Content levels, *in vitro* dissolution and predicted bioavailability of flavonoids from *Sutherlandia frutescens* leaf powder and aqueous extracts

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor Philosophiae in Pharmaceutical Sciences

at the School of Pharmacy, University of the Western Cape

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April 2015

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KEYWORDS

Dissolution

Dissolution profiles

Flavonoids

High performance liquid chromatography (HPLC)

Markers

Quality control

Regulation

Release mechanism

Sutherlandia frutescens

Sutherlandins

ABSTRACT

Content levels, *in vitro* dissolution and predicted bioavailability of flavonoids from *Sutherlandia frutescens* leaf powder and aqueous extracts

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PhD thesis, School of Pharmacy, University of the Western Cape.

Various formulations of the popular South African medicinal plant, *Sutherlandia frutescens*, are commercially available, with no documented specifications for quality assessment. With plans already underway for a clinical trial to assess its efficacy in HIV patients, there is a need for scientifically validated tests for the quality control of products of this plant. Chemical constituents of the plant are many and varied but it is still unclear which might be the most appropriate ones to monitor for activity or to describe the quality of the plant's products. For quality control and regulatory purposes, the content and dissolution of flavonoids in the plant products can be assessed. However, these compounds are not monitored for regulation and there are as yet no HPLC or dissolution methods that can be employed for quality control of herbals like *S. frutescens*. Therefore, the objectives of this study were to assess the suitability of its flavonoid constituents as quality control (QC) marker compounds, and the suitability of content levels and dissolution tests of flavonoids as QC tools for *S. frutescens* products.

To realise the afore-mentioned objectives, non-commercially available flavonoid compounds (sutherlandins) that could be used as marker compounds were isolated from *S. frutescens*. An HPLC assay was developed and validated for determination of flavonoid content in solution. Five *S. frutescens* materials *viz* leaf powder (LP), spray-dried aqueous extract (SDAE) and freeze-dried aqueous extracts (FDAE) were analysed for flavonoid content and dissolution. Dissolution tests were conducted for different *S. frutescens* materials and dissolution profiles of flavonoids in capsules containing these materials were compared using Q-release values, the similarity factor (f_2) and mathematical models. To predict *in vivo* bioavailability of the flavonoids, *in silico* assessment of *in vivo* bioavailability of flavonoids (glycosides and aglycones) that may be contained in different *S. frutescens* materials was conducted.

Sutherlandins A, B, C and D were successfully isolated (percentage purity approximately 99 % for sutherlandins A, C and D, and 90 % for sutherlandin B) and identified, and used, along with other flavonoid compounds, for the development of a simple and robust HPLC method. Content of sutherlandins A, B, C and D, quercetin and kaempferol in different plant materials were 0.4 ± 0.3, 0.8 ± 0.2, 1.3 ± 0.2, 0.6 ± 0.1, 0.01 ± 0.02 and 0.08 ±0.1 %, respectively, and differed significantly ($p < 0.001$). *In vitro* dissolution showed faster dissolution of flavoniod glycosides compared to aglycones. The flavonoids from the LP and SDAE materials showed characteristics of immediate release with Q_{75} in ≤ 45 minutes, and delayed release from the FDAE material, i.e. Q_{75} > 45 minutes. The dissolution profiles of each flavonoid compared from different *S. frutescens* materials were different as signified by their f_2 values which were all below 50. The mathematical models describing release were also different for each flavonoid from the different *S. frutescens* materials. For *in vivo* bioavailability modelling and prediction studies, the flavonoid aglycones met the conditions for oral bioavailability while the flavonoid glycosides did not.

In conclusion, the sutherlandins isolated from *S. frutescens* proved to be good markers for HPLC assay and dissolution tests of *S. frutescens* materials. The HPLC method was suitable for assessing flavonoid levels in *S. frutescens* materials, and also showed differences in flavonoid content in these materials. The dissolution method was simple and reproducible, and Q-release values, the f_2 and mathematical models proved to be good tools for differentiating between *S. frutescens* materials. *In silico* modelling showed that the flavonoid glycosides and aglycones differed in oral bioavailability. Although not presently required by the Medicines Control Council (MCC), quantification, release and dissolution studies and specifications may be employed as tools for routine analysis and for quality control of herbal drug formulations containing *S. frutescens*.

April 2015

DECLARATION

I declare that ***Content levels, in vitro dissolution and predicted bioavailability of flavonoids from Sutherlandia frutescens leaf powder and aqueous extracts*** is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Oluchi Nneka Mbamalu April 2015

Signed: . . . . . .

DEDICATION

To the Almighty, whose mercies never end

and

To CKIC, for their love and support

ACKNOWLEDGEMENTS

A journey of a thousand miles is said to begin with a step. With the right guidance and support, such a journey eventually comes to a fruitful end. I have been blessed to have a wonderful support system in the form of my supervisors, friends, colleagues and acquaintances.

I would like to acknowledge my supervisor, Prof. James Syce, for his tutelage, guidance, mentoring and thorough supervision. His input, advice, criticisms and corrections have improved this project significantly, and brought about its completion.

My co-supervisor, Dr. Halima Samsodien, is acknowledged for being my sounding board, for deciphering complexities, and for her constant encouragement and constructive feedback.

I am thankful to the staff and management of the School of Pharmacy for their assistance, support and encouragement over the years. Each and every one of them has contributed in no small measure to the success of this project. Special thanks to Prof. George Amabeoku for his mentorship over the years, and to Dr. Jacques Joubert for his corrections and advice.

The Staff and management of the South African Herbal Science and Medicine Institute (SAHSMI) are acknowledged for their support and ever-ready assistance in the course of this project. Special thanks to Prof. Gail Hughes, Dr. Seyi Aboyade, Dr. Gustav Styger, Dr. Edith Antunes, Mr. Andre Braaf, Ms. Roche Jeftha and Ms. Alison Borchjes.

I would also like to extend my appreciation to the Department of Science and Technology for financing aspects of this project.

Colleagues, friends and family have encouraged me when the journey got tough. My sincere appreciation goes out to my colleagues and friends – Ms. Abiola Tokosi, Mr. Samuel Egieyeh, Ms. Nolufefe Silosini, Ms. Anjong Nkengla, Ms. Ilana Basson, Ms. Anthea van Wyk, Mrs. Mona Elbasher, Mr. Mohamed Daghman, Mr. Tjokosela Tikiso, Ms. Carmen Buchner and Ms. Genene Stone – for their company, their time, their friendship and contributions during the course of this research project.

A special thank you goes out to all those who partook of this journey with me, who I have not mentioned specifically. Space would not permit me to list you all, but I want you to know that I appreciate your support.

To my family, I appreciate your love, patience and encouragement, and for putting up with me for the duration of this project.

Lastly, but by no means the least, I thank the Almighty God for His love, mercy and favours. He promised that His Grace is more than sufficient for me. Indeed, it has been so.

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ABBREVIATIONS AND DEFINITIONS

% RSD:	Relative standard deviation, expressed as percentage
ADME:	Absorption, distribution, metabolism and excretion
AIC:	Akaike Information Criterion
amu:	Atomic mass unit
API:	Active pharmaceutical ingredient
APPI:	Active phyto-pharmaceutical ingredient
ATM:	African Traditional Medicine
BBO:	Broadband Observe
BCS:	Biopharmaceutics Classification System
BE:	Bioequivalence
cGMP:	Current Good Manufacturing Practices
CoA:	Certificate of analysis
CSIR:	Council for Scientific and Industrial Research
D_2O:	Deuterium
DMSO:	Di-methyl sulphoxide
EurepGAP:	European Supermarkets Organisation Good Agricultural Practices
FDA:	Food and Drug Administration (of the United States)
FDAE:	Freeze-dried aqueous extract (of *S. frutescens*)
GMP:	Good Manufacturing Practices
HACCP:	Hazard Analysis Critical Control Point
HPLC:	High performance liquid chromatography
HR ESI-MS:	High-resolution electrospray ionisation mass spectrometry
ICH:	International Conference on Harmonisation

IR:	Immediate release
IVIVC:	*In vitro in vivo* correlation
LP:	Leaf powder (of *S. frutescens*)
Marker compound:	A natural constituent of a plant material selected for quality assessment when the active component(s) of the plant material are not yet known .
mAU:	Milli absorbance unit
MCC:	Medicines Control Council
MSE:	Mean Square Error
m/z:	Mass to charge ratio
NMR:	Nuclear Magnetic Resonance
PCA:	Principal Component Analysis
ppm:	Parts per million
Reference standard:	A chemical compound of high purity (usually but not always ≥ 99 %) used as a comparator when detecting and evaluating the same chemical compound within a blended formula.
rpm:	Revolutions per minute
SADC:	South African Development Community
SAHSMI:	South African Herbal Science and Medicine Institute
SDAE:	Spray-dried aqueous extract (of *S. frutescens*)
SS:	Sum of squares
TCM:	Traditional Chinese Medicine
USP:	United States Pharmacopoeia
WHO:	World Health Organisation

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PEER-REVIEWED CONFERENCE PRESENTATIONS

The studies reported in this thesis have been presented at the following national and international conferences:

Oral presentations at national conferences:

1. MBAMALU O*, SYCE J & SAMSODIEN H (2012). Comparison of dissolution profiles of selected flavonoid glycosides from two *Sutherlandia frutescens* materials (leaf powder and spray-dried aqueous extract) using a non-pharmacoepoeial method. Presented at the 33rd Annual Conference of the Academy of Pharmaceutical Sciences of South Africa, 12 – 15 September, Grahamstown, Eastern Cape Province, South Africa.

2. MBAMALU O*, SYCE J & SAMSODIEN H (2013). Modelling of flavonoids glycoside dissolution from different *Sutherlandia frutescens* materials. Presented at the 3's a Company Pharmacy Conference, 4 – 6 October, Milnerton, Cape Town, South Africa.

Poster presentations at international conferences:

1. MBAMALU O*, SYCE J & SAMSODIEN H (2014). Prediction of *in vivo* bioavailability of selected flavonols from *Sutherlandia frutescens*. Presented at the 17th World Congress of Basic and Clinical Pharmacology, 13 – 18 July, Cape Town, South Africa.

2. MBAMALU O*, SILOSINI N, ANTUNES E, SYCE J & SAMSODIEN H (2014). High performance liquid chromatographic determination of selected flavonol glycosides and corresponding aglycones in *Sutherlandia frutescens* materials. Presented at the 2nd International Symposium on Natural Products, 23 – 25 September, Cape Town, South Africa.

* Indicates presenting author.

CHAPTER ONE

INTRODUCTION

Sutherlandia frutescens, the famous South African traditionally used medicinal plant, is now also widely available commercially, in a variety of dosage forms and formulations.[1] Like many herbal medicinal products, it contains a myriad of constituent compounds, some of which are reported to be responsible for its many pharmacological effects.[2,3] While many cures have traditionally been linked to this plant, none of the many constituents of *S. frutescens* has been conclusively linked to the pharmacological effects of this product in a clinical trial. Presently, tentative approval has been sought and obtained for a clinical trial to assess its efficacy.[4] However, it is not clear which of the many formulations available is of the best quality and so would be the most appropriate to use. In addition, very little is known of its potential actives and how oral dosage forms containing different forms of the plant material (i.e. leaf powder or extract) compare in the levels, *in vitro* release and dissolution, and oral absorption of such actives. Various marker compounds have been found in this plant, among them the flavonoid glycosides, sutherlandins A, B, C and D. Assessment and comparison of the levels and release of flavonoids from different materials of this plant might show how differences in content and *in vitro* release translate to differences in quality and therapeutic efficacy.

A recently introduced formulation of *S. frutescens*, a freeze-dried aqueous extract, is also claimed to be better and more efficacious than the older formulation (the powdered leaf powder) even though this has not been confirmed.[5] In light of such claims, it is pertinent to assess the different *S. frutescens* formulations commercially available, in order to gain an insight into their differences, and how these may translate to pharmacological effects. Differences in pharmacological effect or efficacy may also be due to differences in quality of different products.

If different products differ in quality, how do we know for certain, how do we assess the quality of *S. frutescens* products? How do we determine a better and more efficacious formulation? Is it the one that releases the drug faster or more slowly? Is it the one with more of the active component or marker compound? Which marker compound is suitable,

and can indicate quality status? What parameters should be assessed for with this marker? The market for herbal medicinal products has grown considerably over the past years but of great concern is the fact that quality control measures that can provide answers to such questions have not kept pace with such growth. With the increasing popularity of herbal medicinal products like *S. frutescens* in recent times, answers are needed to such questions in order to protect consumers who may be at risk of danger from inappropriately prepared and unsafe products.[6,7]

Many *S. frutescens* products are commercially available with no tests or specifications to indicate quality or efficacy. However, the quality control of *S. frutescens* products, as with all herbal products, needs to be more stringent. The European Medicines Agency (EMEA) concurs with this and regards most herbal products as drugs. Therefore, scientifically proven evidence of efficacy is required in order for such products to be registered in Europe.[8] This is not the case in countries like the United States and South Africa where herbal medicinal products are marketed as dietary supplements without proof of quality or efficacy.[9,10] However, the scene is set to change with recent updates as the FDA aims to introduce measures to ensure herbal product approval only if benefits are demonstrated by safety and efficacy studies.[8] Within the past few years, the Medicines Control Council (MCC) of South Africa has also passed guidelines requiring registration of all herbal products in South Africa as a means of ensuring product quality.[11] For such registration, tests that can indicate quality and efficacy status of herbal products like *S. frutescens* are required.

Currently, quality control studies of *S. frutescens* products are non-existent. The setting up of quality control tests for herbals like *S. frutescens* poses great challenges largely because of the multi-component nature of and limited research on herbals.[12,13] This makes it difficult to select an ideal 'active component' which can be used to assess quality. In addition, there is potential for variation with the same herbal product as a result of different growth, harvesting, processing and storage conditions that may have been applied.[14,15] Variation in content of herbals like *S. frutescens* may also translate to variation in quality, and the question still remains: how do we assess and compare the quality of different *S. frutescens* materials?

For quality assessment and comparison of *S. frutescens* materials, suitable marker compounds and analytical assays are required. The marker compounds may be supposed or known actives of the product which are suspected or known to contribute to its activities. For *S. frutescens*, some of its biological effects, specifically the antioxidant effects, have been attributed to the presence of phenolic compounds such as flavonoids[16,17] which exist in this plant in both glycosidic and aglycone forms. Because of differences in chemistry between flavonoid glycosides and aglycones, their presence and levels could be representative of the different forms and conditions under which active components of *S. frutescens* might occur, enabling their use as marker compounds and quality indicators for different materials of this plant.

In addition, appropriate assays for the suitable marker compounds need to be developed for *S. frutescens* quality assessment. Such assays may be conducted using high performance liquid chromatography (HPLC), a technique used to separate, identify and quantify component compounds present in plant matrices.[18] HPLC identification and quantification can be used to differentiate between materials, and between different batches of the same material, enabling its use in quality control and for the setup of regulatory specifications. In order to conveniently set up HPLC assays for quality control of *S. frutescens*, good resolution of peaks representing different compounds of interest is desired, and appropriate marker compounds need to be selected.

Previous HPLC assays of *S. frutescens* focused on the flavonol and cycloartanol glycosides or the sutherlandiosides in aerial parts of *S. frutescens*.[5,19,20] However, the selected marker compounds in these assays did not include related compounds that can indicate quality status by serving as products of marker compound degradation. We propose the use of selected flavonoid glycosides and their corresponding aglycones in quality control and assessment of *S. frutescens* materials. Flavonoid aglycones may be formed from flavonoid glycosides as products of glycoside degradation in unstable conditions;[21] therefore quantification of these two forms in which the flavoniods exist can be used to detect changes in material or product which can translate to changes in quality.

The quality of *S. frutescens* materials can also be evaluated and compared *via* dissolution testing. The dissolution test is a widely used technique employed for quality and bioequivalence assessment, as well as for comparison of release for orally administered conventional pharmaceutical products. As very little is known regarding the release and dissolution of flavonoid glycosides from *S. frutescens* materials, this test can also be employed for release studies, and may detect differences between different *S. frutescens* materials. However, the dissolution test is a novel approach that has not been widely adopted for herbal products,[22] and so its application may present challenges.

The challenges which may be encountered with dissolution testing of *S. frutescens* may include the very low content of flavonoid compounds in plant materials, and extensive within-batch variation which is common in herbal products.[23,24] These shortcomings limit the use of herbal products in conventional settings and may negate the use of standard methods for dissolution testing and dissolution data analyses. In such a case, the standard methods may be modified for analyses of *S. frutescens* dissolution data. These modifications may include changes in the dissolution method as well as in dissolution data comparison methods, and may be used to differentiate *S. frutescens* materials.

For quality comparison of materials using the dissolution test, materials with different dissolution profiles are deemed different. Differences in dissolution profiles of flavonoids may also be due to differences in their release from different matrices of the different plant materials, e.g. leaf powder *versus* extract. As such, mathematical modelling can be employed to compare release from different *S. frutescens* materials. These models describe release curves and elucidate the underlying mechanisms involved in flavonoid release from different *S. frutescens* materials, which has hitherto not been done for *in vitro* release of any marker compound or for any herbal product. The mathematical models may also offer explanations which can be used to predict *in vivo* pharmacokinetics of *S. frutescens* flavonoids from different materials.

It is very likely that some of the flavoniod glycoside compounds found in *S. frutescens* materials may not be orally bioavailable. However, the *in vivo* pharmacokinetics, e.g. bioavailability, of marker compounds in different *S. frutescens* materials can perhaps be

predicted *via in silico* modelling studies, conducted with the use of chemoinformatic resources.

The present study as such focuses on quality control and expected *in vivo* pharmacokinetics of flavonoid glycosides and aglycones of *S. frutescens* leaf powder and aqueous extracts. The leaf powder and aqueous extracts are commonly available commercially, and one of these forms, most likely one of good quality, may be used for the upcoming clinical trial. This study therefore stems from a need for quality control methods for *S. frutescens* products, which can identify *S. frutescens*-containing products as well as differentiate between them. The focus is on the quality of commercially available *S. frutescens* materials, and involves the selection of appropriate markers to use for their quality control, the tools (flavonoid content and dissolution) to use for quality assessment, and the bioavailability of flavonoid marker compounds selected for quality control tests.

Essentially, *S. frutescens* is employed in this study as a template with which to introduce investigations into quality control and regulation of herbal products. The quality control of herbal products is still a work in progress for most countries, largely because the multi-component nature of herbal products presents considerable challenges to quality control efforts. To date, attempts to modernize and integrate these products into the main health care stream have been stalled by these quality control and regulatory challenges. Such limitations to the use of these materials, once addressed, would benefit development and regulation of herbal products to a great extent. In future, guidelines and regulatory specifications will ensure that it will no longer be sufficient to tout a herbal product as possessing different preventive and curative properties, and biological tests and efficacy-indicating tests like the dissolution test may be required to support the health benefits easily claimed for many herbal products. The good quality of the product will need to be ascertained as well in order to confirm purported efficacy as only products of acceptable quality can produce the desired pharmacological effects.

CHAPTER TWO

LITERATURE BACKGROUND

This chapter discusses *Sutherlandia frutescens* as an important South African herbal medicinal product. It also reviews relevant problems associated with the quality of herbal medicinal products and proffers possible solutions that can be used to address these, using *S. frutescens* as a template. The dissolution test as a quality control tool for herbal medicinal formulations is also reviewed. Dissolution profile comparison methods are presented, including the modifications that may be applied for natural products when criteria set for dissolution profiles comparison of conventional drug formulations are violated. In addition, mathematical models of drug release are explored, as well as the relationship between molecular properties and predicted oral bioavailability.

2.1 *Sutherlandia frutescens*, an important herbal medicinal plant

Sutherlandia frutescens (*S. frutescens)*, a common herbal medicinal plant indigenous to Southern Africa, is widely used for the treatment of various ailments.[3,16,25-27] Among its many reputed pharmacological effects, *S. frutescens* is also widely used by HIV patients who attest to improved clinical conditions on using this plant and its products. Such activity against HIV has also been supported by *in vitro* studies.[26] Due to its popularity and the purported activities of the plant, tentative approval has been obtained for a clinical trial to assess its efficacy.[4] To prove or disprove efficacy, a product of suitable quality would be required. However, assessment of *S. frutescens* quality, like that of other herbal materials, is challenging because specifications for such are not readily available.[28] In view of the fact that products of this plant are easily accessible and various forms of different species are widely distributed, there is a need to introduce measures for its quality control. The wide distribution of the plant form, as well as its description, is briefly discussed below.

2.1.1 Distribution and description

Sutherlandia frutescens is widely distributed throughout the dry and stony areas of Southern Africa: in the Western Cape and Lesotho; from the western Karoo to the Eastern Cape and up the West Coast in a northerly direction to Namibia and Botswana. Within this

geographical distribution, it shows great variation in chemistry and genetics.[2] Several species have been identified, which are nevertheless closely related, necessitating a revision of taxonomic classification from six to two species, *S. frutescens* and *S. tomentosa. S. frutescens* is further sub-divided into three subspecies or chemotypes, namely subsp. *frutescens,* subsp. *microphylla* and subsp. *speciosa*.[29] For the purpose of this study, *S. frutescens* subsp. *microphylla* is the specific chemotype under evaluation.

S. frutescens subspecies *microphylla* elite (PN1™) is a small, soft, woody flowering perennial shrub, about 1.4 m high with prostrate to erect stems. It has green, compound-pinnate leaves, with ovate-oblong and glabrous leaflets. The plant produces spectacular red flowers borne in terminal racemes and the fruit from the plant is a large, inflated, green pod producing black, flattened seeds approximately 3mm in diameter (Fig 2.1).[-2,30] The aerial parts of the plant, most commonly the leaves and twigs, are utilized in the treatment of various ailments.

(a) (b)

Figure 2.1: Sutherlandia frutescens showing (a) the fruit and (b) the flowers[30]

2.1.2 Uses and pharmacological effects

S. frutescens has many varied uses, for some of which mechnisms of pharmacological effects have been established. It is used to wash wounds, and as a remedy for everything from colds and inflammation to liver problems and depression.[27] One of its most common names is 'cancer bush', due to its reputation as a cure for cancer. There is as yet no scientific support for the numerous claims and anecdotes that this plant can cure cancer, but there is

preliminary clinical evidence of its immune stimulating properties and its direct anti-cancer effect in some cancers.[3,31] It is also known to decrease anxiety and irritability, to elevate mood, and dramatically improves appetite in cachectic patients who gain weight as a result.[2,26] Its activity against HIV target enzymes and its success in the treatment of septic shock (a development of AIDS progression) may also be possible ways in which this plant improves outcome in HIV /AIDS patients.[26,32-37]

Among its other uses, studies have shown that *S. frutescens* decreases blood sugar levels in diabetic rats possibly due to its pinitol component although a preliminary study with pinitol in type 2 diabetic individuals did not show encouraging results.[38,39] Its antioxidant and anti-inflammatory effects in polar and non-polar extracts has been demonstrated; the latter *in vitro* and *in vivo* possibly through inhibition of cyclooxygenase (COX)-2 expression.[16,27,40-43] Hexane extracts were also found to produce antibacterial effects.[16]

Additionally, *S. frutescens* has shown anticonvulsant and antithrombotic activity.[27,44] Its ability to serve as an adaptogenic agent in stress-related disorders may be by attenuation of adrenal P450 enzymes leading to a reduction in glucocorticoid levels (and by extension stress symptoms).[45,46] These various pharmacological effects of the plant have been attributed to some of its constituent compounds.

2.1.3 Chemical constituents of *S. frutescens*

Various chemical constituents have been identified in *S. frutescens*. The potential actives of the plant are pinitol, *L*-canavanine, GABA, parabens, saponins, arginine, cycloartane glycosides and triterpenoid glucosides.[2,20,45,47-49] The major triterpenoids of this plant are structurally similar to the cycloartane terpenoids which have proven cancer chemopreventive activity[50] and in *in vitro* tests, have been shown to possess evidence of anti-cancer activity.[3,40,43,49]

S. frutescens also contains flavonoids, a group of polyphenolic phytochemicals known to produce many biological effects. Some of these flavonoids in the plant include the flavonol aglycones, quercetin and kaempferol, and their recently identified corresponding glycosides, referred to as sutherlandins A, B, C and D (Figure 2.2).[2,47-49,51] Other glycosides of these

aglycones which may be present in *S. frutescens* include rutin, kaempferol-3-*O*-rutinoside and quercitrin. Sutherlandins A and B, rutin and quercitrin are glycosides of the aglycone, quercetin; while sutherlandins C and D, and kaempferol-3-*O*-rutinoside are glycosides of the aglycone, kaempferol. Can these constituent compounds be employed to address problems associated with quality of *S. frutescens* formulations?

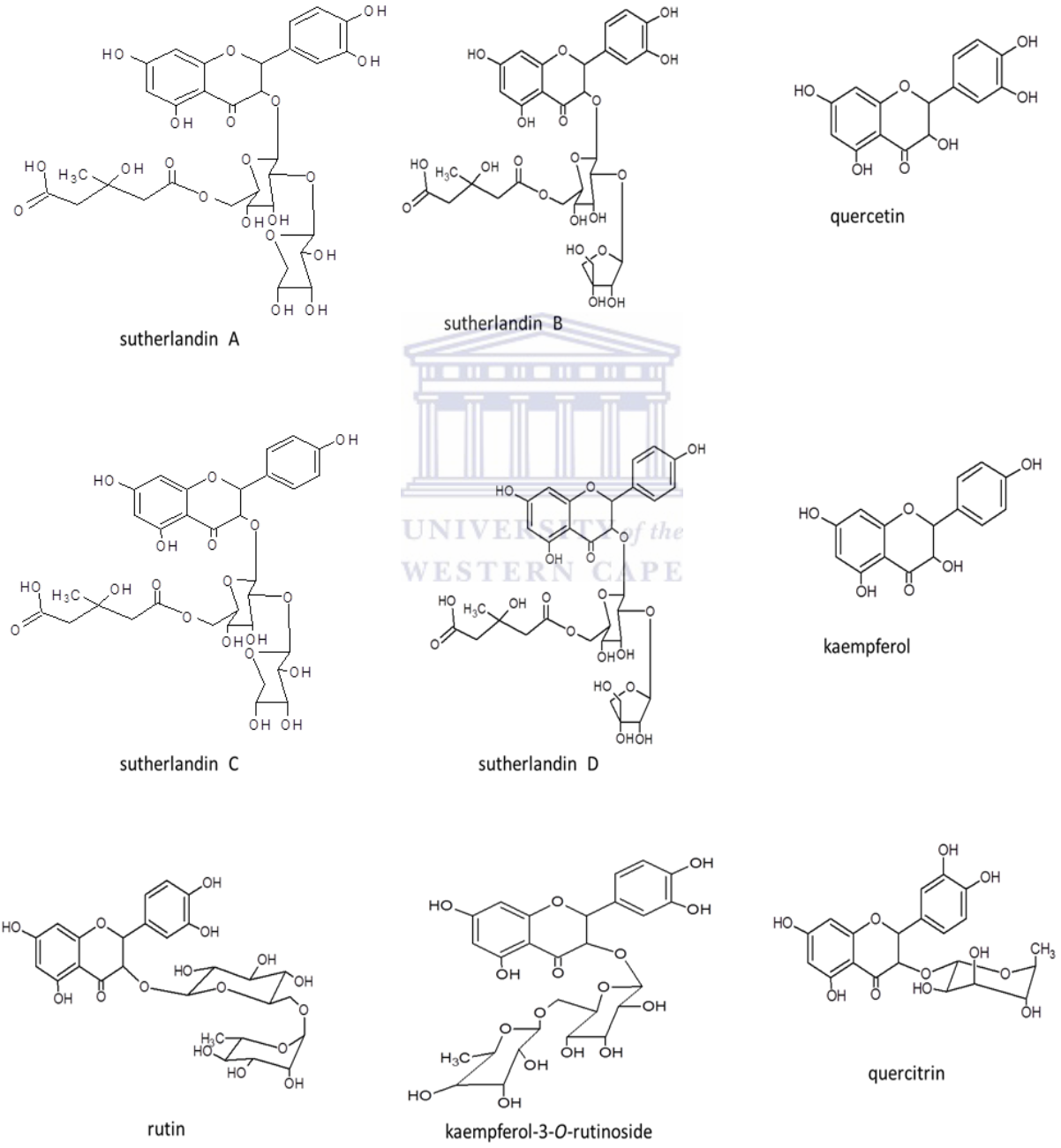


Figure 2.2: Structures of the flavonoid glycosides (sutherlandins A to D), and aglycones (quercetin and kaempferol) possibly present in S. frutescens

2.1.4 Formulations of *S. frutescens* and associated problems

Although not yet registered by the South African regulatory authority (i.e. the Medicines Control Council, MCC), *S. frutescens* is commercially available and used in various forms. Some of these forms include the dried powdered material, extracts, tablets, capsules, tonics, dried powdered material in tea bag for oral consumption, as well as creams and gels for topical administration. A recent entrant into the commercial circle is the dried aqueous plant extract, which is claimed to be closer to the traditionally used dosage forms, and presumably more efficacious than previous formulations. Such claims spur patients to buy the different forms of this plant, as supplements to their diet or as a cure for an illness.

With its increasing popularity, especially among HIV patients who attest to its immune-boosting properties, there is a need for quality control in order to ensure the safety, quality and efficacy of this plant for consumers. The South African Herbal Sciences and Medicines Institute (SAHSMI) has for the past few years conducted extensive research on these products, and a phase I clinical trial on the leaf powder (Trial # TICIPS002_RP01) showed no evidence of toxicity at very high doses.[52] Arrangements are underway for a phase II clinical trial to assess the efficacy of an *S. frutescens* extract in HIV patients.[4] A formulation of appropriate quality is required for this, but how do we assess and compare qualities of different *S. frutescens* formulations? Another limitation to the choice of an appropriate product for the clinical trial is the fact that very little is known concerning the pharmacokinetics of any potential actives such as flavonoids in these preparations, and how oral dosage forms containing different forms of the plant material (i.e. leaf powder or extract) compare in levels, release and oral absorption (i.e. *in vivo* bioavailability) of such actives.

The afore-mentioned quality and other problems of *S. frutescens* are not specific to this plant, but are also encountered with other herbal medicinal products. As such, they can be addressed by the same method(s) that may be proposed for quality control and regulation of other herbal products. With the popular use of herbal medicines today, and the possibility of modified and “improved” versions of such products being introduced as the market for herbals grows, there is a need for quality control measures so as to effectively regulate their use among consumers.

2.2 The need for quality control of herbals

The use of herbal medicinal plants for treating diseases is probably the oldest existing method that humanity has used to cope with illness. Herbal medicinal products are crude plant materials from different parts of the plant as well as extracts from the plant. Recently, the use of herbal medicines has gained popularity, and major pharmaceutical companies have joined in, producing more herbal remedies for the treatment and management of a wide range of conditions. Prominent scientific journals have also devoted significant effort to the publication of both basic and clinical scientific studies on herbal medicines and their products.[53] This has created a scientific platform, internationalising herbal product use and may well enable the physician's prescription of herbal medicinal products.

Internationally, widespread views also support the integration of traditional herbal medicine into conventional health care systems.[35,54] Many organisations such as the World Health Organization (WHO) and the Joint United Nations Programme on HIV/AIDS (UNAIDS) have shown their support for traditional (herbal) medicine integration by developing guidelines for quality control and regulation of these products.[35,54] This has offered some assistance; however, following the introduction of these guidelines, insufficient programmes have so far been established in different countries to attain the set goals.[13,55] Only about four countries – China, the Democratic People's Republic of Korea, the Republic of Korea and Vietnam – are considered to have attained the desired level of integration of traditional herbal medicine into conventional health systems.[56,57]

A considerable challenge to herbal medicine integration is posed by the paucity of research on these products. Insufficient data exist for most herbal products to guarantee their safety, quality and efficacy, and knowledge *cum* resource sustenance of these products still remain challenges.[8,13,58-62] While many pharmaceutical companies invest in the production and marketing of herbal products, they can present their products as dietary supplements and remedies and so do not necessarily have to provide evidence of quality and efficacy for such products.[9,55,63] As such, their products do not have to undergo the extensive quality control and performance tests required for conventional drug formulations, a situation obtained in countries like the USA.[10]

In South Africa, the Medicines Control Council (MCC) is charged with the regulation of medicines. In order to be registered as a medicine in South Africa, a product has to comply with the MCC's standards of safety, quality and efficacy. Despite their popularity, appropriate methods for quality and efficacy assessment of herbal medicinal products are not readily available. While current legislation in South Africa seeks to implement guidelines for the regulation of herbal medicines,[4] developing standards for such regulation is quite time consuming and rife with challenges due to a number of reasons.

Firstly, unlike conventional drugs where activity is due to (a) known active ingredient(s), herbal medicinal products are a combination of several different compounds whose net effect may be agonistic, synergistic, complementary, antagonistic or even toxic to name but a few.[14,15] Secondly, the pharmacological effect of the herbal medicinal product may be from just one component or a combination of several other compounds, whose ratio in the product may also influence safety, quality and efficacy. The safety, quality and efficacy in turn, can be affected by seasonal changes, growth conditions, harvesting, processing and preparation methods.[12,14] Thirdly, there may be challenges with identification of different plants, deliberate adulteration of plant materials as well as multicomponent changes during transportation and storage.[64] This necessitates appropriate standards to monitor these materials and their formulations. Such standards will ensure consistency of formulations, as well as an improvement in their quality.

The Pharmaceutical Manufacturers Association of the United States defines quality as "the sum of all the factors which contribute directly or indirectly to the safety, effectiveness and acceptability of the product".[65] It refers to conformance to requirements and the ability to meet desired standards, and is of utmost significance in relation to drug products or any product with the potential to cure or manage a (perceived) health condition. Quality control measures serve to ensure that each dosage unit of the drug product delivers the same amount of active ingredients within specified limits in order to assure quality and efficacy.[66] This is of importance in herbal product use, as the quality and content of perceived active pharmaceutical ingredients (APIs) in such products can influence safety and efficacy.

There may also be differences in efficacy of different formulations of the same herbal product. This is due to the fact that many herbal products are not necessarily prepared according to current good manufacturing practices (cGMP), and their quality, which can influence their efficacy, may still give room for concern. In view of this, initiatives to address the poor quality of some herbal products are not only necessary, but in the public's best interest. Such quality assessments can be conducted *via* analytical tests which can be introduced as prerequisites for product registration and product introduction into the commercial stream, such as is obtained for conventional drug products. The analytical tests may very well involve studies on the content, stability and dissolution of perceived active ingredients from herbal products, with specifications set for product approval. This will contribute in no small measure to an improvement in herbal products quality, as it will ensure that marketed products meet the specified standards set.

2.3 Towards improving the quality control of herbal medicinal products

Quality control of herbal medicinal products is still in its infancy, and generally very poor. Attempts have been made to draw attention to this problem, with calls being made for improved measures which can translate to better safety, quality and efficacy for these products.[13,67-69] Such improved measures can also be employed to substantiate herbal medicinal product use.

In vitro screening programmes, using the ethnobotanical approach, are important in validating the traditional use of herbal remedies and for providing leads in the search for new active principles. Whereas activity identified by an *in vitro* test does not necessarily confirm that a plant extract is an effective medicine, nor a suitable candidate for drug development, it does however provide a basic understanding of a plant's efficacy and, in some cases toxicity, in a traditional herbal remedy. This is certainly a primary concern of ethnopharmacological research in developing countries. Scientific validation of herbal medicine use also lends support to the continued practice of "traditional medicine" in Africa. Eventually, this may lead to more widespread use of African Traditional Medicine (ATM) in health care systems, as in India and China, provided thorough toxicological investigations are carried out and sufficient guidelines for regulation are put in place.[53,70,71]

Regulation of herbals, though better than non-regulation, is still not as harmonised as regulation of conventional drug formulations. Herbal product regulatory guidelines differ from one country to the next depending on the definition of herbals in the country and also on whether the products are being used for diagnosis, cure, mitigation, treatment or prevention of diseases.[13,14,72] In the United States for instance, herbal products can be sold as "dietary supplements" and so are exempt from regulation.[10,62] More recent guidelines by the FDA however aim to approve herbal mixtures if safety and efficacy data show benefits.[8] Under European standards, herbal products can be licensed as medicines, and are registered after due quality assurance tests. In Korea, proof of quality is required prior to the marketing of herbal medicines, and a licensing system to ensure product safety is in place.[73] The Ayurvedic Pharmacopoeia of India contains monographs for over 250 products; however, the documented standards are deficient for quality assessment. The Government of India has announced regulations for traditional medicines in an effort to improve their quality.[53,74] In China, traditional herbal medicines have been integrated into the primary health care system, and regulations require safety and efficacy evaluation as well as GMP-compliant production. Extensive scientific studies have been conducted, and this has assisted in the promotion of Traditional Chinese Medicine (TCM). The traditional medicine departments are accommodated in almost all the general hospitals in China, and the Pharmacopoeia of the People's Republic of China contained 992 monographs of Chinese crude drugs and traditional Chinese patent medicines as at the year 2000.[8,53,74]

In Africa and many other low income countries, the use of herbal products is largely unregulated, posing a risk to consumer safety.[6] A major challenge in these countries is the slow pace of quality standards and regulatory development compared to the demand for traditional herbal medicines.[7] Guidelines were proposed by the World Health Organisation (WHO) for the quality control and promotion of herbal medicines over a decade ago, and were regularly updated.[75] These guidelines have been adopted by some African countries like Nigeria, Ghana, Mali and South Africa, among others, who are progressing towards attainment of the set goals;[4,74,76] however, there is still considerable room for improvement. In South Africa, the regulatory body, the Medicines Control Council, has set up guidelines for the regulation of herbal medicinal products. Current legislation in South Africa aims to register all herbal products, a means of ensuring product quality.[4,11]

For safe and efficacious use of herbal medicinal products, whether in South Africa or elsewhere, product quality has to be assured. Such quality and efficacy may be attained by standardization of the herbal products. Standardization encompasses all measures taken from plant cultivation to clinical use or application of the final product, which serve to ensure product consistency. This includes assessment of macroscopic and microscopic parameters, chemical assays, moisture content and foreign matter to mention a few.[73,77] HPLC chromatographic fingerprints have also been employed in order to assess the degree of "sameness or difference" in herbal formulations.[78,79] In addition, the quality and efficacy of herbal medicinal products can be monitored by stability and dissolution studies, which have been reported for some herbal medicinal products.[24,67,80,81] Although not a requirement for the regulation of herbal medicinal products in many countries,[81,82] dissolution testing of formulated herbal products can serve as a useful tool for the quality control of these products.

For conventional drug products, dissolution testing is a very important tool employed in drug development and quality control.[22] This is because a drug's rate of dissolution under *in vitro* settings can be employed for bioequivalence assessment of formulations as well as to predict its *in vivo* bioavailability. For this reason, dissolution tests for oral dosage forms are designed to replicate physiological conditions of the gastrointestinal tract,[83] enabling extrapolation of results from such tests to *in vivo* conditions. This quality control test therefore serves as one of the basic tests in the formulation and bioequivalence assessment of drug products containing chemically defined, synthetically produced active ingredients.[14,22,83-85]

With respect to herbal medicinal products however, quality control tests such as dissolution tests are not a prerequisite for their acceptance and introduction into the consumers' circle.[14,22] Even if such quality control tests were to be performed, chemical marker compounds (which can serve as standards) are not readily available for most herbal medicinal products. This is due to the nature of herbal medicinal products, which contain multiple components, the exact ones which are responsible for activity and the mechanisms of such activity not yet fully elucidated. Because of such limitations, setting up quality control tests such as dissolution tests for herbal medicinal products, with specifications, will

present enormous challenges. Such challenges have to be addressed in order to introduce discriminating tests that can be used for quality control and regulation of herbal medicinal products. Among the first challenges to be addressed is the selection of perceived active pharmaceutical ingredients (APIs) of the plant, so-called chemical marker compounds, which can be employed as reference and marker compounds for quality control.

2.4 Chemical marker compounds as quality control tools

The European Medicines Agency (EMEA) defines chemical markers as chemically defined constituents or groups of constituents of a herbal medicinal product which are of interest for quality control purposes regardless of whether they possess any therapeutic activity. A chemical marker can be used to assess the quality of a herbal medicinal product as its concentration can indicate potency and quality status of the product.[12,86] An ideal chemical marker should exhibit therapeutic effects of the herbal medicinal product. Because only a limited number of compounds contained in herbs have been shown to possess pharmacological actions, other chemical components that may or may not exhibit pharmacological effects may also be employed as markers.[12] Such markers could be from any appropriate class of secondary metabolites found in the plant from which the herbal product is sourced. The secondary metabolites in question can be flavonoids, which are known to be abundant in plant materials,[87,88] and are also present in *S.* frutescens.[2,51] As such, flavonoid compounds can serve as chemical marker compounds for *S. frutescens* formulations and materials.

2.4.1 Flavonoids as chemical markers

2.4.1.1 What are flavonoids?

Flavonoids are polyphenolic plant pigments synthesized from the amino acid, phenylalanine. They are common in green plant cells, and are responsible for the bright colours of flower petals.[89] In the last decade, these compounds have become of interest due to their many documented health benefits. They have been reported to prevent and cure many diseases, with very low toxicity to animal cells. Flavonoid use in disease prevention and treatment has been attributed to pharmacological effects produced through antioxidant activity *via* scavenging of free radicals.[90-92] Chemically, they are characterized by two aromatic rings (A and B) linked by a heterocyclic C-ring (Figure 2.3).

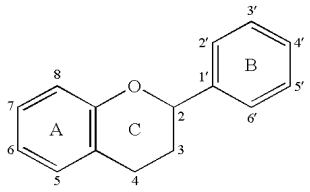


Figure 2.3: Basic structure of a flavonoid

Five major subgroups of the flavonoids are characterised based on the structure of the C-ring (Figure 2.4). These are flavonols (with a hydroxyl group in position C_3), flavones (with a double bond between positions 2 and 3 and a ketone group in C_4), flavanones (with the C-ring saturated), dihydroflavonols/flavanonols (the 3-hydroxy derivatives of flavanones), and flavanols (also called flavan-3ols because of a hydroxyl group almost always at C_3 but with no double bond between C_2 and C_3). In plant materials, some of these different subgroups may be present, and their levels may vary with the type and quality of plant material.

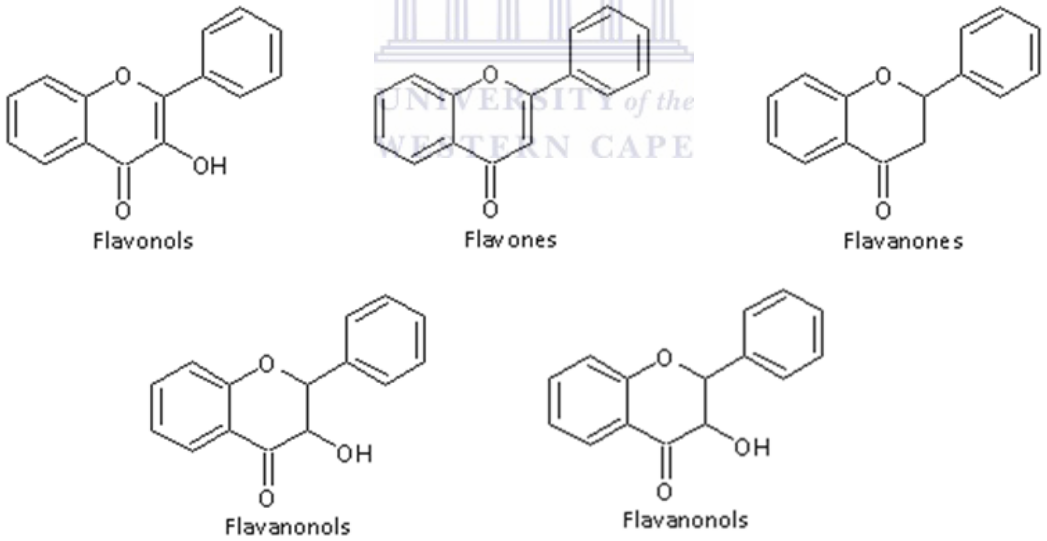


Figure 2.4: Subgroups of the flavonoids

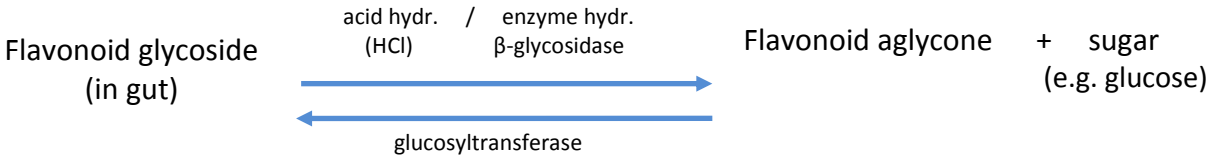
2.4.1.2 Levels and types in herbals

Flavonoids are secondary plant metabolites and are abundant in nature. Over 7000 different flavonoids have been discovered, with the list regularly updated as new ones are discovered.[93] They are present in plants and by default, in most plant-based products. In the

plants, they exist either as the free aglycone or as flavonoid glycosides (where the aglycone is bound to a sugar *via* a glycosidic bond). The more abundant form of flavonoids is the flavonoid glycoside, which is soluble in aqueous media. The aglycone is more soluble in organic solvents, and less soluble in water.

In traditional settings, herbal medicinal plant materials are usually prepared as aqueous-based decoctions and infusions. In these formulations, the different active phytopharmaceutical ingredients (APPI) such as the aqueous-soluble flavonoid glycoside compounds will be released. Thus, the flavonoid glycosides represent the form in which actives from many traditional medicinal products (such as *S. frutescens*) are released when prepared for oral consumption, and are inherently more abundant than the non-soluble flavonoid aglycones in aqueous media used for their extraction.

Despite their abundance, it is difficult to quantify individual flavonoid glycosides due to their inherently large number in plant samples. These glycosides however, are derived from a limited number of flavonoid aglycones, and have actually been described as "sustained-release natural prodrugs of their aglycones".[94]



In quantification studies therefore, the glycosides are usually acid-hydrolysed to the aglycones and glycoside levels quantified in terms of the aglycone content.[87,95] Either form, flavonoid glycoside or flavonoid aglycone, can be employed as marker compound in quality control and pharmacokinetic studies of herbal products as both forms may be available in *in vitro* settings and following human intake.

2.4.1.3 Human intake

Flavonoids are present in fruits (e.g. berries, apples, and oranges), vegetables (onion, spinach, broccoli, green pepper, and tomato), soy beans, herbs, grain and tea. Higher concentrations can be found in fruit peels, leaves and flowers of plants.[94] Recently, due to

their many reported health benefits, they have become a focus of interest, with many consumers purchasing actively developed components marketed as herbal remedies or dietary supplements.[94] From food sources, the mean daily total flavonoid intake by humans ranges from 158.3 to 203.0 mg/day.[96,97] Various amounts are absorbed and eliminated from this intake, and these amounts can be calculated using pharmacokinetic theories developed for such. Due to their abundance in plants and other advantageous characteristics, they can be employed as chemical markers for herbal product research.

2.4.1.4 Suitability of flavonoids as chemical markers

In green plant cells where flavonoids can be detected, they are much diversified.[88,98] The different classes and sub-groups are susceptible to extreme modification and can be readily hydrolysed, oxidized, hydroxylated, methylated, glycosylated, acylated or phenylated, giving rise to the variety of compounds within a class.[99,100] This can be applied in quality control of herbal drug materials as follows.

Generally, most forms of instability in drug products are due to hydrolytic and oxidative reactions.[101,102] These reactions and the subsequent instability caused can lead to poor quality of the affected drugs. In herbal materials which most likely contain flavonoids, flavonoid susceptibility to different chemical processes can be used to monitor product quality. For instance, if the flavonoid glycoside or aglycone content and /or type increases or decreases, this can be an indication of some form of product instability, which can indicate quality status. An understanding of the flavonoid glycoside and aglycone content in herbal materials is however needed for such interpretation.

Oxidative reactions of flavonoids are common, and are catalysed by three major enzymes: catechol oxidases, laccases and peroxidases.[103] As phenolic compounds, the flavonoids are easily oxidized to quinones.[89] The existence of vicinal hydroxyl groups in the flavonoid structure provides a site for oxidative attack that opens up the flavonoid ring.[89] In the presence of ultraviolet light (and sometimes heavy metal ions), oxidation proceeds with a ring opening at C_1. The first step in the oxidative degradation of organic compounds in air is hydroxylation. Hydroxylation reactions of flavonoids occur at positions 3, 5, 7, 3', 4' and 5' which are suitably vacant. Some of the hydroxyl groups may undergo further reactions –

sulphation, methylation, glucuronidation or acetylation to yield other compounds, shown using the flavonoid aglycone, quercetin, as an example (Figure 2.5). Such oxidative reactions of flavonoids in plants can influence their biological effects.[104] For instance, during food processing of plant material and storage, flavonoids can be oxidized by enzymes. This can compromise cell integrity, resulting in discolourations, one of the key symptoms of reduced quality in a product.[105,106]

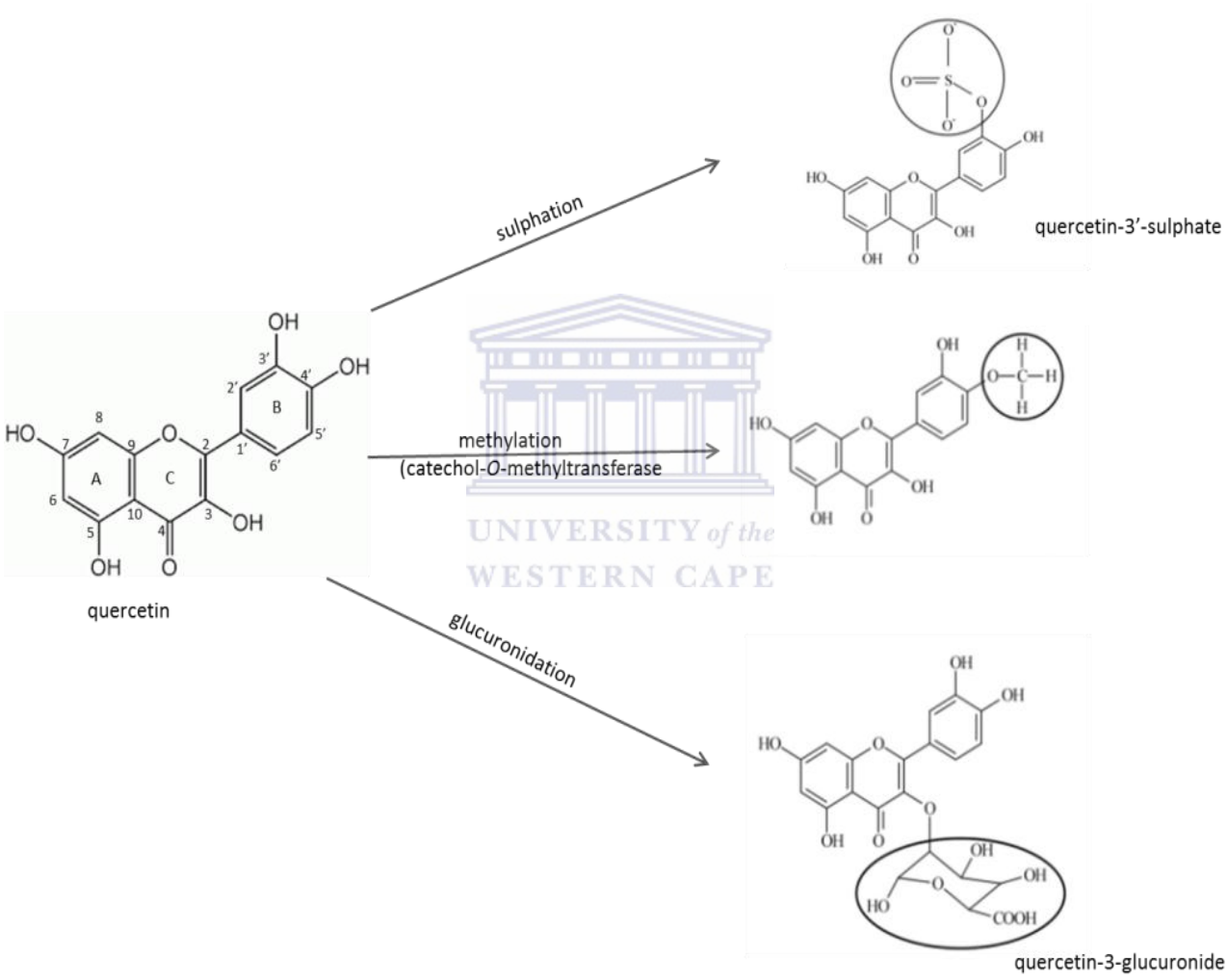


Figure 2.5: Common reactions of flavonoids, using quercetin as a template

Flavonoid glycosides also undergo hydrolysis in the presence of acids or enzymes. The ease of hydrolysis depends on the nature of the flavonoid glycoside (whether an *O*-glycoside, *C*-glycoside, or both), the identity of the sugar and the site of attachment.[107] Flavonoids with an *O*-glycosidic bond are easily hydrolysed by acids while *C*-glycosides exhibit resistance to such hydrolysis.[108] Glucose conjugates of flavonoids are reportedly easier to hydrolyse than

glycosides formed by flavonoid aglycone conjugation with other sugars such as rutin, quercitrin and naringin and galactose.[109,110] On the influence of attachment site to flavonoid glycoside hydrolysis, the C_7 position of the flavonoid aglycone has been found to be more prone to hydrolysis than the C_3 position.

Flavonol glycosides are believed to be hydrolysed to their corresponding aglycones prior to absorption. Because of this, in addition to difficulty in obtaining reference standards for all flavonoid glycosides, the glycosides are usually acid-hydrolysed to the aglycones which are then identified and quantified in analytical procedures.[111]

To summarize, quality assessment of herbal medicinal products can be used to ensure efficacy and batch consistencies. A material of good quality should be able to maintain as much of its original characteristics as possible over its envisaged period of use. Changes in quality and efficacy of herbal medicinal products may result from chemical processes which may be enhanced by changes in temperature, moisture and pH conditions during preparation and or/ storage of the product. The flavonoid compounds may be very sensitive to such changes, and so can serve as quality and by extension, efficacy indicators. Because they can also be affected by changes in the pH of the medium, they can serve as appropriate references for evaluating *in vitro* release from *S. frutescens* materials in different dissolution media. These different media are indicative of different sections of the gastrointestinal tract as well as their prevailing pH conditions, and so are ideal for dissolution testing, a quality control tool which can be employed for *S. frutescens* products.

2.5 Dissolution testing

2.5.1 Brief history of the drug dissolution test

Dissolution has been defined as the amount of substance that goes into solution per unit time under standardised conditions of liquid/solid interface, solvent composition and temperature.[112]

This quality control test originated with the early physical chemists. The first known reference to dissolution testing was made by Noyes and Whitney in their publication, “Rate

of solution of solid substances in their own solution".[113] Their paper suggested that the layer of saturated solution formed around a solid particle during dissolution determines the mechanism and rate of dissolution. Physical and chemical factors as determinants of solid dissolution rate were proposed at the start of the 20th century.[114] Later, the Noyes-Whitney equation, as we know it today, was modified by Nernst and Brunner, relating dissolution rate and diffusion coefficient.[115,116] It was not till 1930 that experiments on dissolution testing of drugs commenced, with *in vitro in vivo* correlations (IVIVC) established by the 1950s. Prior to 1950, experimental work on dissolution as a release mechanism for drugs from dosage forms was limited.[117]

2.5.2 Dissolution testing of herbals

Currently, dissolution testing is a very important tool employed for quality control of conventional drug formulations. It is also used for optimization of formulations, and desired release specifications for drug products can be determined with this test. As a quality control test that assesses release of actives from pharmaceutical formulations and materials, the dissolution test can be used to assess whether an orally administered drug product will realise the objective for which it is formulated, which is to release the API in the right environment (stomach or intestine, depending on formulation) so that it can further be subjected to the pharmacokinetic processes of absorption, distribution, metabolism and /or excretion.[118]

As widely accepted as this test is for conventional drug and active pharmaceutical ingredient (API) formulations, it is still a novelty for herbal medicinal products. Considering the fact that herbal (medicinal) products have gained tremendous popularity in recent years, and may diversify in the pharmaceutical industry, perhaps serving as new formulations or "improved" modified versions of previous formulations, there is a need for the introduction and implementation of new approaches that will improve their quality and efficacy profiles.[67] Dissolution studies of these materials, conducted according to relevant developed guidelines, may well provide the desired advancement. As herbal products are complex multicomponent materials, their dissolution tests may be conducted using one or more

suitable reference marker compounds for development of compendial and regulatory standards.

Development of compendial standards for herbal product dissolution is still in its infancy, and presents considerable challenges. Currently, only four herbal products classified as dietary supplements – arginine, curcuminoids, soy isoflavonoids and turmeric – have monographs with dissolution specifications in the USP.[24] Commercially available herbal products and dietary supplements, like most natural products, are inherently variable in the quantity and quality of constituent "active" and non-active compounds. This, coupled with deficiency in standardisation and processing methods, may result in batch to batch variation and even within batch variation of formulations.[23,24] Such variations may also offer an explanation for the differences in efficacy observed in clinical trials with herbal products.[23,24]

The wide variability in quality and content is in itself a limitation to the use of these products, and methods to address these challenges have become of interest to the stakeholders involved (regulatory authorities, research institutes, manufacturers and marketers alike). Such variability in quality and content may also contribute to significant differences in percentages released and dissolved in dissolution test media at various sampling times, and these factors will need to be considered in method development.

A few studies have reported on dissolution studies of herbal materials, and the percentages of reference compounds released in the medium. Nair and Kanfer reported a minimum of 75 % release of hypoxoside and sterols in one hour, from dissolution studies of African potato (*Hypoxis hemerocallidea*) conducted at pH 1.2 and 5.0 respectively, using the USP basket apparatus.[81] Dissolution tests on *Passiflora* products demonstrated 50 % and almost 100% dissolution of actives from capsules containing the powder and capsules containing the extract, respectively, in 10 minutes (USP paddle method at a speed of 50 rpm, using water at 37 ± 0.5 °C and UV spectrophotometric detection at 340 nm).[22]

A further study by Jackson *et al*. has intimated that the quick release of a drug from its enclosing matrix at earlier time points, which we have encountered with some herbal materials in our labs (unpublished study), may be a disadvantage.[119] For APIs which exhibit quick release, all the active components may be dissolved in the test medium almost

immediately while very little will be absorbed. In such a case, there is a need to determine the bioavailability of the active from the product, in order to assess whether all the released actives can be absorbed, and whether such absorption will maintain effective therapeutic concentrations of the actives in plasma. These and other similar issues may need to be addressed for appropriate dissolution method development.

2.5.3 Method development for dissolution studies

The drug dissolution test is an official quality control test designed to evaluate drug release and dissolution within the gastrointestinal tract. Such a test is conducted according to specifications set out in the pharmacopoeia.[120] These specifications may be modified as necessary, though such modifications need to be validated.[121] Depending on the part of the GIT where drug dissolution is expected, dissolution tests can be conducted using different media and conditions that mimic the gastrointestinal tract (GIT) environment.[83]

Under certain circumstances, the drug dissolution test may also be used to assess bioavailability and bioequivalence. In such cases, *in vitro* dissolution testing is used as a substitute for *in vivo* bioequivalence (BE) studies, and two formulations are deemed therapeutically equivalent if their dissolution profiles are similar. Briefly, a dissolution profile assesses the cumulative percentage of the API that goes into solution over time. For predicting bioavailability and bioequivalence from dissolution data, the Biopharmaceutics Classification Scheme (BCS) class of the drug should be considered.

The Biopharmaceutics Classification Scheme is a scientific classification of drug substances according to their solubility and permeability profiles.[122] Details of this classification are explored under the section on *in vitro in vivo* correlation models (Section 2.7.3.2). For orally administered drugs in BCS class I, and for non *bioproblem* drugs, bioequivalence can be documented using *in vitro* dissolution studies.[123] Non *bioproblem* drugs are products for which bioequivalence may be shown by *in vitro* tests only while *bioproblem* drugs are products that require *in vitro* and *in vivo* tests in order to prove bioequivalence to the innovator or standard drug.[124] Products that may be classified as *bioproblem* drugs include but are not limited to:[124]

- drugs with narrow therapeutic indices,
- drugs known to show intra- and interpatient responses
- drugs contained in dosage forms that may give rise to significantly improved bioavailability (superbioavailability)
- drugs intended for the critically ill, geriatric or paediatric patient

Typically, drugs in BCS class I that do not have *bioproblem* restrictions may be viewed as non *bioproblem* drugs while drugs in the other classes may not be.

