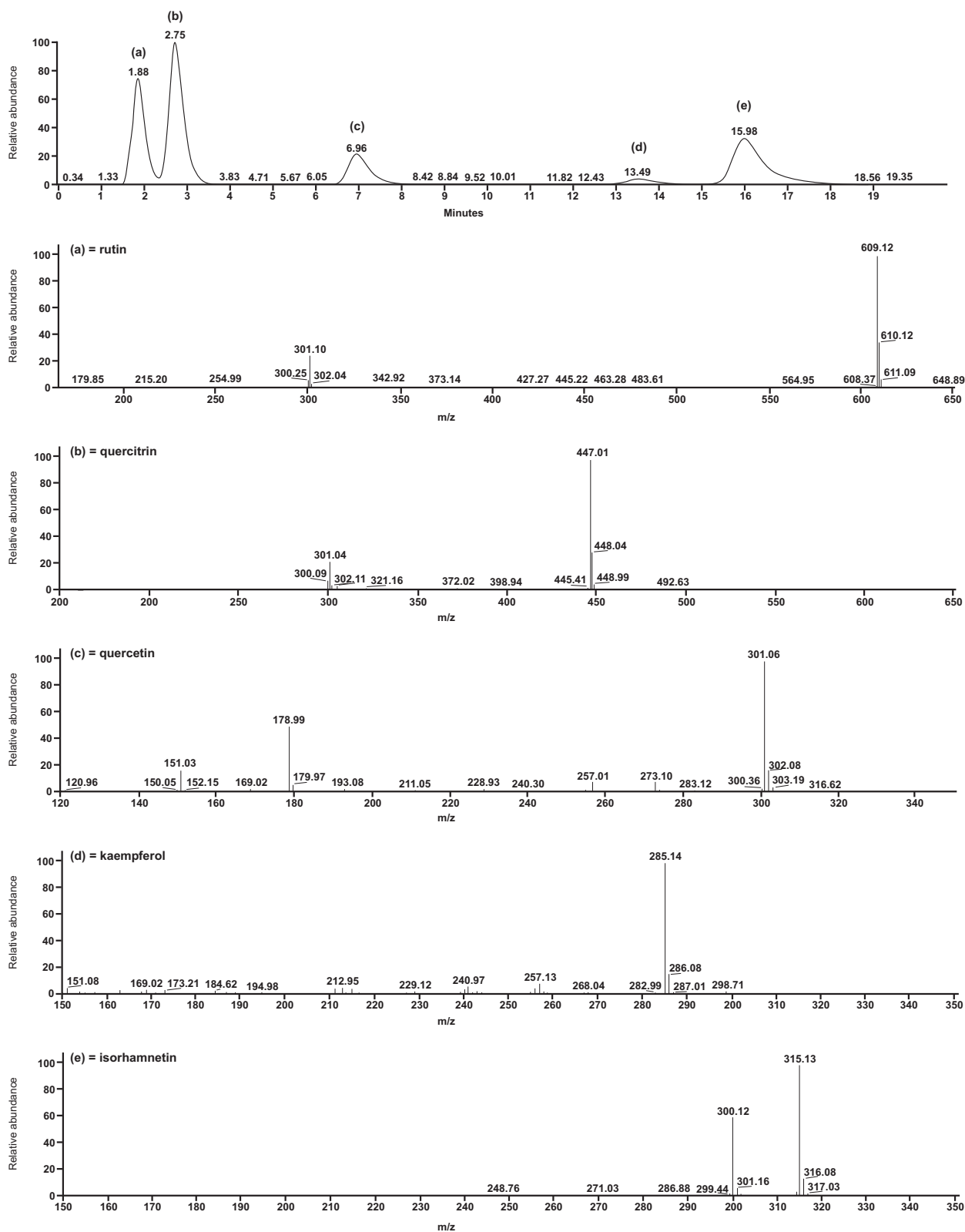
Method validation and marker content analysis in the commercial products was performed in the MS-MS mode using CID. The collision energies for rutin, quercitrin, quercetin, kaempferol and isorhamnetin were 32%, 29%, 35%, 47% and 37% with capillary voltages of -32.5 V, -5.50 V, -46.0 V, -41.0 V and -46.0 V, respectively. The capillary temperature was maintained at 240°C and the sheath and auxiliary gas flow parameters were 80 and 20 arbitrary units, respectively. The spray voltage remained constant for all compounds at 4.5 kV.

#### 5.4.7 HPLC-MS-MS

During the chromatographic run, the mass spectrometer was programmed into five segments, which allowed for optimal detection of each flavonol in the MS-MS mode. A typical total ion chromatogram of the five reference standards is shown in Figure 5.6 together with their corresponding product ion mass spectra. The elution order of the reference standards were as follows: rutin (1.9 minutes),

quercitrin (2.8 minutes), quercetin (7.0 minutes), kaempferol (13.5 minutes) and isorhamnetin (16.0 minutes) together with their corresponding product ion mass spectra.

Figure 5.6. Elution order of the reference standards with their corresponding fragmentation patterns



#### 5.4.8 Method validation

##### 5.4.8.1 *Calibration curves*

Calibration curves were constructed on 3 consecutive days by analysis of a mixture containing each of the flavonols at 4 concentration levels and plotting peak area against the concentration of each reference standard.

##### 5.4.8.2 *Precision and accuracy*

The precision and accuracy of the assay was assessed by spiking an amount of powdered material equivalent to the weight of a single tablet of product I with high, medium and low concentrations of each reference standard. The amount of each analyte was determined in triplicate over three days. Since product I contained a significant amount of rutin (307.3 µg) at the lowest level, recovery of rutin was assessed at medium and high concentrations only.

##### 5.4.8.3 *LOD's and LOQ's*

The LOD's and LOQ's were determined by means of serial dilutions based on a S/N ratio of 3:1 and 10:1 respectively.

#### 5.4.9 Results and discussion

##### 5.4.9.1 *Linearity and range*

Calibration data for each compound were obtained using the optimized LC-MS-MS conditions. The response profile was determined and observed to be linear for each of the flavonols within the concentration ranges 3 - 26 µg/ml. The calibration data are presented in Table 5.4. Each concentration level was analyzed in triplicate, once at the start of the day, midway through the analysis and after the sample set was completed. The RSDs for all data points were less than 4%.

Table 5.4. Linear ranges and co-efficients of determination

Name of compound	Concentration range ( $\mu\text{g/ml}$ )	Day	Equation	Co-efficients of determination ( $R^2$ )
Rutin	3.1 - 25.1	Day 1	$y = 1\text{E}+07x$	0.9942
		Day 2	$y = 1\text{E}+07x$	0.9986
		Day 3	$y = 1\text{E}+07x$	0.9970
Quercitrin	3.2 - 25.7	Day 1	$y = 2\text{E}+07x$	0.9960
		Day 2	$y = 2\text{E}+07x$	0.9989
		Day 3	$y = 2\text{E}+07x$	0.9972
Quercetin	3.1 - 25.0	Day 1	$y = 6\text{E}+06x$	0.9935
		Day 2	$y = 6\text{E}+06x$	0.9936
		Day 3	$y = 7\text{E}+07x$	0.9969
Kaempferol	3.3 - 26.4	Day 1	$y = 1\text{E}+06x$	0.9968
		Day 2	$y = 1\text{E}+06x$	0.9900
		Day 3	$y = 1\text{E}+06x$	0.9937
Isorhamnetin	3.1 - 24.4	Day 1	$y = 2\text{E}+07x$	0.9957
		Day 2	$y = 2\text{E}+07x$	0.9956
		Day 3	$y = 2\text{E}+07x$	0.9974

#### 5.4.9.2 LOD's and LOQ's

The LOD's and LOQ's were determined by means of serial dilution of the reference standards. The LOD's were 0.39  $\mu\text{g/ml}$ , 0.40  $\mu\text{g/ml}$ , 1.56  $\mu\text{g/ml}$ , 1.65  $\mu\text{g/ml}$  and 0.38  $\mu\text{g/ml}$  for rutin, quercitrin, quercetin, kaempferol and isorhamnetin, while their corresponding LOQ's were 0.78  $\mu\text{g/ml}$ , 0.80  $\mu\text{g/ml}$ , 3.13  $\mu\text{g/ml}$ , 3.30  $\mu\text{g/ml}$  and 0.76  $\mu\text{g/ml}$ , respectively.

#### 5.4.9.3 Precision and Accuracy

The repeatability and accuracy of the method was evaluated by analysing a commercial product (Product I) that was spiked at three concentration levels over 2 different days. Prior to spiking, the background levels of the flavonols in Product I were determined so as to calculate actual recoveries. Intra-day RSDs were all less than 10% whilst the inter-day RSDs ranged between 1 and 16%. Isorhamnetin and quercetin showed the highest RSD, 15.61% and 15.32% at the 157  $\mu\text{g/tablet}$  and 437  $\mu\text{g/tablet}$  levels respectively. Such a high variation can be attributed to insolubility which in the case of isorhamnetin has been documented in previous studies [168, 187, 192] as a result of its lower polarity. A higher concentration of quercetin stock solution was used and thus the possibility of precipitation of quercetin cannot be discounted and may have contributed to a larger % RSD at the highest spiking level. Similarly, whilst recoveries greater than 90% were observed for rutin, quercitrin and kaempferol, lower recoveries were observed for quercetin and isorhamnetin. The results of this experiment are presented in Table 5.5.

Table 5.5. Precision and recoveries of flavonols from product I

Constituent	Spiking level ( $\mu\text{g}/500\text{ mg}$ )	Intra-day RSD (%) (n=3)	Inter-day RSD (%) (n=6)	Mean recovery (%) $\pm$ SD (n=6)
Rutin*	-	-	-	-
	312.3	3.0	4.1	94.6 $\pm$ 3.9
	450.0	1.2	6.8	90.4 $\pm$ 6.2
Quercitrin	131.3	1.5	1.7	93.1 $\pm$ 4.9
	287.5	2.8	4.4	93.5 $\pm$ 5.7
	425.0	2.4	4.5	90.6 $\pm$ 6.5
Quercetin	143.8	4.1	4.4	75.3 $\pm$ 1.3
	312.5	1.6	9.5	70.3 $\pm$ 7.9
	437.5	3.0	15.9	69.1 $\pm$ 10.9
Kaempferol	165.0	6.8	1.3	93.5 $\pm$ 8.2
	330.0	3.3	3.7	100.4 $\pm$ 3.6
	475.3	2.8	2.2	100.9 $\pm$ 2.2
Isorhamnetin	157.3	2.7	15.7	58.8 $\pm$ 8.2
	314.5	3.3	11.2	64.1 $\pm$ 7.9
	453.0	2.6	5.4	63.7 $\pm$ 4.2

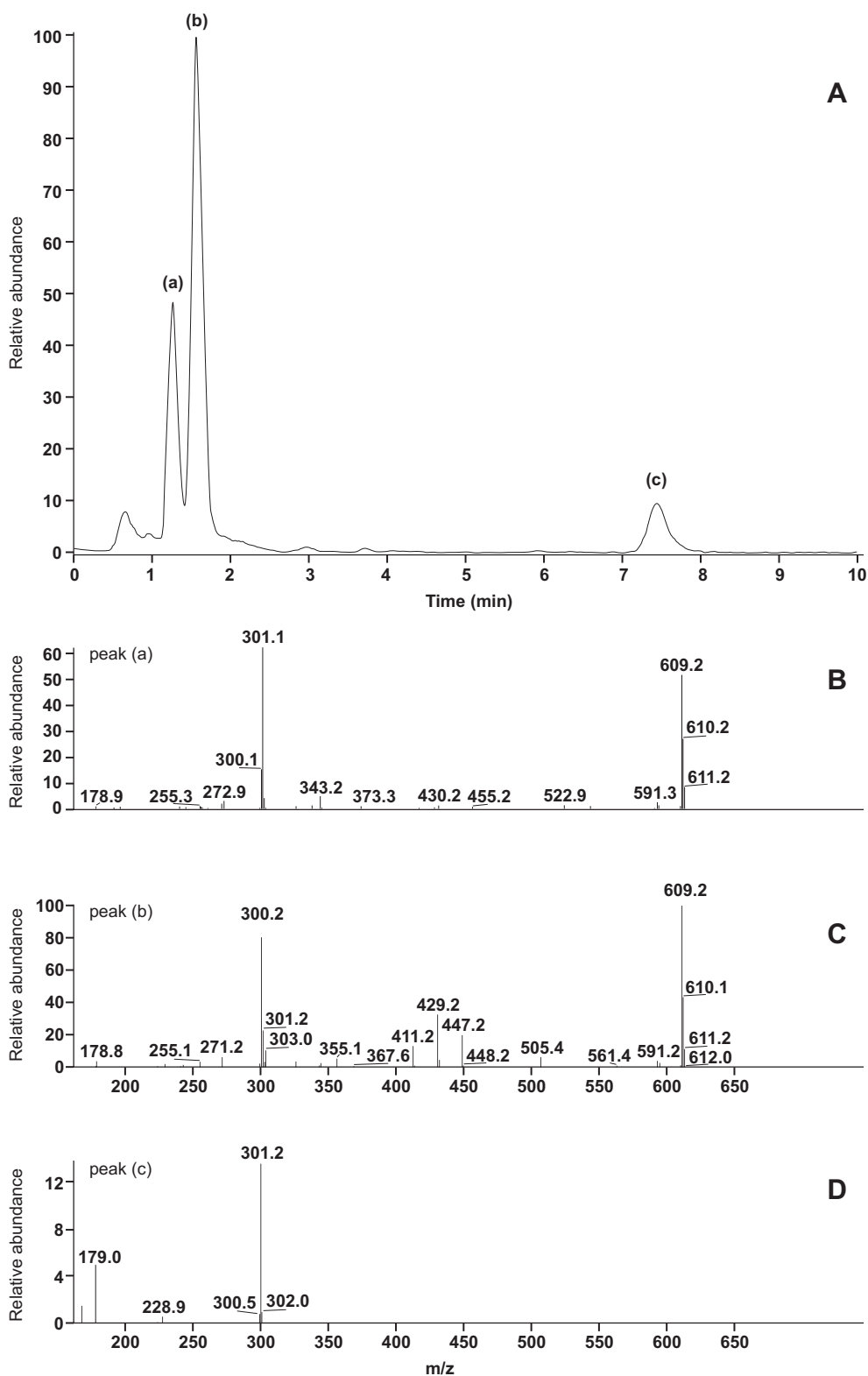
\* Product I contained a quantifiable amount of rutin to fall on the lower linear calibration range and therefore a low spike was not necessary

#### 5.4.9.4 Analysis of commercial products

During the preliminary LC-MS-MS analysis of the same commercially available Ginkgo products described in section 5.3.7, an unknown compound was found (peak b) to be partially resolved from rutin (Figure 5.7). While observing the product ion spectra of rutin and the unknown compound, it was noted that both HPLC peaks yielded deprotonated molecular ions characteristic of rutin ( $m/z$  609). Investigations through MS-MS revealed that the first peak, eluting at 1.27 minutes produced a product ion at  $m/z$  301 resulting from heterolytic cleavage of the rutinoside unit, which also corresponded to the fragmentation profile of the reference standard. The second peak however produced a fragment at  $m/z$  447 resulting from the loss of glucose [M-H-Glu]<sup>-</sup>, followed by dehydration [M-H-Glu-H<sub>2</sub>O]<sup>-</sup> to produce a fragment at  $m/z$  429. A further product ion observed at  $m/z$  300 results from homolytic cleavage of the rhamnose unit from the [M-H-Glu]<sup>-</sup> fragment. While the first peak provided unequivocal confirmation of rutin, the cleavage pattern of the second peak is suggestive of another quercetin glycoside.



