UNIVERSITEIT VAN PRETORIA
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ANALYSIS OF STEROLS AND STEROLINS IN

Hypoxis hemerocallidea AND RELATED HERBAL MEDICINE

by

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Submitted in partial fulfilment of the requirements for the degree of

Magister Scientae

in the Faculty of Medicine (Department of Pharmacology)

University of Pretoria

Pretoria

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July 2001

ACKNOWLEDGEMENTS

Praise the Almighty God.

The author wishes to thank:

Prof. J.N. Eloff, Department of Pharmacology, University of Pretoria, supervisor of this project, for his guidance and exposure to new opportunities.

Dr. R. van Brummelen, BIOMOX PHARMACEUTICALS, Pretoria, co-supervisor of this project, for his guidance, advice, encouragement and support.

Dr. J.J. Retief and Dr. J. Coetzee, SASOL 1, Sasolburg, for performing Nuclear Magnetic Resonance Spectroscopy for structure elucidation.

L. du T. Retief for the computer format of the chemical structures presented in this dissertation, and for advice with computer problems.

Mev. M. van der Walt, Pre-clinical Library, University of Pretoria, for her kindness and willingness with literature requests *etc*.

Her parents, for their love, prayers and support.

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LIST OF ABBREVIATIONS

$[\alpha]_D^{25}$ - specific rotation of plane polarized light at 25 °C

$\in°$ - adsorption solvent strength parameter measured on alumina

AIDS - acquired immuno-deficiency syndrome

B-cells - bone marrow lymphoid cells

BEA - mobile phase consisting of benzene:ethanol:ammonia in the ratios 18:2:0.2

bFGF - basic fibroblast growth factor

BPH - benign prostatic hyperplasia

BSS - β-sitosterol

BSSG - β-sitosterol glucoside (β-sitosterolin)

BuOH – butanol

^{13}C - carbon 13

c.- circa (approximately)

CD4 - T-helper cells

CD8 - T-suppressor cells

CEF - mobile phase consisting of chloroform:ethylacetate:formic acid in the ratios 5:4:1

CS - campesterol

DHEA - dehydroepiandosterone

DHT - 5α-dihydrotestosterone

EGF - epidermal growth factor

EMW - mobile phase consisting of ethylacetate:methanol:water in the ratios 10:1.35:1

EtOH - ethanol

FIV - feline immuno-deficiency virus

FLD - fluorescence detector

G - grated rhizome

GC - gas chromatography

^{1}H - proton

HDL - high density lipoprotein

HIV - human immuno-deficiency virus

HPLC - high performance/pressure liquid chromatography

IC$_{50}$ - 50% inhibition

ID probe - indirect detection probe used in NMR

IFN - interferon

IGF - insulin like growth factor

IL - interleukin

IPPS - international prostate symptom score

KGF - keratinocyte growth factor

KMR – kern magnetiese ressonans spektroskopie

LDL - low density lipoprotein

LUTS - lower urinary tract symptoms

MCW - mixture of methanol:chloroform:water in the ratio 12:5:3

MeOH - methanol

MS - mass spectroscopy

MWD - multiple wavelength detector

NMR - nuclear magnetic resonance spectroscopy

P'- polarity parameter and an indicator of solvent strength in partition chromatography

PGD$_2$ - prostaglandin D$_2$

PSA - prostate specific antigen

PSE - plant stanol ester

PTLC - preparative thin layer chromatography

PVR - Peak urinary volume

Q$_{max}$ - maximal urinary flow

QoL - quality of life

R$_f$ - fractional movement of a solute band, relative to the distance moved by the solvent front

SS - stigmasterol

$t_{10\%}$ - shelf life (time after 10% degradation)

T-cells - thymus lymphoid cells

TG - triglyceride

T$_H$1 - T-helper cell type 1

T$_H$2 - T-helper cell type 2

TLC - thin layer chromatography

t_{max} - time of maximum absorption

TNF-α - tumor necrosis factor alpha

TXB$_2$ - tromboxane B$_2$

UV - Ultra violet

w/w - weight/weight

PUBLICATIONS / PRESENTATIONS EMANATING FROM THIS STUDY

Scientific confrences:

Retief, A.C., Eloff, J.N. 2000. Analysis of sterols and sterolins in *Hypoxis hemerocallidea* and related herbal medicine. Indigenous Plant Use Forum. Nelspruit, South Africa.

Retief, A.C., Eloff, J.N. 2000. Analysis of sterols and sterolins in *Hypoxis hemerocallidea* and related herbal medicine. Annual Congress of the South African Academy for Science and Art. Johannesburg, South Africa.

Retief, A.C., Eloff, J.N. 2001. Quantitative measurement of sterol/sterolins with HPLC. 27th Annual Conference of the South African Association of Botanists. Johannesburg, South Africa.

Retief, A.C., Eloff, J.N., van Brummelen R. 2001. Stability analysis of sterols in *Hypoxis hemerocallidea* and related herbal medicine by TLC and HPLC. 49th Annual Congress of the Society for Medicinal Plant Research. Erlangen, Germany

Retief, A.C., Eloff, J.N., van Brummelen R. 2001 HPLC quantification of phytosterols for industrial and clinical applications. PSE2001 Symposium – Lead compounds from higher plants. Lausanne, Switzerland.

Retief, A.C., Eloff, J.N., van Brummelen R. 2001. HPLC quantification of phytosterols for industrial and clinical applications. Immunopharmacology Confrence. Sun City, South Africa.

Other:

Retief, A.C., Eloff, J.N., van Brummelen R. 2001. Stability analysis of sterols in *Hypoxis hemerocallidea* and related herbal medicine by TLC and HPLC. – Draft publication.

Guest lecturer on sterols/sterolins for BHM Continued Education 5 times during 2000 and 2001. As part of their country wide continued education system for doctors, pharmacists and other health care professionals. Accredited with the Medical and Dental Council of South Africa.

SUMMARY

Phytosterols and their glucosides (sterolins) have many therapeutic indications e.g. immune modulation, hypercholesterolaemia and benign prostatic hyperplasia (BPH). In this study sterols/sterolins in three BPH phytotherapeutics (*Hypoxis hemerocallidea, Prunus africana* and *Serenoa repens*) and related products were investigated.

The aim of this study was to develop, evaluate and apply TLC and HPLC methods for the qualitative and quantitative analyses of sterols and sterolins.

A new optimum TLC method was developed for good visibility and separation of phytosterols and sterolins and could be used to qualitatively compare sterol/sterolin content. A published HPLC method to determine the bioavailability of β-sitosterol in humans was used in a new application to quantitatively determine phytosterols in plant extracts. A new and sensitive method to determine hypoxoside (norlignan diglucoside unique to Hypoxidaceae), by isolation from the crude methanol extract with solid phase extraction (SPE) and HPLC quantification using fluorescence detection (excitation wavelength of 230 nm and emission wavelength of 345 nm), was developed.

The developed TLC and adapted HPLC methods were applied to determine the stability of phytosterols, subjected to increased temperature and gamma irradiation. Phytosterols in isolated form were more stable than the phytosterols in plant material. The data from the accelerated stability tests could be used to estimate the shelf-lives of the BPH phytotherapeutics and related sterol containing products.

The HPLC method to determine β-sitosterol in serum, was evaluated during a pilot study of a clinical trial, to test the bio-equivalence of different phytosterol containing products. The method was found not sensitive enough to determine β-sitosterol in serum, notwithstanding improvements made, i.e. changing the extraction ratio; experimenting with higher dosages, and different products. As result, the proposed clinical trial could not be performed, in the future, serum could rather be analysed by gas chromatographic methods.

TLC and HPLC analyses of medicinal African potato tea, indicated that it contained hypoxoside, but not β-sitosterol or β-sitosterolin. β-Sitosterol (accepted to be the active of *H. hemerocallidea*) might

not be the main active in African potato tea. Hypoxoside and a compound (*red spot compound*), noticed on TLC plates of acetone extracts of *Prunus africana, Serenoa repens*, Moducare®, Harzol®, Immunochoice® and Nutricare®, were extracted with water. This general presence of the *red spot compound* could point to a possible important function. Preparative TLC was unsuccessful to isolate the *red spot compound*, but column chromagraphy was successfully applied. From the proton and carbon NMR spectra, it was concluded, that the compound was definitely not a steroid and could either be a coumarin or an isoflavanoid, with a sugar unit (possibly a rhamose) attached to it. Further analyses to elucidate the structure failed due to decomposition of the compound. Further work on structure elucidation is required and possible therapeutic activity should also be investigated.

The sterols and sterolins in *H. hemerocallidea* and related herbal medicine can be qualitatively and quantitatively analysed with the developed TLC and adapted HPLC methods. This provides natural medicine industry with necessary procedures to ensure proper quality, safety and stability.

OPSOMMING

Plantsterole en glukosiede (steroliene) het verskeie terapeutiese toepassings, byvoorbeeld immunomodulering, hipercholesterolemie en beninge prostaathipertrofie (BPH). In hierdie studie is die sterole/steroliene van drie BPH-kruiemiddels (*Hypoxis hemerocallidea*, *Prunus africana* en *Serenoa repens*) en verwante produkte ondersoek.

Die doel van die studie was om dunlaag- (TLC) en hoë-druk vloeistofchromatografiese metodes (HPLC) te ontwikkel, te evalueer en aan te wend vir kwalitatiewe en kwantitatiewe analises van sterole en steroliene.

'n Nuwe optimale TLC-metode met goeie sigbaarheid en skeiding van sterole en steroliene, is ontwikkel om die sterol/sterolien inhoud kwalitatief te vergelyk. 'n Gepubliseerde HPLC-metode om β-sitosterol se biobeskikbaarheid mee te bepaal, is aangewend om sterole in plantekstrakte te kwantifiseer. 'n Sensitiewe metode om hipoksosied (norlignaan diglukosied uniek aan Hypoxidaceae) te bepaal is ook ontwikkel. Hipoksosied is uit die methanolekstrak van *H. hemerocallidea* geïsoleer met soliede-fase ekstraksie en met fluoressensie meting (eksitasie golflengte van 230 nm en emissie golflengte van 345 nm) gekwantifiseer.

Die nuwe TLC- en aangepaste HPLC-metodes is gebruik om die stabiliteit van pantsterole by verhoogde temperatuur en gammabestraling te bepaal. Klaarblyklik is sterole in geïsoleerde vorm meer stabiel as in plantmateriaal. Die versnelde stabiliteitsdata kon gebruik word om vervaldatums van BPH-kruiemiddels en soortgelyke produkte te voorspel.

Die HPLC-metode vir bepaling van β-sitosterol in serum, was geëvalueer tydens die loodsstudie van 'n bio-ekwivalensie proef van verskillende sterolprodukte. Die metode was egter nie sensitief genoeg om β-sitosterol in serum te meet nie, ongeag verbeteringe aan die metode soos verhoogde doserings, ander produkte en verandering van die ekstraksieverhouding. Gevolglik kon die bio-ekwivalensie studie nie deurgevoer word nie. In die toekoms moet gaschromatografie eerder gebruik word vir serumbepalings van β-sitosterol.

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TLC en HPLC analise het aangetoon dat medisinale Afrika-aartappeltee hipoksosied bevat, maar nie β-sitosterol of β-sitosterolien nie. β-Sitosterol word as die aktiewe bestanddeel van *H. hemerocallidea* beskou, maar is moontlik nie die hoofaktief van die aartappeltee nie. Hipoksosied en 'n interessante *rooikolverbinding* (rooi kol op TLC), sigbaar in asetoonekstrakte van *Prunus africana, Serenoa repens*, Moducare®, Harzol®, Immunochoice® en Nutricare®, word wel met water geëkstraheer. Die feit dat die *rooikolverbinding* in soveel belangrike sterolprodukte voorkom, dui moontlik op 'n belangrike funksie. Preparatiewe dunlaagchromatografie was onsuksesvol om die *rooikolverbinding* mee te isoleer, maar kolomchromatografie was suksesvol. Proton- en koolstof-KMR het aangedui dat die verbinding defnitief nie 'n steroïed is nie, maar moontlik 'n koumarien of isoflavonoïed met 'n suikergroep (moontlik ramnose). Die verbinding het ontbind voordat die analises voltooi kon word om die struktuur volledig op te klaar. Verdere analises is nodig ten einde die *rooikolverbinding* te identifiseer en moontlik terapeutiese aktiwiteit daarvan te bepaal.

Die ontwikkelde TLC- en aangepaste HPLC-metodes kan gebruik word om sterole en steroliene in *H. hemerocallidea* en soortgelyke produkte kwalitatief en kwantitatief te analiseer. Dit bied die natuurlike farmaseutiese industrie die noodsaaklike metodes om die kwaliteit, veiligheid en stabiliteit van sterolprodukte te verseker.

CHAPTER 1

PHYTOTHERAPY AND BENIGN PROSTATIC HYPERPLASIA

1.1 INTRODUCTION

1.1.1 History

Hypoxis hemerocallidea extracts were used traditionally as folk medicine to treat symptoms of cancers, testicular tumours, prostate hypertrophy and urinary disease (Albrecht et al., 1995b) for centuries. A South African, Mr. Roelof Wilke Liebenberg, took note of the African potato in the 1950's when his uncle's prostate cancer was cured after being treated with African potato tea. He made contact with Dr. Scheffel in Germany, who in turn persuaded an urologist, Dr. Ebbinghouse, to treat patients with benign prostatic hyperplasia (BPH) at the Kreis Krankenhaus with *H. hemerocallidea* extracts. Meanwhile Mr. Liebenberg requested a phytochemist from the University of the Witwatersrand, Prof. Karl Pegel, to isolate and identify the active compound in the African potato. Pegel and his assistant extracted a fatty substance which was identified as β-sitosterol. The German clinical trials were successful and in 1974 a patented remedy, Harzol® (10 mg β-sitosterol and 0.1 mg β-sitosterolin per capsule), was released. A few years later the South African equivalent, Moducare®/Sterinol™ (20 mg β-sitosterol and 0.2 mg β-sitosterolin per capsule), was released in South Africa (Vanderhaeghe & Bouic, 1999).

1.1.2 Aim of chapter

In this chapter, literature background on phytosterols and their use in immune modulation; hypercholesterolaemia, and specifically, indications in treating benign prostatic hyperplasia (BPH), is discussed. Three BPH phytotherapeutic plants *Hypoxis hemerocallidea*, *Prunus africana* and *Serenoa repens*, are also considered in terms of their mechanisms of action, the extraction processes, their general compositions, recommended daily therapeutic dosages, efficacy and side effects.

1.2   STEROLS: PHYTOSTEROLS VERSUS CHOLESTEROL

Steroids are members of a large class of lipid compounds called terpenes, which are biogenically derived from the same parent compound, isoprene (C_5H_8). Steroids contain, or are derived from, the perhydro-1,2-cyclopentenophenanthrene ring system (Kroschwitz & Howe-Grant, 1997).

Phytosterols are steroid alcohols (plant fats) similar to the major animal steroid alcohol (sterol), cholesterol (5-cholesten-3β-ol). β-Sitosterol (24β-ethyl-5-cholesten-3β-ol) differs from cholesterol only by an extra ethyl group in its side chain (Bouic et al., 1997). Plant and animal sterols have very distinct biological effects although they are both essential cell membrane components, and phytosterol esters have a similar fatty acid transport and storage function as cholesterol esters (Pegel, 1997). Phytosterols are synthesised in plants whereas humans and animals obtain them through diet (Bouic & Lamprecht, 1999). However, the human absorption of β-sitosterol is 5% or less of the daily intake in comparison with the 50% absorption of cholesterol (Salen et al., 1970). The molecular mechanism regulating the amount of cholesterol retained in the body and selectively excluding other dietary sterols, is poorly understood, but is probably genetically determined (Platel et al., 1998). Sterols never exist on their own in nature, but are always found in combination with their glucosides, which are called sterolins. Thus a sterolin is a sterol with a sugar attached to it. All plants, including fruits; vegetables, seeds, and nuts contain sterols and sterolins (Vanderhaeghe & Bouic, 1999). Approximately 80% of the total phytosterol content of higher plants is composed of β-sitosterol, with about 1% in its glucosidated form (Bouic et al., 1997). Other important phytosterols are campesterol (24α-methyl-5-cholesten-3β-ol) and stigmasterol (3β-hydroxy-24-ethyl-5,22-cholestadiene). See Figure 1.1 for the chemical structures of the sterols and Figure 1.2 for that of β-sitosterolin.

It is important to note that the term "β-sitosterol" on a product usually represents a defined extract of phytosterols with β-sitosterol as the main component. Therefore, even though Harzol®'s container states that every capsule contains 10 mg β-sitosterol and 0.1 mg β-sitosterolin, it in fact contains mainly β-sitosterol and smaller amounts of campesterol, stigmasterol and other sterols along with their glucosides (Berges et al., 1995).