When using *in vitro* tests as substitute for *in vivo* bioavailability and bioequivalence, the Food and Drug Administration (FDA) of the United States and the Southern African Development Community (SADC) recommend that an appropriate USP method be used for such tests.[123,125] If such a method is not available, the FDA method for the reference drug should be used. In cases where neither USP nor FDA methods are available, a dissolution test method should be developed for the products under study. For method development, dissolution profiles should be generated in aqueous-based media with a pH range of 1.2 to 6.8, taking samples at not more than 15-minute intervals (12 units each).

FDA regulatory guidelines further state that for drug dissolution testing, the volume of the medium is usually 500 ml, 900 ml or 1000 ml.[126] Sink conditions are desired (but not mandatory). While aqueous-based media with a pH range of 1.2 to 6.8 are recommended, the use of water as a dissolution medium is not encouraged. This is due to the fact that water has no buffering capacity and test conditions (e.g. pH and surface tension) may vary depending on the water source. In addition, these conditions may change during the actual dissolution test itself, as a result of other ingredients which may be active or inactive,[127] and may return untrue results for dissolution tests and profiles.

Dissolution tests are used to generate dissolution profiles, which can be used to compare products. Two products with similar dissolution, though not necessarily similar release characteristics, will have similar dissolution profiles. When using dissolution tests for bioequivalence assessment, the primary concern is to quantify differences in bioavailabilities of different formulations or products, and to demonstrate the unlikelihood of clinically or therapeutically important differences.[125] This is certainly a primary goal for

the dissolution scientist in dissolution method development, and necessitates the development of a discriminatory dissolution method.

The discriminating power of a dissolution test method is its ability to detect changes (formulation, manufacturing, storage or otherwise) in a product.[128] Discriminatory methods are employed for quality control and can be used to assess batch to batch similarity, for instance, before release of product for a clinical trial. Such discrimination is necessary from a biopharmaceutics point of view, as it will enable detection of changes in product quality that may affect *in vivo* performance.[129]

For dissolution tests of herbal products, component compounds found in the plant can be used for reference purposes. In such a case, they can be referred to as marker compounds because they can be used to characterise the product. Such markers /references can be used as the active (phyto) pharmaceutical ingredient (API or APPI) during dissolution testing. Therefore, in dissolution tests of herbal products, the dissolution of these compounds will be assessed. Such assessment needs a discriminating method that can be used to establish differences between formulations, subtle though such differences might be.

Much as a discriminating method is desired, it may also be necessary to select a discriminating method for dissolution profiles comparison. The previous section described the considerable variation that may be encountered during dissolution testing of herbal materials using reference marker compounds, even with products from the same production batch.[23,24] It then becomes important not only to develop a discriminating dissolution method, but to also compare dissolution profiles with a discriminating comparison method. Some of the dissolution profile comparison methods that have been proposed for their discriminating ability, especially for data that present considerable variation, will be discussed in the following section and may be employed for dissolution profiles comparison of marker compounds from herbal products.

2.6 The dissolution profile and its applications

2.6.1 What is a dissolution profile?

A dissolution profile has been defined as the measured fraction (or percentage) of the labelled amount of drug released from a dosage unit at predetermined time points and

dissolved in the dissolution medium when tested in a dissolution apparatus.[14,130] Dissolution profiles of an API from different formulations can be used to establish the similarity or otherwise of pharmaceutical dosage forms, to confirm dissolution specifications for a specific pharmaceutical dosage form and to predict *in vitro in vivo* correlations. Thus, it prevents the need for costly pharmacokinetic studies in humans as well as speeds up product development procedures. For dissolution profiles of herbal formulations, the release of the suspected active or marker compound is usually assessed.

2.6.2 Methods for dissolution profiles comparison

Dissolution profiles can be compared using one or a combination of graphical methods, statistical analysis, model dependent and model independent methods (Figure 2.6).[120,131-133]

2.6.2.1 Graphical method

The graphical methods plot the concentration of dissolved drug in solution as a function of time, and compare percentages of the drug in solution at each sampling point, as well as the shapes of the curves. Overlapping curves indicate some level of similarity between the curves and the difference between these curves can be assessed to evaluate the level of (dis)similarity. However, there is no knowledge of how small the difference has to be to indicate similarity, or how large it has to be to indicate dissimilarity.

2.6.2.2 Statistical methods

Statistical methods for dissolution profiles comparison include the student's t-test, and the analysis of variance (ANOVA). The one sample t-test, paired or unpaired t-test can be employed in these cases, and t is calculated from the equation:

$$t = \frac{|\, X - \mu \,|}{S/\sqrt{N}}$$

where ***X*** is the sample mean, ***N*** is the sample size, ***S*** is the standard deviation of the sample and ***μ*** is the standard deviation of the population. The calculated "t" value is compared to the tabulated "t" value and the null hypothesis rejected if the calculated exceeds the tabulated value. The ANOVA-based statistical methods are more commonly used than the t-tests.

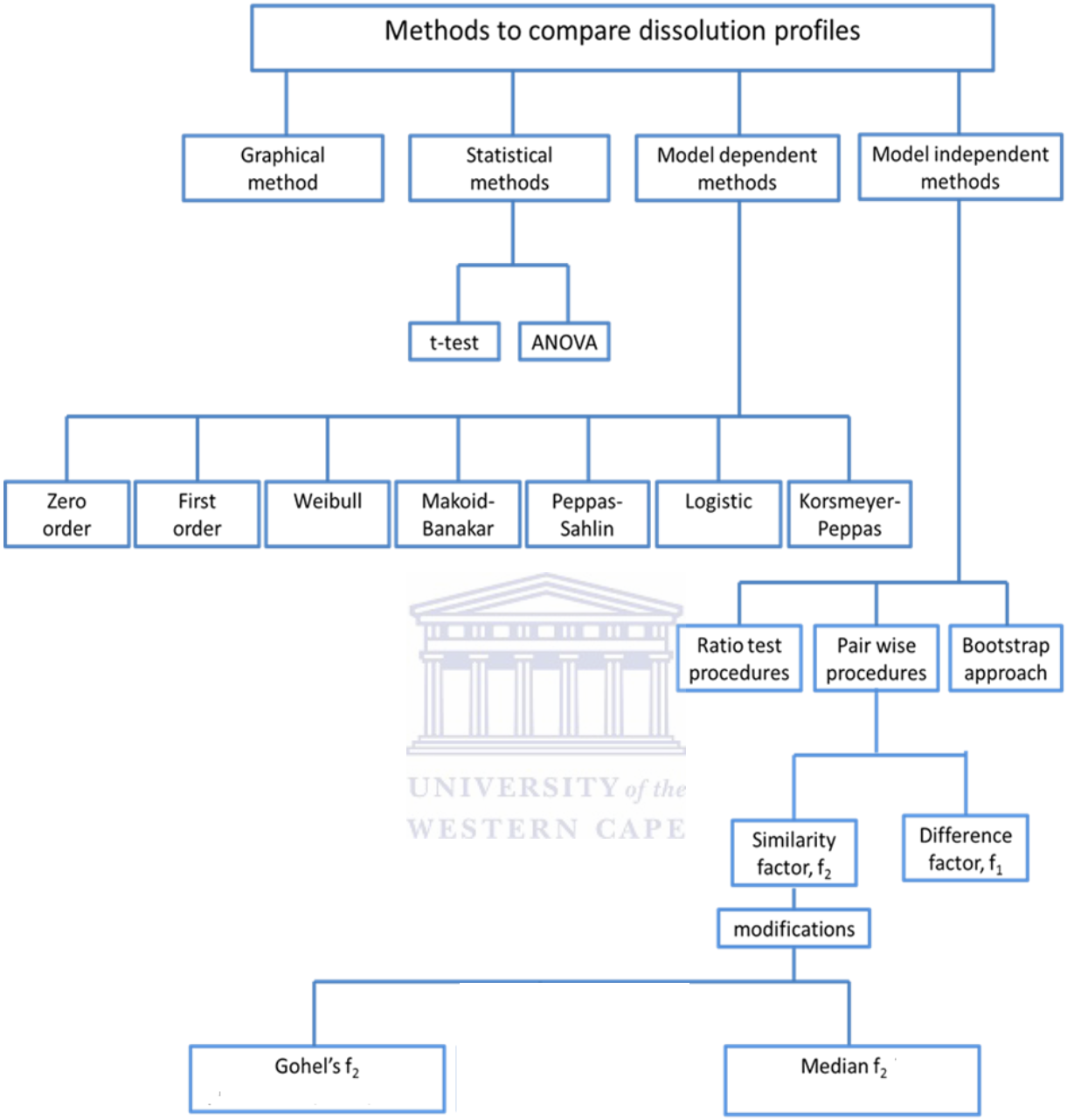


Figure 2.6: Methods for dissolution profiles comparison

For ANOVA-based methods, the variances within the reference and the test groups are calculated as shown in the equation below, and then compared to each other.

$$F = \frac{between - group\ variablity}{within - group\ variability}$$

The F-value (ratio of the variance between the groups to the variance within the groups) calculated is compared to the tabulated (or expected) F-value at specific degrees of freedom and levels of significance. The variation between two profiles is considered insignificant (and hence the profiles declared similar) if the calculated is less than the tabulated F-value. The level of significance is the probability of obtaining a result as rare as the one actually observed, if the null hypothesis is true, and is usually 95 %. These statistical methods may seem good for dissolution profile comparison of active compounds from herbal materials, where within batch variations may be quite significant, as it takes note of the within-batch variation in analysed samples. However, statistical methods for dissolution profiles comparison are not ideal as each dissolution time point is treated as a separate entity, independent of other points, which is not necessarily so. As such, the difference in profiles may be significant at some points and not at others, making interpretation difficult.[134] In addition, statistical methods for dissolution profiles comparison have been found to be over-discriminating from a biopharmaceutical point of view.[135] Such limitations may be addressed with the use of model-dependent methods.

2.6.2.3 Model-dependent methods

Herbal medicinal products like *S. frutescens* are consumed for the prevention and treatment of ailments. As such, they can be viewed as drugs and dissolution processes of their active components can thus be modelled and analysed as is done for conventional drug formulations. Subsequently, the terms 'API' and 'drugs' will be used interchangeably.

Majority of drugs are available as solid dosage forms. For such drugs to be absorbed and hence exert pharmacological action, they have to be released from the dosage form and dissolve in the appropriate section of the gastrointestinal tract.[136] Model dependent methods for dissolution profile analysis and comparison attempt to determine the release of the active from a formulation by employing mathematical equations, which use fitted models to describe dissolution and the kinetics of drug release from a product in different areas of the gastrointestinal tract, each section signified by its appropriate dissolution medium. For instance, in the fasted state, the pH of the stomach is usually below 2. Soon after food intake, gastric pH increases to values between 4 and 7. Along the length of the

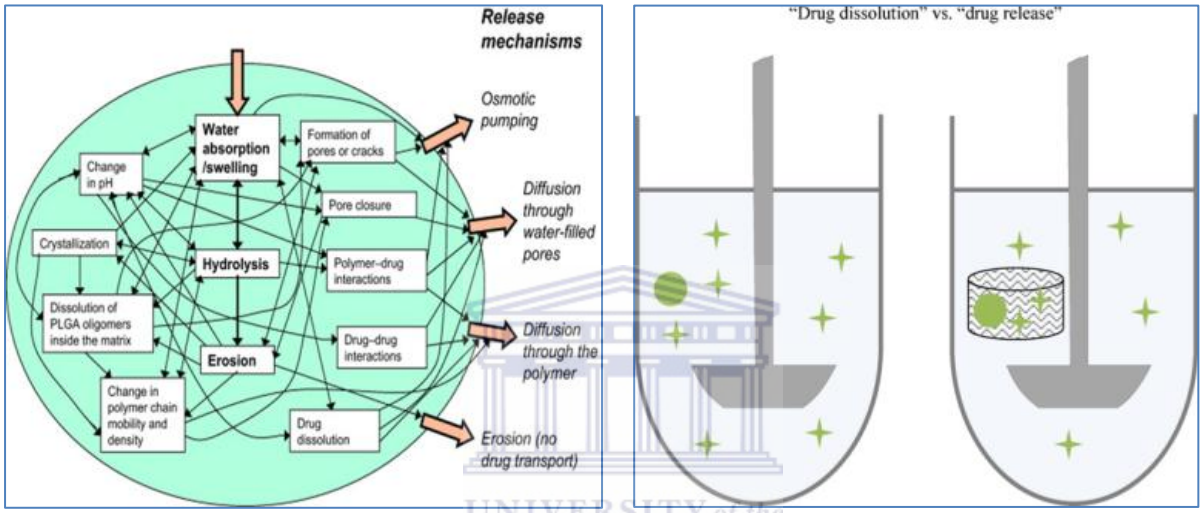
small intestine, the pH fluctuates between 6.5 and 7.5.[137] Dissolution tests in media representative of these pH conditions can therefore be used to evaluate and model API release in various sections of the gastrointestinal tract.

The release of an API from a drug product proceeds *via* a series of processes through which a drug is freed from its enclosing matrix, dissolves to form a homogenous phase, in order to be subjected to the pharmacokinetic processes of absorption, distribution, metabolism and excretion,[138] and ranks as a very important process in development and consumption pharmaceutics. Advantage can be taken of an API's release kinetics in order to produce a formulation with desired release characteristics, translating to optimum bioavailability and efficacy. Prediction of these desired release kinetics can be done by model-dependent methods of dissolution profile analysis *via* mathematical modelling.

In mathematical modelling of drug release, abstract models are used to describe the processes involved in drug release. Many processes are involved in drug release, one of which may be drug dissolution. Dissolution, as defined by the IUPAC, is the mixing of two phases to form one homogenous phase, the solution. Dissolution occurs in a number of stages: the drug is initially wetted with water from the dissolution medium, breaking up solid state bonds in the drug and thereby enabling solvation of individual drug molecules. The individual drug molecules then diffuse through the unstirred boundary layer and eventually, are transferred by convection within the well-stirred bulk fluid.[136] These processes can be represented with appropriate mathematical models.

Drug dissolution is not synonymous with drug release from a matrix (Figure 2.7). [136] In Figure 2.7 (a), the different processes involved in drug release are indicated, and how one process can influence and be influenced by other process(es). Figure 2.7 (b) differentiates drug release from drug dissolution, where the green circles represent the API present in the form of a solid particle, which on contact with the dissolution medium releases individualized drug molecules in solution (the green stars). The polymer matrix enclosing the drug (the shaded cylinder) may also undergo swelling to release some of the drug molecules into solution, well before complete disintegration of the drug product (Figure 2.7 (b): see API, i.e. green circle, still trapped within polymer matrix while some molecules are already in

solution, i.e. the green stars). Thus, various phenomena – such as water diffusion into the polymer enclosing the drug, drug dissolution and diffusion or swelling, dissolution and degradation of the polymer – may be involved in drug release.[136,139] These mechanisms involved in drug release may in turn be affected by other processes such as polymer-drug interactions, pH of the dissolution medium, crystallization and pore closure such that the appropriate underlying mechanism may well involve a number of other processes[140] as shown in Figure 2.7 (a).



(a) (b)

Figure 2.7: Graphics of drug dissolution showing (a) the mechanisms involved in release from matrix (author, not available) and (b) a comparison of drug dissolution to drug release from a matrix[136]

Drug release kinetics and transport processes involved in drug release can be described with suitable mathematical models to estimate drug release from a formulation as well as to elucidate mechanisms involved in drug release.[136,139,141,142] Such models give a good estimate of the geometry and dimensions of the required compound, and can thus be used to optimize drug delivery, and in regulatory settings to assess quality parameters.[136,139,143,144]

It is pertinent to mention that there is no universally acceptable model for release of all drugs, and release data are usually fitted to various models in order to assess goodness of fit prior to selection of the best model. The accuracy of a mathematical model in predicting drug release increases with increasing model complexity, however, theoretical calculations

have to be compared with experimental results before the model of best fit can be selected. Model suitability may also be compromised by prevailing experimental conditions.[139]

When modelling drug release, some equations serve to describe the release profile while some other equations can be used to predict the mechanism of drug release in addition to describing the release profile. Equations and models used to describe the release profile are referred to as empirical. They describe the overall shape of the release curve, not necessarily with any kinetic basis.[145] On the other hand, semi-empirical models describe the shape of the curve as well as offer some explanations for the underlying drug release mechanism.[145] Examples of semi-empirical models of drug /API release employed in this study are the Weibull, Peppas-Sahlin and Korsmeyer-Peppas models.

The Weibull equation, previously criticized for the non-physical nature of its parameters and for having no kinetic basis,[146] has subsequently had its model parameters re-evaluated and related to system geometry. It was documented that the creation of a concentration gradient close to the releasing boundaries of the Euclidian matrix, or the "fractal behaviour" attributed to the environment's fractal geometry, was explained by the Weibull function.[147] Fractal behaviour is one that has a consistent pattern at every scale, whether scaled down or up. It is an integral part of fractal geometry which offers many options for the description, measurement and prediction of natural phenomena (such as may be encountered in dissolution studies) using mathematical equations.[148,149] It can thus be applied to biological systems and processes which typically consist of many levels of substructure, with a consistent pattern at every scale.

A third model classification, the mechanistic models of drug release, can be used to predict dissolution profiles without the need for conduction of dissolution tests. This is due to the fact that physical properties of the API such as solubility and density are components of the model equation.[145] Semi-empirical or semi-mechanistic models are superior to either empirical or mechanistic models alone. This superiority of the semi-mechanistic model holds as long as the capacity for curve fitting and underlying process prediction is not endangered.[150]

Equations for the various models that can be employed to evaluate drug release are given in Table 2.1, and a brief description of each model follows. Descriptions of the parameters in the model equations are explained in Zhang *et al*.[151] In all the model equations, ***F*** represents the fraction of drug (dissolved) in solution, F_{max} represents the percentage of drug released at infinite time, ***t*** represents time and T_{lag} represents the time before onset of drug or API dissolution.

Table 2.1: Mathematical equations for models describing release of active pharmaceutical ingredient (API) from its matrix

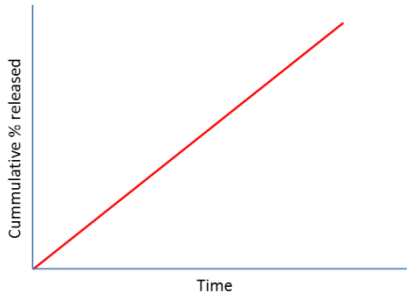
Model	**Equation**	**Parameters**
Zero order	$F = k_0 \,.\, t$	k_0
First order	$F = 100 \,.\, \left(1 - e^{-k_1 t}\right)$	k_1
Weibull_1	$F = 100 \,.\, \left[1 - e^{-\frac{(t-T_i)^{\beta}}{\alpha}}\right]$	α, β, T_i
Weibull_2	$F = 100 \,.\, \left(1 - e^{-\frac{t^{\beta}}{\alpha}}\right)$	α, β
Weibull_3	$F = F_{max} \,.\, \left(1 - e^{-\frac{t^{\beta}}{\alpha}}\right)$	α, β, F_{max}
Weibull_4	$F = F_{max} \,.\, \left[1 - e^{-\frac{(t-T_i)^{\beta}}{\alpha}}\right]$	α, β, T_i, F_{max}
Makoid-Banakar with T_{lag}	$F = k_{MB} \,.\, \left(t - T_{lag}\right)^{n} \,.\, e^{-k.(t-T_{lag})}$	k_{MB}, n, k, T_{lag}
Peppas-Sahlin 1 with T_{lag}	$F = k_1 . \left(t - T_{lag}\right)^{m} + k_2 . \left(t - T_{lag}\right)^{2m}$	k_1, k_2, m, T_{lag}
Logistic_2	$F = F_{max} \cdot \frac{e^{\alpha + \beta . \log(t)}}{1 + e^{\alpha + \beta . \log(t)}}$	α, β, F_{max}
Logistic_3	$F = F_{max} \cdot \frac{1}{1 + e^{-k.(t-\gamma)}}$	K, γ, F_{max}
Korsmeyer-Peppas	$F = k_{KP} \,.\, t^{n}$	k_{KP}, n
Korsmeyer-Peppas with T_{lag}	$F = k_{KP} \,.\, \left(t - T_{lag}\right)^{n}$	k_{KP}, n, T_{lag}

2.6.2.3.1 Zero order kinetics

Release of active pharmaceutical ingredient (API) from a matrix that does not disintegrate, but releases the drug slowly over time can be described by zero order kinetics. The fraction of the API dissolved in time ***t*** is given by the equation:

$$F = k_0 \,.\, t$$

where k_0 is the zero order release constant. The same amount of API is released per unit time, and this model is ideal when the API is required to achieve prolonged pharmacological action.[152]



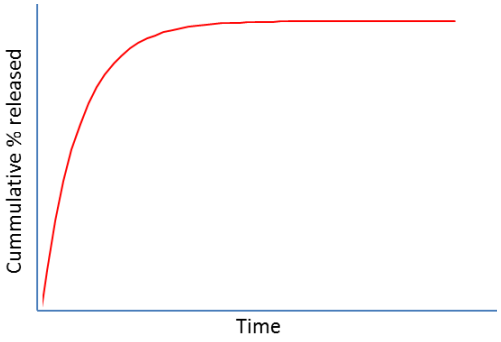
Graphical representation of zero order release model

2.6.2.3.2 First order kinetics

In first order release kinetics, the rate of API release is dependent on a concentration gradient set up between the concentration of the solute and its solubility in time, ***t***. Release of API is concentration dependent, and can be given by the equation:[153,154]

$$F = 100 \,.\, \left(1 - e^{-k_1 t}\right)$$

where k_1 is the first order release constant.



Graphical representation of the first order release model

2.6.2.3.3 The Weibull models

This model was first described by Weibull in 1951 and later modified to suit the release process by Langenbucher.[155] The equation for the Weibull model can be successfully applied to most release curves and is commonly encountered in dissolution studies.[146] According to

this equation, the cumulative fraction of API released from dosage forms in solution, at time ***t***, is given by:

$$F = \left[1 - e^{-\frac{(t-T_i)^{\beta}}{\alpha}}\right]$$

The parameter, T_i, represents the lag time before onset of the release process and is usually zero. The shape parameter, represented by ***b***, describes the release curve as exponential (b=1) (case 1); sigmoidal (b>1) (case 2), when the curve exhibits an S-shape with an upward curve followed by a turning point; or parabolic (b<1) (case 3), when the initial part of the curve is steeper than obtained with the exponential curve; while the scale parameter, ***α***, describes the time scale of the process.

Graphical representation of Weibull release model

This model has been criticised for being too empirical with little or no kinetic basis to explain API release.[156] However, it usually gives very good fit for most dissolution data,[146] and portions of the release curve obtained can be used to evaluate API release by modelling it to the desired semi-empirical model. At least four different modifications of the Weibull model are available for fitting API release data. These differ in terms of the presence or absence of the parameters, T_i and /or F_{max}, and yield slightly different model parameter values for the same data set. The exponent of time, **β**, in the Weibull equations can serve as an indicator of release mechanism of a drug through a polymer matrix, according to the values below:[147]

Interpretation of release mechanisms from polymeric systems using the Weibull function

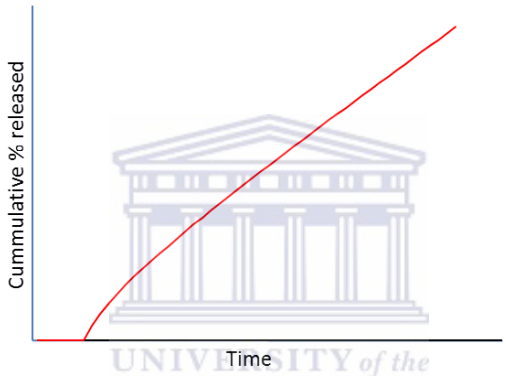
Time exponent, β	**Solute release mechanism**
β ≤ 0.75	Fickian diffusion in fractal or Euclidian spaces
0.75 < β < 1	Combined release mechanism
β > 1	Complex release mechanism

2.6.2.3.4 The Makoid-Banakar with T_{lag} model

This was developed by Dr.s Makoid and Banakar,[157] both pharmaceutical scientists. The Makoid-Banakar function is represented by:

$$F = k_{MB} \cdot (t - T_{lag})^{n} \cdot e^{-k.(t-T_{lag})}$$

where k_{MB}, n and k are empirical parameters of the model. These parameters are limited by the fact that total drug /marker compound dissolution must occur at the same time as the maximum value of the Makoid-Banakar function.[157] The Makoid-Banakar with T_{lag} model differs from the original Makoid-Banakar model by the inclusion of the parameter, T_{lag}, which represents the time before onset of drug release.



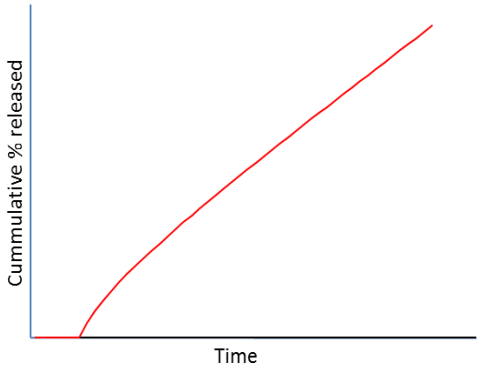
Graphical representation of the Makoid-Banakar release model

2.6.2.3.5 Peppas-Sahlin 1 with T_{lag} model

Peppas and Sahlin (1989) proposed that:[158]

$$F = k_d \cdot (t - T_{lag})^{m} + k_r \cdot (t - T_{lag})^{2m}$$

According to the Peppas-Sahlin equation, drug release can be governed by diffusion or relaxation depending on the values of the parameters k_d and k_r, representing the release rates of polymer diffusion and relaxation respectively, and the coefficient, m, with values between 0 and 1 in most cases.[151,158] Like the Makoid-Banakar with T_{lag} model, this model differs from the original Peppas-Sahlin model by the inclusion of the parameter, T_{lag}.



Graphical representation of the peppas-Sahlin release model

2.6.2.3.6 The Logistic models

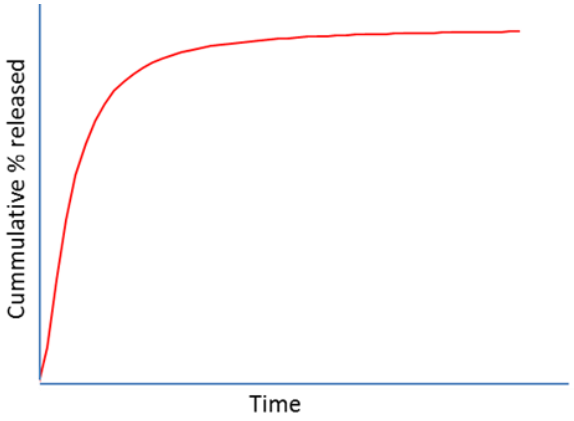
These are also empirical models, and lack significant kinetic properties. These models are valid only when the value of ***t*** exceeds zero.[159] Two of these models, Logistic_2 and Logistic_3 are represented by the respective equations:

$$F = F_{max} \cdot \frac{e^{\alpha + \beta.\log(t)}}{1 + e^{\alpha + \beta.\log(t)}}$$

and

$$F = F_{max} \cdot \frac{1}{1 + e^{-k.(t-\gamma)}}$$

where ***α*** and **β** describe the scale factor and shape parameter respectively, ***k*** is the dissolution rate constant, and ***γ*** is the time at which $F = \frac{F_{max}}{2}$.



Graphical representation of the logistic release model

2.6.2.3.7 Korsmeyer-Peppas with T_{lag} model

Korsmeyer *et al*. developed a relationship describing drug release from polymeric systems.[160] It is widely used to describe the kinetics of drug release from formulations. For the Korsmeyer-Peppas relationship,

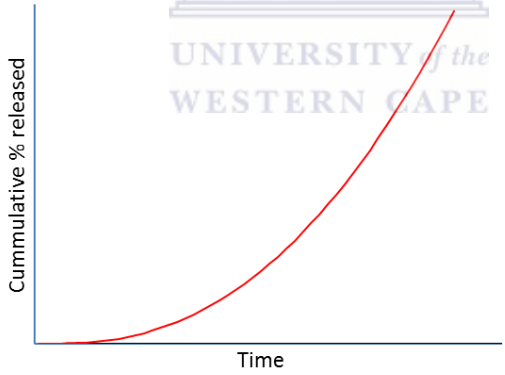
$$\frac{M_t}{M_\infty} = kt^n$$

where $\frac{M_t}{M_\infty}$ = the fraction of drug released at time t,

k = the rate constant

and n = the release exponent

For this model to work effectively, the fraction of drug released at time t, M_t / M_∞, to be fitted to the model, should not exceed 60 %. This is so as to clearly determine the principal mechanism of drug release before a combination of other mechanisms become involved, usually seen beyond the 60 % portion of the curve.



Graphical representation of the Korsmeyer-Peppas release model

With this model, the value of the release exponent *(n)* characterises release mechanism for the compound of interest, depending on the shape of the enclosing matrix. For cylindrical shaped systems, which we can assume are represented by cylindrical tablets and capsule dosage forms, release is according to the mechanisms outlined for the range of values given below. Release exponent values are slightly different for release from polymeric systems of a different shape, e.g. circular flat-topped tablets.

Interpretation of release mechanisms from polymeric systems of cylindrical shape, using the Korsmeyer-Peppas function

Release exponent (n)	Solute release mechanism	Rate as a function of time
0.45	Fickian diffusion	$t^{-0.5}$
0.45 < n < 0.89	Anomalous (non-Fickian) diffusion	t^{n-1}
0.89	Case-II transport	Zero order release
n > 0.89	Super case-II transport	t^{n-1}

No dissolution model is universal for all drug dissolution profiles, and so several models are usually fitted in order to select the model of best fit. Such selections are made after careful consideration of statistical criteria for evaluating a model's goodness-of-fit. Such criteria include the coefficient of determination (R^2), the adjusted coefficient of determination (R^2_{adj}), the Akaike Information Criterion (AIC), the mean square error (MSE) and the sum of squares (SS).

2.6.2.4 Model independent methods

Model independent methods have been described as the most suitable for dissolution profiles comparison when at least three to four dissolution time points are available. They include the ratio tests, the difference and similarity factors *(f_1 & f_2)* which are also referred to as the pair wise procedures, and the multivariate confidence region procedure or bootstrap approach.[126,146]

2.6.2.4.1 Ratio test procedures

The ratio test procedures relate dissolution parameters between reference and test formulations. These parameters may include the percent dissolved at time ***t,*** the area under the dissolution /release curves (AUC) or the mean dissolution time (MDT).[146] For the purposes of this study, the ratio test procedures were not employed in dissolution profiles comparison.

2.6.2.4.2 Pair wise procedures

The pair wise procedures include the *Rescigno index,* the difference factor *(f_1)* and the similarity factor *(f_2).* These procedures calculate the similarity or difference between two dissolution profiles at different time points.[146] To allow for the use of average data when the pair wise procedures are employed for dissolution profiles comparison, the percent coefficient of variation (% CV) should not be more than 20 % at earlier time points and not more than 10 % at other time points. With herbal medicinal products where the ratio of the actives may vary depending on a number of circumstances, maintenance of the % CV limit may pose a challenge. In such cases, where the % CV is more than 15 % within batches, a multivariate model independent procedure may be employed.[126] For the purposes of this study, the *Rescigno index* was not employed in dissolution profiles comparison, and brief descriptions of the pair wise procedures so employed follow.

2.6.2.4.2.1 The difference factor, f_1

The difference factor, f_1, assesses the difference in percentage dissolved from the reference and test products at the sampled times, and can be mathematically computed using the equation:

$$f_1 = \left\{ \frac{\sum |R_t - T_t|}{\sum_{t=1}^{n} R_t} \right\} \times 100$$

where R_t and T_t are the mean percent dissolved at each time point for the reference and test products, respectively, and n is the number of observations /dissolution sample times being considered for the computation. An f_1 value between 0 and 15 indicates similarity of two dissolution profiles while values outside this range indicate dissimilarity.[126]

2.6.2.4.2.2 The similarity factor, f_2

The similarity factor, f_2, is defined as a logarithmic reciprocal square root transformation of the sum of squared error. Unlike the difference factor, the similarity factor is a measurement of the similarity in percent (%) dissolution between two dissolution curves.[126] The similarity factor is computed from the formula:

$$f_2 = 50 \log \left(\frac{100}{\sqrt{\left[1 + \frac{1}{n} \sum_{t=1}^{n} W_t \ (R_t - T_t)^2\right]}} \right)$$

where the parameters, R_t, T_t and n are as described for the difference factor, f_1. Because f_2 has been found to be more sensitive than the f_1 for dissolution profile similarity, it is approved by the FDA for comparison of drug dissolution profiles.[135] An f_2 value between 50 and 100 indicates similarity of two dissolution profiles while values less than 50 indicate dissimilarity.[126]

The calculated value of either f_1 or f_2 depends on which of the formulations is considered the reference; on interchanging the test and reference formulations, different results may be obtained for the f_1 [146] and most likely, the f_2.

2.6.2.4.2.3 Modifications and enhancements of the similarity factor proposed for study

The similarity factor, though suitable for the comparison of dissolution profiles, is dependent on certain conditions: (1) identical sampling times are necessary, (2) only one time point can be considered after 85 % dissolution of one of the products, and (3) of crucial interest to herbal medicinal products, the coefficient of variation may not be greater than 20 or 10 % at earlier and later time points, respectively. The last point has been observed with some herbal products,[23,24] and when this condition is not met, the f_2 value may not be successfully utilized for dissolution profiles comparison as the value obtained may be biased in favour of similarity. The f_2 has also been found not to be discriminating enough, and rather too liberal in concluding similarity between dissolution profiles.[146] Because of these limitations, several authors have proposed modifications to the f_2 equation, and such modified f_2 equations are proposed for dissolution profiles comparison in samples with variation beyond the limit stipulated for original f_2 computation.[131,161-164]

A modified f_2 method may use weighting factors to calculate the effect of variation on the similarity of two dissolution profiles, giving a value for similarity, just like the similarity factor.[161,162,165] Such modifications which make accommodation for within sample variation may present better approaches for dissolution profiles comparison of reference marker

compounds from herbal materials, where wide intra-batch variation has been noted as a limitation to the use and quality control of these products.[23,24] Two of such proposed modifications employed in this study are briefly discussed below.

2.6.2.4.2.3.1 Gohel's f_2

Gohel *et al.* proposed and later modified an equation for the similarity factor.[161,162] This modified equation incorporates a scheme which calculates weight to accommodate for the effect of within sample variability on f_2 values.[161,162] According to this scheme:

$$f_2 = 50 \log \left(\frac{100}{\sqrt{\left[1 + \frac{1}{n} \sum_{t=1}^{n} W_t \, (R_t - T_t)^2\right]}} \right)$$

where w_t is the weight factor due to variability within samples, and is calculated thus:

$$w_t = 1 + \{(\%CV \text{ of } R_t) / (MCV_{E/L})\} + \{(\%CV \text{ of } T_t) / (MCV_{E/L})\}$$

% CV of R_t and ***%CV of T_t*** are the percentage coefficients of variation for reference and test products, respectively; ***$MCV_{E/L}$*** is the maximum allowable % CV; and the percent coefficient of variation, ***% CV*** = (standard deviation /mean) x 100.

2.6.2.4.2.3.2 Median f_2

In this study, we propose another as-yet undocumented modification of the similarity factor for dissolution profiles comparison, especially in cases where variation is beyond the limit stipulated for original f_2 calculation. This modification is the median similarity factor which employs the median (as opposed to the mean) values of the percentages dissolved at each sampling point for dissolution profiles comparison. By virtue of being more robust than the mean to the presence of outliers, the median-f_2 may give a more discriminating value of the similarity factor than the original f_2 for samples with considerable within-batch variation.

2.6.2.5 Principal Component Analysis (PCA) for dissolution data

Principal Component Analysis (PCA) is a multivariate method of data analysis which assesses the underlying structure of a set of data in order to determine the direction(s) responsible

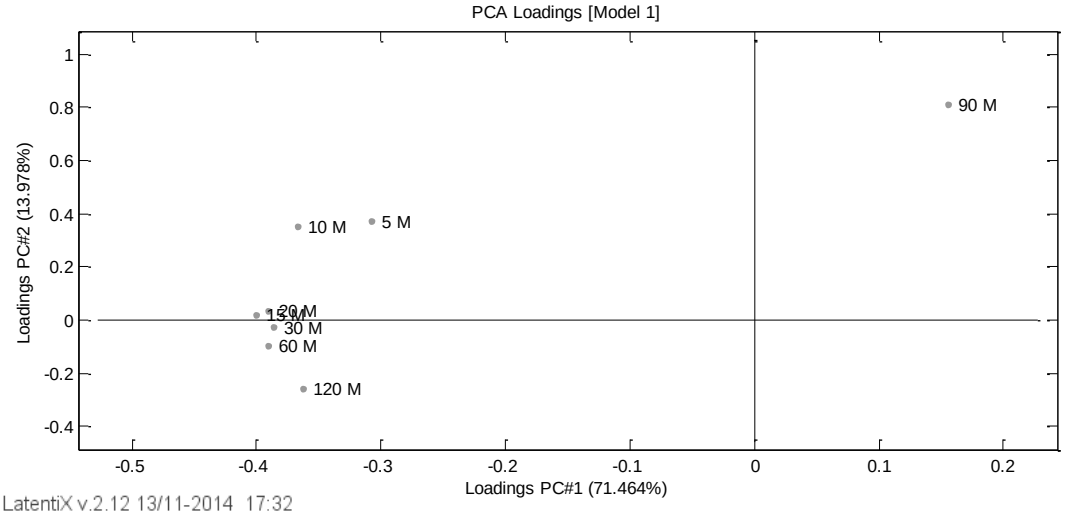
for most of the variation in the data. In PCA, multiple variables determined in a study can be reduced to a minimum number of variables, called principal components, which account for most of the variation in the data. The major aim of PCA is therefore to reduce data dimensionality by extracting the smallest number of components that explain most of the variation in the original multivariate data, summarizing the data with these components and with as little loss of information as possible. This simplifies data for easier interpretation.

The results of a PCA are presented as loadings and scores plots. The loadings plot shows the relationship among variables, assessing the contribution of each variable to data variation; while the scores plot projects the data onto the hyperspace, showing the relationships among data samples. While variables with high correlation are close to each other on the loadings plot, variables with negative correlation are seen on opposite sides (Figure 2.8 a). On the scores plot, objects are clustered according to their attributes (Figure 2.8 b) and objects on one side, e.g. the right side of the score plot, are dominated by variables on that side (Figures 2.8 a & b).

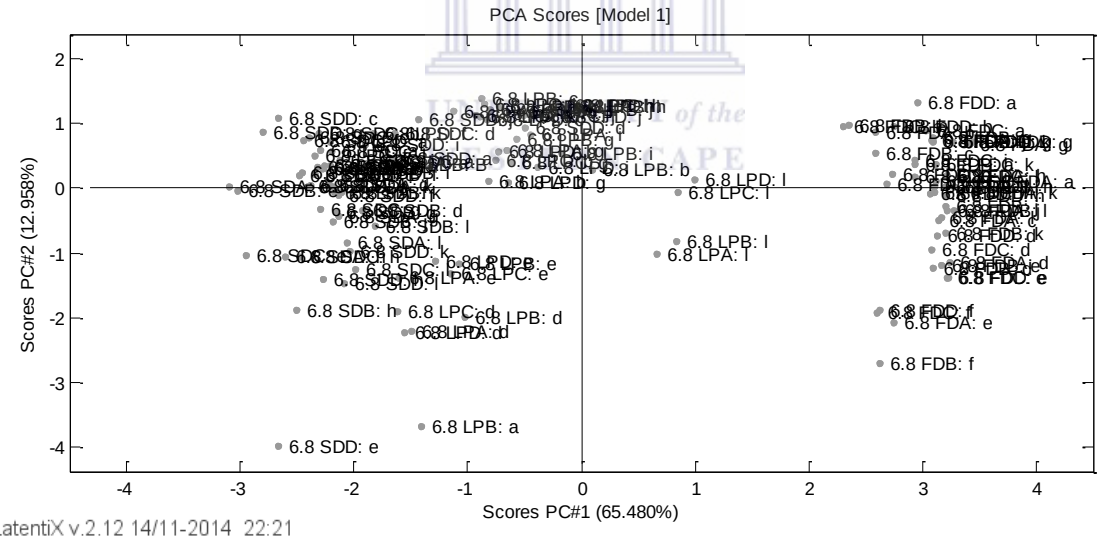
PCA can thus be employed for analysis of dissolution data, where the sampling times can be seen as multiple variables, each variable (sampling time) accounting for a different dimension to the data. As such, the contributions of the variables (different sampling points) to the dissolution profiles of materials can be assessed. Sampling times that are similar will cluster together and a decision can be made to select the most relevant time points from the sample cluster, thereby removing redundant variables (in this case, sampling times) from subsequent dissolution tests. From cluster analysis of the scores plot, the more influential factor determining API release, i.e. type of *S. frutescens* material or the physico-chemical properties of the APIwill be projected.

From literature, it can be seen that no single approach is universally accepted for the comparison of dissolution profiles,[131,134,146] which may be applied to pharmaceutical products. The different methods discussed herein will all be used to analyse dissolution data of marker compounds of *S. frutescens* materials in order to explore the strength of each method, and to determine which of the methods is the most suitable for dissolution data

analysis. However, it is important to ascertain the level of discrimination required prior to selection of any comparison method.



(a): A loadings plot of data after principal component analysis



(b): A scores plot of data after principal component analysis

Figure 2.8: Examples of loadings and scores plot of data after principal component analysis

2.7 Consideration aspects for dissolution tests of *S. frutescens* materials

The drug dissolution test is an official quality control test designed to evaluate drug release and dissolution within the gastrointestinal tract. Generally, it is conducted according to specifications set out in the pharmacopoeia.[120] These specifications may be modified as necessary, though such modifications need to be validated.[121] Depending on the part of the GIT where the drug is expected to be dissolved, dissolution tests can be conducted using different media and conditions that mimic different sections of the gastrointestinal tract (GIT).[83]

With dissolution testing, volumes of dissolution media between 500 and 1000 ml are commonly used, 900 ml being the most common volume. In cases where the label claim is less than 5 mg or if marker compounds have less absorbance at the selected wavelength, the dissolution volume can be reduced in order to enhance the sensitivity of the method.[126,166] The appropriate volume of a dissolution medium to use is however defined based on sink conditions. The ultimate goal in the choice of medium volume for dissolution testing is to obtain a discriminative method, and this is usually done experimentally. Sometimes, the discriminative dissolution test may even be obtained by violating sink conditions.[167]

For dissolution testing of *S. frutescens* materials, the common mode of use of this plant in traditional settings was considered. Traditionally, *S. frutescens* is used in the form of a tea. In a tea, the water soluble flavonoid glycosides would be more easily released than the non-water soluble aglycones, and hence more abundant. In dissolution testing which imitates the traditional conditions of use by virtue of the dissolution medium which is aqueous, the flavonoid glycosides would presumably be more concentrated and hence more available than the flavonoid aglycones. As such, it is envisaged they will serve as better marker compounds than the aglycones, for quality control of herbals containing this plant.

Four of these flavonoid glycoside compounds have been isolated and identified as possible markers for *S. frutescens* products.[51] These are sutherlandins A, B, C and D (Figure 2.7). They contain the same 3-hydroxy-3-methylglutaroyl (HMG) moiety.[51] The major differences are in the aglycone and sugar identities. Sutherlandins A and C are derived from 2 different

aglycones, quercetin and kaempferol, respectively; however, they possess the same sugar molecule attached to an HMG moiety. Similarly, sutherlandins B and D, derived from quercetin and kaempferol respectively, possess the same sugar molecule (though this is different from that of sutherlandins A and C) attached to an HMG moiety. Since the sugar is known to influence aqueous solubility and hence dissolution,[168,169] we hypothesize that the dissolution profiles of sutherlandins A and C from the different *S. frutescens* materials will be similar. Likewise, the dissolution profiles of sutherlandins B and D from the *S. frutescens* materials will be similar, though different from that of sutherlandins A and C. Such differences may also translate to differences in *in vivo* pharmacokinetics, which may influence the outcome of the upcoming clinical trial of *S. frutescens.* As such, the pharmacokinetics of the marker compounds will also be briefly discussed.

2.8 Pharmacokinetic models and pharmacokinetics of flavonoids

Immediately after API release and subsequent dissolution in the appropriate dissolution medium, the API is absorbed, commencing its pharmacokinetic journey. Pharmacokinetics refers to the study of the factors that affect the amount and time course of a substance at its site of action. It encompasses the fate of such a substance, for instance, a drug substance in the body, from absorption to the irreversible loss of the drug or its metabolite *via* excretion. The four pharmacokinetic processes are absorption, distribution, metabolism and excretion (ADME).[170] Parameters representing these processes can be determined with the use of suitable *in vitro in vivo* correlation (IVIVC) models.

The determination of pharmacokinetic parameters is an important part of any API product development study. Such pharmacokinetic parameters can be deduced from *in vivo* human studies such as the upcoming clinical trial of *S. frutescens*. However, *in vivo* studies can be time consuming, expensive (not only financially, but also in terms of human and animal life and health), and in some cases, ethically unjustifiable. Because of these limitations, the pharmaceutical industry is constantly on the lookout for effective alternatives for determination of pharmacokinetic parameters, so-called *in vitro in vivo* correlation tools.[171]

In vitro in vivo correlation is defined by the FDA (1997) as "a predictive mathematical model that describes the relationship between an *in vitro* property of a dosage form and an *in vivo* response". Such models can be used to determine and assess parameters involved in drug /API absorption, as well as other pharmacokinetic processes. Knowledge gained from IVIVC studies can be employed in drug manufacturing, saving time during development and optimization studies.[172] IVIVC is also seen as an alternative to *in vivo* bioequivalence studies. As such, it can be employed by drug regulatory agencies, such as the MCC of South Africa, in product regulation.[172] As helpful as it may be, it is pertinent to mention that an IVIVC relationship is not an absolute correlation, and does not automatically imply causality between *in vitro* and *in vivo* data;[171] it is merely a simulation.

A simulation is an imitation of the operation of a real world process or system over time. The aim of a simulation is to predict expected outcomes, and to give insight into the working of a process that cannot easily be visualized or experienced. For a simulation to work and predict accurately, a model which represents the key characteristics of the process or system, is developed. Such models are designed based on theories that explain the process or system to be simulated. This saves considerable time and money when compared to performing replicate tests on a real life design over time. In addition, extensive details on the workings of a process or system can be obtained from simulations.[173]

Recent simulations that can be used to predict expected pharmacokinetic outcomes in API product development studies are *in vitro* dissolution studies, the Biopharmaceutics Classification System (BCS) as well as various mathematical models and validated simulation programmes such as the *Molecular Operating Environment (MoE).*

2.8.1 The Biopharmaceutics Classification System (BCS)

The Biopharmaceutics Classification System (BCS), devised by Amidon *et al.*, is a scientific framework for classifying drug substances based on their aqueous solubility and intestinal permeability (Figure 2.9).[122] According to this system, APIs are classified into one of four groups based on their solubility and permeability profiles. Class I compounds exhibit high aqueous solubility and high intestinal permeability while class IV compounds exhibit low aqueous solubility and low permeability. Between these two classes are the class II and III

compounds: class II compounds exhibit low solubility but high permeability while class III compounds show and high aqueous solubility and low intestinal permeability.

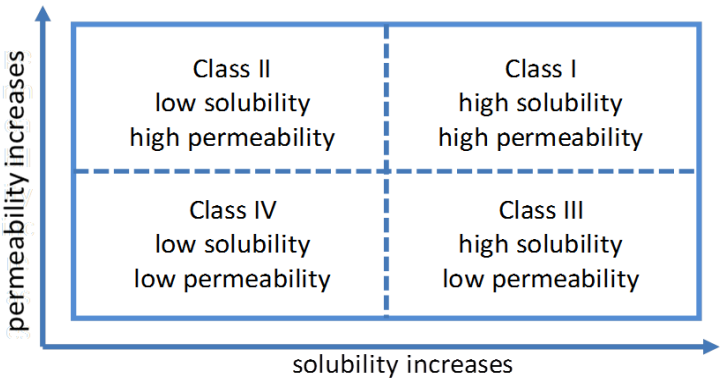


Figure 2.9: The Biopharmaceutics Classification System (BCS)

The BCS finds extensive employment in drug development studies and has been widely recognised and accepted in academic, industrial and regulatory settings.[177] In such settings, it serves as a guide for predicting intestinal drug absorption by estimation of the contributions of three major factors – dissolution, solubility and intestinal permeability – that affect oral drug absorption from immediate release (IR) solid oral dosage forms.[178,179] These factors (dissolution, solubility and intestinal permeability) can therefore be used to predict product bioavailability.

With respect to bioavailability prediction, certain drug products can be considered for biowaivers, i.e. approving the product based on *in vitro* dissolution tests, rather than requiring bioequivalence (BE) studies in human subjects.[180] This is because a product's dissolution rate within the gastrointestinal tract is known to influence its absorption rate; therefore, the drug dissolution test can be used to highlight cases in which different formulations may not be bioequivalent.[181] When using *in vitro* dissolution testing as a substitute for *in vivo* bioequivalence, two formulations are deemed therapeutically equivalent if their dissolution profiles are similar. Thus, for an immediate release (IR) orally administered formulation that is rapidly dissolving *and* contains a class I active pharmaceutical ingredient (API), a biowaiver may be granted for the generic product on the basis of acceptable dissolution data.[123,127] A biowaiver has also been proposed for class III

compounds that do not contain excipients which may interfere with gastrointestinal transit or absorption.[182]

2.8.2 The Molecular Operating Environment *(MoE)*

Due to long held beliefs on its many reputed therapeutic properties, especially in the management of HIV infection,[60] preparations are underway for a clinical trial to assess the efficacy of the popular South African herb, *S. frutescens*, in HIV patients. Prior to this, a phase I pharmacokinetic study of *S. frutescens* will be conducted to assess the level and profiles of appropriate reference and marker compounds in plasma. Previous studies identified flavonoid glycosides as appropriate markers for assessment of products containing this plant;[51] however, the plant is also known to contain aglycones of these flavonoid glycosides, specifically quercetin and kaempferol.

While extensive studies have been conducted on flavonoids and their pharmacokinetics especially with respect to absorption, controversies are still rife over which form of the flavonoid (glycoside, aglycone, both) is actually absorbed. Such absorption is necessary for the 'active components' of the plant to exert pharmacological and hence therapeutic activity. As a product with purported therapeutic effects, *S. frutescens* can be viewed as a pharmacological agent (a drug). Tests and procedures used to assess pharmacological activity, and tests used to assess drug pharmacokinetics can therefore be applied to products of this plant. Such tests can be used to evaluate the bioavailability of *S. frutescens* products.

The oral bioavailabilty of drug candidates is a key parameter in assessing their likelihood to succeed in the drug development process, and recently, is now used to assess the 'drug likeness' of a molecule. For such assessments, chemoinformatics and computational resources are utilized to integrate 'development' activities into early stage drug discovery. Through modelling and simulation, drug candidates with possible theoretical bioavailability are selected for the drug discovery process, while drug candidates that do not meet the common criteria found in most successful drug molecules are withdrawn from the discovery

process, cutting costs and time, as well as reducing new drug molecule attrition rates in the pharmaceutical industry.[183]

The *Molecular Operating Environment (MoE)* is one such chemoinformatic and computational resource for assessment of possible drug molecules. One of the drug properties that can be assessed by the *MoE* is the bioavailability of a substance under consideration as a new drug molecule. For bioavailability assessment, specific physico-molecular characteristics of the compound of interest are calculated, and used to predict the bioavailability or otherwise of compounds. The desired physico-molecular characteristics for oral drug bioavailability were summed up by Lipinski *et al.* in their popular proposal, "The Rule of 5".[184] According to this rule, successful new drug candidates for oral administration should not violate more than one of the following criteria:

- *Not more than 5 hydrogen bond donors (the total number of nitrogen-hydrogen and oxygen-hydrogen bonds)*
- *Not more than 10 hydrogen bond acceptors (all nitrogen or oxygen atoms)*
- *A molecular mass of less than 500 Daltons*
- *An octanol-water partition coefficient, log P, not greater than 5*

This rule has also given rise to other extensions. An example of such an extension is "The Rule of 3" proposed by Congreve *et al*.[185] The Rule of 3 proposes that for new drug candidates to succeed in the drug development process, they should possess the following characteristics:[185]

- *Molecular weight not more than 300*
- *Hydrogen bond donors not more than 3*
- *Hydrogen bond acceptors not more than 3*
- *C log P value of not more than 3*

The desired characteristics so-listed by Lipinski and others can be calculated from the structural formula of each compound by *MoE* and used to predict *in vivo* pharmacokinetics (e.g. bioavailability) of selected marker compounds such as the flavonoids from *S. frutescens.*

2.8.3 General ADME profiles of flavonoids

Extensive studies have been conducted on flavonoid absorption; however, controversies are still rife over which form of the flavonoid (glycoside, aglycone, both) is actually absorbed. Gastrointestinal absorption is thought to be dependent on intestinal microflora, which hydrolyse glycosides into readily absorbable aglycones.[111,186] While it has been a long held belief that flavonoid aglycones are more readily absorbed, unlike the flavonoid glycosides which are poorly so, some reports have indicated otherwise and even reported that the sugar moiety, to a great extent, determines the absorption of dietary flavonoid glycosides in man.[187,188]

Animal studies and *in vitro* studies suggest that flavonoid glycosides and aglycones are rapidly and intensively metabolized. Phase I metabolic reactions are likely, however, conjugation reaction with sulphate and /or glucuronic acid seem to be the most common metabolic reaction for flavonoid metabolism.[94,189]

Very low amount of flavonoids have been recovered in the urine of animals after administration, indicating extensive metabolism to other compounds, while suggesting that the renal route is not a major pathway for excretion of intact flavonoids.[190]

2.8.4 Pharmacokinetics of flavonoids present in *S. frutescens*

The flavonoid aglycones under study, quercetin and kaempferol, belong to the subclass of flavonoids called the flavonols. These two compounds exhibit low solubility in aqueous-based solvents.[191,192] Due to their amphipathic nature, flavonoids can partition into or bind lipid layers;[193] kaempferol is known to exhibit good permeability, driven by concentration gradient, across the membranes in Caco-2 studies while quercetin has its permeation limited only by extensive intestinal and /or hepatic glucuronidation.[194-197] With their low solubility and high permeability, they may be classified as BCS class II agents.

The flavonoid glycosides, on the other hand, exhibit high solubility, possibly due to the presence of the sugar molecule attached to the aglycone. These compounds are thought to be transported paracellularly. This may account for their low permeability, as most flavonoids are known to be transported transcellularly, either by passive diffusion or carrier-mediated flux. Furthermore, the addition of a sugar moiety increases the molecular size and

number of hydroxyl groups, hindering flavonoid glycoside transport across cell membranes.[198,199] With their high solubility and low permeability, the flavonoid glycosides may be classified as BCS class III agents. In the presence of *fast-dissolving or uncritical excipients,* it is envisaged that they will readily penetrate membranes.[182] However, the different matrices within which the flavonoids are trapped in different *S. frutescens* formulations (i.e. leaf powder, spray-dried and freeze-dried aqueous extract) may affect their candidature for class III BA/BE waiver.

In summary, herbal medicinal products have contributed to new drug discovery, both in the past and recently. As such, their use in the treatment and management of human ailments is becoming more popular. One of such products is the popular South African medicinal plant, *S. frutescens,* which has been used for over a century in South Africa for a wide range of illnesses. Its use has been boosted by *in vitro* studies that validate its use in treatments as well as *in vivo* studies that have reportedly shown no undue toxic effects from its consumption. Due to its many reputed benefits, arrangements are underway for a clinical trial to assess its efficacy. However, it is necessary to be able to assess and compare the quality of different *S. frutescens* materials in order to find the most appropriate one to be used in such a trial, therefore, the need to develop quality control tests and specifications for S*. frutescens* products. Such quality assessment will also be of use in setting regulatory specifications for *S. frutescens* materials. The theories behind such quality control tests have been presented in this chapter. The next chapters detail the rationale for each test chosen and how these will be conducted.

CHAPTER THREE

PLAN OF WORK

In this chapter, the main research question(s) and specific objectives that are to be pursued, as well as the hypotheses to be tested are presented. The study approach to be followed is also discussed.

3.1 The main research questions

Various forms of *Sutherlandia frutescens* materials, e.g. leaf powder, aqueous extracts, gels and tonics to mention a few, are commercially available and popularly used for the treatment of many ailments. Despite its popularity, not much is known about the quality and stability of its various forms available in commercial settings. The quality and stability may also have a marked influence on the content, release and *in vitro* dissolution of actives and subsequent performance of its various forms. The different forms of the plant contain flavonoids which might contribute to the possible pharmacological activities of the plant, and hence can be used to assess quality and by extension, efficacy.

The primary research questions for which answers are needed are as follows:

- Would the flavonoids be suitable marker compounds for assessment of *S. frutescens* quality?
- Can the quality of different *S. frutescens* materials be assessed or compared based on the flavonoid content and release /dissolution of such flavonoids?

To answer these questions, a suitable assay which can be used to assess flavonoids in *S. frutescens* materials and in dissolution media would be required. A major objective in the assay development is the isolation and identification of non-commercially available flavonoid glycosides which can serve as markers for products of this plant. In addition, there exists a need for a suitable dissolution test which can differentiate between *S. frutescens* materials. A suitable dissolution test in this context refers to the actual dissolution test itself as well as the methods employed for analysis of dissolution data. There is also a need to

predict oral bioavailability of selected flavonoids as a means of assessing bioequivalence of different *S. frutescens* products.

3.2 Study aims and objectives

The overall aim of the study was to develop quality control tests which can be employed in regulatory settings for assessment of *S. frutescens* materials. The specific objectives of the study were:

- to isolate and identify non-commercially available flavonoid glycoside compounds for *S. frutescens* assay,
- to develop and validate an HPLC assay for flavonoids in *S. frutescens*,
- to determine and compare flavonoid levels in different *S. frutescens* materials (leaf powder or extract, also called active herbal ingredient [AHI] or active phyto-pharmaceutical ingredient [APPI]),
- to compare dissolution profiles and underlying release mechanisms of flavonoids from different *S. frutescens* materials, and
- to predict *in vivo* bioavailability of flavonoids.

3.3 Hypotheses

The following specific hypotheses are postulated:

a. Flavonoid content: The percentage content of the flavonoid glycosides will be more than that of the flavonoid aglycones in each *S. frutescens* material.

b. Flavonoid dissolution:
    i. Irrespective of the *S. frutescens* material, the flavonoid glycosides will have a higher dissolution rate than the flavonoid aglycones, i.e.
    $Q_{75}^{\text{(flavonoid glycosides)}} < Q_{75}^{\text{(flavonoid aglycones)}}$
    ii. With respect to the *S. frutescens* materials, flavonoid dissolution rate from the extracts (SDAE and FDAE) will be higher than flavonoid dissolution rate from the LP material, i.e.
    $Q_{75}^{SDAE} = Q_{75}^{FDAE} < Q_{75}^{LP}$

c. Mechanism of flavonoid release prior to dissolution: This will depend more on the *S. frutescens* material than on the physicochemical properties of the flavonoid marker compounds, i.e. from each *S. frutescens* material, release of different flavonoid marker compounds will be by the same mechanism irrespective of the physicochemical properties of each specific marker compound.

3.4: Study approach and justification

3.4.1 Selection of different *S. frutescens* materials

- **What materials and why those?**

The different materials chosen were the leaf powder (LP), spray-dried aqueous extract (SDAE) and freeze-dried aqueous extract (FDAE) materials. These were selected because they were produced by different methods and hence constitute part of the different *S. frutescens* materials that are commercially available. The material to be used for the upcoming clinical trial will also be selected from one of these.

3.4.2 Selection of marker compounds to monitor

- **Why flavonoids?**

Flavonoids were chosen because some of the biological effects of *S. frutescens*, especially the antioxidant effects, have been attributed to flavonoid compounds.[16,200,201] As such, they can be seen as active components of *S. frutescens*, and have the potential to serve as marker compounds for its products.[16,17,51] Both the glycosidic and aglycone forms of flavonoids are known to exist in this plant. Because of differences in flavonoid glycoside and aglycone chemistry, they can be said to be representative of the different forms under which active components of *S. frutescens* might occur in different conditions, e.g. storage and environmental conditions, and so can serve as appropriate references for quality, stability and efficacy assessment.