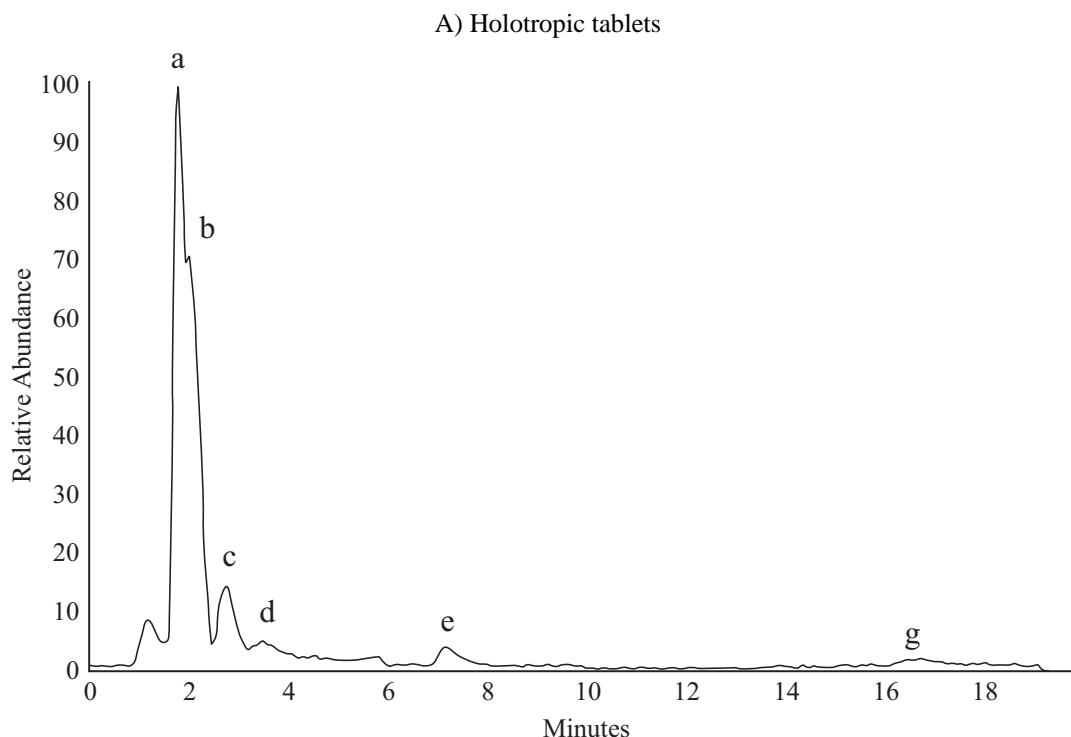
Figure 5.7. A negative total ion chromatogram (TIC) of Vital (A) with corresponding mass spectra of rutin (B), a quercetin glycoside (C) and quercetin (D)



When these experiments were repeated in our laboratory using the LC-MS-MS conditions described in sections 5.4.5 and 5.4.6, the partial resolution between the rutin peak (peak a) and peak b was not as pronounced (Figure 5.8). Five parameters had however changed since the preliminary studies, the

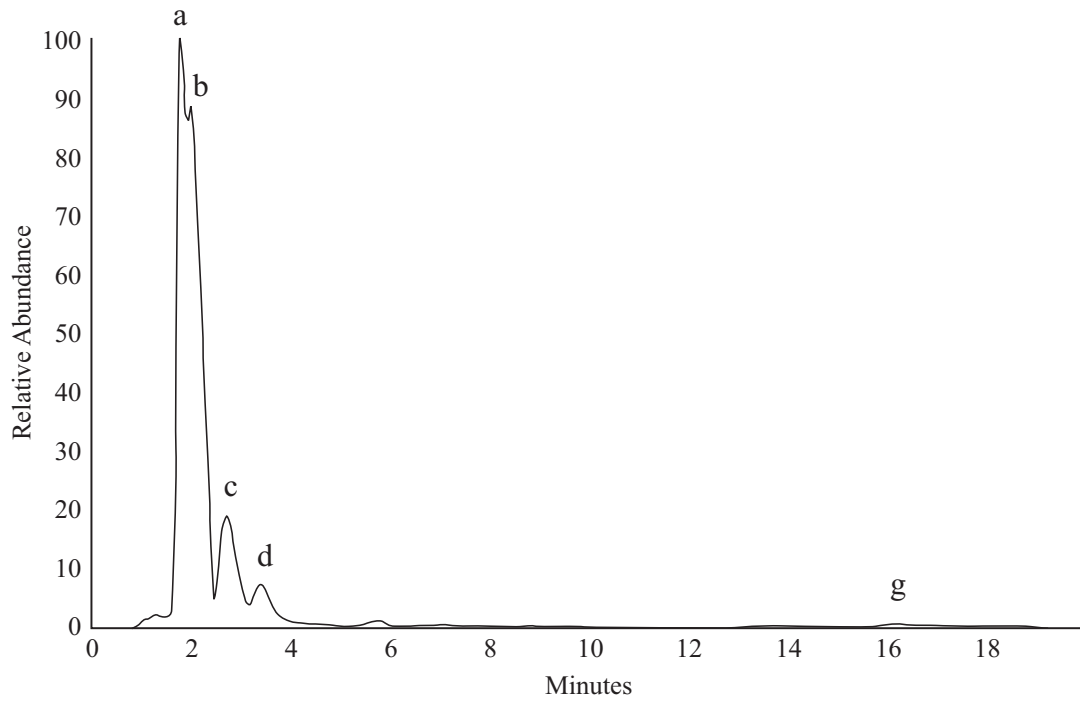
mobile phase flow rate, the tablet batches, a newer column, the injection volume and the instrument. It was possible on our SpectraSYSTEM HPLC pump to decrease with flow rate below 700  $\mu\text{l}/\text{min}$  and maintain a stable eluent flow which was not possible on the HPLC pump used during the preliminary studies. However an increase in flow rate above 500  $\mu\text{l}/\text{min}$  resulted in back-pressures above 3 000 p.s.i. and lower flow rates were thus preferred. The improved resolution obtained between rutin and peak b for all 4 products in the preliminary study and lower resolution observed for the same products in the subsequent study eliminated the possibility constitutional differences between batches. In addition, preparation and injection of extracts from both new and old batches of the Naturelle dosage form using LC-MS-MS showed no difference in peak resolution. The use of a newer column was expected to improve the resolution and this was indeed the case when the previous column was used as a comparison. A decrease in sample loading is usually paired with lower peak areas with improved resolution and the change from a fixed 20  $\mu\text{l}$  to 5  $\mu\text{l}$  loop was necessary to prevent overloading on the minibore column. The causative factor for the deteriorated resolution therefore suggested potential instrumental differences (including the plumbing) between the system where the preliminary studies were conducted and our equipment.

Figure 5.8. Negative TIC of the *Ginkgo biloba* commercial products which shows the poor resolution between rutin (a) and peak (b)

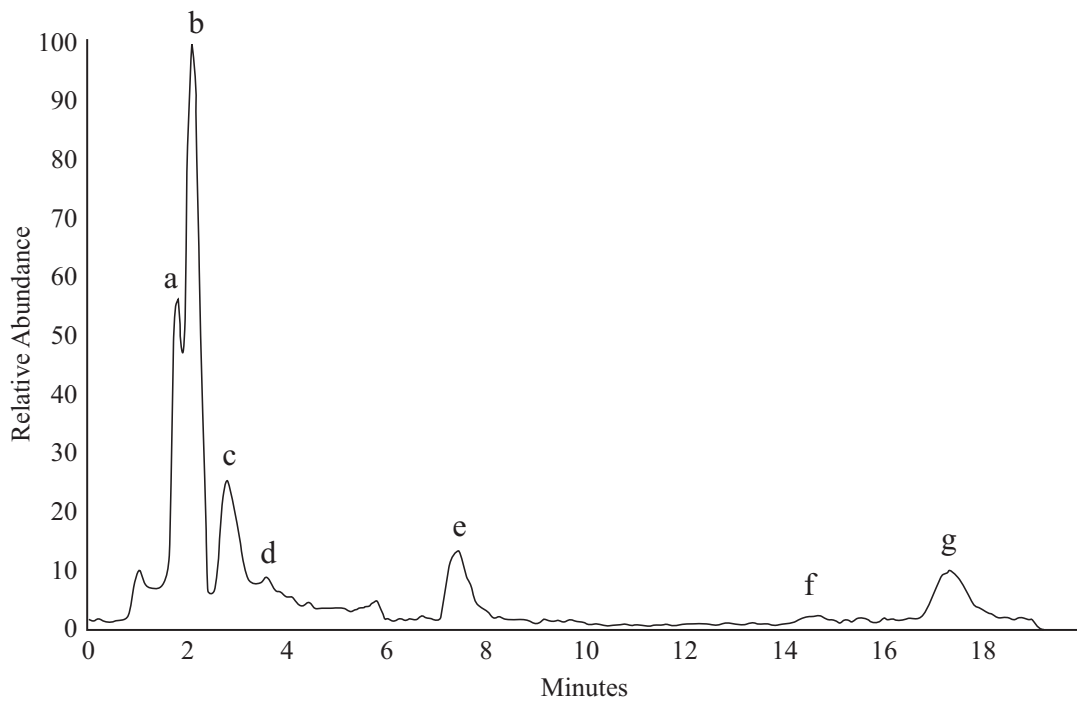


Conditions: Column: Phenomenex Luna (5  $\mu\text{m}$ )  $\text{C}_{18}$  (150 x 2.0 mm I.D.); Column temperature: 45  $^{\circ}\text{C}$ ; Mobile phase: MeCN-0.3% formic acid (75:25); Flow rate: 500 $\mu\text{l}/\text{min}$ ; Injection volume: 5  $\mu\text{l}$ ; Peak labels: a= rutin, c=quercitrin, d=unknown glycoside, e=quercetin, f=kaempferol, g=isorhamnetin.

B) Naturelle tablets

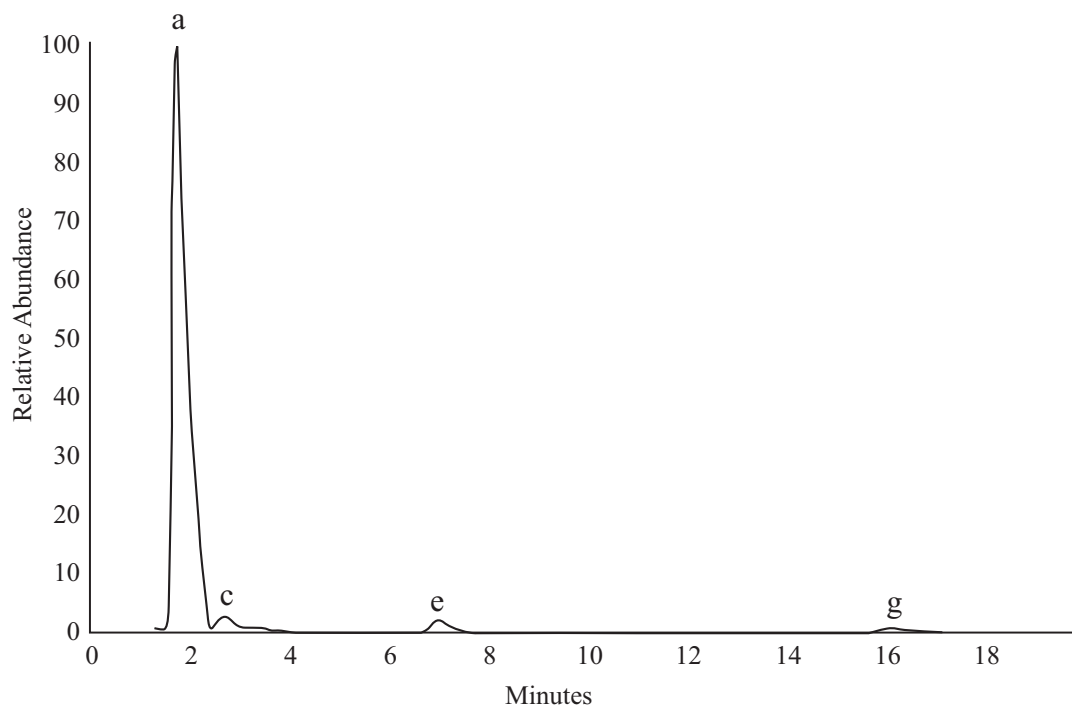


C) Ginkgoforce tablets

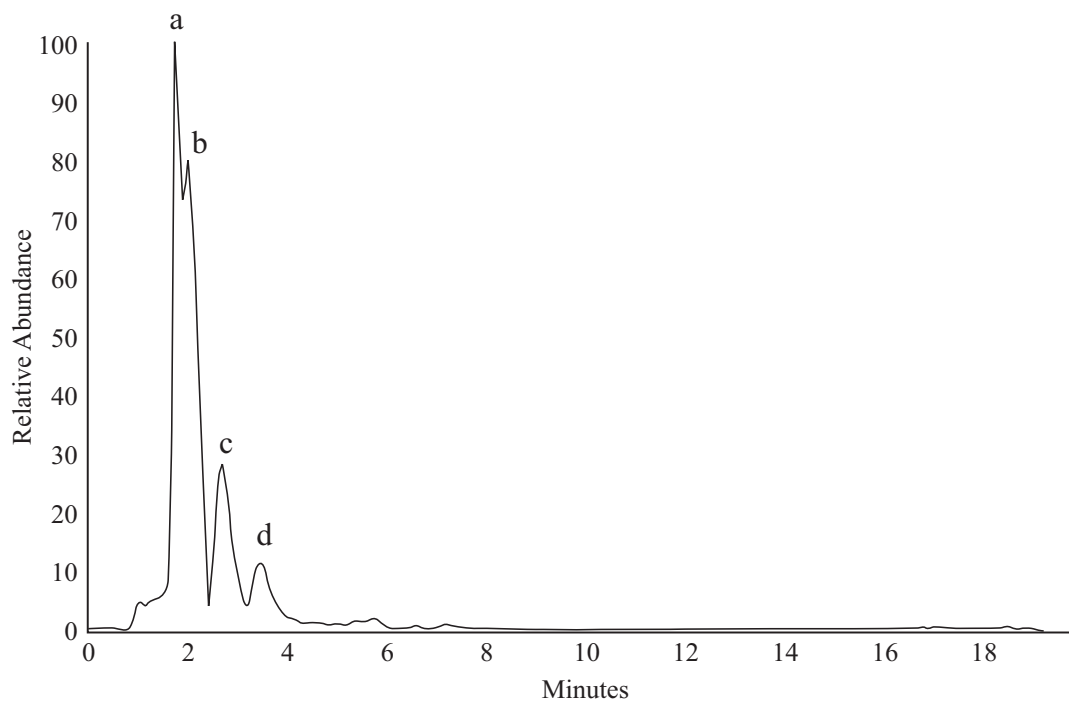


Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (150 x 2.0 mm I.D.); Column temperature: 45 °C; Mobile phase: MeCN-0.3% formic acid (75:25); Flow rate: 500  $\mu$ l/min; Injection volume: 5  $\mu$ l; Peak labels: a= rutin, c=quercitrin, d=unknown glycoside, e=quercetin, f=kaempferol, g=isorhamnetin.

D) Nrf tablets

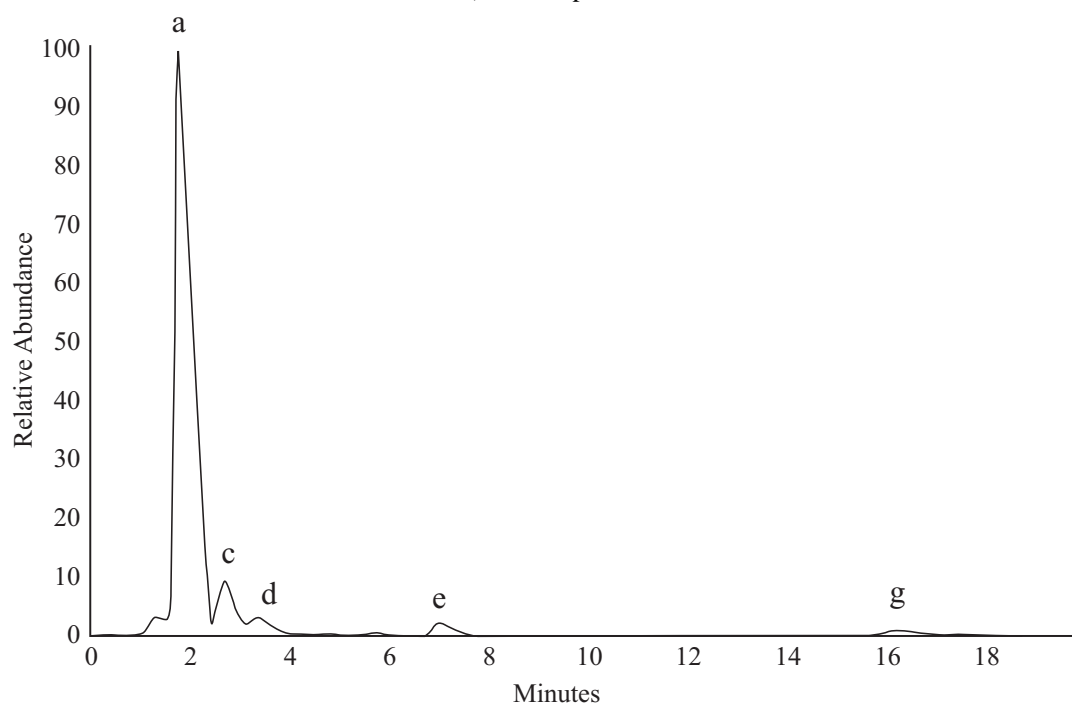


E) Bioharmony capsules



Conditions: Column: Phenomenex Luna (5  $\mu$ m)  $C_{18}$  (150 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic acid (75:25); Flow rate: 500 $\mu$ l/min; Injection volume: 5  $\mu$ l; Peak labels: a= rutin, c=quercitrin, d=unknown glycoside, e=quercetin, f=kaempferol, g=isorhamnetin.

## F) Vital capsules

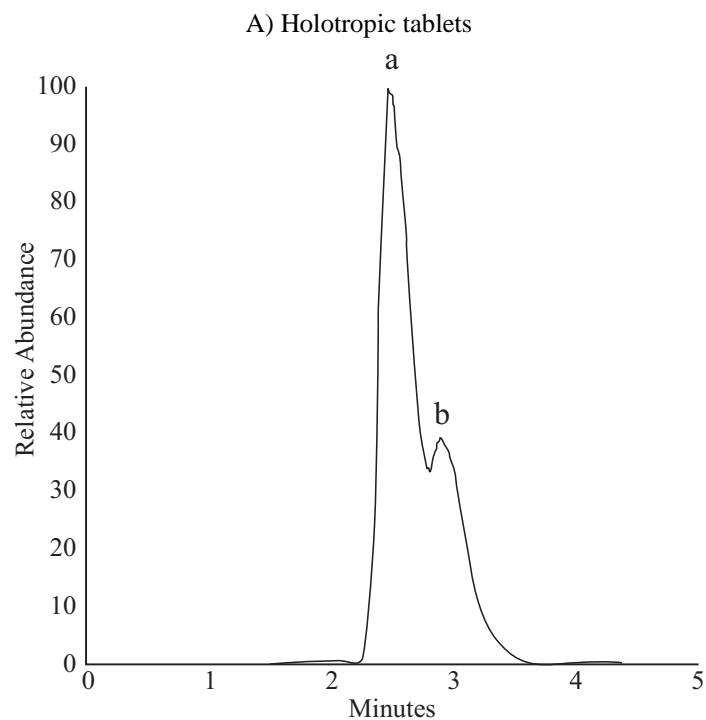


Conditions: Column: Phenomenex Luna (5  $\mu\text{m}$ )  $\text{C}_{18}$  (150 x 2.0 mm I.D.); Column temperature: 45  $^{\circ}\text{C}$ ; Mobile phase: MeCN-0.3% formic acid (75:25); Flow rate: 500  $\mu\text{l}/\text{min}$ ; Injection volume: 5  $\mu\text{l}$ ; Peak labels: a= rutin, c=quercitrin, d=unknown glycoside, e=quercetin, f=kaempferol, g=isorhamnetin.