Cholesterol

$(C_{27}H_{46}O;$ mol wt 386.7 g/mol)

β-sitosterol

$(C_{29}H_{50}O;$ mol wt 414.7 g/mol)

Campesterol

$(C_{28}H_{48}O;$ mol wt 400.7 g/mol)

Stigmasterol

$(C_{29}H_{48}O;$ 412.7 g/mol)
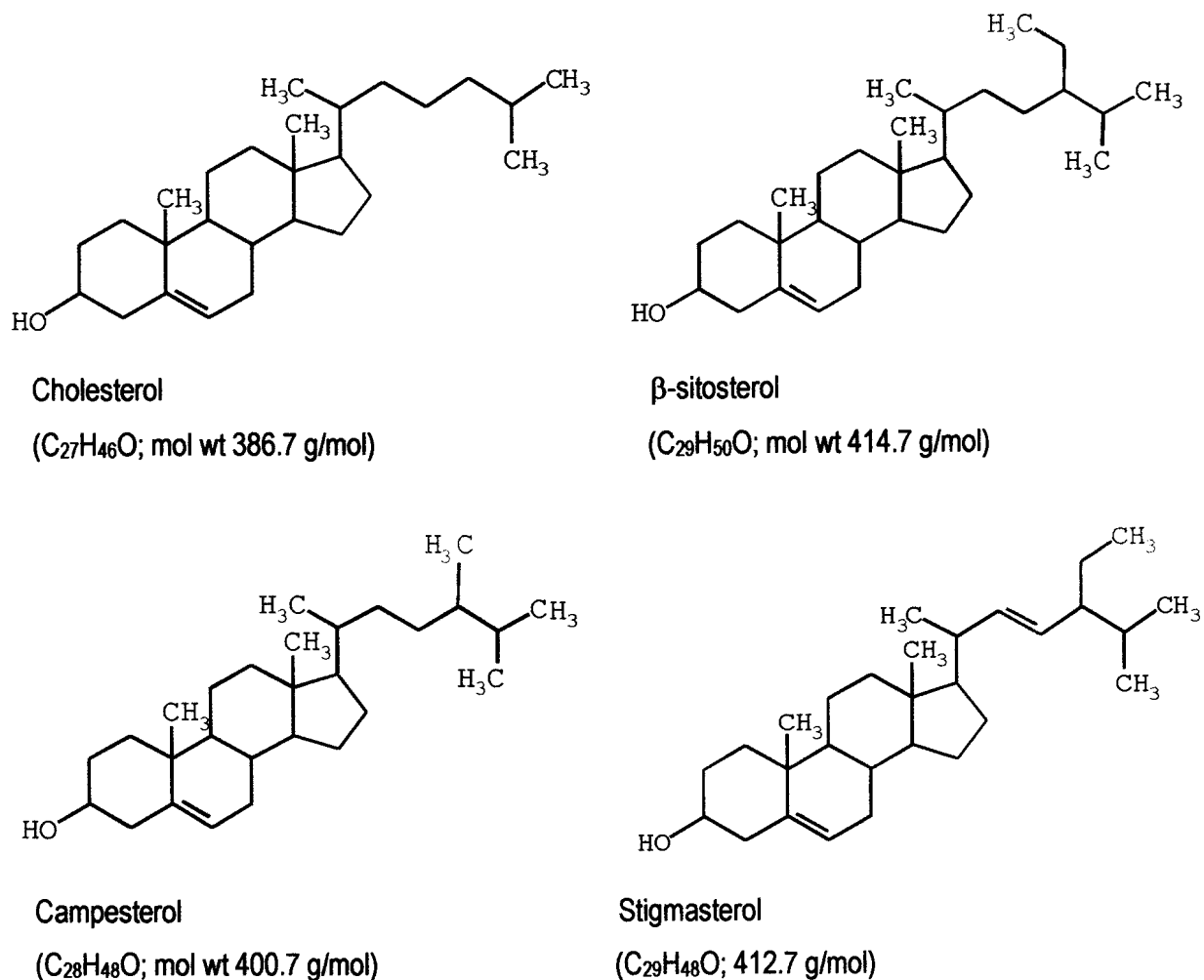
Figure 1.1: The chemical structures, molecular formulae and weights of cholesterol, β-sitosterol, campesterol and stigmasterol. The red groups indicate the differences from cholesterol.
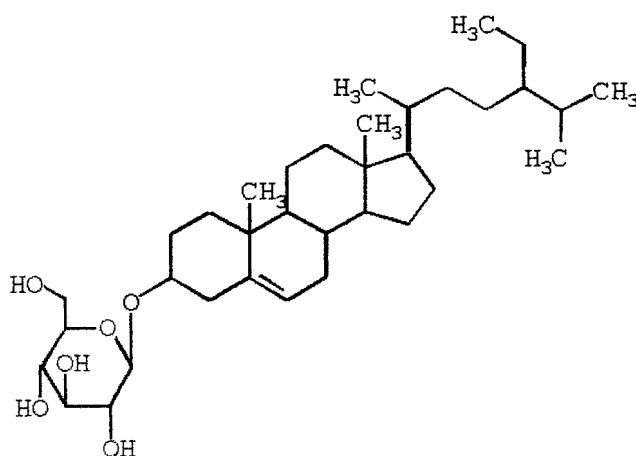
Figure 1.2: Chemical structure of β-sitosterolin $(C_{35}H_{60}O_6;$ mol wt 576.9 g/mol)

(β-sitosteryl β-*D*-glucoside)

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1.3   THERAPEUTIC INDICATIONS OF PHYTOSTEROLS

The three major indications for phytosterols are immune modulation (Bouic et al., 1999), hypercholesterolaemia (Nguyen, 1999) and benign prostatic hyperplasia (Wilt et al., 1998), although, research on β-sitosterol also revealed antihyperglycemic and insulin-releasing effects (Ivorra et al., 1988), anti-inflammatory (similar to cortisone)- and antipyretic (similar to aspirin, but not analgesic) effects (Gupta et al., 1980).

1.3.1   Immuno-modulation – Reinstating the balance in the immune system

1.3.1.1 The immune response

T-cells (thymus lymphoid cells) are divided into T-helper cells (CD4+), T-suppressor cells (CD8+) as well as cytotoxic T-cells (CD8+), all of which   mediate cellular immunity. B-cells (bone marrow lymphoid cells) form antibodies and mediate humoral immunity. The T-cells recognise antigens e.g. pathogens, and release chemical substances (cytokines) to regulate the immune systerm – stimulation or suppression. T-helper cells (CD4+) are divided into type 1 (T_H1) and type 2 (T_H2) cells (Ingraham & Ingraham, 1995).

T_H1 releases interleukin 2 (IL2) and gamma interferon (IFN-γ), that bind to, and activate, the (CD8+) cytotoxic T-cells (T_c) to become killer T-cells. These killer cells attack and remove intracellular pathogens (Vanderhaeghe & Bouic, 1999) e.g. viruses and *Mycobacterium tuberculosis*, cancerous cells and unfortunately, donor transplant cells (Ingraham & Ingraham, 1995).

T_H2 secretes interleukin 4 (IL4), IL6 and IL10, that stimulate B-lymphocyte differentiation into antibody forming cells (forming immunoglobulins e.g. IgE, IgG *etc*). Other important immune factors are phagocytes (granulocytes and macrophages) and natural killer cells. The natural killer cells form the first line of defence against pathogens and cancer cells by releasing toxic enzymes and do not need activation by T-cells (Vanderhaeghe & Bouic, 1999).

Too high levels of IL4 promote allergic responses, and too high levels of IL6 are associated with auto-immune disorders, inflammatory and allergic conditions. In health there is a delicate balance between T_H1 and T_H2 which cross-regulate each other's activity. Cortisol, the stress hormone,

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enhances T_H2 activity and increases the release of IL6 which promotes inflammation and destruction of body tissues. DHEA (dehydroepiandosterone) antagonizes cortisol and enhances T_H1 activity which balances the immune system (Vanderhaeghe & Bouic, 1999).

Table 1.1: The dichotomy of T-helper cells based on their defining cytokine profiles and functions (Bouic et al., 1999)

Cell	Cytokine profile	Function of TH subset
T_H1	IL2, IFN-γ	Activation of cytotoxic cells Antagonism of T_H2
T_H2	IL4, IL6, IL10	Activation and maturation of B-cells Antagonism of T_H1

If T_H1-levels drop (due to cortisol release, as with unrelenting stress, DHEA decrease or chronic viral and bacterial diseases) T_H2-levels will rise. A humoral, but non-protective immune response, will result and chronic conditions such as cancer, allergies ($\uparrow$IgE, $\uparrow$IL6, $\uparrow$IL4) and auto-immune disorders ($\uparrow$IL6 - rheumatoid arthritis, lupus erythematosus, pernicious anaemias, Type 1 diabetes mellitus, Crohn's disease *etc.*) can appear (Vanderhaeghe & Bouic, 1999).

1.3.1.2 Immune stimulation and suppression by sterols/sterolins

Both β-sitosterol and β-sitosterolin enhance T-cell proliferation *in vitro* and *in vivo*. However, they increase T_H1 (immuno-stimulation) and, therefore, IL2 and γ-interferon secretion and citotoxic T-cell activity is augmented, but T_H2 is reduced (immuno-suppression) resulting in reduced IL4, IL6 and IL10 levels, Thus reinstating the balance (immuno-modulation). A mixture of β-sitosterol and β-sitosterolin in a ratio of 100:1 is claimed to be more effective than either sterol or its glucoside alone – synergism (Vanderhaeghe & Bouic, 1999).

Indications:

$\uparrow$ T_H1 ($\uparrow$IL2, $\uparrow$IFN-γ) – Immuno-stimulation

- Cancer - $\uparrow$IL2, $\uparrow$IFN-γ, $\uparrow$T$_c$ (Vanderhaeghe & Bouic, 1999). - HT-29 colon cancer (Pegel, 1997).
- AIDS - $\uparrow$IL2, $\uparrow$IFN-γ - FIV (cats) and HIV (humans) CD4 counts remained stable with no progression of the disease - up to date (36 months). Plasma viral loads also declined as result of

an enhanced cell-mediated immune response. Sterols and sterolins do not have anti-viral properties (Bouic et al., 1999).

- Tuberculosis - $\uparrow$eosinophil & $\uparrow$lymphocyte counts, faster weight gain (Donald et al., 1997)

- Post-marathon or high intensity exercise immuno-suppression - $\uparrow$DHEA , $\uparrow$ T_H1, IL2 & IFN-γ and $\downarrow$cortisol, $\downarrow T_H2$, $\downarrow$IL6 (Bouic et al.,1999)

- Others: Chronic fatigue syndrome ($\uparrow T_c$, $\uparrow$IL2, $\uparrow$IFN-γ),

$\downarrow$ T_H2 ($\downarrow$IL4, $\downarrow$IL6) – Immuno-suppression

- Rheumatoid arthritis ($\downarrow$IL6, $\downarrow$ B-cell antibody formation) and other auto-immune diseases e.g. Type 1 diabetes mellitus ($\downarrow$IL6, $\downarrow$ B-cell antibody formation, $\uparrow$DHEA & $\downarrow$cortisol) – can decrease insulin requirement (Vanderhaeghe & Bouic, 1999).

- Allergies ($\downarrow$IL4, $\downarrow$IL6 → $\downarrow$IgE) – asthma, allergic eczema, chronic rhinitis and sinusitis, hives and rashes (Vanderhaeghe & Bouic, 1999).

- Hepatitis C – ($\downarrow$IL6, $\downarrow$ B-cell antibody formation) - Reduce attacks on liver cells by antibodies ($\uparrow$ IL2, $\uparrow$IFN-γ) increased protection against viral infection (Vanderhaeghe & Bouic, 1999).

1.3.2 Hypercholesterolaemia

β-sitosterol is absorbed less efficiently (1/10) than cholesterol, but in high dosages ($\geq$10 g/d) it lowers the absorption of cholesterol by 10–20%, and lowers total cholesterol and low density lipoprotein (LDL) levels. Plant sterols appear to decrease the solubility of cholesterol in oil and micellar phases, thus displacing cholesterol from bile salt micelles and interfering with its absorption (Nguyen, 1999). They can, therefore, be used to treat mild cases of hypercholesterolaemia (Pegel, 1997).

However saturated derivatives of plant sterols, called plant stanols (Δ4 double bond absent), can reduce serum cholesterol at lower doses (2-3 g esterified sitostanol lowers LDL cholesterol by 10-15%). They reduce total cholesterol and LDL, but not high-density lipoprotein (HDL) or triglyceride (TG) levels. These plant stanols are virtually unabsorbable and remain in the intestinal lumen where they can interfere continuously and more efficiently with micellar solubility of cholesterol. The most common dietary plant stanol is sitostanol, the saturated derivative of sitosterol. It occurs naturally in wood pulp, tall oil and in lesser amounts in soybean oil. The Western daily diet contains 100-300 mg

plant sterols and 20-50 mg plant stanols. In 1995 the Finnish introduced plant stanol esters (PSE) into margarine as dietary adjuncts, to lower cholesterol (Nguyen, 1999).

Sitosterolaemia (phytosterolaemia) is a rare, autosomal, recessively inherited disorder characterised by premature coronary artery disease, skin xanthomas and increased plasma plant sterols, stanols and cholesterol. Affected individuals show an increased absorption of both cholesterol and sitosterol from the diet, which distinguishes it from homozygous familial hypercholesterolaemia. Furthermore, the excretion of both cholesterol and phytosterols into the bile is also greatly impaired. The molecular defect in sitosterolaemia has not been identified, but it is probably determined genetically (Platel et al., 1998). The role of the increased serum phytosterol levels in the resulting premature coronary artery disease and skin xanthomas has not been elucidated.

1.3.3 Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is a common medical condition in older men affecting as many as 40% of men aged 70 years and older (Wilt et al., 1998). Symptoms associated with the disease include urgency, frequency, hesitancy, weak stream and incomplete bladder voiding (Segars, 1999). In the United States of America, treatment exceeds $2 billion in costs; accounts for 1.7 million physician office visits, and results in more than 100 000 prostatectomies annually (Wilt et al., 1998).

1.3.3.1 Allopathic treatment

Western medicines are seen as those opposing the so-called alternative, complementary or unconventional medicines (including phytotherapy). These western medicines have to be critically evaluated for safety, efficacy etc. and approved by the American Food and Drug Administration or related council (the Medicines Control Council in South Africa) before production or marketing. Alternative medicines on the other hand are regarded as dietary supplements and regulated under the 1994 Dietary Supplement Health and Education Act in the USA (Food supplements, Cosmetics and Disinfectants Act 54 of 1972, in South Africa) (Marks & Tyler, 1999). Though it seems as if certain of these herbal medicines, especially those for which medicinal claims are made, will have to be registered under the Medicines and Related Substances Control Act 101 of 1965, in South Africa in the future.

Transurethral resection of the prostate in men with symptoms of obstruction is the standard treatment (Berges et al., 1995). The most commonly used western medicines for the treatment of BPH are α_1-adrenergic antagonists such as prazosin, terazosin, doxazosin and trimazosin, as well

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as 5α–reductase inhibitors e.g. finasteride (Hardman & Limbird, 1996). At least two isomeric forms of 5α–reductase exist. Type I is mainly expressed in the skin and liver, but it is also present in the normal and pathological prostate. Type II is mainly expressed in the prostate, in particular the stromal compartment and in the male genital tract (Di Silverio et al., 1998). Finasteride inhibits the Type II 5α–reductase. The α_1-adrenergic blockers cause relaxation of the smooth muscle in the bladder neck, prostate capsule and prostatic urethra, with a rapid improvement in urinary flow as a result; 5α–reductase inhibitors, on the other hand, inhibit the conversion of testosterone to 5α-dihydrotestosterone (DHT) (Hardman & Limbird, 1996). See Figure 1.3. DHT represents approximately 95% of the total androgen content within the prostate cell nuclei (Di Silverio et al., 1998) and is a critical factor in the growth of the prostate and the development of BPH (Bayne et al., 1999). In contrast to the rapid effects of the α_1-adrenergic blockers, the actions of finasteride can be delayed for months (Hardman & Limbird, 1996).
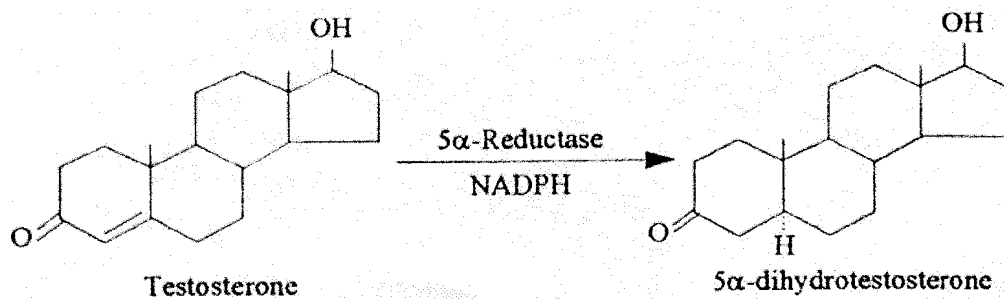
Figure 1.3: Transformation of testosterone into 5α-dihydrotestosterone by 5α-reductase (Toth et al.,1996)

1.3.3.2 Phytosterols and BPH

Phytotherapeutic agents represent more than 90% of all drugs prescribed for BPH in Germany and Austria (Wilt et al., 1998). The two BPH phytotherapeutics most often used in Germany are two β-sitosterol products, Azuprostat®, which contains 65 mg β-sitosterol per capsule, and Harzol®, which contains 10 mg β-sitosterol and 0.1 mg β-sitosterolin per capsule (Schultz et al., 1998).

Although the exact biochemical mechanism of action is not yet known, β-sitosterol shows a significant effect in patients with symptomatic BPH. It decreases the International Prostate Symptom Score (IPSS – questionnaire) and the modified Boyarsky score; improves the quality of life score

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(QoL); increases the peak urinary flow rate (Q_{max}) and decreases the post void residual urinary volume (PVR). (Klippel et al, 1997). These results are comparable with those achieved by α_1-blockers or 5-α-reductase inhibitors, but in contrast to finasteride, β-sitosterol does not reduce prostate size. Adverse effects, such as dizziness, decreased blood pressure, tachycardia *etc.* associated with α_1-blockers (Berges et al., 1995), and erectile dysfunction, ejaculatory disturbance or altered libido associated with finasteride (Marks & Tyler, 1999), are absent with β-sitosterol (Berges et al., 1995).

However, the problem of drug therapy (allopathic or phytotherapeutic) in the treatment of BPH is complicated by the fact that the natural history of the untreated disease is one of fluctuating symptoms and spontaneous improvements (Kadow & Abrams, 1986). It is well known that placebo effects occur in pharmacological therapies in general and particularly in patients with BPH who wish to avoid operative intervention; responses of up to 40% or more have been reported (Klippel et al., 1997). Ideally, BPH treatment and follow-up should be for years rather than months and placebo control is essential (Kadow & Abrams, 1986).

1.3.3.3 Phytosterols' mechanism of action in BPH

It is speculated that the activity of phytosterols can be ascribed to enzymatic effects (inhibition of 5α-reductase leading to a reduction in dihydrotestosterone levels) or to decreased binding of dihydrotestosterone within the prostate (Van Wyk et al., 1997). Other possible mechanisms of action are competition with androgen precursors and inhibition of prostaglandin biosynthesis (anti-inflammatory) by the phytosterols in the extract. The pentacyclic terpenes exhibit anti-inflammatory activity by inhibiting glucosyl transferase and β-glucuronidase enzymes involved in the depolymerisation of proteoglycans in the connective tissues (Shulz et al., 1991).

1.3.3.4 β-Sitosterol, β-sitosterolin or combination?

Dr. Ebbinghouse of the Kreis Krankenhaus, Germany, (See 1.1.1 History) experienced that β-sitosterol alone was not as effective as the extract of *H. hemerocallidea* on patients with BPH. Prof. Karl Pegel, who isolated and identified β-sitosterol, realised that sterolglucoside, the sterolin, was possibly better absorbed than the sterol, but that during the process of isolation of the sterol, the sterolin was removed via hydrolysis, yielding sterols and glucose. This was the reason why the β-sitosterol used on Dr. Ebbinghaus's patients did not work as well – it was lacking the "key to the immune-enhancing effect", the sterolin. Together with Prof. C.B. Rodgers, a product containing

sterols and sterolins was developed and capsules were sent to Dr. Ebbinghaus in Germany. Several clinical trials were performed there, resulting in the release of a patented BPH remedy, Harzol® (10 mg β-sitosterol and 0.1 mg β-sitosterolin per capsule) in 1974 (Vanderhaeghe & Bouic, 1999). However, a double blind, placebo-controlled urodynamic study on 53 patients with proven outflow obstruction, has failed to prove that β-sitosterolin (formula WA184) is superior to a placebo in the treatment of outflow obstruction due to BPH, when administered at a dose of 0.3 mg/day (Kadow & Abrams, 1986). Thus, β-sitosterolin alone is also not effective in treating BPH, but a combination of β-sitosterol and β-sitosterolin has been found to be effective.