- **Which flavonoids were chosen? Why were these flavonoid compounds chosen?**

A total of nine flavonoid compounds were selected for assay development. Five of the compounds (the glycosides: rutin, kaempferol-3-*O*-rutinoside, quercitrin; and the aglycones: quercetin and kaempferol) were purchased commercially. Three of these five compounds (rutin, kaempferol-3-*O*-rutinoside and quercitrin), though not yet conclusively identified in *S.*

frutescens, were selected because they are glycosides of the aglycones present in *S. frutescens* and so may also be present in the plant. The other four compounds selected were the flavonoid glycosides, sutherlandins A to D, which though not yet commercially available, have been isolated and identified from *S. frutescens* material as possible quality control markers.[51]

- **What did these choices entail (e.g. also required isolation of marker compounds)?**

The flavonoid glycosides, sutherlandins A to D, were isolated from *S. frutescens* SDAE. Isolation of flavonoid glycosides for analytical studies has not always been common practice in herbal product analysis. Flavonoid glycosides arise from a limited number of flavonoid aglycones; however, the flavonoid glycosides derived from the few aglycones are varied and extensive in number. Because of their large number, it is not always possible to obtain commercial quantities of the flavonoid glycosides for analytical purposes. To address this shortcoming, previous studies usually quantified flavonoid glycosides in terms of their corresponding aglycones, to which the glycosides have been acid-hydrolysed for such purpose.[87,95] Such hydrolysis reduces the number and variety of flavonoid compounds available, simplifying analysis.[202]

However, plant material may be adulterated with pure flavonoid glycoside or aglycone compounds, or even other flavonoid-containing plant materials, which may not be detected following acid hydrolysis of flavonoid glycosides. Acid hydrolysis of flavonoid glycosides may also result in degradation of other phenolic compounds present, with the result that the true flavonoid content and arrangement in plant materials may be misrepresented.[107,203] In addition, acid removal following hydrolysis of flavonoid glycosides to aglycones may pose considerable challenges. These limitations of flavonoid glycoside assay *via* the flavonoid aglycones therefore behoves us to identify and quantify individual flavonoid glycosides and not just the flavonoid aglycones present in plant samples, as a means of assessing quality of the plant materials.[204]

An ideal approach may therefore be to isolate and purify the appropriate glycosides from the plant, and then use these for quantification purposes. This would also be a better option especially for aqueous-based quantification studies where the flavonoid glycosides are more

concentrated than the aglycones in the extracting solvent and so may be easier to detect, making them more suitable marker compounds for reversed phase high performance liquid chromatographic (HPLC) analysis.

- **Why HPLC (and what criteria should the HPLC method comply with)?**

For this study, an assay that will separate flavonoid glycosides from aglycones and all detectable flavonoids from each other had to be developed. It should be noted that as different formulations and dosage forms of *S. frutescens* are commercially available, so also are their matrices. Because of this, conditions have to be selected and optimized in order to allow for separation of the compounds of interest in the presence of other chemical constituents. The HPLC assay technique was employed due to its ability to separate and analyse multiple components present in plant matrices.[18]

A good HPLC method for *S. frutescens* materials should be simple and reproducible. It should be able to separate all nine selected compounds as this will result in good separation in plant material where other yet unknown compounds may also be present in the plant matrix.

3.4.3 Determination of content levels and dissolution profiles of marker flavonoids from different *S. frutescens* materials

- **Why content levels?**

Flavonoid glycosides and aglycones possess different structures and chemistries. In plant material, most flavonoids exist in the glycosidic form with a sugar molecule attached to the flavonoid aglycone structure. In unstable conditions, e.g. exposure to acid, heat or light, the flavonoid glycosides may be degraded or converted to the aglycones.[21] Therefore, assessment of flavonoid glycosides and aglycones, and their levels in products, will give an indication of the stability of a plant product.

- **Why dissolution (and the selected method thereof)?**

For this study, dissolution tests for three different *S. frutescens* materials (LP, SDAE and FDAE 1) were conducted in three different dissolution media at pH 1.2, 4.5 and 6.8, to simulate conditions obtained in different sections of the gastrointestinal tract. Even though

all the products are presumably derived from one source, there are subtle differences in appearance, processing methods and processing dates. The three products used for dissolution were therefore selected in order to assess the discriminating power of the dissolution method.

For the dissolution tests, the standard USP basket method, with modifications, was employed. The first modification was in the volume of the dissolution medium which was reduced to 450 ml. The second modification was in the number of dosage units to be employed which was increased to two capsules containing each plant material. This was done to increase flavonoid concentration in the dissolution medium and so enhance detection.

- **How were profiles to be compared and interpreted? Why that way?**

Dissolution profiles of flavonoids from the different materials were compared using Q-release values. Q-values give an indication of the time taken for the specified percentage of actives to dissolve in the dissolution medium. Q-75 % and Q-85 % were evaluated for each flavonoid marker compound at different pH conditions in order to characterise each flavonoid as rapidly dissolving or otherwise from the plant material. Although there are no pharmacopoeial specifications for *S. frutescens* materials, the acceptance criterion employed for this study was arbitrarily set at a minimum of 75 % flavonoid dissolution in 45 minutes. A Q-value of 75 % in 45 minutes suggests that the marker compound exhibits the characteristics of immediate release from its enclosing material. Immediate release formulations may be further classified as rapidly dissolving (Q-value of ≥ 85 % dissolution in ≤ 30 minutes) or very rapidly dissolving (Q-value of ≥ 85 % dissolution in ≤ 15 minutes). This parameter (Q 85 %) was also assessed for the flavonoid markers from the *S. frutescens* materials.

Dissolution data were further compared using the original similarity factor, f_2. Because the data had percent coefficient of variation (% CV) in excess of 20 % at earlier time points (≤ 15 minutes) and 10 % at later time points (> 15 minutes), and to assess the suitability of original f_2 use, the dissolution profiles were also compared using a modification of the original f_2 proposed for dissolution profiles comparison in cases of extensive variation.[161,162]

We also propose another as-yet undocumented modified method for dissolution profiles comparison: the median-f_2. This is considered because the median value, being more robust to the presence of outliers, may be expected to give a more accurate value of the f_2 than the original f_2 method which uses mean values of percentages dissolved for f_2 calculation, in cases where samples present with extensive variation.

Finally, to determine the minimum crucial number of sampling points necessary for flavonoid dissolution from *S. frutescens* materials, as well as to assess the relationship between dissolution data from different materials, dissolution data was further subjected to principal component analysis (PCA). As a data reduction tool, PCA analyses data by extracting the smallest number of components to explain most of the variation in the original data.

- **Why dissolution data modelling (e.g. to compare release mechanisms)?**

It was also of interest to determine if the dissolution mechanisms were different for flavonoid markers form different *S. frutescens* materials. Therefore, to describe the release and elucidate the underlying mechanism of release for each flavonoid marker compound, dissolution data was fitted to various mathematical models. The release of an API from its enclosing material occurs in dissolution media prior to API dissolution, and is a prerequisite for such dissolution. Such release can be described using appropriate mathematical models. The dissolution data was thus fitted to various models in order to obtain the mathematical models of best fit which can be used to describe the dissolution curve mathematically as well as to elucidate the kinetics of marker compound release.

Three semi-empirical models – Weibull, Peppas-Sahlin and Korsmeyer-Pepas models – were utilized in order to characterise the release mechanisms of markers from different *S. frutescens* materials. The Weibull model describes release by diffusion or a complex mechanism depending on the value of the time exponent, **β**; the Peppas-Sahlin model describes API release as governed by diffusion or polymer relaxation depending on the values of $\boldsymbol{k_d}$ and $\boldsymbol{k_r}$ while the Korsmeyer-Peppas model describes API release as *via* diffusion or a complex mechanism according to the value of the release exponent, ***n***.

For Korsmeyer-Peppas modelling, the fraction of drug released at time ***t*** (M_t /M) utilised for modelling should not be more than 60 % in order to appropriately elucidate release mechanism before complete dissolution of the dosage form, by which time other mechanisms not involved in release may contribute to a wrong inference.[160] A minimum of three data points are required for Korsmeyer-Peppas modelling.[160] For all the sutherlandins, the portion of the curve $M_t/M \leq 60$ % did not have enough sampled data points for the analysis especially for glycosides from the LP and SDAE; where in some cases close to 100% had been released by the second or third time point. To obtain enough data to fit $M_t/M \leq 60$ %, the predicted values from the empirical model of best mathematical fit, for each flavonoid glycoside, which generated more data points before the portion of the curve $M_t/M \leq 60$ %, were used for fitting to the Korsmeyer-Peppas equation.

3.4.4 Prediction of *in vivo* bioavailability of *S. frutescens* flavonoids

- **Why was this done? How (and why that way)?**

The essence of this section is to evaluate a selection of marker compounds in order to predict the ones that would most likely be bioavailable. For the upcoming clinical trial on *S. frutescens*, an analytical test has to be developed and validated for proposed marker compounds (flavonoids) in plasma and other biological fluids. However, prior to such assay development, possible markers need to be evaluated in order to select the ones most likely to be bioavailable. This was done using the molecular properties of each marker compound and the computational resource, *Molecular Operating Environment, MoE*. An assay will then be developed for the bioavailable marker compounds in plasma since these are the compounds that are likely to be detected. Prediction of *in vivo* bioavailability offers advantage over the conduction of several experiments to determine such as it saves time and resources, and removes the need for ethical justification of possible *in vivo* studies.

CHAPTER FOUR

MATERIALS AND METHODS

In this chapter, the materials, equipment and procedures used to determine the comparative levels of flavonoids in, and their dissolution from, a selection of *S. frutescens* materials are presented. Included among the procedures explained are those for the selection and isolation of the relevant flavonoids, development and validation of a suitable HPLC assay, conduct of dissolution studies and the analysis of dissolution data. In addition, the methods employed for modelling of *in vitro* release using computerized software, and the prediction of *in vivo* bioavailability from molecular properties, are also presented.

4.1 Materials

The following herbal and chemical materials were sourced and used:

- *S. frutescens*-containing products, i.e. leaf powder ***(LP, batch number: E16794, Afriplex, Paarl)***; spray-dried aqueous extract ***(SDAE, batch number: Ferl-DST/001-1210; CSIR,Bellville)***; freeze-dried aqueous extract 1 ***(FDAE 1, batch number: 1674, Ferlot Manufacturing & Packaging Pty (Ltd))***; freeze-dried aqueous extract 2 ***(FDAE 2, batch number: E62265, Afriplex, Paarl)***; freeze-dried aqueous extract 3 ***(FDAE 3, batch number: E63067, Afriplex, Paarl)***. The certificates of analysis (CoA) for the different products are available in Appendix 1.

- Reference compounds: Commercially available reference standards, with purity as indicated in the manufacutrer's CoA, and source, in parantheses: rutin (≥ 94 %) ***(Sigma Aldrich)***; quercetin dihydrate (≥ 99 %), quercitrin (≥ 98.5 %), kaempferol-3-O-rutinoside (≥ 98 %), kaempferol (≥ 99 %) ***(Extrasynthese, Genay Cedex, France)***;
  and non-commercially available marker compounds: flavonoid glycosides isolated from *S. frutescens* SDAE (as described in section 4.3.1), with purity determined by HPLC analysis as ≥ 98.9 %, ≥ 89.7 %, ≥ 99.6 % and ≥ 98.6 % for sutherlandins A, B, C and D, respectively.

- Solvents and reagents: Acetonitrile, methanol ***(Sigma Aldrich)***; *f*ormic acid, concentrated hydrochloric acid *(**Merck (Pty) Ltd**)*; butanol ***(Associated Chemicals Enterprises, Johannesburg, RSA)***; distilled water *(**Millipore, Milford, MA)***; sodium hydroxide pellets, potassium dihydrogen orthophosphate, di-sodium hydrogen orthophosphate ***(Merck, SA)***; monobasic potassium phosphate ***(Sigma Aldrich, USA)***; distilled water ***(Millipore, Milford, MA, USA)***.

- HPLC and other consumables: Strata™ 1 ml C_{18} solid phase extraction (SPE) cartridges, Luna® C_{18} preparative column (250 x 10 mm, 10 µm), Luna® C_{18} analytical column (250 x 4.6 mm, 5 µm), Kinetex® C_{18} analytical column (150 x 2.1 mm, 5 µm) ***(Phenomenex, Torrance, CA, USA)***; Discovery C_{18} preparative column (250 x 10 mm, 10 µm), Discovery® C_{18} analytical column (250 x 10 mm, 5 µm), Discovery® C_{18} guard column (1 cm x 10 mm, 5 µm) ***(Supelco, Bellefonte, PA, USA)***; HPLC vials ***(1.8 ml, AA Tech, CT, RSA)***; latex gloves, nose masks, pipettes – 20, 200 & 1000 µl ***(Lasec, SA)***; pipette tips - 200 & 1000 µl ***(Bio-smart Scientific, London, UK)***; nylon 0.45 µm syringe filters ***(25 mm, StarLab Scientific)***; 0.45 µm membrane filters ***(Millipore, Ireland)***; parafilm ***(Pechiney Plastic Packaging, Chicago, IL-60631)***; syringe needles ***(Avacare, Sunray Medical Co., China)***; eppendorf tubes ***(Eppendorf, Germany)***; centrifuge tubes ***(Biologix, USA)***.

4.2 Equipment

The following equipment were used:

- Centrifuge ***(Digicen 21, Orto alresa, United Scientific)***; rotary evaporator ***(Büchi, Labotec, SA)***; -86°C upright ultralow freezer ***(NU-9668E, NuAire, USA)***, dissolution apparatus ***(VanKel VK 700, USA)***.

- Chromatographic equipment: HPLC-DAD analyses using an Agilent 1200 series HPLC system, equipped with an in-line degassing system ***(G1322A, Japan)****;* quaternary pump ***(G1311A, Germany)****;* auto loading sampler *(**G1329A, Germany)****;* thermostatted column compartment ***(G1316A, Germany)*** and photodiode array detector ***(G1315B, Germany)***;

and LC-MS and LC-MS/MS analyses using a Waters Synapt G2 quadrupole time-of-flight mass spectrometer ***(Milford, MA, USA)***.

- NMR spectrometer ***(Bruker Avance IIID Nanobay, Bruker BioSpin GmbH, Rheinstetten, Germany)***.

- Small scale instruments: Weighing balance *(max 210 g, Ohaus, NJ, USA)*; vacuum pump ***(Rocker, Singhla Scientific, Haryana, India)***; heater ***(MS7-H550-Pro, DragonLAB)***; Vortex mixer ***(VM-400, Gemmy Industrial Corp., Taiwan)***, pH meter ***(Model PL-700PV, Taipei, Taiwan)***, size 0 capsule filler ***(Cap.M.Quik™, S L Sanderson & Co., Berry Creek, CA, USA)***.

- Computer modelling software: OpenLAB™ CDS ChemStation edition HPLC data acquisition software ***(Agilent Technologies, Palo Alto, CA, USA)***; LatentiX© data analytical software ***(Version 2.12, www.latentix.com, Latent5, Copenhagen, Denmark)***; DDSolver ***(Microsoft Excel add-in program, Zhang et al., 2010)***; Molecular Operating Environment ***(MoE) (Chemical Computing Group Inc., Montreal, Canada)***.

4.3 Methods

The methods involved in this study are shown in the flow diagram (Figure 4.1) and discussed in this sub-section.

4.3.1 Acquisition and isolation of flavonoid compounds from *S. frutescens*

Briefly, nine reference marker compounds (two flavonoid aglycones and seven of their corresponding glycosides) were selected for suitability assessment as marker compounds for *S. frutescens (1a and 1b).* Five of these compounds, *viz.* rutin, kaempferol-3-*O*-rutinoside, quercitrin, quercetin and kaempferol were purchased, while the other four, *viz* the flavonoid glycosides, sutherlandins A to D, were not commercially available and had to be isolated from *S. frutescens* plant material *(1b)*. An HPLC assay for the flavonoid reference compounds in *S. frutescens* solution was developed and validated *(2a and 2b)* and used for the quantification of the reference compounds in the different *S. frutescens* materials *(3)*.

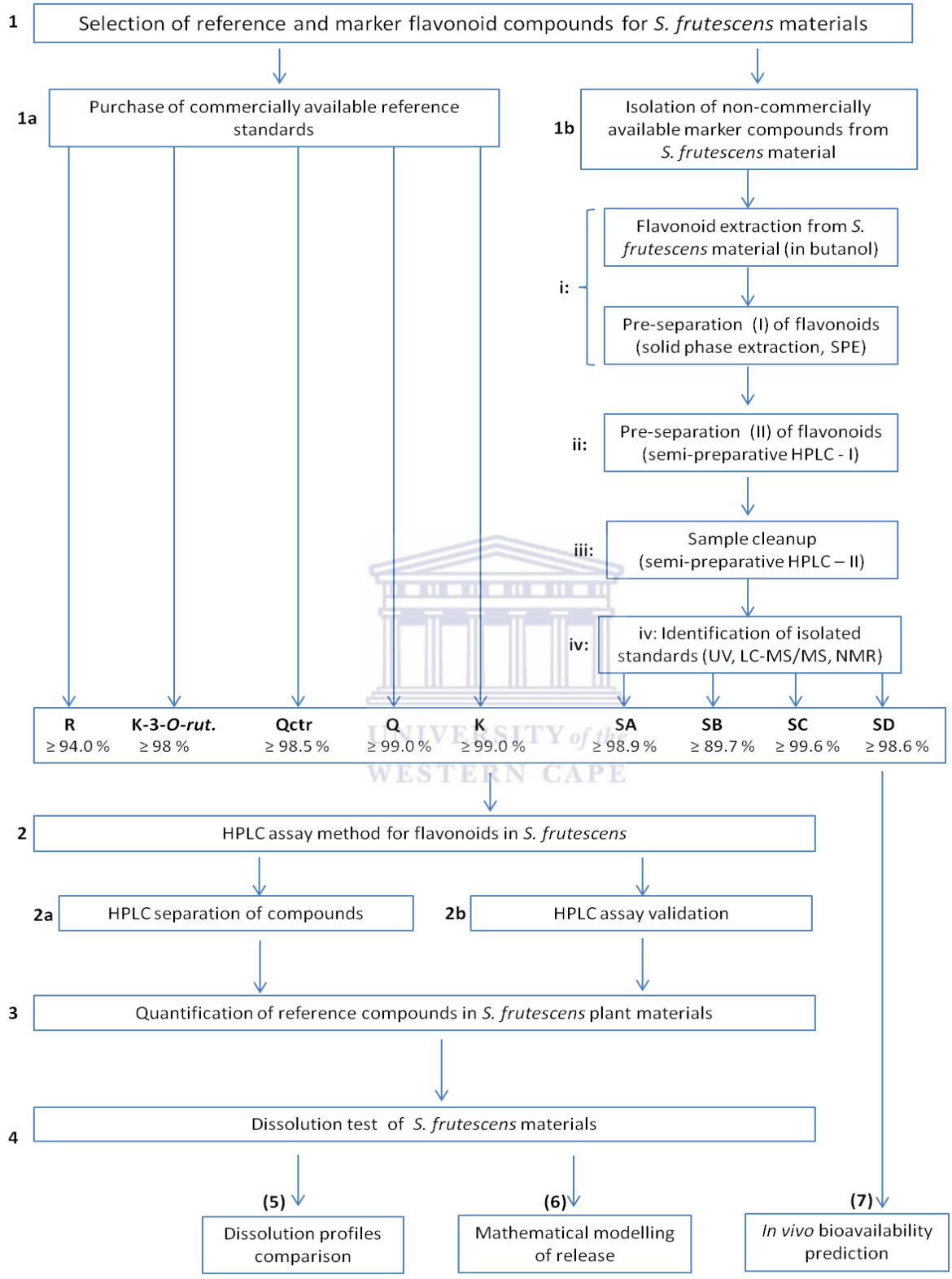


Figure 4.1: *Flow diagram of step-wise approach to determine flavonoid content, dissolution characteristics and in vivo bioavailability of flavonoids in S. frutescens phytomedicinal materials*

KEY: *SA: sutherlandin A* *SB: sutherlandin B* *SC: sutherlandin C*

SD: sutherlandin D	*R: rutin*	*K-3-O-rut: kaempferol-3-O-rutinoside*
Qctr: quercitrin	*Q: quercetin*	*K: kaempferol*

A dissolution test was also developed and validated for the *S. frutescens* materials *(4)* and the flavonoids released in dissolution media were detected and quantified using a valildated HPLC method. Dissolution profiles of marker compounds were compared using standard and modified methods *(5)*, modelled to determine release characteristics of flavonoid markers from different *S. frutescens* materials *(6)* and the *in vivo* bioavailability of selected marker compounds predicted *(7)*.

4.3.1.1 Flavonoid extraction and pre-separation (solid-phase extraction)

Powdered *S. frutescens* SDAE material was washed, first with hexane, and then with ethyl acetate. The ethyl acetate fraction was partitioned by liquid-liquid extraction into butanol, and the butanol extract concentrated using the rotary evaporator under reduced heat to obtain a dark viscous product.

The crude butanol extract was subjected to reversed phase column chromatography using C_{18} Strata™ solid phase extraction (SPE) cartridges for the isolation of flavonoid fractions. First, the SPE cartridge was conditioned with 100 % acetonitrile followed by water containing 0.01 % formic acid. The butanol fraction (dissolved in 50 % acetonitrile in water) was applied uniformly to the SPE cartridges and the column eluted using a five step gradient elution with water: acetonitrile mixtures (0.01 % formic acid) of decreasing polarity (0 to 100% acetonitrile). Six major fractions were obtained (Figure 4.2). Fraction 3 was found to contain the sutherlandins (Figure 4.2), identified using UV-spectroscopy, and was further fractionated using the methods discussed below.

4.3.1.2 Pre-separation of flavonoids by semi-preparative HPLC # 1

Fraction 3 obtained from SPE gradient elution was subjected to HPLC, using a Supelco® Discovery semi-preparative C_{18} column (250 x 10 mm, i.d. 10 µm) with a compatible guard column, both maintained at 45°C. The mobile phase consisted of water (0.01 % formic acid) (A) and acetonitrile (0.01 % formic acid) (B), and was filtered through a 0.45 µm filter and degassed prior to use. The flow rate of the mobile phase was 3 ml /min, injection volume 50 µl and peaks were separated according to the following linear gradient elution:

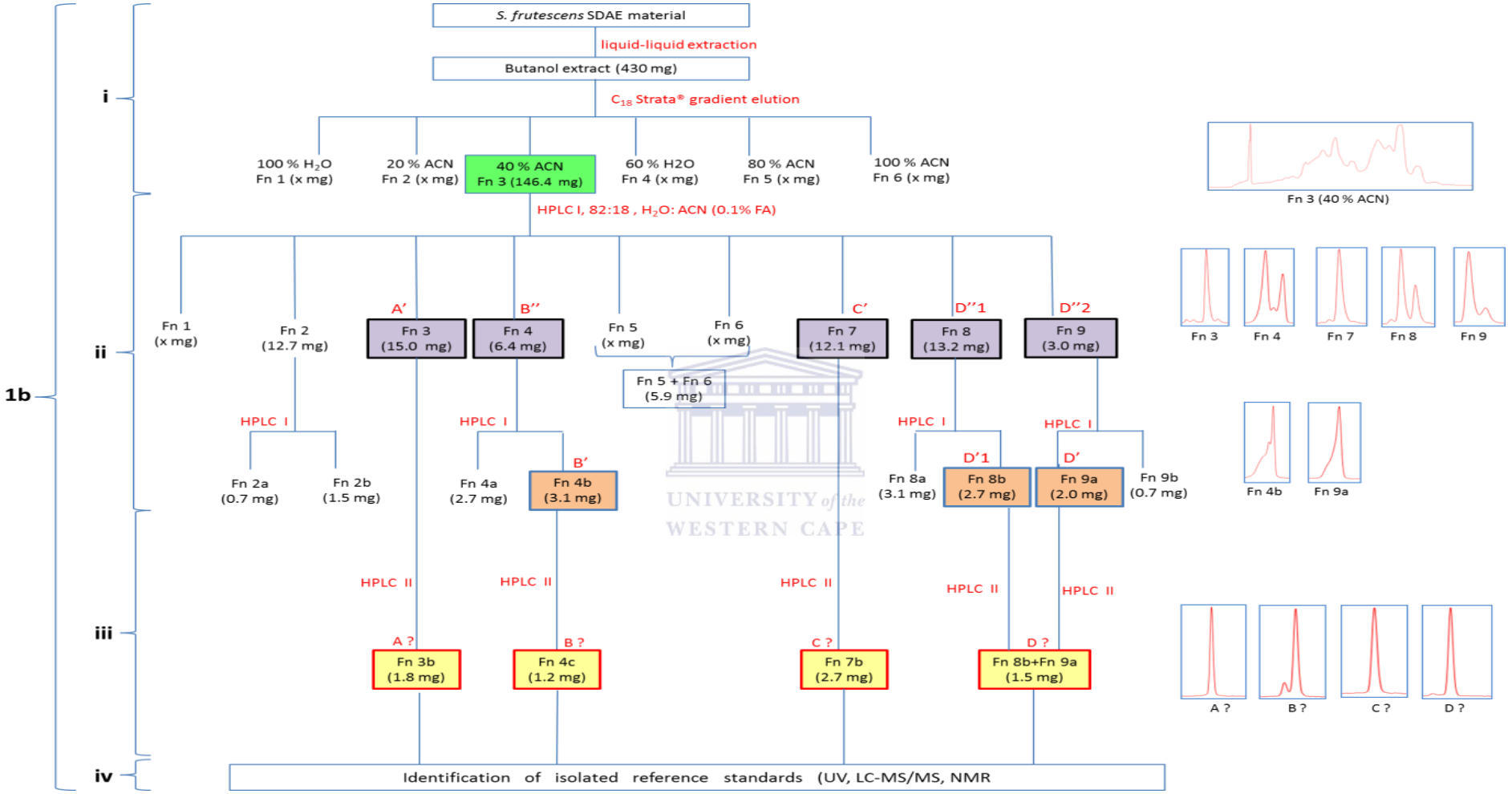


Figure 4.2: Flow diagram showing step-wise approach to isolation of marker compounds (sutherlandins A, B, C and D) from S. frutescens

0 to 1 min, 82 % A/ 18 % B; 1 to 15 min, 82 % A/ 18 % B to 75 % A/ 25 % B; 15 to 20 min, 75 % A/ 25 % B to 65 % A/ 35 % B; 20 to 25 min, 65 % A/ 35 % B to 40 % A/ 60 % B; 25 to 26 min, 40 % A/ 60 % B to 82 % A/ 18 % B; followed by an equilibration with 82 % A/ 18 % B from 26 to 35 minutes. The eluent was monitored at several wavelengths over a range from 250 to 380 nm, the specific wavelength of interest being 370 nm.

Preliminary identification of the four flavonoid glycosides for collection during isolation was based on retention times and UV-spectral characteristics.

Preliminary quantities obtained for the semi-pure compounds were as follows:

Fraction 3:	Sutherlandin A ?:	15.0 mg
Fraction 4:	Sutherlandin B ?:	6.4 mg
Fraction 7:	Sutherlandin C ?:	12.1 mg
Fraction 8b+9a:	Sutherlandin D ?:	16.2 mg

Fractions 4, 8 and 9 were further subjected to HPLC using the same method described above (semi-preparative HPLC # 1) to further separate component compounds. Fraction 4 yielded two different compounds, *viz.* 4a and 4b and fraction 8 two different compounds, i.e. 8a and 8b, while compounds 9a and 9b were obtained from fraction 9. Further HPLC separation and purification of fractions 3, 4b, 7, 8b and 9a was carried out using the method described below (semi-preparative HPLC # 2).

4.3.1.3 Sample clean-up (semi-preparative HPLC # 2)

Portions of fractions 3, 4b, 7, 8b and 9a above were subjected to HPLC for further clean up, using the Phenomenex Luna® semi-preparative column (250 mm x 10 mm, i.d. 10 μm) with a compatible guard column, both maintained at 45°C. The other method parameters were the same as in HPLC # 1 with the exception of the injection volume which was 100 μL.

The isolated compounds were identified based on retention times and UV-spectral characteristics, and their percentage purity calculated based on analytical HPLC. The final isolated products were dried, weighed and stored in amber coloured vials at 4°C until LC-MS/MS and NMR analysis to confirm their identities.

4.3.1.4 Identification of isolated compounds

LC-MS and LC-MS/MS analyses were conducted using a Waters Synapt G2 quadrupole time-of-flight mass spectrometer, connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) and photo diode array (PDA) detector. For the LC analysis, a Waters UPLC BEH C_{18} column (100 x 2.1 mm, 1.7 µm) and gradient elution was used. The mobile phase, consisting of water (1.0 % formic acid) (A) and acetonitrile (B), was filtered and degassed prior to use. The flow rate of the mobile phase was maintained at 0.35 ml/min, and peaks representing the different marker compounds were separated according to the following gradient elution prior to a holding period of 0.5 minute: 0 to 0.5 min, 95 % A/ 5 % B; 0.5 to 20 min, 95 % A/ 5 % B to 56 % A/ 44 % B; 20 to 21 min, 56 % A/ 44 % B to 0 % A/ 100 % B; 21 to 22 min, 0 % A/ 100 % B to 0 % A/ 100 % B; 22 to 23 min, 0 % A/ 100 % B to 95 % A/ 5 % B; followed by an equilibration with 95 % A/ 5 % B from 23 to 26 minutes. The injection volume was 3 µL and MS data was acquired *via* high resolution electrospray ionisation (HR-ESI) performed in the positive ion mode with nitrogen gas as nebulizer, using further details of the method described by Albrecht *et al.*[19] to generate LCMS chromatograms and fragmentation data of the marker compounds.

The ^{1}H-NMR spectra were recorded in *methanol-d4 (CD_3OH)* using a Bruker Avance IIID Nanobay 400 MHz NMR spectrometer equipped with a 5 mm broadband observe (BBO) probe, and chemical shifts were recorded in parts per million (ppm).

4.3.2 HPLC assay method for quantification of flavonoids in *S. frutescens*

An HPLC assay for nine flavonoid compounds was developed and validated (Figure 4.1, *2a & 2b*).

4.3.2.1 HPLC system and conditions.

The HPLC system used for the acquisition of chromatograms and UV spectra was an Agilent 1200 series HPLC system, equipped with an in-line degassing system, quaternary pump, auto loading sampler, thermostatted column compartment and photodiode array detector. Chromatographic separation was obtained using a Phenomenex Luna® C_{18} column (25 cm x 4.6 mm, 5 µm i.d.) with a compatible guard column, both maintained at 45°C. The mobile phase, consisting of water (0.01 % formic acid) (A) and acetonitrile (0.01 % formic acid) (B),

was filtered through a 0.45 µm filter and degassed prior to use. The flow rate of the mobile phase was maintained at 0.8 ml/min, injection volume was 20 µl, and peaks were separated according to the following linear gradient elution: 0 to 1 min, 82 % A/ 18 % B; 1 to 15 min, 82 % A/ 18 % B to 75 % A/ 25 % B; 15 to 20 min, 75 % A / 25 % B to 65 % A/ 35 % B; 20 to 25 min, 65 % A/ 35 % B to 40 % A/ 60 % B; 25 to 26 min, 40 % A/ 60 % B to 82 % A/ 18 % B; followed by an equilibration with 82 % A/ 18 % B from 26 to 35 minutes. The eluent was monitored at several wavelengths over a range from 250 to 380 nm, the specific wavelength of interest at 370 nm. Samples were collected based on retention times and UV-spectral characteristics of compounds. Data acquisition and processing was carried out using the OpenLAB™ CDS ChemStation Edition software.

4.3.2.2 Validation of HPLC assay

The following parameters were determined to validate the HPLC assay according to ICH guidelines.[205]

For linearity assessment, stock solutions for the calibration curves and quality control assays were prepared by weighing out specific amounts of the nine reference and marker compounds, which were dissolved in methanol: water (50:50) to achieve concentrations between 0.5 and 2 mM. The linearity of the calibration curve for each of the reference and marker compounds was assessed in triplicate over a six sample concentration range, and calibration curves constructed by a linear regression of plots of peak area against concentration.

The limit of detection (LOD) is defined at a signal-to-baseline ratio of 3:1, and is taken as the lowest concentration of standard, which under the described HPLC conditions, will produce a peak height at least three times higher than the base line noise, while the limit of quantification (LOQ) is defined at a signal-to-baseline ratio of 10:1.[205] For the LOD and LOQ determination, the peak area was used as the signal response and, the mean baseline noise was 0.4 mAU (n = 3).

For stability assessment, samples of the reference and marker compounds were subjected to a variety of conditions (acid /base hydrolysis, freeze /thaw cycles, ambient light and heat

exposure) and injected into the HPLC system to assess the analyte in the presence of any degradants which may arise under these conditions. For acid /base hydrolysis, reference and marker compounds were treated with 2 M solution of hydrochloric acid or sodium hydroxide, respectively, heated in a water bath at 60°C for 60 minutes (for acid hydrolysis), or left at room temperature for two hours (for basic hydrolysis), and reaction products observed. Freeze-thaw cycles were conducted by five consecutive freezing (at –80°C for 30 minutes) and thawing (at room temperature for 30 minutes) sessions. For ambient light analysis, the reference and marker compounds were exposed to sunlight for one month; while for assessment of heat stability, the reference standards were heated for 3 hours at 60°C.

The precision of the analytical method was determined by assaying six spiked samples at the lower and upper limits of the concentration range studied for each of the reference or marker compounds. The accuracy of the method was determined by the mean concentrations obtained for the replicates and the percentage difference. Samples were prepared by dilution of the stock solutions with solvent for each standard. The relative standard deviations (% RSD) were then calculated for the reference and marker standard samples at the lower and upper limits of the concentration range.

Evaluation of HPLC method robustness was considered in the mobile phase development. The robustness and ruggedness of the method were investigated by varying chromatographic parameters such as the flow rate and temperature.

Finally, system suitability parameters[206] were calculated from the chromatograms obtained for each of the reference and marker compounds during the studies. The Agilent ChemStation software was used to calculate these parameters thus:

Capacity factor: $k' = \frac{t_a}{t_b} - 1$, where t_a is the retention time from the time of injection to the time of the elution of the peak maximum and t_b is the elution time of the void volume or non-retained compounds. Peaks should be well resolved from the void volume and k’ > 2 is acceptable.

Resolution, R_s: $R_S = 2 \times \frac{(t_2 - t_1)}{(W_2 + W_1)}$, where t_2 and t_1 are retention times of the two components, and W_1 and W_2 are corresponding widths of the bases of the peaks, obtained by extrapolating the sides of the peaks to the baseline. $R_s > 2$ between the peak of interest and the closest potential interfering peak is desirable.

Tailing factor, T: $T = \frac{W \times 0.05}{2f}$, where f is the distance from the front edge of the peak to a perpendicular line drawn from the peak maximum to the baseline. T < 2 is acceptable.

4.3.3 Determination of presence and levels of reference and marker compounds in selected *S. frutescens* materials

Five *S. frutescens* products were selected for analyses. These materials were the leaf powder, spray-dried aqueous extract (SDAE) and three freeze-dried aqueous extract materials described in Section 4.1.

Samples of the five *S. frutescens* materials selected were prepared thus: about 100 mg of each *S. frutescens* material, i.e. leaf powder (LP), spray-dried aqueous extract (SDAE), freeze-dried aqueous extract 1 (FDAE 1), freeze-dried aqueous extract 2 (FDAE 2) and freeze-dried aqueous extract 3 (FDAE 3), was vortexed in 2 ml of 50 % aqueous methanol, sonicated for 30 minutes and then centrifuged at 3500 rpm for 15 minutes. The extraction procedure was repeated thrice, respective supernatants combined and the final volume adjusted to 8 ml with 50 % aqueous methanol and mixed thoroughly. Prior to injection, appropriate dilutions were made, and the final solution filtered using a 0.45 µm nylon membrane filter. Aproximately 1.0 ml of filtrate was discarded and the remaining volume collected in an LC sample vial. Triplicate sample solutions of each *S. frutescens* material were made, injection volume was 50 µl and samples were analysed using the validated HPLC method already described under section 4.3.2. The average peak areas of the flavonoids of interest in each sample were used to quantify and determine concentration of the sample from the calibration curve and flavonoid content in the different *S. frutescens* materials

were compared. Data was presented as average percentage content (mg flavonoid x minimum purity of flavonoid reference /mg plant material) ± SD (n = 3).

4.3.4 Dissolution test for *S. frutescens* materials

Three *S. frutescens* materials (i.e. LP, SDAE and FDAE 1) were selected for the dissolution tests conducted in three different dissolution buffers at pH 1.2, 4.5 and 6.8. The selected *S. frutescens* materials were filled into size *0* capsules using Cap.M.Quik™ size 0 capsule filler. A hundred capsules of each material were prepared, and the capsules weighed before and after filling in order to determine the uniformity of weight in compliance with USP guidelines.

The USP basket apparatus method was used to determine the dissolution of the flavonoids from the *S. frutescens*-containing capsules in the following media – 0.1 N hydrochloric acid (pH 1.2), acetate buffer (pH 4.5) and phosphate buffer (pH 6.8). Standard procedures were used to prepare the media and the final pH confirmed using a calibrated pH meter. Initially, 1 or 2 or 3 capsules were immersed in 900 ml of dissolution medium for the test. Due to the low content of the flavonoids in plant material (< 5mg /capsule) as well as to improve the sensitivity of the method by increasing marker compound concentration, the dissolution medium was reduced to 450 ml, maintained at 37 ± 0.5 °C. Two capsules containing the appropriate *S. frutescens* material were placed in each dissolution vessel basket, and vessel contents stirred at 100 rpm. Apart from the change in volume and number of dosage units, all other conditions were in accordance with standard USP guidelines.

At predetermined time intervals (5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes), 3 ml of sample was withdrawn from each vessel through a 0.45 µm nylon membrane syringe filter. The samples were filtered during withdrawal to prevent continuation of dissolution, and replaced with an equal volume of the same pre-warmed medium in order to maintain constant volume and temperature. The filtered withdrawn samples were analysed using the validated HPLC method reported in 4.3.2 and the amount and percentage of reference compounds dissolved calculated from the peak area response of the HPLC chromatograms, using the highest peak response obtained from each vessel as the 100 % value. Data was

plotted as percentage dissolved ± standard deviation *versus* dissolution time, and Q-release values were determined for the flavonoid marker compounds of interest.

The samples were filtered during withdrawal to prevent continuation of dissolution, and replaced with an equal volume of the same pre-warmed medium in order to maintain constant

As a category III analytical procedure, i.e. analytical procedure for determination of performance characteristics, the dissolution test method was validated for precision by means of repeatability.[207] The precision was evaluated for repeatability by analysing six individual samples (six replicates) collected from the dissolution apparatus at a time of 60 minutes (pH 1.2), and expressed as relative standard deviation (% RSD) between samples.

Principal component analysis (PCA) was conducted using *LatentiX version 2.12*. Dissolution data was uploaded and autoscaled and the PCA model for the data calculated. The loadings and scores plots were generated from the calculated PCA model and used to assess the relationship between different sampling times and the relationship between dissolution of marker compounds from the different materials. Clustering of variables in PCA analysis indicates that all the variables in the cluster provide the same information, and this was applied to identify redundant variables.

4.3.5 Comparison of flavonoid glycoside dissolution profiles from different S. frutescens materials

To compare dissolution profiles of flavonoids from the different *S. frutescens* materials in three different dissolution media, three methods were employed. These were the original similarity factor and two modifications of this: Gohel *et al.*'s similarity factor and a new method which we propose, the median similarity factor. The objective of comparing dissolution profiles with these different methods was to assess how the modifications compared to the original similarity factor, and to determine which method was the most discriminating for the profiles. The most discriminating method for dissolution profiles comparison is the one which gives the lowest f_2 value.

4.3.5.1 Similarity factor, f_2 method

For each combination of product dissolution profiles (e.g. LP *versus* SDAE, etc.), the similarity factor (f_2) was calculated using the equation:

$$f_2 = 50 \log \left(\frac{100}{\sqrt{\left[1 + \frac{1}{n} \sum (R_t - T_t)^2\right]}} \right)$$

where R_t and T_t are the average percentages dissolved at each time point for the reference and test products, respectively; and n is the number of observations. An f_2-value between 50 and 100 indicates similarity of two dissolution profiles while values below 50 indicate dissimilarity[179], and this criterion was used to compare the product profiles.

4.3.5.2 Gohel's modified similarity factor method

The similarity factor was calculated using the equation:

$$f_2 = 50 \log \left(\frac{100}{\sqrt{\left[1 + \frac{1}{n} \sum_{t=1}^{n} W_t \ (R_t - T_t)^2\right]}} \right)$$

which includes a weight factor proposed by Gohel *et al.*[161,162] This weight factor was calculated using the following equation:

weight, w_t = 1 + {(% CV of R_t) /($MCV_{E/L}$)} + {(% CV of T_t)/($MCV_{E/L}$)}

where w_t is weight; ***% CV of*** R_t and ***% CV of*** T_t are the percentage coefficient of variation of reference and test products, respectively; $MCV_{E/L}$ is the maximum allowable % CV and co-efficient of variation was calculated thus: ***% CV = (standard deviation /mean) × 100***. All other symbols are as represented in the original similarity factor, f_2, equation.

Again, if the f_2 values are between 50 and 100, it indicates similarity of two dissolution profiles while values below 50 indicate dissimilarity[179], and this criterion was used to compare the product profiles.

4.3.5.3 Median similarity factor

The median similarity factor, median f_2, was calculated, using the same equation used for ordinary f_2 calculation:

$$f_2 = 50 \log \left(\frac{100}{\sqrt{\left[1 + \frac{1}{n} \sum (R_t - T_t)^2\right]}} \right)$$

All symbols are as represented in the original similarity factor equation with the exception of R_t and T_t, which represent the median percent dissolved at each time point for the reference and test products, respectively. As in the previous cases, a median f_2-value between 50 and 100 indicates similarity of two dissolution profiles while values below 50 indicate dissimilarity, and this criterion was used to compare the product profiles.

4.3.6 Determination of release characteristics

For this, the methods used to fit release data for description of release curves, as well as semi-empirical model fitting for release mechanism elucidation are presented.

4.3.6.1 Model fitting for description of flavonoid release from *S. frutescens* materials

From dissolution data of *S. frutescens* LP, SDAE and FDAE in three different dissolution buffers, reference and marker compound release were assessed by different mathematical models of API release in order to select the model of best fit. Data was fitted to various models using DDSolver. For fitting drug dissolution models to non-transformed data, DDSolver determines the parameter values that minimize the sum of squares (SS) or the weighted sum of squares (WSS) by use of the non-linear least-squares curve-fitting technique: [151]

$$WSS = \sum_{i=1}^{n} W_i \cdot \left(y_{i\_obs} - y_{i\_pre}\right)^2$$

where n is the number of observations; w_i is the weighting factor; y_{i_obs} refers to the ith observed y value and y_{i_pre} refers to the ith predicted y value. Mathematical expressions describing these models are as summarized in Table 2.1.

First, in order to determine the best mathematical equation for description of flavonoid glycoside release from different *S. frutescens* materials, dissolution data was fitted to different models. Descriptions of the parameters in the model equations are explained in literature.[151] Goodness of fit was analysed for the models and the best mathematical model for flavonoid release from *S. frutescens* material was chosen after careful consideration of the coefficient of determination (R^2) and the adjusted coefficient of determination (R^2_{adj}). For release models with the same number of parameters, the R^2 value was used to determine the model of best fit (with higher R^2 value indicating better fit) while for release models with different number of parameters, R^2_{adj} was used to select the best model (with higher R^2_{adj} value indicating better fit).[151] In cases of over fitting, R^2_{adj} may decrease, giving an indication of model suitability, while R^2 will always increase or remain constant as more parameters are included in a model.[146]

Although there are no specifications for modelling of flavonoid glycoside release from *S. frutescens,* the specification for goodness of fit of a model was arbitrarily set at a minimum value of 0.98 for R^2. Besides R^2 and R^2_{adj}, model suitability was also confirmed by assessment of the Akaike Information Criterion (AIC), Model Selection Criterion (MSC), mean square error (MSE) and sum of squares (SS) values.

4.3.6.2 Evaluation of flavonoid release mechanisms from *S. frutescens* materials

To determine the mechanisms involved in flavonoid release from the different *S. frutescens* materials, dissolution data were fitted to three semi-empirical models: Weibull, Peppas-Sahlin and the Korsmeyer-Peppas models. Release mechanisms were determined according to values of the time exponent (**β**) for the Weibull model (as discussed in Section 2.6.2.3.3).

To assess if release was via diffusion or relaxation, dissolution data was fitted to the semi-empirical equation proposed by Peppas and Sahlin:[158]

$$F = k_d \,.\left(t - T_{lag}\right)^m + k_r \,.\left(t - T_{lag}\right)^{2m}$$

The parameters, k_d and k_r, represent the release rates of polymer diffusion and relaxation, respectively.[158] A higher value of k_d than k_r implies that API release is more by diffusion than relaxation, and vice versa.

For fitting to the Korsmeyer-Peppas model, the first 60 % or less of the release data was fitted to the Korsmeyer-Peppas equation and release mechanism determined by the value of the release exponent.

4.3.7 Prediction of flavonoid *in vivo* bioavailability

For the prediction of *in vivo* bioavailability of the flavonoid compounds employed in this study, the chemoinformatic and computational software, *MoE*, was used. Structural formulae of the reference or marker compounds employed were exported to the operating environment. Values were generated for different molecular properties such as molecular weight, number of hydrogen bond acceptors, octanol-water partition coefficient, rotatable bonds, to mention a few. Such molecular properties enabled the characterisation of each compound's bioavailability according to documented literature theories proposed by Lipinski *et al.* and Congreve *et al.*[184,185]

In the next chapter, results obtained after application of these methods are discussed.

CHAPTER FIVE

RESULTS AND DISCUSSION

In this chapter, the results obtained in isolation of marker compounds (sutherlandins) from *S. frutescens* material, development and validation of HPLC assay for flavonoid compounds in solution, assessment of marker compound profiles and levels in different *S. frutescens* materials, and dissolution testing and dissolution profiles comparison of flavonoids from different *S. frutescens* materials, are presented. In addition, flavonoid dissolution and molecular properties were modelled for release mechanism elucidation and *in vivo* bioavailability prediction, respectively, and results from these are also presented and discussed.

5.1 Isolation and identification of sutherlandins A, B, C and D from *S. frutescens*

The use of flavonoid glycosides as marker compounds for plant materials is usually limited by their commercial non-availability. This section reports on the isolation of four flavonoid glycosides (sutherlandins A, B, C and D) from *S. frutescens* material and their subsequent identification by appropriate analytical techniques.

5.1.1 Isolation of sutherlandins A, B, C and D

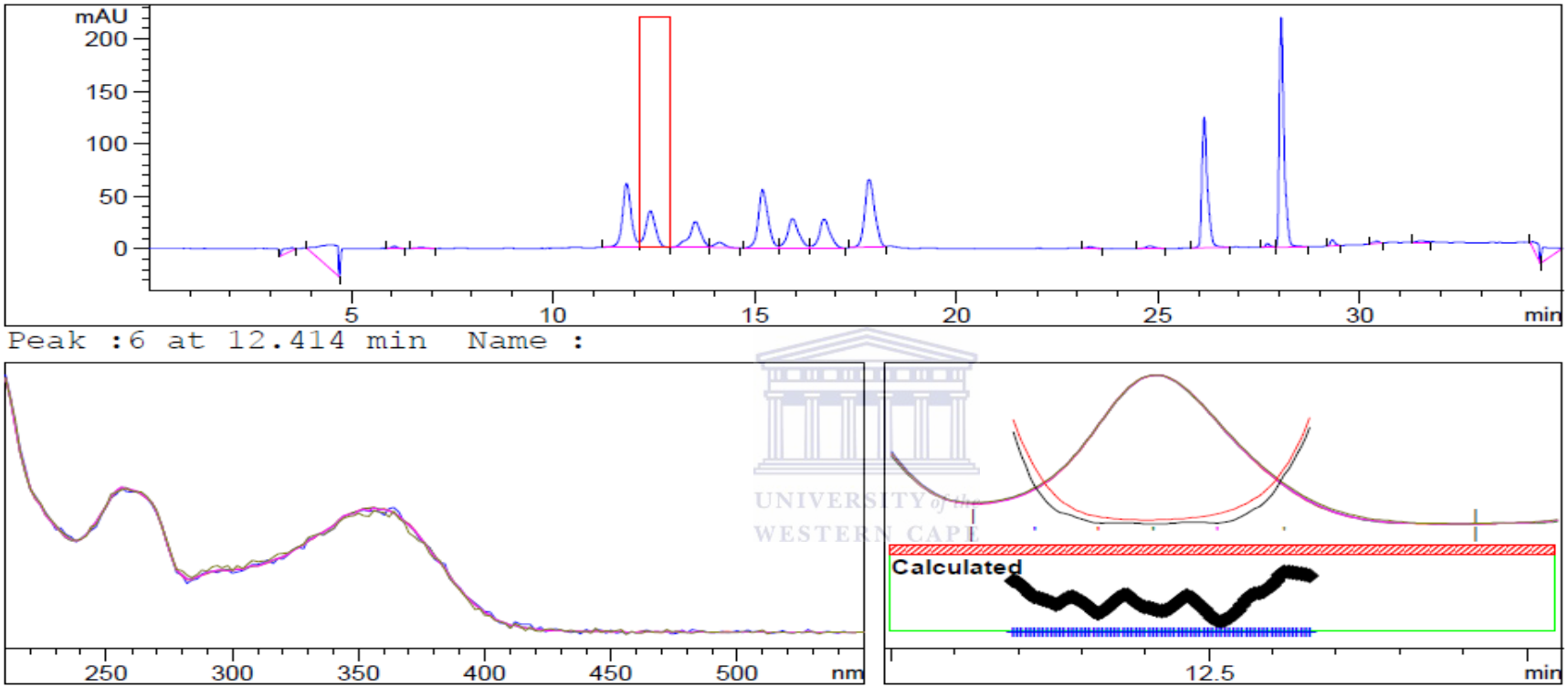
Fractionation of the crude butanol extract yielded six fractions eluted with varying acetonitrile-water concentrations. The UV spectra of the six fractions (Appendix 2, Figures A 2.1 to A 2.4) were compared and fraction 3 (Chapter 4, Figure 4.2, green highlight) was found to be rich in flavonoid compounds. The other fractions did not contain much of the flavonoid glycoside compounds as can be seen from the quantities reflected on the y-axis of their HPLC chromatograms (Appendix 2, Figures A 2.1 to A 2.4).

5.1.2 Identification of sutherlandins A, B, C and D

The component compounds in fraction 3 were further separated and samples cleaned up using semi-preparative HPLC. Use of a photodiode array detector (PDA) in HPLC analysis enabled the acquisition of the UV/Vis spectral data for the sutherlandins. The four marker compounds contained in 40 % ACN solution (Figure 4.2, green highlight) were isolated; preliminary identification while they were still in solution was *via* UV-spectral characteristics as shown in the spectrum index plot (Figure 5.1.1). These matched those previously reported for the same compounds.[20]

The isolated compounds were irregularly shaped, non-crystalline and yellow to peach coloured. Final quantities obtained for sutherlandins A, B, C and D were 1.8 mg, 1.2 mg, 2.7 mg and 1.5 mg, respectively. Minimum values for percentage purity (as assessed by analytical HPLC) were 98.9 %, 89.7 %, 99.6 % and 98.6 % for sutherlandins A, B, C and D, respectively.

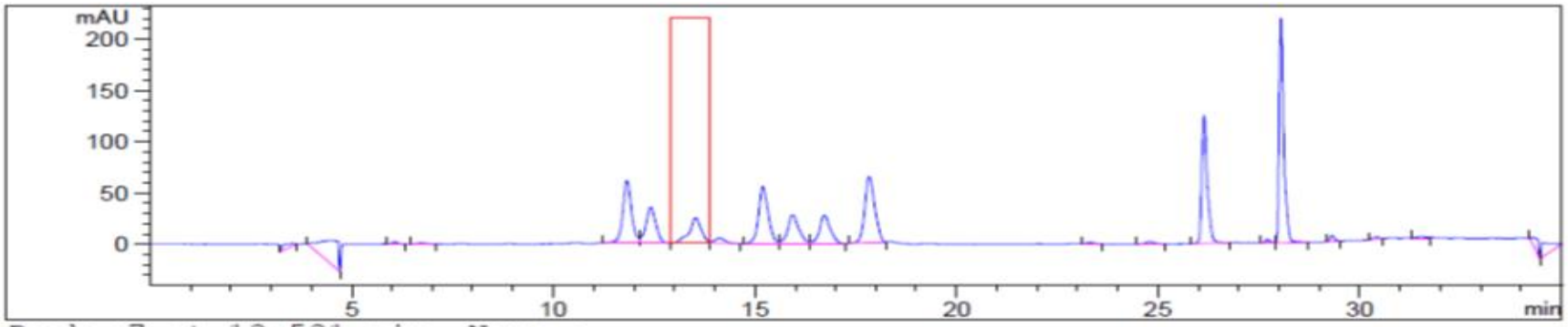
Figure 5.1.1: Spectrum index plot of HPLC-PDA analyses of isolated compounds, sutherlandins A, B, C and D, isolated from *S. frutescens*



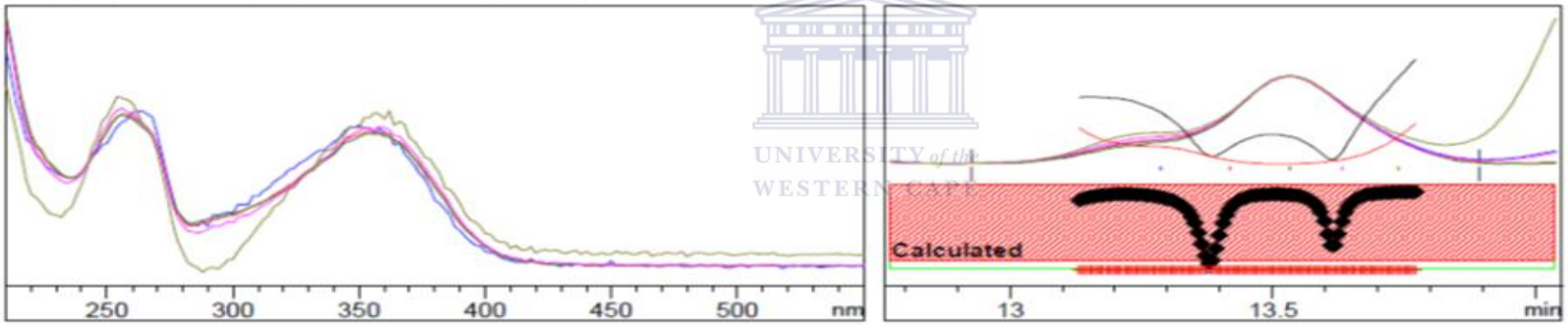
-> The purity factor is within the calculated threshold limit. <-

Purity factor : 999.293 (71 of 71 spectra are within the calculated threshold limit.)
Threshold : 998.924 (Calculated with 71 of 71 spectra)
Reference : Peak start and end spectra (integrated) (12.125 / 12.919)
Spectra : 5 (Selection automatic, 5)
Noise Threshol: 0.109 (12 spectra, St.Dev 0.0527 + 3 * 0.0187)

Sutherlandin A



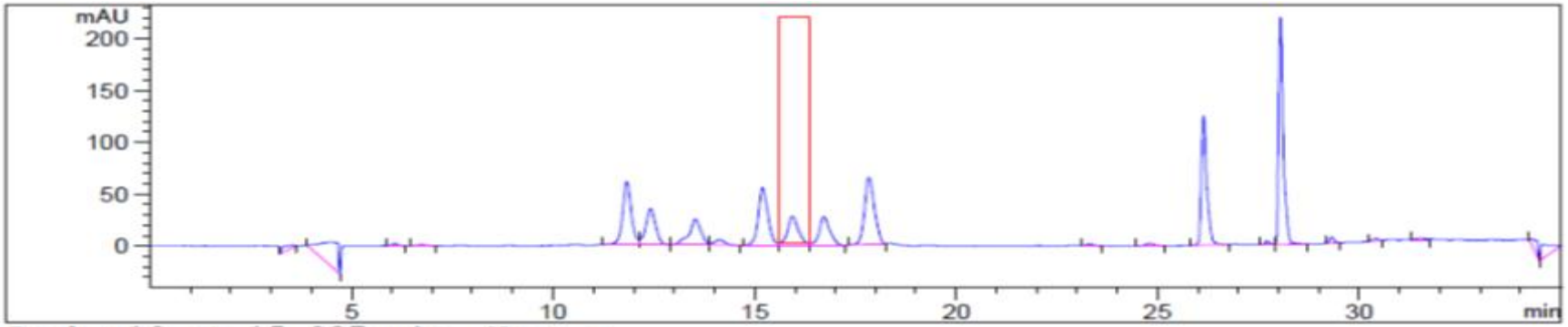
Peak :7 at 13.531 min Name :



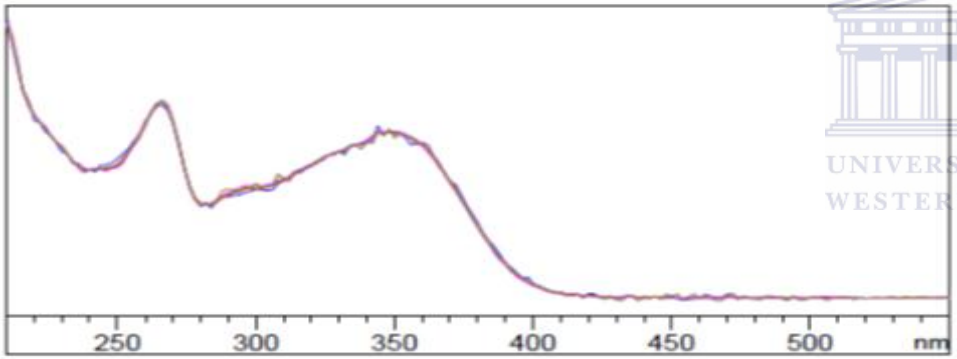
-> The purity factor exceeds the calculated threshold limit. <-

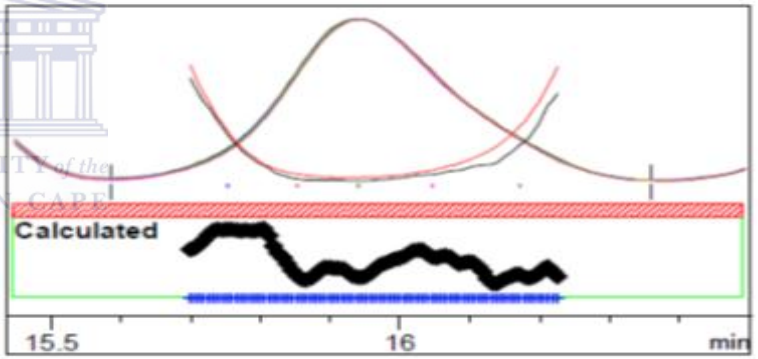
Purity factor : 969.259 (96 of 97 spectra exceed the calculated threshold limit.)
Threshold : 998.639 (Calculated with 96 of 97 spectra)
Reference : Peak start and end spectra (integrated) (12.925 / 13.892)
Spectra : 5 (Selection automatic, 5)
Noise Threshol: 0.109 (12 spectra, St.Dev 0.0527 + 3 * 0.0187)

Sutherlandin B



Peak :10 at 15.937 min Name :

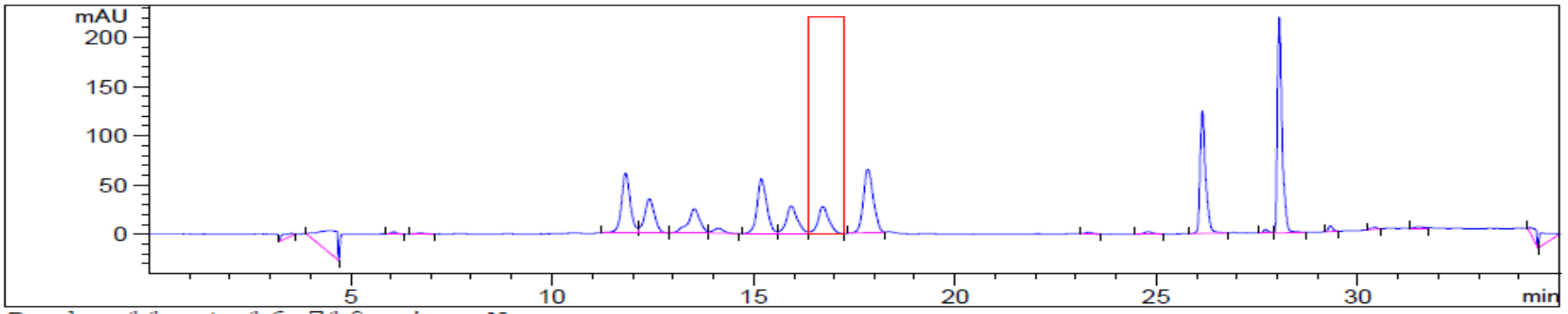




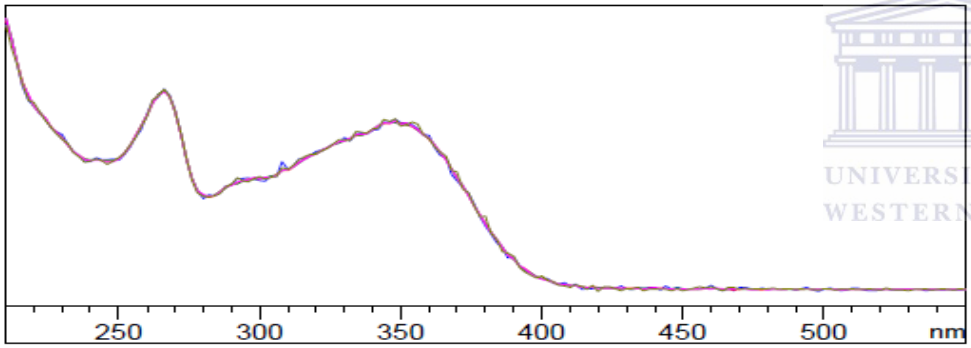
-> The purity factor is within the calculated threshold limit. <-

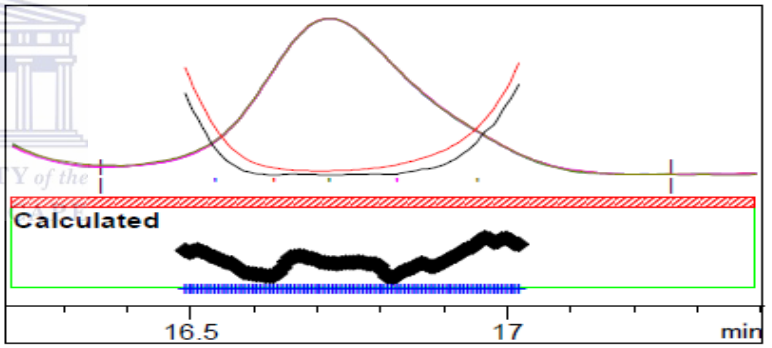
Purity factor : 999.412 (80 of 80 spectra are within the calculated threshold limit.)
Threshold : 999.131 (Calculated with 80 of 80 spectra)
Reference : Peak start and end spectra (integrated) (15.585 / 16.359)
Spectra : 5 (Selection automatic, 5)
Noise Threshol: 0.109 (12 spectra, St.Dev 0.0527 + 3 * 0.0187)

Sutherlandin C



Peak :11 at 16.719 min Name :





-> The purity factor is within the calculated threshold limit. <-

Purity factor : 999.561 (80 of 80 spectra are within the calculated threshold limit.)
Threshold : 999.187 (Calculated with 80 of 80 spectra)
Reference : Peak start and end spectra (integrated) (16.359 / 17.259)
Spectra : 5 (Selection automatic, 5)
Noise Threshol: 0.109 (12 spectra, St.Dev 0.0527 + 3 * 0.0187)

Sutherlandin D

To confirm the identity of the isolated compounds, they were analysed using mass spectrometry (MS), and the MS data is shown in Figure 5.1.2. High-resolution electrospray ionisation mass spectrometry (HR ESI-MS) analysis provided the $[M+H]^+$ molecular ions of the respective compounds in the positive ion mode. The protonated species $[M+H]^+$, similar to those previously reported,[20] were therefore observed at m/z 741.1862, 741.1857, 725.1900 and 725.1913 atomic mass units (amu), corresponding to and confirming the molecular identities of sutherlandins A, B, C and D, respectively (Figure 5.1.2 and Table 5.1.1).