In order to improve the resolution between the unresolved peaks, a Phenomenex Luna  $\text{C}_{18}$  5  $\mu\text{m}$  (ODS 2) with dimensions 250 x 2.0 mm I.D. was used. Rutin was then quantified in MS-MS mode with SRM of the ions at  $m/z$  609 and 301. Based on the rutin standard, a separate calibration curve between the range 0.39 - 25.1  $\mu\text{g}/\text{ml}$  was constructed ( $R^2 = 0.9964, 0.9971$  and  $0.9972$  over the 3 days, respectively). In this manner, rutin was selectively and accurately quantified in extracts. The improved separation between rutin and peak b in the products is shown in Figure 5.9. The second peak with an  $[\text{M}-\text{H}]^-$  ion at  $m/z$  609 was also suggestive of a quercetin glycoside. Based on the biochemical pathway of the flavonol glycosides, the most likely positions for glycosylation to take place is at the 3- and 7-hydroxyl group, while the 5-hydroxyl group of quercetin is protected by hydrogen bonding with the adjacent carbonyl group and is therefore never apparently involved in glycosylation [230]. Hence this would indicate the possibility of one of two quercetin glycoside analogs *viz.*, quercetin 3-*O*-glucoside-7-*O*-rhamnoside or quercetin 3-*O*-rhamnoside-7-*O*-glucoside. The former compound has been found in the leaves of sea buckthorn (*Hippophae rhamnoides* ssp. *Mongolica*) where it was found to be the dominant flavonol in the leaves and accounted for approximately 24% of the flavonoids [231]. The latter compound has been found to be the most abundant glycoside in the leaves of *Ligustrum vulgare* L. (Oleaceae) and the mass spectral pattern matches that observed in the current study [232]. Structural investigations of flavonol glycosides from *H. rhamnoides* recently carried out have demonstrated that the loss of the sugar moiety from C-7 of the aglycone is more favoured than fission of the glycosidic linkage at the C-3 position [233]. Hence, based on literature evidence and the mass spectral pattern, the

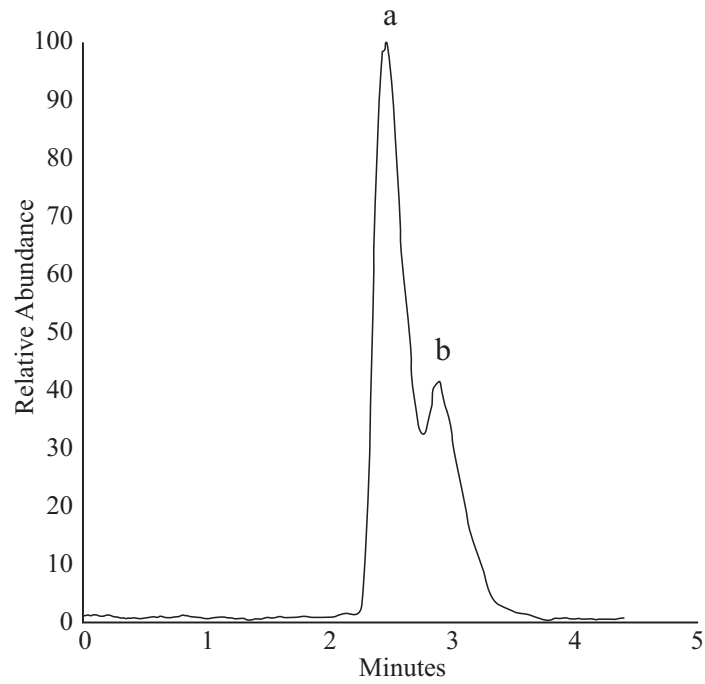
second peak is suggestive of the 7-*O*-glucoside, however NMR spectroscopy of the isolated compound is required to conclusively confirm this speculation and was not within the scope of this work.

Figure 5.9. The resolution between rutin (a) and peak (b) using the Phenomenex Luna C<sub>18</sub> 5 $\mu$ m (ODS 2) column with dimensions 250 x 2.0 mm I.D.

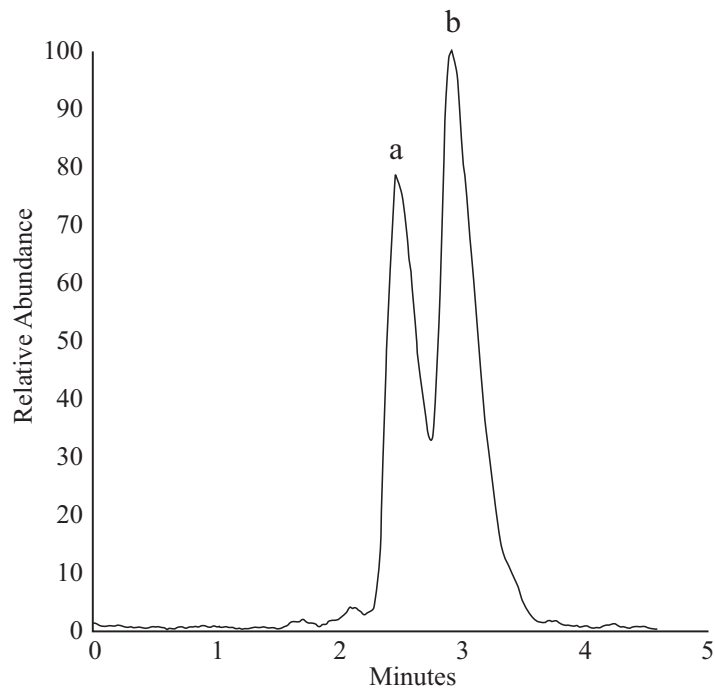


Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45 °C; Mobile phase: MeCN-0.3% formic acid (75:25); Flow rate: 700 $\mu$ l/min; Injection volume: 5  $\mu$ l; Peak labels: a=rutin, b=unknown glycoside.

B) Naturelle tablets

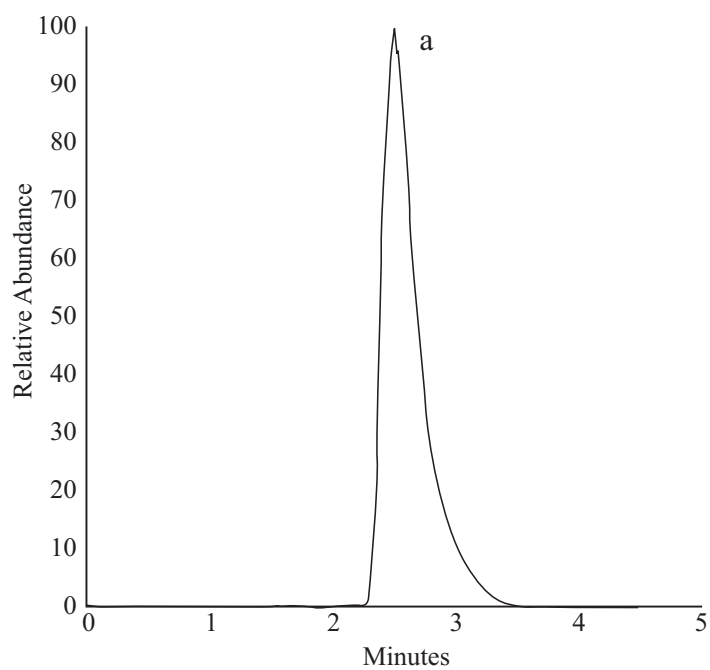


C) Ginkgoforce tablets

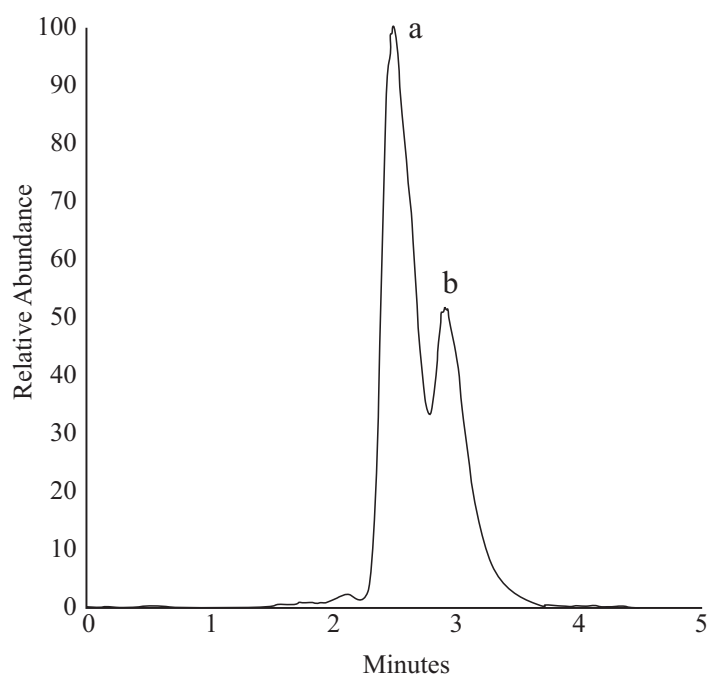


Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic acid (75:25); Flow rate: 700 $\mu$ l/min; Injection volume: 5  $\mu$ l; Peak labels: a=rutin, b=unknown glycoside.

D) Nrf tablets



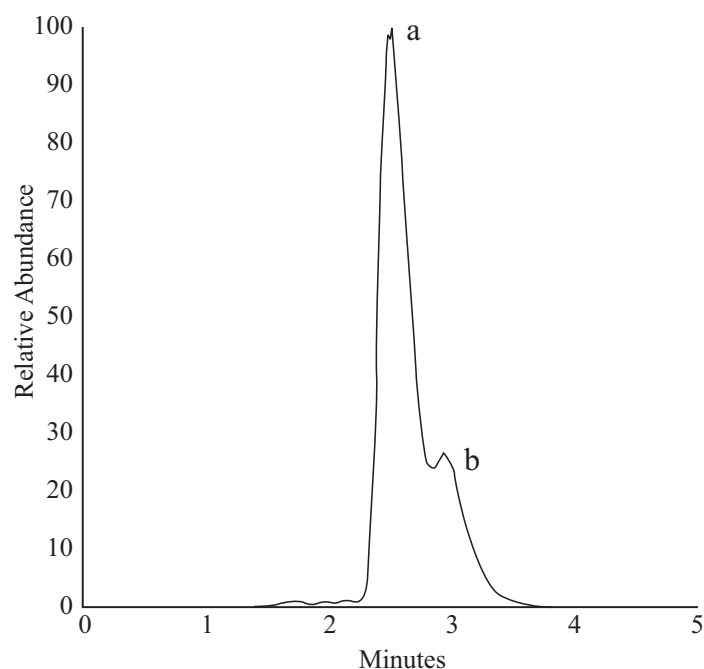
E) Bioharmony capsules



Conditions: Column: Phenomenex Luna (5  $\mu$ m)  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic acid (75:25); Flow rate: 700 $\mu$ l/min; Injection volume: 5  $\mu$ l; Peak labels: a=rutin, b=unknown glycoside.



## F) Vital capsules



*Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic acid (75:25); Flow rate: 700 $\mu$ l/min; Injection volume: 5  $\mu$ l; Peak labels: a=rutin, b=unknown glycoside.*

In 5 of the commercial products analyzed using LC-MS-MS (Figure 5.8), an additional peak (d) was observed in the quercitrin scanning segment (150.0 - 650.0). The mass spectra of peak (d) indicated on the TIC of Bioharmony (Figure 5.8 E) is shown in Figure 5.10. The fragmentation ions of  $m/z$  447 and 285 are indicative of the presence of either luteolin or a luteolin glycoside such as luteolin-7-*O*-glucoside or luteolin-7-*O*-galactoside. Kaempferol-3-*O*-glucoside also has fragmentation ions of  $m/z$  447 and 285 [218, 221]. The presence of this additional peak demonstrates that this method could potentially be used for the analysis of other flavonol compounds present in Ginkgo extracts. Unfortunately, reference standards were not available for confirmation of this peak's identity.

Figure 5.10. Mass spectra of peak (d) present in 5 of the Ginkgo solid oral dosage forms

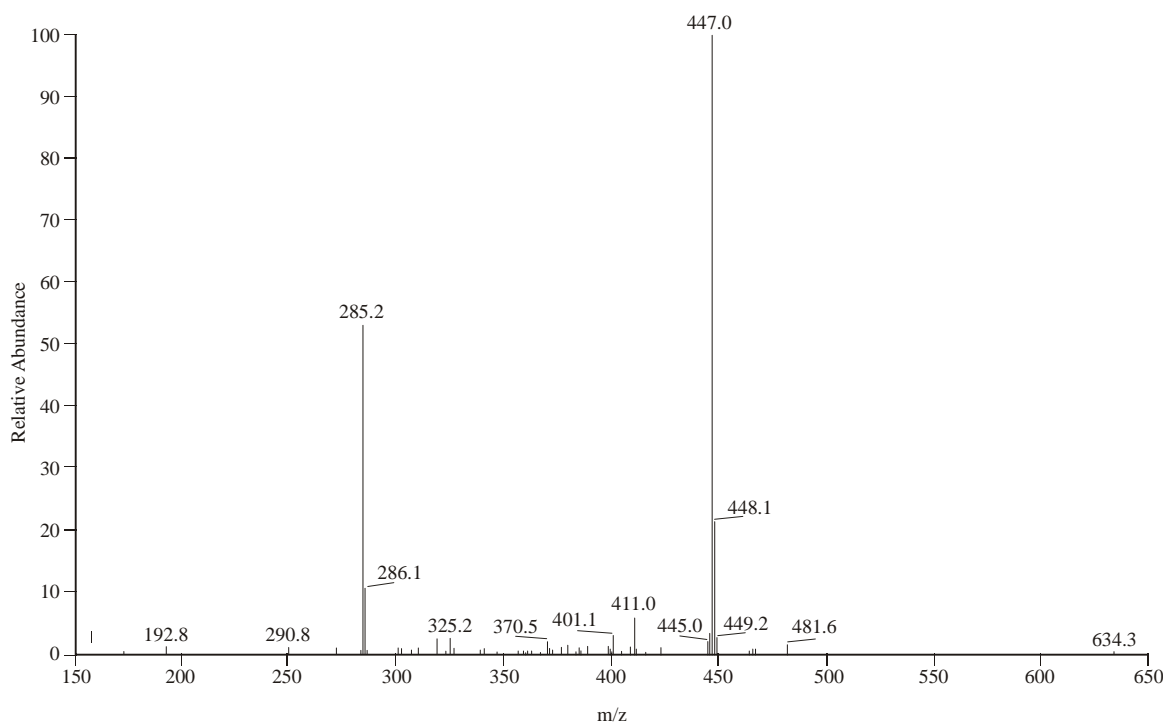


Table 5.6 contains the assay results of each of the five flavonols expressed in  $\mu\text{g}$  per tablet/capsule. Product VI was formulated as a liquid in a soft gelatin capsule and some loss of material during the extraction procedure was evident by the higher % RSD values, hence only 2 of the 3 assays were included in the final assay results. Concerning the aglycones, quantifiable amounts of quercetin, kaempferol and isorhamnetin were found in Product IV whereas Product VI showed the presence of quercetin and kaempferol, with a trace amount of isorhamnetin. All the products analyzed were observed to contain quantifiable amounts of the two flavonol glycosides, rutin and quercitrin. Once again from a product quality aspect, the presence of significant amounts of glycosides compared to aglycones is preferable, however conspicuously large discrepancies in the flavonol glycoside content (particularly rutin) between products is indicative of a lack of adequate QC. Product IV showed the highest overall marker content followed by Product VI. Product III had the lowest glycoside content with the detection of all 3 aglycones, albeit below the LOQ. This is indicative of poor storage conditions and/or rigorous extraction procedures. Based on the recommended daily dosage displayed on the product labels (Table 5.3), the intake of rutin for each product would be 804  $\mu\text{g}$ , 3300  $\mu\text{g}$ , 232  $\mu\text{g}$ , 12080  $\mu\text{g}$  (if the maximum dosage is followed), 2082  $\mu\text{g}$  and 4750  $\mu\text{g}$  for products I, II, III, IV, V and VI, respectively. With these random values, significantly different therapeutic responses related to the ingestion of these products can be expected and the label claims prove to have little relevance.

Table 5.6. Flavonol marker content (per tablet/capsule) in 6 dosage forms

Product (n=3) <sup>1</sup>	Rutin <sup>2</sup>	Quercitrin	Quercetin	Kaempferol	Isorhamnetin
I (Holotropic)	268.0 ± 77.3 µg	15.9 ± 0.9 µg	+	-	-
II (Naturelle)	1100.0 ± 9.1 µg	78.6 ± 3.7 µg	+	-	+
III (Ginkgoforce)	38.6 ± 0.9 µg	4.6 ± 0.5 µg	+	+	+
IV (Nrf)	6040.0 ± 109.0 µg	76.5 ± 1.5 µg	328.0 ± 6.3 µg	85.0 ± 3.7 µg	42.9 ± 2.5 µg
V (Bioharmony)	347.0 ± 15.9 µg	35.5 ± 2.5 µg	-	-	-
VI (n=2) (Vital)	4750.0 ± 500.0 µg	143.7 ± 19.2 µg	224.0 ± 16.1 µg	113.2 ± 7.2 µg	+

<sup>1</sup>n = number of assays conducted per dosage form; <sup>2</sup>Calculated using Selected Reaction Monitoring (SRM); (+) indicates that the marker could be identified but was below the LOQ; (-) indicates that the marker was not detected

### 5.5 LC-MS METHODS USED FOR THE ANALYSIS OF TERPENE LACTONES

Since the ginkgolides have poor chromophores, alternative detection techniques to UV have been extensively explored. These include RI [42, 195], ELSD (Chapter 3), GC-MS [192, 199] and LC-MS [119, 170, 234-238]. Of the LC-MS methods, 3 were developed for the qualitative analysis of *Ginkgo biloba* leaf extracts [170, 214, 235] using ESI as the API mode except for Chen *et al.* [238] who used an APCI interface. The details of some recent methods used for the quantitative analysis of the terpene lactones are shown in Table 5.7.