1.4 PHYTOSTEROL CONTAINING BPH PHYTOTHERAPEUTICS

Phytotherapy, the use of plant extracts, for treating BPH symptoms, was first described in Egypt in the 15th century BC. Currently phytotherapy is common in Europe and increasing in usage in the western hemisphere (Wilt et al., 1998). The most widely used and thoroughly tested of the BPH phytotherapies is saw palmetto, scientifically known as *Serenoa repens* or *Sabal serrulata* (Marks & Tyler, 1999). There may be some confusion regarding the names used for the particular plants in this study as earlier synonyms may be used in addition to the widely accepted scientific names. Only one of these names will be used. To avoid confusion the recognised name, synonyms, common name, plant part used for extraction and trade name of some plants, often used as BPH phytotherapeutic agents, are tabled in Table 1.2. Besides saw palmetto, other plant extracts that are used for the treatment of lower urinary tract symptoms (LUTS) associated with BPH, include the following: *Hypoxis hemerocallidea* (African potato), *Prunus africana* (African plum), *Urtica dioica* (stinging nettle), *Secale cereale* (rye pollen), *Cucurbita peopo* (pumpkin seeds), *Picea* (spruce) and *Pineas* (pine) (Lowe et al., 1998).

In this chapter three of the above mentioned plants will be considered: *H. hemerocallidea, P. africana* and *S. repens*, in terms of their mechanism of action in BPH, the extraction process, their general composition, recommended daily therapeutic dosage in BPH, efficacy and side effects.

Table 1.2: Different names and trade names of some important BPH phytotherapeutic agents (composed from data by Lowe et al., 1998)

Recognised name	Synonym	Common name	Part of plant used	Tradename
Serenoa repens	*Sabal serrulata*	Saw palmetto	Berries	Permixon® Strogen® etc.
Hypoxis hemerocallidae	*Hypoxis rooperi*	African potato	Corms (rhizomes)	Harzol®
Prunus africana	*Pygeum africanum*	African plum/ Red stinkwood	Bark	Tadenan®
Urtica dioica		Stinging nettle	Roots	Prostagutt Forte® (with Saw palmetto)
Secale cereale		Rye pollen	Pollen	Cernilton®
Cucurbita peopo		Pumpkin seed	Seeds	

1.4.1   *Hypoxis hemerocallidea* (African potato)

1.4.1.1 General information

The family, Hypoxidaceae, includes fifty *Hypoxis* species (Arnold & De Wet, 1993) of which *H. hemerocallidea* (previously also known by the name *H. rooperi*) is probably the most important in terms of medicinal use. The plants are herbaceous perennials with long, strap-shaped leaves and yellow, star-shaped flowers. The species can be distinguished by the size, shape and orientation of the leaves and the size and shape of the flowers (Van Wyk et al., 1997). They have tuberous rhizomes or corms (up to 10 cm in diameter and length and up to 2 kg in weight) and abundant adventitious roots that enable them to survive under high-stress conditions. Members of Hypoxidaceae are found mainly in the southern hemisphere, especially in Africa (Albrecht et al., 1995b).

Traditional uses in folk medicine have been reported to include treatment of cancers, testicular tumours, prostate hypertrophy and urinary disease (Albrecht et al., 1995b).

In Germany a lipophilic extract of *H. hemerocallidae* is marketed as a phytomedicine Harzol® and has been used for the treatment of prostate hypertrophy since 1974 (Pegel, 1997). Harzol® contains β-sitosterol and its glucoside in the ratio 10:0.1 per capsule. In South Africa, the dried rhizomes are marketed (Prostamin®) for the preparation of an aqueous potion for the same purpose (Nicoletti et al., 1996). Another German β-sitosterol preparation, Azuprostat®, contains 65 mg of a chemically defined extract of phytosterols, with β-sitosterol as the main component, per capsule. These β-sitosterols are derived from different plants, for example, species of *Pinus*, *Picea* or *Hypoxis spp.* (Klippel et al., 1997).

1.4.1.2 General composition

From the rhizomes of *H. obtusa*, a norlignan diglucoside named hypoxoside was isolated and later the occurrence of hypoxoside in several *Hypoxis spp.* was reported (Nicoletti et al., 1996). Hypoxoside is the trivial name for (E)-1, 5-bis (4'-β-D-glucopyranosyloxy-3'-hydroxyphenyl) pent-4-en-1-yne. It is a pale yellow, water-soluble, crystalline compound which is readily converted to the more lipophilic aglucone rooperol by beta-glucosidase action (Albrecht et al., 1995b). See Figure 1.4.
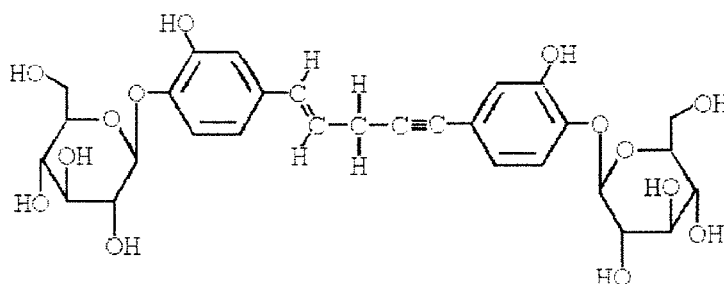
Figure 1.4: Chemical structure of hypoxoside ($C_{29}H_{34}O_{14}$; mol wt 606.6g/mol)

Albrecht et al. (1995a), analysed a standardised *Hypoxis* extract, supplied by ESSENTIAL STEROLIN PRODUCTS in capsule form, each capsule containing 200 mg plant extract. Routine HPLC analyses assured uniform quality of each batch of plant extract within the limits as shown in Table 1.3:

Table 1.3: Composition and limits of a standardized *Hypoxis* plant extract detected by HPLC analyses (composed from data by Albrecht et al., 1995a)

Component	Percentage of contents
Hypoxoside	50-55%
β-sitosterol	8-12%
β-sitosterolin	0.2 – 0.3%
Complex carbohydrates with traces of reducing sugars, amino acids, tannins and flavonoids (not detectable by HPLC)	33-42%
Alkaloids	0%

1.4.1.3 Mechanism of action

The activity of the drug against prostate adenoma and prostatic hypertrophy is ascribed mainly to phytosterol glucosides, mainly β-sitosterol. Anti-cancer (Vanderhaeghe & Bouic, 1999; Pegel, 1997), anti-HIV (Bouic et al., 1999) and anti-inflammatory (Gupta et al., 1980) activity are ascribed to β-sitosterol and rooperol (aglucone of hypoxoside) (Van Wyk et al., 1997).

As regards, cytotoxicity, *In vitro* hypoxoside is non-toxic to cancer cells at concentrations up to 100 μg/ml. However, when it is hydrolysed to its aglucone, rooperol, by β-glucosidase, cytotoxicity is found at concentrations ranging from 2 to 10 μg/ml (Albrecht et al., 1995a). Hypoxoside given orally to mice is deconjugated to form rooperol by bacterial β-glucosidase enzymes in the caecum and colon. The rooperol is then absorbed and converted to new conjugates (sulphates and glucuronides) by phase II biotransformation and then excreted into the bile and absorbed into mice portal blood. Even though no hypoxoside, rooperol, or rooperol metabolites entered the general circulation of the mice, the situation in humans was strikingly different and metabolites appeared in the serum of humans (Albrecht et al., 1995b). See Figure 1.5 for the biotransformation of hypoxoside in mice and primates. Albrecht et al. (1995b) demonstrates that these metabolites could be activated to become cytotoxic to melanoma cells, in culture, in the presence of β-glucuronidase as monosulphates of rooperol and dehydroxyrooperol (with glucuronic acid) are formed. They also found that extracts of human tumour (aqueous supernatant of human melanoma) could also deconjugate the metabolites and thus

activate them for their cytotoxic action. Hypoxoside could thus be a non-toxic pro-drug for cancer therapy (Albrecht et al., 1995b).
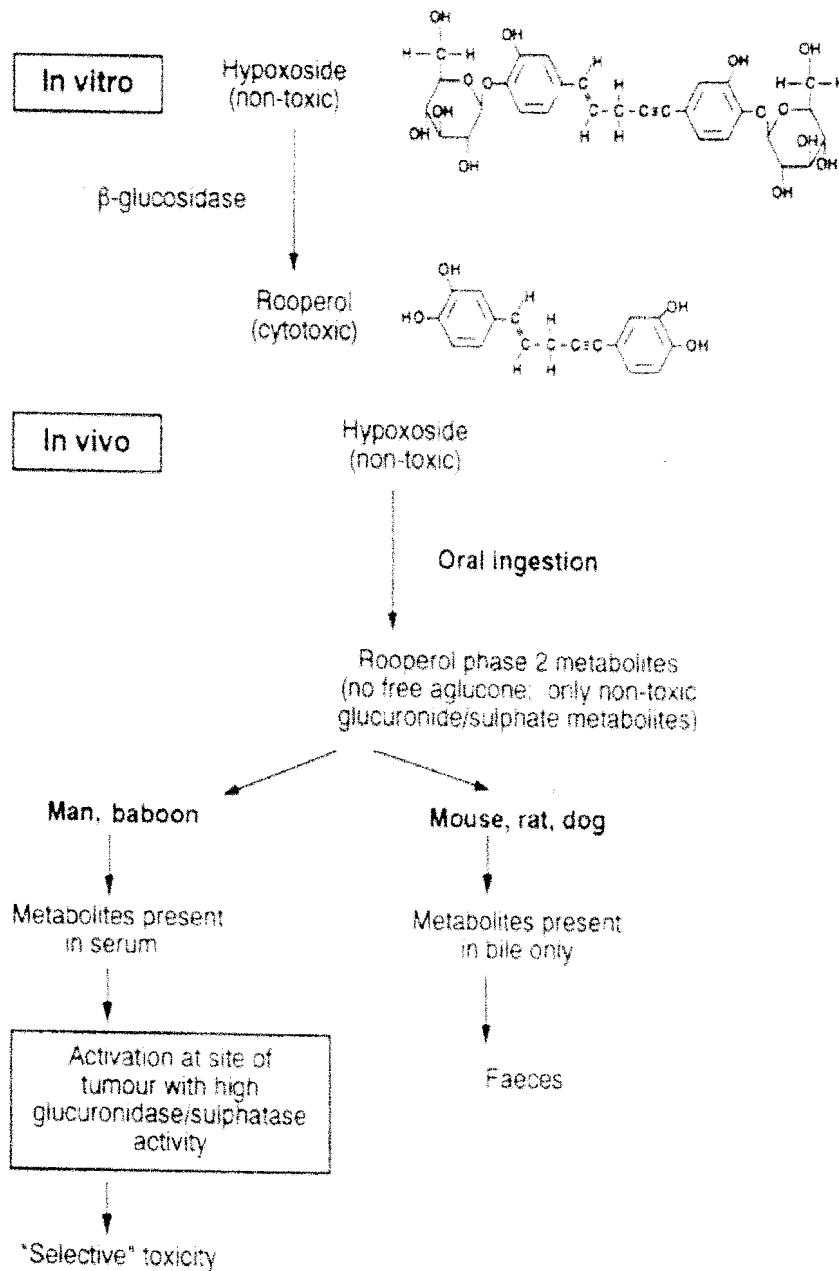
Figure 1.5: The biotransformation of hypoxoside to cytotoxic rooperol (Smit et al., 1995)

The cytotoxic effect of rooperol manifested as vacuolisation of the cytoplasm and formation of pores in the plasma membrane. The molecular basis of rooperol cytotoxity still needs to be clarified, but

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previous biochemical studies have shown that rooperol is a potent inhibitor of leukotriene synthesis in polymorphonuclear leukocytes at concentrations of 1 μM or less. The synthesis of cyclooxygenase products, TXB_2 (tromboxane B_2) and PGD_2 (prostaglandin D_2), are inhibited only at concentrations between 10 and 100 μM. Rooperol-induced growth inhibition occurs at concentrations ranging from about 0.6 to 8 μg/ml which is equivalent to about 1 – 13 μM. It is, therefore, possible that the cytotoxicity is triggered by inhibition of leukotriene synthesis. Another possibility is that rooperol may be oxidised to form reactive semiquinone- and quinone radicals that could damage cell membranes directly (Albrecht et al., 1995b). This is known to occur with dicatechols such as the closely related cytotoxic agent, nor-dihydroguaiaretic acid (Albrecht et al., 1995b).

1.4.1.4 Extraction

Shredded *Hypoxis* corms are dehydrated in a convection oven at 70°C for 3.5 hours and then milled to a powder, which can be extracted with 25*l* of methanol at room temperature for 30 minutes (Kruger et al., 1994). The corms can also be freeze-dried with liquid nitrogen and extracted with 80% ethanol in the dark at 23 °C for 4 hours (McAllister & van Staden, 1994).

Another extraction method used locally, is that of Santjie Marx African potato product (promotional pamphlet). She proposes that c. 6.4 g of dried *Hypoxis* be simmered in 1200 ml water for 15 to 20 minutes and 600 ml should be taken daily.

1.4.1.5 Presentation and dosage

Santjie Marx Product's dosage is 200 ml {200 ml equals 416 mg dry extract as water only extracts 39% (Eloff, 1999)} three times daily (promotional pamphlet). Local preparations that are available in most pharmacies are Moducare® and Immunochoice®. These preparations contain only phytosterols and their glucosides (Immunochoice® contains absorption enzymes as well), without any hypoxoside or rooperol. Moducare® is available as capsules containing 20 mg phytosterols and 0.2 mg sterolins per capsule in an internationally patented formulation. The daily dosage is 60 mg daily (one capsule three times daily). Immunochoice® is available as capsules containing a 20 mg mixture of phytosterols (β-sitosterol, campesterol and stigmasterol) and sterol glucosides (sterolins), as well as 50 mg of enzymes (formula GR8CF-77) to promote the absorption of the phytosterols and sterolins per capsule. The absorption of β-sitosterol is only about 5% in comparison with the 50% absorption of cholesterol (Pegel, 1997). The dosage of Immunochoice® is also one capsule, three times daily.

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Nutricare 2000® is also a South African phytosterol preparation (400 mg capsules, containing 58 mg fat, including sterols) but it differs from Moducare® and Immunochoice® in that hydroponically grown oilseed sprouts are harvested at their peak and freeze-dried in less than an hour, thus no chemical extractions are necessary (Anon, 2000). It is claimed that the absorption of the natural sterols in plants, especially in sprouts, is about 80% in comparison with the 5% absorption of sterol extracts (Personal communication with Dr. van Brummelen, BIOMOX PHARMACEUTICALS; Maria Ascencao, spokesperson of Bioharmony). Nutricare 2000® also contains digestive enzymes that are derived from wheat, barley, lupins and fenugreek sprouts and blended with the phytosterols derived from sprouted oilseeds. These enzymes are supposed to increase the bio-availibility of the sterols (Anon, 2000). The dosage is one capsule three times a day, as well.

1.4.1.6 Efficacy

A study was performed by Berges et al. (1995) on 200 randomized patients who received either a placebo or β-sitosterol, at a dose of 20 mg, three times daily for 6 months. The results of the treatment group, in comparison with the placebo group, were promising. The IPSS decreased from 14.9 to 7.5 and the maximal urinary flow (Qmax) increased from 10.0 to 15.2 ml/s (Fitzpatrick, 1999).

1.4.1.7 Side effects

A study done to assess the toxicity of hypoxoside taken orally by 24 patients with lung cancer found no toxic effects in clinical examinations or biochemical or haematological measurements. Only one occasion of possible drug intolerance, at a dosage as high as 2400 mg standard *Hypoxis* plant extract (200 mg capsules) daily, with anxiety, nausea, vomiting and diarrhoea, was noted. The dosage of the particular patient was then reduced to 1200 mg per day, which was well tolerated without other incidents for another 36 days, after which the patient had cardio-respiratory failure (not attributable to the drug) (Smit et al., 1995).

1.4.2 *Prunus africana* (African plum)

1.4.2.1 General information

P. africana is the evergreen African plum tree (Lowe et al., 1998). This tall forest tree may reach a height of more than 30 metres. Buttress roots are often present and the bark is coarse, with a dark brown and black colour. It has dark green glossy leaves, small white flowers in elongated clusters and reddish-brown berries of about 10 mm in diameter (Van Wyk et al., 1997).

The bark is reported to be of value in treating chest pain (Van Wyk et al., 1997). Since the first registration by LABORATORIES DEBAT (France, 1969), an extract of the bark of *P. africana* has been used successfully in Europe and elsewhere for the treatment of BPH symptoms. LABORATORIES DEBAT marketed the *P. africana* extract as Tadenan® (Breza et al., 1998), but it is currently manufactured by LABORATORIES FOURNIER S.A., FONTAINE les Dijon, France. Other formulations (Prostata® *etc.*) are available throughout the United States, but almost all the research and clinical trials on *P. africana* bark extract have been done using the product Tadenan® (Lowe & Fagelman, 1999).

1.4.2.2 General composition

Lipophilic extracts of *P. africana* bark have been shown to contain at least three classes of active constituents that exert a beneficial effect on BPH. Phytosterols (present in both free and conjugated form); pentacyclic terpenes (including oleanolic, crataegolic and ursolic acids); and ferulic acid esters of fatty alcohols (Schulz et al., 1998).

1.4.2.3 Mechanism of action

P. africana bark extract (Tadenan®) has been used for the past 25 years in the treatment of micturition disorders associated with BPH at a non-surgical stage. Unfortunately the mechanism of action had not been clearly elucidated (Yablonsky et al., 1997).

Growth factors and their receptors' activation play an important role in the mechanisms of normal and pathological development of the prostate (Yablonsky et al., 1997). The effects of *P. africana* bark extract (Tadenan®) on the proliferation of rat prostatic stromal cells were studied by Yablonsky et al. (1997). The inhibitory activity of the extract was tested either on unstimulated or on basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I) and keratinocyte growth factor (KGF), stimulated cells. The ability of Tadenan® to inhibit ³H-thymidine incorporation into stromal cells was also investigated. When increasing concentrations of the extract were added to either non-stimulated or growth factor stimulated cells, a dose-dependant inhibition of DNA synthesis was observed. However, Levin et al. (1996) found, in their experiments on rabbits with partially obstructed bladders, that the magnitude of increases in bladder mass and the level of DNA synthesis (³H-thymidine incorporation) were similar for the Tadenan® treated and untreated animals. Levin et al. (1996), therefore, concluded that Tadenan® had no effect on either the increase in bladder mass or the stimulation of DNA-synthesis induced by partial outlet obstruction. The

inhibition of DNA synthesis, according to Yablonsky et al. (1997), was significant at 1 μg/ml of the extract in bFGF; EGF and IGF-I treated cells and 5 μg/ml in non-treated cells. According to their results, stromal cells appeared to be more sensitive to bFGF and EGF than to IGF-I, while they did not show any sensitivity to KGF.

Using a rabbit model of partial outlet obstruction, Levin et al. (1997) identified three major cellular changes in the bladder as result of such obstruction: progressive denervation; mitochondrial dysfunction and disturbances of calcium storage, and release from the sarcoplasmic reticulum. Their hypothesis is that outlet obstruction results in bladder hypertrophy which induces ischaemia. This leads to a release of intracellular calcium, leading to activation of specific enzymes (calcium-activated protease and phospholipase A_2) and to generation of free radicals. These then attack the membranes of nerves, sarcoplasmic reticulum and mitochondria. By pretreating rabbits with *P. africana* extract (Tadenan®), Levin et al. (1997) found that the severity of both the contractile and metabolic dysfuntions, induced by partial outlet obstruction, were reduced. They, therefore, suggest that Tadenan® might either prevent the activation of degradative enzymes (or generation of free radicals), or protect the intracellular membranes against the destructive effects of free radicals or degradative enzymes.