The isolated compounds were further characterized using the fragmentation patterns observed in the mass spectrum. Sutherlandins A and B showed characteristic fragments at *m/z* 609.1487 ($[M+H\text{-sugar}]^+$, i.e. $[M+H-132]^+$) and 303.0484 (base peak, quercetin), while sutherlandins C and D showed characteristic fragments at *m/z* 593.1472 ($[M+H\text{-sugar}]^+$, i.e. $[M+H-132]^+$) and 287.0554 (base peak, kaempferol) (Table 5.1.1). These ions reflect the loss of sugar from the flavonoid glycoside skeleton, and confirm the identity of the isolated compounds as sutherlandins A, B, C and D.

Table 5.1.1: LC-ESI-MS of isolated compounds identified using positive ion mode and key fragment analysis

Reference compound	**Exact mass (amu)**	**$[M+H]^+$ (amu)**	**$[M+H\text{-sugar}]^+$ (amu)**	**Key fragment (amu)**
Sutherlandin A	740.1800	741.1862	609.1487	303.0484
Sutherlandin B	740.1800	741.1857	609.1448	303.0498
Sutherlandin C	724.1851	725.1900	593.1472	287.0545
Sutherlandin D	724.1851	725.9130	593.1486	287.0554

In addition to HPLC-DAD and LC-ToF-MS, ^{1}H NMR was also performed to ensure structural elucidation of the isolated compounds. The results are shown in Appendix 3: the chemically distinct hydrogens are shown downfield to the left while the sugar moieties of the isolated sutherlandins are upfield to the right in the ^{1}H NMR spectra.

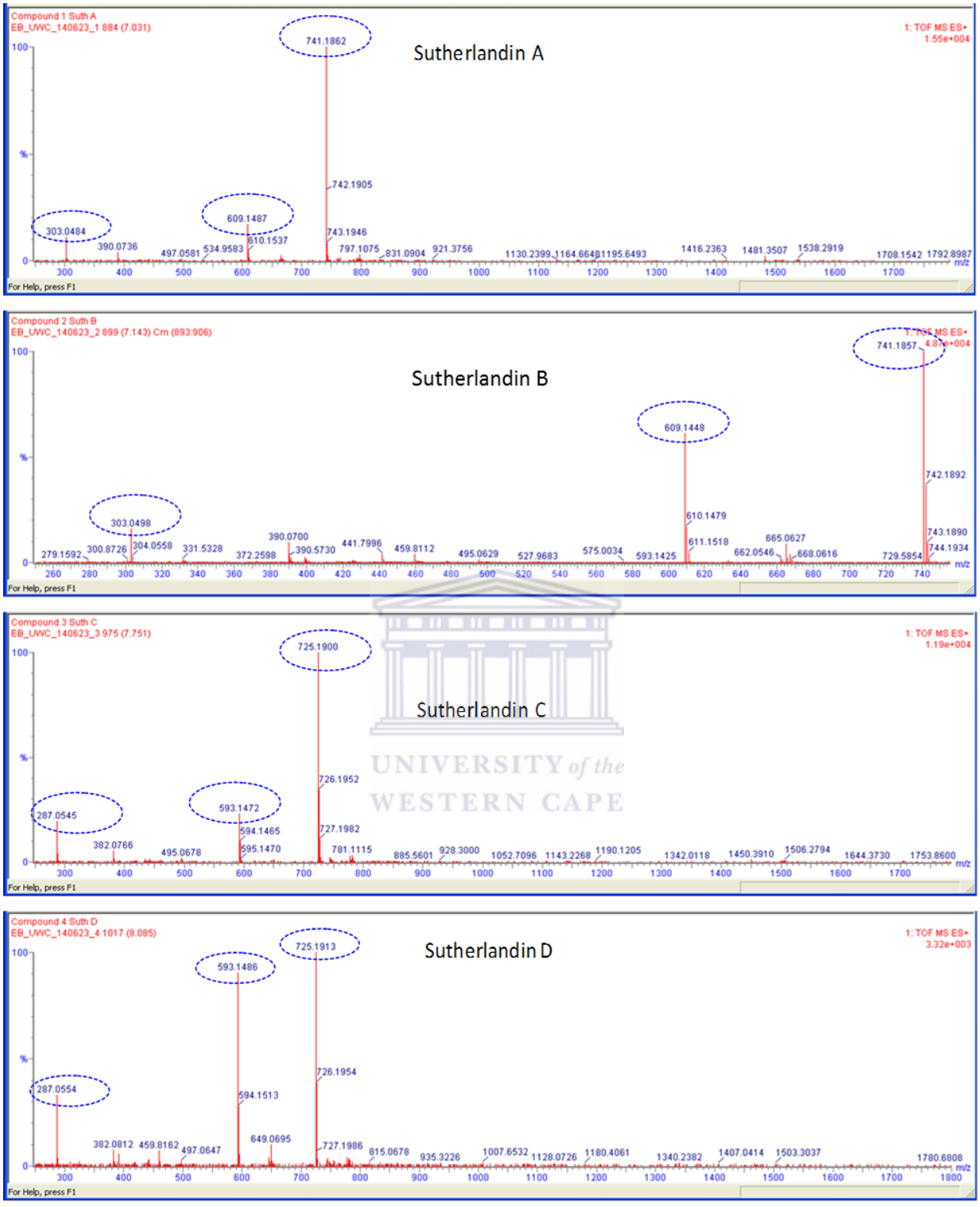


Figure 5.1.2: Mass spectra of sutherlandins A to D, isolated from S. frutescens (separation of the reference standards was on a Waters UPLC BEH 1.7 µm C18 column with dimensions 100 x 2.1 mm, using gradient elution and flow rate of 0.35 ml/min. Mobile phases A and B were water (1% formic acid) and acetonitrile respectively, injection volume was 3 µL and total run time was 26 minutes). The protonated species $[M+H]^+$ were observed at m/z 741.1862, 741.1857, 725.1900 and 725.1913 amu, for sutherlandins A to D respectively.

5.1.3 Conclusions

A crucial part of this study was the isolation of non-commercially available marker compounds (sutherlandins A, B, C and D) for *S. frutescens* assay. Chromatographic purity of the isolated compounds were found to be 98.9 %, 89.7 %, 99.6 % and 98.6 % for sutherlandins A, B, C and D, respectively. Based on the described experiments and results obtained, these compounds were qualified for use as reference standards to estimate concentrations of sutherlandins A, B, C and D in various *S. frutescens* products.

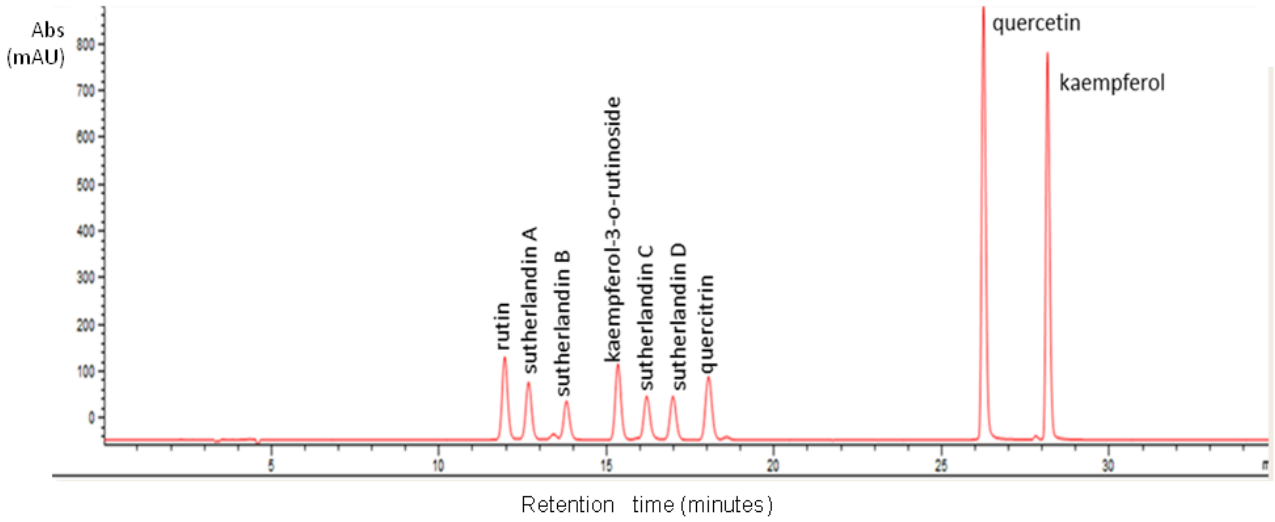
5.2 Development and validation of HPLC assay for flavonoid compounds in *S. frutescens*

This section reports the development and validation of a reversed phase HPLC method to assess content and dissolution of reference and marker compounds in *S. frutescens* products. Nine flavonoid compounds (consisting of seven flavonoid glycosides: sutherlandins A, B, C and D, rutin, kaempferol-3-*O*-rutinoside and quercitrin; and their two corresponding flavaonoid aglycones: quercetin and kaempferol) were selected for assay development and validation.

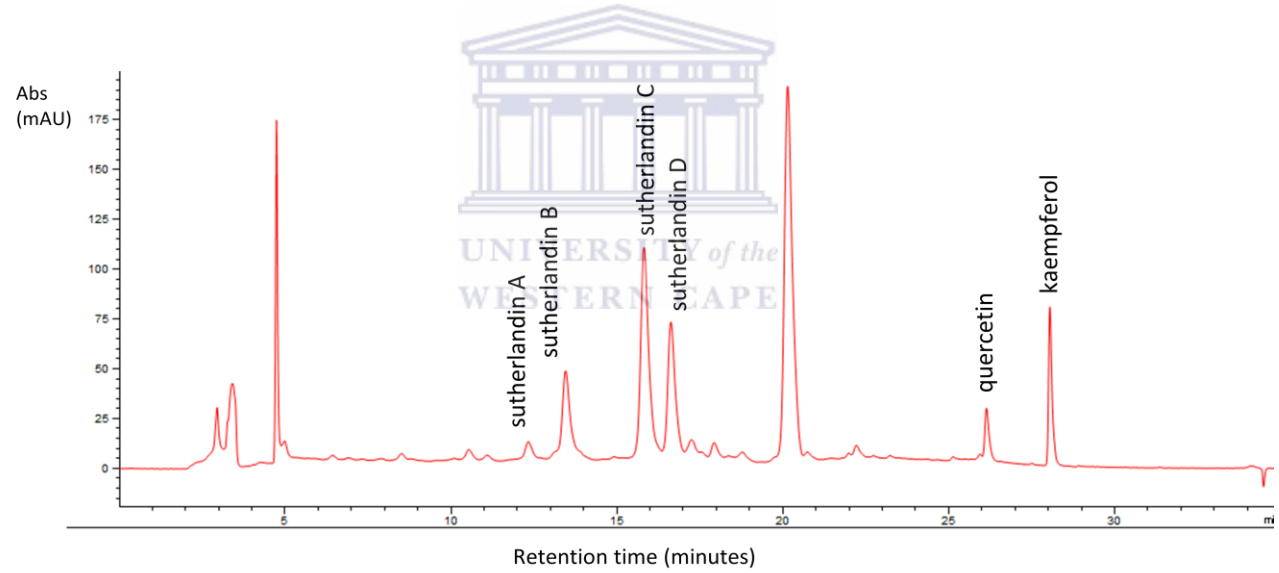
5.2.1 HPLC method development

To assess the profiles and levels of flavonoid glycosides and aglycones in *S. frutescens* plant materials, an assay was required. For this purpose, an HPLC assay was developed and validated for flavonoid analysis. The assay method involved a reverse phase HPLC separation with PDA detection, and the nine reference compounds earlier mentioned were used for the assay.

Different HPLC conditions were analysed in order to identify conditions with satisfactory resolution of chromatograms within the shortest possible time. Final chromatographic conditions selected are as described in Section 4.3.2.1. The use of acetonitrile-water was suitable for satisfactory resolution of the chromatograms, and the addition of 0.01 % formic acid to both solvents improved peak separation. The reference and marker compounds were separated by isocratic followed by gradient elution, and this was found to be suitable for the analysis of flavonoid glycosides and their corresponding aglycones in *S. frutescens* materials. Ultraviolet (UV) radiation was employed for the detection of the flavonoids due to the presence of flavonoid chromophores that absorb UV-light,[107] as well as the availability of UV-detection systems. Although absorption maximum was 350 nm for the selected compounds, 370 nm was the preferred wavelength due to its greater selectivity for the flavonoid compounds of interest. The reference compounds were separated within 30 minutes by HPLC-DAD technique. Chromatograms of standards and an *S. frutescens* sample are depicted in Figure 5.2.1 *a* and *b.* The glycosides eluted between 11 and 19 minutes, and the aglycones between 26 and 29 minutes.



(a) Typical HPLC chromatogram of a mixture of the nine reference and marker compounds in 50 % aqueous methanol solution



(b) Typical HPLC chromatogram showing reference and marker compounds in S. frutescens plant material

Figure 5.2.1: HPLC chromatograms of reference and marker compounds in (a) 50 % aqueous methanol and (b) plant material, measured at λ = 370 nm. Retention times for rutin, sutherlandin A, sutherlandin B, kaempferol-3-O-rutinoside, sutherlandin C, sutherlandin D, quercitrin, quercetin and kaempferol were 11.9, 12.7, 13.8, 15.3, 16.2, 17.0, 18.0, 26.2 and 28.1 minutes, respectively. Separation of the reference and marker compounds was achieved at 45°C on a Phenomenex Luna® 5 µm C_{18} column with dimensions 250 x 4.6 mm, using gradient elution and a flow rate of 0.8 ml/min. Mobile phases A and B were acetonitrile (0.01 % formic acid) and water (0.01 % formic acid), respectively, injection volume was 20 µL and total run time was 35 minutes.

5.2.2 HPLC assay validation

The following are the results of the HPLC assay validation for flavonoids in solution.

5.2.2.1 Calibration curves and linear concentration range

To determine linearity, various concentrations of the reference compounds were set up and calibration curves analysed. The equations for calibration curve analyses are presented in Table 5.2.1. The (six point calibration) curves for the nine reference compounds showed a linear correlation, i.e. $R^2 > 0.99$, between concentration and peak area of the detector response (Table 5.2.1).

Table 5.2.1: Summary of selected calibration parameters for reference and marker compounds by HPLC-DAD method

Analyte	Regression equation	Linearity range (µg/ml)	R^2	LOD (µg/ml)	LOQ (µg/ml)
Sutherlandin A	y = 22.252x – 9.5909	4.0 to 180.0	0.9998	2	4
Sutherlandin B	y = 14.543x + 13.149	4.0 to 200.0	0.9996	4	4
Sutherlandin C	y = 17.890x + 11.763	4.0 to 200.0	0.9990	4	15
Sutherlandin D	y = 17.45x - 1.5755	4.0 to 180.0	0.9996	2	4
Quercetin	y = 108.51x + 104.95	4.0 to 120.0	0.9967	< 2	9
Kaempferol	y = 91.752x + 140.98	4.0 to 120.0	0.9983	2	9
Rutin	y = 27.88x + 35.835	4.0 to 120.0	0.9988	< 2	15
Quercitrin	y = 27.472x - 12.148	0.4 to 60.0	0.9998	5	10
K-3-*O*-rutinoside	y = 26.729x + 54.301	4.0 to 200.0	0.9991	< 2	9

5.2.2.2 Limit of detection (LOD) and limit of quantitation (LOQ)

For this assay, the limit of detection (LOD) was defined at a signal-to-baseline ratio of 3:1 and the limit of quantitation (LOQ) at a signal-to-baseline ration of at least 10, with acceptable accuracy and precision (< 10 % for each criterion).[205] Values for the LOD and LOQ are presented in Table 5.1.2. The LOD was lowest for quercetin, rutin and kaempferol-3-*O*-rutinoside, and highest for quercetin. The LOQ was lowest for sutherlandins A, B and D, and highest for Sutherlandin C and rutin.

These values showed some similarity to those from another study with different flavonoid compounds.[208] However, they are higher than the results obtained with a few other flavonoid studies,[209,210] possibly due to the wavelength chosen for analysis, 370 nm. This wavelength, though not the absorption maxima for the flavonoids, was chosen in order to enhance selectivity for the flavonoid compounds of interest at the expense of other non-flavonoid compounds that may be present in plant material. The LOD and LOQ values obtained are therefore deemed suitable for this assay. Some other studies on flavonoids in plant material have also reported higher LOD and LOQ values than were obtained in this study.[211,212]

5.2.2.3 Stability

Stability assessment involved acid-base hydrolysis, consecutive freeze-thaw cycles and exposure to ambient light and heat.

5.2.2.3.1 Acid /base hydrolysis

The results of acid-base hydrolysis are presented in Figure 5.1.4. The original chromatogram of each unhydrolysed compound showed only one noticeable peak (in green) in the flavonoid glycoside region for all seven flavonoid glycosides and in the aglycone region for the aglycones. On hydrolysis, the flavonoid glycosides were hydrolysed to the corresponding flavonoid aglycones (in purple), identified by comparison of their retention times and UV spectra with those of reference flavonoid aglycone compounds.

A wide range of flavonoid glycosides are available in nature. Because of their abundance, quantification of each and every flavonoid glycoside in samples will present a Herculean task. These flavonoid glycosides are however derived from a very small number of flavonoid aglycones; therefore, the starting point for most analytical reactions involving flavonoid glycosides is the identification of the flavonoid aglycone. For this, acid is used to cleave the sugar from the aglycone.[107] Clearly, all the flavonoid glycosides of interest in this study are sensitive to acid hydrolysis, confirming their identity as *O*-glycosides. They can thus serve as markers for quality control as their reactions under acid-base conditions can indicate instability of *S. frutescens* products.

As expected, the flavonoid aglycones were not affected by acid hydrolysis. Alkaline hydrolysis of the flavonoid glycosides led to the production of the deacylated flavonoid glycosides (navy traces, Figure 5.2.2).

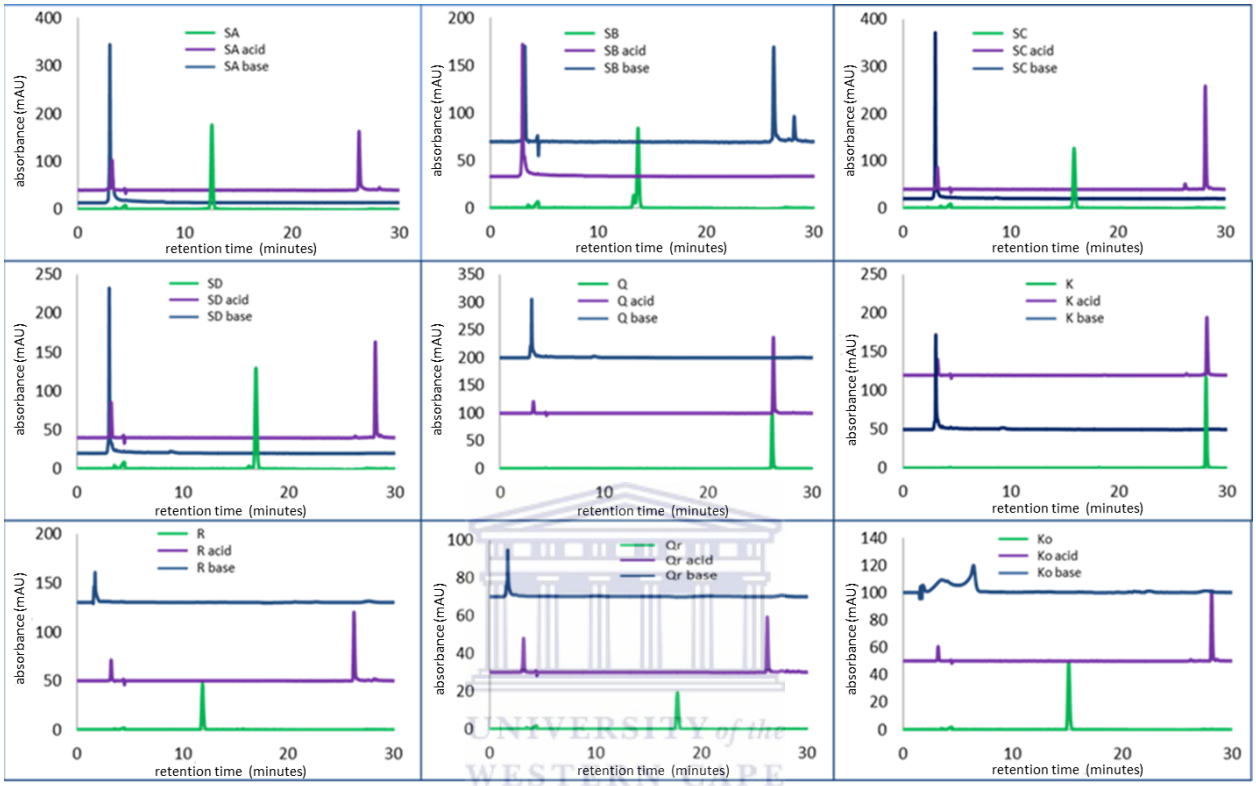


Figure 5.2.2: HPLC chromatograms (at 370 nm) of reference and marker compounds before and after acid or base hydrolysis

KEY: *SA: sutherlandin A* *SD: sutherlandin D* *R: rutin*
SB: sutherlandin B *Q: quercetin* *Qr: quercitrin*
SC: sutherlandin C *K: kaempferol* *Ko: kaempferol-3-O-rutinoside*

5.2.2.3.2 Freeze-thaw cycle

The results of the freeze-thaw cycle after five consecutive freezing (at -80°C) and thawing (at room temperature) exercises are presented in Figure 5.2.3. The reference standards showed relative stability to five successive freezing and thawing cycles, as signified by the retention times and peak sizes before and after the freeze-thaw cycle.

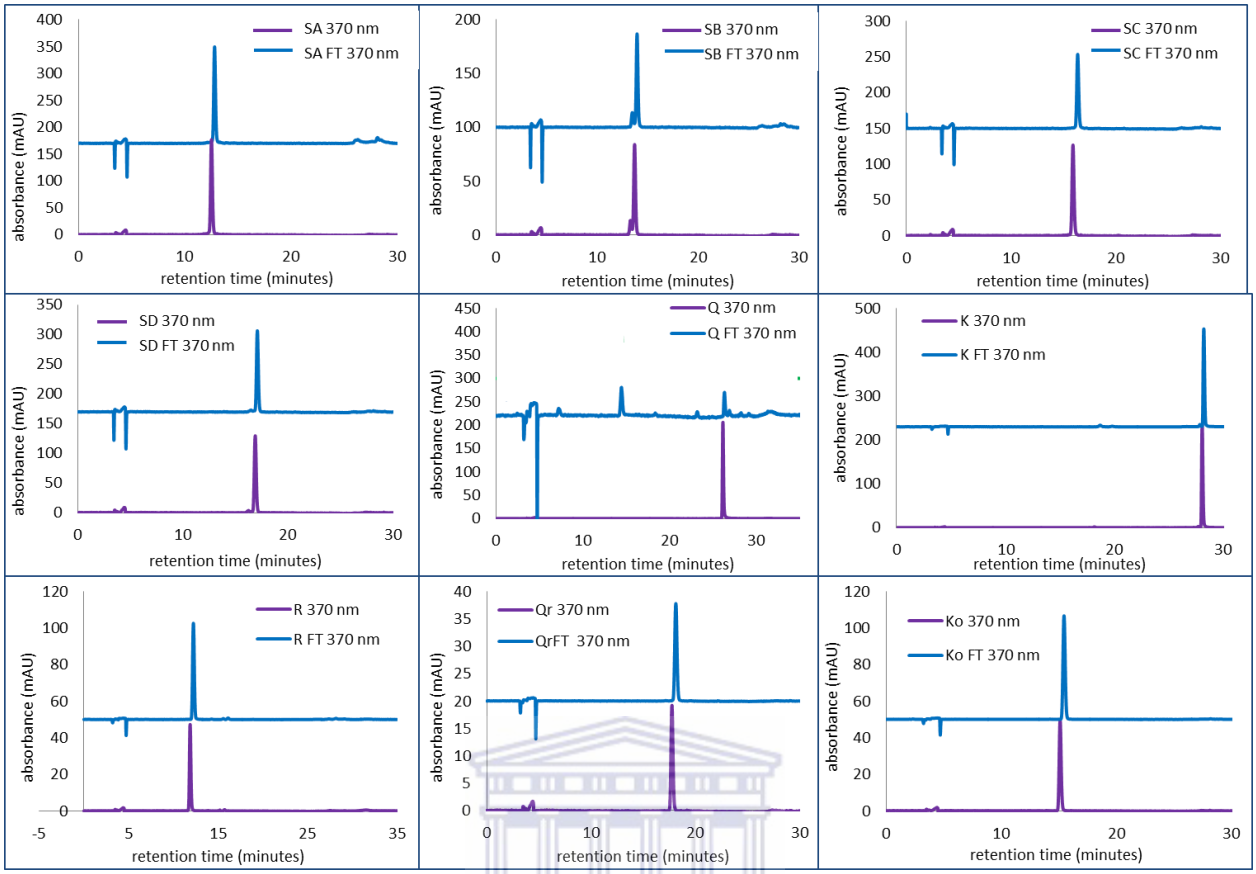


Figure 5.2.3: HPLC chromatograms (at 370 nm) of reference and marker compounds before and after undergoing five consecutive freeze-thaw cycles

KEY: *SA: sutherlandin A* *SB: sutherlandin B* *SC: sutherlandin C* *SD: sutherlandin D* *Q: quercetin* *K: kaempferol* *R: rutin* *Qr: quercitrin* *Ko:kaempferol-3-O-rutinoside*

An exception was quercetin, exhibiting instability, shown by decrease in peak size, and formation of other products. This was in contrast to previous studies that demonstrated quercetin stability to freeze-thaw cycles, though the said studies evaluated quercetin stability in biological fluids,[213,214] and not in aqueous-based solutions as presented here. Since this study utilized plant samples containing mostly flavonoid glycosides, the samples can be stored for prolonged periods in the freezer prior to analysis as their stability on thawing has been demonstrated. This however may not be ideal if quercetin is to be used as a marker as it is prone to instability under freeze-thaw conditions as investigated herein, and may also be formed in cases of product instability.

5.2.2.3.3 Ambient light exposure

HPLC chromatograms of samples subjected to sunlight exposure for one month are shown in Figure 5.2.4. The traces indicate that the flavonoid glycosides (sutherlandins A, B, C and D) showed relatively good stability with respect to the retention times (i.e. still eluted at previous retention times observed prior to sunlight exposure). However, the peak response for all seven glycosides decreased to between 45 and 97 % of the original.

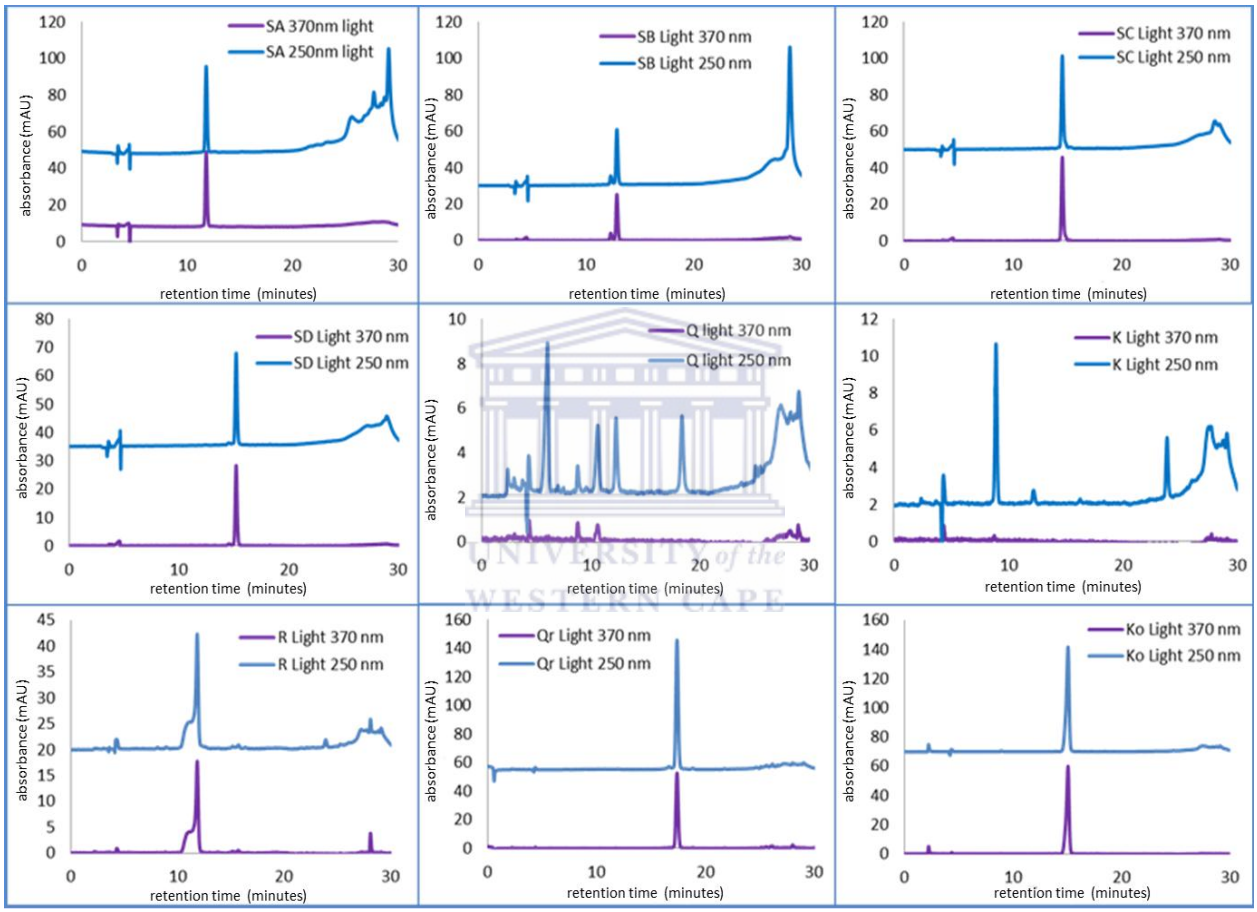


Figure 5.2.4: HPLC chromatograms (at 370 and 250 nm) of reference and marker compounds after sunlight exposure for one month

SA: sutherlandin A	*SD: sutherlandin D*	*R: rutin*
SB: sutherlandin B	*Q: quercetin*	*Qr: quercitrin*
SC: sutherlandin C	*K: kaempferol*	*Ko: kaempferol-3-O-rutinoside*

The flavonoid aglycones on the other hand were not stable, and additional peaks representing other compounds were observed. These other compounds were probably degradation products of the aglycones, and were observed in the chromatograms for

quercetin and kaempferol. The actual peaks representing quercetin and kaempferol were however not detected after light exposure (Figure 5.2.4), implying that these compounds had been degraded, possibly due to photo-oxidation.[215] The stability of flavonoids to light is known to depend on the nature of the hydroxyl group attached to C_3 of the flavonoid structure (please see Figure 2.2, Chapter 2), where the absence or glycosylation of the hydroxyl group confers some degree of stability not seen in flavonoid molecules with a hydroxyl group at C_3 as is the case with quercetin and kaempferol.[216] The seven flavonoid glycosides under study are all glycosylated at position C_3 (please refer to flavonoid structures in Figure 2.2, Chapter 2), conferring on them some degree of photostability not obtained with the non-glycosylated flavonoid aglycones, quercetin and kaempferol. This may explain why the flavonoid glycosides, though affected by ambient light exposure which served to reduce the peak response, were affected to a lesser degree than the flavonoid aglycones of lower photostability due to the presence of a free hydroxyl group at C_3. The 'lower-stability' flavonoid aglycones underwent degradation to such an extent that their peaks were not detected in the chromatograms obtained following ambient light exposure. The lower phtostability of the flavonoid aglycones is ascribed to a greater triplet state population *cum* higher singlet oxygen reactivity.[216]

All the compounds exhibited instability on exposure to light; ideally, samples containing these compounds should not be exposed to ambient light conditions as tested here. This also shows that the flavonoid compounds are susceptible to light and can therefore be used as markers to monitor quality and stability of *S. frutescens* products exposed to light.

5.2.2.3.4 Heat exposure

After exposure to heat (for 3 hours at 60°C), all the flavonoid glycosides and aglycones were still observed at their respective retention times (Figure 5.2.5), indicating that heat did not cause exhaustive degradation of the compounds. The peak response was however reduced to between 4 and 84 % of the original for the flavonoid glycosides and aglycones under study. This is in line with a study by Manach *et al.* [217] which reported a 60 % loss of quercetin in apple juice stored for 9 months at 25°C.

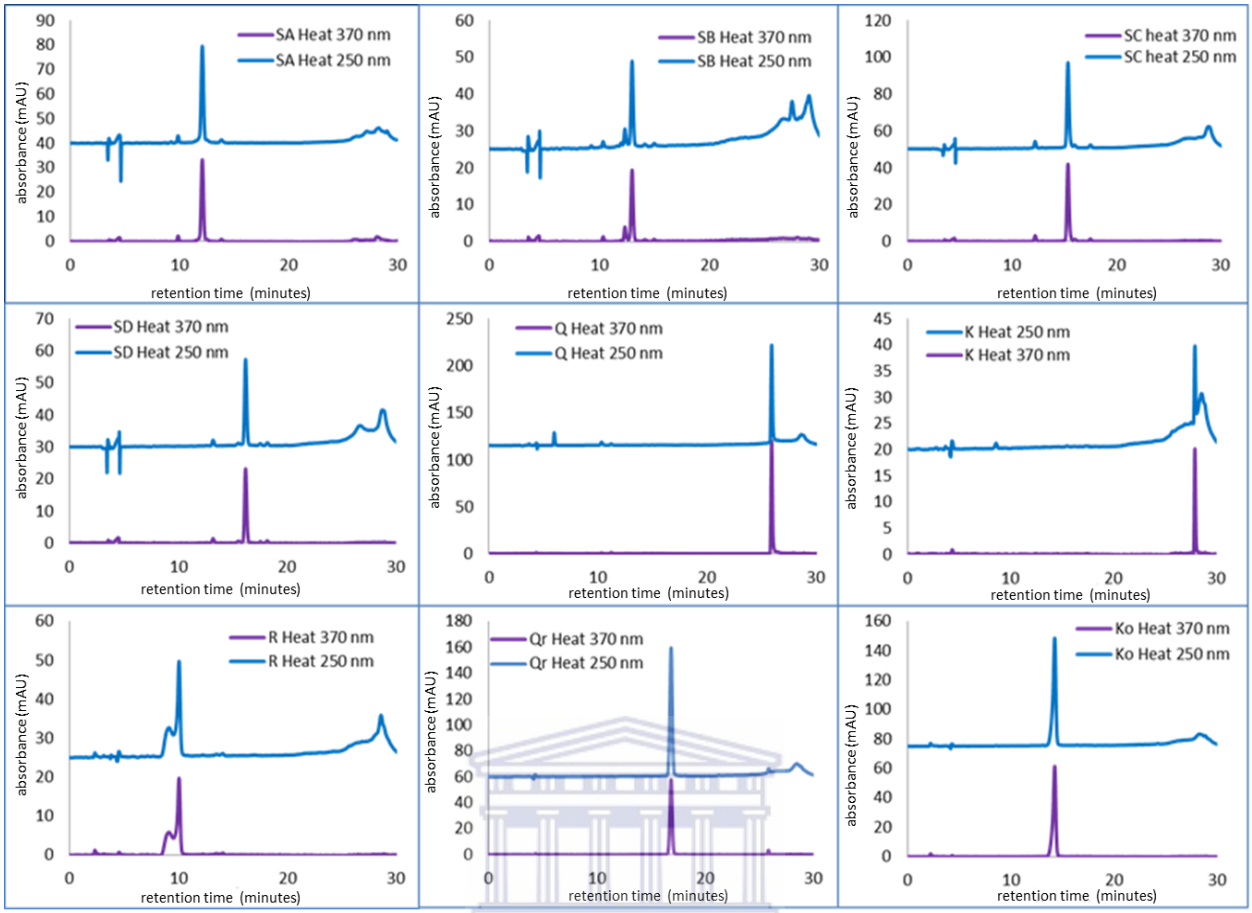


Figure 5.2.5: HPLC chromatograms (at 370 and 250 nm) of reference and marker compounds after heat exposure for 3 hours at 60°C

SA: sutherlandin A	*SD: sutherlandin D*	*R: rutin*
SB: sutherlandin B	*Q: quercetin*	*Qr: quercitrin*
SC: sutherlandin C	*K: kaempferol*	*Ko: kaempferol-3-O-rutinoside*

The flavonoid glycoside, kaempferol-3-*O*-rutinoside, where peak response increased to 106.8 % after heat exposure was an exception from the other flavonoids in the group, though such increase can also be viewed as being due to some form of instability or an indication of compromised quality. Ideally, samples containing these compounds should not be exposed to heat conditions as tested here. The flavonoid compounds, having shown susceptibility to heat, can therefore be used as markers for quality and stability assessment of *S. frutescens* products exposed to heat.

5.2.2.4 Precision and accuracy

Precision and accuracy data for the reference and marker compounds are shown in Tables 5.2.2 and 5.2.3. The RSD for the compounds ranged from 1.01 to 5.75 % but were less than 3 % for most of the compounds (Table 5.2.2). Precision data less than 5 % RSD is acceptable,[218] more so for herbal materials where variation in actives may be quite considerable.[24,186] This suggests that the proposed method is sensitive enough for the assay of flavonoid glycosides in *S. frutescens*. Results of intra-day and inter-day assays were consistent, with RSD less than 1 % for all reference and marker compounds (Table 5.2.3). The precision and accuracy data are therefore within acceptable limits for *S. frutescens* assay.

Table 5.2.2: Precision and accuracy data for quantification of reference and marker compounds (λ = 370 nm)

	Reference compound	**% RSD**
Lower conc. (µg/mL): 15 µg/mL	Sutherlandin A	2.01
Higher conc. (µg/mL): 33 µg/mL	Sutherlandin A	2.54
Lower conc. (µg/mL): 15 µg/mL	Sutherlandin B	5.75
Higher conc. (µg/mL): 35 µg/mL	Sutherlandin B	2.83
Lower conc. (µg/mL): 15 µg/mL	Sutherlandin C	3.80
Higher conc. (µg/mL): 35 µg/mL	Sutherlandin C	2.14
Lower conc. (µg/mL): 15 µg/mL	Sutherlandin D	2.36
Higher conc. (µg/mL): 33 µg/mL	Sutherlandin D	2.29
Lower conc. (µg/mL): 8 µg/mL	Quercetin	2.84
Higher conc. (µg/mL): 35 µg/mL	Quercetin	1.28
Lower conc. (µg/mL): 9 µg/mL	Kaempferol	2.13
Higher conc. (µg/mL): 35 µg/mL	Kaempferol	0.85
Lower conc. (µg/mL): 9 µg/mL	Rutin	2.39
Higher conc. (µg/mL): 35 µg/mL	Rutin	2.21
Lower conc. (µg/mL): 9 µg/mL	Quercitrin	2.01
Higher conc. (µg/mL): 33 µg/mL	Quercitrin	1.01
Lower conc. (µg/mL): 9 µg/mL	Kaempferol-3-*O*-rutinoside	3.20
Higher conc. (µg/mL): 35 µg/mL	Kaempferol-3-*O*-rutinoside	1.63

Table 5.2.3: Intra- and inter-day precision analyses for reference and marker compounds (λ = 370 nm)

Reference/ marker compound	Ave	% RSD	Intra-day (Ave)	Intra-day (% RSD)	Inter-day (Ave)	Inter-day (% RSD)
Sutherlandin A	1325.99	0.998	1341.60	0.061	1328.03	0.442
Sutherlandin B	916.62	0.442	933.47	0.469	909.18	0.818
Sutherlandin C	1167.74	0.111	1130.98	0.452	1136.91	0.629
Sutherlandin D	1085.46	0.205	1087.62	0.056	1087.89	0.959
Quercetin	7134.14	0.146	7063.00	0.433	6987.52	0.327
Kaempferol	5952.61	0.242	5967.14	0.090	5989.19	0.494
Rutin	1836.46	0.738	1861.07	0.161	1853.79	0.355
Quercitrin	1646.19	0.045	1659.16	0.048	1662.54	0.457
Kaempferol-3-o-rutinoside	1759.65	0.019	1761.84	0.204	1782.92	0.502

'Ave' represents average peak area in mAU

5.2.2.5 Robustness and ruggedness freezer

Evaluation of method robustness was considered when the suitability of the mobile phase was assessed. The robustness and ruggedness of the method were investigated by varying chromatographic parameters such as the flow rate and temperature, and the results obtained are given in Figures 5.2.6 and 5.2.7.

A decrease in the flow rate of the mobile phase (from 0.8 to 0.5 ml/minute) resulted in an increase in the retention time for all the reference compounds (Figure 5.2.6). A decrease in flow rate generally increases the retention time of eluting compounds. In this case, the increase in retention time was significant. A significant increase in retention times may result in peak broadening, and lead to compromised chromatographic output. While a faster flow rate generally results in decreased retention times and sharper peaks for the eluted compounds, this is not always the case. Faster is not always better; a faster flow rate may result in insufficient time for the analyte to interact with the stationary phase, and so the best (optimal) flow rate for optimal separation of the analytes of interest is usually sought. For this study, the optimal flow rate was 0.8 ml/minute.

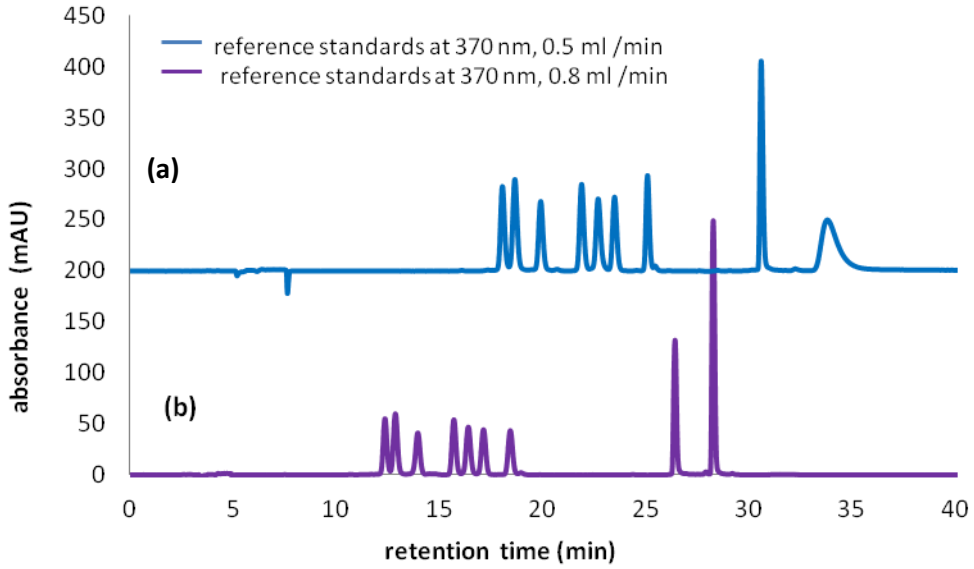


Figure 5.2.6: HPLC chromatograms for a mixture of the nine reference and marker compounds, demonstrating the effect of variation in flow rate: (a) 0.5 ml/min (b) 0.8 ml/min

As shown in Figure 5.2.7 below, changing the temperature of the column compartment from 40°C to room temperature did not cause significant changes in the peak sizes and retention times, showing that the developed method is robust to temperature changes.

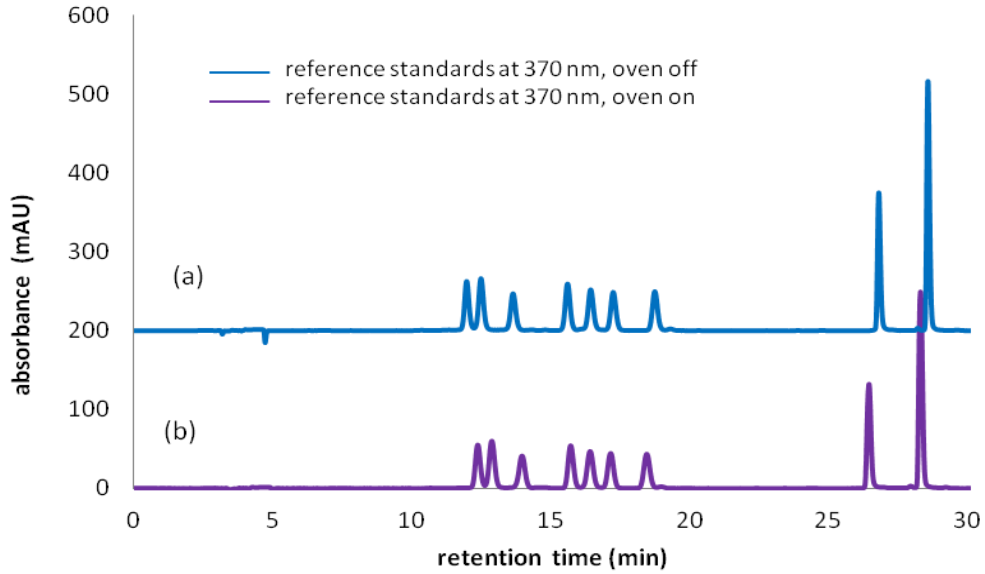


Figure 5.2.7: HPLC chromatograms for a mixture of the nine reference and marker compounds demonstrating the effect of variation in column temperature

5.2.2.6 System suitability testing

The Agilent ChemStation® software was used to calculate system suitability parameters and results obtained are summarized in Table 5.2.4. Values of the capacity factor (k') ranged from 30 to 71, resolution (R_s) from 49 to 245 and tailing factor (T) from 1.0574 to 1.2563. The suitability of the chromatographic system in terms of column efficiency, resolution and precision, for flavonoid analysis in *S. frutescens* is therefore assured.

Table 5.2.4: HPLC retention times and selected system suitability parameters for the reference and marker compounds

Compound	**k'**	**R_s**	**T**
Sutherlandin A (SA)	31.5870	81.594	1.0714
Sutherlandin B (SB)	34.4686	83.906	1.0574
Sutherlandin C (SC)	40.6013	61.483	1.1421
Sutherlandin D (SD)	42.6056	48.999	1.0843
Quercetin (Q)	66.3686	244.577	1.1981
Kaempferol (K)	71.2636	-	1.2563
Rutin (R)	29.7370	85.084	1.1117
Kaempferol-3-*O*-rutinoside (Ko)	38.3600	97.067	1.1065
Quercitrin (Qr)	45.3070	97.910	1.0903

5.2.3 Conclusions

To date, analysis of flavonoid glycosides, together with their corresponding flavonoid aglycones, which can be used for the assay of *S. frutescens* materials, has not yet been investigated. There are many flavonoids in this plant which may be used for development of its HPLC assay. For this assay, we chose nine flavonoids (seven flavonoid glycosides and two corresponding flavonoid aglycones) which were all found to be suitable for the assay. The marker and reference compounds were well separated, resulting in good separation of component flavonoids in plant materials. The developed assay employed a combination of isocratic and gradient elution systems, with DAD-detection, and provided a simple method with relatively good linearity, accuracy and robustness, which can be suitably employed for the assay of *S. frutescens* materials.

5.3 Profiles and levels of flavonoid compounds in different *S. frutescens* materials

For this section of the study, a validated HPLC method was employed for the analysis of reference and marker compounds in samples of different *S. frutescens* materials. The chromatograms obtained were analysed for the presence of any flavonoid compounds and specifically the marker compounds, sutherlandins A, B, C and D, in different *S. frutescens* materials *viz* LP, SDAE and FDAE.

5.3.1 Quantification of reference compounds in plant materials

The HPLC separation of reference and marker compounds in the different *S. frutescens* materials analysed is presented in Figure 5.3.1. The flavonoid glycosides, sutherlandins A, B, C and D, represented by peaks 1, 2, 3 and 4, respectively, were detected in all the samples assayed. The flavonoid aglycones, quercetin and kaempferol, represented by peaks 5 and 6, respectively, were only detected in two of the FDAE materials and not in the LP or SDAE materials. Three of the nine compounds, rutin, quercitrin and kaempferol-3-*O*-rutinoside were not conclusively identified in the plant materials assayed. While peaks with similar retention times and UV-spectra as these three compounds were detected in some of the *S. frutescens* materials assayed, they were not isolated and so their identities could not be conclusively confirmed by further analytical techniques.

The percentage content (w/w) of the reference and marker compounds detected in the different *S. frutescens* materials is shown in Table 5.3.1. The average peak area was used to quantify each compound in each of the five *S. frutescens* materials. Of the two possible forms in which the flavonoids can exist in plant materials (glycoside and aglycone), the flavonoid glycosides were more abundant than the flavonoid aglycones in each of the assayed materials, in support of our first hypothesis (Section 3.3).

The flavonoid aglycone content (0.01 to 0.21 %) was found to be lower than that of the flavonoid glycosides (0.07 to 1.58 %), and in some cases, below the quantification limit. The freeze-dried extracts contained 0.01 to 0.20 % of the flavonoid aglycones, while these compounds were not detected in either the LP or SDAE samples. This suggests that the flavonoid aglycone content may be increased by processing parameters, such as is used in

the preparation of the freeze-dried extracts. However, no collaboration of this was found in existing literature.

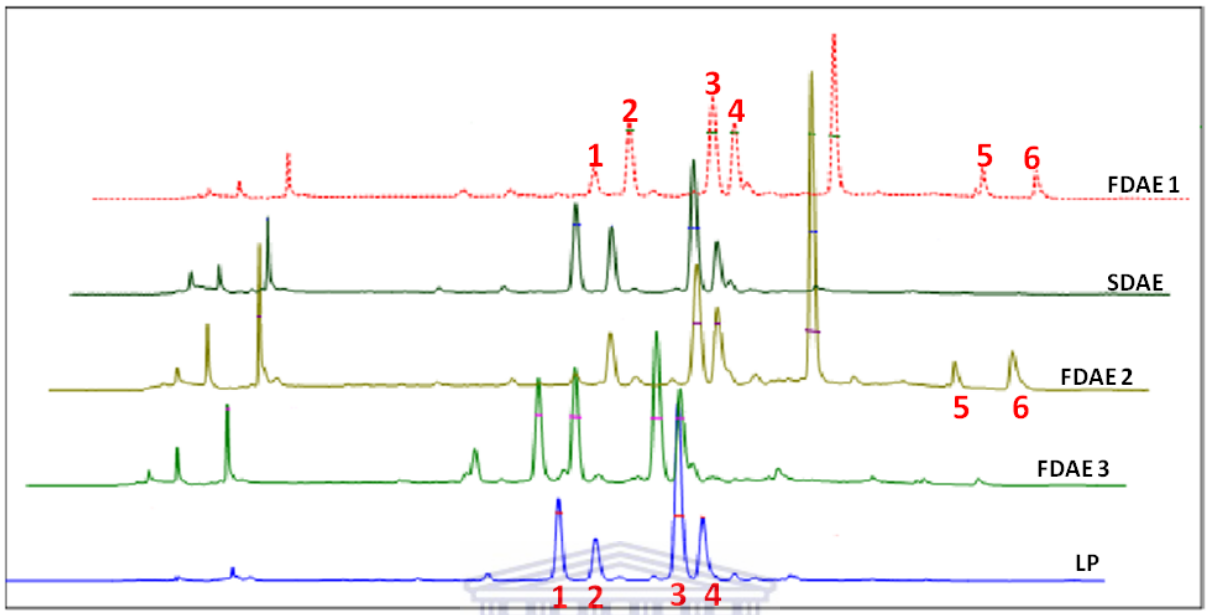


Figure 5.3.1: HPLC chromatograms of the five S. frutescens materials in 50 % aqueous methanol solution. The numbers 1 to 6 represent sutherlandin A, sutherlandin B, sutherlandin C, sutherlandin D, quercetin and kaempferol, respectively.

In all the plant materials assayed, sutherlandin C was found to have the highest abundance, and so can be assayed for in all the *S. frutescens* materials. The least abundant flavonoid glycoside was not the same in all the plant samples assayed, with different materials showing non-uniformity in the flavonoid of least abundance. In the LP and SDAE materials, the flavonoid glycosides of least abundance were sutherlandin B and sutherlandin D, respectively, while in all the freeze-dried materials, the flavonoid glycoside of least abundance was sutherlandin A.

Neither the LP nor SDAE material contained detectable levels of the aglycones, quercetin and kaempferol. In the freeze-dried materials, kaempferol content was more than quercetin content for FDAE 1 and FDAE 3, and below quantifiable limits for FDAE 2. The sum of the analysed flavonoids in the plant materials exceeds that obtained in other studies of total flavonoids[219,220,221] and suggests that the flavonoid content in the *S. frutescens* materials is sufficient for flavonoid use as marker compounds. Overall, the levels of the flavonoid

glycosides and aglycones in the different *S. frutescens* materials assessed varied significantly ($p < 0.001$). The assessment of flavonoid glycoside (sutherlandin A, B, C and D) content can therefore be used to differentiate between *S. frutescens* products.

Table 5.3.1: *Percentage (w/w) of reference and marker compounds found in 50 % aqueous methanol extracts of the different S. frutescens materials. Results are presented as percentage content (w/w)*

Reference compound	**LP**	**SDAE**	**FDAE 1**	**FDAE 2**	**FDAE 3**
SA	0.526 ± 0.005	0.579 ± 0.011	0.200 ± 0.002	0.647 ± 0.022	0.072 ± 0.001
SB	0.450 ± 0.034	0.597 ± 0.006	0.760 ± 0.005	1.004 ± 0.018	0.560 ± 0.002
SC	1.576 ± 0.009	1.204 ± 0.002	1.084 ± 0.000	1.343 ± 0.032	1.110 ± 0.013
SD	0.513 ± 0.018	0.442 ± 0.001	0.701 ± 0.018	0.777 ± 0.036	0.662 ± 0.020
Q	ND	ND	0.024 ± 0.000	< LOQ	0.014 ± 0.000
K	ND	ND	0.210 ±0.000	< LOQ	0.055 ± 0.000

Each value is expressed as average percentage content (mg flavonoid x minimum purity of flavonoid reference*/mg plant material) ± SD (n = 3). Means within a row for each flavonoid compound are significantly different (p < 0.001).*

Key: *SA: sutherlandin A* *SB: sutherlandin B* *ND: not detected* *SC: sutherlandin C* *SD: sutherlandin D* *< LOQ: less than LOQ* *Q: quercetin* *K: kaempferol*

In addition, the levels of the flavonoid aglycones (quercetin and kaempferol) in the analysed samples were quite low and in some cases, absent, thus making quantification of these compounds a challenge. The ratio of the flavonoid glycosides to the total flavonoids (glycoside + aglycone) was at least 90 % in the samples studied. Therefore, the use of the flavonoid aglycones (and not the glycosides) as markers for quality control and stability assessment of *S. frutescens* LP, SDAE or FDAE materials may not be justifiable or feasible using the specific assay employed in the present study. This however, can be expected in samples extracted using more polar solvents like methanol and water. It may well be that extraction with less polar solvents would yield more of the aglycones than the glycosides.

5.3.2 Conclusions

The validated HPLC-DAD method previously reported in Section 5.2 was found to be appropriate for separation and quantification of flavonoid glycosides and their corresponding aglycones in different formulations of *S. frutescens*. The LP, SDAE and FDAE materials all contained all four marker compounds, i.e. sutherlandins A, B, C and D, with sutherlandin C being the most abundant in all the five materials. The two flavonoid aglycones, quercetin and kaempferol, were detected in two of the FDAE materials, and not in the LP or SDAE material. There were more flavonoid glycosides than aglycones in all the plant materials, suggesting that the former may be more suitable as marker compounds. The use of the flavonoid glycosides as markers is therefore recommended for assessment of *S. frutescens* materials.

The aglycones can also be used as marker compounds because their presence, as products of flavonoid glycoside breakdown, can indicate instability of the plant materials. However, use of the flavonoid aglycones levels alone, without the flavonoid glycosides levels as well, is not recommended. Assay of all six flavonoids employed here is preferred to the assay of a single flavonoid as it gives the profiles and levels of the flavoniods in the different materials. This in turn can indicate quality status of different *S. frutescens* products.

5.4 Comparison of flavonoids dissolution in *S. frutescens* materials

The objective of this section was to assess if dissolution studies of *S. frutescens* can be done, how they can be done and the suitability of dissolution testing for assessment and comparison of *S. frutescens* materials. The USP basket method was used with modifications, and dissolution profiles of the marker compounds were compared using standard and modified methods.

5.4.1 Characteristics of *S. frutescens* materials and packed capsules used for analysis

For this study, the materials investigated were *S. frutescens* LP, SDAE and FDAE packaged in capsules. The *S. frutescens* materials (LP, SDAE and FDAE) complied with set specifications such as plant material appearance, odour and taste, foreign matter, moisture and microbiology at the time of delivery. Details of their specifications are presented in the Certificates of Analyses (CoA) in Appendix 1. The LP was a coarsely-milled, free-flowing, light green powder with light green stalk bits. The extracts (SDAE and FDAE), of a smaller particle size than the LP, were homogenous and hygrocopic materials. The SDAE was a light brown colour while the FDAE was a darker shade of brown.