Table 5.7. LC-MS methods used for the quantitative analysis of the terpenes

Matrix analyzed	Analyzed terpene lactones	LC eluent	API mode	Type of MS	LOD	Ref
Human plasma after administration of 2 <i>Ginkgo</i> commercial products	GA, GB, BB	Gradient: A) MeOH B) water	APCI (-)	IT	3 ng/ml	119
<i>Ginkgo biloba</i> leaf extracts and commercial products	GA, GB, GC, GJ, BB	Gradient: A) water B) MeOH	APCI (-) (SIM)	QqQ	0.5-2.0 ng/ml	194
<i>Ginkgo biloba</i> commercial products	GA, GB, GC, GJ, BB	Gradient: A) water B) MeOH	ESI (-)	IT	0.13-5.0 ng/ml	236
<i>Ginkgo biloba</i> leaf extracts	GA, GB, GC, GJ, BB	Gradient: A) water B) MeOH	SSI	IT	2.5-10 ng*	237

MeOH, methanol; GA, ginkgolide A; GB, ginkgolide B; GC, ginkgolide C; GJ, ginkgolide J; BB, bilobalide; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; SIM, single ion monitoring; IS, ion spray; IT, ion trap; QqQ, triple quadrupole; (-), negative ion mode; (+), positive ion mode; SSI, sonic spray ionization; Ref, reference; \*on column load

Two of these papers are of particular relevance with respect to the QC of *Ginkgo biloba* dosage forms. Jensen *et al.* [194] developed and validated a rapid, sensitive LC-APCI-MS method for the detection

and quantification of the terpene lactones in 10 Swiss products extracted in triplicate with methanol. Although the run time was short (14 minutes, including column stabilization), separation was not achieved using LC alone and SIM was required for accurate quantitation. GJ was quantified using the calibration curve of GB and recovery studies were performed by adding standardized extract to the relevant preparations due to a lack of reference standards. Nevertheless, this method demonstrated superior sensitivity compared to UV with minimal sample clean-up and was deemed suitable for the rapid, routine analysis of Ginkgo dosage forms. The assay results revealed inconsistent terpenoid content between the products with only 4 products demonstrating adequate levels corresponding with those used in clinical trials. In addition, the recommended daily dosages differed significantly between manufacturers and the authors suggested that submission of additional pharmacological and clinical data should be instated for those products which contained lower terpenoid content before authorization of product registration.

Sun *et al.* [236] recently published a paper where 9 Ginkgo commercial products were analyzed LC-MS-MS for terpenoid content. Baseline separation of the standards was achieved within 14 minutes and an internal standard was included in the assay. The LOD's were exceptionally low (0.13 - 1.5 ng/ml) except for GJ which was 5 ng/ml. Recovery ranged between 90.0 - 99.4% and intra and inter-day RSDs of the QC samples were <5% and <8%, respectively. The terpene trilactone content of 6 of the commercial products were shown to deviate from the amount disclosed on the product labels. This method was superior to previously published LC-MS-MS methods in terms of sensitivity and the authors suggested using this method for the routine analysis of Ginkgo products.

## 5.6 CONCLUSIONS

A rapid, accurate, specific, precise and reproducible LC-MS-MS method for the identification and quantification of some relevant marker compounds in *Ginkgo biloba* solid oral dosage forms was developed. The use of an isocratic LC method contributed to the simplicity and speed of the method by avoiding additional column equilibration between consecutive sample injections. In addition, the lower flow rate design of the minibore column reduced solvent consumption and ensured efficient vaporization of the LC eluent in the ESI source.

The simultaneous quantitative determination of rutin and quercitrin together with the aglycones, quercetin, kaempferol and isorhamnetin permitted the detection of those flavonol glycosides, facilitating the provision of useful stability information which is circumvented when samples are hydrolyzed to back calculate flavonol glycoside content. This method also has the advantage that a hydrolysis step, previously used for the standardization of flavonols in Ginkgo extracts is not required. To our knowledge, this is the first validated LC-MS method published [169] for the quantification of the flavonols in Ginkgo dosage forms using this QC approach.

Tandem mass spectrometric analysis of Ginkgo extracts revealed, in addition to rutin, the possible presence of other quercetin analogues, quercetin-3-*O*-rhamnoside-7-*O*-glucoside or quercetin-3-*O*-glucoside-7-*O*-rhamnoside, previously unreported in *Ginkgo biloba* leaf extracts or dosage forms. Recent literature findings and the mass fragmentation pattern observed in this study favour the presence of the former analogue although NMR elucidation studies are required to confirm this speculation.

The assay results of the Ginkgo solid oral dosage forms showed major disparities in marker content and daily dosages did not compensate for these radical differences. Product labels contained minimal relevant information and only 1 product (Naturelle) was standardized to contain 24% Ginkgo “flavonglycosides”. The results of this study clearly indicated that QC procedures need to be implemented to ensure the QSE of *Ginkgo biloba* products currently available to consumers.

Although LC-MS is a sophisticated and relatively expensive technique in terms of capital and operating costs, it is extremely valuable for the accurate quantitative analysis of flavonoid compounds in plant extracts since separation and unequivocal identification of the flavonoids using the more common UV/PDA detection systems cannot be guaranteed. With regards to the terpene lactones however, adequate LC-MS methods are available for the analysis of these analytes and since ELSD is a less expensive and relatively uncomplicated detection technique, it was chosen to assay commercial products for terpene lactone content (Chapter 4). LC-MS does, however, demonstrate the necessary sensitivity and selectivity to quantify terpenes in human and animal plasma where the detection of trace amounts of the target analytes is essential.

## CHAPTER 6

### QUALITY CONTROL

#### 6.1 INTRODUCTION

In order to ensure safety and efficacy of a medicine, the quality of that medicine should be consistent. This quality is directly related to the manufacturing process or so-called GMP. In addition, the quality of the finished product is determined by various official tests such as identification, dissolution, uniformity of dosage units, assay, moisture and heavy metal determinations in order to confirm the products' identity, content and purity as well as various other chemical, physical and biological properties [9].

The biopharmaceutical characterization of herbal preparations is not as perspicuous as proprietary medicines since in many cases the activity of the preparation is attributed to the synergistic effect of multiple components or the preparation in its entirety. In many countries, long-standing use usually precludes the standard requirements for biopharmaceutical characterization (Chapter 1) [14]. As a result, some of the usual QC tests are deemed unsuitable or unnecessary or are of insignificant value.

WHO has issued general guidelines to assist in ensuring the safety and efficacy of CAM products. These include suitable plant identification tests (physical and/or chemical) and if possible, chromatograms of the purported active/s or marker compound/s present in products should be provided. Alternatively, characteristic fingerprints of the plant material used in the products are essential to prove botanical authenticity [9].

The USP [240] has included dry Ginkgo leaf in a section devoted to dietary supplements. Although a monograph for Ginkgo solid oral dosage forms has not been included, the USP does specify that some formulated dietary supplements such as Glucosamine tablets should undergo dissolution and weight variation testing or in some cases, only weight variation and disintegration testing are required such as for the specifications for American Ginseng tablets. The general test scheme for dietary supplements and pharmaceutical medicines are shown in Table 6.1 [239]. Hence, whilst several of the tests for the QC of dietary supplements in the USA have recently been described in the USP as shown in the table below, mandatory testing for those criteria has not yet been implemented. This situation is similar in other countries as well making some of those tests optional rather than mandatory.

Table 6.1. Pharmaceutical test scheme for pharmaceuticals and dietary supplements [239]

<b>Pharmaceuticals</b>	<b>Dietary supplements</b>
<301> Acid-neutralizing capacity	<1216> Tablet friability
<701> Disintegration	<2040> Disintegration and dissolution of dietary supplements
<711> Dissolution	<2091> Weight variation
<724> Drug release	<2750> Manufacturing practices of dietary supplements
<785> Osmolarity	<2090> Weight variations of dietary supplements
<905> Uniformity of dosage forms	
<1087> Intrinsic dissolution	
<1088> <i>In vitro</i> and <i>in vivo</i> dissolution evaluation of dosage forms	
<1090> <i>In vivo</i> bioequivalence guide	
<1216> Tablet friability	

The numbers in <> refer to the specific sections in the USP 28 [240]

In Germany, comprehensive quality criteria for each marketed phytopharmaceutical are specified in individual monographs according to a general guideline (Table 6.2). In addition, those preparations where the active constituents have been identified with certainty (e.g. Opium, Belladonnae folium) are required to undergo compulsory stability and *in vitro* dissolution testing [9].

Table 6.2. Quality criteria for herbal preparations in Germany [9]

<b>Criteria</b>	<b>Description</b>
<b>Clear scientific definition</b>	Plant specie, part of plant used, origin, time of harvesting and collection, processing procedures
<b>Proof of identity</b>	Authenticity of raw material using TLC*, HPLC, GC to produce comparative fingerprints Sensory features such as smell and colour
<b>Purity</b> General  Foreign materials  Radioactivity  Adulteration testing	Moisture, ash, physical constants, solvent residues, microbial contamination Lead: ≤ 5.0 ppm, cadmium: ≤ 0.2 ppm, mercury: ≤ 0.1 ppm, pesticides: ppm range (varies), Aflatoxin B1: ≤ 2 µg/kg, Aflatoxins B1, B2, G1, G2: ≤ 4 µg/kg Compulsory for phytopreparations which originate in contaminated locations If applicable/common occurrence
<b>Content</b>  Lead compounds or active constituents  Biological activity	Analytical methods for quantitative determination such as photometric analysis, TLC, HPLC, GC Biological assay required if appropriate analytical methods do not exist or are unacceptable

\*Thin layer chromatography

In Canada, NHP's are required to undergo identification, assay, heavy metal, microbial contamination and disintegration testing while leak rate and leachability, dissolution, uniformity of dosage units and moisture content testing is voluntary [241].

## 6.2 CLASSIFICATION OF HERBAL PREPARATIONS

The International Pharmaceutical Federation (FIP) has published guidelines for the classification of herbal preparations according to the amount of information available on the efficacy and chemical composition of the product. According to these guidelines, herbal preparations can be broadly classified into the following categories:

- A Extracts in which the known/accepted pharmacological activity is assigned solely to a single or group of constituents. Standardization may be achieved by adjusting the level of actives by addition of inert excipients or extracts which have higher or lower levels of the desired active compounds.
- B Extracts where the pharmacological effects are associated with constituents or groups of constituents which synergistically contribute to the desired effect of which the mechanism is largely unknown i.e. active marker/s have been identified. Standardization can be achieved by blending batches of either raw botanical materials or herbal preparations of higher and lower quality but the addition of inert excipients is not permitted.
- C Extracts where there is no documented evidence of an identified active constituent which is responsible for the therapeutic effect. Chemical compounds which may not contribute to any pharmacological activity are then selected as markers for GMP purposes [14, 242].

According to the European Agency for the Evaluation of Medicinal products (EMA), preparations which fall under categories B and C as well as immediate-release formulations are exonerated from dissolution testing. In addition, in preparations where the active constituent is highly soluble in aqueous media which have pH values consistent with that of the GIT, disintegration testing is then a sufficient indicator of GMP. Germany requires that all herbal preparations in category A undergo dissolution testing while those belonging to groups B and C are exempt, provided they are immediate-release formulations [242].

## 6.3 DISSOLUTION

*Ginkgo biloba* falls under category B and although the USP does not specify dissolution testing, 2 papers have appeared in the literature using this valuable QC tool.



Kressmann *et al.* [42] determined the release rate of terpene trilactones and flavonol glycosides in 14 commercial products using 0.1 M HCl as the dissolution medium at  $37 \pm 0.5^\circ\text{C}$  and a rotation speed of 100 rpm. The methodology was however poorly described and exactly how the flavonol glycoside content was determined is questionable. It can be assumed that each sample was hydrolyzed before injecting onto the HPLC system in order to determine the aglycone content and the dissolution profiles of 3 products showed total 'flavone glycoside' release. The manner in which the data were obtained for this profile is unclear. It is well known that the flavonoids are not soluble in acidic media. Moreover, the authors referred to the release rate of the glycosides in 14 products on at least 2 occasions without showing any data whilst a detailed table showed the release rates of the terpene trilactones at 15, 30 and 60 minutes sampling intervals. Since the dissolution rate may relate to the bioavailability of the active compounds and 3 of the products' dissolution profiles released less than 75% of the purported active constituents within 30 minutes compared to the other products, the authors questioned the efficacy of the various brands.

Two commercial products which demonstrated dissimilar *in vitro* quality in the above study were used for an *in vivo* clinical trial using 12 subjects to determine the pharmacokinetic parameters of GA, GB and BB as well as the significance of the differing profiles. One product contained EGb 761® which was used as the reference and the other was a standardized test product. Dissolution studies were performed to confirm the results of the first study under the same conditions and a second test using acetate buffer (pH 4.5) reiterated the original profiles. Each subject was given a 120 mg dose of either the test or reference product which was previously assayed in order to determine the GA, GB and BB content (Section 2.9 in Chapter 2). The bioavailability results were congruent with the dissolution data where the test product showed a poor dissolution profile in comparison to the innovator product. It was thus concluded that 2 products which contain the same amount of desired actives (i.e. they are pharmaceutically equivalent) do not necessarily have identical bioavailability [120].

Since dissolution testing of flavonols in Ginkgo formulations is conspicuously absent from the literature, one of the objectives was to determine optimal aqueous dissolution conditions to determine the release rate of rutin from 3 *Ginkgo biloba* solid oral dosage forms.

## **6.4 METHOD DEVELOPMENT**

### **6.4.1 Instrumentation**

A Hanson SR8 PLUS Autoplus™, Multifill™ and maximiser syringe fraction collector (Chatsworth, CA, USA) was used for the dissolution studies. A Waters 2690 Separations Module and Waters 2996 Photodiode Array equipped with an auto sampler, on-line degasser system and column heater was used for the HPLC analysis (Milford, MA, USA). A Mettler Dual Range Electronic Balance, Type AE 163 (Mettler Instruments AG, Griefensee, Zurich, Switzerland) was used for the weighing of reagents and standards and a Crison GLP21 pH Meter (Crison, Barcelona, Spain) was used to measure and adjust

the pH of relevant solutions. A Phenomenex Luna (5 µm) C<sub>18</sub> column with dimensions 250 x 2.0 mm I.D. was used to effect the separation.

#### 6.4.2 Materials and reagents

Formic acid, hydrochloric acid and phosphoric acid (analytical grade) were purchased from Merck (Darmstadt, Germany) and acetonitrile (HPLC grade) and sodium hydroxide pellets were obtained from BDH chemicals (Poole, UK). Rutin (95%) and quercetin (95%) were purchased from Sigma (Missouri, USA). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA) and PVDF filters were purchased from the same supplier. Three commercial products were procured from a local pharmacy in Grahamstown, South Africa (see Table 6.3).

Table 6.3. *Ginkgo biloba* commercial products selected for dissolution profiling

Manufacturer	Name of product	Dosage form	Label claim	Daily dose	Batch number	Extract
Nrf Herbal <sup>1</sup>	<i>Ginkgo biloba</i>	Tablet	Each tablet contains 350mg <i>Ginkgo biloba</i> , 50mg <i>Ginkgo biloba</i> extract 24% and 240mg FoodMatrix™	1 - 2 tablets daily	NTN092PW	K
Formule Naturelle <sup>2</sup>	<i>Ginkgo biloba</i>	Tablet	Standardized to contain 24% Ginkgo Flavonglycosides	1 tablet tds <sup>4</sup> , ac <sup>5</sup>	3011	L
Vital <sup>3</sup>	<i>Ginkgo biloba</i>	Capsule	Each capsule contains <i>Ginkgo biloba</i> extract equivalent to 6 000 mg of herb powder	1 capsule daily	BO400209B	M

<sup>1</sup>Nrf Herbal, Centurion, South Africa; <sup>2</sup>Formule Naturelle, Hout Bay, South Africa; <sup>3</sup>Vital Health Foods (EDMS) BPK, Kuils River, Western Cape, South Africa; <sup>4</sup>tds, three times daily; <sup>5</sup>ac, before meals

#### 6.4.3 HPLC conditions

Separation of rutin and quercetin was achieved within 9 minutes using a mobile phase of acetonitrile-0.3% formic (25:75) at a flow rate of 400 µl/min. For the analysis of rutin only, the acetonitrile fraction was decreased to 14% in order to increase the elution time to 6.5 minutes to ensure peak purity. The column temperature was maintained at 45°C and the PDA scanning range was from λ 200 - 400 nm.

#### 6.4.4 Dissolution conditions

Dissolution tests were performed using the Type 2 (Paddle) apparatus of the USP 28 (<711>) [240]. Each of the 8 vessels contained 900 ml of the appropriate dissolution media and the temperature of the vessel contents was maintained at 37 ± 0.5°C. The rotation speed of the paddles was 100 rpm. Volume

adjustments of the media were made by replacement of the withdrawn sample volume with fresh buffer at the same pH.

#### 6.4.5 Selection of pH

##### 6.4.5.1 *Experimental procedure*

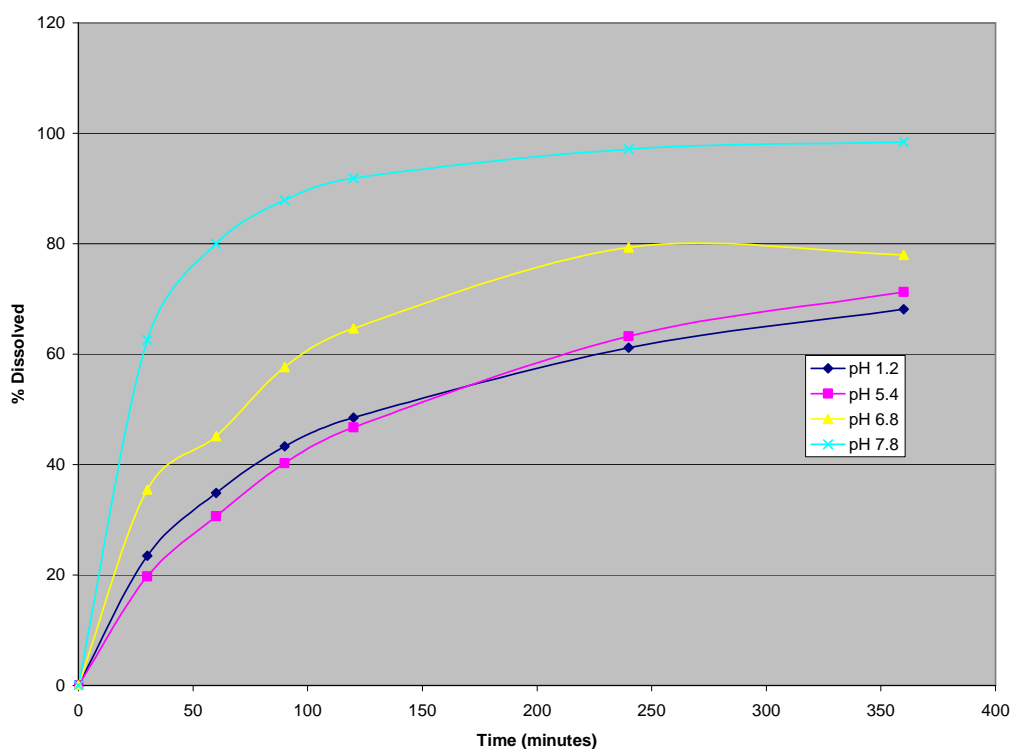
Four pH values of 1.2, 5.4, 6.8 and 7.8 were selected for the initial experiment. A 0.1 M HCl solution was used for the dissolution medium at pH 1.2 and 0.1M phosphoric acid was used for preparation of the remaining media. Adjustments of the pH were made with sodium hydroxide pellets. Nrf was selected for method development since it consistently demonstrated higher rutin and quercetin content compared to the other available commercial products. Eight vessels were available and for each pH value 2 vessels were used. One of each of the duplicate vessels was allocated for the reference standard (weighed amounts of rutin and quercetin) whereas 4 Nrf tablets were added to each of the other vessels at the different pH's. The reference standards, rutin (23 mg) and quercetin (1.4 mg) representing typical amounts present in 4 Nrf tablets were added to investigate the solubility of these components under the conditions of the dissolution tests.