Other possible mechanisms of action are competition with androgen precursors and inhibition of prostaglandin biosynthesis (anti-inflammatory) by the phytosterols in the extract. The pentacyclic terpenes exhibit anti-inflammatory activity by inhibiting glucosyl transferase and β-glucuronidase enzymes involved in the depolymerisation of proteoglycans in the connective tissues. Ferulic acid esters of fatty alcohols reduce the level of cholesterol in the prostate thereby limiting androgen synthesis (Schulz et al., 1998). When investigating the mechanism of action of selected plant extracts compared to finasteride, Rhodes et al. (1993), found that a concentration of as high as 63 ng/ml Tadenan® caused 50% inhibition (IC_{50}) of 5α-reductase. Only 1 ng/ml of finasteride was needed for 50% inhibition of the enzyme, thus *P. africana* bark extract, in the form of Tadenan®, has weak 5α-reductase inhibitory activity.

1.4.2.4 Extraction

In Natal, the powered bark of the African plum tree was used in the form of a milk suspension for micturition problems (Schulz et al., 1998).

As Tadenan® is usually the product on which all the research and clinical trials have been conducted, no extraction is needed in these experiments, therefore, very little information regarding extraction of *P. africana* could be found. Tadenan® is manufactured by LABORATORIES DEBAT by solvent extraction of *P. africana* barks and the extract is dispersed in peanut oil. Tadenan® is administered orally in capsule form (Levin et al., 1996).

1.4.2.5 Dosage

The currently recommended daily dosage regimen is 50 mg of *P. africana* bark extract twice daily. However, Chatelain et al. (1999) compared the efficacy and safety of 50 mg Tadenan® twice daily and 100 mg Tadenan® once daily, and found the results to be similar.

1.4.2.6 Efficacy

Breza et al. (1998) did an open three-centre efficacy and safety study on Tadenan® in urology clinics in the Czech and Slovak Republics and in Poland. They used the IPSS and Quality of Life (QoL) as subjective assessments and urine flowmetry for objective evaluations. After a two-month treatment period with 50 mg Tadenan® twice daily, the mean IPSS was reduced by 40% and the QoL increased by 31%. The mean frequency and nocturia had decreased from 2.62 times per night before treatment to 1.66 times per night after two months of treatment with Tadenan®. Their evidence suggests that the beneficial effects of Tadenan® developed after one month of treatment, with a further improvement by the end of the second month. These rapid effects are advantageous in comparison with finasteride of which the onset of full activity appears to be slow (6-12 months) (Breza et al., 1998).

1.4.2.7 Side Effects

Acute and chronic toxicity tests in small animals showed *P. africana* to be devoid of severe side effects. Tests for mutagenesis and teratogenesis were negative. The extract appears to be well tolerated in humans following long-term administration (Schulz et al., 1998).

1.4.3   *Serenoa repens* (saw palmetto)

1.4.3.1 General information

Serenoa repens, also known as the American dwarf palm or saw palmetto, it's lipoidal berry extract are the most widely used and most thoroughly tested BPH phytotherapeutic agent. The main source of *S. repens* is plantations in the south-eastern United States, especially in the state of Florida, where the plant was previously regarded as a weed (Marks & Tyler, 1999).

1.4.3.2 General composition

In general, *S. repens* extracts are composed of fatty acids [(90 wt %), 90% of which is lauric acid, oleic acid, myristic acid and palmitic acid] and a variety of alcohols e.g. n-alcohols; isoprenoidal alcohols; acyl glycerides; sterols (campesterol, dihydrobrassicasterol, stigmasterol, β-sitosterol, cycloartenol, 24-methylenecycloartenol), and a pentacyclic triterpenoid, lupeol (Quirke et al., 1998). Fractionation of *S. repens* extract IDS 89 (Strogen®) by Weisser et al. (1996), led to a non-saponifiable, a saponifiable and a hydrophilic subfraction. Weisser et al. (1996) found that IDS 89 inhibits 5α-reductase (See Figure 1.3), but it was mainly ascribed to the saponifiable subfraction, whereas the non-saponifiable and the hydrophilic subfractions showed only a slight inhibition and no inhibition of 5α-reductase respectively. As the fatty acids accumulate in the saponifiable subfraction, the inhibitory actions were attributed to the fatty acids in the extract. Only myristic and lauric acid, however, were capable of significantly inhibiting 5α-reductase (Weisser et al., 1996).

1.4.3.3 Mechanism of action

The use of phytotherapy in the rational medical treatment of patients with LUTS has been limited by the lack of knowledge regarding their possible mechanism of action (Goepel et al., 1999). Because the extracts contain many different molecules, more than one mechanism may be involved (Marks & Tyler, 1999). *S. repens,* however, is one of the few plants on which substantial research has been done and some of the previously proposed mechanisms of action have been experimentally confirmed.

Goepel et al. (1999) demonstrate that *S. repens* extract inhibits radioligand binding to human α_1-adrenergic receptors concentration dependently. Another well-researched mechanism of action is inhibition of the prostatic 5α-reductase enzyme, thus reducing the conversion of testosterone to DHT. According to Bayne et al. (1999), Permixon® (*S. repens* extract) is an effective inhibitor of both

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isomeric forms of 5α-reductase expressed in the prostate. Di Silverio et al. (1998) find that, at the recommended dose (320 mg/day), Permixon® induces a 50% reduction of DHT-levels as result of this inhibition of 5α-reductase. Di Silverio et al. (1998) state that Rhodes et al. (1993) questioned the capacity of this compound to inhibit 5α-reductase because serum DHT levels did not decrease in their experiments, however, Rhodes et al. (1993) did a very short study (7-day) on healthy volunteers (age range 20-30 years), whose hormonal status differs from that of BPH patients. Furthermore, it has been clearly demonstrated by Toscano and Horton (1987) that circulating DHT does not reflect its peripheral formation. Di Silverio et al. (1998) also state that the levels of epidermal growth factor (EGF) are androgen-dependent and that a marked decrease of DHT causes EGF reduction, which is important, if one considers that DHT acts on cell proliferation mainly indirectly through growth factors.

Other possible mechanisms of action have been proposed, including inhibition of cyclo-oxygenase and lipoxygenase (leading to an anti-inflammatory and anti-oedemous effects), inhibition of sex hormone-binding globuline (Goepel et al., 1999), as well ascompetitive binding to cytosolic androgen receptors and antiestrogenic activity in prostatic tissue (Glenn et al., 1998). As far as I could ascertain not one of these mechanisms has yet been convincingly demonstrated.

1.4.3.4 Extraction

There are different extraction processes for *S. repens* e.g. supercritical fluid extraction with carbon dioxide as solvent, and lipophilization by 90% ethanol. The most extensively investigated preparation is an n-hexane liposterolic extract previously manufactured in France and sold as Permixon®, however, Permixon® is currently manufactured by GERMANIA PHARMAZEUTICA, Vienna, Austria. One result of the different extraction processes used, is that most plant extract preparations have different components, thereby complicating a direct comparison between various preparations from different companies (Lowe et al., 1998).

1.4.3.5 Dosage

The recommended therapeutic dosage of *S. repens* extract is 320 mg daily (160 mg twice per day) (Bayne et al., 1999 & Gerber et al., 1998).

1.4.3.6 Efficacy

Serenoa repens, taken over a period of six months by 50 patients, resulted in a significant improvement in their International Prostate Symptom Score (IPSS). However, there was no

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significant change in peak urinary flow rate, serum PSA (prostate-specific antigen) levels or in any of the measured urodynamic parameters (Gerber et al., 1998). In a major European study comparing the effects of saw palmetto and finasteride, the two were found to have nearly equal effects, causing parallel and statistically significant symptom scores and increases in maximal flow rates (Carraro et al, 1999). When comparing the prices of Proscar® (5 mg finasteride) with an over-the-counter saw palmetto product (160 mg saw palmetto, as well as beta-carotene, vitamins B1, B2, B6, C and E, and zinc), one realises the cost-effective benefit of saw palmetto as phytotherapeutic agent against BPH. A packet of 28 Proscar® tablets (one tablet daily) costs c. R660 in comparison with a packet of 60 Formule Naturelle's Saw Palmetto® capsules (one capsule three times daily) for c. R75. Even though effective over-the-counter therapy is available for BPH, pharmacists and health shop personnel should advise their clients with LUTS to see a physician for a proper diagnosis and not to assume that it is BPH. It could be more serious e.g. carcinoma, sclerosis, fibrosis of the bladder neck, urethral stricture disease or urethral valves, smooth or striated sphincter disynergia (Levin et al., 1996).

1.4.3.7 Side effects

The general safety profile of *S. repens* compares favourably with that of finasteride and sexual side-effects (erectile dysfunction, ejaculatory disturbance or altered libido) were less common with the plant product than with the drug. Aside from an occasional instance of gastro-intestinal upset, side-effects of *S. repens* extracts have not been reported (Marks & Tyler, 1999).

1.5   SIDE EFFECTS OF PHYTOSTEROLS

Adverse effects caused by β-sitosterol are usually mild and in most studies comparable to those of the placebo in frequency (Wilt et al., 1998). Gastro-intestinal side-effects (constipation and diarrhoea) are the most common and are more likely to occur higher doses as used for hypercholesterolaemia (3-30 g/d) (Pegel, 1997).

The fact that phytosterols and sterolins have a steroid structure, but no side-effects, is interesting, because steroids are generally associated with many adverse effects. However, critical chemical groups responsible for e.g. corticosteroidal activity (and its side-effects) are absent in phytosterols and their glucosides (See Figure 1.6), and, therefore, their long term use are not associated with the

side-effects of long-term corticosteroidal use e.g. metabolic side-effects (hyperglycemia; fat redistribution; skeletal muscle wasting; skin atrophy, and gastro-intestinal ulcers etc.), water retention, hypertension, immuno-suppression etc. (Hardman & Limbird, 1996).

Betamethasone (Celestone®)   β-Sitosterol
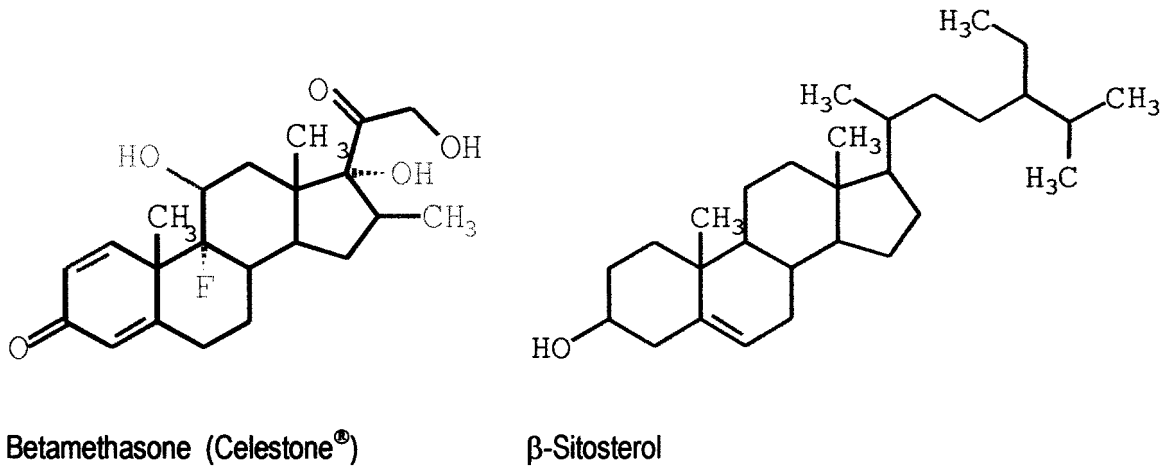
Figure 1.6: Structural differences between betamethasone and β-sitosterol. Red groups enable activity on the steroid receptor (intrinsic activity), and green groups increase activity on the steroid receptor (affinity). These critical functional groups are absent in β-sitosterol (composed from data from Hardman & Limbird, 1996).

1.6 CONCLUSION

Although the prejudice against phytotherapy still remains in many medical circles (probably because of a lack of knowledge regarding herbal therapy), treatment of BPH with certain scientifically-tested phytotherapeutic agents (e.g *H. hemerocallideae*, *P. africana* and *S. repens*) is a definite option. Not only is the efficacy of *S. repens* extract comparable with finasteride, but the price is about a tenth of that of Proscar® for a month's supply. There are also very few side effects with the phytotherapeutics as opposed to allopathic medicines (dizziness, asthenia and hypotension with α-blockers; decreased libido and impotence with finasteride (Breza et al., 1998). Effective over-the-counter phytotherapeutics for BPH are available. However, pharmacists and health shop personnel should emphasise to their clients with lower urinary tract symptoms (LUTS), that they must consult their physicians first for proper diagnosis, and not assume that BPH is the cause of their micturition problems, as the cause could be more severe e.g. prostatic cancer.

As plant extracts are complex mixtures containing different active and inactive components, more than one mechanism is often involved in achieving the therapeutic effect. This is perhaps the main reason why nine years after the First International Consultation on BPH, in 1991, where the Scientific Committee proposed the standardisation of the evaluation of BPH pharmacological management, there still are uncertainties regarding the precise mechanism of action of many phytotherapeutics.

Despite the resistance by some conservative western physicians, the popularity of phytotherapy is increasing every year and, therefore, formal instruction in complementary and alternative medicine is now being offered in 64% of American medical schools (Marks & Tyler, 1999).

1.7 AIM OF THE STUDY

As BPH is a common medical condition in older men affecting as many as 40% of men aged 70 years and older (Wilt et al., 1998) and phytosterols and sterolins are regarded as the active compounds in many BPH phytotherapeutics, a study on the analysis of sterols and sterolins is undertaken.

The aim of the study is to develop and apply two analysis techniques for the qualitative and quantitative analyses of sterols and sterolins: thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). A new TLC method will be developed and an existing HPLC method will be evaluated and adapted for plant extract analyses. The methods will be applied to investigate the sterols and sterolins in three BPH phytotherapeutics: *H. hemerocallidea, P. africana* and *S. repens;* as well as different sterol/sterolin containing products, qualitatively and quantitatively. As *H. hemerocallidea* is the main phytotherapeutic of interest, the analysis of hypoxoside (unique to Hypoxidaceae) will also be investigated. A study by Eloff (1999) revealed that water does not extract sterols or sterolins, and uncertainty remains regarding the actives in African potato tea. Water extraction of *H. hemerocallidea* as performed by Eloff (1999), will be repeated and if his finding is confirmed, other possible actives will be investigated.

Little data is available on the shelf-life of sterols and their stability against gamma irradiation, used to prevent microbial contamination of raw material and products. Therefore, the TLC and HPLC methods will also be applied to determine the stability of sterols. Information on the bioavailability of β-sitosterol from certain products is known, but no bio-equivalence study on the bioavailability of β-sitosterol from South African products has been published yet. Some companies claim that their products have very high bioavailability due to added enzymes or specialised growth and harvesting methods. Furthermore, there is a large discrepancy in bioavailability values found in literature. Therefore, a HPLC method used to determine the "bioavailability of β-sitosterol from *Pygeum africanum* extract in humans" (Emara et al., 1999), will be evaluated and applied to determine the bio-equivalence of four South African and three European sterol containing products. A clinical trial consisting of three parts will be performed: part 1 (pilot study), part 2 (South African products) and part 3 (European products).

It is important to develop analysis techniques, because up to now very few companies in natural medicine industry have quality control procedures in place and little information is available on the stability and/or bioavailability of phytotherapeutic products. This lack of knowledge is mainly due to the lack of proper methods to perform these analyses. By developing and improving methods to analyse phytosterols, the industry will be supplied with the necessary techniques that ensure the quality, safety and efficacy of their sterol/sterolin containing products.

CHAPTER 2

MATERIAL, STANDARDS AND METHODS

2.1. MATERIAL

2.1.1 Plant material

African potato 1 - Dried grated rhizomes of *Hypoxis hemerocallidea,* collected on the farm Knopfontein, in Nylstroom (Northern province) were obtained from the distributor, Mrs. Santjie Marx.

African potato 2 - Powder samples of African potato capsules (Batch 9908202).

African potato 3 – *Hypoxis* sp. powder (Batch 990910).

African potato 4 – SAVANNAH FOODS African potato powder (Batch 9910062).

Prunus africana bark was obtained from the botanical garden at Nelspruit.

Prunus africana leaves were obtained from the botanical garden at Nelspruit.

Prunus africana extract (5:1) (Batch 30240P429 & P11199011503) from WARREN CHEM.

Serenoa repens berry powder (Batch 9807506 & S02000020101).

Serenoa repens berry extract (4:1) (Batch S02199083017) from WARREN CHEM.

WARREN CHEM Betasitosterol (Batch 28757P94).

All of the above except African potato 1, *P. africana* bark and leaves were gifts from BIOMOX PHARMACEUTICALS, Pretoria.

2.1.2 Products

African potato 5 (627 mg of *H.hemerocallidea* powder per capsule) - DISCOVERIES OF NATURE, Pretoria.

African potato 6 (300mg *H.hemerocallidea* powder per capsule) - PLANTANICAL MEDICINE, Pretoria.

Harzol® (10 mg β-sitosterol and 0.1 β-sitosterolin per capsule) - HOYER GmbH & Co, Monheim, Germany.

Immunochoice® (20 mg plant sterols and sterolins and 50 mg absorption enzymes per capsule) - PHARMA CHOICE, Fourways.

Moducare® (20 mg plant sterols and 0.2 mg sterolins per capsule) - PHYTO LABS, Midrand.

Nutricare® (400 mg freeze-dried enzymes and sterol compound per capsule) - NUTRIGREEN Hydroponics, Umhlanga Rocks.

Permixon® (160 mg *S. repens* extract per capsule) - GERMANIA PHARMAZEUTICA, Vienna, Austria.

Phytogard® (10 mg glutathione, 75 mg food state selenium and 60 mg β-sitosterol per capsule) - SPORTRON INTERNATIONAL, Paulshof.

Prostol® (200 mg of *S. repens* berry powder, 200 mg of *P.africana* extract (5:1) and 100 mg pumpkin seed extract (4:1), per capsule) - BIOHARMONY, Wynberg.

Tadenan® (50 mg *P. africana* extract) - LABORATORIES FOURNIER S.A., FONTAINE les Dijon, France.

2.2 STANDARDS

Campesterol (c. 65%) was bought from ALDRICH.

Cholesterol (99+%) from SIGMA, was received as a gift from Magda Roseman, Dept. of Chemical Pathology, University of Pretoria.

β-Sitosterol (c. 40%, containing 20-30% campesterol and 10-30% dihydrobrassicasterol), derived from soybeans, was bought from ALDRICH.

Synthetic β-sitosterol (95.7%) was bought from SIGMA.

Stigmasterol (c. 95%) was bought from SIGMA.

Isolated hypoxoside was received as a gift from Prof. J. van Staden, Dept. of Botany, University of Natal.