Three of the five *S. frutescens* materials used for quantification studies in Section 5.3 *viz* LP, SDAE and FDAE 1, were selected for dissolution tests of *S. frutescens* materials. The three materials were filled into size *0* capsules, which were weighed before and after filling in order to assess uniformity of weight. The weights obtained for the LP, SDAE and FDAE materials were 322.5 ± 8.7 mg, 324.9 ± 7.6 mg and 322.5 ± 8.5 mg, respectively, which complied with the USP requirements for weight uniformity, i.e. between 85 and 115 % of the mean.

5.4.2 Validation of dissolution method

To validate the dissolution method as a category III procedure, i.e. analytical procedure for determination of performance characteristics, six individual samples (six replicates, each containing the SDAE material) collected from the dissolution apparatus at a time of 60 minutes (pH 1.2) were analysed to validate method precision by repeatability, and the values expressed as relative standard deviation (RSD) between samples.[206] For sutherlandins A, B and C, the RSD values were 4.4, 4.8 and 2.7 % respectively, all within specified criterion

of ≤ 5 %.[218] This suggests that the dissolution method described herein can be used for dissolution of sutherlandins A, B and C from different plant materials. The RSD for sutherlandin D was however 17.0 %, outside the specification limits, possibly due to degradation of this marker compound at different rates in the different vessels containing the acidic dissolution medium (please see Appendix 4, Figure A 4.2, pH 1.2: SDAE). This suggests that sutherlandin D may not be a good marker compound for analysis of plant samples at pH 1.2. Nevertheless, this does not necessarily negate its use for assessment at other pH conditions.

5.4.3 Dissolution of flavoniod compounds from *S. frutescens* materials

The Q-release values for flavonoids dissolution from different *S. frutescens* materials are presented in Tables 5.4.1 to 5.4.6. Although there are no pharmacoepoeial specifications for dissolution of *S. frutescens* materials, the acceptance criterion employed for this study was arbitrarily set at a minimum of 75 % flavonoid dissolution in 45 minutes. For comparison using the similarity factor (original similarity factor and its modifications), the acceptance criterion was f_2 < 50. Following are the results of Q-release and dissolution profiles comparison for flavonoids from *S. frutescens* LP, SDAE and FDAE at different pH conditions. In the figures and tables, '*NA*' refers to not available, 'f_2' is the original similarity factor, '*wt* f_2' refers to the similarity factor modified to allow for impact of within batch variability on f_2, and *'f_2-median'* refers to the similarity factor calculated using median values of the percentages dissolved at each sampling point.

5.4.3.1 Dissolution profiles of sutherlandin A

The dissolution profile of sutherlandin A from different *S. frutescens* materials, at three pH conditions, is shown in Figure 5.4.1. A perusal of the profiles at pH 1.2 shows that the dissolution curves for sutherlandin A are different from the three different materials at each specific pH, suggesting that release of this marker compound is different from all three materials.

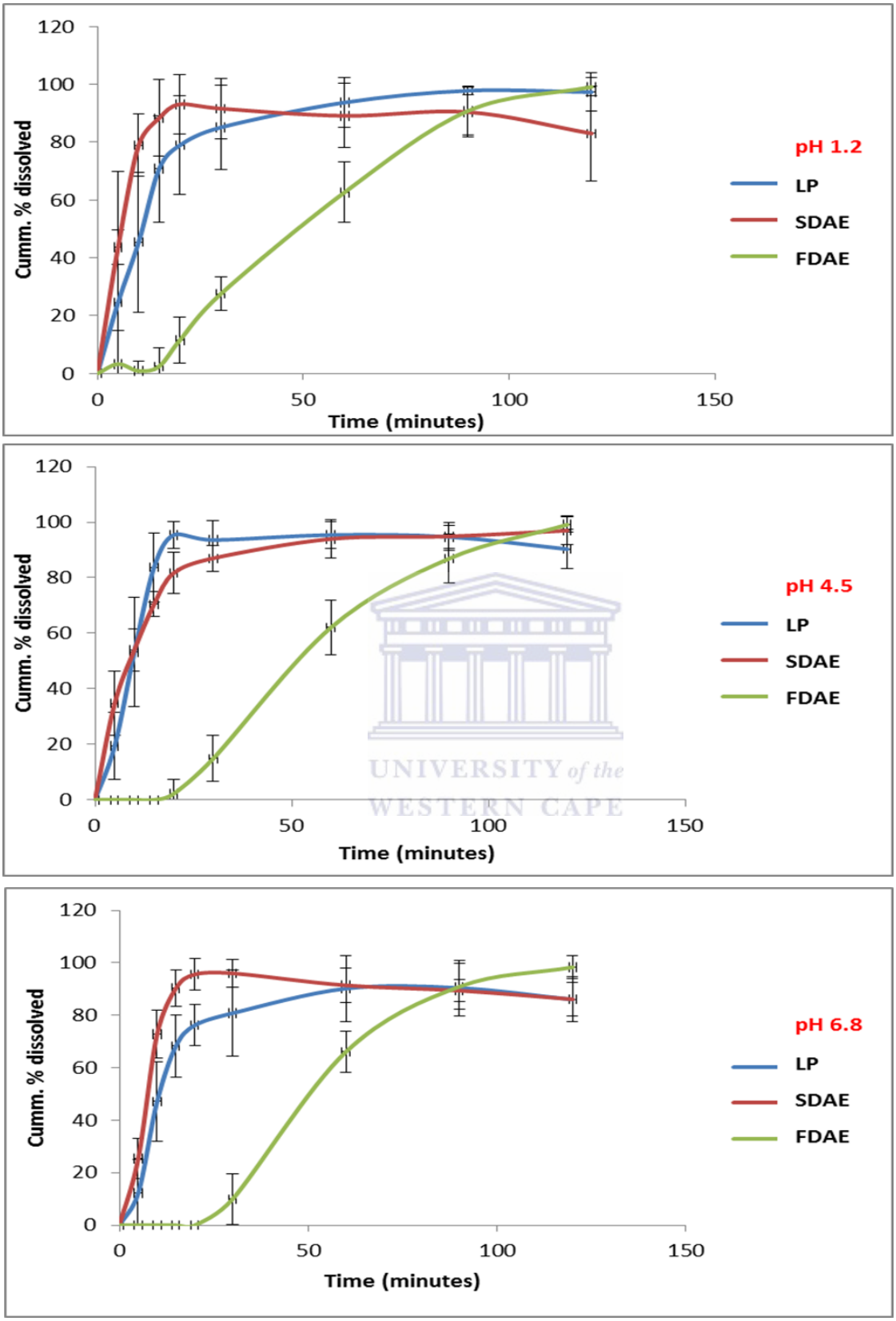


Figure 5.4.1: Dissolution profiles of sutherlandin A from different S. frutescens materials at pH 1.2, 4.5 and 6.8. Data is presented as mean ± SD

The Q-values for 75 % and 85 % release were attained at different times from the three materials (Table 5.4.1), implying different dissolution characteristics. Dissolution from the LP and SDAE materials were immediate while dissolution of the marker from the FDAE displayed a lag period of about 15 minutes. This trend was also noticed at pH 4.5 and 6.8; the dissolution profiles for sutherlandin A were all different as judged by the shape of the curves and the Q-75 and Q-85 release values.

The dissolution profiles of sutherlandin A from all three materials were compared using the original f_2 equation. The results obtained for comparison of any two of the three materials were all below 50, indicating dissimilarity of all profiles, therefore implying differences in the dissolution of the different materials (Table 5.4.2).

Table 5.4.1: Classification of sutherlandin A in S. frutescens materials by dissolution rate

	Plant material & pH		Q = 75 % (minutes)	Dissolution characteristic	Q = 85 % (minutes)	Dissolution characteristic
Suth A	1.2	**LP**	45	Immediate release	60	Delayed dissolution
		SDAE	10	Immediate release	20	Rapidly dissolving
		FDAE	90	Not immediate release	90	Delayed dissolution
	4.5	**LP**	20	Immediate release	30	Rapidly dissolving
		SDAE	15	Immediate release	20	Rapidly dissolving
		FDAE	90	Not immediate release	90	Delayed dissolution
	6.8	**LP**	45	Immediate release	120	Delayed dissolution
		SDAE	15	Immediate release	15	Very rapidly dissolving
		FDAE	90	Not immediate release	90	Delayed dissolution

The variances obtained with the dissolution data were however beyond that stipulated for use of the original f_2 equation and therefore, dissolution data were also compared using Gohel's modified f_2 and the median f_2. Comparison of sutherlandin A dissolution profiles at pH 4.5 and 6.8 for LP *versus* SDAE, LP *versus* FDAE and SDAE *versus* FDAE materials gave

original and modified f_2 values as shown in Table 5.4.2. The results confirm dissimilarity of sutherlandin A dissolution from all three *S. frutescens* materials, i.e. $f_2 < 50$.

Table 5.4.2: Comparison of f_2-values for sutherlandin A dissolution profiles at pH 1.2, 4.5 and 6.8

(a) LP (reference) versus SDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	34.7	22.3	32.8
4.5	47.2	33.6	43.3
6.8	37.3	24.3	36.7

(b) LP versus FDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	16.4	-9.2	15.4
4.5	12.7	-12.2	12.5
6.8	35.6	18.0	14.1

(c) SDAE versus FDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	11.6	-15.7	9.8
4.5	10.6	-15.3	10.2
6.8	8.7	-5.9	8.5

The dissolution of sutherlandin A is therefore clearly different from the three materials at pH 1.2, 4.5 and 6.8. As such, the dissolution test can be used for quality control of *S. frutescens* materials at any of the three pH conditions as it can distinguish between different materials.

5.4.3.2 Dissolution profiles of sutherlandin B

The dissolution profiles of sutherlandin B from different *S. frutescens* materials, at three different pH conditions, are shown in Figure 5.4.2.

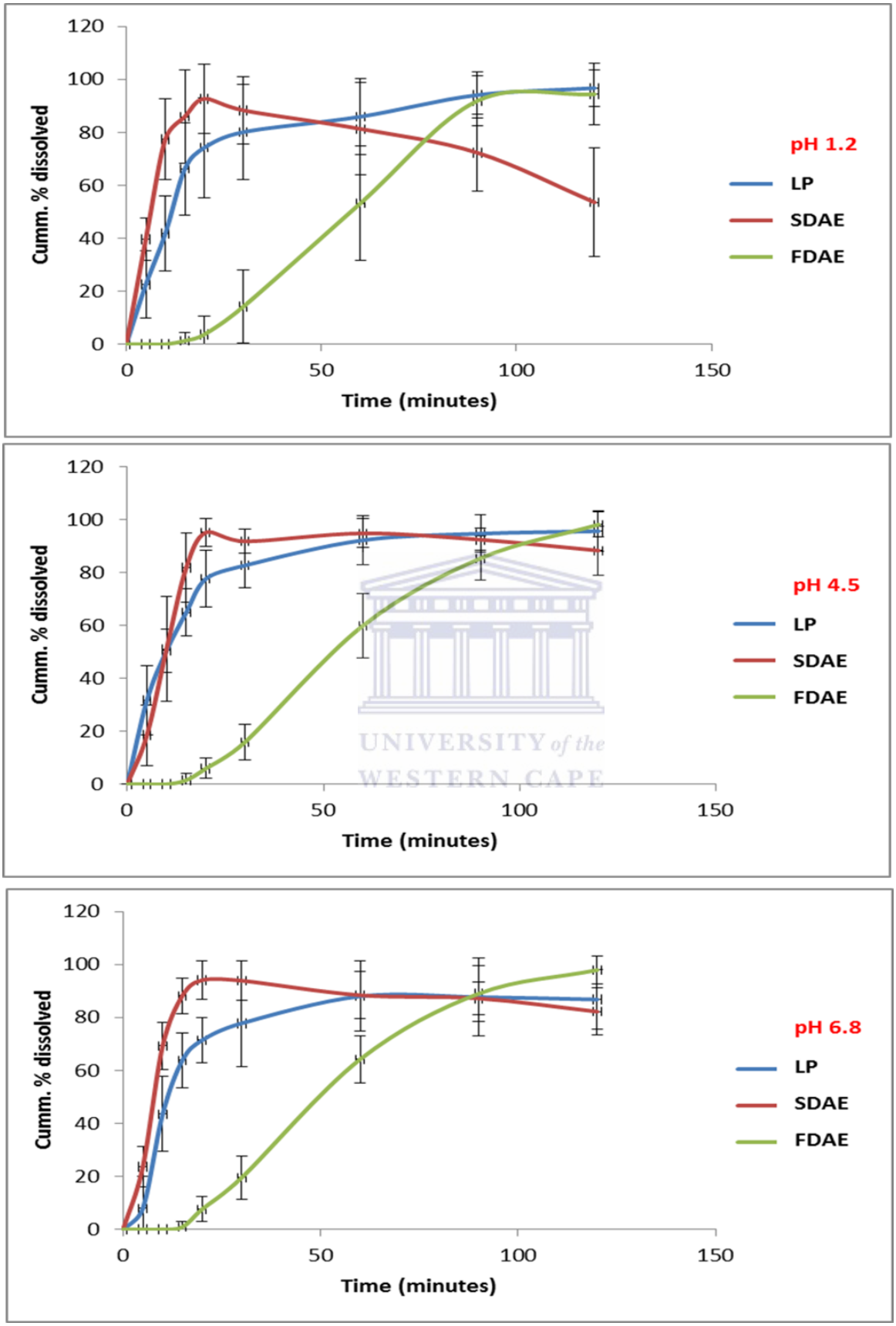


Figure 5.4.2: Dissolution profiles of sutherlandin B from different S. frutescens materials at pH 1.2, 4.5 and 6.8. Data is presented as mean ± SD

The shapes of the profiles show differences in sutherlandin B dissolution from the different materials at the three pH conditions. However, dissolution characteristics overlapped for some of the materials; the times to attain Q-values of 75 % and 85 % were the same for the LP and FDAE materials at pH 1.2, but differed for all three materials at pH 4.5 and 6.8 (Table 5.4.3). This implies that Q-release values of sutherlandin B may not be ideal for distinguishing between the *S. frutescens* materials at pH 1.2, though the reverse may well be obtained at pH 4.5 and 6.8.

Table 5.4.3: Classification of sutherlandin B in S. frutescens materials by dissolution rate

	Plant material & pH		Q = 75 % (minutes)	Dissolution characteristic	Q = 85 % (minutes)	Dissolution characteristic
Suth B	1.2	**LP**	90	Not immediate release	90	Delayed dissolution
		SDAE	20	Immediate release	> 120	Delayed dissolution
		FDAE	90	Not immediate release	90	Delayed dissolution
	4.5	**LP**	30	Immediate release	45	Delayed dissolution
		SDAE	15	Immediate release	20	Rapidly dissolving
		FDAE	90	Not immediate release	90	Delayed dissolution
	6.8	**LP**	45	Immediate release	45	Delayed dissolution
		SDAE	15	Immediate release	15	Very rapidly dissolving
		FDAE	90	Not immediate release	120	Delayed dissolution

From the SDAE, the dissolution curve of this marker compound displayed a downward trend after peak dissolution, which was not observed with the LP or FDAE materials. The downward trend may possibly be due to degradation of this marker compound in the SDAE material at pH 1.2. As was obtained with sutherlandin A, dissolution of sutherlandin B from the FDAE showed a lag period, with Q-75 % not attained till the 90-minute sampling point.

Comparison of dissolution profiles of sutherlandin B from any two of the three materials gave f_2 values less than 50 for all combinations (Table 5.4.4), indicating that the dissolution profiles of sutherlandin B from all three materials were different. While Q-values may not

differentiate between materials at pH 1.2, values of f_2 and its modifications can be employed to differentiate between the *S. frutescens* materials at any of the three pH conditions assessed.

In addition, for differentiation between *S. frutescens* materials using sutherlandin B as a marker compound, the Q-values at pH 4.5 and 6.8 are preferred to the Q-value at pH 1.2 as the latter may not be able to differentiate between the LP and the SDAE materials.

Table 5.4.4: Comparison of f_2-values for sutherlandin B dissolution profiles at pH 1.2, 4.5 and 6.8

(a) LP (reference) versus SDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	35.3	19.2	31.1
4.5	46.1	32.9	44.6
6.8	35.4	21.1	35.3

(b) LP versus FDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	15.5	-12.6	16.1
4.5	14.6	-6.7	15.2
6.8	38.5	-6.0	17.0

(c) SDAE versus FDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	10.6	-16.3	9.0
4.5	11.5	-10.4	12.6
6.8	10.4	-11.7	10.5

5.4.3.3 Dissolution profiles of sutherlandin C

The dissolution profiles of sutherlandin C from different *S. frutescens* materials, at three different pH conditions, are shown in Figure 5.4.3. As with dissolution of sutherlandins A

and B, the shapes of the profiles looked different for the three materials at each of the three pH conditions.

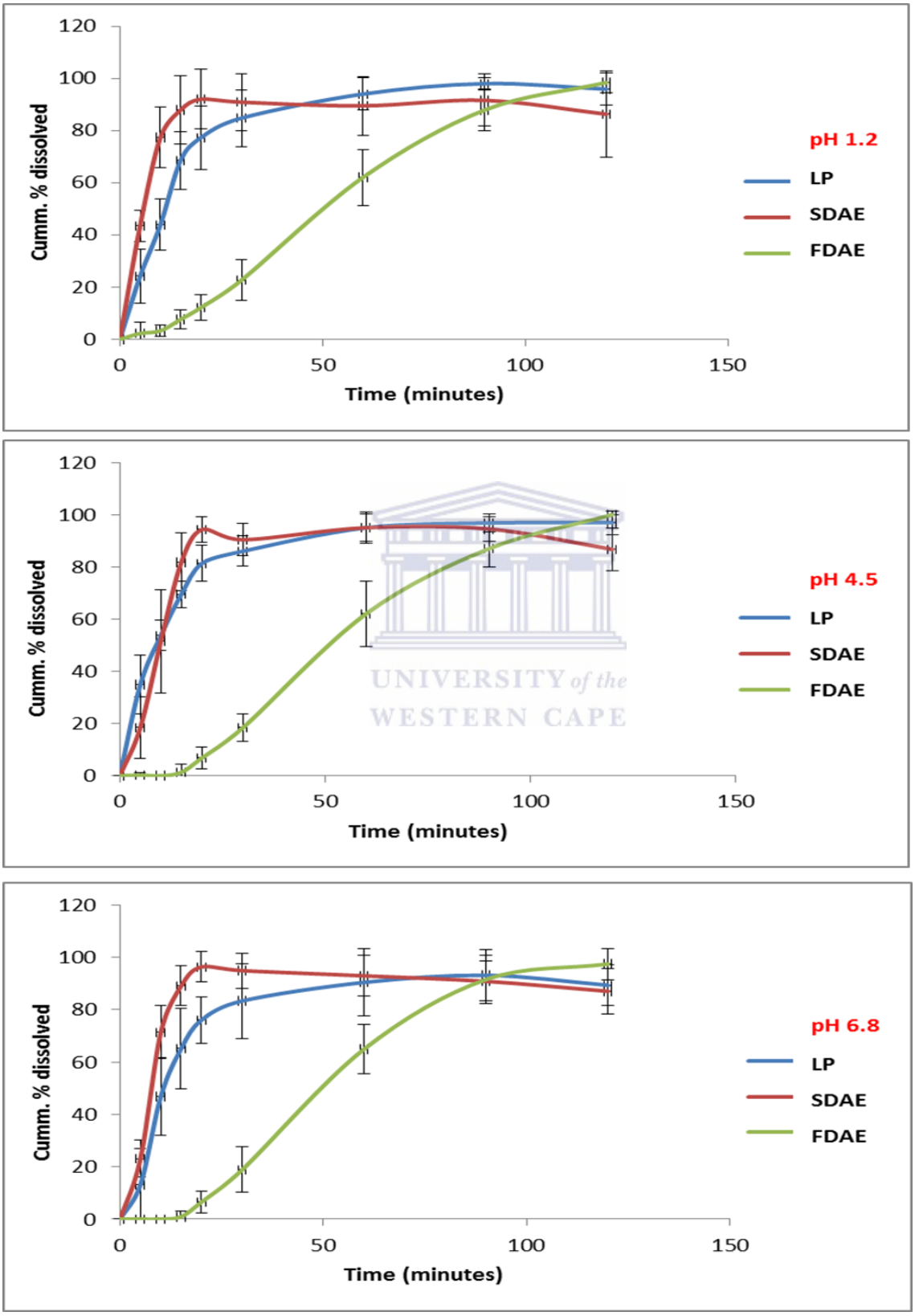


Figure 5.4.3: Dissolution profiles of sutherlandin C (from S. frutescens materials) at pH 1.2, 4.5 and 6.8. Data is presented as mean ± SD

The dissolution characteristics were the same as with sutherlandin A; however, the time to attain 75 % dissolution from the SDAE at pH 1.2 and 85 % dissolution from the LP at pH 6.8 differed for the two marker compounds: sutherlandins A and C (Table 5.4.5). The times to attain the specified Q-values were different for sutherlandin C from all three materials at all three pH conditions, even though the dissolution characteristics overlapped for some of the materials.

Dissolution profiles comparison using the f_2 and its modifications returned values below 50 for any two of the three materials at all three pH conditions (Table 5.4.6), indicating that the dissolution profiles were not similar. Therefore, for assessment and comparison of *S. frutescens* materials using sutherlandin C as a marker compound, the times to attain specified Q-values for sutherlandin C dissolution as well as the f_2 can be used to differentiate between *S. frutescens* materials.

Table 5.4.5: Classification of sutherlandin C in S. frutescens materials by dissolution rate

	Plant material & pH		**Q = 75 % (minutes)**	**Dissolution characteristic**	**Q = 85 % (minutes)**	**Dissolution characteristic**
Suth C	1.2	**LP**	45	Immediate release	60	Delayed dissolution
		SDAE	15	Immediate release	20	Rapidly dissolving
		FDAE	90	Not immediate release	90	Delayed dissolution
	4.5	**LP**	20	Immediate release	30	Rapidly dissolving
		SDAE	15	Immediate release	20	Rapidly dissolving
		FDAE	90	Not immediate release	90	Delayed dissolution
	6.8	**LP**	45	Immediate release	45	Delayed dissolution
		SDAE	15	Immediate release	15	Very rapidly dissolving
		FDAE	90	Not immediate release	90	Delayed dissolution

Table 5.4.6: Comparison of f_2-values for sutherlandin C dissolution profiles at pH 1.2, 4.5 and 6.8

(a) LP (reference) versus SDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	33.9	21.6	30.6
4.5	47.6	33.1	45.0
6.8	37.9	25.3	34.6

(b) LP versus FDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	16.7	-1.7	16.6
4.5	13.6	-8.9	13.0
6.8	16.0	-6.8	15.2

(c) SDAE versus FDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	11.7	-7.2	11.5
4.5	11.9	-10.8	11.8
6.8	10.0	-11.6	9.3

5.4.3.4 Dissolution profiles of sutherlandin D

The graph of cumulative percent dissolved over time for marker compound, sutherlandin D, is shown in Figure 5.4.4. As with sutherlandins A, B and C, the shapes of the dissolution curves looked different for the different materials, and the dissolution characteristics overlapped for some of the materials (Table 5.4.7).

With the exception of sutherlandin D dissolution from the LP and FDAE materials at pH 1.2, the time to attain specified Q-values were different for all the materials at all pH conditions. This is a similarity to the case obtained with sutherlandin B dissolution.

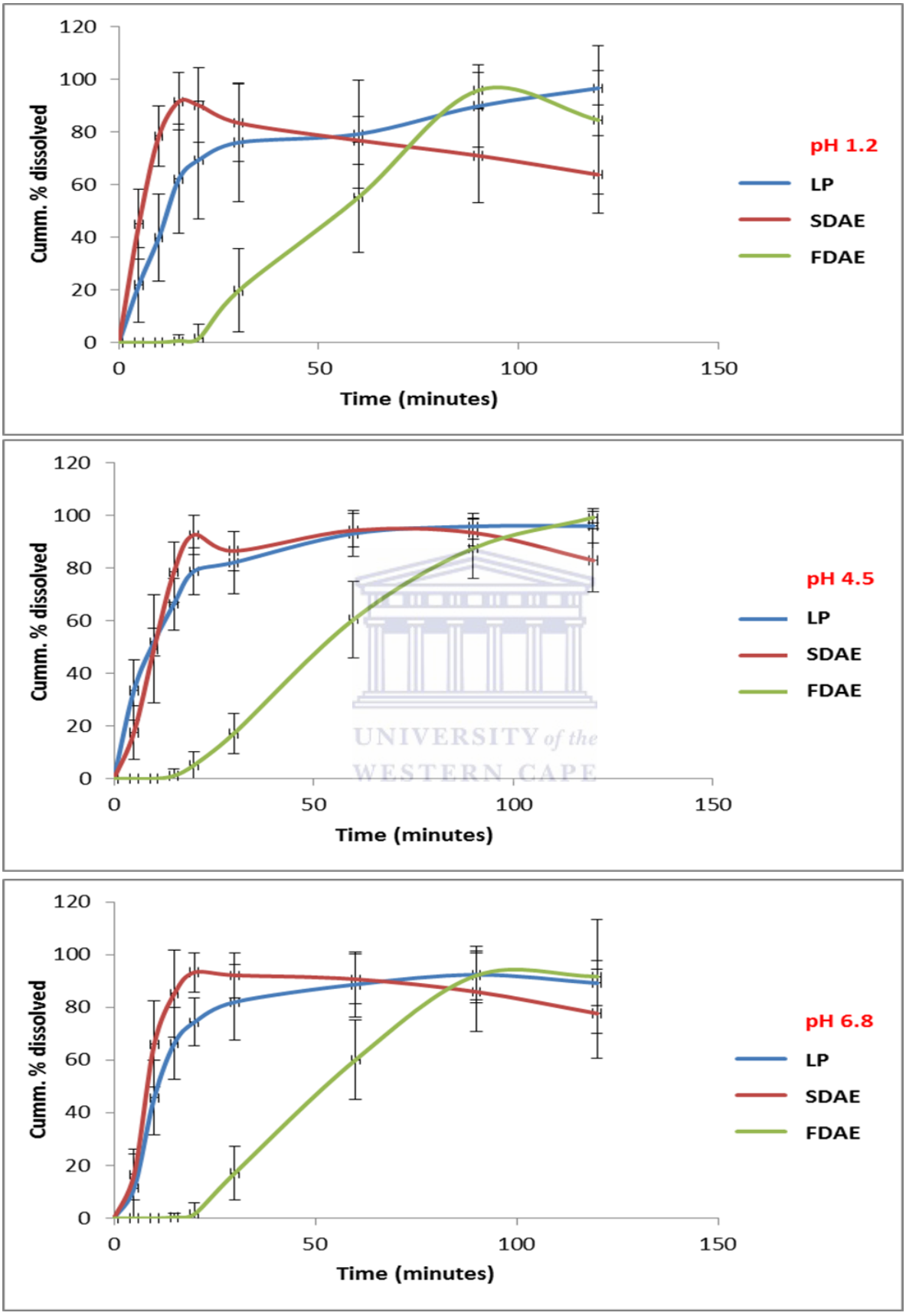


Figure 5.4.4: Dissolution profiles of sutherlandin D from different S. frutescens materials at pH 1.2, 4.5 and 6.8. Data is presented as mean ± SD

As with sutherlandin B, Q-release values of sutherlandin D cannot be used to differentiate between different *S. frutescens* materials at pH 1.2, but can be used at pH 4.5 and 6.8. The dissolution of this marker compound from the SDAE, as with sutherlandin B, displayed a downward trend in the SDAE at pH 1.2. As suggested with sutherlandin B dissolution, it is possible that this marker compound is not stable for prolonged periods at pH 1.2. The two marker compounds (sutherlandins B and D) are also known to contain the same sugar moiety, which perhaps influences their dissolution. Because the same trend was not noticed for the LP and FDAE materials, such instability may perhaps be enabled by properties of the SDAE material. As with sutherlandins A, B and C, dissolution of sutherlandin D from the FDAE material displayed a characteristic lag, with with Q-75 % not attained till the 90-minute sampling point.

Table 5.4.7: Classification of sutherlandin D in S. frutescens materials by dissolution rate

	Plant material & pH		Q = 75 % (minutes)	Dissolution characteristic	Q = 85 % (minutes)	Dissolution characteristic
Suth D	1.2	**LP**	90	Not immediate release	120	Delayed dissolution
		SDAE	15	Immediate release	> 120	Delayed dissolution
		FDAE	90	Not immediate release	90	Delayed dissolution
	4.5	**LP**	20	Immediate release	45	Delayed dissolution
		SDAE	15	Immediate release	20	Rapidly dissolving
		FDAE	90	Not immediate release	120	Delayed dissolution
	6.8	**LP**	45	Immediate release	60	Delayed dissolution
		SDAE	15	Immediate release	20	Rapidly dissolving
		FDAE	90	Not immediate release	90	Delayed dissolution

Comparison of sutherlandin D dissolution profiles between any two *S. frutescens* materials at all three pH conditions using the f_2 and its modifications returned values indicative of dissimilarity of profiles (Table 5.4.8). As with sutherlandin B, Q-values at pH 4.5 and 6.8 (but not at pH 1.2, with respect to the LP and FDAE materials) and f_2 values at any of the three pH conditions can be used to differentiate *S. frutescens* materials.

Table 5.4.8: Comparison of f_2-values for sutherlandin D dissolution profiles at pH 1.2, 4.5 and 6.8

(a) LP (reference) versus SDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	33.1	17.0	27.8
4.5	45.6	11.8	46.5
6.8	40.0	22.0	35.6

(b) LP versus FDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	17.5	-15.1	20.1
4.5	14.3	-35.2	13.6
6.8	15.4	-16.2	15.0

(c) SDAE versus FDAE materials

pH	f_2	$W_t f_2$	f_2-median
1.2	10.5	-20.4	9.3
4.5	13.9	-10.7	13.6
6.8	10.4	-19.5	9.4

5.4.3.5 Dissolution profiles of quercetin

The graph of cumulative percent dissolved over time for the reference compound, quercetin, from *S. frutescens* materials is shown in Figure 5.4.5. There was no dissolution of quercetin from the LP material, at any of the three pH conditions. This supports the data from the study on flavonoid content, which showed no detectable peaks for the flavonoid aglycone, quercetin, in the LP material.

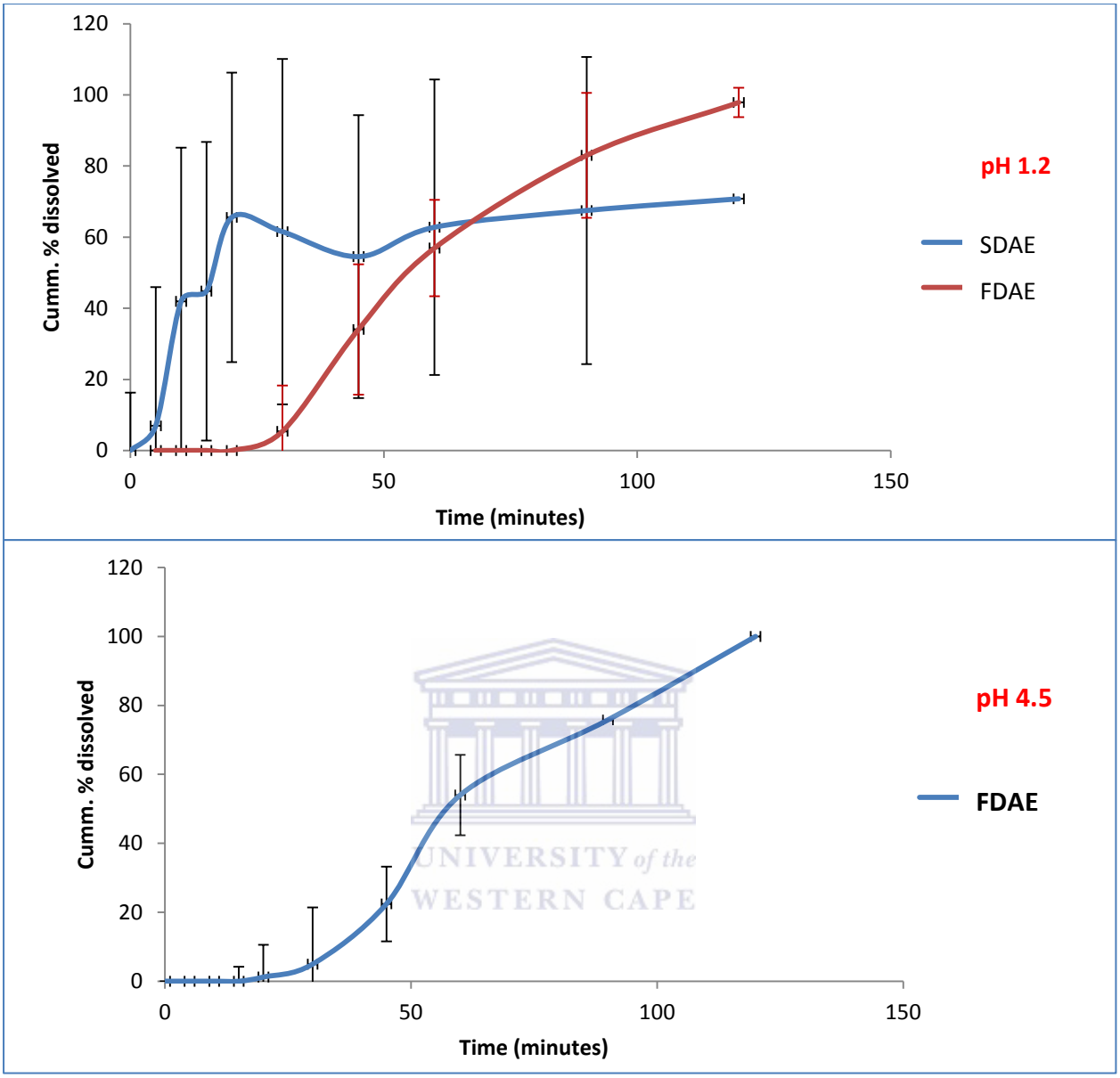


Figure 5.4.5: Dissolution profiles of quercetin from different S. frutescens materials at pH 1.2 and 4.5. Data is presented as mean ± SD

From the SDAE materials, there was a dissolution profile of this compound only at pH 1.2, characterised by very wide deviations, and no dissolution of quercetin at pH 4.5 and 6.8. The study on flavonoid content also showed no presence of quercetin from the SDAE material; however, it is possible that a very acidic pH enhances its dissolution, resulting in the profile observed at pH 1.2. This marker compound is known to be sparingly soluble and chemically unstable in aqueous-based gastrointestinal fluids. Acidic conditions are reported to offer quercetin some protection from degradation, while its chemical instability increases as the condition becomes more alkaline.[222] These may explain the dissolution of quercetin from

the SDAE at pH 1.2, albeit with wide deviations, and the absence of any detectable levels at pH 4.5 and 6.8. Such deviations and the non-detection of quercetin from the LP material and in the dissolution media at pH 4.5 and 6.8 also indicate that this compound may not be a good marker compound for dissolution tests of *S. frutescens* materials.

Dissolution of quercetin from the FDAE also showed some variation, though not as extensive as that obtained with the SDAE. A lag time of 10 minutes was noticed before the detection of peaks at the 15-minute sampling time. Even though the dissolution characteristics were similar for the SDAE and FDAE materials at pH 1.2 (Table 5.4.9), their f_2 values were below 50; original f_2, Gohel's f_2 and the median f_2 values were 21.3, -6.8 and 14.4, respectively, indicating dissimilarity of dissolution profiles.

Table 5.4.9: Classification of quercetin in S. frutescens materials by dissolution rate

	Plant material & pH		**Q = 75 % (minutes)**	**Dissolution characteristic**	**Q = 85 % (minutes)**	**Dissolution characteristic**
Quercetin	1.2	**LP**	NA	NA	NA	NA
		SDAE	> 120	Not immediate release	> 120	Delayed dissolution
		FDAE	> 120	Not immediate release	> 120	Delayed dissolution
	4.5	**LP**	NA	NA	NA	NA
		SDAE	NA	NA	NA	NA
		FDAE	120	Not immediate release	120	Delayed dissolution
	6.8	**LP**	NA	NA	NA	NA
		SDAE	NA	NA	NA	NA
		FDAE	NA	NA	NA	NA

Thus, the Q-values cannot be used to differentiate between the SDAE and FDAE materials, while the f_2 values showed differences in dissolution profiles of quercetin from the two materials. At a pH of 4.5, dissolution of quercetin was detected only from the FDAE material. This reference compound cannot therefore be used to differentiate between the *S. frutescens* materials employed in this study as it is not common to all.

5.4.3.6 Dissolution profiles of kaempferol

The graph of cumulative percent dissolved over time for the reference compound, kaempferol, from *S. frutescens* materials is shown in Figure 5.4.6. There was no dissolution of kaempferol from the LP at any of the three pH conditions. From the SDAE material, dissolution of kaempferol was detected at only one of the pH conditions assessed (pH 1.2), with wide deviations, such as was seen for quercetin. Dissolution of kaempferol was also detected from the FDAE material at pH 1.2, though there was a lag time, with the first peak only detected at the 30-minute sampling time.

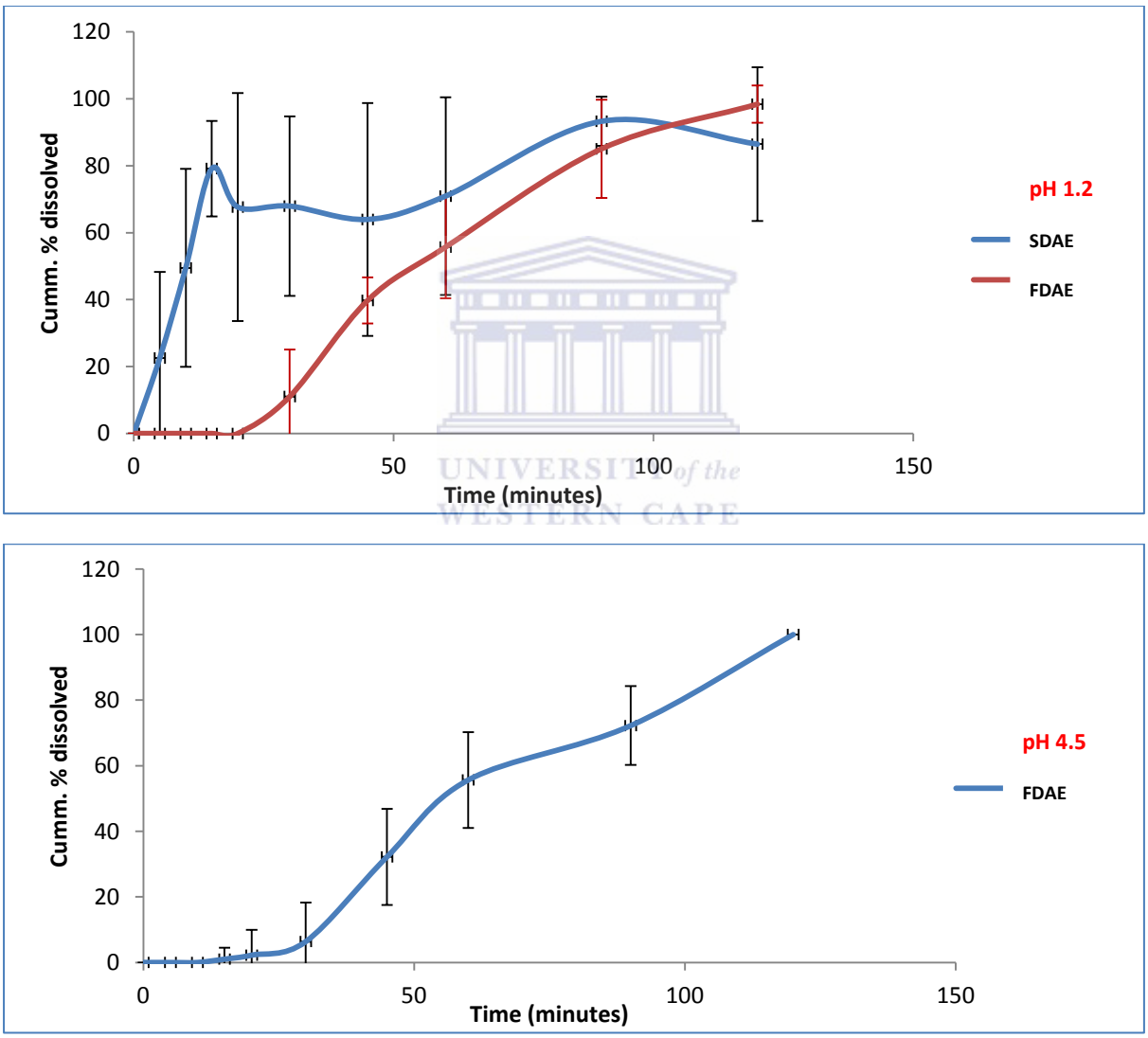


Figure 5.4.6: Dissolution profiles of kaempferol from different S. frutescens materials at pH 1.2 and 4.5. Data is presented as mean ± SD

The Q-release values and dissolution characteristics were mostly similar for the two materials, i.e. the SDAE and the FDAE at pH 1.2 (Table 5.4.10) and so cannot be used to

differentiate *S. frutescens* materials when the reference compound is kaempferol. At pH 4.5, kaempferol dissolution was observed only from the FDAE material.

Comparison of dissolution profiles of kaempferol from the SDAE and FDAE materials using the original f_2, Gohel's f_2 and median f_2 returned values of 16.2, -4.1 and 27.3, respectively, indicating dissimilarity of dissolution profiles. However, this marker compound may not be as good as the flavonoid glycosides for differentiating *S. frutescens* materials as it was not detected in some of the materials and at some pH conditions.

Table 5.4.10: Classification of kaempferol in S. frutescens materials by dissolution rate

	Plant material & pH		**Q = 75 % (minutes)**	**Dissolution characteristic**	**Q = 85 % (minutes)**	**Dissolution characteristic**
Kaempferol	1.2	**LP**	NA	NA	NA	NA
		SDAE	90	Not immediate release	> 120	Delayed dissolution
		FDAE	> 120	Not immediate release	> 120	Delayed dissolution
	4.5	**LP**	NA	NA	NA	NA
		SDAE	NA	NA	NA	NA
		FDAE	120	Not immediate release	120	Delayed dissolution
	6.8	**LP**	NA	NA	NA	NA
		SDAE	NA	NA	NA	NA
		FDAE	NA	NA	NA	NA

The findings from the dissolution tests indicate that flavonoid glycosides are better marker and differentiating compounds than the flavonoid aglycones for dissolution studies of *S. frutescens* materials; they were detected in the different dissolution media and also projected different dissolution properties for different *S. frutescens* materials. Glycosylation of a flavonoid decreases its reactivity while improving its water solubility,[223,224] enhancing its dissolution in aqueous-based media. This may serve to explain the dissolution of the flavonoid glycosides, to different degrees, in the dissolution media assessed. The flavonoid aglycones, on the other hand, are not ideal as marker compounds for dissolution tests but

may be used to monitor instability as they are sometimes formed as degradation products of the glycosides.

The Q-release values for the flavonoid aglycones, though not available in all instances, were more than the Q-release values for the flavonoid glycosides, supporting the first of our second hypotheses (Chapter Three, Section 3.3, b (i)), which states that flavonoid glycoside dissolution will proceed faster than flavonoid aglycone dissolution. The second part of our second hypotheses, b(ii), states that flavonoid dissolution from the two extracts (SDAE and FDAE) will be the same but faster than flavonoid dissolution from the LP material. We had reasoned that being aqueous extracts, the SDAE and FDAE materials will be fully soluble in the aqueous-based dissolution media, and release their flavonoid content faster than the LP, which in its aqueous-unextracted from would be less soluble. However, the dissolution rates of the SDAE and FDAE did not match and the FDAE material was found to have the slowest dissolution rate as seen from the Q-values (Tables 5.4.1 , 5.4.3, 5.4.5, 5.4.7, 5.4.9 and 5.4.10). Dissolution seemed fastest from the SDAE material but was equally matched by the rate obtained from the LP material, while the freeze-dried aqueous extract presented with the slowest release. This may stem from its processing parameters which will be investigated in further studies.

To conduct dissolution studies and differentiating tests on *S. frutescens* materials therefore, any of the four flavonoid glycosides but not the flavonoid aglycones may be used in any of the three media assessed as their Q-release and f_2 values are differentiating of the materials. However, the use of sutherlandin D at pH 1.2 is not recommended as its precision under dissolution conditions did not meet validation specifications.

It is interesting to note that in the SDAE material, sutherlandin B and D displayed a downward trend after attaining maximum dissolution, while this was not the case for sutherlandins A and C. Sutherlandins B and D are different in that they are flavonoid glycosides of the aglycones, quercetin and kaempferol respectively, while the presence of the apiofuranosyl group, is a similarity. It is very likely that the breakdown of the sugar moiety within the SDAE material is responsible for the decrease in percentage observed after peak dissolution. Likewise, sutherlandins A and C are different in that they are flavonoid glycosides of the aglycones, quercetin and kaempferol respectively, while the

presence of the xylopyranosyl group, is a similarity. It is also very likely that the resistance of sutherlandins A and C to breakdown in the dissolution medium, especially at pH 1.2, can be attributed to this similarity – the xylopyranosyl group. This downward trend in dissolution profiles of sutherlandins B and D was not the case for the LP and FDAE material though. It seems that while the similarity in the sugar group may be the reason for the decrease in cumulative amount of sutherlandins B and D dissolved over time, the SDAE material also provided an enabling environment for this to occur.

The dissolution characteristics of each flavonoid compound from the different *S. frutescens* materials, reported in Tables 5.4.1 , 5.4.3, 5.4.5, 5.4.7, 5.4.9 and 5.4.10, show that the same flavonoid glycoside may not necessarily be released at the same rate from different materials. Thus, the dissolution test reported here has established differences in flavonoid dissolution from different materials, and so can be used as a tool for quality control.

5.4.4 Analysis of dissolution data using Principal Component Analysis (PCA)

The results of PCA analysis of dissolution data are presented in Figures 5.4.7 to 5.4.10. The loadings plot of dissolution data for *S. frutescens* LP, SDAE and FDAE materials are presented in Figure 5.4.7. It can be seen from the cluster that sample collection at 20, 30, and 60 minutes does not make much difference to the results, and one of these time points can be selected in order to save time and resources. The sampling time at 120 minutes was different at all the pH conditions, however, sampling at 5 and 10 minutes may not be necessary and only one of the two sampling times may suffice. At all the pH conditions assessed, a total of 4 to 6 sampling points can therefore be adequately used to construct a dissolution profile of flavonoid glycoside release from any of the three *S. frutescens* materials.

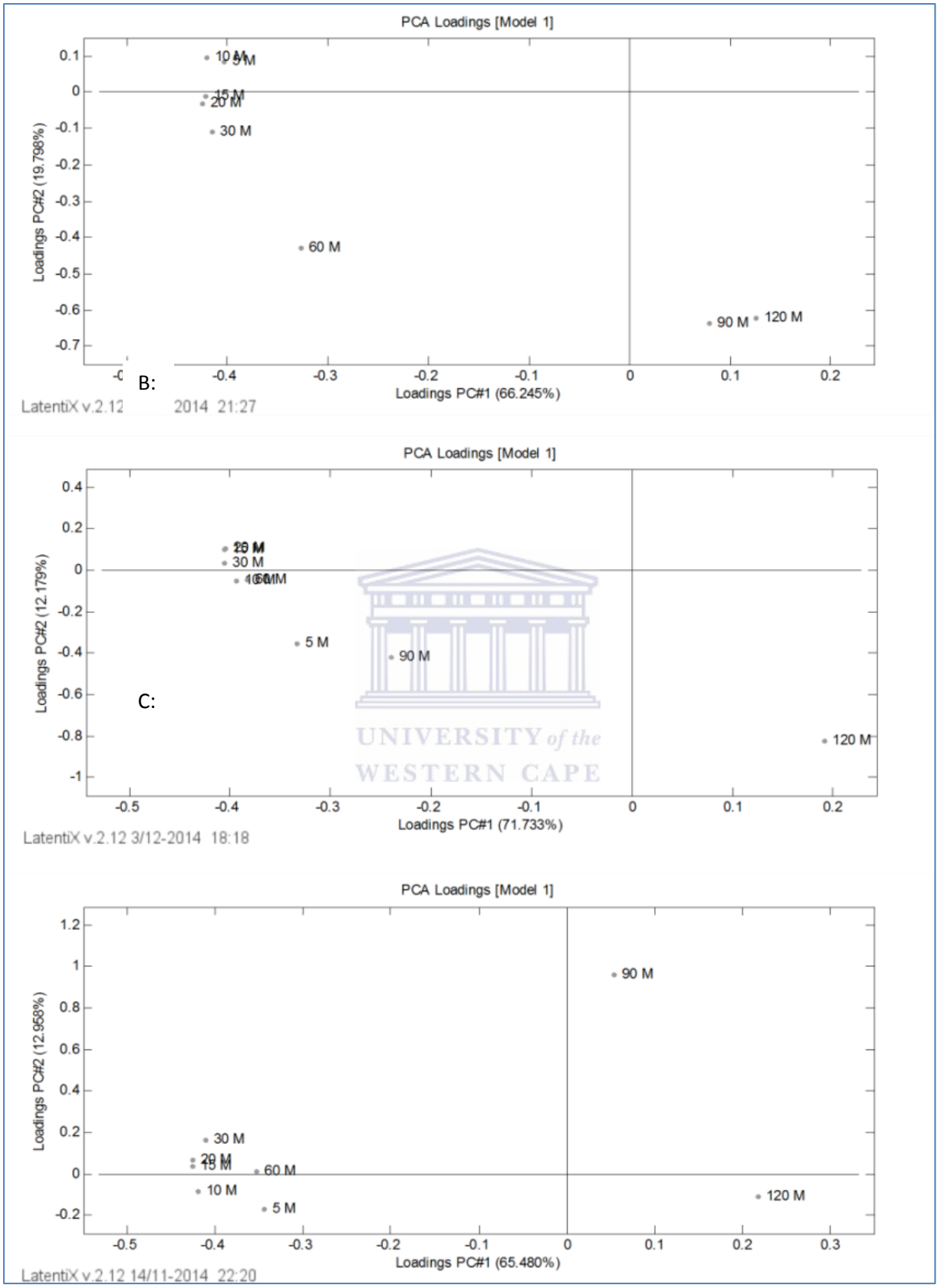


Figure 5.4.7: PCA loadings for the dissolution of sutherlandins A, B, C, D, from three S. frutescens materials (LP, SDAE and FDAE) at (A) pH 1.2 (B) pH 4.5 and (C) pH 6.8

The scores plot of dissolution data for *S. frutescens* LP, SDAE and FDAE materials are presented in Figures 5.4.8 to 5.4.10. The scores plot of a PCA portrays the relationship between observations, and observations that are similar cluster more than observations that exhibit significant differences. The scores plots projected show that dissolution of flavonoid glycosides from *S. frutescens* materials at pH 1.2, 4.5 and 6.8 depends mainly on the *S. frutescens* material and less on the pH of the dissolution medium or the properties of the flavonoid glycoside itself. Note the clustering of *S. frutescens* materials (LP, SDAE or FDAE) in the highlighted sections of Figures 5.4.8 *a*, 5.4.9 *a* and 5.4.10 *a*. Such clustering shows similarity in dissolution of different marker compounds from the same material, while the non-clustering observed in the highlighted sections (of Figures 5.4.8 *b*, 5.4.9 *b* and 5.4.10 *b*) indicates that the pH of the medium or characteristics of the marker compounds contribute less than the *S. frutescens* material in the dissolution characteristics of the markers from *S. frutescens* materials.

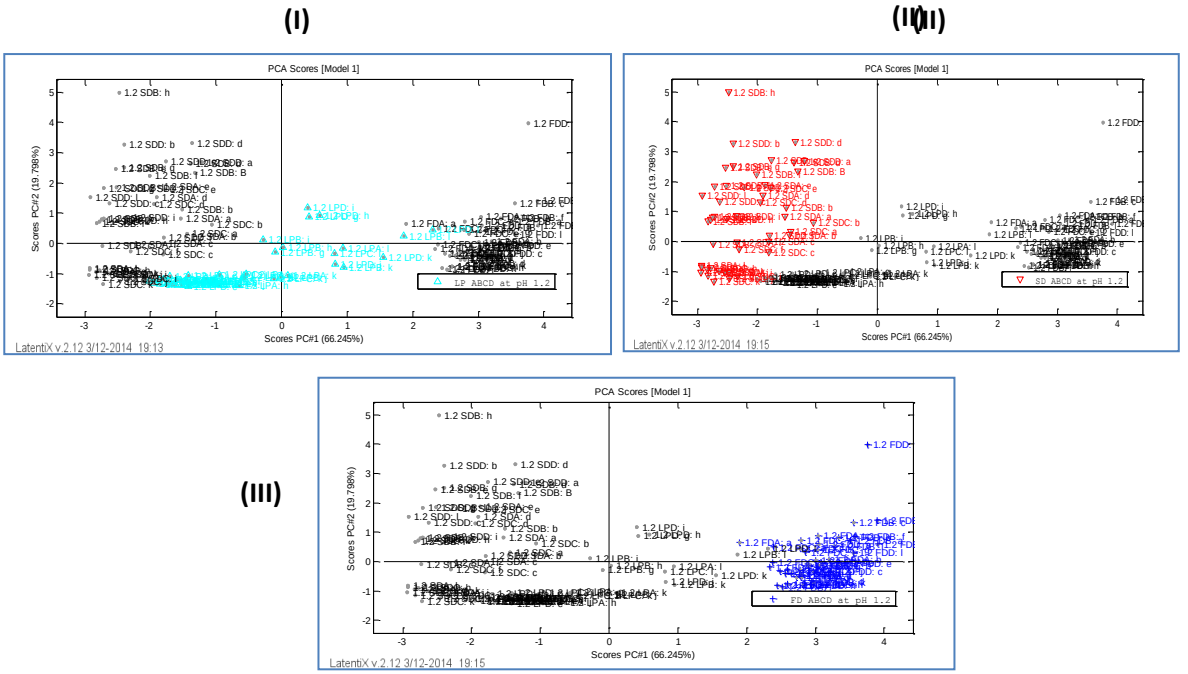


Figure 5.4.8 (a): *PCA scores plot of marker compounds dissolution from S. frutescens materials at pH 1.2, with highlights on the different materials: (I) LP material, (II) spray-dried aqueous extract material, and (III) freeze-dried aqueous extract material*

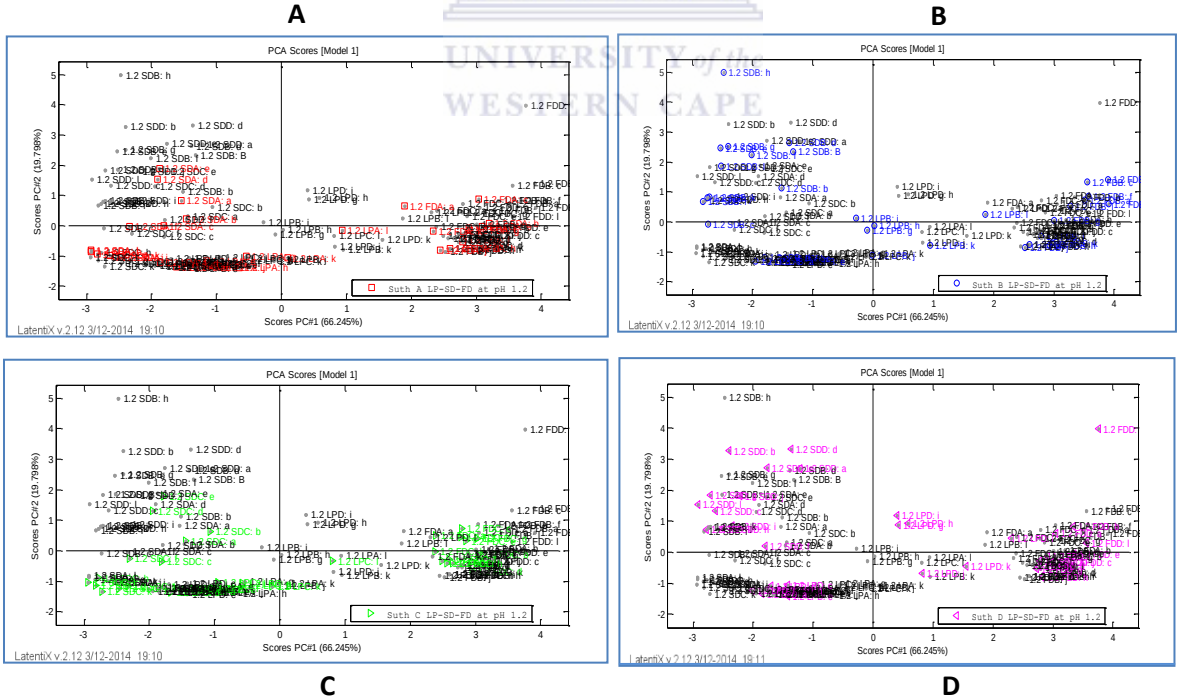


Figure 5.4.8 (b): *PCA scores plot of marker compounds dissolution from S. frutescens materials at pH 1.2, with highlights on the different marker compounds: (A) sutherlandin A, (B) sutherlandin B, (C) sutherlandin C, and (D) sutherlandin D*

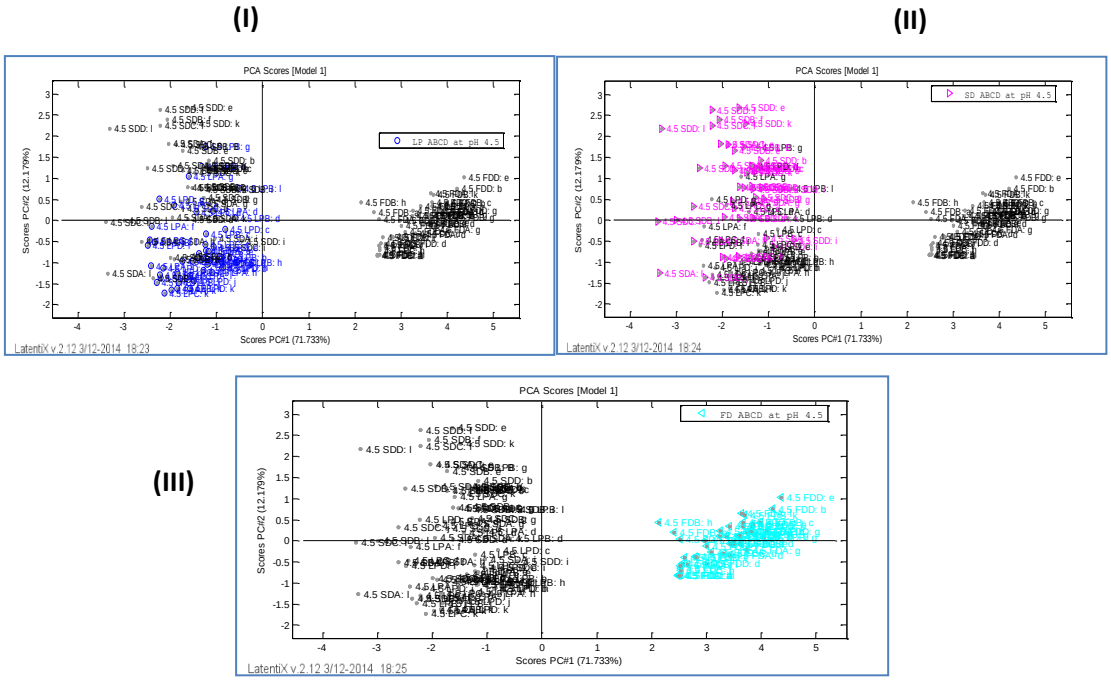


Figure 5.4.9 (a): *PCA scores plot of marker compounds dissolution from S. frutescens materials at pH 4.5, with highlights on the different materials: (I) LP material, (II) spray-dried aqueous extract material, and (III) freeze-dried aqueous extract material*

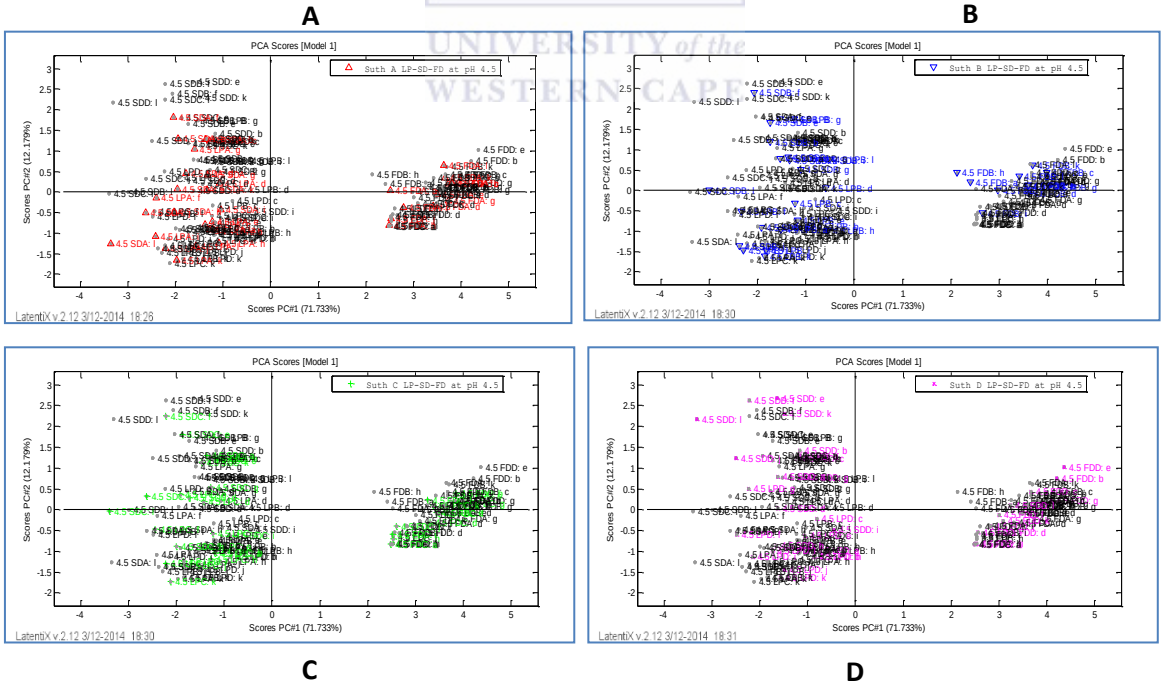


Figure 5.4.9 (b): *PCA scores plot of marker compounds dissolution from S. frutescens materials at pH 4.5, with highlights on the different marker compounds: (A) sutherlandin A, (B) sutherlandin B, (C) sutherlandin C, and (D) sutherlandin D*

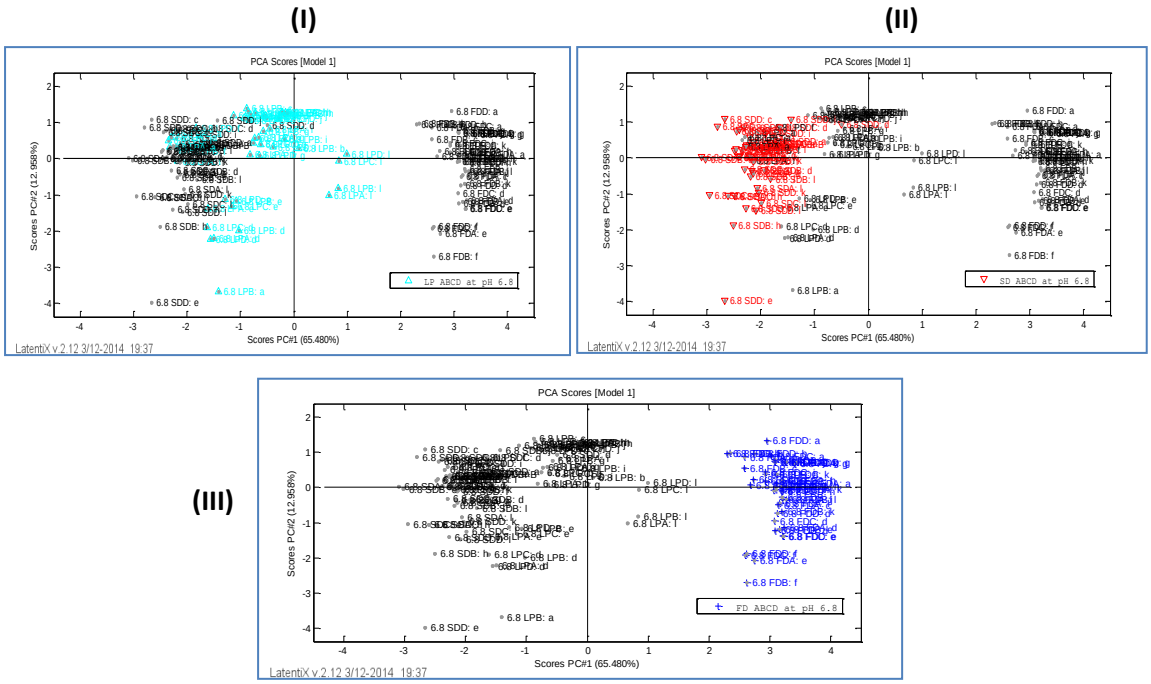


Figure 5.4.10 (a): *PCA scores plot of marker compounds dissolution from S. frutescens materials at pH 6.8, with highlights on the different materials: (I) LP material, (II) spray-dried aqueous extract material, and (III) freeze-dried aqueous extract material*

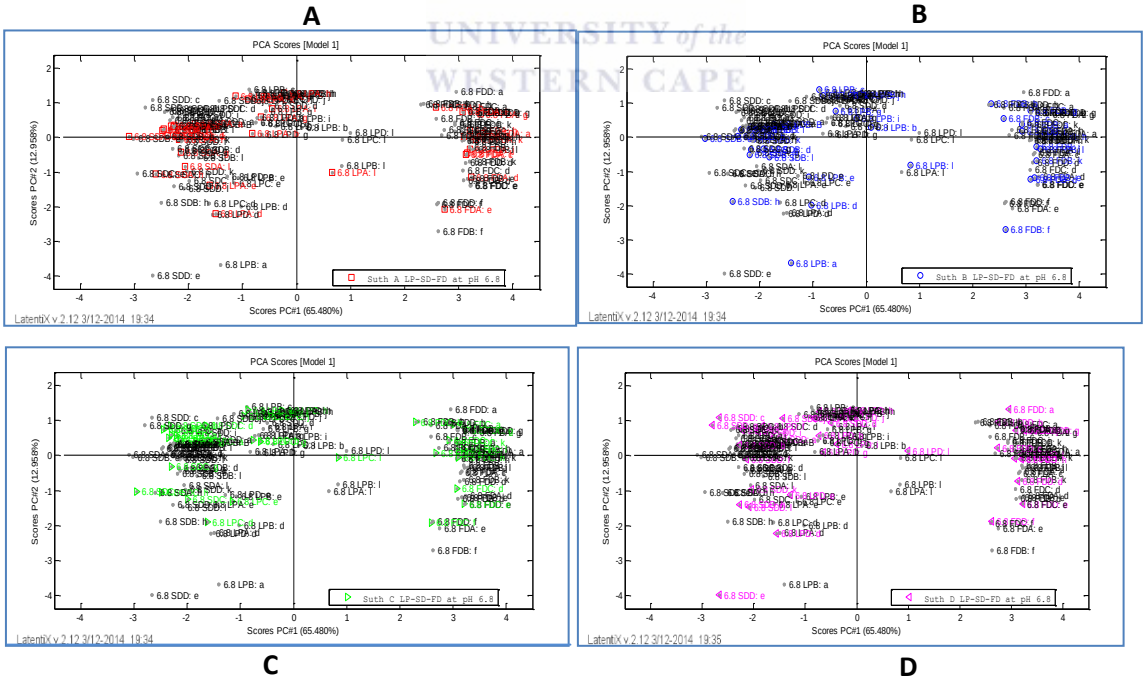


Figure 5.4.10 (b): *PCA scores plot of marker compounds dissolution from S. frutescens materials at pH 6.8, with highlights on the different marker compounds: (A) sutherlandin A, (B) sutherlandin B, (C) sutherlandin C, and (D) sutherlandin D*

5.4.5 Conclusions

A dissolution test was developed and utilized for *S. frutescens* materials, with selected flavonoid glycosides as marker compounds. The flavonoid glycoside compounds were soluble in aqueous-based media, enabling the assessment of dissolution profiles at three different pH conditions. The dissolution profiles for each flavonoid glycoside marker compound were found to differ in all three *S. frutescens* materials.