The results of this study provided information for use for a subsequent study to examine the release profiles of rutin from Nrf at pH 6.8, 7.4 and 7.8. Three vessels were allocated for each of pH 6.8 and 7.8 and 2 vessels for pH 7.4 in order to obtain average values and % RSDs by generating data at 6 different intervals of time for each sample (30 - 360 minutes). All withdrawn samples were analyzed in triplicate.

##### 6.4.5.2 *Results*

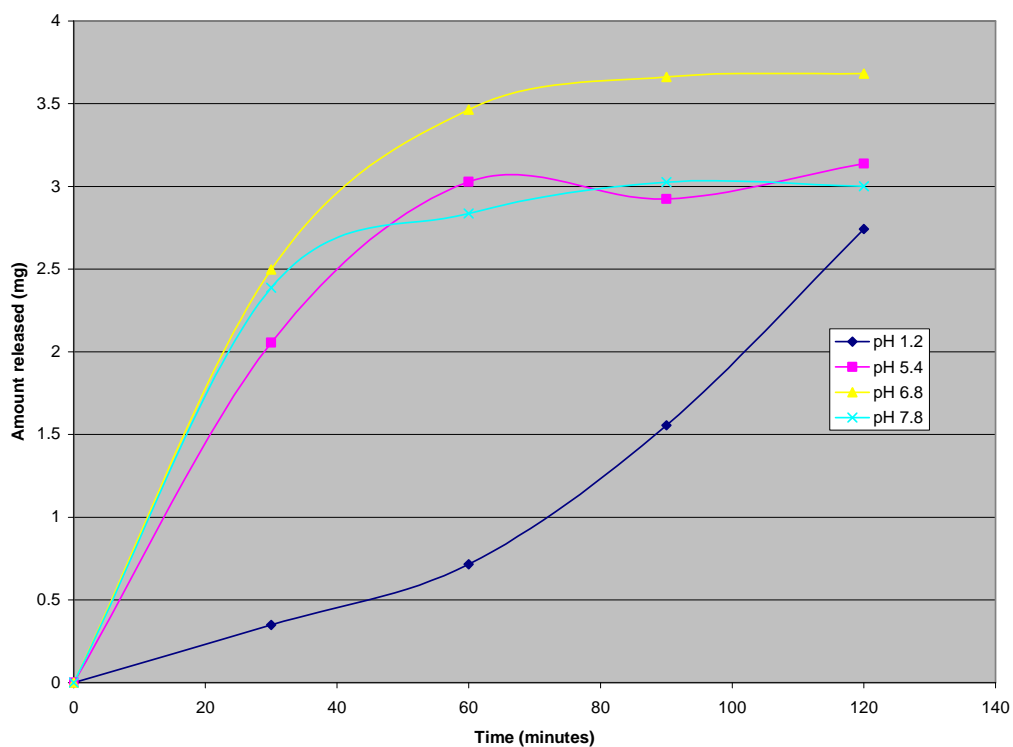
Figure 6.1 shows the results obtained from the rutin reference standard at 4 different pH's. The solubility and the dissolution rate of rutin is seen to increase with an increase in pH. However, solid reference material was still visible in the dissolution media at pH 1.2 after 24 hours. At pH 7.8, 80.0% of the rutin was dissolved after 60 minutes whereas at the other pH values, much less rutin was dissolved at that same time. Quercetin, in the amounts present in 4 Nrf tablets (1.4 mg total) was not detectable in the concentrations present in the volume of dissolution media in each vessel (900 ml).

Figure 6.1. Dissolution profiles of rutin in various dissolution media



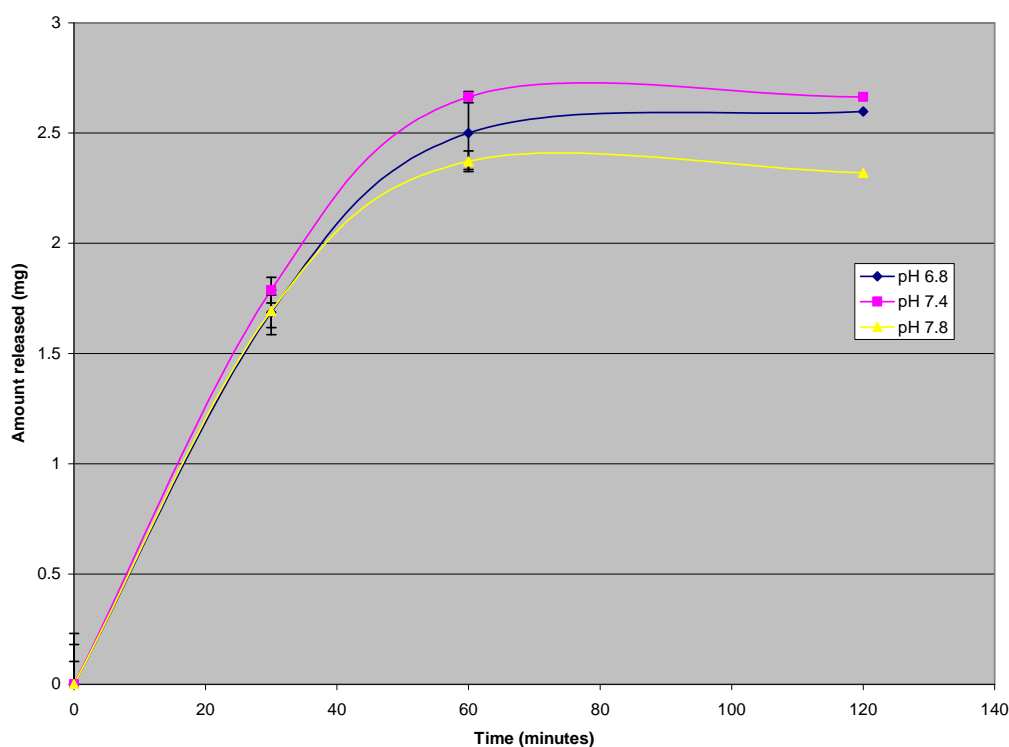
Similar release profiles of rutin were observed from the Nrf tablets monitored over a shorter time frame (Figure 6.2). However, rutin showed a relatively higher release at pH 6.8 compared to H 7.8. The profile at pH 1.2 showed a much slower release compared to the rutin reference standard profile at the same pH.

Figure 6.2. Dissolution profiles of rutin in dissolution media of various pH values



Based on the above results, 3 pH values (6.8, 7.4 and 7.8) were selected and release profiles were once again determined. A new Nrf product with the same batch number was used for this experiment. Less rutin was released than the previous Nrf lot (Figure 6.3) over the same time period. The release rate was marginally better at pH 7.4 than at the other 2 pH values.

Figure 6.3. Dissolution profiles of rutin from Nrf at pH 6.8, 7.4 and 7.8



#### 6.4.5.3 Discussion

The pH range was selected according to the FDA guidance for industry where the recommended range is 1 - 7.5. The pH values were chosen according to the  $pK_a$  of the drug [243]. The flavonols have  $pK_a$  values of approximately 6.8 and solubility was determined at  $pH = pK_a$  (6.8),  $pH = pK_a + 1$  (7.8),  $pH = pK_a - 1$  (5.4) and then at pH 1.2. As previously mentioned, the flavonols are soluble in aqueous alkaline solutions [60] and it was therefore predictable that the best solubility of the reference standards was observed at higher pH values. The release of rutin from the tablet matrix was however better at  $pH = pK_a$  rather than at pH 7.8 which was optimal for the standards.

The second experiment also showed more release at pH 6.8 than 7.8. However, pH 7.4 showed a marginal improvement in release of rutin and although only 2 vessels were used, the % RSD values were lower. The optimal buffer pH was therefore selected as pH 7.4. Possible reasons why less rutin was released from the tablets compared to the previous experiment may be due to the fact that the previous Nrf lot had been opened approximately 1 month prior to dissolution assessment and the tablets (which were stored without the presence of a desiccant) may therefore have absorbed moisture. The possibility that the 2 containers actually contained different batches of raw material and/or different excipients were used which possibly altered the release of rutin cannot be discounted. Both experiments were conducted for at least 8 hours with the maximum amount of rutin being released after 2 hours in the first experiment as opposed to 1 hour in the second experiment.

#### 6.4.6 Dissolution profiles of commercial *Ginkgo biloba* products

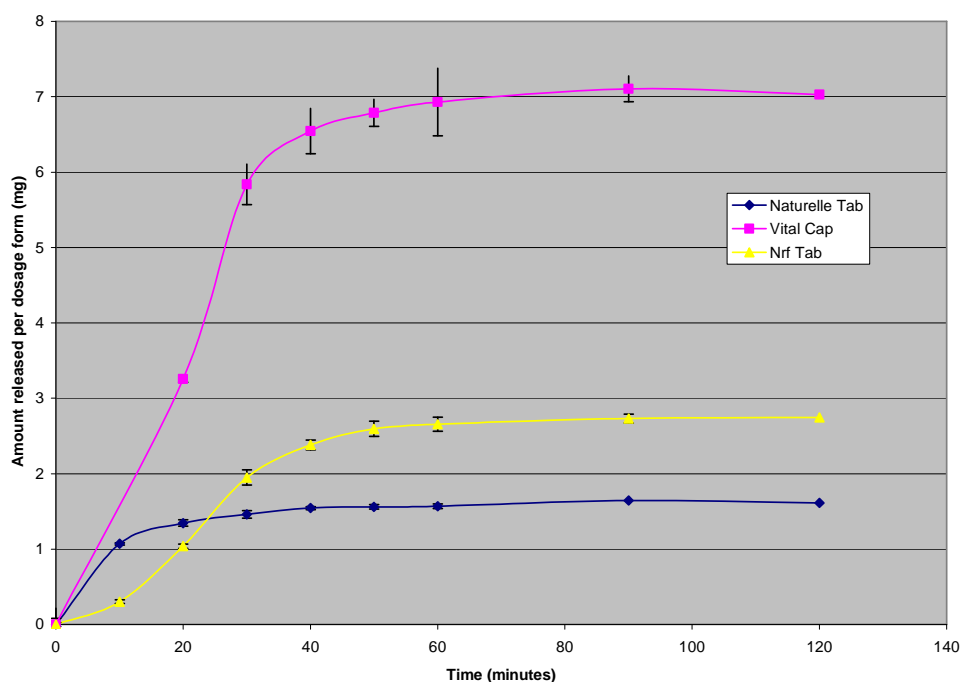
##### 6.4.6.1 *Experimental conditions*

The HPLC and dissolution conditions were as described in sections 6.4.3 and 6.4.4. The dissolution medium consisted of a 0.1M phosphoric acid (pH 7.4). Either 4 tablets or 4 capsules were placed into each of 4 vessels at 37°C. Four vessels were allocated for each of the 3 products, Naturelle, Vital and Nrf. Samples were withdrawn at 10 minute intervals for the first hour, followed by half hourly intervals for the next hour and then samples were taken every 2 hours until 8 hours. Fresh buffer was used to replace the volume removed by sampling and corrections for dilution were made in the calculations.

##### 6.4.6.2 *Results*

Figure 6.4 A shows the dissolution profiles of each of the 3 dosage forms. The RSDs for all data points ranged between 1.1 and 8.6%. The results from the 10 minute sampling time for Vital was not reported due to the high RSD (>20%). Vital is formulated as an oily liquid in a soft gelatin capsule and variable data were therefore expected especially during the first few sampling times as the capsule disintegrated almost immediately, releasing the oily mass into the aqueous media. The Nrf tablet gave results similar to those observed in the previous section with a 3.1% difference in the maximum amount of rutin released within 60 minutes. There were major disparities in amount of rutin released (Figure 6.4). However, each profile reached a plateau after 60 minutes and minimal further release of rutin was seen for all dosage forms.

Figure 6.4. Dissolution profiles of 3 commercial *Ginkgo biloba* products



### 6.3.6.3 Discussion

From the release profiles it can be assumed that all 3 dosage forms were immediate release formulations since most of the rutin, if not all, was released after 60 minutes. None of the product label's expressed the rutin content present in the dosage forms and comparisons using the label claims were therefore not possible.

The solubility of rutin in aqueous alkaline solutions was demonstrated by determining the dissolution of rutin reference standard in the various media over an initial 24 hour period. It was obvious that the matrix affected the release of rutin and although the optimal pH was selected based on the release of this marker from Nrf, profiles of 2 other dosage forms, a plain coated tablet (Naturelle) and a liquid extract in a soft gelatin coat (Vital) were additionally obtained. Similar profiles and acceptable % RSDs were obtained at each sampling interval hence showing applicability of the method for determining dissolution of rutin in *Ginkgo biloba* formulations.

This dissolution test was simple and economical to perform and no sample hydrolysis was required. Since rutin is probably the most dominant single contributor to the quercetin aglycone content in *Ginkgo biloba* dosage forms assayed and its absorption, distribution, metabolism and excretion (ADME) mechanisms are not yet fully known, its release characteristics provide valuable information relating to the biopharmaceutical properties of *Ginkgo* formulations.

Since *Ginkgo biloba* falls under category B according to the FIP classification system and these products are immediate-release formulations, these commercial products would be exempt of



dissolution testing in both Europe and Germany and disintegration testing would suffice. The USP does not include Ginkgo formulated as dosage forms in its monograph.

## 6.5 TABLET HARDNESS AND DISINTEGRATION

### 6.5.1 Instrumentation

Tablet hardness was determined using an Erweka TBH 28 No 50691 and an Erweka 2T61 version 1.45 of 12.08.96 (both supplied by Optolabor, Randburg, South Africa) was used for tablet disintegration assessment.

### 6.5.2 Materials

Two products were selected for the determination of tablet hardness and disintegration, Ginkgoforce and Nrf. Nrf contained consistently higher levels of positive markers when analyzed both qualitatively and quantitatively while Ginkgoforce demonstrated much lower content. A similar trend was seen when these products were qualitatively analyzed for negative marker content in section 3.15 of Chapter 3 although the content disparity was less apparent. Table 6.4 provides information on each of these products.

Table 6.4. *Ginkgo biloba* products selected for tablet hardness and disintegration testing

Company	Name of product	Dosage form	Label claim	Daily dose	Batch	Extract
Bioforce <sup>1</sup>	Ginkgoforce	Tablet	Each 250mg tablet is equivalent to 10 drops of Ginkgoforce liquid	2 tablets tds*	406088E	K
Nrf Herbal <sup>2</sup>	<i>Ginkgo biloba</i>	Tablet	Each tablet contains 350mg <i>Ginkgo biloba</i> , 50mg <i>Ginkgo biloba</i> extract 24% and 240mg FoodMatrix™	1 to 2 tablets or as directed by your healthcare practitioner	2H2044PW	L

<sup>1</sup>Bioforce S.A. (Pty) Ltd., Midrand, South Africa; <sup>2</sup>Nrf Herbal, Centurion, South Africa; \*three times daily

### 6.5.3 Tablet hardness

The USP does not specify tablet hardness parameters for dietary supplements. Twenty tablets of each of Nrf and Ginkgoforce tablets were determined and tablet hardness was found to be 70.0 and 69.8 Newton, respectively. The RSD was 23.23% for Nrf and 7.12% for Ginkgoforce and the high RSD of Nrf may be interpreted as a lack of suitable manufacturing QC and/or inappropriate storage conditions.

#### 6.5.4 Tablet disintegration

Tablet disintegration was determined according to the specifications for uncoated and film-coated tablets in the USP 28 (<2040>) [240], using Apparatus A. Distilled water, maintained at  $37 \pm 0.5^\circ\text{C}$  was used as the immersion fluid. One tablet was placed in each of the six baskets and then raised and lowered vertically at a constant frequency within the disintegration medium for the allocated time period. According to the USP, 6 tablets should disintegrate completely within 20 minutes. If 1 or 2 of these tablets fail to disintegrate, the same test should be repeated on an additional 12 tablets. Of the 18 tablets tested, 16 must disintegrate completely within 20 minutes in order to meet the requirements. The results for both Nrf and Ginkgoforce are shown in Table 6.5.

Table 6.5. Disintegration results of 2 *Ginkgo biloba* dosage forms

Nrf		Ginkgoforce	
Test	Disintegration time (minutes)	Test	Disintegration time (minutes)
1 (20 minutes)	None of the tablets disintegrated	1 (20 minutes)	3.22 2.36 2.54 4.50 3.36 3.16
2	39.03 31.36 32.07 43.03 44.24 22.03	2	4.53 3.15 4.55 4.30 5.34 4.01
3	27.43 24.33 24.31 29.03 25.07 28.33	3	3.56 1.56 4.48 4.54 -* 2.36
4	44.46 58.48 46.44 40.04 38.46 28.23	4	Not performed
<b>Average (n=18)</b>	34.80	<b>Average (n=17)</b>	3.62
<b>SD</b>	9.9	<b>SD</b>	1.0
<b>% RSD</b>	28.5	<b>% RSD</b>	28.4

\* No result was recorded

Not a single Nrf tablet disintegrated within the specified USP time of 20 minutes and the disintegration time was then lengthened to 60 minutes in order to obtain values. Disintegration times for 18 tablets ranged from 22.0 to 58.5 minutes with a RSD of 28.52%.