Isolated β-sitosterolin was received as a gift from Prof. C.B. Rodgers, Dept. of Chemistry, University of Durban-Westville.

2.3 METHODS

The methods used in this study, are discussed in the individual chapters.

CHAPTER 3

TLC METHOD DEVELOPMENT AND APPLICATION

3.1   INTRODUCTION

3.1.1   History

Chromatography is a method of analysis in which a mobile phase passes over a stationary phase in such a way that a mixture of substances is separated into its components (Hanh-Deinstrop, 2000). Chromatography began in 1903 in Russia, when Michael Tsvett separated chlorophyll pigments in a tube filled with calcium carbonate. Long after Tsvett's adsorption chromatography, partition chromatography with silica gel columns was developed in 1941 by Martin and Synge to separate hydrophilic substances like amino acids. Three years later they experimented with filter paper and paper chromatography was born. The next separation technique, introduced in 1945, was gas chromatography developed by Hesse, Cremer, James and Martin. However, a milestone in the history of chromatography was reached in 1956 when a new concept, thin layer chromatography (TLC), was developed by Egon Stahl and his collaborators working on glandular hairs of plants. (Von Zahn & Rheinholz, 1980).

3.1.2   Aim of chapter

In this chapter a TLC method is developed and optimised to investigate the phytosterols and sterolins in *H. hemerocallidea, P. africana, S. repens*, and related herbal products. By evaluating different extractants, mobile phases and spray reagents, the best combination to investigate sterols and sterolins is selected. By comparison with standards, the sterol/sterolin contents of the three plant species and related products are qualitatively determined and compared. The information obtained from this technique, regarding the best extractant, method of extraction *etc.*, can then be applied in preparation of samples for analyses with high performance liquid chromatography (HPLC).

The results in this chapter will be discussed in two parts:

A – Method development

B – Application.

3.2   MATERIAL AND METHODS

3.2.1   Material

Plant material, products and standards as described in Chapter 2, 2.1 and 2.2 were used.

3.2.2   Methods

3.2.2.1 Extraction

3.2.2.1 (a) Selecting extractants according to polarity and solvent strength

The total interaction of a solvent molecule (mobile phase) with a sample molecule (solid phase) is the result of a combination of dispersion, dipole, hydrogen bonding and dielectric interactions. The larger this combined interaction is, the stronger is the attraction of solvent and solute molecules. The ability of a sample or solvent molecule to react in all four of these ways is referred to as the "polarity" of the compound. The "strength" of a solvent is directly related to its polarity. Solvent strength increases with solvent polarity in normal phase partition and adsorption liquid chromatography, but solvent strength decreases with increasing polarity in reverse phase liquid chromatography (Snyder & Kirkland, 1979). See Table 3.1.

Table 3.1: Solvent strengths, polarities and boiling points of the extractants used (from Snyder & Kirkland, 1979)

Solvent	Polarity (P')	Solvent strength ($\in°$)	Boiling Point (°C)
Acetone	5.1	0.56	56
Methanol	5.1	0.95	65
Ethanol	4.3	0.88	78
Chloroform	4.1	0.40	61
Dichloromethane	3.1	0.42	40
Diethylether	2.8	0.38	35
Hexane	0.1	0.01	69

Table 3.1 indicates the polarities, solvent strengths and boiling points of the extractants used, and evaluated, in the study. The polarity parameter P' is based on the experimental solubility data reported by Rohrschneider and is an indicator of solvent strength in partition chromatography. The experimental adsorption solvent strength parameter $\epsilon°$, measured on alumina, is a better index of solvent strength for adsorption chromatography (Snyder & Kirkland, 1979).

Note that acetone and methanol has the same polarity P', according to Snyder and Kirkland (1979), whereas methanol is generally regarded as a solvent with much higher polarity than acetone. The table of Snyder and Kirkland (1979) was, however, used as an indication of polarity and solvent strength.

3.2.2.1 (b) Types of extraction used

3.2.2.1 (b) (i) Simple extraction

The plant material was usually extracted, with a ratio of 10 ml of extractant per gram plant powder e.g. 500 mg extracted with 5 ml extractant, by shaking the test tube vigorously on a horizontal shaking machine, for 5 minutes. The extract was then centrifuged in a Heraeus Labofuge 200 centrifuge at 3000 g for 5 minutes and decanted into a weighed amber vial (volume c. 8 ml). However, in the case of dense solvents e.g, chloroform, even high speed centrifugion did not result in sedimentation and the extracts had to be filtered. Qualitative filter paper (Whatman type 1 or 4) was wet before filtration and rinsed afterwards with the extractant, and the extract collected in a weighed amber vial. The extraction process was usually repeated three times with the originally weighed material and the supernatant was collected either in three different vials, for quantitative purposes or combined in the same vial. In the case of dense solvents, filtration was performed and the extraction process could only be repeated once, as the material was poured with the extract onto the filter paper. The extractant was usually removed by a stream of cold air in a fume cupboard, but in some cases (oily precipitates) to confirm the dryness after 24 hours, the extract was dried under vacuum in a dessicator. The dry extract was then redissolved, either in the extractant or in another solvent with particular polarity, to yield a known concentration of extract, usually 10 mg/ml.

All the extractants and solvents used for TLC purposes were analytical quality reagents: Acetone, methanol, ethanol, chloroform, dichloromethane, diethylether and hexane.

3.2.2.1 (b) (ii) Soxhlet extraction

In some cases of simple extraction, very little material was extracted per gram plant material exposed to the extractant, and a soxhlet extraction apparatus was used for continuous and complete extraction in a closed system. In these cases 20 g of plant material was placed in two porous paper thimbles (J Green's 603) and extracted, with excess extractant boiled on a heating mantle. The extraction period was two to three hours. The extract was dried and redissolved to yield a 10 mg/ml concentration, as with simple extraction.

3.2.2.1 (b) (iii) Water extraction of *H. hemerocallidea*

According to African potato 1's promotional pamphlet, one tablespoon (6.4 g) dried, grated corm of *H. hemerocallidea* must be simmered in six cups (1200 ml) water for 15 to 20 minutes and refrigerated until use.

Water extraction of the grated corm of African potato 1 was performed according to this method. The water extract was divided into four parts, one of which was dried to determine the concentration of the total volume; to the second ethylacetate was added as a water immiscible solvent with relatively high solvent strength ($\epsilon° = 0.58$), to remove the sterolins; to the third, chloroform was added as a water immiscible solvent with relative low solvent strength ($\epsilon° = 0.40$), to remove the sterols; and the fourth was kept unchanged as a control. See Table 3.1. Simple water extraction of powdered African potato 1 was also performed in duplicate. The extracts dried and redissolved in acetone and chloroform respectively.

To ascertain that ethylacetate does extract sterolins, the solubility of sterolins in ethylacetate was investigated. Simple extraction with methanol was performed (as methanol definitely extracts the sterolin as demonstrated in 3.3.1.2 (b) and Figure 3.3). The extract was redissolved in acetone as control (contains stigmasterolin after redissolving the methanol extract), methanol and ethylacetate. To determine whether methanol does not perhaps extract a low concentration of sterols, the methanol extract was also redissolved in chloroform.

3.2.2.2 Preparation of standards

The sterol standards were dissolved in chloroform to yield the required concentration, but β-sitosterolin was dissolved in acetone, and the hypoxoside in methanol.

3.2.2.3 Thin layer chromatography (TLC)

Pre-coated 0.25 mm silica 60 glass and 0.2 mm silica 60 aluminium plates were used. Glass plates (Macherey-Nagel DURASIL-25) were used to investigate the *red spot compound* (as will be discussed at a later stage) and cheaper aluminium plates (Macherey-Nagel ALUGRAM® SIL G) were used for sterols/sterolins and hypoxoside. Volumes of 5 to 10µl were applied with a pipette to the plates. In all cases 100 µg of the extracts was applied (10 µg/µl x 10 µl). The mass of the standards applied, depended on the concentration, but in all cases 5 µl of the standards was applied. The plates were placed in a glass tank with porous paper on the sides and saturated with the particular mobile phase, for development. All mobile phase chemicals were analytical reagents:

BEA: Benzene:Ethanol:Ammonia in the ratios 18:2:0.2 or

CEF: Chloroform:Ethylacetate:Formic acid in the ratios 5:4:1 or

EMW: Ethylacetate:Methanol:Water in the ratios 10:1.35:1.

3.2.2.3 (a) Visualisation of separated components

After development, the plates were examined under ultraviolet (UV) light and usually sprayed with *p*-anisaldehyde spray (1 ml *p*-anisaldehyde, 18 ml ethanol and 1 ml sulphuric acid). Other spray reagents investigated were vanillin (0.1 g vanillin, 28 ml methanol and 1 ml sulphuric acid), phosphoric acid (15 ml 85% phosphoric acid diluted to 100ml with methanol), *p*-toluene sulphonic acid (20% *p*-toluene sulphonic acid in methanol), perchloric acid (20% aqueous perchloric acid), trichloroacetic acid (25% solution of trichloroacetic acid in chloroform) and a mixture of equal volumes of sulphuric acid and methanol (Stahl, 1969). The plates were heated in a Gallenkamp Hotbox oven at c. 110 °C until the colour bands were optimally visible and examined under UV light for a second time. With the phosphoric spray, it takes 15-30 minutes at 120 °C for development.

3.2.2.3 (b) The retardation factor (R$_f$)

The position of a substance zone in TLC can be described with the aid of the retardation factor R$_f$. This is defined as the quotient obtained by dividing the distance between the substance zone and the starting line, by the distance between the solvent front and the starting line (Hahn-Deinstrop, 2000).

By comparing the fractional movement of a solute band, relative to the distance moved by the solvent front of the separated components with that of the standards, sterols and sterolins could be identified. R$_f$ values are usually reproducible under a given set of circumstances. Differences in

mobile phase composition, room temperature, and saturation of the development tank with the solvent system, could however cause R_f-values to vary.

3.2.2.4 Fluorescence

To detect fluorescent compounds, the TLC plates were observed with a CAMAG TL-900/U UV lamp, with wavelengths of 254 nm and 350 nm before and/or after they were sprayed with the reagent. Nothing could be seen before spraying with the reagent. With *p*-anisaldehyde spray, which was used most often, the sterols and sterolins were not found to be fluorescent and UV examination was usually not performed. However, with other spray reagents e.g. phosphoric and especially *p*-toluene sulphonic acid, the sterols and sterolins were clearly visible at the longer UV wavelength.

3.2.2.5 Hydrolysis of "stigmasterolin"

To determine qualitatively whether an extra low intensity band seen with TLC in the 95% stigmasterol standard was really the stigmasterolin, it was attempted to hydrolyse it to the sterol. Acid hydrolysis with 5% hydrochloric acid were performed. A mixture of methanol:chloroform:water (MCW) in the ratios 12:5:3, was used to dissolve stigmasterol standard (50 mg stigmasterol dissolved in 5 ml mixture) and 5% hydrochloric acid was added. At this ratio the methanol make the chloroform and water miscible, with water being necessary for hydrolysis.

The experiment was repeated with African potato 1, as a component with similar R_f and colour as the "stigmasterolin", was noticed in its acetone and methanol extracts with TLC. In the rest of the study this unknown substance is referred to as the *red spot compound*. African potato 1 was extracted with the same MCW mixture and acidified. The acidified stigmasterol solution and African potato 1 MCW extract was refluxed on a heating mantle for an hour and then dried under vacuum (Büchi Rotavapor R-114 and KNF Neuberger Laboport). The dried stigmasterol residue was then washed with distilled water to remove the acid, dried again and redissolved in chloroform to yield a concentration of 10 mg/ml. An unhydrolysed control of stigmasterol in MCW was prepared in the same way. The African potato 1 extract however, was not dried but a $^1/_6$ of the volume methanol and a ¼ of the volume chloroform were added, to form two immiscible phases in which the sterols and sterolins could separate. To determine the concentration of the African potato 1 extract, a small volume was dried and the concentration of the total extract was calculated. This was done because it is often difficult to redissolve the material after it has been dried completely, even if it is redissolved in the same solvent as was used for extraction. Again, an unhydrolysed control was prepared using the same method.

3.2.2.6 Isolation of the *red spot compound* with preparative thin layer chromatography (PTLC)

A pre-coated 2 mm silica 60 glass plate (Merck precoated PLC silica gel 60 F-254) was used. The *H. hemerocallidea* acetone extract was concentrated and c. 200 µl was applied over the length of the PTLC plate and developed in CEF. After development, a 2 cm strip on the right of the plate was sprayed with *p*-anisaldehyde spray and overheated for maximum visibility of *red spot compound*. The identified zone was scraped off the plate using a spatula, and collected. The silica scrapes were ground to a fine powder and then placed into a glass tube. Acetone was passed through the silica to dissolve the *red spot compound*. The solution was dried (to remove the TLC mobile phase components) and redissolved in 0.5 ml deuterated acetone.

3.2.2.7 Structure elucidation of the *red spot compound* with Nuclear Magnetic Resonance Spectroscopy (NMR)

The samples were analysed by Dr. J.J. Retief, SASOL Technology, Research and Development. A 400 MHz Varian Unity Inova NMR spectrometer was used for proton (^{1}H) analysis of the *red spot compound*. For the small volume with low concentration, an indirect detection (ID) probe was used and the sample was moved upwards so that the centre of the sample and the centre of the NMR coils coincided. To increase the concentration, only 0.5 ml of deuterated acetone was used, instead of the usual 0.7 ml.

3.3   RESULTS AND DISCUSSION

A: METHOD DEVELOPMENT

3.3.1   Extractant evaluation

3.3.1.1 The solubility of phytosterols in acetone, methanol and chloroform

The solubility of the 95% stigmasterol standard and Warrenchem Betasitosterol were determined in acetone, methanol and chloroform. It was found that acetone dissolved 17.4% stigmasterol and 66.2% Warrenchem Betasitosterol. Methanol dissolved only 3.2% stigmasterol and 8.4% Warrenchem Betasitosterol, while chloroform dissolved 90.6% of the stigmasterol and 98.8% Warrenchem Betasitosterol. This is consistent with Merck index (Stecher et al., 1968), which states that stigmasterol is insoluble in water, but soluble in usual organic solvents. The stereochemical angle of rotation $[\alpha]_D^{25}$ of β-sitosterol, campesterol and stigmasterol is measured in chloroform in the Merck Index (Stecher et al., 1968). It seems that stigmasterol is less soluble than β-sitosterol in all three solvents.

It is important to note that the term β-sitosterol frequently represents a defined extract of phytosterols with β-sitosterol as the main component (Berges et al., 1995). According to the certificate of analysis, Warrenchem Betasitosterol contains 46.6% β-sitosterol, 28.8% campesterol and 17.6% stigmasterol.

3.3.1.2 Extraction

The extractants are discussed here in order of polarity as indicated in Table 3.1 in 3.2.2.1 (a).
Extraction will be discussed under the following headings:

   (a) (i) Acetone extraction
   (a) (ii) Acetone extract redissolved in methanol, diethylether and water
   (b) Methanol, ethanol and chloroform extraction
   (c) Dichloromethane and diethylether extraction
   (d) Hexane extraction
   (e) Best extractant evaluation

3.3.1.2 (a) (i) Acetone extraction

Acetone is a solvent with an intermediate polarity. It dissolves many polar and non-polar substances and gives a good indication of the polarity of the components in a plant, as well as which other solvents to consider (with regards to their polarities). Furthermore, acetone is miscible with water, volatile and evaporates quickly when drying the extract and when applying it on a TLC plate, to give a neat thin line.

The African potato 1 and 2; *S. repens* berry powder; *P. africana* bark extract (5:1), and Warrenchem Betasitosterol (composed of mainly β-sitosterol, but also campesterol and stigmasterol) were extracted with acetone and chromatographed on TLC, developed in BEA and sprayed with *p*-anisaldehyde and vanillin respectively. See Figure 3.1.

(a)   **(b)**
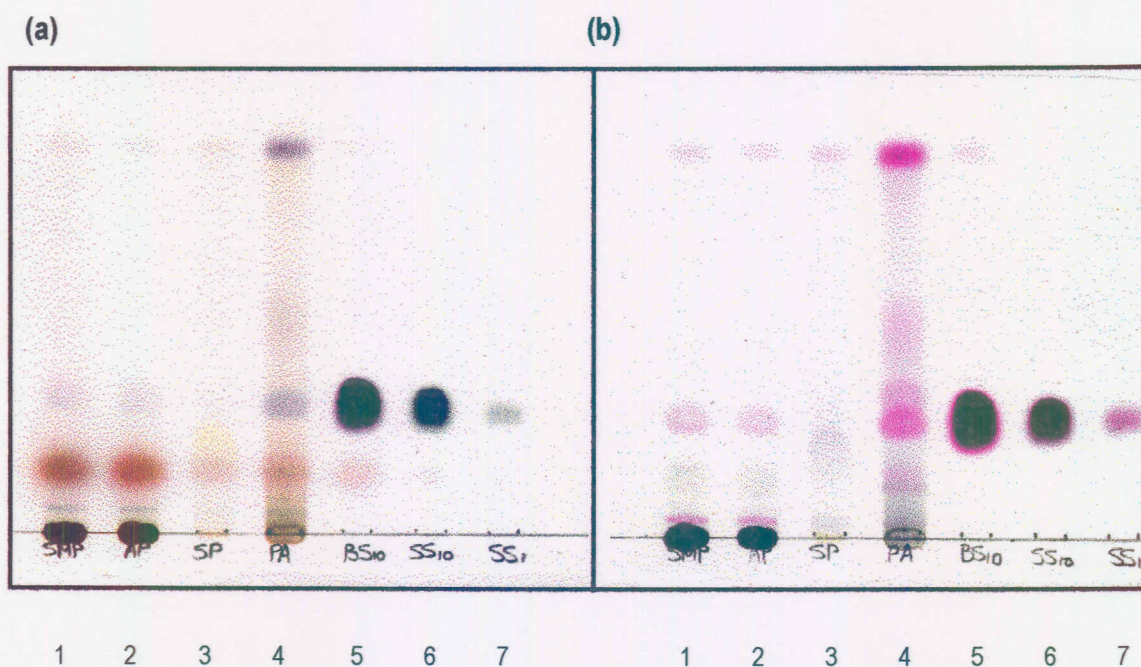
Figure 3.1: TLC on aluminium plates of acetone extracts of African potato 1(1) and 2 (2), *S. repens* berry powder (3), *P. africana* bark extract (4) and Warrenchem Betasitosterol (5). The plates were developed in BEA and sprayed with **(a)** *p*-anisaldehyde and **(b)** vanillin. On the right are 10 mg/ml 95% stigmasterol standard (6) and 1 mg/ml stigmasterol standard (7).
In all cases 100 μg of the extracts was applied.

With BEA the majority of the compound bands were on the lower half of the plate, which is not ideal. Warrenchem Betasitosterol and the stigmasterol standards had the same R_f-value and colour

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reaction with the spray reagents. Warrenchem Betasitosterol (composed of three phytosterols) and 95% stigmasterol standard had the same retardation factor (R_f = 0.26). TLC with BEA could not separate the different phytosterols in Warrenchem Betasitosterol – a blue "phytosterol-complex" was visible. When the chemical structures of these sterols are considered, it is understandable why their R_f-values were the same (See Chapter 1, 1.2). A similar blue spot with R_f = 0.26 was visible in all the samples in Figure 3.1, except *S. repens* berry powder, and it was postulated to be a "phytosterol-complex" as well.