For quality control and regulation of *S. frutescens* products then, the flavonoid glycosides, along with their Q-release and f_2-values can be employed as differentiating tools in dissolution tests of different materials. The dissolution test as well as the profile comparison methods here reported can be applied to the quality control and bioequivalence assessment of *S. frutescens* products using the flavonoid glycosides as marker compounds. The same can also be applied in the quality control of other herbal materials containing flavonoid glycosides provided suitable marker compounds are selected for such materials. The flavonoid aglycones, on the other hand, could not be detected in some of the materials and so may not be ideal for quality control studies by dissolution tests.

The differences observed in the dissolution of each flavonoid glycoside marker compound from the different materials may be due to differences in their release from the enclosing material, a prerequisite for API dissolution. This shall be explored in the next section.

5.5 Modelling of flavonoid glycoside release from different *S. frutescens* materials

To elucidate the mechanisms behind release of flavonoid glycoside marker compounds from *S. frutescens* materials, dissolution data of flavonoid glycosides from different *S. frutescens* materials were fitted to mathematical models. This section describes the results obtained from mathematical modelling of marker compound release from the different materials.

5.5.1 Release profiles and kinetics of flavonoid glycosides from *S. frutescens* materials (LP, SDAE and FDAE)

5.5.1.1 Release profiles and kinetics at pH 1.2

From the leaf powder (LP) material at pH 1.2, release profile of sutherlandins A and B best fit the Weibull_1 model (R^2 = 0.9818 and 0.9818, respectively), while the best fit for sutherlandins C and D (R^2 = 0.9845 and 0.9871, respectively) was obtained with the Weibull_4 model (Figure 5.5.1, and Appendix 6: Table A 6.1). Although there is no universally acceptable empirical model for dissolution curves, the Weibull model has been found to fit most dissolution data curves.[151] The β-values of the Weibull function were 1.827 ± 1.453, 2.434 ± 1.757, 1.509 ± 1.517 and 2.384 ± 1.540 for sutherlandins A, B, C and D, respectively. Based on the β-values, release of sutherlandin A from the LP material at pH 1.2 is by a complex mechanism.

From the spray-dried aqueous extract (SDAE), the *in vitro* release data for sutherlandins A to D did not fit any of the assessed models. The models of best fit were the Peppas-Sahlin and Makoid-Banakar models (Figure 5.5.2); however, the R^2 values were only between 0.4000 and 0.8000, and so the profiles were not deemed good enough for fitting to the Korsmeyer-Peppas model for release mechanism elucidation.

For release of the reference standards from the FDAE material, the plot showed relatively good R^2 values, with goodness of fit for the Weibull models, as well as the Makoid-Banakar with T_{lag}, Peppas Sahlin 1 and Logistic models. Based on the R^2 and R^2_{adj} values, the Weibull_4 model for sutherlandins A (R^2 = 0.9902) and C (R^2 = 0.9909), and the Makoid-Banakar model for sutherlandin B (R^2 = 0.9949) were selected as the models of best fit (Figure 5.5.3).

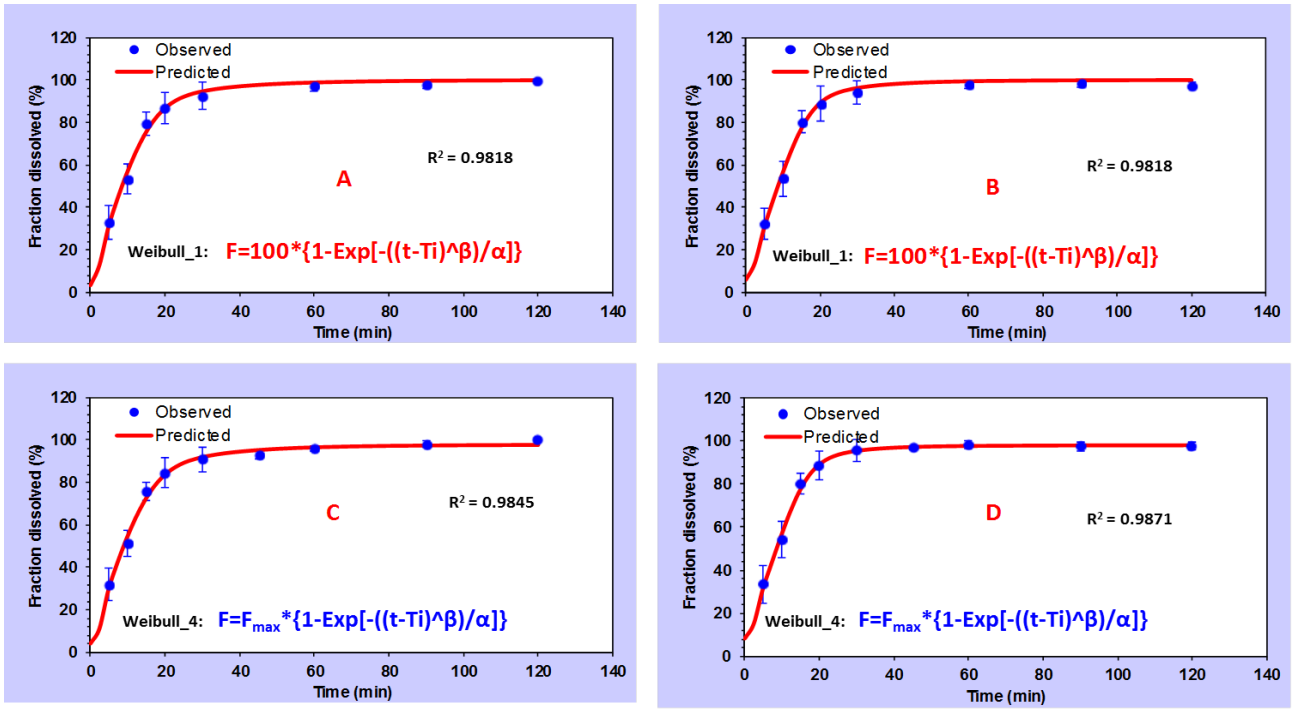


Figure 5.5.1: Models of best fit for in vitro release of sutherlandins A–D from S. frutescens LP at pH 1.2. Results are Mean ± SD. Equations are for models of best fit for release of marker compounds from S. frutescens material.

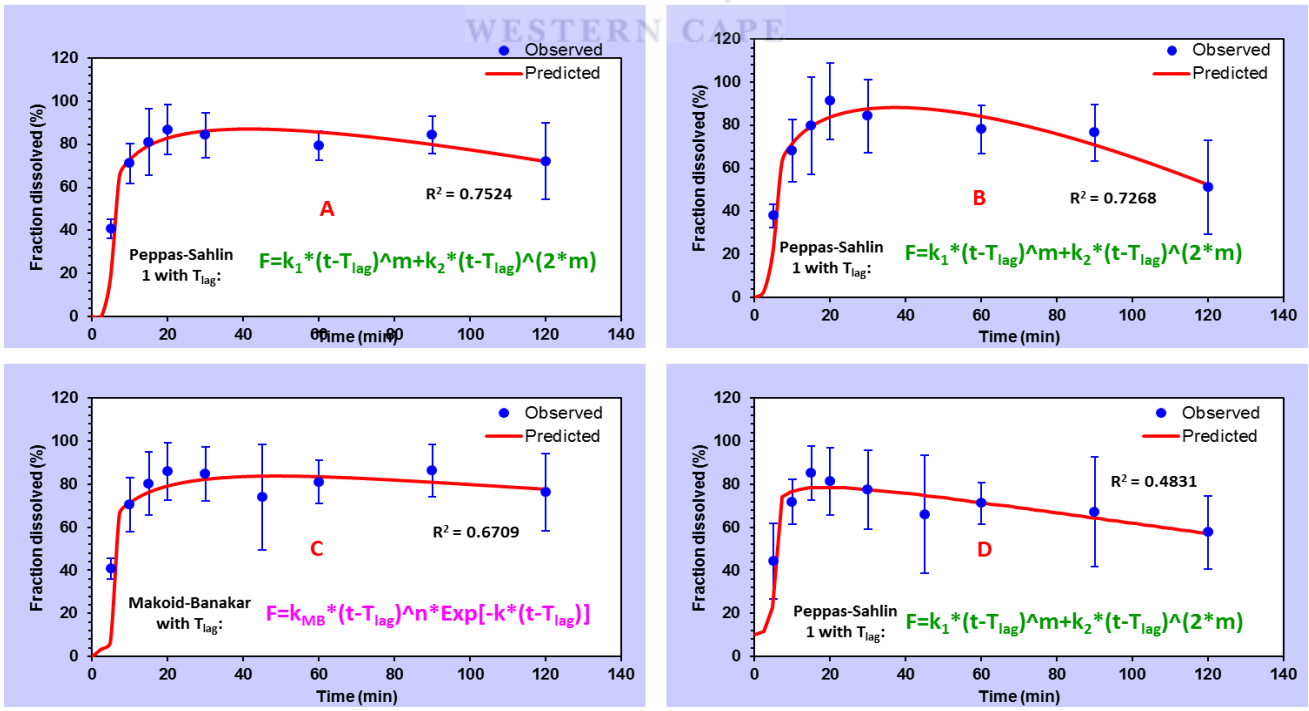


Figure 5.5.2: Models of best fit for in vitro release of sutherlandins A–D from S. frutescens SDAE at pH 1.2. Results are Mean ± SD. Equations are for models of best fit for release of marker compounds from S. frutescens material.

Values of β in the Weibull models for sutherlandins A and C release were all above 1 (Appendix 4: Table 1), suggesting a complex release mechanism. The release of sutherlandin D from the FDAE showed best fit for the Peppas-Sahlin 1 with T_{lag} model (R^2 = 0.9714) though such fit did not meet the minimum acceptable criterion of 0.9800 for R^2, and so could not be fit to the Korsmeyer-Peppas model for release mechanism elucidation (Figure 5.5.3 and Appendix 4: Table 1).

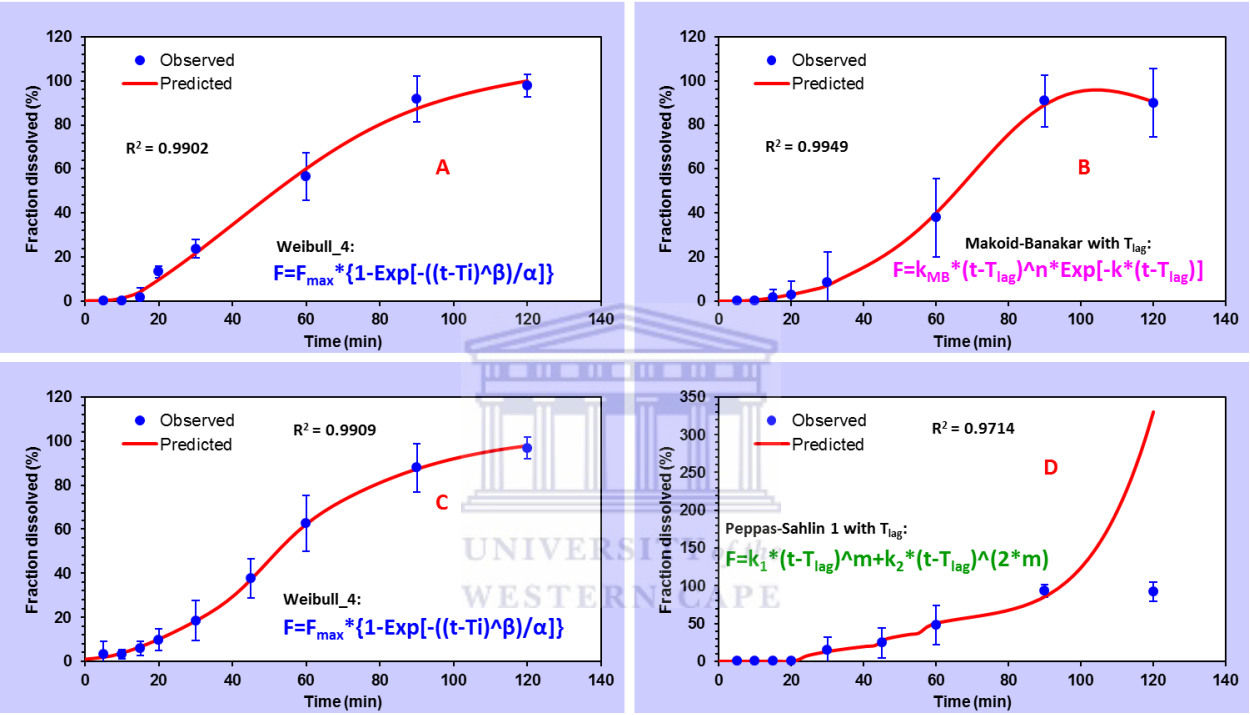


Figure 5.5.3: Models of best fit for in vitro release of sutherlandins A–D from S. frutescens FDAE at pH 1.2. Results are Mean ± SD. Equations are for models of best fit for release of marker compounds from S. frutescens material.

The Korsmeyer-Peppas release exponent, ***n***, for sutherlandin A, B, C and D in the LP material at pH 1.2 were 1.183 ± 0.727, 1.183 ± 0.727, 1.145 ± 0.654 and 1.594 ± 0.890, respectively (Appendix 6: Table A 6.1). From the FDAE materials at pH 1.2, values of ***n*** were 1.455 ± 0.853, 1.709 ± 2.252 and 1.663 ± 0.588 for sutherlandins A, B and C, respectively (Appendix 4, Table 1). Such values indicate that release of the marker compounds from the two materials was due to polymer relaxation as a result of gel swelling, so-called super case-II transport.[225] This also seems to agree with the β values obtained from the Weibull models for marker compounds release from the LP and FDAE materials, which were above 1 and

indicated that release of the reference standards was not by diffusion but rather by a complex mechanism. For the release of reference standards from the SDAE material at pH 1.2, and sutherlandin D from the FDAE at pH 1.2, empirical mathematical models of good fit could not be obtained, and so these were not modelled to the Korsmeyer-Peppas equation for release mechanism elucidation.

The release of an API in the gastrointestinal tract (GIT), a prerequisite for API dissolution, usually takes place in the acidic gastric region, hence the use of dissolution data obtained at pH 1.2. However, because some marker compounds exhibited delayed dissolution at some pH conditions, as seen in the previous section (Tables 5.4.1 , 5.4.3, 5.4.5, 5.4.7, 5.4.9 and 5.4.10), which may be due to their delayed release, release profiles and kinetics were also assessed at pH 4.5 (representing gastric pH soon after food intake, which may cause delayed release and dissolution) and 6.8 (representing intestinal pH), and are here presented.

5.5.1.2 Release profiles and kinetics at pH 4.5

At pH 4.5, release profiles of sutherlandins A to D from the LP material did not meet the set requirement for model assignment (i.e. $R^2 \geq 0.9800$). The models of best fit were the Weibull_3 model for sutherlandins A, C and D (R^2 = 0.9702, 0.9720 and 0.9581, respectively) and the Weibull_4 model for sutherlandin B (R^2 = 0.9729) (Figure 5.5.4, and Appendix 6: Table A 6.2). Because none of these models met the minimum specification set for R^2, they were not fit to the Korsmeyer-Peppas model for elucidation of release mechanism.

From the SDAE material, release profiles of the marker compounds (sutherlandins A to D), like that from the LP material, did not meet the minimum specifications set for the R^2 value. As such, they were not fitted to the Korsmeyer-Peppas model for release mechanism elucidation. The model of best fit for the reference standards was the Weibull_3 model, with R^2 values of 0.9623, 0.9599, 0.9598 and 0.9448 for sutherlandins A, B, C and D respectively, (Figure 5.5.5).

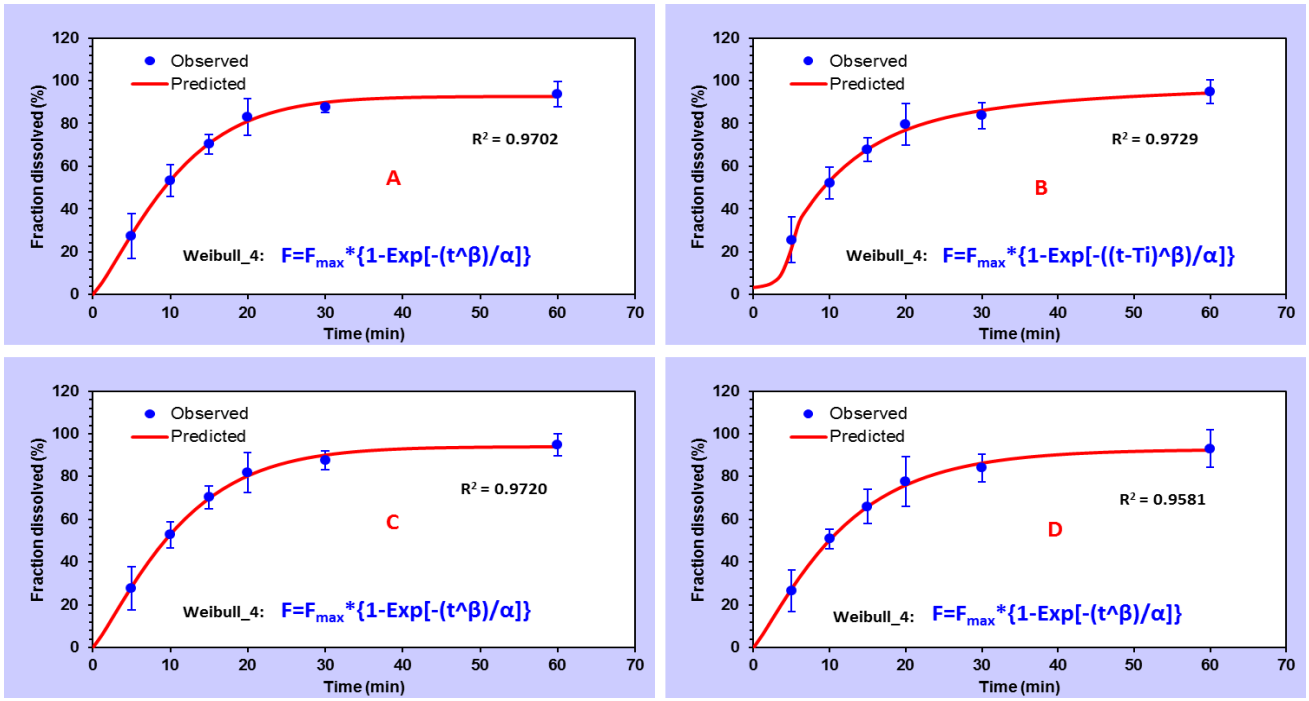


Figure 5.5.4: Models of best fit for in vitro release of sutherlandins A–D from S. frutescens LP at pH 4.5. Results are Mean ± SD. Equations are for models of best fit for release of marker compounds from S. frutescens material.

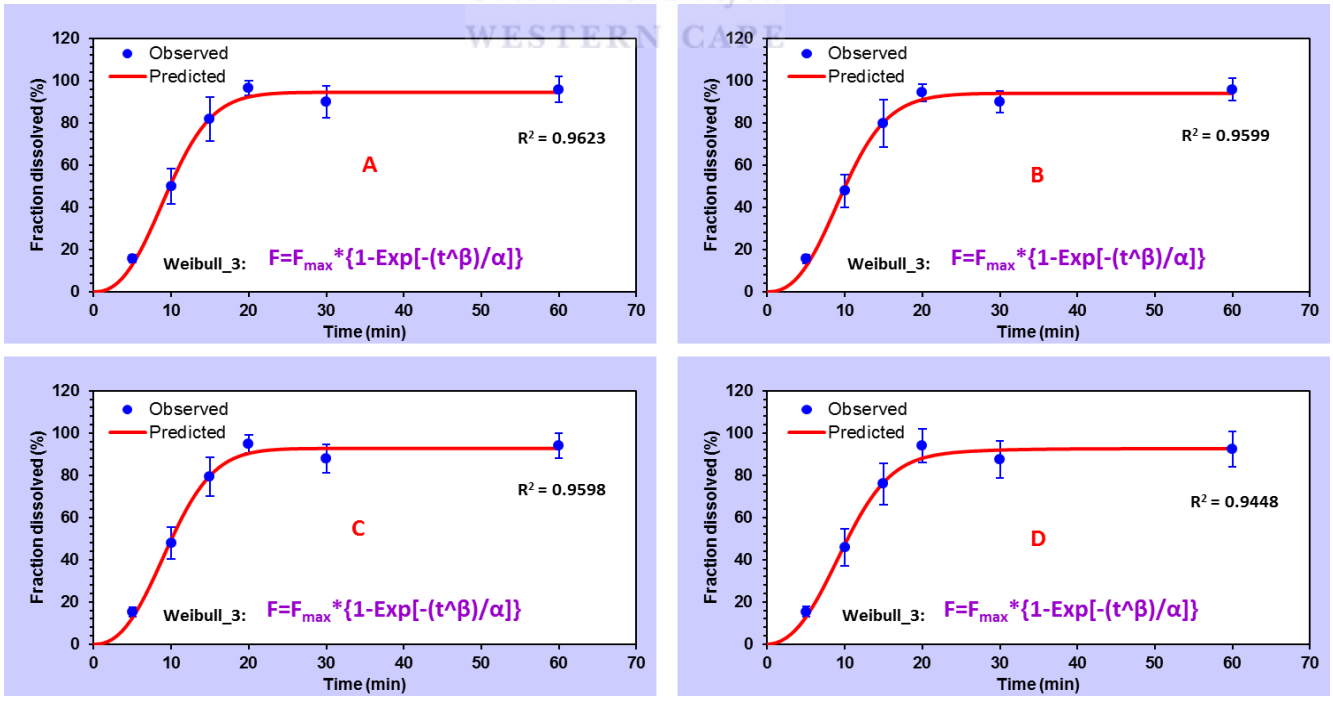


Figure 5.5.5: Models of best fit for in vitro release of sutherlandins A–D from S. frutescens SDAE at pH 4.5. Results are Mean ± SD. Equations are for models of best fit for release of marker compounds from S. frutescens material.

From the FDAE material at pH 4.5, most of the models assessed were good fits for description of marker compound release. With the exception of the zero and first order models, all the other models assessed showed almost perfect fit ($R^2 \geq 0.9900$) for the release of the four marker compounds (Appendix 6: Table A 6.2). The mathematical models of best fit selected for marker compounds release are shown in Figure 5.5.6, and were the Peppas-Sahlin_1 with T_{lag} model for sutherlandin A ($R^2 = 0.9982$), the Makoid-Banakar with T_{lag} model for sutherlandin B ($R^2 = 0.9983$) and the Weibull_4 model for sutherlandins C (R2 = 0.9988) and D ($R^2 = 0.9932$).

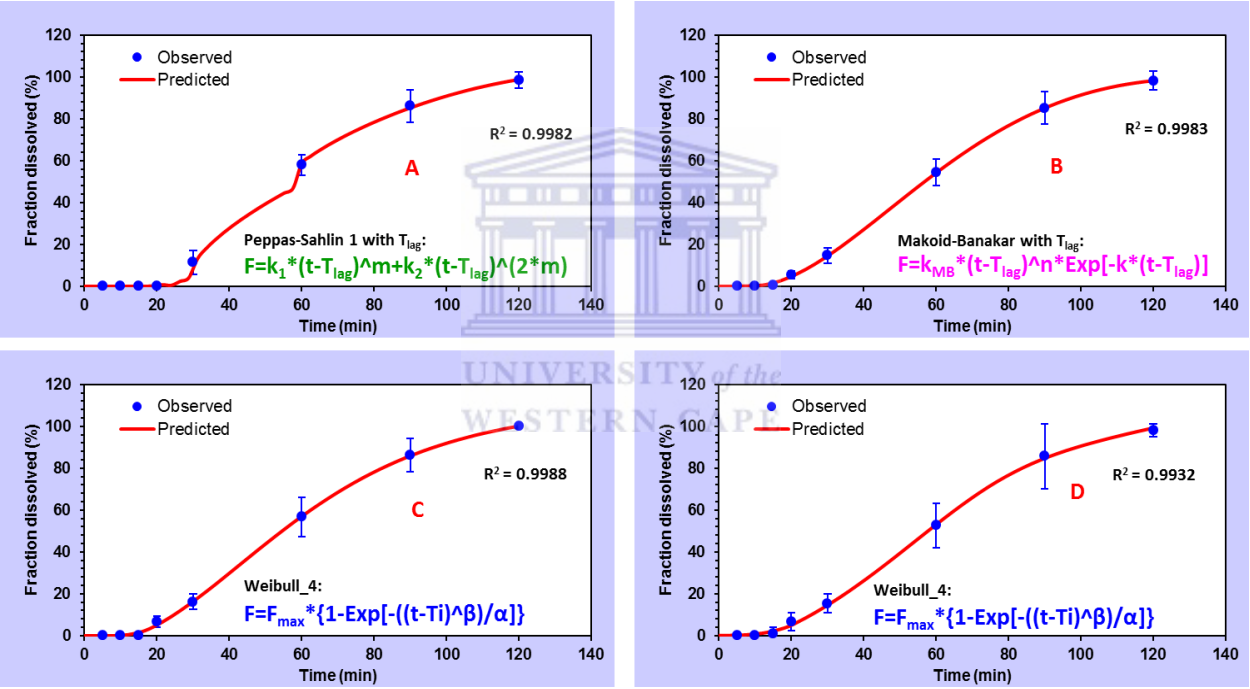


Figure 5.5.6: Models of best fit for in vitro release of sutherlandins A–D from S. frutescens FDAE at pH 4.5. Results are Mean ± SD. Equations are for models of best fit for release of marker compounds from S. frutescens material.

The Peppas-Sahlin model can be seen as semi-empirical since in addition to describing the shape of the curve, it also gives some information on the underlying mechanism of API release. The vakues of k_d and k_r represent the extent of API release by polymer diffusion and polymer relaxation, respectively.[158]

For sutherlandin A release from the FDAE material, which was best described by the Peppas-Sahlin model, the values of k_d and k_r were 5.562 ± 29.21 and 7.135 ± 12.41, respectively. These values are significantly different ($p < 0.05$), suggesting that they differ for sutherlandin A release from the FDAE material. The value of k_r is higher than the value of k_d, suggesting that release of sutherlandin A from the FDAE material may be more by polymer relaxation and less by polymer diffusion; however, with the wide deviations in the values of k_d and k_r, it may well be by a combination of both mechanisms.

Sutherlandin B release from the FDAE material was best described by the Makoid-Banakar with T_{lag} model. Model description for sutherlandins C and D release from the FDAE material was best with the Weibull_4 model (Figure 5.5.6). The β-values (1.688 ± 0.386 and 2.420 ± 1.681 for sutherlandins C and D, respectively) indicate that release is governed by a complex mechanism. Even though the Weibull models were not the models of best fit for sutherlandins A and B from the FDAE material, they can also be used to model and describe sutherlandin A and B release from this material as they proved to fit these models (R^2 > 0.9900). The value of β from the Weibull models (greater than 1 in all cases) suggests a complex release mechanism for all the marker compounds from the FDAE material at pH 4.5.

The Korsmeyer-Peppas release exponents for sutherlandins A, B, C and D from the FDAE were 1.059 ± 1.011, 1.681 ± 0.718, 1.647 ± 0.770 and 2.044 ± 1.049, respectively (Appendix 4, Table 2), indicating that release of the reference standards from the FDAE material at pH 4.5 was by super case-II transport, a confirmation of the inference drawn from the β values in the Weibull models of good fit which suggested a complex release mechanism.

5.5.1.3 Release profiles and kinetics at pH 6.8

For release profiles of sutherlandins A to D from the LP material at pH 6.8, none of the models assessed fit well enough (R^2 was less than 0.9800) to be selected as a good fit for describing API or marker compound release, and so dissolution data of these markers were not fit to the Korsmeyer-Peppas model for elucidation of release mechanism. The models of best fit were the Makoid-Banakar with T_{lag} model for the release of sutherlandins A, B and D (R^2 = 0.9245, 0.9189 and 0.9389, respectively; Appendix 4, Table 3) and the Peppas-Sahlin 1

with T_{lag} model for the release of suterhlandin C (R^2 = 0.9376). These models are shown in Figure 5.5.7.

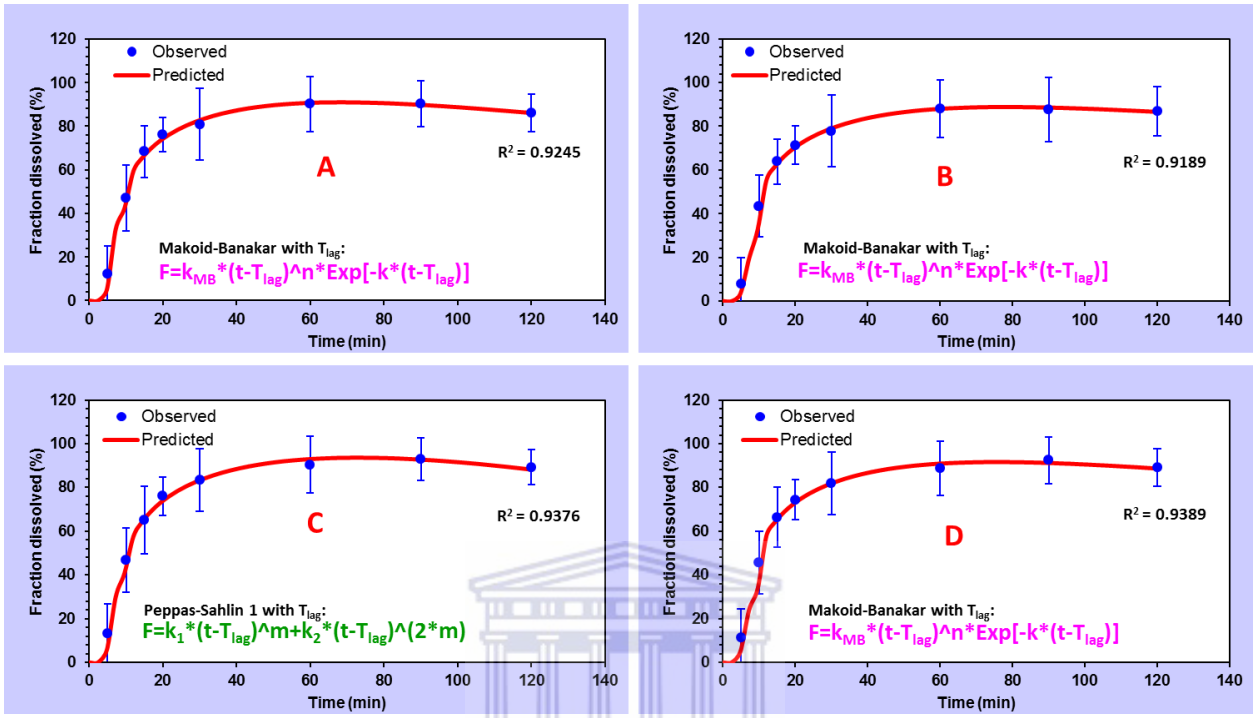


Figure 5.5.7: Models of best fit for in vitro release of sutherlandins A–D from S. frutescens LP at pH 6.8 . Results are Mean ± SD. Equations are for models of best fit for release of marker compounds from S. frutescens material.

From the SDAE material, release profiles of the marker compounds (sutherlandins A to D), like that from the LP material, did not meet the minimum specifications set for the R^2 value. The models of best fit for marker compounds release were the Weibull_4 model for sutherlandins A and C (R^2 = 0.9448 and 0.9399, respectively), the Weibull_3 model for sutherlandin B (R^2 = 0.9163), and the Peppas-Sahlin 1 with T_{lag} model for sutherlandin D (R^2 = 0.8889) (Figure 5.5.8). Due to the low R^2 values, dissolution data from the SDAE at this pH were not fit to the Korsmeyer-Peppas model for release mechanism elucidation.

The FDAE material showed good fit to many of the assessed models (R^2 > 0.9800) for the release of three out of the four marker compounds (sutherlandins A, B and C) at pH 6.8. The release of the markers was best described by the Makoid-Banakar with T_{lag} model for

sutherlandins A, B and D (R^2 = 0.9979, 0.9938 and 0.9955, respectively) and the Weibull_4 model for sutherlandin C (R^2 = 0.9948).

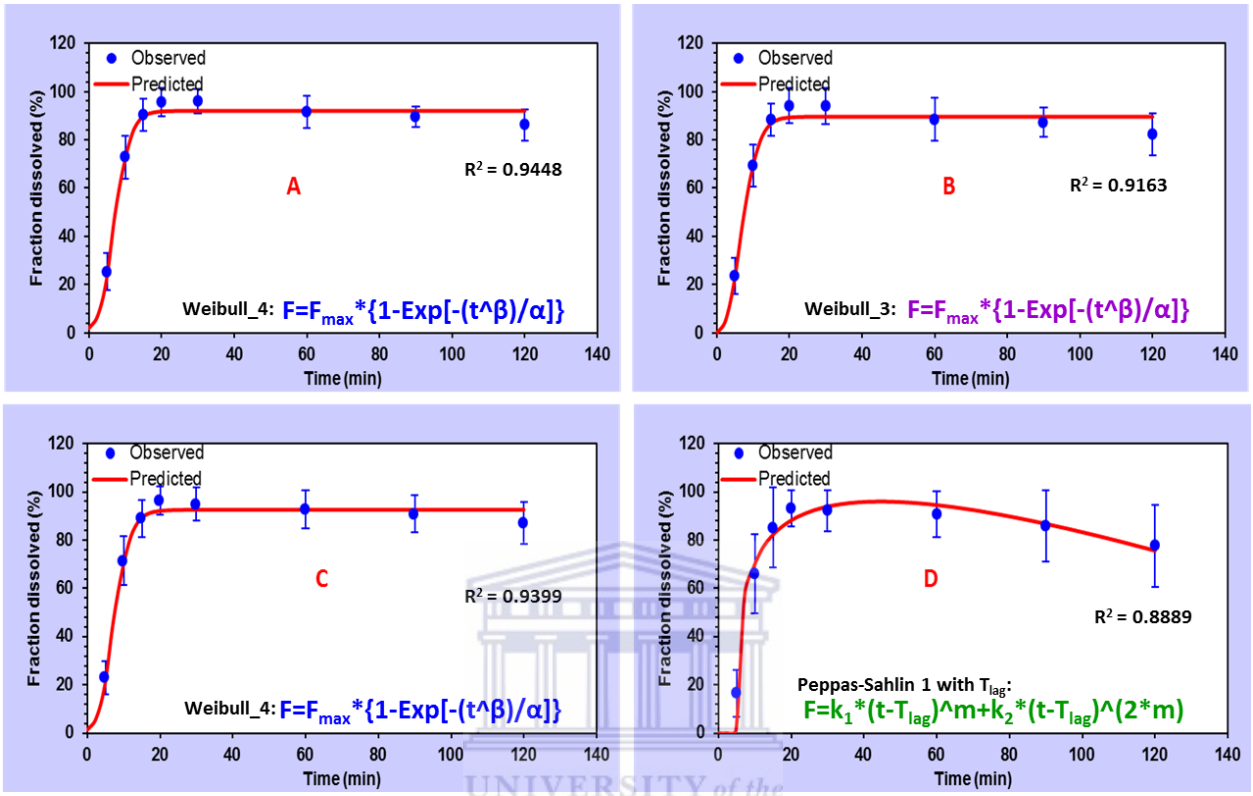


Figure 5.5.8: Models of best fit for in vitro release of sutherlandins A–D from S. frutescens SDAE at pH 6.8. Results are Mean ± SD. Equations are for models of best fit for release of marker compounds from S. frutescens material.

For the release of sutherlandins A, B and C, the Weibull models also met the required minimum R^2 value (i.e. 0.9800) for good model selection. Values of β for this model were all above 1 for sutherlandin A, B and C release from the FDAE material, implying that their release is by a complex mechanism. Release was however best described by the Makoid-Banakar model for sutherlandins A, B and D and by the Weibull_4 model for sutherlandin C (Figure 5.5.9).

Values of the Korsmeyer-Peppas release exponent for sutherlandins A, B, C and D were all above 0.89 (Appendix 4, Table 3), suggesting that release of these compounds from the FDAE material at pH 6.8 was neither *via* diffusion nor zero order kinetics but possibly by super case-II transport, a complex release mechanism.

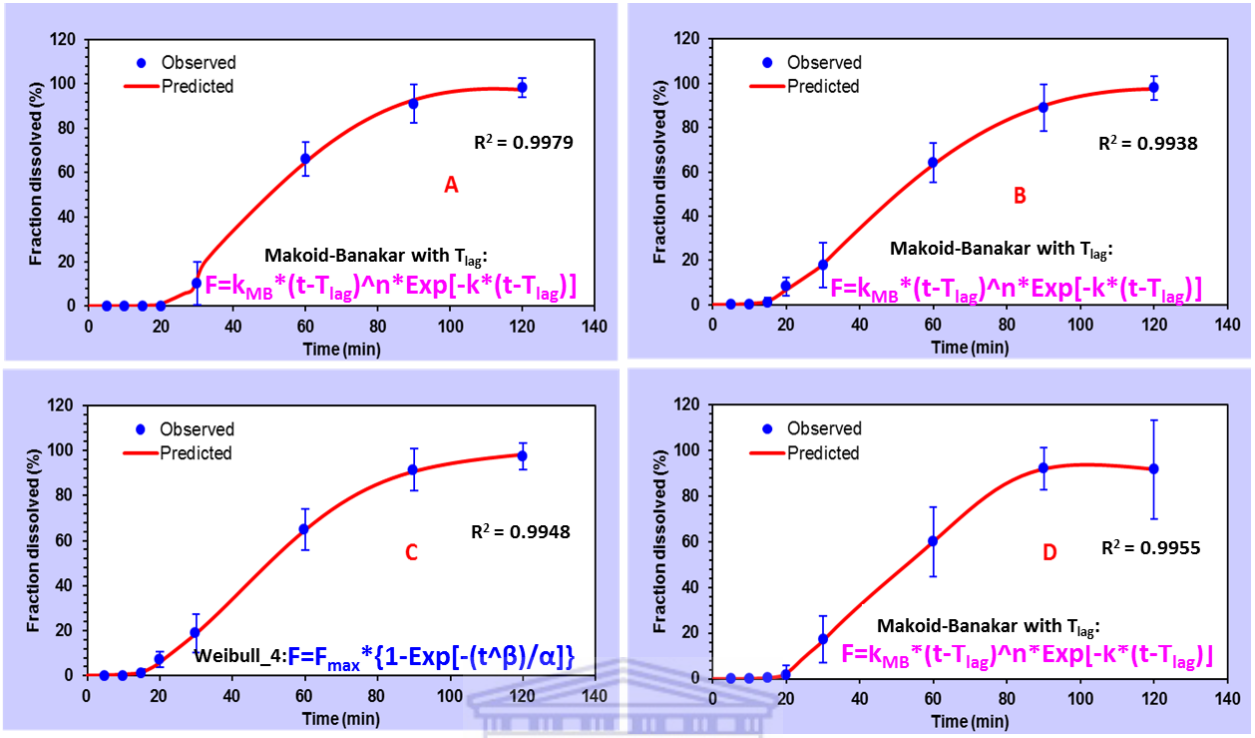


Figure 5.5.9: Models of best fit for in vitro release of sutherlandins A–D from S. frutescens FDAE at pH 6.8. Results are Mean ± SD. Equations are for models of best fit for release of marker compounds from S. frutescens material.

The results of curve-fitting studies suggest that the Weibull and Makoid-Banakar models are good mathematical models for describing sutherlandin release from *S. frutescens* materials, even in cases where the coefficient of determination (R^2) did not meet the minimum specifications set. Release characteristics of the marker compounds from the LP material at pH 4.5 and 6.8, and from the SDAE material at all three pH conditions, did not fit optimally to any of the models assessed and so could not be described.

The three pH conditions – 1.2, 4.5 and 6.8 – can be said to represent the prevailing pH conditions in the stomach prior to feeding, in the stomach after feeding and in the small intestine, respectively. As such, they give an indication of the release mechanisms in these regions of the gastrointestinal tract and how pH affects such release.

5.5.2 Conclusions

This section reports on the mechanism of release for the marker compounds, sutherlandins A to D, from *S. frutescens* LP, SDAE and FDAE materials. Generally, the empirical models for release description of each marker compound at a specific pH differed in the different *S. frutescens* materials. However, the release mechanisms were not Fickian but ranged from combined to complex mechanisms that could all be described by super-case II transport. The results showed that release was similar for different markers from the same material, in support of our third postulated hypothesis, and further confirming results of the PCA analysis reported in the previous section.

Based on the elucidated mechanisms of release, the marker compounds were all released from the different materials *via* a complex mechanism. It may thus be concluded that such release studies can be used in the analysis and verification of products that claim to contain *S. frutescens*. To differentiate *S frutescens* materials however, it may be necessary to determine the model of best fit as this, unlike the release mechanism, differed for different materials. Such differences may further serve to explain the differences in Q-release values and dissolution profiles of markers from different *S. frutescens* materials, and may likely translate to a difference in *in vivo* bioavailability and efficacy of formulations containing different materials. There may therefore be a need to implement tests such as these for quality control, especially in the formulation stages of *S. frutescens* products, in order to obtain products with desired release characteristics.

5.6 Prediction of *in vivo* bioavailability of selected flavonoids

The objective of this section was to assess the likelihood of flavonoid absorption after oral administration in humans. Chemical structures of reference and marker compounds employed in this study, and the computational resource, *Molecular Operating Environment (MoE*), were utilized to generate molecular properties which were assessed by Lipinski's Rule of 5 and the Rule of 3 in order to predict each reference or marker compound's expected bioavailability.[184,185] Such predictions will be compared to actual *in vivo* results obtained from the upcoming clinical trial.

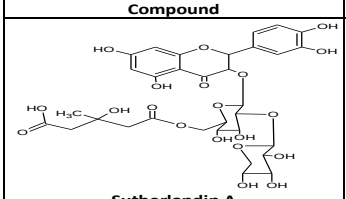
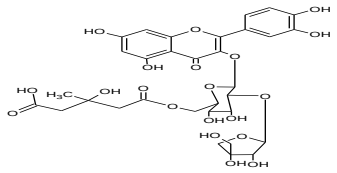
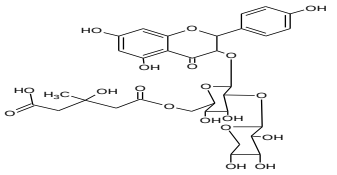
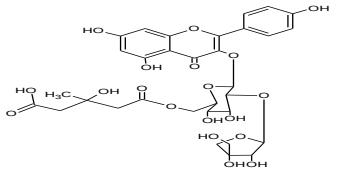
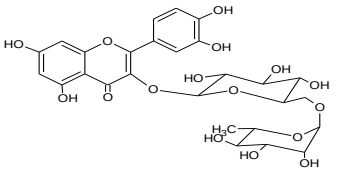
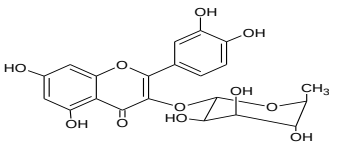
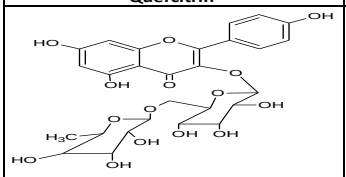
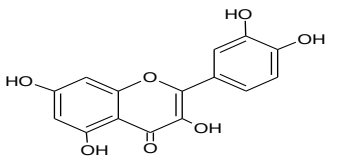
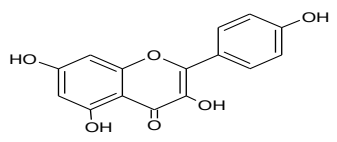
5.6.1 Molecular properties of flavonoids

Table 5.6.1 depicts the molecular properties of *S. frutescens* reference and marker compounds, which can be used to assess their bioavailability following oral administration.

The first four flavonoid glycosides (sutherlandins A, B, C and D) have 11 to 12 hydrogen bond donors, 17 to 18 hydrogen bond acceptors, molecular weights greater than 500 Daltons, and log P values less than 5. They can be seen to have violated more than one criterion in the Rule of 5, which makes them unlikely candidates for new drug development. Even with log P values less than 5, more than one rule has been violated and so these compounds may not be likely drug candidates. In any case, the said log P values for all the flavonoid glycosides are negative, indicating that they did not actually meet the criterion set for log P (≤ 5); compounds with negative log P values are too hydrophilic and unable to pass through membranes, since they hardly enter the hydrophobic interior (exemplified by the octanol phase in the octanol /water system) of the lipophilic lipid bilayer.[226] The three other flavonoid glycosides (rutin, quercitrin and kaempferol-3-*O*-rutinoside) also have molecular properties in excess of the specifications set by Lipinski *et al*.[184] The glycoside, quercitrin, even though it complied with the molecular mass and log P values, may not be bioavailable as more than one criteria of the Rule of 5 has been violated.

The flavonoid aglycones, on the other hand, have 4 to 5 hydrogen bond donors, 5 to 6 hydrogen bond acceptors, molecular mass of less than 500 Daltons and log P values less than 5, which makes them likely candidates for new drug development according to the Rule of 5.

Table 5.6.1: Molecular properties of reference and marker compounds

Compound	H-bond donors	H-bond acceptors	Molecular mass (Da)	log P	Rotatable bonds	Total polar surface area	Bioavailability status
 **Sutherlandin A**	12	18	740.62	-1.85	11	0	Not bioavailable
 **Sutherlandin B**	12	18	740.62	-1.90	12	0	Not bioavailable
 **Sutherlandin C**	11	17	724.62	-1.57	11	0	Not bioavailable
 **Sutherlandin D**	11	17	724.62	-1.63	12	0	Not bioavailable
 **Rutin**	10	16	612.54	-1.35	6	0	Not bioavailable
 **Quercitrin**	7	11	450.4	0.57	3	0	Not bioavailable
 **Kaempferol-3-*O*-rutinoside**	9	15	596.54	-1.08	6	0	Not bioavailable
 **Quercetin**	5	6	302.24	2.03	1	0	Bioavailable
 **Kaempferol**	4	5	286.24	2.3	1	0	Bioavailable

Applying the Rule of 3 for oral bioavailability assessment, all seven flavonoid glycosides can also be seen to have violated the rule in terms of the listed characteristics, while the flavonoid aglycones did not.

While the flavonoid glycosides have been shown to be good markers for content and dissolution studies of *S. frutescens,* they may not be orally bioavailable. This means that in plasma samples analysed during the forthcoming clinical trial, the flavonoid glycosides may not be detected. However, flavonoid glycosides can be converted to the aglycones on hydrolysis, contributing to the plasma profiles of the bioavailable aglycones. We can therefore hydrolyse flavonoid glycosides to aglycones, and determine the equivalent level of flavonoid aglycones represented by the flavonoid glycosides in each *S. frutescens* material. This can be compared to the actual levels of flavonoid aglycones detected in plasma in order to calculate the percentages of the marker compounds that are absorbed.

5.6.2 Conclusions

Identification of lead molecules with drug-like properties is still a major challenge in drug discovery settings. A computerised model incorporating various guidelines for drug discovery can assist in the identification of lead molecules with favourable properties early on in drug discovery, minimizing the chances of misses, and perhaps by-passing the need for *in vivo* tests. From the results obtained, the flavonoid aglycones of *S. frutescens* (quercetin and kaempferol), but not the flavonoid glycosides (sutherlandins A, B, C and D) met the Rule of 5 and the Rule of 3 criteria for *in vivo* bioavailability of orally administered compounds. They (the flavonoid aglycones) can thus be employed as marker compounds for *in vivo* studies of *S. frutecens* products. While the flavonoid glycosides may not be orally bioavailable, they can be quantified from flavonoid aglycone levels in hydrolysed samples.

CHAPTER SIX

CONCLUSIONS & RECOMMENDATIONS

Various formulations of the popular South African medicinal plant, *Sutherlandia frutescens*, are commercially available, with no documentation for their quality assessment. This study set out to develop quality control measures that can be employed in the control and regulation of *S. frutescens* products.

The objectives of this study were:

- to isolate and identify flavoniod glycosides (sutherlandins A, B, C and D) as marker compounds for *S. frutescens*,
- to develop and validate an HPLC assay for flavonoids in *S. frutescens*,
- to compare flavonoid content of different *S. frutescens* materials,
- to compare *in vitro* dissolution profiles and release mechanisms of flavonoids from different *S. frutescens* materials, and
- to predict *in vivo* bioavailability of *S. frutescens* flavonoids.

From the results obtained, the following findings were made:

- Sutherlandins A, B, C and D were successfully isolated from *S. frutescens* SDAE material, with percentage purity approximately 99 % for sutherlandins A, C and D, and 90 % for sutherlandin B, as determined from HPLC analysis. These compounds can be used for HPLC method development, as well as for quantification studies of sutherlandins in different materials.

- An HPLC assay was successfully developed and validated for flavonoid glycosides and their corresponding aglycones in *S. frutescens* materials. The developed HPLC method is simple, precise and robust, and was further applied for flavonoid quantification in different *S. frutescens* materials. Validation parameters for the developed HPLC method were within acceptable limits. The flavoniod glycosides

were found to be good marker compounds for *S. frutescens* materials, and their levels were more than the levels of the flavonoid aglycones in the materials assessed, supporting our proposed hypothesis that flavonoid glycoside content exceeds flavonoid aglycone content. The percentages of the flavonoid glycosides were at least 0.5 % in all the samples assayed with the exception of sutherlandin A content in FDAE 1 and FDAE 3 which were 0.203 ± 0.002 % and 0.073 ± 0.00 %, respectively. The difference in content of each flavonoid compound in the different materials was found to be statistically significant ($p < 0.001$), and so flavoniod content levels can be used to differentiate *S. frutescens* materials.

- A dissolution test was utilized for *S. frutescens* materials, with the flavonoids as marker compounds. A Q-value of 75 % was attained within 45 minutes for all the flavonoid glycosides from the LP and SDAE materials, implying that release and dissolution from these materials was immediate. From the FDAE sample, a Q-value of 75 % was attained in 90 minutes at the earliest, implying delayed release and dissolution from this material. The f_2 values for each flavonoid glycoside compound from the different materials were all below 50, thus implying dissimilarity of dissolution profiles. The flavonoid aglycones, on the other hand, could not be detected in some of the materials and so may not be ideal markers for *S. frutescens* dissolution studies. The dissolution test established differences in flavonoids dissolution from different *S. frutescens* materials and so can be employed for their quality control.

- Different mathematical models described the release of each flavonoid glycoside (sutherlandin) from the different *S. frutescens* materials. Release mechanisms also differed for each sutherlandin in the different *S. frutescens* materials, but were similar for different sutherlandins from the same material. This indicates that release of the flavonoid glycosides depends more on the *S. frutescens* material than on the characteristics of the marker compounds. Therefore, release mechanisms of all four sutherlandins (A, B, C and D) can be employed for identification of *S. frutescens* materials as they will be similar in all *S. frutescens* materials. Following such

identification, the mathematical model of best fit for any of the sutherlandins can be used to differentiate between *S. frutescens* materials.

- The flavonoid aglycones of *S. frutescens* (quercetin and kaempferol), but not the flavonoid glycosides (sutherlandins A, B, C and D, rutin, quercetrin and kaempferol-3-*O*-rutinoside) met the criteria for *in vivo* bioavailability of orally administered compounds. This shows that the flavonoid aglycones, rather than the flavonoid glycosides, will be orally bioavailable. In *S. frutescens* samples, bioavailability of the flavonoid glycosides can thus be assessed by hydrolysing such flavonoid glycosides to their corresponding flavonoid aglycones.

Overall, this study provides methods and valuable preliminary data that can be used for quality assessment of *S. frutescens* materials. In line with our findings, the following are recommended:

- For quality assessment of *S. frutescens* materials, the HPLC method here reported can be employed as it is sensitive enough to detect differences in *S. frutescens* materials.
- The dissolution test so reported also establishes differences between different *S. frutescens* materials, and gives an indication of the release mechanisms of flavonoid marker compounds from these materials. As such, it can be employed for the quality control of *S. frutescens* materials.

The problems encountered with quality control and assessment of *S. frutescens* materials are however not peculiar to *S. frutescens* alone, but can be experienced with any other herbal material or product. Therefore, the HPLC and dissolution methods herein described for flavonoids in *S. frutescens* can be employed for the quality assessment and control of other flavonoid-containing herbal materials. Flavonoids are known to be very common in plant materials and so such methods and tests as reported here will assist to a great extent in herbal product quality control and regulation, thereby improving the quality and efficacy of such products.