All 6 tablets of the first test for Ginkgoforce disintegrated within 4.5 minutes and Test 1 was therefore sufficient to meet the USP specifications. An additional 2 tests were performed in order to obtain a comparative % RSD to Nrf. It is interesting to note that the RSDs of both products were very similar with 28.5% reported for Nrf and 28.4% for Ginkgoforce.

Since the average mass of the Nrf tablets were twice that of the Ginkgoforce tablets and they were also larger, the thickness and diameters of 20 tablets from both dosage forms were then compared using a Mitutoyo MFG CO.LTD PB-1B caliper in order to explain the large variations in the disintegration results. These measurements are shown in table 6.6.

Table 6.6. Diameter and thickness measurements of the 2 dosage forms

Parameter	Nrf (n=20)		Ginkgoforce (n=20)	
	Diameter (cm) (mm)	Thickness	Diameter (cm) (mm)	Thickness
Average	1.38	0.79	0.95	0.87
SD	0.02	0.10	0.01	0.02
% RSD	1.5	12.5	1.1	2.3

The high variability in the thickness of the Nrf tablets may explain the large range of disintegration times observed for this formulation. Although unlikely, Nrf had a convex surface which might also have contributed to its longer disintegration times.

## 6.6 WEIGHT VARIATION

The weight variation tolerance for uncoated and film-coated botanical dosage forms as specified in the USP 28 (<2091>) [240] was used to compare various batches of tablets used in the validation of previously described methods. The results are shown in Table 6.7.

Table 6.7. Weight variation of some *Ginkgo biloba* dosage forms

Product	Validation	Batch number	Average $\pm$ SD (mg) (n=20)	% RSD	Inter-batch % RSD
Ginkgoforce	HPLC LC-MS	210120E	251.2 $\pm$ 2.3	0.9	-
Holotropic	HPLC LC-MS, ELSD	0318	493.8 $\pm$ 11.4	2.3	1.7 (n=2)
		0319	481.8 $\pm$ 7.3	1.5	
Naturelle	HPLC LC-MS, ELSD	82060	551.2 $\pm$ 5.2	0.9	0.1 (n=2)
		82060	552.9 $\pm$ 4.5	0.8	
Nrf	HPLC LC-MS, ELSD, CE	200303047	569.8 $\pm$ 17.5	3.1	1.9 (n=3)
		MTM092PW	549.3 $\pm$ 22.4	4.1	
		NTN042PW	563.0 $\pm$ 6.3	1.1	

According to the USP, 20 tablets of each dosage form must be weighed individually and not more than 2 of the tablets are permitted to deviate from the mean by more than 7.5%. All of the tested dosage forms met the criteria of weight variation.

## 6.7 DISCUSSION AND CONCLUSION

Dissolution, tablet hardness, disintegration and weight uniformity testing were performed on various *Ginkgo biloba* dosage forms.

Dissolution testing is becoming increasingly important in the QC of CAM since it gives an indication of the rate and amount of the active compound/s which is/are released over a specific time frame. Dissolution of 3 different formulations revealed that all were immediate-release dosage forms and therefore would normally be exempt from dissolution testing (*vide infra* p. 236). In such cases, disintegration testing may be considered a suitable indicator of GMP.

Disintegration tests were performed on 2 products, one which consistently contained higher levels of positive markers (Nrf) and one which contained very little flavonol components (Ginkgoforce). Nrf failed to meet the USP 28 disintegration specifications with an average disintegration time of 34.8 minutes (n=18) while the Ginkgoforce tablets (n=17) disintegrated well within 20 minutes. It is obvious from these results that a lack of QC is implied for the Nrf formulation. The size and shape of the formulations may have had an influence on the disintegration rate while tablet hardness did not directly influence this experiment.

All tablet batches tested for weight uniformity complied with the USP 28 specifications.

The issue of bioavailability and bioequivalence of herbal preparations has gained much interest in countries such as Germany where herbal products are regulated as proprietary medicines. The majority of Ginkgo clinical trials have used the innovator product, Egb 761® and the question of bioequivalence has arisen since many products have been subsequently formulated based on the standardized content requirements of the German and European monographs i.e. they are pharmaceutically equivalent. As shown by Kressman *et al.* [120], pharmaceutically equivalent products do not necessarily imply bioequivalence since different extraction and manufacturing procedures may alter the release characteristics of the active markers. The correlation between *in vitro* dissolution testing and *in vivo* bioavailability if shown to be consistent, proves that dissolution is an extremely economical and valuable tool to assess the quality of the finished product and may be an important indicator of the bioavailability of the preparation.

## 6.8 HYDROLYSIS METHOD VERSUS INTACT FLAVONOL GLYCOSIDE DETERMINATION

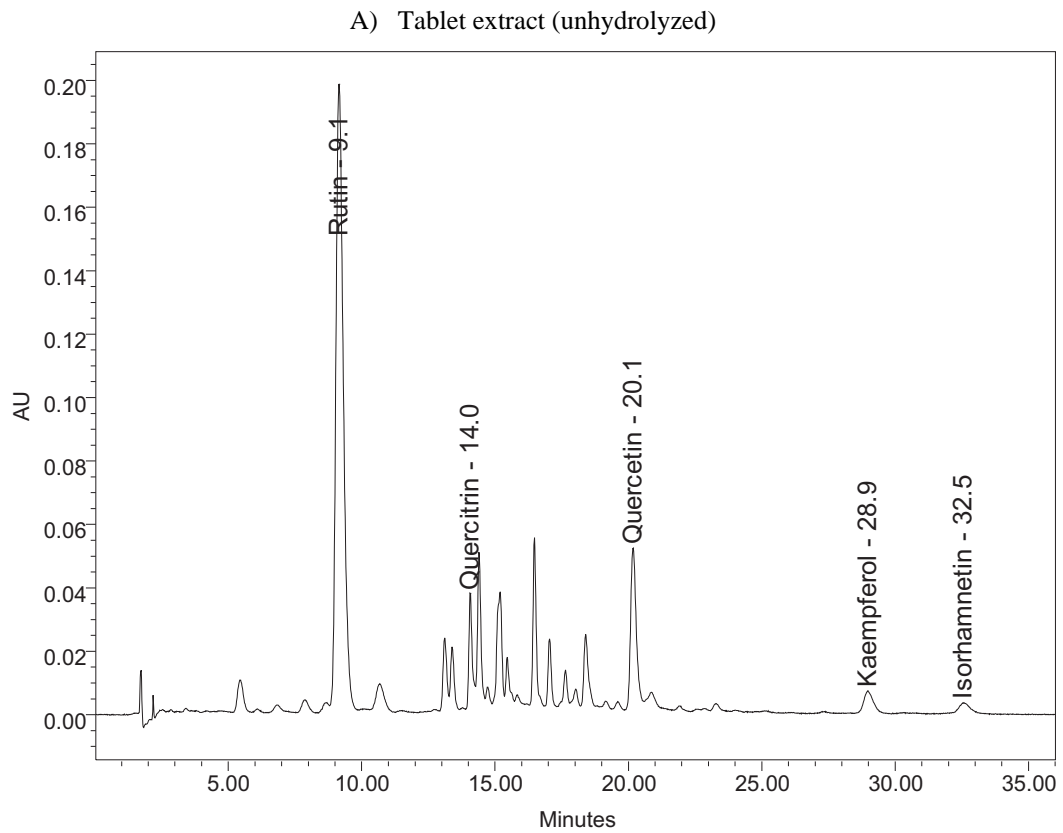
As mentioned previously, conventional quantification of the flavonol glycosides for marketing purposes is determined indirectly from the aglycone content after acid hydrolysis of the sample. The presence of small amounts of other aglycones such as apigenin and luteolin are also often present [52, 61]. However, the true flavonol content following hydrolysis may be exaggerated as previously

discussed (Chapter 3). In the past, these shortcomings were not considered a serious problem provided all manufacturers adopted the same QC technique and, according to Sticher *et al.* [61], “As long as there is no exact knowledge on the biological activity of individual flavonol glycosides, standardization based on these (aglycone) compounds is acceptable from a pharmaceutical point of view.”

Although sparse, evidence exists which suggests that rutin does in fact contribute to the activity of herbal preparations [84]. Moreover, recent articles have also suggested that commercial products may be adulterated with rutin in order to register higher “Ginkgo flavonol” glycoside content, especially when QC involves spectroscopic analysis [22, 162, 170]. Although conclusive evidence concerning the individual biological activity of the flavonol glycosides is still amiss, these recent studies on rutin have demonstrated that rapid advancement is being made in this area and to exclude the glycosides, particularly rutin from routine QC analysis is considered presumptuous.

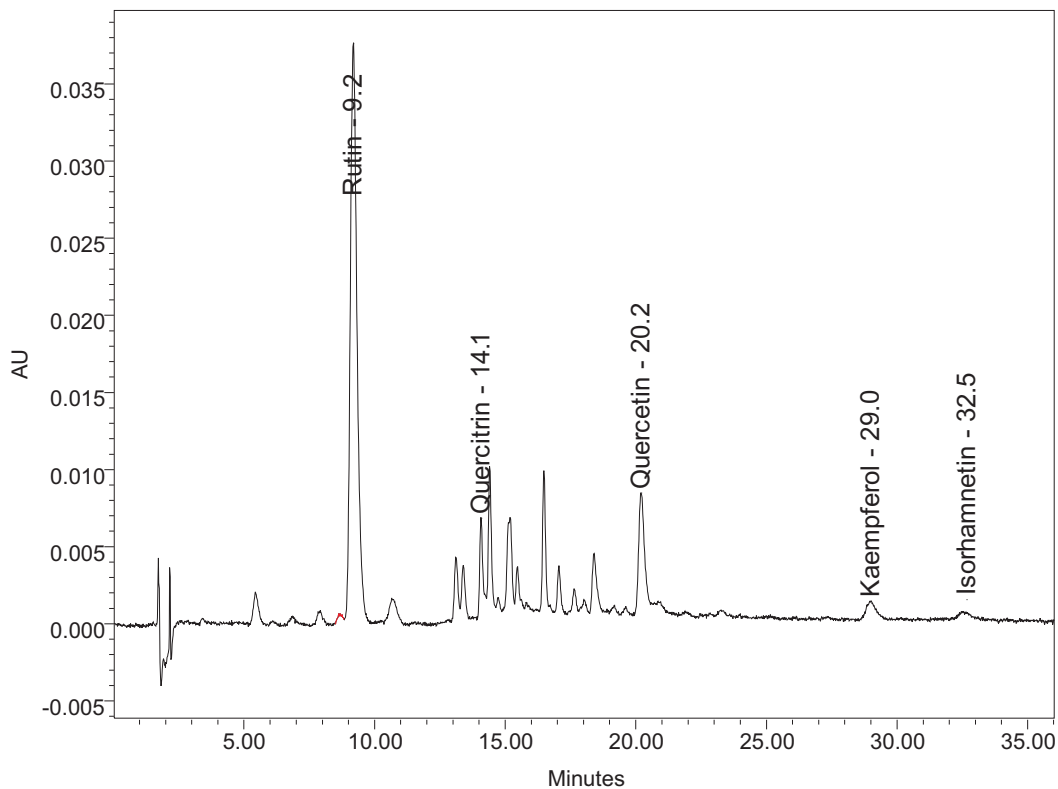
To illustrate this point, a commercial product used in the CE validation of rutin and quercetin (Nrf: batch number: NTN042PW) was extracted with methanol as described in section 3.10.3.1 and reconstituted in 25 ml methanol. Five millilitres of the extract was then hydrolyzed based on a similar procedure described by Li and Fitzloff [188] where 5 ml of the extract was refluxed with 20 ml 4N HCl:MeOH (1:4) (v/v) for 30 minutes. The hydrolysis was however incomplete and the acid strength was increased to 6N HCl:MeOH (1:4) (v/v). Complete hydrolysis was achieved after 90 minutes. A blank sample was also prepared by hydrolysis with methanol only. The results are shown in Figure 6.5.

Figure 6.5. HPLC-UV chromatograms of an unhydrolyzed sample (A), blank standard (B) and acid hydrolyzed tablet (C) extract ( $\lambda$  370 nm)

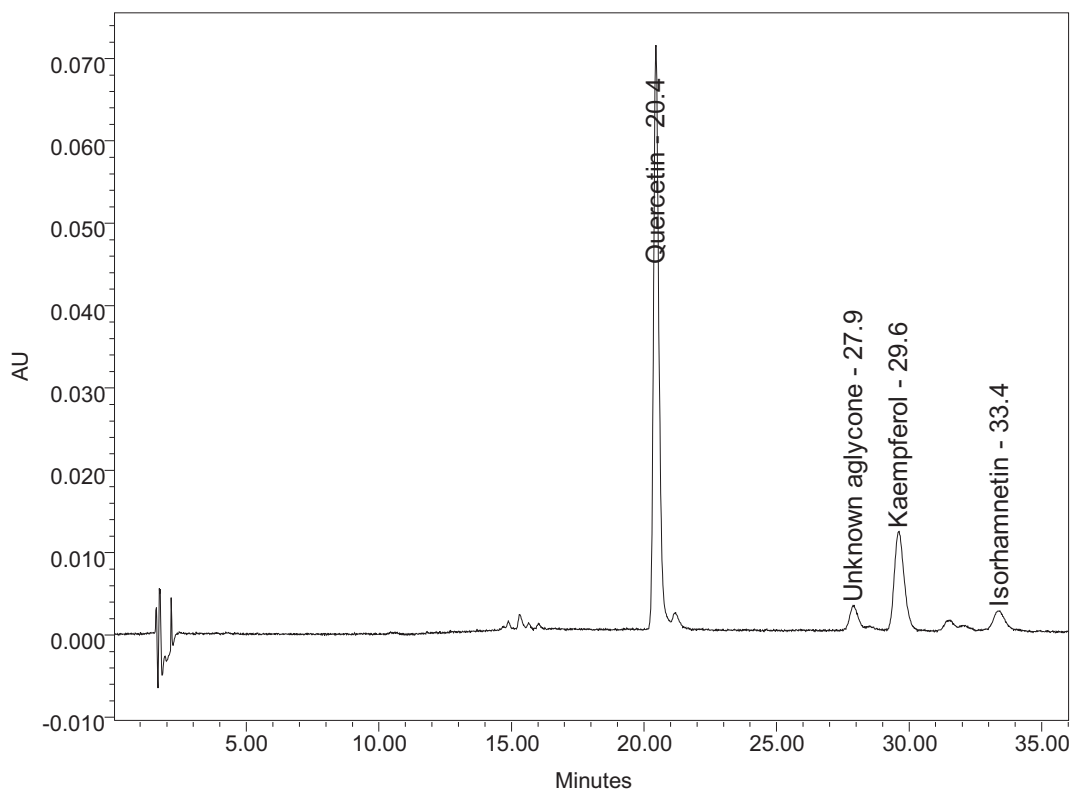


Conditions: Column: Phenomenex Luna ( $5\ \mu\text{m}$ )  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature:  $45^\circ\text{C}$ ; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate:  $400\ \mu\text{l}/\text{min}$ ; Injection volume:  $5\ \mu\text{l}$ ; Detection  $\lambda$ : 350 nm.

B) Tablet refluxed in methanol only- no acid (blank)



C) Acid hydrolyzed tablet



Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 400 $\mu$ l/min; Injection volume: 5  $\mu$ l; Detection  $\lambda$ : 350 nm.

From these results it can be seen that a quantifiable amount of quercetin, kaempferol and isorhamnetin were intrinsically present in the extract before hydrolysis (Figure 6.5 A) and that rutin was the main contributor to the quercetin peak after acid hydrolysis (Figure 6.5 B and C). Identification of the aglycones after acid hydrolysis was facilitated by the UV spectra since a slight shift in the retention times of kaempferol and isorhamnetin was seen. A small peak eluted at 27.9 minutes which was not present in the blank and could also possibly be a flavonol aglycone compound such as apigenin, myricetin or luteolin.

Of the 22 flavonol glycosides identified in *Ginkgo biloba* leaf extracts, 8 are quercetin glycosides, 7 kaempferol glycosides, 3 isorhamnetin glycosides, 2 glycosides of myricetin and 1 luteolin and 1 apigenin glycoside have been found [62]. Although all are not present in equal amounts, based on this composition, the quercetin peak should be the most prominent, followed by kaempferol and then isorhamnetin. The large discrepancy between the quercetin and kaempferol peak heights therefore raises concern and the unhydrolyzed sample reveals that the greatest contributor to the large quercetin peak is in fact rutin and not any of the other 7 quercetin glycosides. Although substantial amounts of rutin have been identified in leaf extracts, nowhere in the published literature has rutin been identified as the most dominant flavonol and in the fingerprint of the 33 identified flavonoids by Haslet *et al.* [62], the rutin peak is of similar intensity compared to the other glycosides. Moreover, in the HPLC-PDA profiles of the various *Ginkgo biloba* dosage forms analyzed in Chapter 4 (Figure 4.5), it is fairly obvious that rutin in Nrf out-scales the other flavonol glycosides in the chromatogram and based on these findings, this product may be suspected of adulteration. Figure 4.6 also illustrates that fingerprinting of crude *Ginkgo biloba* extracts is essential for identifying product fortification.

Since the ratio of glycosides to aglycones is an important stability indicator of suitable storage and extraction conditions, analysis of the intact glycosides is certainly warranted for comprehensive QC. For example, Ginkgoforce has consistently shown a high aglycone to glycoside ratio which is clearly illustrated by quantification of the selected flavonol aglycone and glycoside markers in *Ginkgo biloba* commercial products in Chapters 4 and 5 where all aglycone marker compounds were detected and glycoside content was remarkably low in comparison to the other commercial products. As the aglycones contribute to only 0.2 - 0.4% (w/w) of Ginkgo leaves [61] and since the results of the HPLC analyses revealed that the content of quercetin was comparable to both rutin and quercitrin (Figure 4.5 B and Table 4.7), the quality of this preparation is of great concern. Moreover, acid hydrolysis results alone would have given an unrealistic and gross exaggeration of the true glycoside content.

While criticism of the conventional quantification method is indeed warranted at this point, the replacement of this well-accepted method is not contested. Instead it is suggested that comprehensive routine QC of *Ginkgo biloba* commercial products should include one or more flavonol glycosides and that rutin currently proves to be an excellent QC indicator.