On the moderately heated aluminium plates, a pink-red spot with R_f = 0.12 in BEA was noticed below the "phytosterol-complex" in Warrenchem Betasitosterol and 10 mg/ml stigmasterol standard (not 1 mg/ml). This was, however, only seen on the plates sprayed with *p*-anisaldehyde, but not on the plates sprayed with vanillin. The compound responsible for the second spot in stigmasterol standard was thought to be stigmasterolin. A similar pink-red spot was visible in African potato 1 and 2; *S. repens* berry powder, and *P. africana* bark extract (5:1). At the time β-sitosterolin standard was not available, but it was identified retrospectively as the blue band with R_f = 0.053 (BEA) in African potato 1 and 2 and in *P. africana* bark extract (5:1). See Figure 3.2 where the isolated β-sitosterolin is included. If the pink-red spot was a complex of sterolins (similar to the "phytosterol-complex"), it did not include β-sitosterolin. In the rest of the study, the "pink-red spot" mentioned, is referred to as the *red spot compound*. The pink-red spot in stigmasterol standard could be stigmasterolin, as the standard was 95% pure and sterols never exist on their own in nature, they are always found in combination with their glucosides (Vanderhaeghe & Bouic, 1999). Despite the same R_f-value, the *red spot compound* however, is not necessarily stigmasterolin, but it will be discussed as a possible sterolin in the rest of the study.

Vanillin did not reveal the "stigmasterolin" band in stigmasterol standard, but the type of TLC plate (aluminium vs. glass) also determines the visibility of "stigmasterolin" and the *red spot compound*. The *red spot compound* was substantially more visible when the TLC plates were overheated, but in the case of aluminium plates sprayed with *p*-anisaldehyde, the background turned pink when overheated and this reduced the visibility of the *red spot compound*.

Conclusion: Glass TLC plates (overheated) are best for investigation of the red spot compound.

Assuming that the *red spot compound* is a sterolin (glucoside), alkaline conditions would cause the hydroxyl groups to be in undissociated form (the sugar hydroxyl in the –OH instead of –O⁻), which would reduce the polarity and increase the solubility in non-polar extractants. To determine the effect

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of increased pH on extraction of African potato 1, 20% ammonium hydroxide was added to the acetone extractant. The result was an increase in the intensity of the *red spot compound* on TLC. It is also would appear that as if the removal of the extractant and redissolution (in the same or another solvent), does not cause degradation (e.g. hydrolysis) of the *red spot compound*. See Figure 3.2.
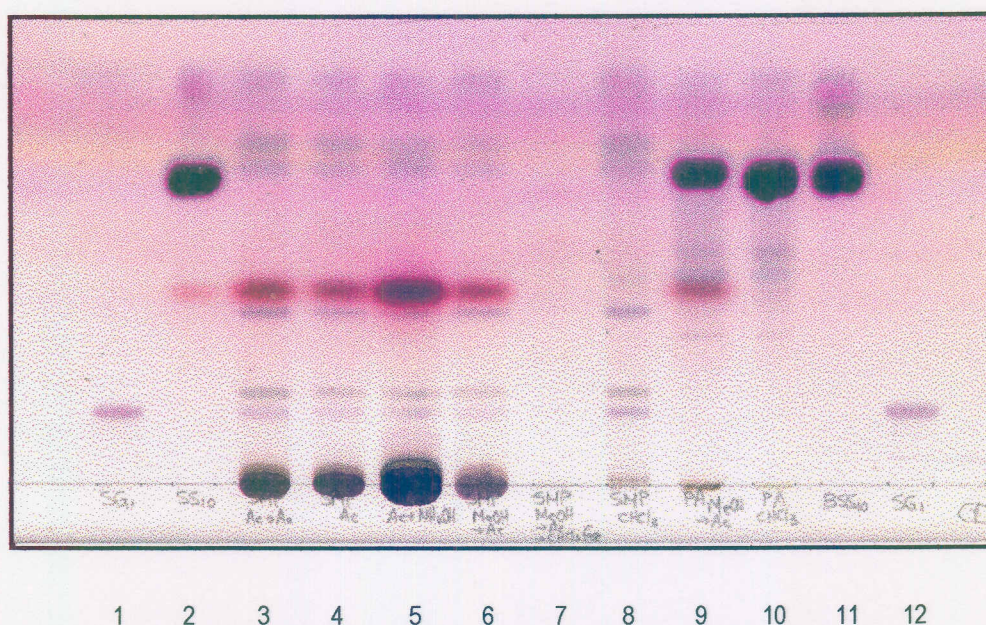
Figure 3.2: Overheated TLC on a glass plate, of different extracts of *H. hemerocallidea* powder and *P. africana* extract (5:1), developed in CEF and sprayed with *p*-anisaldehyde. On the left are 1 mg/ml isolated β-sitosterolin, 10 mg/ml 95% stigmasterol standard and on the right are 10 mg/ml 40% β-sitosterol standard and 1 mg/ml isolated β-sitosterolin. In all cases 100 μg of the extracts was applied.

In Figure 3.2 from the left are 1 mg/ml isolated β-sitosterolin (1) and 10 mg/ml 95% stigmasterol standard (2). The order of the African potato 1 extracts are as follows: Acetone, dried and redissolved in acetone again (3), acetone, not dried (4), alkaline acetone (5), methanol, dried and redissolved in acetone (6), acetone insolubles from 6 redissolved in 50/50 $CHCl_3/H_2O$ – chloroform phase (7) and chloroform, dried and redissolved in chloroform (8). The extracts of *P. africana* extract (5:1) are methanol, dried and redissolved in acetone (9) and chloroform, dried and redissolved in

chloroform (10). On the right are 10 mg/ml 40% β-sitosterol standard (11) and 1 mg/ml isolated β-sitosterolin (12).

Conclusion: The red spot compound is more soluble and better extracted at an alkaline pH.

3.3.1.2 (a) (ii) Acetone extract redissolved in methanol, diethylether and water

Acetone extracted a substantial amount of African potato 1, African potato 2, *P. africana* extract (5:1) and *S. repens* berry powder, but when the dried acetone extract was redissolved in acetone, a large quantity of material did not dissolve. This was true of all the samples, except *S. repens* berry powder where 96.5% of the dried extracted material dissolved in acetone. The reason for the insoluble residue could be complex formation during drying.

The acetone-insoluble residue of the other three samples, was then redissolved in methanol. Methanol dissolved 72.9% and 75.9% respectively of the acetone-extracted material of African potato 1 and African potato 2 (filtered), but only 10.3% of *P. africana* extract (5:1). See Table 3.2. Subsequently the *P. africana* extract (5:1)'s acetone and methanol insoluble material was redissolved in a non-polar solvent, diethylether. None of the material dissolved. It was decided to try using a very polar solvent, water, to redissolve the acetone-, methanol- and diethylether-insoluble material of *P. africana* extract (5:1) – 63.8% material dissolved.

TLC of the extracts in Table 3.2 (results not shown), sprayed with *p*-anisaldehyde, indicated that acetone removed certain polar compounds from both African potato samples, which were not present in the methanol solution. The acetone-insoluble compounds that dissolved in methanol, were so polar, that the methanol solution did not separate in CEF (although it separated in EMW).

The acetone solution of *P. africana* extract (5:1) separated in CEF, but EMW was too polar. On the other hand the water solution of *P. africana* extract (5:1) did not separate in either CEF or EMW. Even EMW was too non-polar in this case.

Table 3.2: Percentage of material extracted with acetone from *H. hemerocallidea, P. africana* bark extract (5:1) and *S. repens* berry powder and residue redissolved in different solvents

Plant material	% (w/w) Extracted with Acetone	% (w/w) Residue redissolved in Acetone	% (w/w) Residue redissolved in MeOH	% (w/w) Residue redissolved in Ether	% (w/w) Residue redissolved in Water	Best Solvent
African potato 1 powdered	2.4%	23.7%	72.9%	-	-	MeOH
African potato 2 capsules filtered	2.3%	16.6%	75.9%	-	-	MeOH
P. africana ext.	2.3%	31.0%	10.3%	0	63.8%	Water
S. repens pwd.	5.8%	96.5%	-	-	-	Acetone

Conclusion: Methanol is the best solvent for redissolution to yield a final concentration of H. hemerocallidea acetone extracts, water for P. africana (5:1) acetone extracts and acetone for S. repens acetone extracts.

3.3.1.2 (b) Methanol, ethanol and chloroform extraction

As mentioned above, in many cases acetone extracted a substantial amount of material, but when the dried extract was redissolved in acetone, a significant quantity was acetone-insoluble. This resulted in a low concentration of the extract. Due to this and because alcohols, especially ethanol, are often used in the extraction of *H. hemerocallidea* (McAllister & van Staden, 1994), *S. repens* (Lowe et al., 1998) and *P. africana* (Yablonsky et al., 1997), methanol and ethanol were also applied for extraction. As it was shown with stigmasterol standard and Warrenchem Betasitosterol that sterols are very soluble in chloroform, this non-polar solvent was applied as well. Three different samples of *H. hemerocallidea* powder were extracted with methanol, ethanol and chloroform respectively and evaluated.

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Methanol extracted the highest quantity of material from all three *H. hemerocallidea* samples, ethanol c. 50% and chloroform c. 1% of the quantity extracted by methanol. See Table 3.3. It was noted that very little material was extracted by chloroform. Although absorption onto the filter paper during filtration of these extracts, could have been a reason. It was postulated that perhaps chloroform selectively extracts sterols. This was confirmed with TLC.

Table 3.3: Percentage of material extracted with methanol, ethanol and chloroform from three sources of *H. hemerocallidea* powder and residue redissolved in different solvents

Plant material	Extractant	% Extracted (w/w)	% (w/w) Residue redissolved in Acetone	% (w/w) Residue redissolved in MeOH	% (w/w) Residue redissolved in EtOH	% (w/w) Residue redissolved in CHCl$_3$
African potato 1 powdered	Methanol	4.2%	6.7%	77.1%	–	–
"	Ethanol	1.8%	10.9%	–	54.3%	–
"	Chloroform	0.06%	–	–	–	100%
African potato 2 capsules	Methanol	6.0%	6.6%	64.2%	–	–
"	Ethanol	3.6%	3.3%	–	59.3%	–
"	Chloroform	0.06%	–	–	–	100%
African potato 3 powder	Methanol	6.8%	5.3%	65.5%	–	–
"	Ethanol	4.2%	0	–	77.4%	–
"	Chloroform	0.08%	–	–	–	100%

Instead of dissolving the extract in the extractant to give a particular concentration, the ethanol and methanol extracts were dissolved in sufficient acetone to yield 10 mg/ml concentrations, but afterwards the remaining acetone insoluble precipitates were redissolved in ethanol and methanol respectively, to yield again 10 mg/ml concentrations.

The chloroform extract was dried and redissolved in chloroform for a 10 mg/ml concentration.

TLC of the 10 mg/ml extracts in Table 3.3 and 5 mg/ml stigmasterol standard was performed, using CEF and EMW solvent systems and *p*-anisaldehyde as spray reagent. It showed, that although the percentage of material extracted by methanol, ethanol and chloroform differed for the three African potato samples, the components extracted are similar and all three samples can, therefore, be considered as representing *H. hemerocallidea*. See Figure 3.3.

(a)
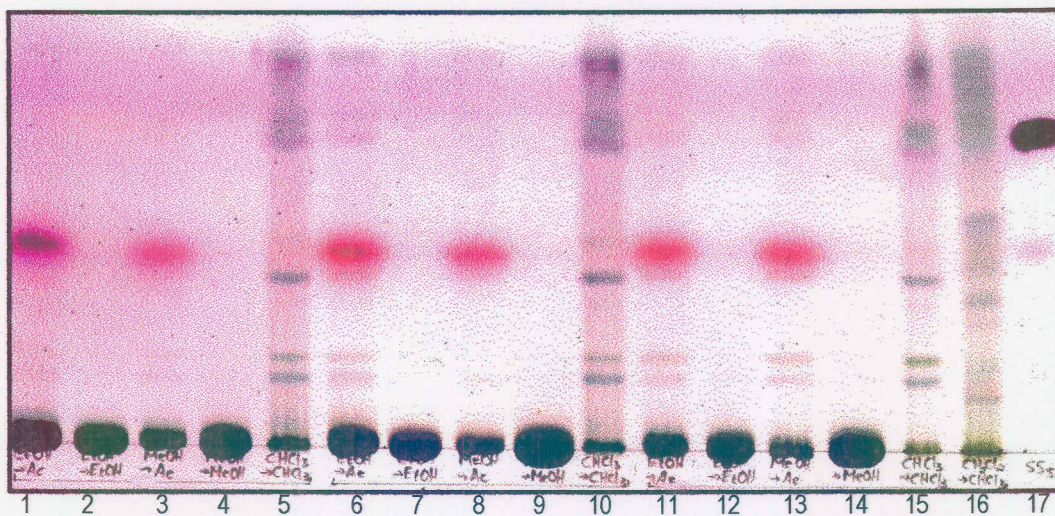
(b)
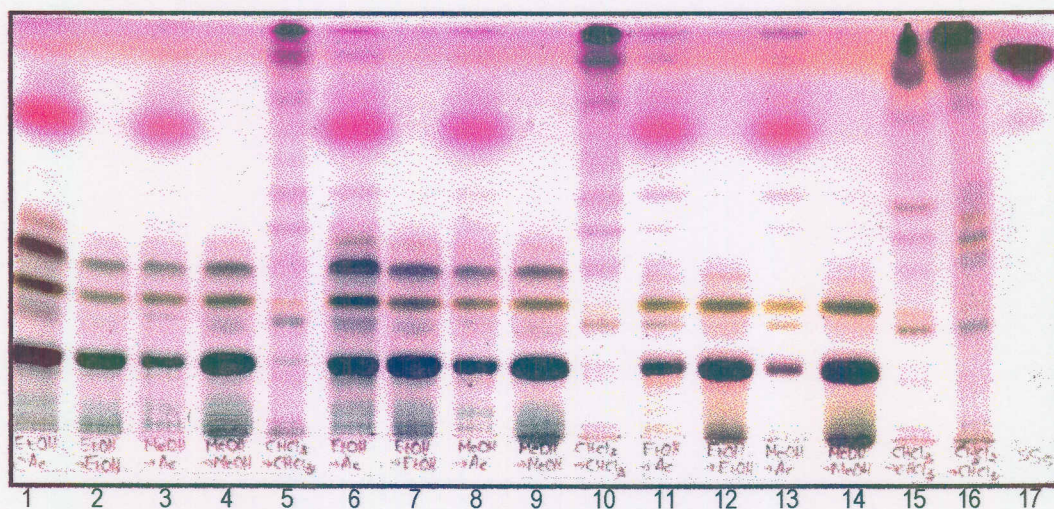
Figure 3.3: Overheated TLC on glass plates of ethanol, methanol and chloroform extracts of *H. hemerocallidea* powder from three sources (African potato 1, 2 and 3). The plates were developed in CEF (**a**) and EMW (**b**), using *p*-anisaldehyde as spray reagent.

In all cases 100 µg of the extracts was applied.

In Figure 3.3 the identity of the different lanes are as follows: African potato 1 (lanes 1-5), African potato 2 (lanes 6-10) and African potato 3 (lanes 11-15). From the left: ethanol, dried and redissolved in acetone (1, 6 & 11), ethanol, dried and redissolved in ethanol (2, 7 & 12), methanol, dried and redissolved in acetone (3, 8 & 13), methanol, dried and redissolved in methanol (4, 9 & 14), chloroform, dried and redissolved in chloroform (5, 10 & 15). On the right a soxhlet dichloromethane extract of African potato 1, which was redissolved in chloroform (16) and 5 mg/ml 95% stigmasterol standard (17).

At the time, hypoxoside standard was not available, but retrospectively hypoxoside was identified as the black compound (R_f = 0.17) in all the methanol and ethanol extracts (dissolved in acetone or the particular alcohol) on the overheated plate, developed in EMW. On moderately heated plates sprayed with p-anisaldehyde, the compound was purple. Hypoxoside is a norlignan diglucoside unique to the Hypoxidaceae. Due to the two glucose molecules in its structure, the molecule is polar and therefore only separates in the highly polar EMW. On the TLC plates, it seemed that methanol and ethanol extracted the same components: the *red spot compound*; a low concentration β-sitosterolin (visible in the alcohol extracts dissolved in acetone in CEF) and also hypoxoside. Methanol, however, extracted substantially more material. See Table 3.3. The isolated hypoxoside dissolved extremely well in methanol, and a concentration of 10 mg/ml was achieved without shaking. Koch and Brosch-Starzengruber (1991), and Marini-Bettolo and Galeffi (Nicoletti et al., 1992) also used methanol extracts for hypoxoside analyses.

Conclusion: Methanol was identified as the best extractant for extracting hypoxoside.

TLC in Figure 3.3 shows that the acetone removed all the *red spot compound* and β-sitosterolin (not all the hypoxoside) from the methanol and ethanol extracts (lanes 1, 3, 6, 8, 11 & 13) and, therefore, the alcohols used to rinse the vials after acetone (lanes 2, 4, 7, 9, 12 & 14), did not contain the *red spot compound*. The *red spot compound* was yellow-brown on the normally heated glass plate sprayed with p-anisaldehyde, but when overheated it turned a beautiful, pink-red. When aluminium plates were used, the *red spot compound* was quickly visible, but with overheating the background turned pink so that the pink-red compound could no longer be distinguished.

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Chloroform extracted a radically different spectrum of components, among others, the "phytosterol-complex" (R_f = 0.72 in CEF and 0.91 in EMW), which was not visible in the alcohol extracts, and even β-sitosterolin (R_f = 0.17 in CEF, but not clear in EMW). As β-sitosterolin is a glucoside (polar), it was not expected to be extracted by chloroform (non-polar).

In both CEF and EMW solvent systems the sterols and sterolins were visible. Stigmasterol (standard) and the "phytosterol-complex" had a R_f = 0.72 in CEF and 0.91 in EMW, and stigmasterolin and the *red spot compound* had R_f-values = 0.44 in CEF and 0.73 in EMW. However the sterolin of the 5 mg/ml stigmasterol standard (as opposed to the 10 mg/ml concentration discussed previously) could not be seen when heating the glass plates as usual at 105 °C. Overheating the plates until the background turned pink-red (*p*-anisaldehyde), made even the lower concentrations of sterolins (e.g. in 5 mg/ml stigmasterol standard) visible. This led to a theory that overheating the glass plate caused hydrolysis of the sterolin to the sterol. Assuming that the sterol reacts more readily with the spray reagent (*p*-anisaldehyde), the hydrolysed sterolin is more clearly visible. This formed the basis of the experiments as described in 3.2.2.5

3.3.1.2 (c) Dichloromethane and diethylether extraction

As simple chloroform extraction of African potato 1, 2 and 3 [described in 3.3.1.2 (b)] only extracted 0.06 – 0.08% plant material (See Table 3.3), it was decided to apply soxhlet extraction for continuous and complete extraction in a closed system. The plant material of African potato 1 was placed in two porous paper thimbles and extracted with excess extractant. Solvent strength ($\in°$), instead of solvent polarity (*P'*) was used as polarity parameter: Dichloromethane's solvent strength ($\in°$) is 0.42, which is very close to that of chloroform ($\in°$= 0.40), was used as extractant because it boils at 40 °C whereas chloroform boils at 61 °C. It was felt that the active extract constituents could possibly be degrade at the higher temperature. The solvent polarity (*P'*) of dichloromethane and chloroform, however, differ by one unit (3.1: 4.1). See Table 3.1.