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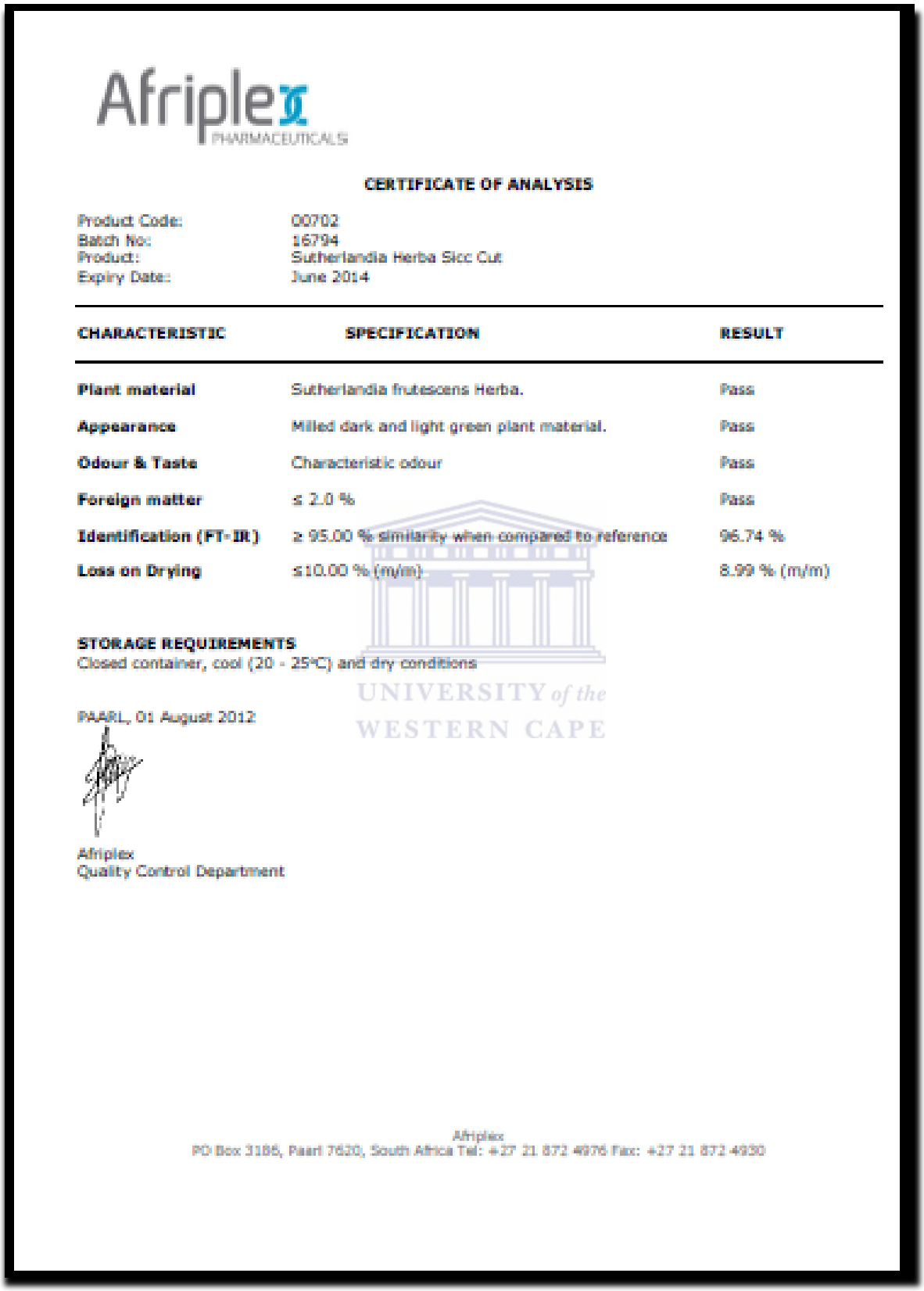
Appendix 1: Certificates of analysis (CoA) for *S. frutescens* materials



Afriplex
PHARMACEUTICALS

CERTIFICATE OF ANALYSIS

Product Code: 00702
Batch No: 16794
Product: Sutherlandia Herba Sicc Cut
Expiry Date: June 2014

CHARACTERISTIC	SPECIFICATION	RESULT
Plant material	Sutherlandia frutescens Herba.	Pass
Appearance	Milled dark and light green plant material.	Pass
Odour & Taste	Characteristic odour	Pass
Foreign matter	≤ 2.0 %	Pass
Identification (FT-IR)	≥ 95.00 % similarity when compared to reference	96.74 %
Loss on Drying	≤10.00 % (m/m)	8.99 % (m/m)

STORAGE REQUIREMENTS
Closed container, cool (20 - 25°C) and dry conditions

PAARL, 01 August 2012

Afriplex
Quality Control Department

Afriplex
PO Box 3185, Paarl 7620, South Africa Tel: +27 21 872 4976 Fax: +27 21 872 4930

Figure A 1.1: Certificate of analysis for leaf powder (LP) material, batch number: E 16794

South African Herbal Science and Medicine Institute (SAHSMI)

Tel: +27-21-959 3033
Fax: +27-21-959 1383

Certificate of Analysis

Product: **Spray-dried Sutherlandia capsules**
Product Code: Active Material (Batch - CSIR-SUT/UWC of 30/11/2010)
Manufacturer: Ferlot Manufacturing & Packaging (Pty) Ltd, Jeffreys Bay, SA
Production Batch #: FMPL 123
Trial Batch No: Ferl-DST/001-1210
Trial Batch Size: 18 700
Date of Manufacture: 07 December 2010
Expiry Date: December 2012
Pharmaceutical evaluation by: SAHSMI & Pharmaceutics Lab, UWC and CSIR Bioscience

Characteristic	Method /Test	Result/ Comment
1. Description of Dosage form	Visual inspection:	Size 0 with white body and green cap
2. Description of Contents	Visual inspection:	Odour: slight mouldy plant odour Texture: homogenous fine powder, not free flowing, dry but becomes sticky upon exposure to air (hygroscopic) Colour: light brown
3. Contents Formula	Taken from production Sheets	Spray- dried Sutherlandia extract 100%
4. Mass uniformity	Weighed contents of randomly selected capsules (n = 11) *Filled weight* *Capsule contents*	Ave Mass ± SD = 0.4626 ± 0.03023g %RSD = 6.53 % *Complies* Ave Mass ±SD = 0.3682 ± 0.03006g %RSD = 6.67 % *Complies*
5. Moisture Content (MC)	Lab: School of Pharmacy, UWC Determined with HR Halogen moisture analyzer (Mettler Toledo)	Ave MC ± SD = 5.37 ± 0.45 % *Complies*

UWC: A Place of Quality, A Place to Grow
C:\11James Syce L1 -11-08-10\11James Syce L1\DST\DST Sutherlandia Biopharm Project DST001\11DST001 Trial Product CoA Spray Dried Sutherlandia caps - 17 aug.doc 2011
Page 1 of 3
Confidential 8/18/2011

Figure A 1.2: Certificate of analysis for spray-dried aqueous extract (SDAE) material, batch number: Ferl-DST/001-1210

Afriplex
SOURCE TO SHELF

CERTIFICATE OF ANALYSIS

Product Code	00814
Batch No.	62265
Product	Sutherlandia PE
Expiry date	May 2015

Characteristic	Specification	Result
Plant material	*Sutherlandia frutescens* herba sicc	Pass
Appearance	A hygroscopic powder with a mustard brown colour	Pass
Odour & Taste	Characteristic with a bitter taste	Pass
Country of origin	South Africa	Pass
Solubility	≥95% Soluble in water	Pass
Moisture	<7% (m/m)	6% (m/m)
Bulk Density	0.2 – 0.5 g/ml	0.4 g/ml
Identity (FT-IR)	≥95.00% similarity when compared to reference	97.57%
Heavy metals		
Lead	<5.0 mg/kg	Not Detected
Cadmium	<0.5 mg/kg	Not Detected
Mercury	<0.1 mg/kg	Not Detected
Arsenic	<1.00 mg/Kg	Not Detected
Microbiological		
Total viable aerobic count	<2000cfu/g	1305 cfu/g
Yeast & Moulds	<100cfu/g	No growth/g
Escherichia coli	Absent/g	No growth/g
Staph. aureus	Absent/g	No growth/g
Salmonella	Absent/25 g	Absent/25 g

STORAGE REQUIREMENTS
Closed container, cool (10 - 25°C) and dry conditions

PAARL, 20 Jun 2013

Afriplex
Quality Control Department

Afriplex
PO Box 3186, Paarl 7620, South Africa Tel: +27 21 872 4976 Fax: +27 21 872 4930

Figure A 1.3: Certificate of analysis for freeze-dried aqueous extract (FDAE) material 2, batch number: E 62265

Afriplex
SOURCE TO SHELF

CERTIFICATE OF ANALYSIS

Product Code	00814
Batch No.	63067
Product	Sutherlandia PE
Expiry date	Jun 2016

Characteristic	Specification	Result
Plant material	*Sutherlandia frutescens* herba sicc	Pass
Appearance	A hygroscopic powder with a mustard brown colour	Pass
Odour & Taste	Characteristic with a bitter taste	Pass
Country of origin	South Africa	Pass
Solubility	≥95% Soluble in water	Pass
Moisture	<7.0% (m/m)	2.9% (m/m)
Bulk Density	0.20 – 0.50 g/ml	0.45 g/ml
Identity (FT-IR)	≥95.00% similarity when compared to reference	97.30%
Heavy metals		
Lead	<5.0 mg/kg	Not Detected
Cadmium	<0.5 mg/kg	Not Detected
Mercury	<0.1 mg/kg	Not Detected
Arsenic	<1.00 mg/Kg	Not Detected
Microbiological		
Total viable aerobic count	<2000cfu/g	9 cfu/g
Yeast & Moulds	<100cfu/g	No growth/g
Escherichia coli	Absent/g	No growth/g
S. aureus	Absent/g	No growth/g
Salmonella	Absent/25 g	Absent/25 g

STORAGE REQUIREMENTS
Closed container, cool (10 - 25°C) and dry conditions

PAARL, 29 Jul 2013

Afriplex
Quality Control Department

Afriplex
PO Box 3186, Paarl 7620, South Africa Tel: +27 21 872 4976 Fax: +27 21 872 4930

Figure A 1.4: Certificate of analysis for freeze-dried aqueous extract (FDAE) material 3, batch number: E 63067

Appendix 2: UV spectra of the first four fractions from *S. frutescens* material

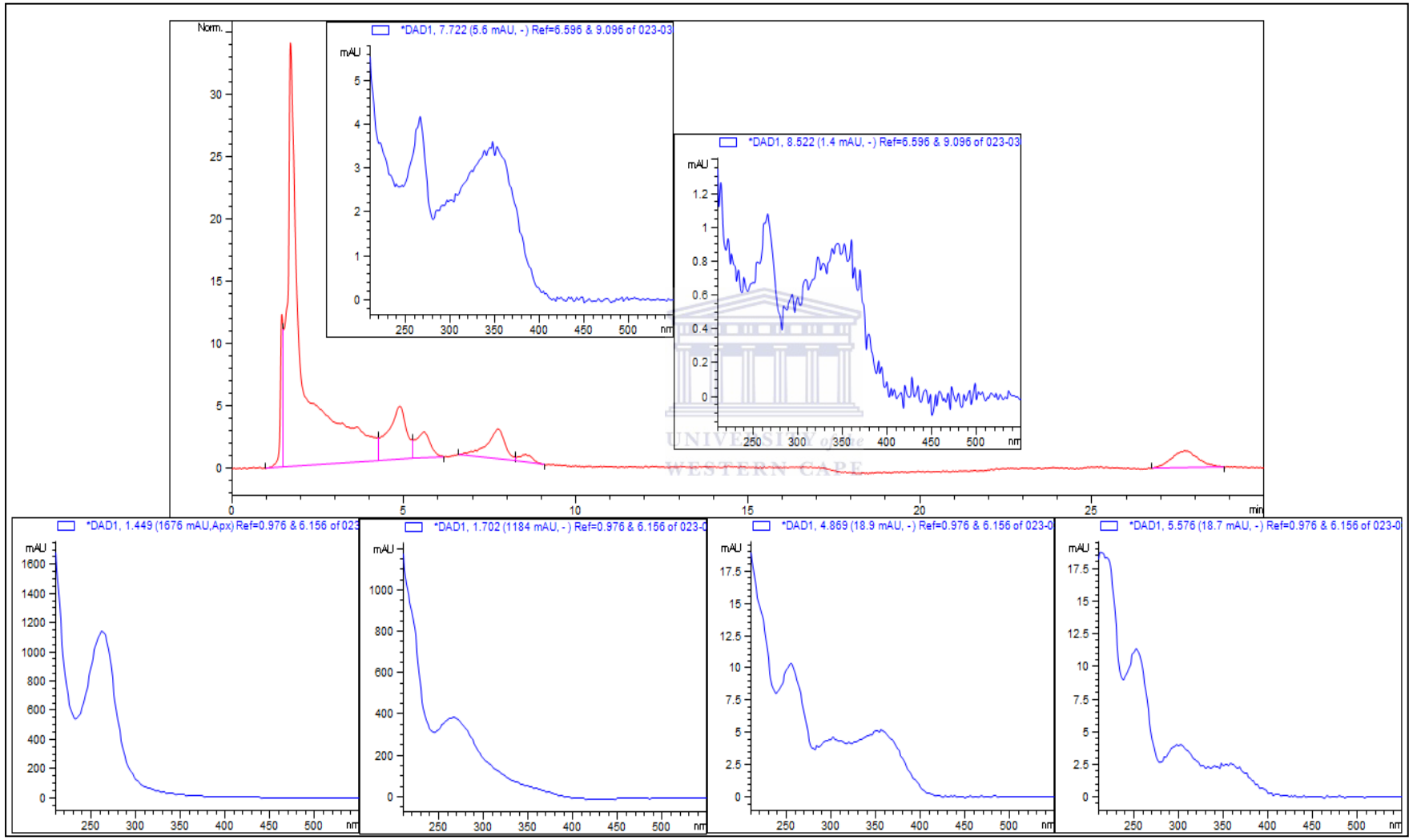


Figure A 2.1: Fraction 1; 0% acetonitrile

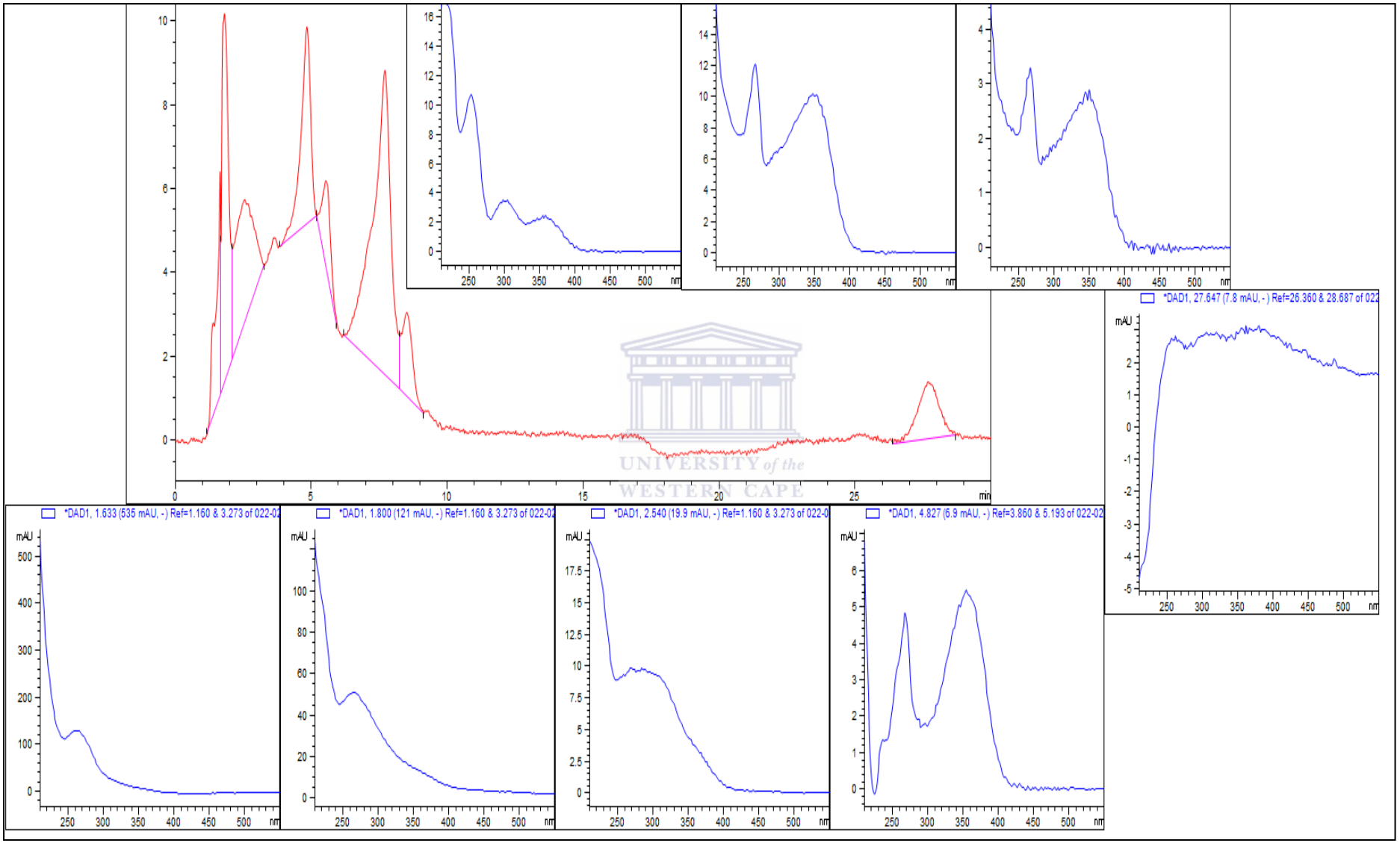


Figure A 2.2: Fraction 2; 20% acetonitrile

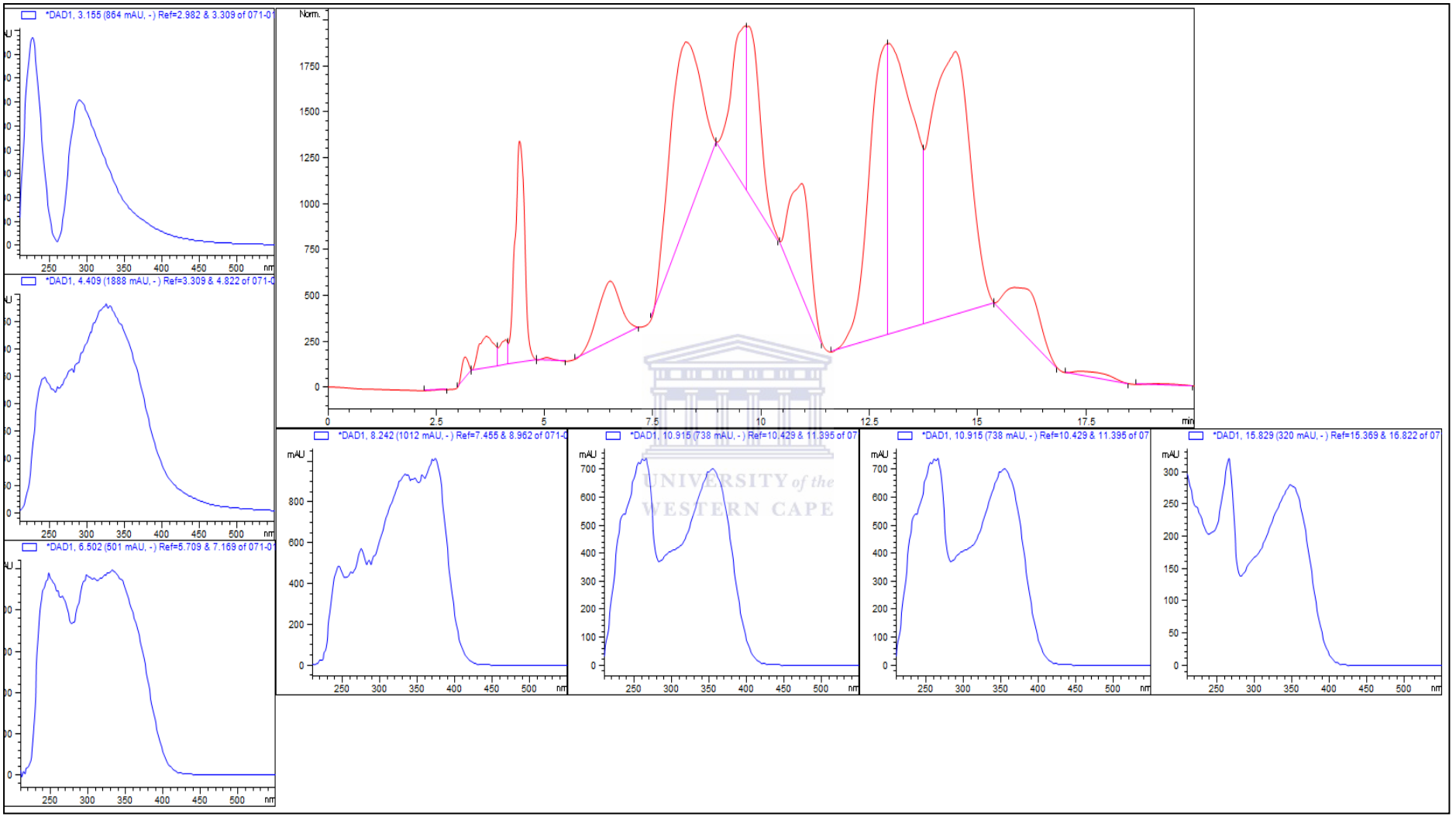


Figure A 2.3: Fraction 3; 40% acetonitrile

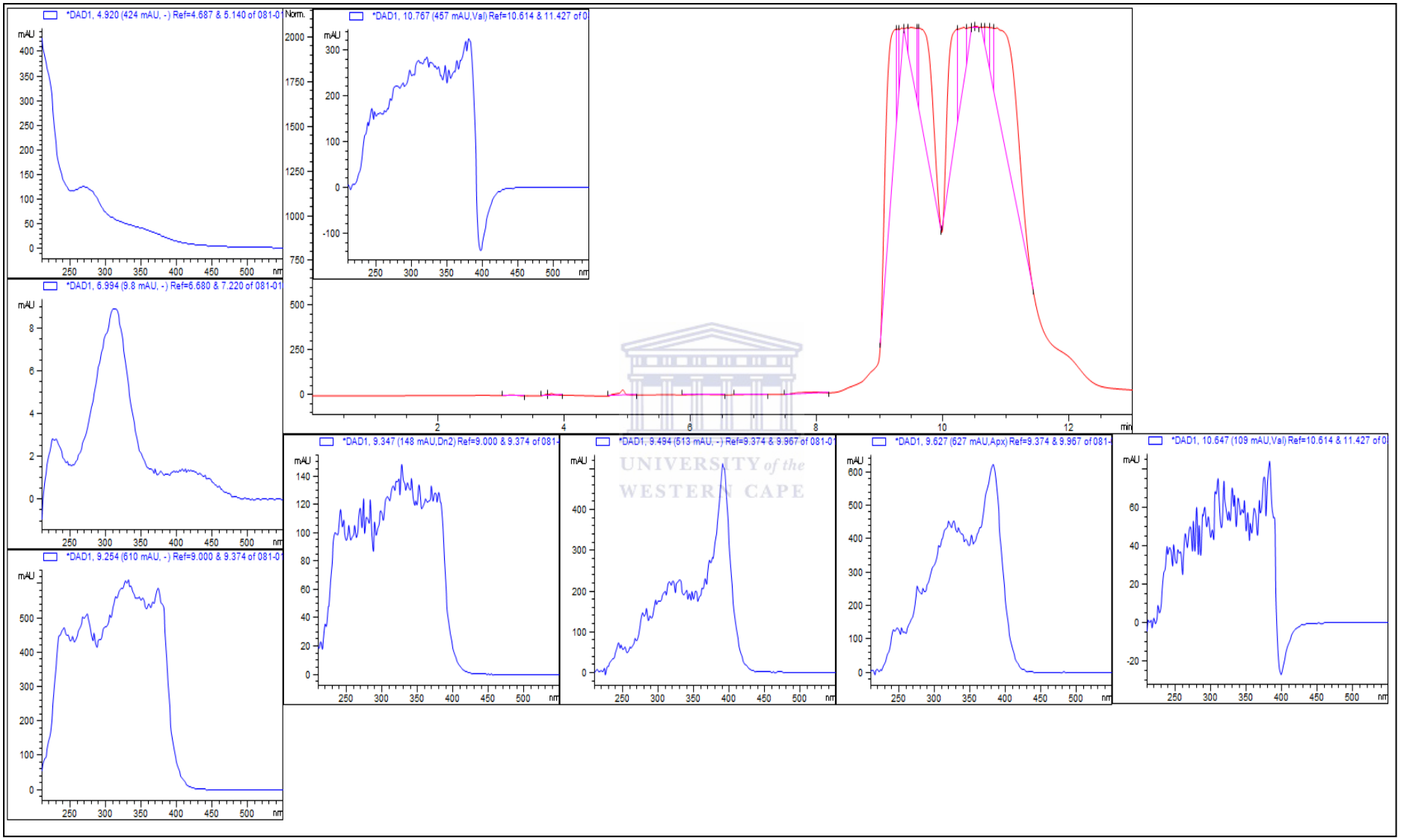


Figure A 2.4: Fraction 4; 60% acetonitrile

Appendix 3: NMR spectra of flavonoid glycosides isolated from *S. frutescens* matrerials

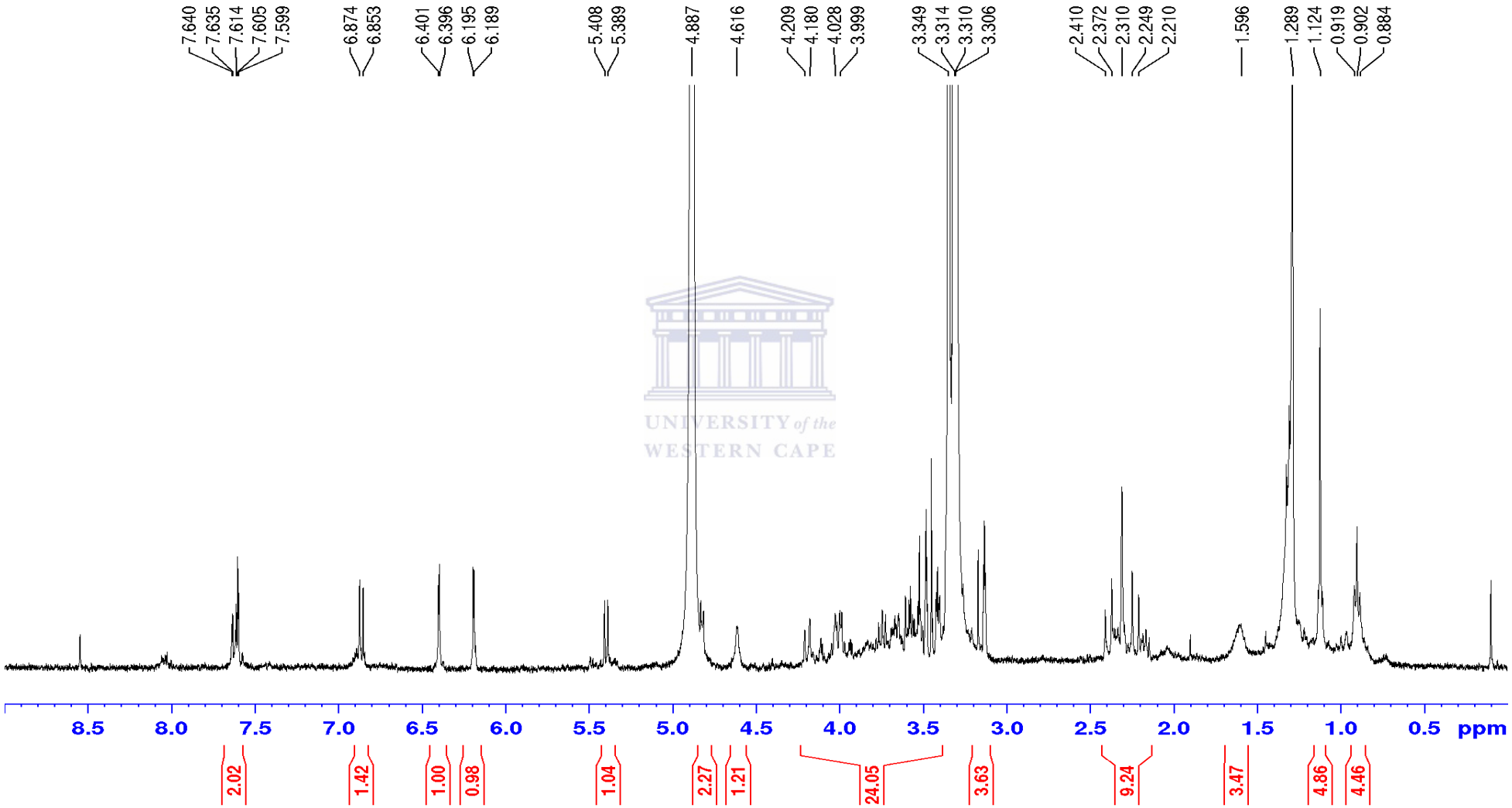


Figure A 3.1: NMR spectrum of sutherlandin A in methanol-d4 (CD_3OH)