## 6.9 ANALYTICAL METHOD COMPARISON FOR QC OF *GINKGO BILOBA* DOSAGE FORMS

### 6.9.1 CE Analysis

Although CE is a fairly new technique which was only introduced in the 1980s [162], it presents distinct and unique advantages compared to HPLC and is particularly useful for the analysis of complex mixtures of compounds such as herbal preparations due to its versatility, high resolution and efficiency, speed and low cost [162, 216]. A limitation of this analysis method is that typically small sample volumes (5 - 10 nl [136]) are required to be injected onto the capillary in order to maintain high efficiency with a subsequent loss of sensitivity [139]. Furthermore, use of optical detection is also associated with reduced sensitivity due to the very small pathlength provided by small capillary diameters. Problems relating to repeatability and absolute precision are also frequently documented [162].

In terms of the analysis of *Ginkgo biloba* solid oral dosage forms, this technique proved to be extremely useful for the fingerprinting of both positive and negative markers particularly due to its versatility, high resolution and low cost. The actual analysis of the separations demonstrated shorter run times compared to HPLC, however conditioning of the capillary between consecutive injections required to achieve acceptable reproducibility detracted from its overall speed. Sensitivity was enhanced by using a square capillary to increase the optical path length through the detector cell and the terpene trilactones were detected in the extracts without interferences by using RF-CDMEKC where the hydrophilic components migrated at a slower velocity, facilitating maximal separation. The poor response of the terpene trilactones is only partially due to CE constraints however and the low UV absorbance of these compounds is mostly responsible for their difficult detection.

The high efficiency of this technique facilitated detailed profile comparisons of the flavonoids present in *Ginkgo biloba* extracts, however unequivocal identification of the peaks of interest using UV detection alone was difficult. Although a micellar marker was used to calculate relative migration velocity in order to eliminate shifts in retention time, slight changes in elution times may possibly have resulted in erroneous peak identification and spiking of the extracts was pivotal to ensure confirmation of the peaks of interest during analysis. Due to these constraints and the fact that the terpenoids possess poor chromophores, it is suggested that simple UV detection is replaced with more sophisticated detection systems when analyzing *Ginkgo biloba* leaf extracts or dosage forms. PDA detection, for example, would allow spectral confirmation of peaks. Clearly, hyphenated CE techniques with more complex detectors such as MS would be preferable [162].

Two marker compounds were successfully used for the validation of the RF-CDMEKC method and notorious precision and accuracy problems were successfully overcome by inclusion of salicylic acid as the IS.

The use of non-aqueous CE for the fingerprinting of the ginkgolic acids in *Ginkgo biloba* dosage forms (section 3.14 in Chapter 3) is ideal for these compounds as their structural similarity and hydrophobicity resulted in more complicated and/or longer analysis times when using RP-HPLC and exorbitant expenditure on analysis of these compounds using LC-MS is currently unwarranted. From a QC perspective, a simple, rapid and economic fingerprinting method is sufficient to evaluate whether an exclusion step was part of manufacturing process and this method demonstrated definite potential to be used for routine QC analysis of the negative markers in *Ginkgo biloba* solid oral dosage forms.

Although CE does present definite challenges, some consideration should be given to the fact that it is a relatively new technique and initial difficulties are thus inevitable. Advances are continuously being made in this field and CE has irrefutably demonstrated in the last decade that it has enormous potential in the future for analysis of herbal preparations [216].

## 6.9.2 HPLC and LC-MS

### 6.9.2.1 Analysis of the flavonols

More direct comparisons can be made between LC-MS-MS and HPLC-PDA analysis of *Ginkgo biloba* commercial products since the same samples were used for both methods. However, samples were frozen at -60°C after the HPLC determinations for a period of 1 month before the LC-MS-MS analysis. The dosage form contents were determined on each of the 3 validation days using HPLC-PDA detection while that of LC-MS was determined on the last day only. The HPLC-PDA data are shown in Table 6.8 and the LC-MS data in Table 6.9.

Table 6.8. Marker content of the various dosage forms analyzed by HPLC-PDA detection

Product (n=9)	Rutin (µg ± SD)	Quercitrin (µg ± SD)	Quercetin (µg ± SD)	Kaempferol (µg ± SD)	Isorhamnetin (µg ± SD)
Holotropic	213.0 ± 7.4	38.4 ± 2.2	24.4 ± 4.2	-	-
Formule Naturelle	1240.0 ± 5.2	508.0 ± 9.5	+	-	-
Ginkgoforce <sup>1</sup>	35.4 ± 2.2	29.5 ± 1.4	26.35 ± 5.2	15.4 ± 2.1	+
Nrf Herbal	5690.0 ± 5.1	318.2 ± 16.8	352.7 ± 7.7	95.2 ± 2.4	51.5 ± 2.6
Bioharmony	293.2 ± 8.8	205.50 ± 6.2	ND	-	-
Vital	4200.0 ± 377.2	1074.2 ± 96.2	307.4 ± 11.0	137.0 ± 8.1	49.9 ± 8.7

<sup>1</sup>Amount expressed per 2 tablets; ND, not determined due to interferences

Table 6.9. Marker content of the various dosage forms analyzed by LC-MS-MS

Product (n=3)	Rutin ( $\mu\text{g} \pm \text{SD}$ )	Quercitrin ( $\mu\text{g} \pm \text{SD}$ )	Quercetin ( $\mu\text{g} \pm \text{SD}$ )	Kaempferol ( $\mu\text{g} \pm \text{SD}$ )	Isorhamnetin ( $\mu\text{g} \pm \text{SD}$ )
Holotropic	268.0 $\pm$ 77.3	15.9 $\pm$ 0.9	+	-	-
Formule Naturelle	1100.0 $\pm$ 9.1	78.6 $\pm$ 3.7	+	-	+
Ginkgoforce	38.6 $\pm$ 0.9	4.6 $\pm$ 0.5	+	+	+
Nrf Herbal	6040.0 $\pm$ 109.0	76.5 $\pm$ 1.5	328.0 $\pm$ 6.3	85.0 $\pm$ 3.7	42.9 $\pm$ 2.5
Bioharmony	347.0 $\pm$ 15.9	35.5 $\pm$ 2.5	-	-	-
Vital	4750.0 $\pm$ 500	143.7 $\pm$ 19.2	224.0 $\pm$ 16.1	113.2 $\pm$ 7.2	+

From these results, where the markers were successfully quantified using both LC-MS and HPLC-PDA, the average, standard deviation and % RSDs were calculated in order to directly compare the results (Table 6.10).

Table 6.10. Percentage RSD of the marker compounds between the 2 analytical methods

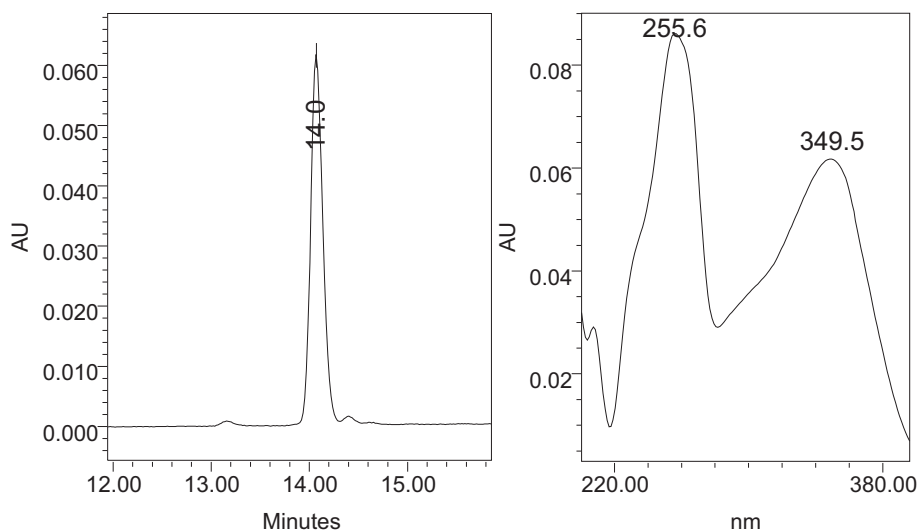
Product	Rutin % RSD	Quercitrin % RSD	Quercetin % RSD	Kaempferol % RSD	Isorhamnetin % RSD
Holotropic	16.2	58.6	+	-	-
Formule Naturelle	8.5	103.5	+	-	+
Ginkgoforce	54.2	74.1	+	+	CD
Nrf Herbal	4.2	86.6	5.1	8.0	12.9
Bioharmony	11.9	99.8	ND	-	-
Vital	8.7	108.1	22.2	13.5	+

CD, content discrepancy (detection using LC-MS only); ND, not determined since quantification was obtained using LC-MS only

From these results it can be seen that for rutin, quercetin, kaempferol and isorhamnetin, similar content trends were observed using both detection methods except for rutin in Ginkgoforce which was quantified near the LOQ which explains the high % RSD. Isorhamnetin in Naturelle was also only detected using LC-MS-MS. The RSDs were all well below 20.0% except for quercetin in Vital (22.2%). Slightly higher amounts were expected using HPLC-PDA detection due to background absorbance which is essentially nullified when using LC-MS-MS due to exclusive detection of the target analyte.

On the other hand, the quercitrin content was not remotely comparable and since LC-MS-MS is a highly selective detection technique which facilitates unequivocal peak identification, the HPLC results were re-evaluated to find possible reasons for this large discrepancy. The HPLC-PDA peaks were then carefully double-checked and identified using retention times, spectral comparisons with reference standards and peak purity testing with 3 dimensional viewing for verification of peak homogeneity. The HPLC-UV chromatogram ( $\lambda$  350 nm) of the quercitrin reference standard and its corresponding spectrum is shown in Figure 6.6.

Figure 6.6. The HPLC-UV chromatogram of quercitrin reference standard ( $\lambda$  350 nm) and corresponding UV spectrum

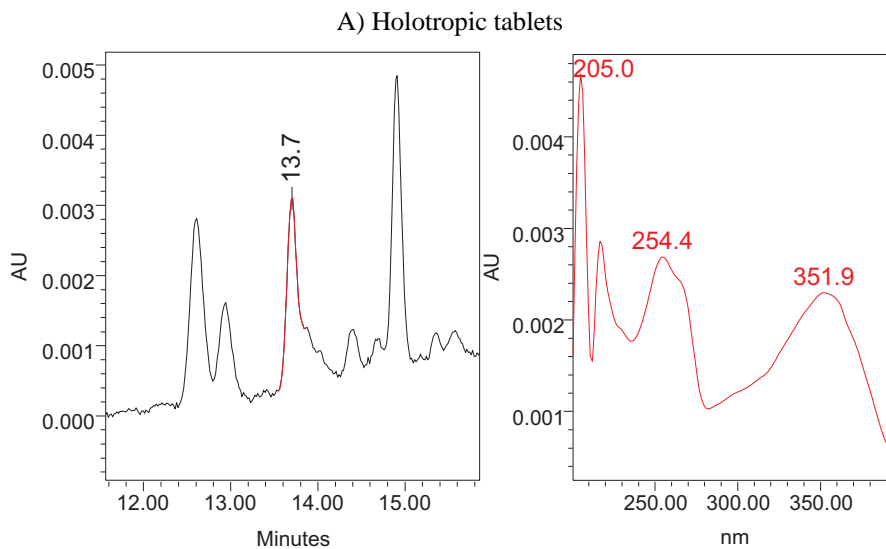


Conditions: Column: Phenomenex Luna ( $5\ \mu\text{m}$ )  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 400 $\mu\text{l}/\text{min}$ ; Injection volume: 5  $\mu\text{l}$ ; Detection  $\lambda$ : 350 nm.

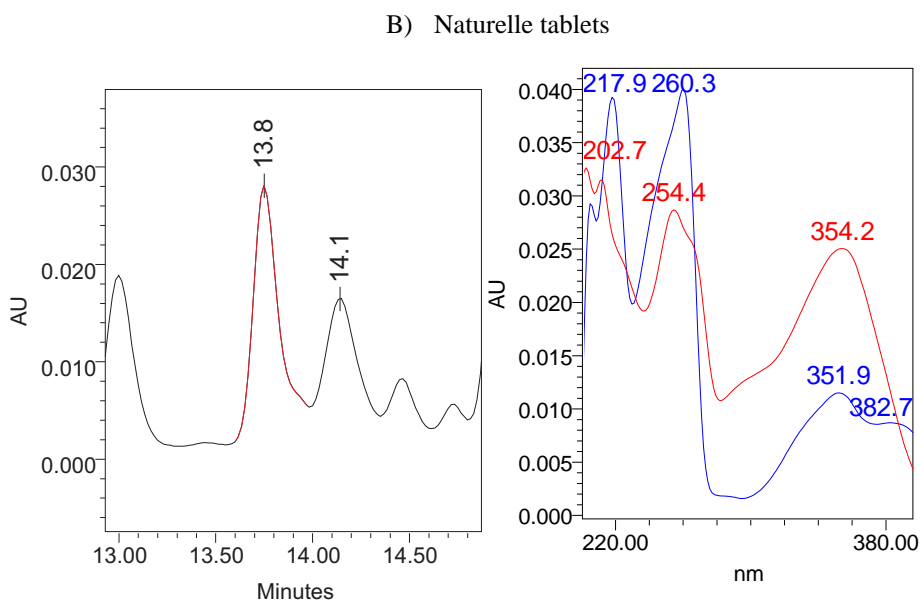
In the extracts it appeared that there was a slight change in the retention time of quercitrin which is not unusual when analyzing complex matrices and the spectra of the peaks closest to the retention time of the reference standard were therefore reviewed. In Holotropic (Figure 6.7 A), a single peak was observed at 13.7 minutes and although the retention time was exactly 1 minute early, the spectrum was very similar to that of quercitrin. The peak had a long tail and the 3-dimensional (3-D) spectrum showed minor unresolved peaks in this area. Similar scenarios were observed for Ginkgoforce, Bioharmony and Vital (Figures 6.7 C, E and F, respectively) however in each of those chromatograms, the profile of a second peak was more evident in the peak tailing zones. Definitive spectra of these peaks were not possible due to their presence in low concentrations and the first peak's spectrum in all 3 cases seemed to correspond reasonably closely to that of the quercitrin reference standard.

For Naturelle (Figure 6.7 B), a peak eluted before and after the expected quercitrin elution time. The spectra were compared and the first spectrum (in red) closely matched that of quercitrin while the other was obviously that of another related flavonoid. In Nrf (Figure 6.7 D), 3 clear peaks were observed in the quercitrin elution zone. The spectrum of the middle peak resembled that of quercitrin while the remaining 2 were also of other unidentified compounds, also probably flavonoids.

Figure 6.7. HPLC-UV chromatograms ( $\lambda$  350 nm) of the identified quercitrin peaks in extracts

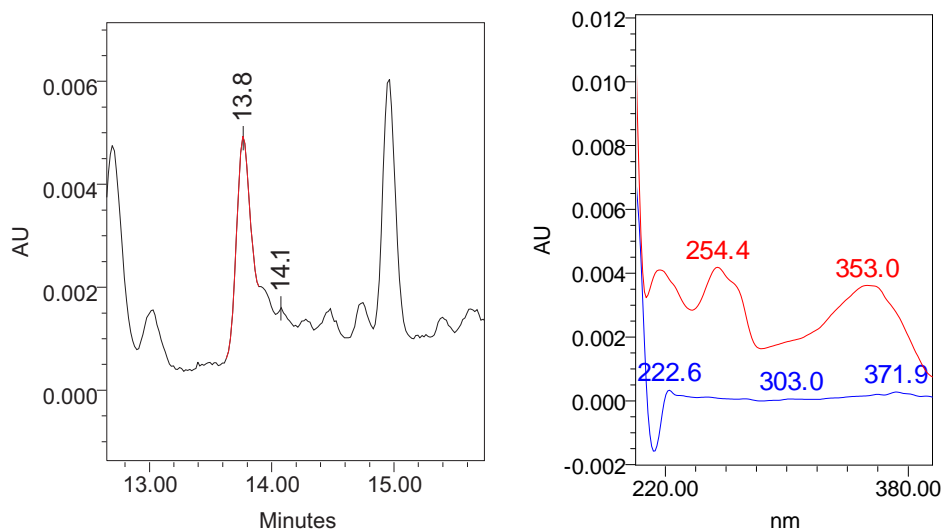


Conditions: Column: Phenomenex Luna ( $5\ \mu\text{m}$ )  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature:  $45^\circ\text{C}$ ; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate:  $400\ \mu\text{l}/\text{min}$ ; Injection volume:  $5\ \mu\text{l}$ ; Detection  $\lambda$ : 350 nm.



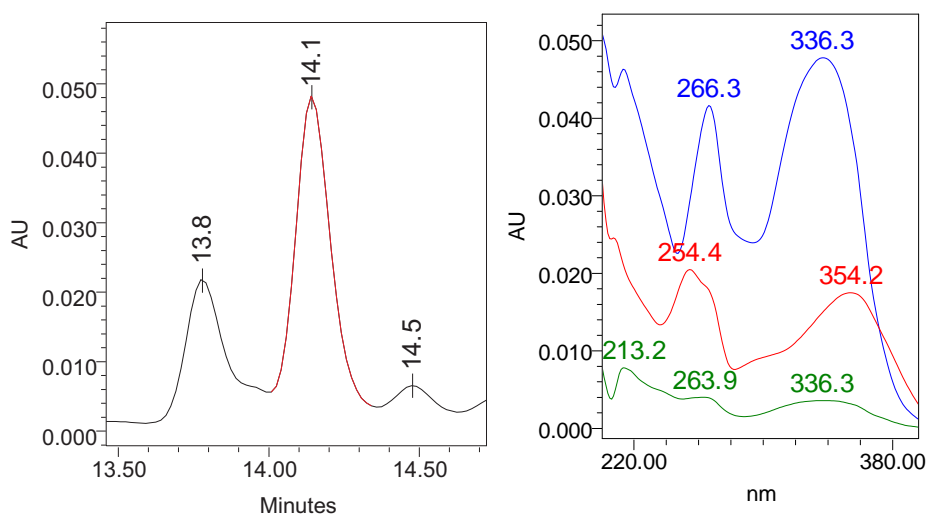
Conditions: Column: Phenomenex Luna ( $5\ \mu\text{m}$ )  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature:  $45^\circ\text{C}$ ; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate:  $400\ \mu\text{l}/\text{min}$ ; Injection volume:  $5\ \mu\text{l}$ ; Detection  $\lambda$ : 350 nm; Red spectrum=peak 1 (13.8 minutes), Blue spectrum=peak 2 (14.1 minutes).