TLC of the soxhlet dichloromethane African potato 1 extract. compared to the simple chloroform extract, indicated that the dichloromethane extracted a greater variety of compounds (See Figure 3.3). Soxhlet dichloromethane extraction of African potato 1, extracted 1.705% compared to only 0.04% with simple extraction.

Soxhlet extraction of African potato 1 was repeated with diethylether. Again solvent strength was used as polarity parameter: As previously explained with dichloromethane, diethylether's solvent strength ($\epsilon° = 0.38$) is close to that of chloroform ($\epsilon° = 0.40$) as well, although the boiling point is much lower (35 °C: 61 °C). Yet again the solvent polarity P' (as opposed to the solvent strength, $\epsilon°$) differs substantially, with $P' = 4.1$ for chloroform and $P' = 2.8$ for diethylether. See Table 3.1.

TLC of the soxhlet diethylether extract compared to the simple chloroform extract, indicated that diethylether extracted the same components as chloroform. Soxhlet diethylether extraction however, extracted a substantially higher concentration of each component than simple chloroform extraction. Soxhlet diethylether extraction of African potato 1, extracted 0.125% compared to the 0.06% with simple extraction.

3.3.1.2 (d) Hexane extraction

Hexane is a very non-polar solvent and its extraction capability of sterols was tested and compared with chloroform. Hexane extracted 0.04% material from African potato 1, compared to the 0.06% extracted by chloroform [See 3.3.1.2 (b)]. TLC of the chloroform and hexane extracts revealed that chloroform is in fact a better extractant than hexane (results not shown).

Conclusion: Chloroform was identified as the best extractant for sterols.

3.3.1.3 Best extractant evaluation

When the acetone extracts of African potato 1 are compared with the methanol extract (redissolved in acetone) in Figure 3.2, it would seem that as if the composition and contents of the same concentration of methanol (lane 6) and acetone extracts (lane 3 & 4) of African potato 1, are very similar. However, methanol extracts substantially more material from African potato 1 than acetone (4.2%:2.2%) See Table 3.2 and 3.3. Furthermore, the methanol extract (redissolved in methanol) in Figure 3.3 does not separate in CEF, as it contains very polar compounds (it separates in EMW) thus, the extracting properties of acetone and methanol are not the same, despite the fact that their solvent polarity ($P' = 5.1$) is the same, according to the data of Snyder and Kirkland (1979). See Table 3.1.

However, it is important to realise that other factors e.g. large dipole and proton donating-and-accepting properties influence solvent selectivity. The data of Rohrschneider, used to define solvent polarity values P', was plotted on a triangular diagram by Snyder & Kirkland (1979). This diagram

grouped various solvents into clusters of similar selectivity and, according to these groups, methanol is a good proton acceptor with a large dipole (group II), and acetone is a better proton donor with a smaller dipole (group VIa). This partly explains the different extracting properties. On the other hand, acetone and methanol's solvent strength ($\epsilon°$) differs significantly, with $\epsilon°= 0.56$ for acetone and $\epsilon°= 0.95$ for methanol (Snyder & Kirkland).

Thus acetone extracts less material from African potato 1 but it does extract the *red spot compound* and β-sitosterolin. Therefore, acetone is seen as a more selective extractant (w/w) for sterolins (including the *red spot compound*) than methanol. Furthermore, acetone is easier to work with, as the extracts dry quickly for concentration determination and, the volatility also results in neat thin lines on TLC plates.

Conclusion: Acetone is the best extractant for sterolins (including the red spot compound).

Chloroform was shown to be the best extractant for sterols and methanol for hypoxoside. If required that all three compounds, that is phytosterols, sterolins and hypoxoside be indicated with one extract, methanol should be used as extractant.

3.3.2 Mobile phase evaluation

Three different solvent systems were used as mobile phases for the development of TLC plates. In order of polarity:

*BEA: Benzene:Ethanol:Ammonia in the ratios 18:2:0.2 (separates non-polar compounds).

*CEF: Chloroform:Ethylacetate:Formic acid in the ratios 5:4:1 (separates compounds of intermediate polarity).

*EMW: Ethylacetate:Methanol:Water in the ratios 10:1.35:1 (separates polar compounds).

All three the systems showed the phytosterols clearly, although none of the systems could separate the individual sterols (β-sitosterol, campesterol and stigmasterol) and a "phytosterol-complex" was visible. In BEA (non-polar system), the sterols had low R_f-values and the compounds of interest were, therefore, on the lower half of the chromatogram. The *red spot compound* and β-sitosterolin were visible as well. In CEF (intermediate polarity) the sterols and sterolins of interest had intermediate R_f-values and were spread over the entire chromatogram. Both the *red spot compound* and β-sitosterolin were visible. In EMW (polar system), the sterols and sterolins had high R_f-values and the compounds of interest dominated the upper half of the chromatogram. Therefore, with

balance in distribution on the chromatogram and its general appearance, as the main reason, CEF was chosen as the best mobile phase for separation of the sterols and sterolins. However, only EMW was able to separate hypoxoside from the polar methanol extracts and was used for hypoxoside determinations.

Conclusion: CEF is the best mobile phase to separate sterols and sterolins and EMW for hypoxoside determination.

3.3.3   Spray reagent evaluation

3.3.3.1 *P*-Anisaldehyde spray reagent

P-Anisaldehyde reacts with the sterols, the *red spot compound* and β-sitosterolin to reveal the coloured substances in daylight. When glass plates are moderately heated, the *red spot compound* is brown-yellow, but becomes a pink-red when the plate is overheated.   β-Sitosterolin is a light purple line and the phytosterols form a dark, blue-purple mass – "phytosterol complex".
Sterols and sterolins do not fluoresce at 254 nm or 350 nm when sprayed with *p*-anisaldehyde.

3.3.3.2 Vanillin spray reagent

Eloff (1999) found in his experiments that vanillin does not reveal the sterolins. However, when these experiments were repeated, it was found that vanillin does show the sterolins present in high concentrations e.g. isolated β-sitosterolin and "stigmasterolin" in 10 mg/ml stigmasterol standard. Overheating glass plates made the "stigmasterolin" in the 10 mg/ml standard more visible. The *red spot compound* and β-sitosterolin in African potato 1 and *P. africana* extract (5:1) were also visible when sprayed with vanillin. See Figure 3.4 and compare with Figure 3.2 (sprayed with *p*-anisaldehyde).

Sterols and sterolins fluoresce slightly at 350 nm when sprayed with vanillin. Five compounds were visible under 350 nm UV light that were not visible in daylight or with *p*-anisaldehyde. Three of them had R_f-values higher than the *red spot comound* and lower than the "phytosterol-complex" and were present in the alkaline acetone (lane 5) and in the methanol extracts (lane 6 and 7) respectively. Two compounds were present in the *P. africana* extract (5:1) methanol and chloroform extracts respectively, with R_f-values lower than the *red spot compound.*
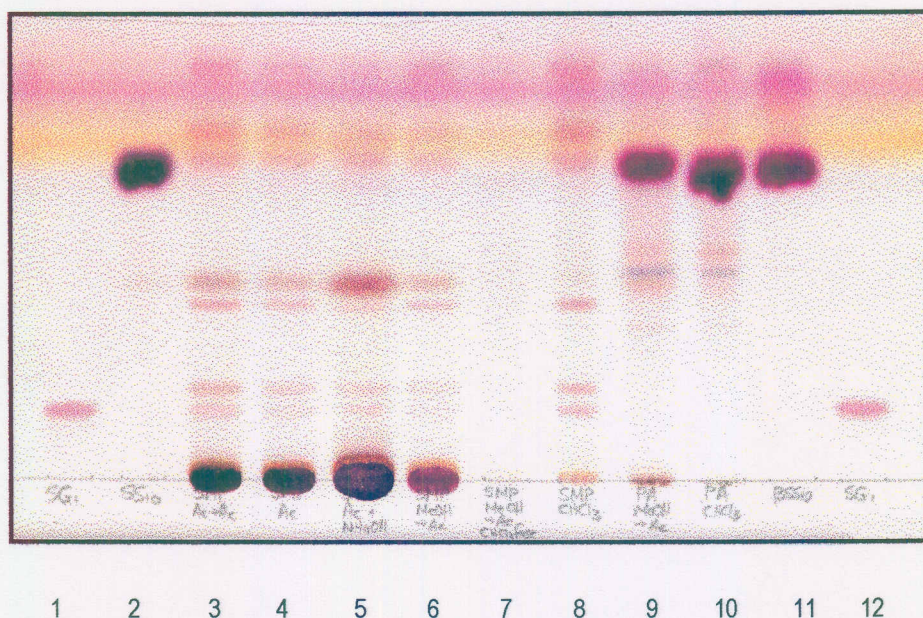
Figure 3.4: Overheated TLC on a glass plate of different extracts of *H. hemerocallidea* powder and *P. africana* extract (5:1), developed in CEF and sprayed with vanillin. On the left are 1 mg/ml isolated β-sitosterolin, 10 mg/ml 95% stigmasterol standard and on the right are 10 mg/ml 40% β-sitosterol standard and 1 mg/ml isolated β-sitosterolin. In all cases 100 μg of the extracts was applied.

In Figure 3.4 from the left are 1 mg/ml isolated β-sitosterolin (1) and 10 mg/ml 95% stigmasterol standard (2). The order of the African potato 1 extracts are as follows: Acetone, dried and redissolved in acetone again (3), acetone, not dried (4), alkaline acetone (5), methanol, dried and redissolved in acetone (6), acetone insolubles from 6 redissolved in 50/50 CHCl₃/H₂O – chloroform phase (7) and chloroform, dried and redissolved in chloroform (8). The extracts of *P. africana* extract (5:1) are methanol, dried and redissolved in acetone (9) and chloroform, dried and redissolved in chloroform (10). On the right are 10 mg/ml 40% β-sitosterol standard (11) and 1 mg/ml isolated β-sitosterolin (12).

3.3.3.3 Perchloric acid spray reagent

Perchloric acid chars the organic compounds on the TLC plate and they turn brown or black depending on the quantity present. It only charred the compounds that were revealed by *p*-anisaldehyde, which indicates that there are not other organic compounds present in high

concentrations. The sterols and sterolins (*red spot compound* and β-sitosterolin) fluoresce at 350 nm, when sprayed with perchloric acid. The sterols fluoresced orange and β-sitosterolin light-pink

3.3.3.4 50% Sulphuric acid in methanol spray reagent

50/50 H_2SO_4/MeOH also chars the organic compounds. Again no other compounds than those revealed by *p*-anisaldehyde, were shown, therefore, it can be assumed that *p*-anisaldehyde reveals all the organic compounds present in high concentrations.

The sterols and sterolins do not fluoresce when sprayed with 50/50 H_2SO_4/MeOH at either 254 nm or 350 nm.

3.3.3.5 Phosphoric acid spray reagent

The colour reaction, that takes place when spraying the plates with phosphoric acid, was not satisfactory. However, under 350 nm UV light, apart from the usual compounds revealed by *p*-anisaldehyde, seven bands could be seen in the African potato 1 methanol extracted, acetone-insoluble, chloroform phase (lane 7 in Figure 3.2 and 3.4, but now sprayed with phosphoric acid). *P*-Anisaldehyde did not reveal anything in this extract, but vanillin showed one compound at 350 nm UV. the sterols fluoresced orange and β-sitosterolin light-pink, as with perchloric acid.

3.3.3.6 *P*-Toluene sulphonic acid spray reagent

Similar to phosphoric acid, the colour reaction, that reveals compounds in daylight, was not satisfactory. However, two blue bands with R_f-values higher than the *red spot compound*, were visible in the *P. africana* extract (5:1)'s methanol and chloroform extracts. As with perchloric and phosphoric acid, at 350 nm the sterols fluoresced orange and β-sitosterolin was light pink. The *red spot compound* was not clearly visible.

3.3.3.7 Trichloroacetic acid spray reagent

After spraying the plates with trichloracetic acid, nothing could be seen in daylight. However, at 350 nm UV the sterols looked light blue, but β-sitosterolin was not clear.

3.3.3.8 Best spray reagent evaluation

Apart from *p*-anisaldehyde and vanillin, the other spray reagents made the sterols/sterolins visible under UV light, but not in daylight. Two other important spray reagents widely used in herbal

medicinal TLC, fast blue and natural product reagent, also gave no colour reaction in daylight, when used by Eloff (1999) for sterol/sterolin analyses, and were, therefore, not applied.

P-Anisaldehyde was most often used as spray reagent. Although not all compounds (that were visible under UV light) were revealed by it, they were not the major compounds of interest. The important compounds, e.g. the sterols/sterolins (including the *red spot compound*) and even hypoxoside, were revealed by *p*-anisaldehyde and were clearly visible in daylight.

Conclusion: P-Anisaldehyde was regarded as the best reagent tested to indicate sterols and sterolins and hypoxoside.

B.   APPLICATION

3.3.4   TLC Application

3.3.4.1 Analyses of different plants used for benign prostatic hyperplasia (BPH)

3.3.4.1 (a) *H. hemerocallidea* from three different sources – extracts compared

Comparison of methanol, ethanol and chloroform extracts of three sources of African potato revealed no differences on a qualitative basis with TLC. Although the percentage (w/w) of material extracted by methanol, ethanol and chloroform differed for the three African potato samples, the components extracted were similar, and all three samples could be considered as pure *H. hemerocallidea*. TLC can, therefore, be used to identify qualitatively unknown corms as *H. hemerocallidea* by comparing and matching with the TLC template of a known corm. Furthermore, TLC can indicate batches that do not comply to set standards e.g. minimum sterol/hypoxoside levels, again by comparison with a satisfactory TLC template of *H. hemerocallidea*. The compound levels in plants can be influenced by e.g. time of harvest, absence/availability of essential ground nutrients, climate *etc*. See 3.3.1.2 (b) and Figure 3.3.

3.3.4.1 (b) Comparison of sterols/sterolins in BPH phytotherapeutics: *H. hemerocallidea* powder, *P. africana* extract and *S. repens* powder/extract

The aim was to compare the sterol/sterolin contents of different plants, specifically African potato 1, *P. africana* extract (5:1), *S. repens* powder and extract (4:1). Furthermore the composition and concentration differences of the actives in the powder and extract were also compared in the case of *S. repens*.

Methanol extracts were used to compare sterolins (including the *red spot compound*) and chloroform extracts for the sterols. The methanol extracts were redissolved in acetone and the chloroform extracts in chloroform. The TLC plate was developed in CEF with *p*-anisaldehyde as spray reagent. See Figure 3.5.

1   2   3   4   5   6   7   8   9   10   11   12
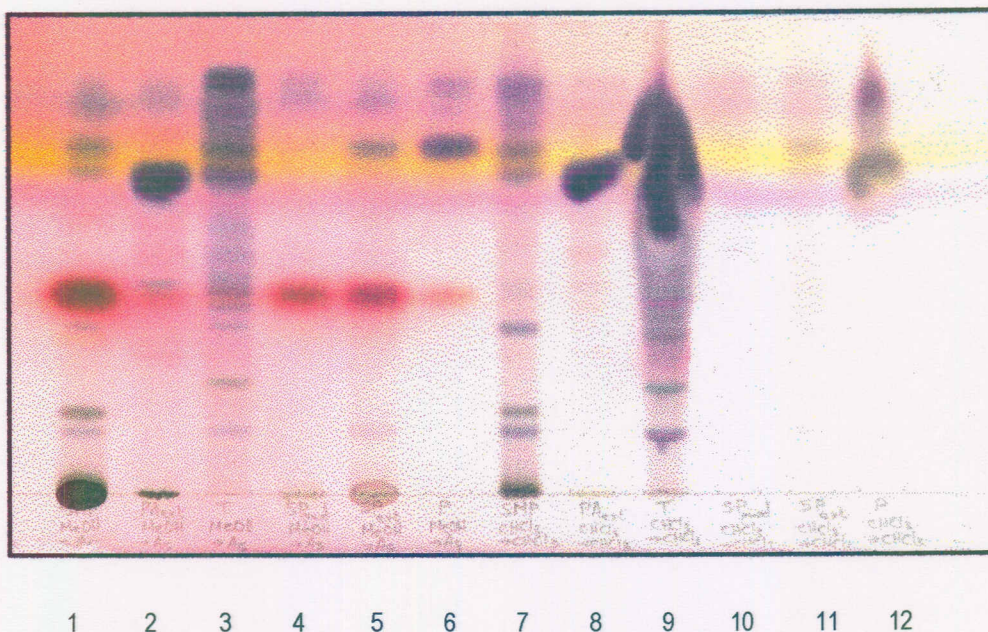
Figure 3.5: Overheated TLC on a glass plate of methanol (lanes 1-6) and chloroform (lanes 7-12) extracts of African potato 1 (1 & 7) *P. africana* extract (2 & 8), Tadenan® (*P. africana* extract) (3 & 9), *S. repens* powder (4 & 10) and extract (5 & 11) and Permixon® (*S. repens* extract) (6 & 12). The plate was developed in CEF and sprayed with *p*-anisaldehyde. In all cases 100 μg of the extracts was applied.

Methanol extracted a high concentration of phytosterols and a low concentration of the *red spot compound* and β-sitosterolin *from P. africana* extract (5:1). See that the methanol extract of *P. africana,* in the form of Tadenan® (lane 3), contained β-sitosterolin (R_f = 0.14). Methanol, however, extracted a low concentration of phytosterols, a substantial amount of the *red spot compound*, as well as hypoxoside (not visible in CEF, but visible in EMW – not shown) and β-sitosterolin from African potato 1. Methanol extracted the *red spot compound* from *S. repens* powder and extract (4:1), and a low concentration β-sitosterolin and sterols from the extract (4:1). Apparently chloroform extracted the same quantity of sterols from *P. africana* extract (5:1) than methanol, which was surprising. On the other hand chloroform extracted almost nothing from either *S. repens* powder or the extract (4:1). According to literature *S. repens* extracts are composed of 90% fatty acids and a variety of alcohols (including sterols) and a pentacyclic triterpenoid, lupeol (Quirke et al., 1998). Therefore, sterols were not expected, but it was strange that the fatty acids were not noticeable in the chloroform extracts of *S. repens*.