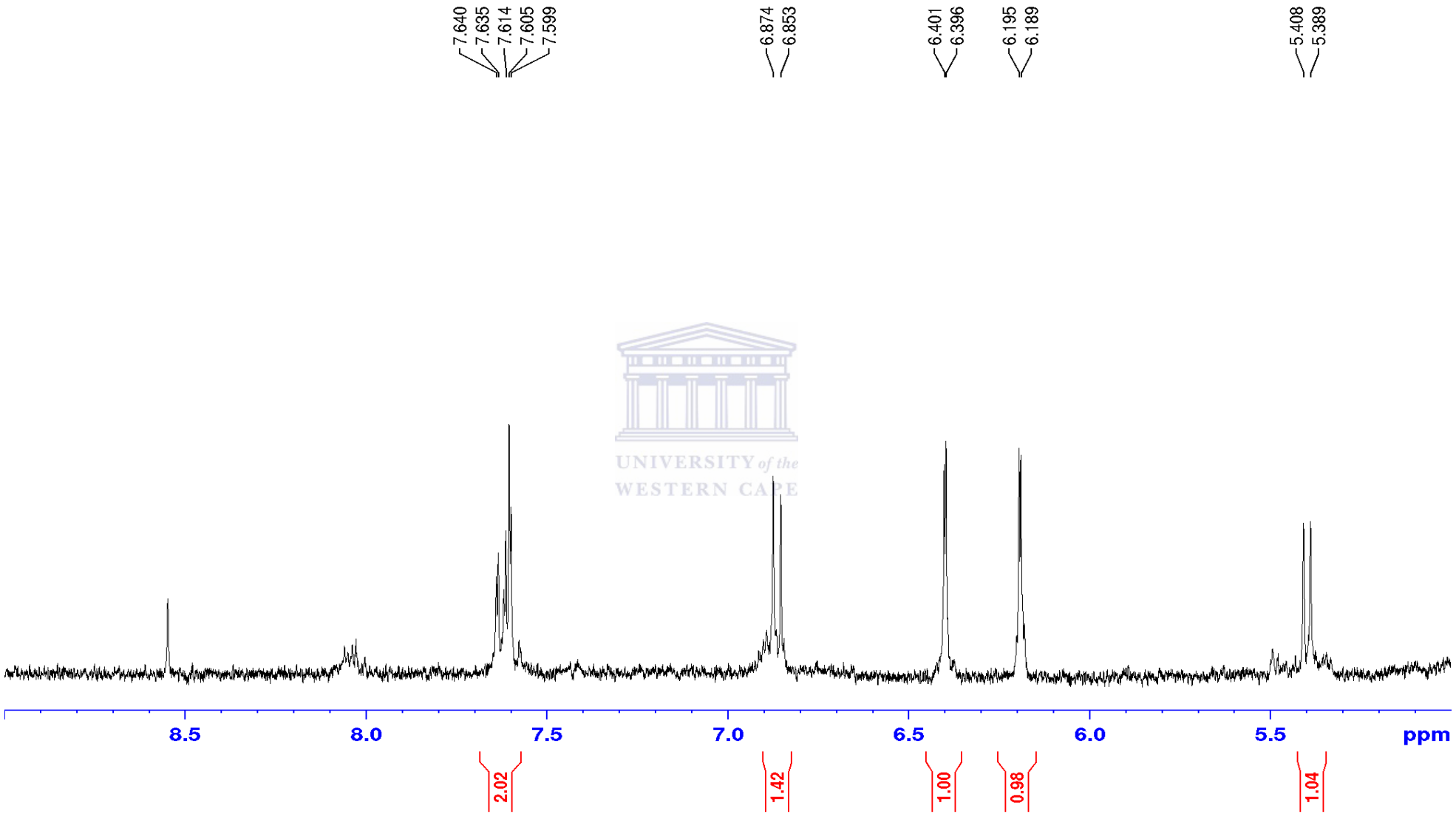


Figure A 3.2: **NMR spectrum of sutherlandin A in methanol-d4 (CD_3OH)**

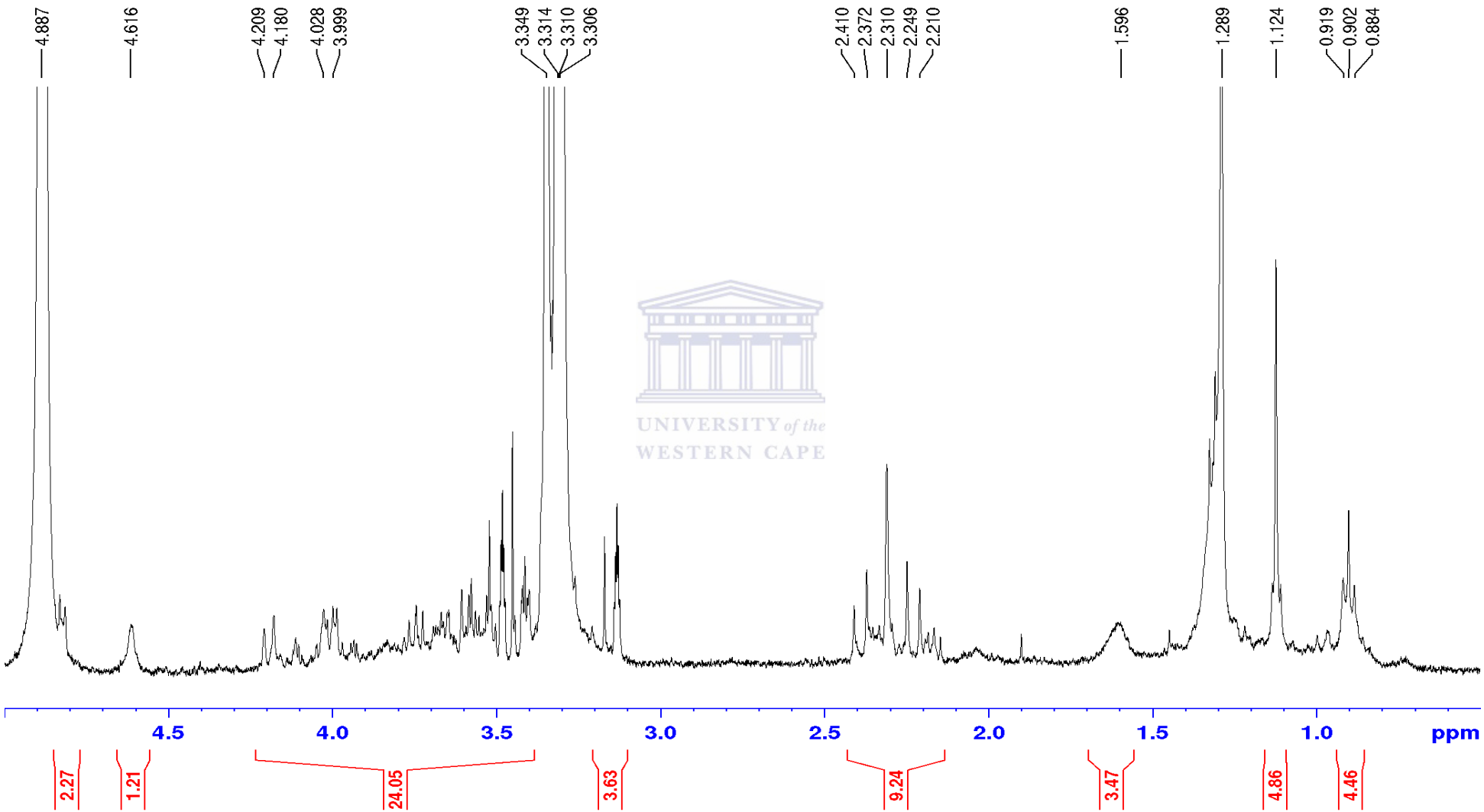


Figure A 3.3: **NMR spectrum of sutherlandin A in methanol-d4 (CD_3OH)**

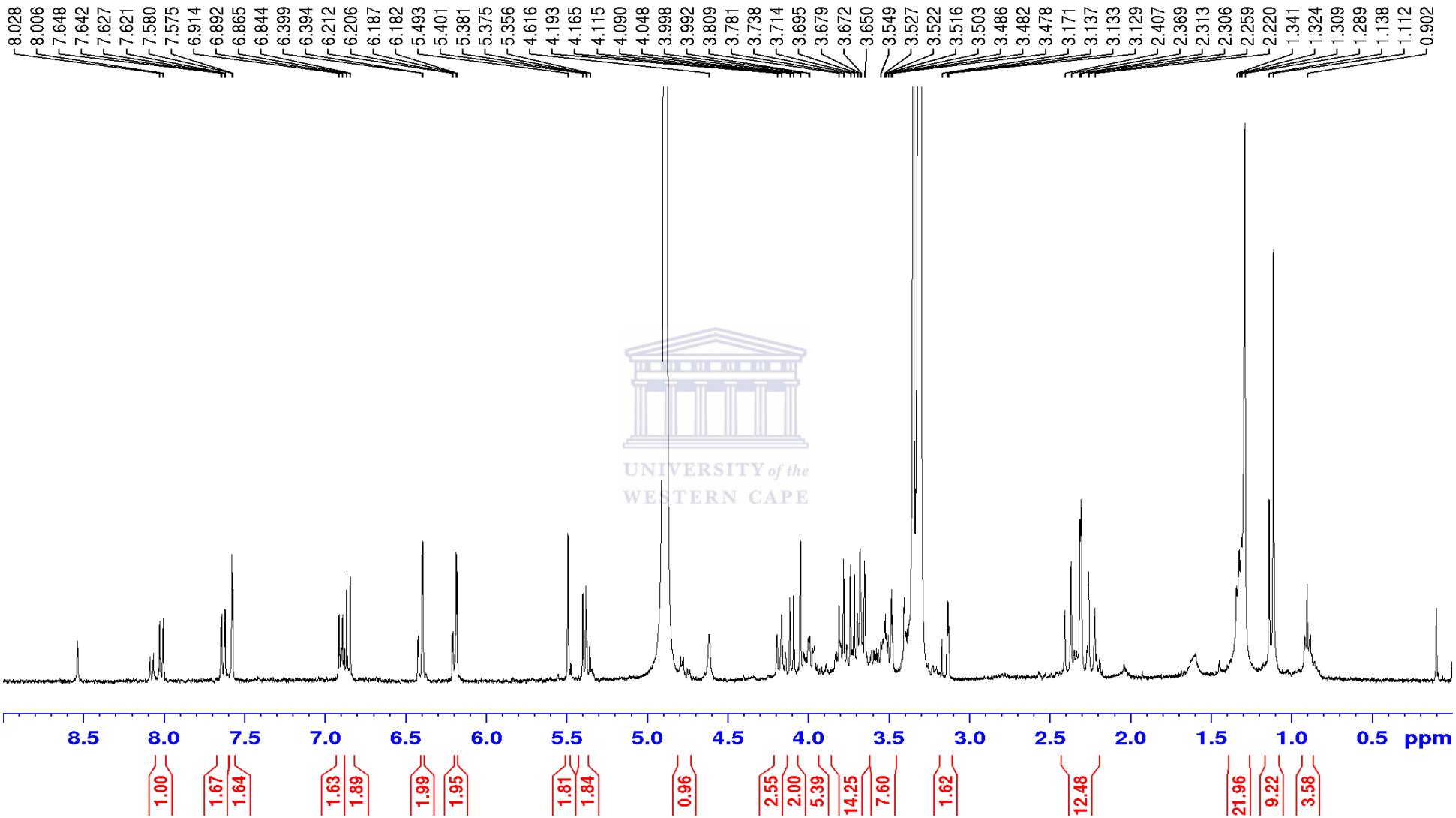


Figure A 3.4: **NMR spectrum of sutherlandin B in methanol-d4 (CD_3OH)**

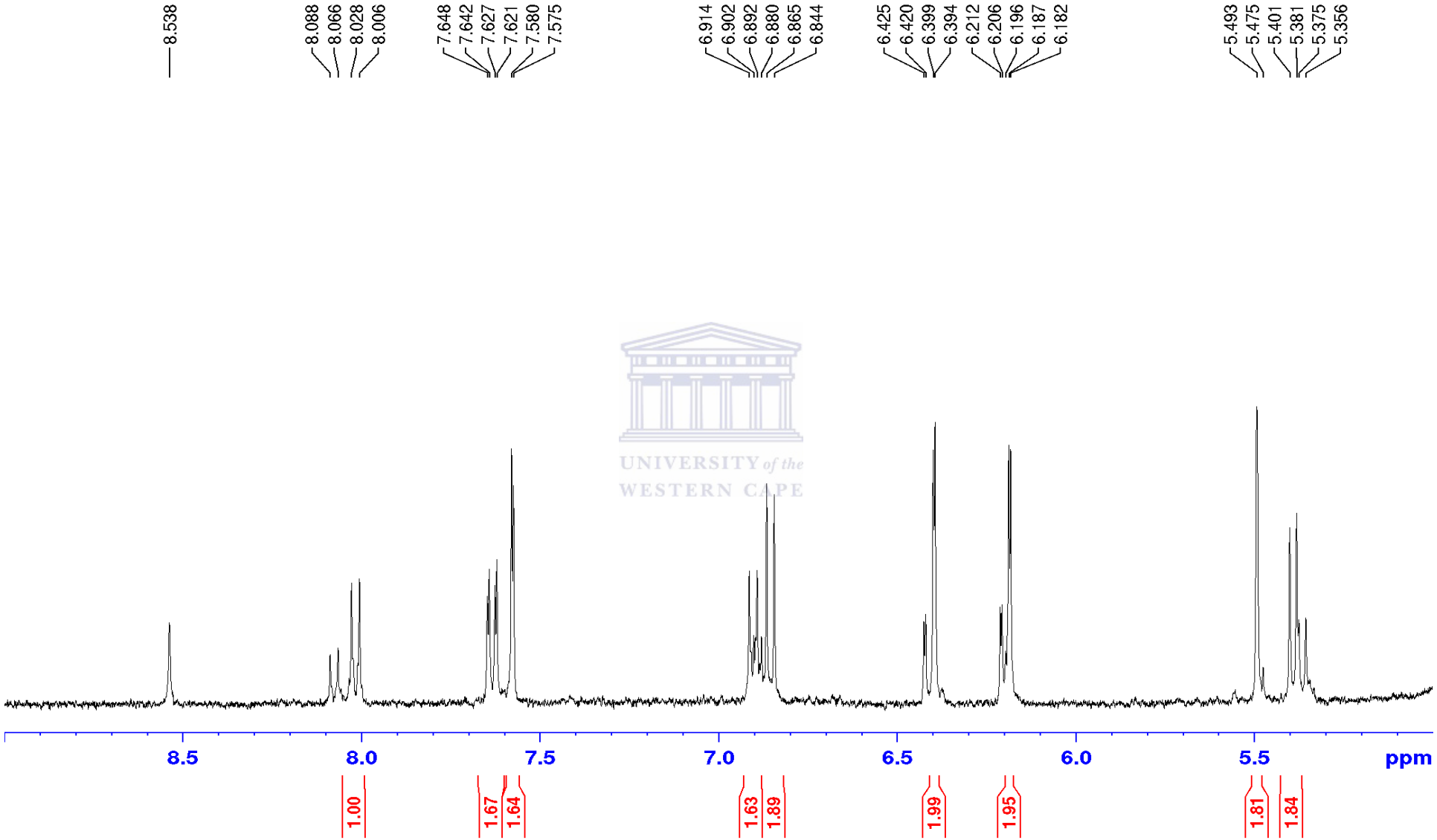


Figure A 3.5: **NMR spectrum of sutherlandin B in methanol-d4 (CD_3OH)**

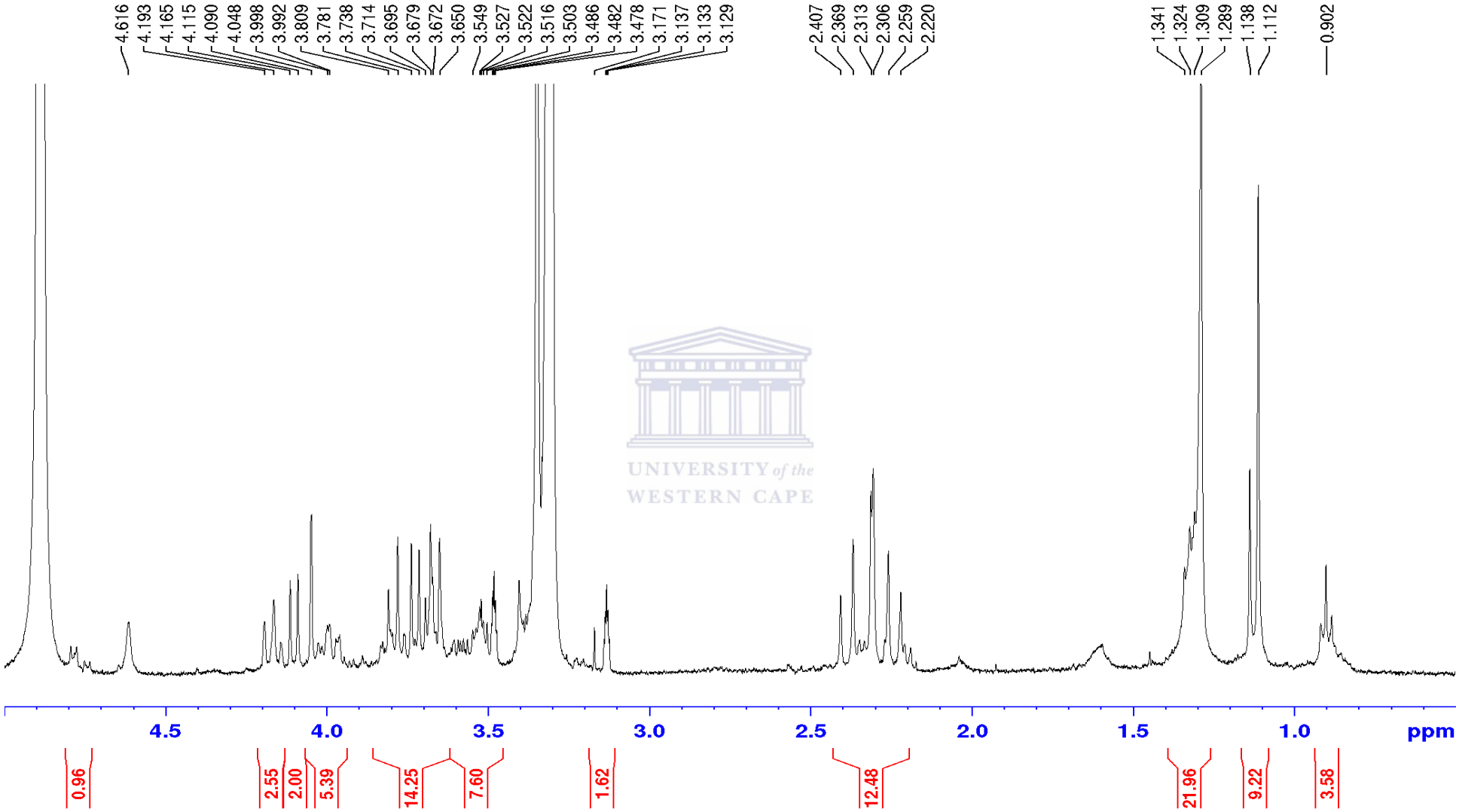


Figure A 3.6: **NMR spectrum of sutherlandin B in methanol-d4 (CD_3OH)**

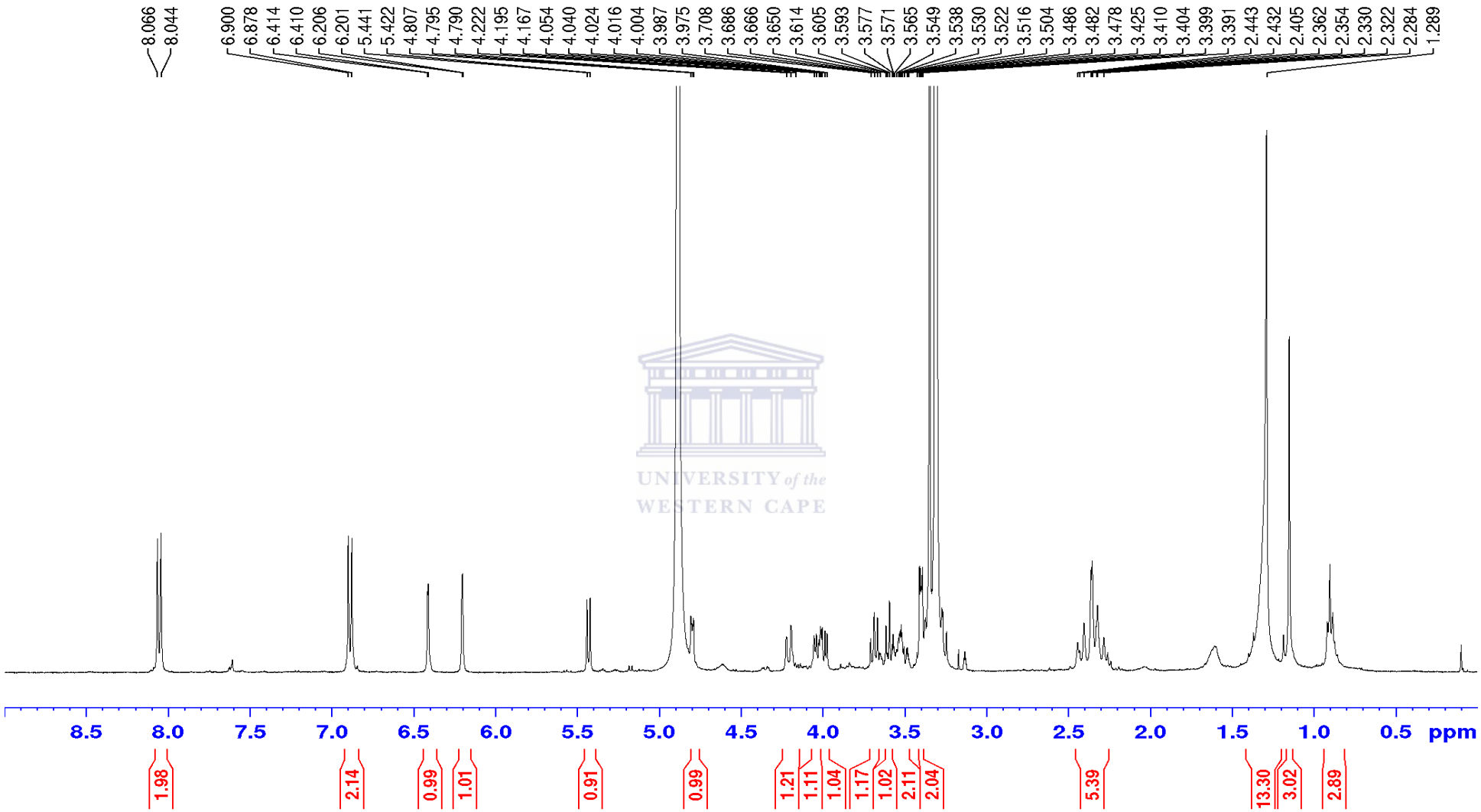


Figure A 3.7: **NMR spectrum of sutherlandin C in methanol-d4 (CD_3OH)**

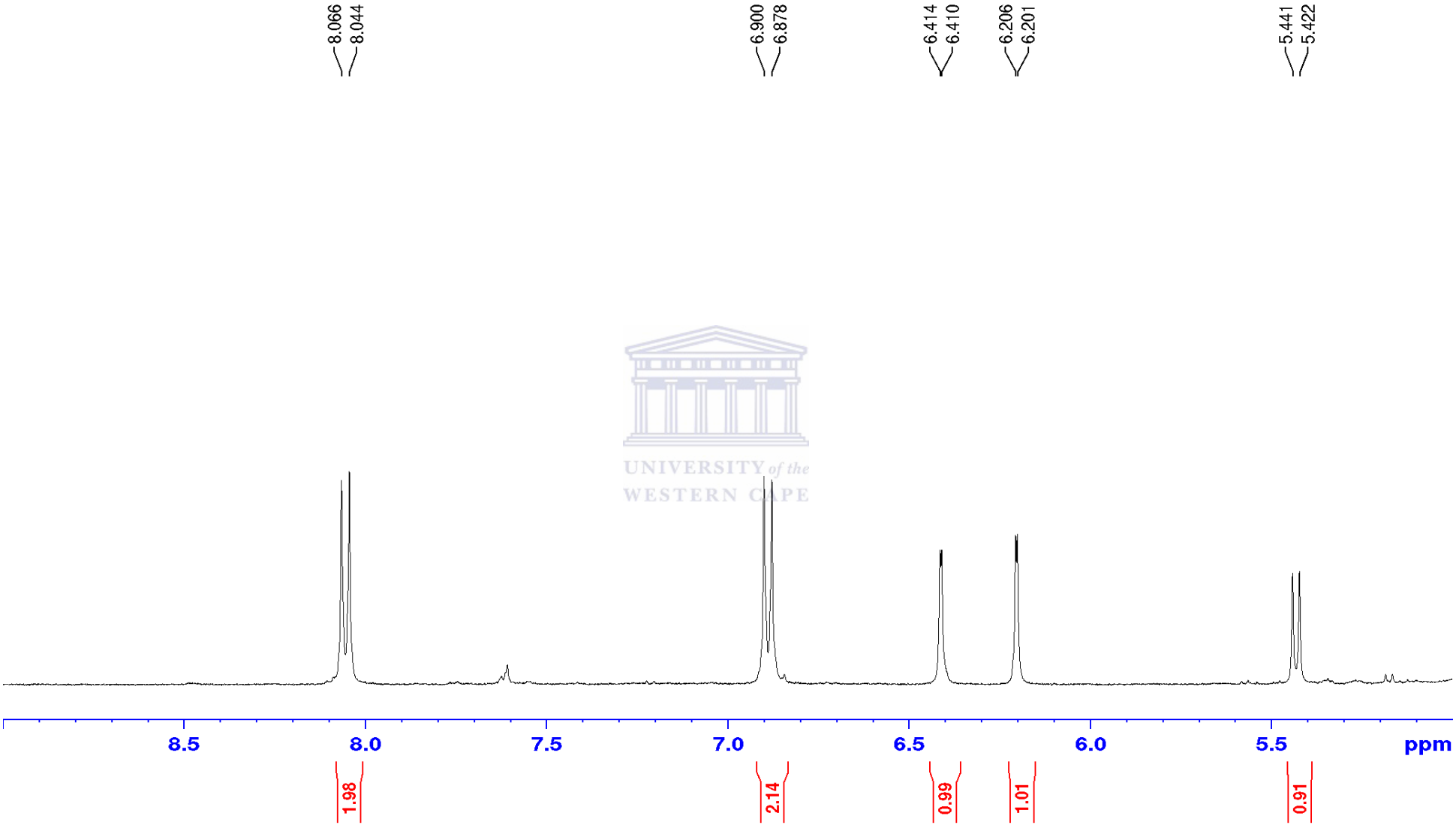


Figure A 3.8: **NMR spectrum of sutherlandin C in methanol-d4 (CD_3OH)**

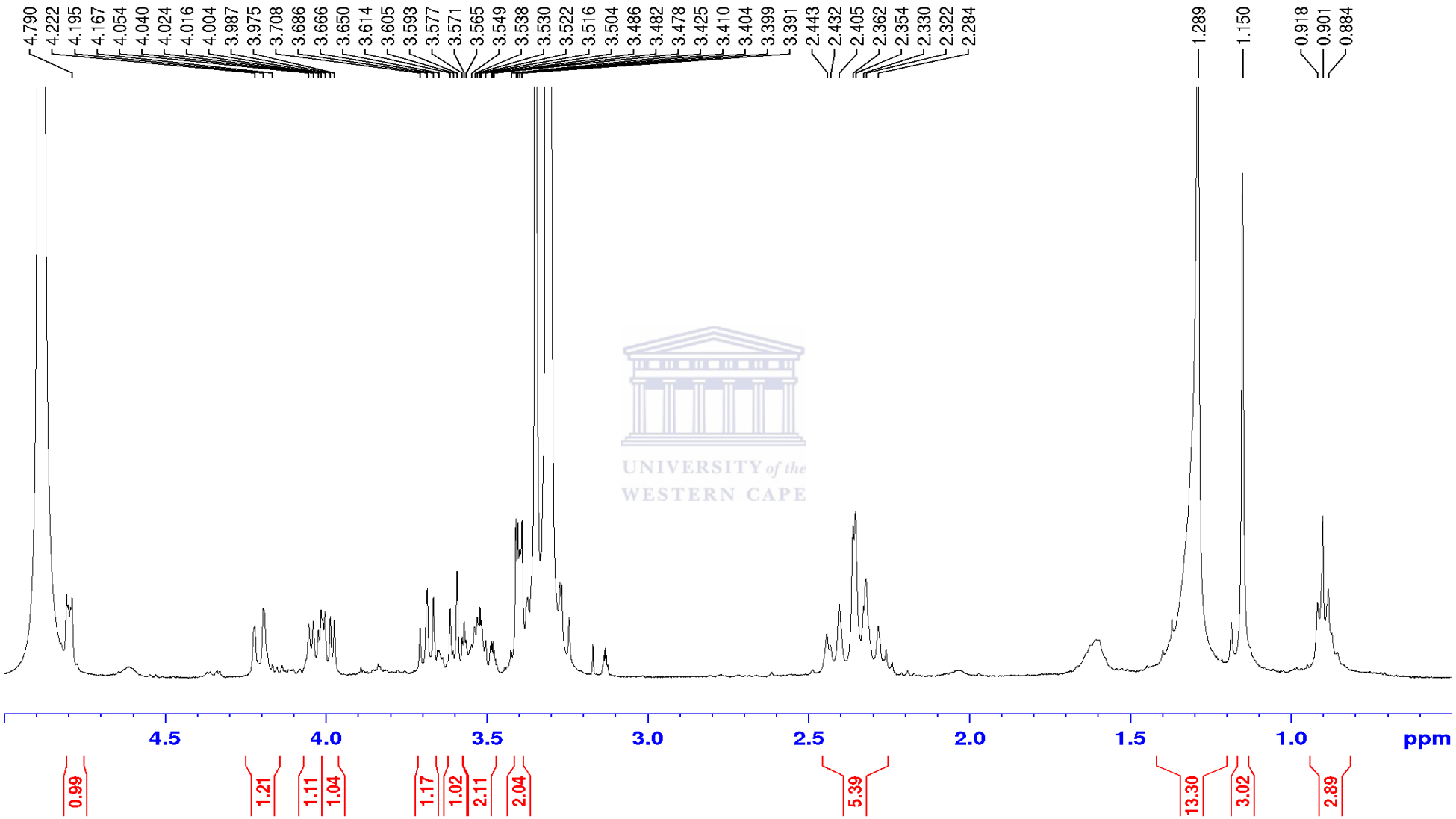


Figure A 3.9: **NMR spectrum of sutherlandin C in methanol-d4 (CD_3OH)**

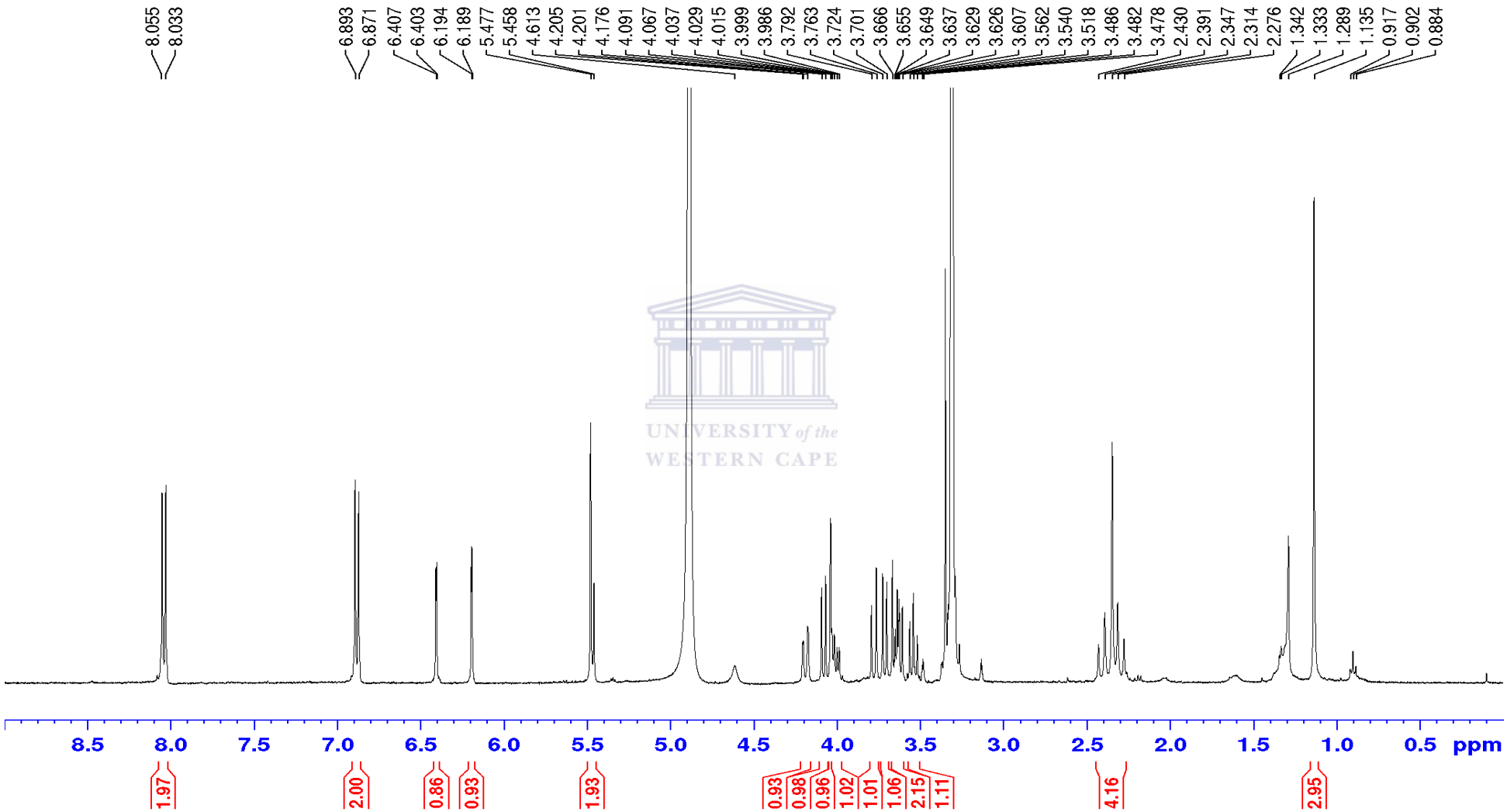


Figure A 3.10: **NMR spectrum of sutherlandin D in methanol-d4 (CD_3OH)**

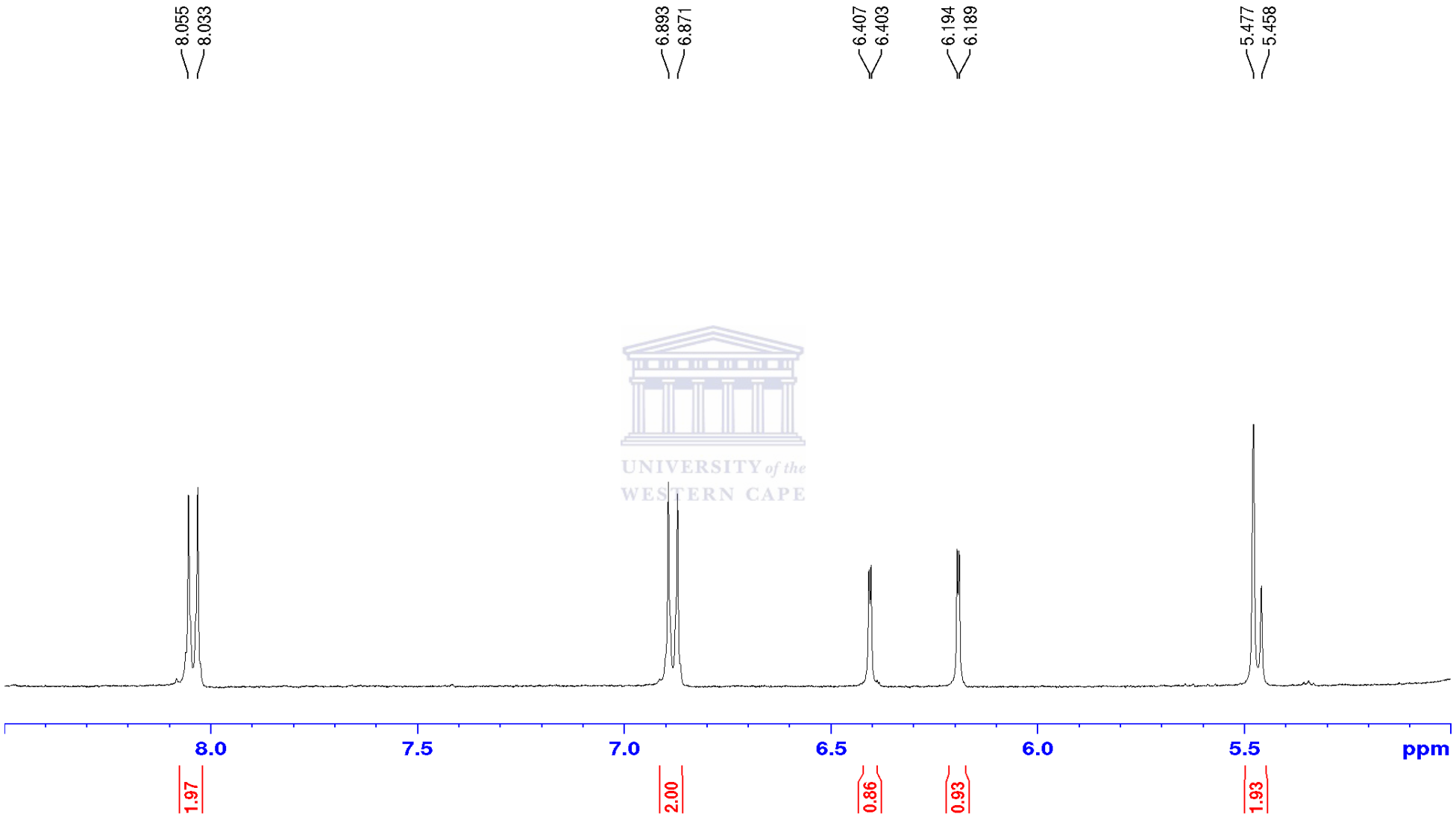


Figure A 3.11: **NMR spectrum of sutherlandin D in methanol-d4 (CD_3OH)**

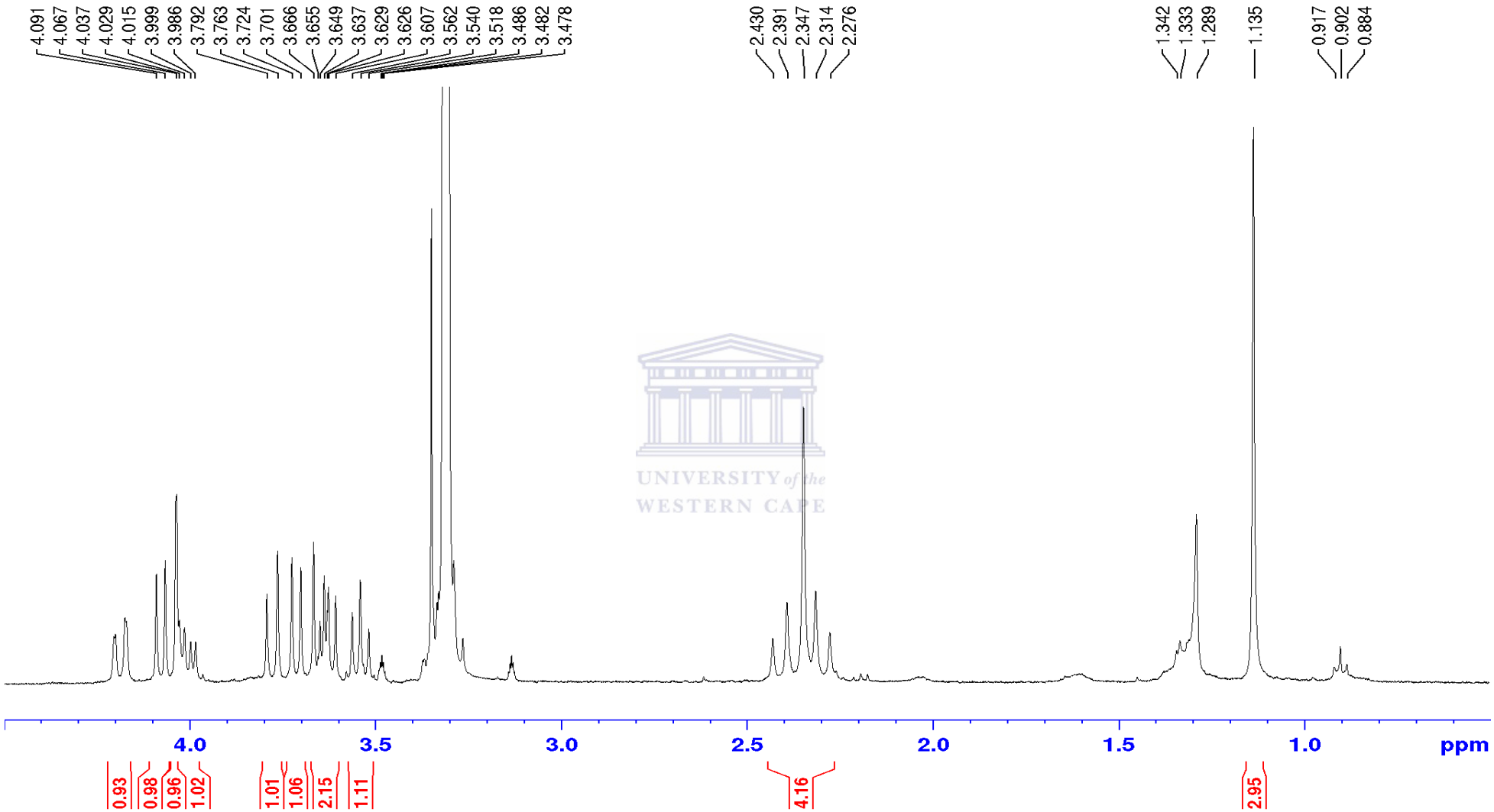


Figure A 3.12: **NMR spectrum of sutherlandin D in methanol-d4 (CD_3OH)**

Appendix 4: Dissolution profiles of sutherlandins A, B, C and D from *S. frutescens* materials at different pH conditions

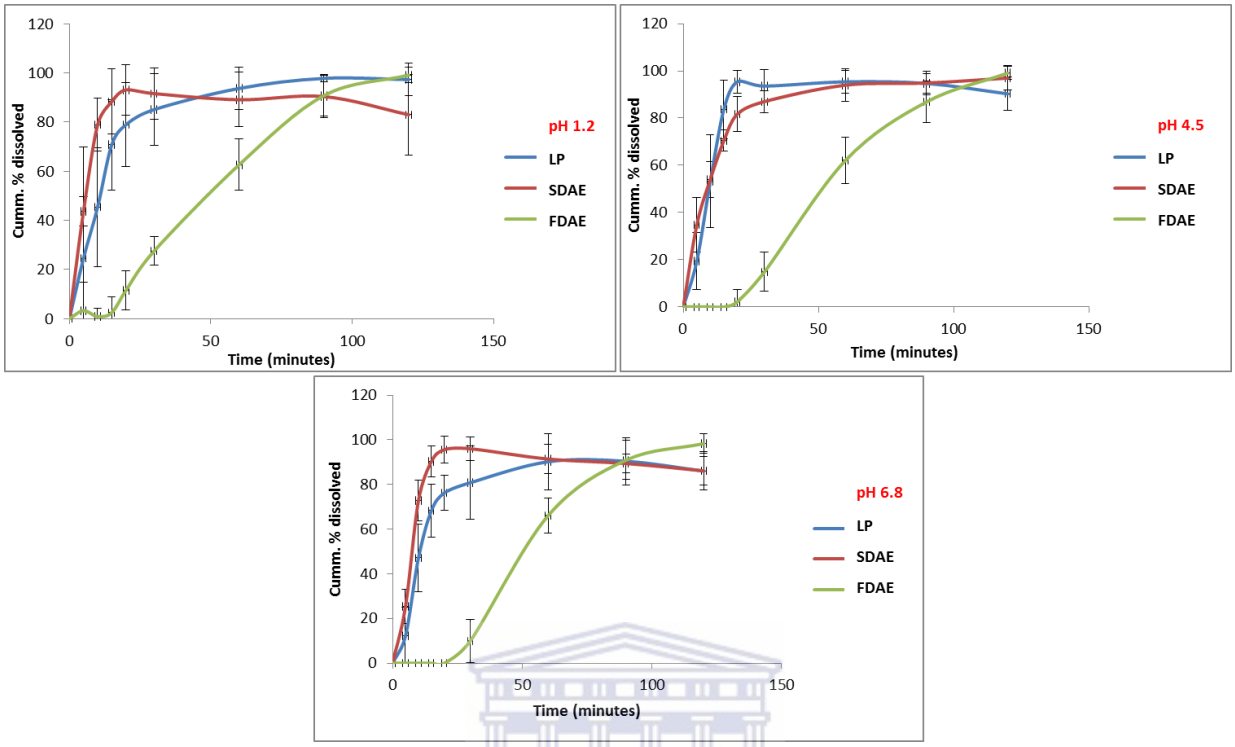


Figure A 4.1: Dissolution profiles of sutherlandin A (from S. frutescens materials) at pH 1.2, 4.5 and 6.8. Data is presented as mean ± SD.

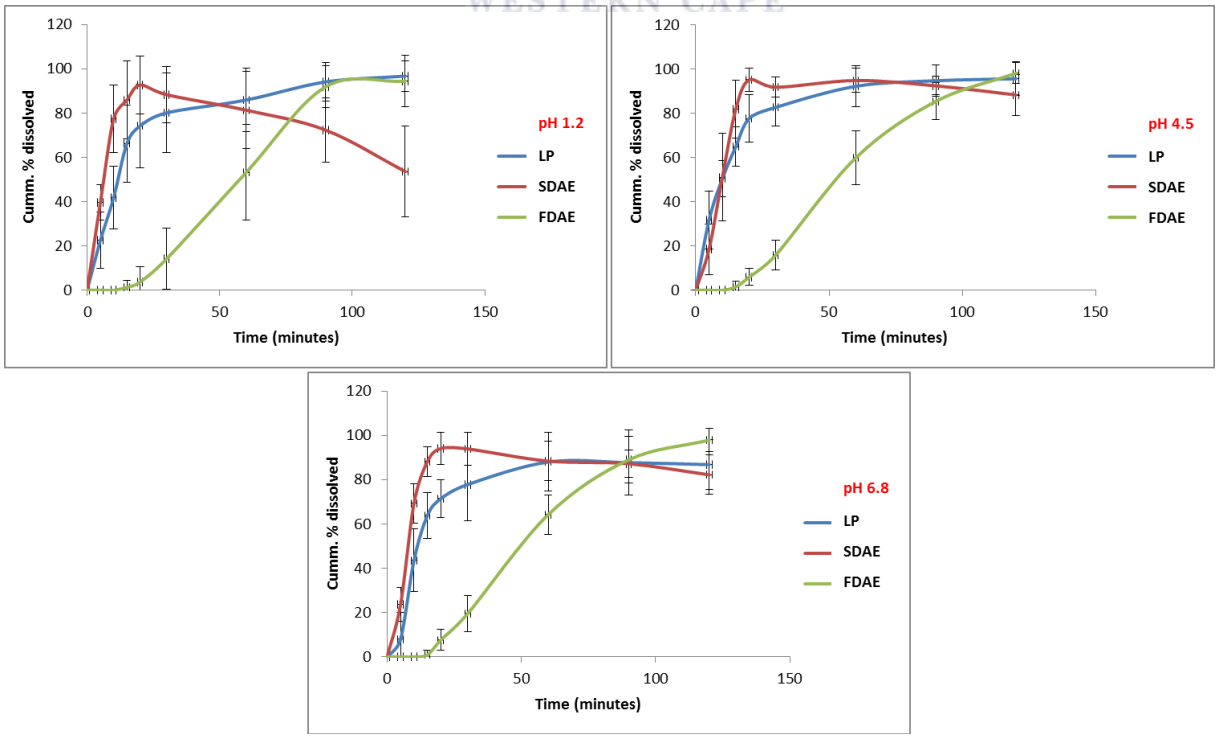


Figure A 4.2: Dissolution profiles of sutherlandin B (from S. frutescens materials) at pH 1.2, 4.5 and 6.8. Data is presented as mean ± SD.

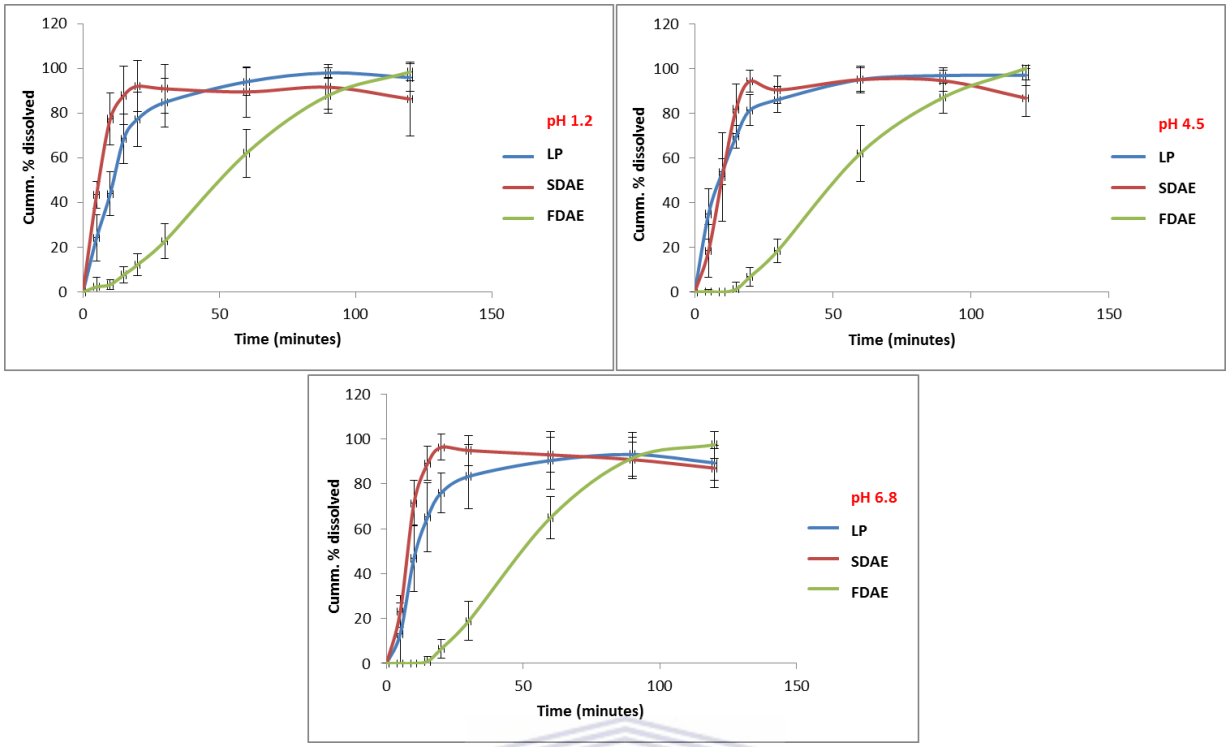


Figure A 4.3: Dissolution profiles of sutherlandin C (from S. frutescens materials) at pH 1.2, 4.5 and 6.8. Data is presented as mean ± SD.

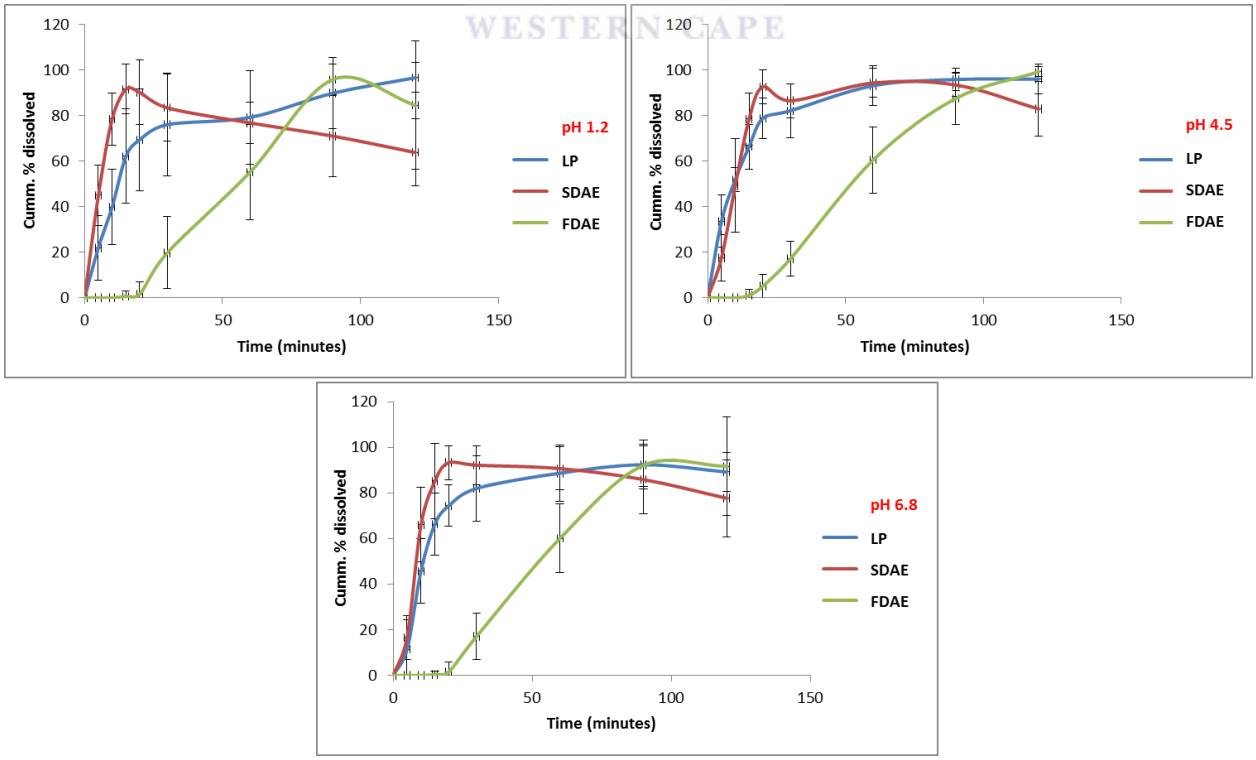


Figure A 4.4: Dissolution profiles of sutherlandin D (from S. frutescens materials) at pH 1.2, 4.5 and 6.8. Data is presented as mean ± SD.

Appendix 5: Dissolution data for sutherlandins from different *S. frutescens* materials *at pH 1.2, 4.5 and 6.8*

Table A 5.1: Dissolution data for sutherlandin A

LP									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	CV %	Ave	SD	CV %
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	24.6	11.1	45.1	19.4	12.1	62.4	12.2	12.8	104.9
10	45.4	11.0	24.2	53.2	19.7	37.0	47.1	15.1	32.1
15	70.8	13.0	18.4	83.7	12.4	14.8	68.3	11.8	17.3
20	78.9	13.4	17.0	95.4	4.7	4.9	76.2	7.9	10.4
30	85.1	12.5	14.7	93.6	7.0	7.5	80.8	16.4	20.3
60	93.7	8.0	8.5	95.4	4.9	5.1	90.2	12.6	14.0
90	97.8	1.5	1.5	94.7	4.1	4.3	90.4	10.5	11.6
120	97.3	6.4	6.6	90.3	7.0	7.8	86.0	8.6	10.0
SDAE									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	CV %	Ave	SD	CV %
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	43.7	5.9	13.5	34.7	11.5	33.1	25.4	7.6	29.9
10	78.9	10.7	13.6	54.0	7.7	14.3	72.8	9.0	12.4
15	88.3	13.3	15.1	70.4	4.5	6.4	90.3	6.8	7.5
20	93.1	10.2	11.0	81.7	7.5	9.2	95.6	6.0	6.3
30	91.6	10.4	11.4	87.0	4.6	5.3	96.0	5.2	5.4
60	89.1	11.1	12.5	94.0	7.0	7.4	91.4	6.6	7.2
90	90.4	8.7	9.6	94.9	5.0	5.3	89.4	4.3	4.8
120	83.0	16.5	19.9	97.0	5.1	5.3	86.1	6.5	7.5
FDAE									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	CV %	Ave	SD	CV %
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	3.3	11.4	345.5	0.0	0.0	0.0	0.0	0.0	0.0
10	0.9	3.2	355.6	0.0	0.0	0.0	0.0	0.0	0.0
15	2.5	6.4	256.0	0.0	0.0	0.0	0.0	0.0	0.0
20	11.5	8.0	69.6	2.2	5.1	231.8	0.0	0.0	0.0
30	27.6	5.8	21.0	14.8	8.3	56.1	10.0	9.7	97.0
60	62.6	10.4	16.6	62.1	9.8	15.8	66.1	7.8	11.8
90	90.8	8.2	9.0	86.9	8.7	10.0	90.9	8.7	9.6
120	99.1	3.2	3.2	99.1	2.7	2.7	98.3	4.4	4.5

Table A 5.2: Dissolution data for sutherlandin B

LP									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	% CV	Ave	SD	% CV
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	22.5	12.6	56.0	31.6	13.0	41.1	7.9	12.0	151.9
10	41.8	14.2	34.0	50.4	8.2	16.3	43.5	14.2	32.6
15	66.2	17.5	26.4	64.8	8.8	13.6	63.8	10.3	16.1
20	74.2	18.9	25.5	77.5	10.8	13.9	71.4	8.6	12.0
30	80.1	17.9	22.3	82.6	8.6	10.4	77.8	16.3	21.0
60	85.9	14.3	16.6	92.1	9.1	9.9	88.0	13.3	15.1
90	94.1	8.6	9.1	94.6	7.2	7.6	87.7	14.7	16.8
120	96.7	6.8	7.0	95.5	7.5	7.9	86.8	11.4	13.1

SDAE									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	% CV	Ave	SD	% CV
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	39.6	7.9	19.9	18.5	11.4	61.6	23.7	7.5	31.6
10	77.4	15.3	19.8	51.0	19.8	38.8	69.3	8.8	12.7
15	85.9	17.5	20.4	81.7	13.1	16.0	88.2	6.7	7.6
20	92.7	13.0	14.0	95.0	5.1	5.4	94.1	7.4	7.9
30	88.3	12.6	14.3	91.7	4.6	5.0	93.9	7.4	7.9
60	81.3	17.5	21.5	94.7	5.5	5.8	88.4	8.9	10.1
90	72.3	14.6	20.2	92.3	4.2	4.6	87.2	6.1	7.0
120	53.6	20.5	38.2	88.2	9.2	10.4	82.2	8.9	10.8

FDAE									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	% CV	Ave	SD	% CV
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	1.3	3.2	246.2	1.5	2.7	180.0	0.9	2.2	244.4
20	3.7	6.7	181.1	5.9	3.8	64.4	7.6	4.8	63.2
30	14.2	13.9	97.9	15.9	6.6	41.5	19.5	8.2	42.1
60	53.2	21.6	40.6	59.8	12.1	20.2	64.2	8.9	13.9
90	91.9	9.6	10.4	85.3	8.4	9.8	89.0	10.5	11.8
120	94.4	11.6	12.3	98.0	4.8	4.9	97.9	5.3	5.4

Table A 5.3: Dissolution data for sutherlandin C

LP									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	% CV	Ave	SD	% CV
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	24.2	10.2	42.1	34.9	11.2	32.1	13.2	13.6	103.0
10	43.9	9.9	22.6	53.7	5.8	10.8	46.8	14.8	31.6
15	68.6	11.1	16.2	69.5	5.1	7.3	65.1	15.3	23.5
20	77.3	12.2	15.8	81.3	6.9	8.5	76.0	8.8	11.6
30	84.8	10.9	12.9	86.0	5.8	6.7	83.3	14.3	17.2
60	94.0	6.2	6.6	95.0	6.0	6.3	90.4	13.0	14.4
90	98.0	2.3	2.3	96.8	3.3	3.4	93.1	9.7	10.4
120	95.9	6.3	6.6	96.9	4.5	4.6	89.3	7.9	8.8

SDAE									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	% CV	Ave	SD	% CV
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	43.5	6.0	13.8	18.4	11.7	63.6	23.1	7.0	30.3
10	77.4	11.5	14.9	51.5	19.8	38.4	71.3	10.1	14.2
15	87.9	13.2	15.0	81.8	11.1	13.6	89.0	7.6	8.5
20	92.0	11.4	12.4	94.3	4.9	5.2	96.3	5.8	6.0
30	90.9	10.8	11.9	90.4	6.1	6.7	94.9	6.7	7.1
60	89.5	11.3	12.6	95.0	5.1	5.4	92.9	7.9	8.5
90	91.6	10.0	10.9	94.5	4.7	5.0	90.8	7.6	8.4
120	86.3	16.4	19.0	86.7	8.2	9.5	87.0	8.7	10.0
FDAE									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	% CV	Ave	SD	% CV
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	2.3	4.3	187.0	0.2	0.8	400.0	0.0	0.0	0.0
10	3.3	2.3	69.7	0.0	0.0	0.0	0.0	0.0	0.0
15	7.7	3.7	48.1	1.3	3.2	246.2	0.9	2.1	233.3
20	12.2	4.9	40.2	6.8	4.2	61.8	6.5	4.1	63.1
30	22.7	7.9	34.8	18.4	5.2	28.3	18.8	8.7	46.3
60	62.1	10.7	17.2	62.0	12.5	20.2	64.9	9.3	14.3
90	87.9	8.0	9.1	87.0	7.2	8.3	91.5	9.2	10.1
120	98.4	3.8	3.9	100.0	0.0	0.0	97.4	6.0	6.2

Table A 5.4: Dissolution data for sutherlandin D

LP									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	% CV	Ave	SD	% CV
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	21.9	14.1	64.4	33.5	11.4	34.0	11.4	13.1	114.9
10	39.8	16.6	41.7	51.8	5.2	10.0	45.7	14.2	31.1
15	62.0	20.7	33.4	66.2	9.9	15.0	66.3	13.6	20.5
20	69.3	22.4	32.3	78.7	9.0	11.4	74.4	9.1	12.2
30	75.9	22.6	29.8	82.0	11.9	14.5	82.0	14.3	17.4
60	79.1	20.6	26.0	93.0	8.6	9.2	88.7	12.3	13.9
90	89.7	15.5	17.3	95.7	4.9	5.1	92.4	10.7	11.6
120	96.5	6.5	6.7	95.9	6.5	6.8	89.2	8.6	9.6
SDAE									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	% CV	Ave	SD	% CV
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	44.9	13.4	29.8	17.5	10.2	58.3	16.6	9.7	58.4
10	78.3	11.3	14.4	49.1	20.5	41.8	66.0	16.3	24.7
15	91.4	10.9	11.9	78.3	11.5	14.7	85.2	16.5	19.4
20	90.0	14.1	15.7	92.5	7.4	8.0	93.3	7.5	8.0
30	83.3	14.6	17.5	86.4	7.4	8.6	92.2	8.5	9.2
60	76.7	9.1	11.9	94.2	6.4	6.8	90.7	9.5	10.5
90	70.9	18.0	25.4	93.3	5.1	5.5	85.9	14.9	17.3
120	63.7	14.8	23.2	82.8	11.9	14.4	77.7	16.9	21.8
FDAE									
	pH 1.2	pH 1.2	pH 1.2	pH 4.5	pH 4.5	pH 4.5	pH 6.8	pH 6.8	pH 6.8
Time (mins)	Ave	SD	% CV	Ave	SD	% CV	Ave	SD	% CV
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	0.7	2.4	342.9	1.1	2.5	227.3	0.4	1.3	325.0
20	1.6	5.5	343.8	5.1	5.2	102.0	1.6	4.1	256.3
30	19.7	15.8	80.2	17.1	7.6	44.4	17.1	10.2	59.6
60	55.1	20.8	37.7	60.4	14.5	24.0	60.1	15.2	25.3
90	95.7	6.9	7.2	87.4	11.5	13.2	92.1	9.3	10.1
120	84.4	28.1	33.3	99.1	2.2	2.2	91.7	21.7	23.7

Appendix 6: Fitting of dissolution data to mathematical models

Table A 6.1: Fitting of dissolution data at pH 1.2 for mathematical modelling, best values are highlighted

		LP: A	LP: B	LP: C	LP: D	SDAE: A	SDAE: B	SDAE: C	SDAE: D	FDAE: A	FDAE: B	FDAE: C	FDAE: D
Zero order	k_0	1.188±0.016	1.186±0.011	1.242±0.014	1.252±0.013	0.988±0.094	0.870±0.125	1.058±0.127	0.886±0.145	0.876±0.052	0.778±0.099	0.869±0.033	0.787±0.114
	R 2	-3.0575	-3.1309	-2.7536	-3.6018	-8.2291	-6.0427	-7.5255	-9.5853	0.9262	0.8570	0.9348	0.7976
	R^2_{adj}	-3.0575	-3.1309	-2.7536	-3.6018	-8.2291	-6.0427	-7.5255	-9.5853	0.9262	0.8570	0.9348	0.7976
First order	k_1	0.090±0.011	0.092±0.011	0.083±0.008	0.094±0.012	0.108±0.016	0.107±0.033	0.102±0.032	0.103±0.047	0.015±0.002	0.012±0.003	0.014±0.001	x
	R 2	0.9482	0.9427	0.9514	0.9450	-0.1140	-0.5441	-0.2837	-1.5726	0.8509	0.7680	0.8575	x
	R^2_{adj}	0.9482	0.9427	0.9514	0.9450	-0.1140	-0.5441	-0.2837	-1.5726	0.8509	0.7680	0.8575	x
Weibull_1	β	1.827±1.453	2.434±1.757	1.509±1.517	2.384±1.540	0.054±0.039	-0.078±1.415	0.083±0.068	-0.259±0.550	2.720±1.450	3.296±1.367	2.635±0.942	x
	Ti	-4.037±9.935	-6.648±9.583	-2.490±10.479	-7.197±9.230	5.000±0.000	-51.368±135.230	4.996±0.006	-69.206±181.766	-10.993±22.495	12.991±32.665	-8.717±24.988	x
	R 2	**0.9818**	**0.9818**	0.9805	0.9819	0.6085	0.3747	0.5725	0.2722	0.9874	0.9665	0.9892	x
	R2adj	**0.9745**	**0.9745**	0.9740	0.9759	0.4519	0.1245	0.4300	0.0296	0.9824	0.9531	0.9856	x
Weibull_2	β	1.232±0.416	1.325±0.414	1.169±0.345	1.302±0.397	0.540±0.522	0.360±0.685	0.722±0.828	0.038±0.132	2.032±0.253	4.462±2.375	2.683±1.912	x
	R 2	0.9765	0.9745	0.9795	0.9755	0.3240	0.1519	0.4460	0.0568	0.9579	0.9651	0.9867	x
	R^2_{adj}	0.9726	0.9702	0.9761	0.9714	0.2113	0.0105	0.3536	-0.1005	0.9509	0.9592	0.9849	x
Weibull_3	β	1.281±0.434	1.376±0.442	1.224v0.388	1.352±0.431	1.909±1.000	1.034±1.833	1.666±0.537	0.587±1.911	2.035±0.337	4.613±2.778	2.633±2.139	x
	R 2	0.9787	0.9773	0.9800	0.9796	0.6553	0.4632	0.5988	0.2203	0.9873	0.9797	0.9890	x
	R^2_{adj}	0.9701	0.9683	0.9733	0.9728	0.5174	0.2485	0.4651	-0.0396	0.9822	0.9715	0.9854	x
Weibull_4	β	2.108±1.382	2.428±1.477	1.655±1.123	2.510±1.418	0.461±0.844	-0.177±0.613	0.539±1.030	-0.473±0.567	1.936±1.325	2.830±1.265	2.427±0.949	x
	T_i	-4.959±6.945	-6.134±6.547	-2.759±6.660	-7.146±6.411	3.689±3.209	-53.410±143.074	3.008±4.858	-229.738±213.282	0.945±17.979	20.584±24.727	-5.116±23.064	x
	R 2	0.9841	0.9853	**0.9845**	**0.9871**	0.6252	0.3787	0.5879	0.1934	**0.9902**	0.9816	**0.9909**	x
	R^2_{adj}	0.9722	0.9742	**0.9751**	**0.9793**	0.3441	-0.0872	0.3407	-0.2906	**0.9828**	0.9677	**0.9854**	x
Makoid-Banakar with T_{lag}	k_{MB}	36.798±7.402	35.722±8.411	35.521±5.688	37.201±8.244	53.981±21.429	47.364±24.389	58.155±18.440	64.655±12.038	0.702±1.274.	0.702±1.658	0.116±0.220	x
	n	0.322±0.065	0.346±0.079	0.316±0.056	0.329±0.075	0.220±0.242	0.566±0.928	0.149±0.187	0.101±0.088	1.829±0.954	6.831±6.380	3.039±2.291	x
	k	0.005±0.001	0.006±0.002	0.004±0.001	0.005±0.002	0.005±0.006	0.014±0.015	0.003±0.006	0.006±0.002	0.014±0.011	0.067±0.071	0.024±0.027	x
	T_{lag}	4.195±0.671	4.195±0.601	4.203±0.640	4.227±0.545	3.943±2.132	2.000±6.915	4.407±1.405	3.881±2.556	6.577±6.115	7.714±11.134	-7.082±12.312	x
	R 2	0.9742	0.9482	0.9548	0.9473	0.7513	0.7131	**0.6709**	0.4662	0.9898	**0.9949**	0.9815	x
	R^2_{adj}	0.9493	0.9094	0.9276	0.9156	0.5648	0.4980	**0.4735**	0.1460	0.9821	**0.9911**	0.9705	x
Peppas-Sahlin 1 with T_{lag}	k_1	39.702±7.984	39.169±8.751	37.435±5.854	40.311±8.533	65.837±21.693	66.464±39.516	70.731±21.329	115.347±63.518	-22.915±27.496	-0.089±51.167	-41.395±81.385	4.133±28.991
	k_2	-4.011v1.648	-3.908±1.873	-3.556±1.031	-4.153±1.853	-12.130±6.882	-17.332±21.039	-14.023±7.183	-49.016±46.217	19.258±20.005	14.105±20.359	16.235±25.915	13.834±22.051
	T_{lag}	4.116±0.666	4.168±0.571	4.085±0.654	4.161±0.518	0.265±0.140	4.100±1.558	4.538±0.906	3.726±2.674	12.430±3.413	17.554±49.610	-16.171±48.055	37.105±18.592
	R 2	0.9496	0.9469	0.9558	0.9468	**0.7524**	**0.7268**	0.6658	**0.4831**	0.9800	0.9555	0.9541	**0.9714**
	R^2_{adj}	0.9118	0.9070	0.9292	0.9149	**0.5667**	**0.5220**	0.4653	**0.1729**	0.9651	0.9221	0.9266	**0.9542**
Logistic_2	α	-3.943±1.421	-4.247±1.436	-3.794±1.257	-4.161±1.442	-10.473±13.600	2.462±21.355	-5.336±1.523	63.836±105.864	-9.257±0.938	-78.095±95.617	-14.007±14.052	x
	β	4.313±1.365	4.652±1.361	4.052±1.214	4.595±1.325	14.967±19.357	3.542±12.707	7.628±2.123	-24.584±57.416	5.179±0.720	43.560±53.733	7.940±8.463	x
	R 2	0.9718	0.9660	0.9620	0.9665	0.6418	0.4591	0.5894	0.2361	0.9850	0.9794	0.9881	x
	R^2_{adj}	0.9605	0.9524	0.9683	0.9553	0.4985	0.2428	0.4526	-0.0186	0.9790	0.9712	0.9841	x
Logistic_3	k	0.202±0.067	0.217±0.065	0.187±0.060	0.212±0.062	0.492±1.045	-0.140±0.329	0.189±0.404	-0.038±0.339	0.066±0.009	0.218±0.220	0.085±0.062	x
	γ	8.407±1.432	8.503±1.408	8.685±1.472	8.264±1.697	1905.214±4201.0	106.289±51.195	336.261±480.146	115.929±57.965	55.111±6.892	63.409±9.467	52.739±4.902	x
	R 2	0.9791	0.9797	0.9780	0.9824	0.4900	0.2911	0.4560	0.2046	0.9813	0.9775	0.9865	x
	R^2_{adj}	0.9708	0.9716	0.9707	0.9766	0.2860	0.0075	0.2747	-0.0605	0.9738	0.9685	0.9820	x
Korsmeyer-Peppas with T_{lag}	kKP	10.196±11.431	10.196±11.431	9.821±11.852	6.678±12.657	x	x	x	x	0.889±0.840	0.753±1.526	0.300±0.393	x
	n	1.183±0.727	1.183±0.727	1.145±0.654	1.594±0.890	x	x	x	x	1.455±0.853	1.709±2.252	1.663±0.588	x
	T_{lag}	-1.729±5.278	-1.729±5.278	-1.320±4.129	-3.949±3.904	x	x	x	x	5.068±12.448	5.269±13.446	-4.691±11.053	x
	R 2	1.0000	1.0000	0.9999	0.9953	x	x	x	x	0.9998	0.3519	0.9998	x
	R2adj	0.9999	0.9999	0.9998	0.9858	x	x	x	x	0.9998	0.2799	0.9998	x

Table A 6.2: Fitting of dissolution data at pH 4.5 for mathematical modelling, best values are highlighted

		LP: A	LP: B	LP: C	LP: D	SDAE: A	SDAE: B	SDAE: C	SDAE: D	FDAE: A	FDAE: B	FDAE: C	FDAE: D
Zero order	k_0	2.216±0.098	2.182±0.104	2.225±0.103	2.150±0.153	2.320±0.129	2.302±0.072	2.272±0.110	2.233±0.145	0.842±0.026	0.838±0.027	0.857±0.050	0.839±0.068
	R 2	-1.2932	-0.9461	-1.1699	-0.9492	-0.4607	-0.3849	-0.4234	-0.3777	0.9223	0.9424	0.9469	0.9373
	R^2_{adj}	-1.2932	-0.9461	-1.1699	-0.9492	-0.4607	-0.3849	-0.4234	-0.3777	0.9223	0.9424	0.9469	0.9373
First order	k_1	0.079±0.017	0.073±0.015	0.078±0.018	0.071±0.018	0.085±0.013	0.081±0.009	0.081±0.011	0.077±0.013	0.013±0.001	0.013±0.001	0.013±0.002	0.013±0.002
	R 2	0.9269	0.9066	0.9425	0.9064	0.8369	0.8447	0.8420	0.8345	0.8233	0.8468	0.8496	0.8417
	R^2_{adj}	0.9269	0.9066	0.9425	0.9064	0.8369	0.8447	0.8420	0.8345	0.8233	0.8468	0.8496	0.8417
Weibull_1	β	1.102±0.902	0.729±0.199	1.189±1.015	1.200±1.241	2.995±1.814	3.398±2.102	2.730±1.991	2.539±1.874	1.563±0.184	2.225±0.403	2.119±0.222	3.272±1.710
	Ti	-0.461±9.485	3.077±1.438	-1.028±10.236	-1.314±11.327	-3.851±7.578	-5.342±8.112	-2.539±7.714	-2.027±7.317	18.981±2.481	3.656±6.216	4.623±3.358	-12.521±21.432
	R 2	0.9665	0.9588	0.9690	0.9542	0.9628	0.9639	0.9576	0.9455	0.9968	0.9965	0.9979	0.9927
	R2adj	0.9441	0.9313	0.9483	0.9236	0.9381	0.9399	0.9293	0.9092	0.9955	0.9951	0.9971	0.9898
Weibull_2	β	1.099±0.236	1.069±0.312	1.089±0.212	0.989±0.226	2.209±0.409	2.185±0.404	2.108±0.476	2.032±0.559	2.641±0.213	2.385±0.105	2.371±0.091	2.548±0.480
	R 2	0.9434	0.9388	0.9551	0.9278	0.9569	0.9567	0.9499	0.9362	0.9932	0.9961	0.9976	0.9913
	R^2_{adj}	0.9292	0.9235	0.9439	0.9097	0.9461	0.9459	0.9374	0.9203	0.9921	0.9954	0.9972	0.9899
Weibull_3	β	1.301±0.147	1.227±0.351	1.240±0.128	1.187±0.143	2.427±0.294	2.462±0.377	2.403±0.321	2.296±0.498	2.709±0.276	2.329±0.149	2.289±0.150	2.397±0.661
	R 2	**0.9702**	0.9547	**0.9723**	**0.9581**	**0.9774**	**0.9759**	**0.9759**	**0.9669**	0.9938	0.9969	0.9981	0.9935
	R^2_{adj}	**0.9503**	0.9244	**0.9538**	**0.9302**	**0.9623**	**0.9599**	**0.9598**	**0.9448**	0.9913	0.9957	0.9973	0.9909
Weibull_4	β	1.371±0.947	1.067±1.044	1.352±0.998	1.566±1.275	3.896±0.750	4.040±1.027	3.747±1.028	3.273±1.509	1.173±0.430	1.907±0.888	1.668±0.386	2.420±1.681
	T_i	-1.362±9.005	0.413±8.335	-1.297±8.867	-2.659±9.150	-6.305±3.827	-6.923±4.569	-5.806±4.347	-3.941±4.899	23.141±5.025	7.474±12.232	10.233±4.646	-0.186±19.207
	R 2	0.9790	**0.9729**	0.9805	0.9708	0.9816	0.9819	0.9804	0.9713	0.9982	0.9983	**0.9993**	**0.9961**
	R^2_{adj}	0.9474	**0.9322**	0.9512	0.9270	0.9541	0.9547	0.9509	0.9283	0.9969	0.9970	**0.9988**	**0.9932**
Makoid-Banakar with T_{lag}	k_{MB}	22.759±3.263	27.002±11.581	21.783±3.497	20.561±6.194	23.792±8.912	21.678±9.395	22.491±8.840	19.632±9.795	0.036±0.049	0.070±0.064	0.093±0.109	1.047±1.900
	n	0.526±0.100	0.443±0.197	0.532±0.078	0.535±0.130	0.598±0.168	0.634±0.188	0.614±0.186	0.685±0.254	2.996±1.347	2.284±0.848	2.139±0.549	2.176±1.544
	k	0.012±0.002	0.008±0.007	0.012±0.003	0.011±0.005	0.017±0.006	0.018±0.006	0.018±0.007	0.020±0.010	0.027±0.011	0.018±0.010	0.017±0.005	0.016±0.019
	T_{lag}	3.392±1.286	3.863±1.318	3.307±1.158	3.142±1.361	4.435±0.431	4.298±0.513	4.403±0.467	4.175±0.633	6.090±9.480	7.255±6.244	7.111±5.092	9.417±9.353
	R 2	0.9718	0.9665	0.9733	0.9610	0.9446	0.9441	0.9449	0.9448	0.9958	**0.9990**	0.9988	0.9961
	R^2_{adj}	0.9296	0.9161	0.9331	0.9025	0.8615	0.8603	0.8623	0.8621	0.9927	**0.9983**	0.9980	0.9931
Peppas-Sahlin 1 with T_{lag}	k_1	25.240±6.295	25.422±5.356	23.893±3.878	22.076±6.718	28.046±8.336	33.822±17.780	26.664±8.074	23.943±8.896	5.562±29.914	-34.891±35.208	-34.413±47.912	32.270±51.202
	k_2	-1.797±1.103	1.545±5.386	-1.520±0.501	-1.354±0.745	-2.045±1.109	-3.544±3.916	-1.885±1.074	-1.575±1.102	7.135±12.412	26.892±30.769	28.091±42.439	26.647±44.565
	T_{lag}	3.565±0.975	3.970±1.026	3.455±0.843	3.226±1.069	4.589±0.262	5.379±2.097	4.574±0.266	4.436±0.371	30.926±14.109	15.000±3.162	15.000±0.000	13.885±4.915
	R 2	0.9708	0.9654	0.9724	0.9603	0.9404	0.9413	0.9405	0.9391	**0.9990**	0.9906	0.9929	0.9833
	R^2_{adj}	0.9269	0.9134	0.9309	0.9008	0.8510	0.8533	0.8512	0.8478	**0.9982**	0.9836	0.9876	0.9708
Logistic_2	α	-4.033±0.743	-3.915±1.242	-3.841±0.664	-3.690±0.598	-7.625±1.416	-7.583±1.386	-7.638±1.499	-7.764±2.227	-13.991±1.505	-11.364±0.661	11.075±0.649	-11.433±2.663
	β	4.330±0.428	4.036±1.087	4.062±0.319	3.811±0.508	7.839±1.457	7.727±1.510	7.805±1.522	7.817±2.210	7.967±0.914	6.331±0.582	6.196±0.639	6.235±1.832
	R 2	0.9692	0.9580	0.9705	0.9556	0.9647	0.9637	0.9636	0.9548	0.9959	0.9968	0.9983	0.9910
	R^2_{adj}	0.9486	0.9300	0.9508	0.9260	0.9412	0.9395	0.9394	0.9246	0.9943	0.9955	0.9976	0.9874
Logistic_3	k	0.210±0.014	0.190±0.050	0.195±0.016	0.183±0.042	0.373±0.071	0.369±0.090	0.365±0.067	0.343±0.079	0.088±0.008	0.072±0.007	0.072±0.009	0.069±0.017
	y	8.666±1.999	9.044±1.909	8.885±1.884	9.377±2.111	9.733±0.725	9.973±0.916	9.818±0.645	10.094±0.736	55.701±3.493	57.951±5.308	57.051±5.684	61.299±9.127
	R 2	0.9673	0.9405	0.9682	0.9534	0.9786	0.9784	0.9770	0.9669	0.9901	0.9918	0.9928	0.9873
	R^2_{adj}	0.9455	0.9008	0.9470	0.9223	0.9643	0.9640	0.9616	0.9448	0.9862	0.9886	0.9899	0.9822
Korsmeyer-Pep	kKP	x	x	x	x	x	x	x	x	6.488±5.223	0.266±0.228	0.392±0.463	0.410±0.582
	n	x	x	x	x	x	x	x	x	1.059±1.011	1.681±0.718	1.647±0.770	2.044±1.049
	Tlag	x	x	x	x	x	x	x	x	20.295±16.355	7.253±10.849	7.645±11.770	1.848±14.134
	R 2	x	x	x	x	x	x	x	x	0.9942	0.9976	0.9972	0.9958
	R^2_{adj}	x	x	x	x	x	x	x	x	0.9936	0.9974	0.9969	0.9953

Table A 6.3: Fitting of dissolution data at pH 6.8 for mathematical modelling, best values are highlighted

		LP: A	LP: B	LP: C	LP: D	SDAE: A	SDAE: B	SDAE: C	SDAE: D	FDAE: A	FDAE: B	FDAE: C	FDAE: D
Zero order	k_0	1.059±0.073	1.038±0.075	1.083±0.078	1.074±0.078	1.112±0.035	1.076±0.055	1.122±0.057	1.051±0.080	0.874±0.020	0.877±0.038	0.884±0.036	0.8450.120
	R 2	-1.6599	-1.0765	-1.5455	-1.3726	-4.5829	-4.2639	-3.9857	-3.1304	0.8980	0.9250	0.9223	0.8740
	R^2_{adj}	-1.6599	-1.0765	-1.5455	-1.3726	-4.5829	-4.2639	-3.9857	-3.1304	0.8980	0.9250	0.9223	0.8740
First order	k_1	0.063±0.020	0.055±0.015	0.064±0.023	0.062±0.022	0.114±0.011	0.107±0.012	0.110±0.012	0.098±0.016	0.014±0.001	0.014±0.001	0.014±0.001	0.013±0.002
	R 2	0.7103	0.6993	0.7866	0.7754	0.7287	0.6486	0.7259	0.5641	0.8055	0.8459	0.8445	0.7971
	R^2_{adj}	0.7103	0.6993	0.7866	0.7754	0.7287	0.6486	0.7259	0.5641	0.8055	0.8459	0.8445	0.7971
Weibull_1	β	0.797±1.179	0.456±0.338	0.929±1.172	0.611±0.399	1.403±1.393	1.341±1.291	x	1.260±1.923	1.490±0.739	1.852±0.911	2.365±1.407	1.865±1.345
	Ti	4.760±4.850	6.595±2.279	4.144±5.200	5.566±2.704	2.124±4.236	1.689±5.220	x	3.339±6.081	23.843±8.231	9.730±14.990	1.791±20.138	12.814±13.623
	R 2	0.8925	0.8910	0.9188	0.9173	0.8922	0.8434	x	0.8031	0.9957	0.9908	0.9923	0.9531
	R2adj	0.8495	0.8474	0.8863	0.8842	0.8491	0.7807	x	0.7243	0.9940	0.9871	0.9892	0.9343
Weibull_2	β	1.394±0.846	1.329±0.795	1.404±0.795	1.397±0.676	2.122±0.678	1.715±0.756	2.193±0.670	2.596±1.885	4.923±2.743	2.844±1.659	2.873±1.707	2.997±1.781
	R 2	0.8093	0.7874	0.8660	0.8546	0.8406	0.7429	0.8410	0.6501	0.9907	0.9874	0.9893	0.9437
	R^2_{adj}	0.7776	0.7520	0.8437	0.8304	0.8140	0.7000	0.8145	0.5918	0.9892	0.9853	0.9875	0.9343
Weibull_3	β	2.384±1.647	2.435±1.694	1.704±0.735	1.808±0.703	2.561±1.000	2.572±0.965	2.591±0.999	3.312±1.576	4.565±2.071	2.906±1.544	6.915±2.814	3.523±2.035
	R 2	0.8842	0.8723	0.9079	0.9072	0.9431	**0.9163**	0.9390	0.8586	0.9930	0.9894	0.9912	0.9609
	R^2_{adj}	0.8379	0.8212	0.8711	0.8701	0.9203	**0.8828**	0.9145	0.8020	0.9902	0.9852	0.9876	0.9453
Weibull_4	β	1.278±1.244	0.655±0.509	1.252±1.024	1.234±1.141	2.909±1.253	2.914±1.294	2.849±1.004	x	1.572±0.874	1.761±0.906	2.150±1.306	1.823±1.331
	T_i	2.880±5.025	5.740±2.720	2.046±5.445	2.642±6.257	-2.295±3.824	-2.108±4.346	-1.850±3.534	x	24.471±8.548	12.006±11.478	5.056±17.600	18.277±17.599
	R 2	0.9154	0.9043	0.9363	0.9364	**0.9448**	0.9093	**0.9399**	x	0.9974	0.9927	**0.9948**	0.9661
	R^2_{adj}	0.8519	0.8326	0.8886	0.8886	**0.9035**	0.8412	**0.8948**	x	0.9954	0.9872	**0.9909**	0.9407
Makoid-Banakar with T_{lag}	k_{MB}	41.073±13.192	41.024±13.335	34.504±17.971	41.223±13.929	59.387±8.810	55.269±9.625	57.004±11.508	51.260±15.642	8.528±15.582	1.887±2.790	1.242±2.058	2.878±3.034
	n	0.266±0.160	0.250±0.177	0.442±0.423	0.262±0.153	0.188±0.061	0.209±0.083	0.205±0.095	0.258±0.140	1.810±1.480	1.879±1.270	2.315±1.245	2.383±2.359
	k	0.004±0.004	0.003±0.005	0.006±0.004	0.004±0.004	0.005±0.001	0.005±0.002	0.005±0.002	0.007±0.004	0.018±0.019	0.017±0.015	0.021±0.012	0.030±0.046
	T_{lag}	6.228±2.454	7.364±2.573	2.249±13.706	7.116±2.651	4.975±0.034	4.959±0.062	4.975±0.036	5.299±1.199	19.320±9.821	11.696±11.247	7.528±11.714	16.864±11.173
	R 2	**0.9245**	**0.9189**	0.9216	**0.9389**	0.9368	0.9131	0.9319	0.8855	**0.9979**	**0.9938**	0.9944	**0.9955**
	R^2_{adj}	**0.8679**	**0.8580**	0.8628	**0.8930**	0.8895	0.8479	0.8808	0.7995	**0.9964**	**0.9891**	0.9901	**0.9922**
Peppas-Sahlin 1 with T_{lag}	k_1	42.643±15.884	39.670±18.358	39.766±15.933	40.056±14.956	73.062±24.062	68.465±22.598	72.750±31.113	62.291±23.503	-71.361±198.033	-2.316±44.040	-53.699±119.974	-34.168±147.298
	k_2	-4.286±5.501	-3.488±6.180	-3.993±4.493	-2.374±7.178	-15.502±14.174	-13.996±12.264	-16.219±18.682	-11.779±9.935	70.198±146.34	21.917±49.288	55.473±100.40	44.532±124.96
	T_{lag}	6.217±2.410	6.551±2.386	6.119±2.494	6.637±2.516	4.941±0.057	4.933±0.077	4.956±0.044	5.310±1.261	20.607±31.264	26.201±15.452	19.685±8.281	27.281±11.700
	R 2	0.9226	0.9149	**0.9376**	0.9351	0.9397	0.9169	0.9347	**0.8889**	0.9778	0.9869	0.9838	0.9543
	R^2_{adj}	0.8645	0.8511	**0.8908**	0.8864	0.8945	0.8457	0.8858	**0.8055**	0.9611	0.9770	0.9716	0.9200
Logistic_2	α	-6.976±2.616	-7.324±2.978	-6.040±2.787	-6.225±2.601	-10.046±10.711	-9.596±8.798	-9.838±9.52	-13.835±13.290	-32.377±45.583	-13.475±5.474	-13.435±5.672	-40.931±62.704
	β	7.103±2.006	7.257±2.854	5.948±2.332	6.116±2.230	12.802±15.307	12.037±12.571	12.297±13.592	16.169±16.535	18.606±25.616	7.850±3.252	7.874±3.356	23.250±35.066
	R 2	0.8925	0.8803	0.9131	0.9139	0.9326	0.9033	0.9298	0.8463	0.9943	0.9904	0.9910	0.9617
	R^2_{adj}	0.8495	0.8324	0.8783	0.8795	0.9057	0.8646	0.9017	0.7848	0.9920	0.9866	0.9874	0.9464
Logistic_3	k	0.372±0.171	0.367±0.208	0.249±0.102	0.265±0.106	0.623±0.725	0.650±0.695	0.660±0.732	0.561±0.804	0.162±0.112	0.102±0.054	0.101±0.051	0.130±0.110
	y	9.829±2.472	10.858±2.701	11.972±6.021	12.180±6.693	2.566±15.927	7.225±1.004	7.364±1.180	33.093±60.514	53.267±5.235	50.931±8.247	50.756±7.541	52.895±6.041
	R 2	0.8832	0.8635	0.8941	0.8939	0.8714	0.9154	0.9378	0.7576	0.9908	0.9853	0.9875	0.9566
	R^2_{adj}	0.8365	0.8088	0.8517	0.8514	0.8199	0.8816	0.9129	0.6607	0.9872	0.9794	0.9825	0.9392
Korsmeyer-Peppas with Tlag	kKP	x	x	x	x	x	x	x	x	2.987±6.772	1.842±3.020	1.249±1.689	2.737±3.237
	n	x	x	x	x	x	x	x	x	3.437±2.800	1.973±1.420	2.638±2.109	9.957±28.423
	Tlag	x	x	x	x	x	x	x	x	7.293±16.043	6.062±14.101	1.302±14.286	11.040±12.791
	R 2	x	x	x	x	x	x	x	x	0.9795	0.9936	0.9970	0.9874
	R2adj	x	x	x	x	x	x	x	x	0.9765	0.9928	0.9966	0.9858

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