C) Ginkgoforce tablets



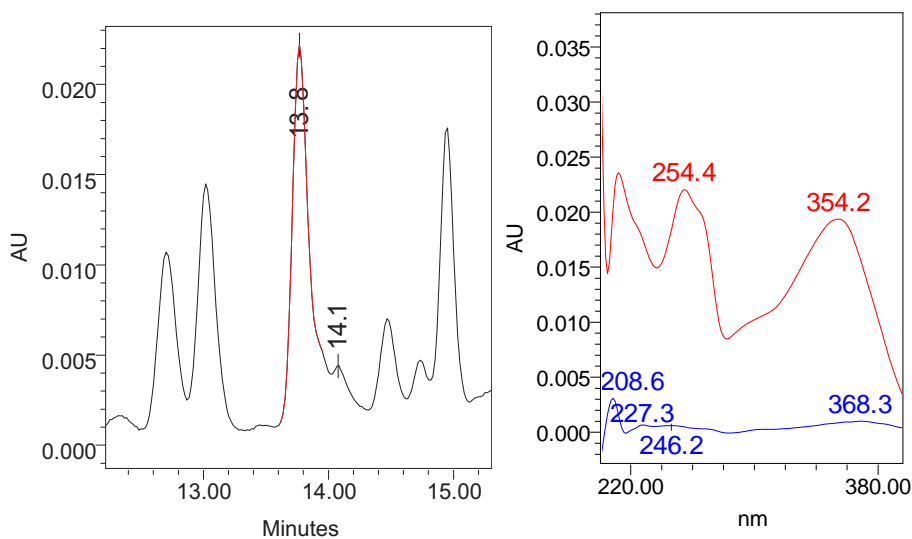
Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 400 $\mu$ l/min; Injection volume: 5  $\mu$ l; Detection  $\lambda$ : 350 nm; Red spectrum=peak 1 (13.8 minutes), Blue spectrum=peak 2 (14.1 minutes).

D) Nrf tablets



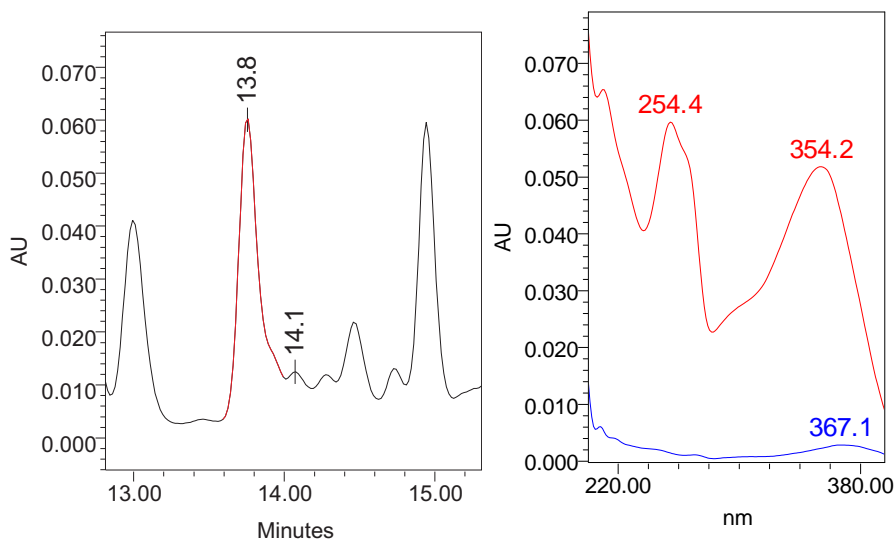
Conditions: Column: Phenomenex Luna (5  $\mu$ m) C<sub>18</sub> (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 400 $\mu$ l/min; Injection volume: 5  $\mu$ l; Detection  $\lambda$ : 350 nm; Red spectrum=peak 1 (13.8 minutes), Blue spectrum=peak 2 (14.1 minutes), Green spectrum=peak 3 (14.5 minutes).

### E) Bioharmony capsules



Conditions: Column: Phenomenex Luna (5  $\mu$ m)  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 400 $\mu$ l/min; Injection volume: 5  $\mu$ l; Detection  $\lambda$ : 350 nm; Red spectrum=peak 1 (13.8 minutes), Blue spectrum=peak 2 (14.1 minutes).

### F) Vital capsules



Conditions: Column: Phenomenex Luna (5  $\mu$ m)  $C_{18}$  (250 x 2.0 mm I.D.); Column temperature: 45°C; Mobile phase: MeCN-0.3% formic (gradient change 7-12 minutes); Flow rate: 400 $\mu$ l/min; Injection volume: 5  $\mu$ l; Detection  $\lambda$ : 350 nm; Red spectrum=peak 1 (13.8 minutes), Blue spectrum=peak 2 (14.1 minutes).

Since quercitrin is a flavonol glycoside it was expected to be present in substantial quantities in the dosage forms and based on this premise, the peak highlighted in red in Figure 6.7 in all of the extracts were determined quantitatively to give the same results as in Table 6.8. The LC-MS-MS data however revealed that the dosage forms contained relatively low levels of quercitrin and it was concluded that the UV detection of quercitrin was negated due to its presence in small quantities in the *Ginkgo biloba*

dosage forms and its response was therefore easily compromised by interfering flavonoid compounds with similar spectra.

It obvious that numerous flavonoids with similar spectra are present in *Ginkgo biloba* leaf extracts and unambiguous identification of marker compounds using UV detection alone has shown to be not possible. HPLC-PDA detection is however well suited for the fingerprinting of *Ginkgo biloba* solid oral dosage forms where emphasis is on the qualitative profile rather than on actual content. HPLC-UV is still the most common system available in pharmaceutical laboratories, is fairly cost efficient and easily to maintain and operate compared to more sophisticated systems. At present, it should be considered as the first choice for the qualitative analysis of the selected flavonols in *Ginkgo biloba* commercial products.

LC-MS-MS, on the other hand, proved to be indispensable for the accurate quantification of the flavonol markers in *Ginkgo biloba* dosage forms within a reasonable analysis time. In addition to peak identification using retention times afforded by liquid chromatography, the molecular mass of the analyzed compounds as well as efficient structure elucidation was possible using CID which resulted in the mass detection of the characteristic daughter ions. Moreover, selective detection of the target analytes ensured exclusion of any possible interference and background absorbance and the marker compounds were accurately quantified within a relatively short run time of between 10 and 20 minutes. This is not possible using HPLC-UV detection where long run times and complex gradient systems are normally required for separation. LC-MS-MS is generally not the first choice for fingerprint analysis due to high operational costs compared to HPLC. However, it is highly recommended for quantification of compounds in complex matrices such as natural products and according to Careri *et al.* [197], this hyphenated technique has demonstrated both maturity and robustness for this application. These authors predicted that with greater availability of automated bench-top systems, LC-MS will be the method of choice for the routine analysis of analytes present in complex matrices such as food.

#### 6.9.2.2 *The terpene trilactones*

It has already been established that HPLC-UV analysis is not suitable for the determination of the terpene trilactones due to their poor UV absorbing properties and the presence of unknown interfering compounds in crude *Ginkgo* leaf extracts and formulations. Two alternative detection methods that have frequently been used are HPLC-ELSD and LC-MS. As previously mentioned, ELSD provides advantages above UV detection in terms of selectivity and is relatively inexpensive and easily operable compared to MS equipment. LC-MS-MS is a more sensitive detection technique and also demonstrates the necessary selectivity to quantify the terpenes in human and animal plasma where the detection of trace amounts of the target analytes is essential. Minimal sample clean up is also a distinct advantage as well as reduced analysis times. For the routine QC analysis of *Ginkgo biloba* solid oral dosage forms, ELSD detection is however sufficiently sensitive and is favored due its simplicity and economic attributes. Moreover, recent publications have reported excellent validation data with better recoveries



than some of those achieved using LC-MS. This has also been demonstrated in the ELSD study presented in this thesis.

## 6.10 CONCLUSIONS

Direct data comparison using CE, HPLC and LC-MS for the analysis of the flavonols and terpene trilactones in *Ginkgo biloba* dosage forms is not possible since method development and analyses were conducted over a 3 year period and batch-to-batch consistency is not guaranteed. Products were purchased when required and those that were available at that time were analyzed. Due to an obvious lack of standardization and QC criteria, definitive product evaluation is not currently possible. General trends between products could however be discerned and will be briefly summarized.

### 6.10.1 Ginkgoforce

Two different batches of Ginkgoforce were qualitatively analyzed using CE, one at high pH and the other using RF-CDMEKC and in both fingerprint profiles, very few peaks were observed. These results were confirmed when the profiles of a selective and non-selective extraction solvent were compared for the determination of the ginkgolic acids using non-aqueous CE. Both profiles were remarkably similar and it was concluded that this product contained little extractable constituents. The latter fingerprints also revealed the presence of substantial amounts of negative marker compounds (Figure 3.34 in Chapter 3).

Quantitative analyses using HPLC-PDA and LC-MS-MS merely confirmed the above results. All 5 of the flavonol markers were detected using either detection mode. In both cases, the flavonol marker content was shown to be extremely low and the ratio of aglycones to glycosides was exaggerated. This provided substantial evidence of unfavorable raw material extraction and/or storage conditions.

This product was not selected for dissolution studies since its rutin concentration (as illustrated by previous HPLC-PDA and LC-MS-MS analyses) was predicted to be below the LOD. Contrary to what was expected, this product complied with the USP 28 criteria of tablet disintegration and weight variation and the tablet hardness was 69.8 Newton with a RSD of 7.12% (n=20).

In summary, although this formulation did meet some USP 28 specifications, positive marker content was grossly lacking regardless of which batch was analyzed and the ginkgolic acids were also determined to be present in substantial amounts in the batch which was fingerprinted. Moreover, the high ratio of flavonol aglycones to glycosides indicated product instability and this formulation was therefore deemed to be of poor quality.

### 6.10.2 Holotropic

Holotropic was also fingerprinted using CE at low and high pH. A remarkably different fingerprint was obtained for the batch analyzed at high pH in comparison with the 3 other products and its botanical authenticity was considered questionable. The batch analyzed at low pH had a profile similar to that of Ginkgoforce where very few peaks were detected, however the peak responses were somewhat higher.

Although a different batch was analyzed, the HPLC-PDA and LC-MS analyses were in agreement with the qualitative results obtained at low pH. The flavonol glycoside levels were low in comparison with the other products but significantly higher than in Ginkgoforce and the aglycone to glycoside ratio was also more acceptable. Quercetin was detected using both methods while kaempferol and isorhamnetin concentrations were below the LOD. Although all 5 terpene trilactones were quantifiable, the overall content was lower than the products discussed in the following sections.

Rutin's release profile was not determined since its rutin content was predicted to be below the LOD. Holotropic met the USP specifications for weight variation testing.

In summary, this product contained little positive marker content and its quality was considered marginally better than Ginkgoforce due to its improved aglycone to glycoside ratio.

### 6.10.3 Bioharmony

Bioharmony was fingerprinted using CE at low pH and rutin and quercetin were quantified using RF-CDMEKC. All the flavonol markers and terpene trilactones were detected in the fingerprint analysis while rutin was quantified in the batch used for method validation.

HPLC-PDA and LC-MS-MS data showed similar glycoside content to Holotropic but none of the flavonol aglycones were detected. The quercetin content was not determined using HPLC-PDA detection due to interferences. HPLC-ELSD analysis of the terpene trilactones revealed an average content trend and all were present in quantities above the LOQ.

In summary, this product contained moderate flavonol and terpene trilactone marker content and the ratio of the glycoside and aglycone content reflected favourable storage conditions.

### 6.10.4 Naturelle

Informative fingerprint profiles of this product were also obtained at both high and low pH using CE. Both profiles were relatively complex and indicated high flavonol marker content. Of the terpenoids, GA and GB were detected in the RF-CDMEKC fingerprint.

Once again, the HPLC-PDA and LC-MS-MS results confirmed the qualitative data and the 2 flavonol glycosides were quantified in the extracts. Quercetin was detected using both techniques but kaempferol was absent. Conflicting results were obtained for isorhamnetin which was detected using LC-MS-MS only. The HPLC-ELSD results showed that Naturelle also contained relatively high amounts of all of the terpene trilactones. The BB content of 0.72 mg per tablet was slightly less than the label claim (1.0 mg) while the ginkgolide content (3.73 mg) exceeded the specified claim of 1.2 mg.

From the release profile of rutin, it can be deduced that Naturelle is an immediate release formulation with 97.2% release after 60 minutes. Naturelle met the USP 28 weight variation specifications and the product label included a list of “typical” content values of active ingredients per tablet which also specified the vitamin B and folic acid content. Standardization of the “Ginkgo flavonglycosides” to 24% was claimed which was conspicuously displayed on the product packaging.

In summary, this product contained a relatively high flavonol and terpene lactone marker content and is an immediate release formulation. The ratio of flavonol aglycones to glycosides suggested favourable raw material extraction and/or storage conditions and this product was considered of higher quality than Ginkgoforce, Holotropic and Bioharmony. Although not entirely accurate, this product voluntarily supplied more consumer information on its container than any of the other products.

#### 6.10.5 Nrf

Nrf tablets were unfortunately not available for CE fingerprinting however, its rutin and quercetin content was quantified using the CD-RFMEKC method where the content of both marker compounds were found to be superior to that of Ginkgoforce, Holotropic and Naturelle (using HPLC-PDA and LC-MS determinations) as well as Bioharmony which was also quantified using CE, HPLC-PDA and LC-MS-MS.

This was the only product in which all 5 flavonol marker compounds were quantified using both HPLC-PDA and LC-MS determinations. The HPLC-PDA fingerprint profile showed that rutin was present in a disproportionate quantity and this batch was suspected of fortification. CE quantification of rutin in a subsequent batch showed lower rutin content which was also reflected in the dissolution data where the maximal rutin content corresponded to the previous batched analyzed using RF-CDMEKC.

The HPLC-ELSD data showed that the terpene content was dramatically higher compared to the content in all the other analyzed products barring that of Vital. Based on the disclosed label content, Nrf also exceeded the German Pharmacopeia’s QC standard for *Ginkgo biloba* leaf extracts.

Since this product appeared to be of exceptionally quality, it was fingerprinted for the presence of the ginkgolic acids. The content was determined to be higher than that observed for Ginkgoforce and it was deduced that little or no attempts had been made to reduce the negative marker content in this product.

Nrf was used for determining optimal dissolution conditions due to its exceptionally high rutin content and its final dissolution results demonstrated that this product was an immediate release dosage formulation. Although a different batch was used, disintegration tests failed to comply with USP 28 specifications. Nrf met the USP's criteria for weight variation and tablet hardness was determined to be 70.0 Newton with a RSD of 23.23% (n=20).

In summary, although batch-to-batch consistency seemed to be somewhat lacking with 1 batch showing evidence of rutin fortification, high levels of all the selected marker compounds were evident and this product was distinctly superior in terms of positive marker content. The flavonol aglycone to glycoside ratio suggested suitable storage and extraction conditions. This product revealed the presence of a substantial amount of negative marker compounds and did not pass the USP 28's tablet disintegration test. In addition, a high RSD (23.2%) was noted when the tablet hardness was tested.

#### 6.10.6 Vital

Vital was fingerprinted at alkaline pH and the high flavonoid peak density required detection to be performed at  $\lambda$  350 nm in order to minimize interferences (Figure 3.9 C in Chapter 3).

All 5 flavonol markers were quantified using HPLC-PDA detection and all except isorhamnetin were also present in concentrations above the LOQ using LC-MS-MS. The rutin content was somewhat lower than in Nrf, quercitrin content was marginally higher and the aglycone content was comparable in both Nrf and Vital products. The terpene trilactone levels were the highest of all the products (Holotropic, Bioharmony, Naturelle and Nrf) analyzed by HPLC-ELSD.

Dissolution testing revealed that this was an immediate release formulation with 98.6% of the maximum amount of rutin being released at the 60 minute sampling time.

In summary, this product was not subject to as many QC tests as Nrf but nevertheless demonstrated consistent product quality in terms of positive marker content with acceptable storage and extraction procedures as indicated by the favourable flavonol glycoside to aglycone ratio.

### 6.11 GENERAL CONCLUSIONS

*Ginkgo biloba* is a well-known phytomedicine which has been extensively studied as shown by numerous publications which have appeared in the literature. However, QC of Ginkgo (and other phytomedicines) is severely lacking. Although a number of papers have been published addressing the

quality of *Ginkgo biloba* formulations available on the market, discrepancies in the marker content between products were observed and in many cases there was an urgent call for the implementation of effective QC criteria.

This thesis has met the timely call for practical and economical separation methods using leading edge technology and equipment such as CE and MS as well as the more usual and accepted HPLC techniques for the determination of appropriate marker compounds postulated to contribute to the pharmacological activity in Ginkgo. These methods are predicted to be of significant future assistance to companies involved in the manufacture and QC of Ginkgo-containing products as well as to regulatory authorities in their quest to assure QSE of such products.

The major contribution of the novelty of this work lies in the RF-MEKC method used for the simultaneous determination of the terpenes and flavonols as well as the non-aqueous method for the determination of the negative markers, the ginkgolic acids. Of the HPLC methods available in literature, the most recent were selected and improved in order to deliver rapid and inexpensive analysis of the positive marker compounds present in Ginkgo and also included some glycosides in order to detect product fortification as well as providing information to ensure suitable storage conditions. The LC-MS-MS study revealed novel information regarding the fragmentation of some flavonols specific to Ginkgo.

The results in this thesis regarding the flavonol and terpene trilactone content in *Ginkgo Biloba* commercial products available on the South African market emphasized the lack of QC on one level or another. These results reiterate justified concerns regarding the QSE of these products and implementation of effective QC criteria is thus urgently necessary to ensure consistent product quality.

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**THE RELEVANT STANDARD OPERATING PROCEDURES AND REFERENCED WEBSITES ARE INCLUDED ON THE COMPACT DISC PROVIDED**