Table 3.4: Relative amounts of sterols, sterolins and hypoxoside (indicated by 1+ to 4+) extracted by methanol and chloroform respectively from *H. hemerocallidea* powder, *P. africana* extract (5:1), *S. repens* powder and extract (4:1), deduced from Figure 3.5

Methanol extraction				
	Phytosterols	β-Sitosterolin	*Red spot compound*	Hypoxoside
H. hemerocallidea pwd.	+	+ +	+ + + +	+ + +
P. africana ext	+ + + +	+	+	-
S. repens pwd.	-	-	+ +	-
S. repens ext.	+	+	+ + +	-
Chloroform extraction				
	Phytosterols	β-Sitosterolin	*Red spot compound*	Hypoxoside
H. hemerocallidea pwd.	+ +	+ + +	-	+
P. africana ext	+ + + +	-	-	-
S. repens pwd.	+	-	-	-
S. repens ext.	+	-	-	-

Conclusion: From the results one can conclude that H. hemerocallidea contains sterols, sterolins (including the red spot compound) and also hypoxoside. P. africana extract (5:1) contains a very high concentration of sterols and a low concentration of β-sitosterolin and the red spot compound. S. repens extract (4:1) contains hardly any sterols, low concentrations β-sitosterolin and relatively high concentrations of the red spot compound (lower than H. hemerocallidea).

**Note that the extractability of compounds and their sensitivity to the spray reagent may differ. For example, in Table 3.4 the concentration of phytosterols in *H. hemerocallidea* may be higher than the concentration of the *red spot compound*. However, if the *red spot compound* is more soluble in the extractant used and it reacts more readily with *p*-anisaldehyde than the phytosterols, the *red spot compound* band will have a higher intensity on TLC and wrongly give the impression of a higher

concentration in the plant. Therefore only comparisons of the same compound using the same extractant should be made.

3.3.4.2 Analysis of different phytosterol products

The aim was to compare the phytosterol levels in four different phytosterol containing products (Moducare®, Immunochoice®, Phytogard® and Nutricare®), four *H. hemerocallidea* products (African potato 1, 4, 5, 6), a BPH product [Prostol® contains *S. repens* berry powder, *Prunus africana* extract (5:1) and pumpkin seed extract (4:1)] and *S. repens* extract (4:1) and *P. africana* extract (5:1). For phytosterol comparison, chloroform extraction was performed and aluminium TLC plates were suitable. See Figure 3.6.
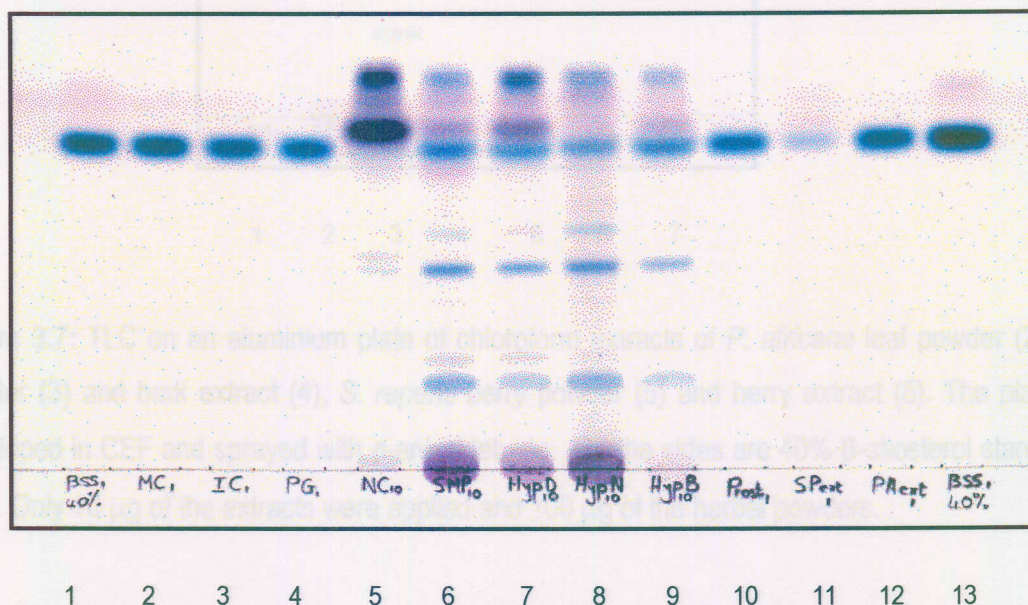
Figure 3.6: TLC on an aluminium plate of chloroform extracts of different phytosterol products. From the left are 40% β-sitosterol standard (1), Moducare® (2), Immunochoice® (3), Phytogard® (4) and Nutricare® (5), African potato 1 (6), African potato 4 (7), African potato 5 (8), African potato 6 (9), Prostol® (10), *S. repens* extract (11), *P. africana* extract (12) and 40% β-sitosterol standard (13). The plate was developed in CEF and sprayed with *p*-anisaldehyde.

In the case of Nutricare® and the *H. hemerocallidea* samples, 100 µg was applied, but in all other cases 10 µg was applied.

TLC of the chloroform extracts of Moducare®, Immunochoice®, Phytogard®, Prostol® and *P. africana* extract (5:1) were similar - only the "phytosterol-complex", as seen in the 40% β-sitosterol standard, was visible. Nutricare® appeared different from the other phytosterol products and the usual "phytosterol-complex" was not visible. The four *H. hemerocallidea* products appeared similar although the band intensities differed. *S. repens* extract (4:1) contained a very low concentration phytosterols, compared to *P. africana* extract (5:1). This confirmed the results of 3.3.4.1 (b). The phytosterol complex of Prostol® could probably also be attributed to *P. africana* extract (5:1) – the phytosterol contents of pumpkin seed was not investigated.

3.3.4.3 Analysis of herb powder versus the herbal extract of *P. africana* and *S. repens*

In the natural medicine industry herbal extracts are claimed to contain substantially higher concentrations of the particular active components when compared to the herb powders from which they have been extracted. These extracts are often also very expensive. This was investigated by comparing the phytosterol contents of chloroform extracts of *P. africana* bark powder extract (5:1) and *S. repens* berry powder and extract (4:1). The phytosterol contents of *P. africana* leaves were investigated as well. See Figure 3.7.

The *P. africana* leaf powder contained phytosterols ("phytosterol-complex") in similar concentrations as the bark powder, but both contained less than *P. africana* bark extract (5:1). The chloroform extract of *P. africana* bark powder is complex and more than fourteen different bands were visible on TLC, whereas there was only one band ("phytosterol-complex") visible in the bark extract (5:1). The "phytosterol-complex" in *P. africana* bark extract (5:1) is at least five times as intense as that of the bark powder.

The chloroform extract of *S. repens* berry powder is much simpler than that of *P. africana* bark powder and approximately four bands are visible on TLC. Only two bands of *S. repens* berry extract (4:1) are visible on TLC. The "phytosterol-complex" in *S. repens* berry extract (4:1) appears lighter than that of *S. repens* berry powder, but the concentration was ten times less than that of the powder. However, the total quantity extracted should also be taken into consideration with these comparisons.

1   2   3   4   5   6   7
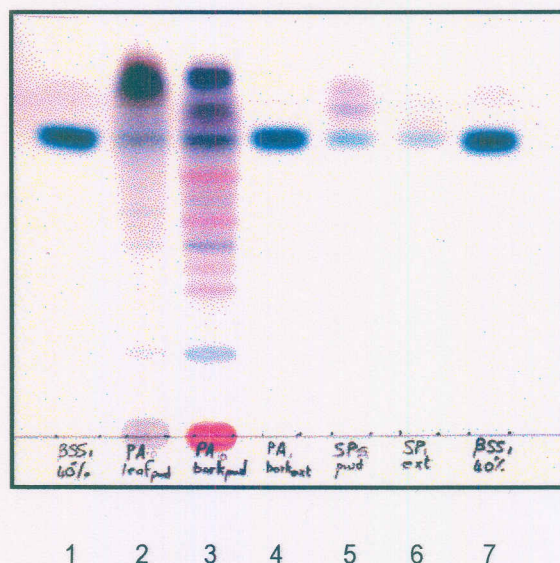
Figure 3.7: TLC on an aluminium plate of chloroform extracts of *P. africana* leaf powder (2), bark powder (3) and bark extract (4), *S. repens* berry powder (5) and berry extract (6). The plate was developed in CEF and sprayed with *p*-anisaldehyde. On the sides are 40% β-sitosterol standard (1 & 7). Only 10 μg of the extracts were applied and 100 μg of the herbal powders.

3.3.4.4 Hydrolysis of "stigmasterolin" and the *red spot compound*

See the method of hydrolysis in section 3.2.2.5.

"Stigmasterolin" was visible in the unacidified 10 and 5 mg/ml stigmasterol standards (lanes 2 & 4), but it disappeared in the acidified stigmasterol solutions (lanes 1 & 3). Thus, as expected the hydrochloric acid hydrolysed the "stigmasterol glucoside" and cleaved the sugar bond to form stigmasterol. See Figure 3.8

However, hydrolysis of the *red spot compound* in the MCW extract of African potato 1 (water-methanol phase) was not observed (results not shown). It is possible that the *red spot compound*'s concentration was too high in proportion to the HCl added and that the reaction was not complete. In the MCW-chloroform phase of African potato 1, new bands appeared after hydrolysis, while others disappeared.

SS_{10} SS_{H10} SS_5 SS_{H5}

1   2   3   4

Figure 3.8: Overheated TLC on a glass plate of unhydrolysed (1) and hydrolysed 10 mg/ml 95% stigmasterol standard (2) and unhydrolysed (3) and hydrolysed 5 mg/ml 95% stigmasterol standard (4).

3.3.4.5 Isolation of the *red spot compound* with preparative thin layer chromatography (PTLC)

See the PTLC method in section 3.2.2.6.

After dissolving the *red spot compound* on the silica powder in acetone, the acetone was removed and c. 500 mg precipitate remained. Usually at least 1 mg, but preferably 10 mg pure sample is needed for NMR structure elucidation. The sample was sent for structure elucidation to SASOL.

3.3.4.6 Structure elucidation of the *red spot compound* with Nuclear Magnetic Resonance Spectroscopy (NMR)

See the method in section 3.2.2.7.

From SASOL's ACD/HNMR Dictionary the ^{1}H spectra of β-sitosterol, campesterol and stigmasterol could be obtained. See Appendix A.

As there was very little sample, special measures were taken to improve the sensitivity of NMR (indirect detection probe *etc.*) as discussed in 3.2.2.7. However, NMR ^{1}H spectrum revealed that the sample was impure and there was too little to identify the major compound present.

TLC of the NMR analysed samples (CEF with *p*-anisaldehyde) revealed that they contained at least three different components. Apparently, the separation of the extract on the thicker preparative plate (2 mm), as discussed in 3.2.2.7, was not as good as on the ordinary glass TLC plates (0.25 mm). The bad separation on the preparative plate is consistent with Cannell (1998), who states that when attempting to separate a mixture with PTLC (preparative TLC) it should not contains more than four main components. The acetone extract of *H. hemerocallidea* (African potato 1) contained at least nine different compounds, therefore, NMR analysis of the *red spot compound*, isolated with PTLC, was unsuccessful.

3.3.4.7 Water extraction of African potato 1

African potato 1 is marketed for various medicinal indications, e.g. immunity stimulation, anti-cancer effects, arthritis, *etc.* (promotional pamphlet). These effects are attributed to the sterols and sterolins in the plant (Van Wyk et al., 1997 & Bouic et al., 1999). The extraction method suggested by the distributor [See this chapter, 3.2.2.1 (b) (iii)] hardly extracts any phytosterols or sterolins (Eloff, 1999). To determine whether sterols/sterolins are extracted by water, the work of Eloff (1999) was repeated as it is possible that the particular sample analysed by Eloff (1999) had a very low concentration of sterols/sterolins. For the complete method see this chapter, 3.2.2.1 (b) (iii). The prepared extracts were chromatographed and developed in EMW (*p*-anisaldehyde as spray reagent) as the water extract is polar and does not separate well in CEF. See Figure 3.9.

From TLC, it seemed that water extracted the *red spot compound* and a little hypoxoside from African potato 1, but not β-sitosterolin – as retrospectively identified when isolated β-sitosterolin was obtained (lane 4). It does not appear as if water extracted the sterols, as to be expected (lanes 8 & 9), as the sterols are too non-polar. The methanol extract, dried and redissolved in methanol (lanes 3 & 11), contained hypoxoside but not the *red spot compound.* The methanol extract, dried and redissolved in acetone (lane 12), on the other hand, contained hypoxoside and the *red spot compound.* Possible reasons were that the *red spot compound* did not dissolve in the methanol again after drying the methanol extract, or that the methanol extract did not separate completely and that the *red spot compound* was present, but was not visible. Note that all that is extracted does not necessarily dissolve again, due to possible complex formation *etc.*

59
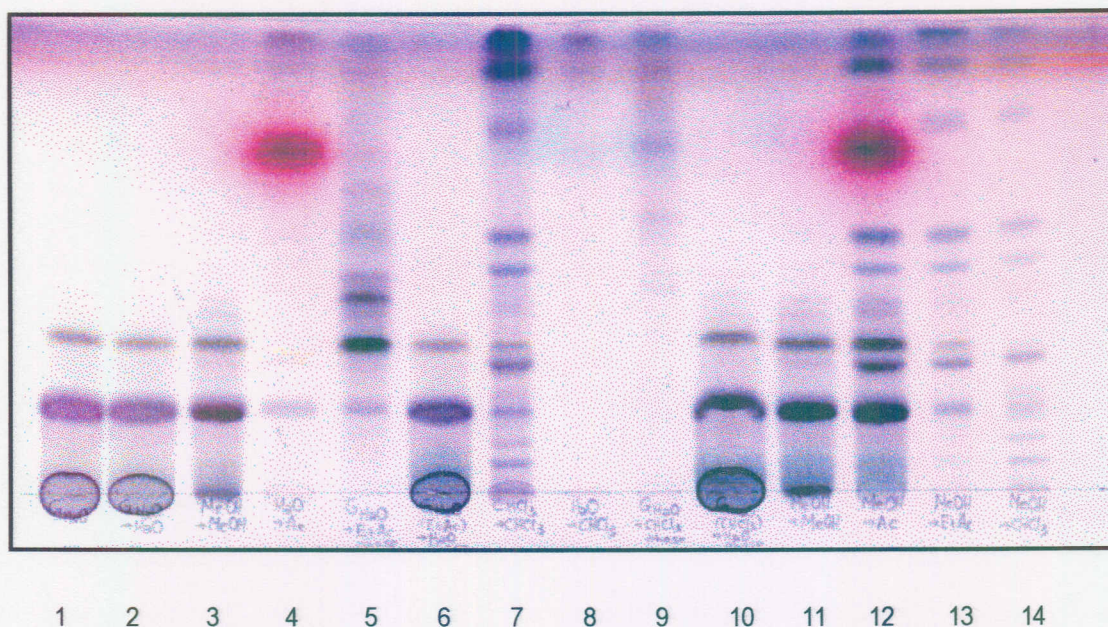
Figure 3.9: Overheated TLC on a glass plate of water and other extracts of grated African potato 1 corms (G) and powdered African potato 1, developed in EMW and sprayed with *p*-anisaldehyde.

In Figure 3.9 the identity of the different lanes are as follows: Grated (G) corms water extract (1); G water extract dried and redissolved in water (2); powder methanol extract (redissolved in methanol) (3); powder water extract (redissolved in acetone) (4); G water extract (ethylacetate's) organic phase (5); G water extract (ethylacetate's) watery phase (6); powder chloroform extract (redissolved in chloroform) (7); powder water extract (redissolved in chloroform) (8); G water extract (chloroform's) organic phase (9); G water extract (chloroform's) watery phase (10); powder methanol extract (redissolved in methanol) (11); powder methanol extract (redissolved in acetone) (12); powder methanol extract (redissolved in ethylacetate) (13), and powder methanol extract (redissolved in chloroform) (14).

The therapeutic effect of African potato 1, extracted with water, may be ascribed to hypoxoside, or perhaps even the *red spot compound* or other novel compounds present, but apparently not to β-sitosterol.

3.4 CONCLUSION

In order to develop an optimum TLC method for phytosterols and sterolins, the best extractant, mobile phase and spray reagent were determined. The developed TLC method could be applied to qualitatively compare *H. hemerocallidea*, *P. africana* and *S. repens*, as well as different phytosterol containing products, in terms of their phytosterol and sterolin contents.

Acetone was the best extractant for sterolins (β-sitosterolin and the *red spot compound*), chloroform for phytosterols and methanol for hypoxoside. The best separation of the sterols/sterolins was found with chloroform:ethylacetate:formic acid (5:4:1) as mobile phase and the best spray reagent was *p*-anisaldehyde.

Apart from the extractant, mobile phase and spray reagent, it seems that even the type of TLC plate (aluminium vs. glass) can influence the visibility of compounds on TLC. Overheated plates are not always negative, as the *red spot compound* was more prominent and therefore noticed for the first time, after the glass plates (sprayed with *p*-anisaldehyde) were overheated by accident.

In our results, *H. hemerocallidea* powder contained sterols, sterolins (including the *red spot compound*) and hypoxoside; *P. africana* extract (5:1) contained a very high concentration of sterols and a low concentration of β-sitosterolin and the *red spot compound*, but *S. repens* extract (4:1) contained hardly any sterols, low concentrations β-sitosterolin and relatively high concentrations of the *red spot compound* (lower than *H. hemerocallidea*).

The developed TLC method thus provides the natural medicine industry with a simple, cost-effective and reproducible method to impose the necessary quality control procedures.

As African potato tea was used traditionally for centuries and is still marketed for medicinal application, the water extract of *H. hemerocallidea* was also investigated. From the TLC results on water extraction of *H. hemerocallidea* powder, it appeared that water does not extract phytosterols or β-sitosterolin. This was concerning, as currently the phytosterols (especially β-sitosterol) and sterolins, are regarded as the main active components of *H. hemerocallidea* and also of African potato tea. These results should however be confirmed with further research, possibly using more sensitive techniques. The water extract did contain the *red spot compound* and hypoxoside.

Hypoxoside is metabolised *in vivo* to rooperol and has anticancer activity and could thus possibly be considered as one of the main actives. See Chapter 1, section 1.4.1.3.

Whether the *red spot compound* has therapeutic effect is unknown, as the structure could not be identified. Apparently it is a common compound in phytosterol-containing material, as it could be detected in acetone, methanol and water extracts of *H. hemerocallidea* and in acetone extracts of *P. africana*, *S. repens*, Moducare®, Harzol®, Immunochoice® and Nutricare®. Although the R_f-value was the same as that of, or what was considered to be, "stigmasterolin" in the 95% stigmasterol standard, the *red spot compound* is not necessarily stigmasterolin and its identity has to be determined. In this study, however, it was regarded as a sterolin and reported as such. An attempt to hydrolyse the *red spot compound* did not cause the band intensity to decrease, as could be shown with "stigmasterolin" in the 95% stigmasterol standard. PTLC isolation of the *red spot compound* was unsuccessful as the separation on the PTLC plate was not as effective as on TLC. Column chromatography could perhaps provide a clean sample, but a substantial quantity of *H. hemerocallidea* will have to be extracted with acetone.

It is important to note that the compared *P. africana* and *S. repens* extracts (and other products containing extracts) were produced and/or extracted by different companies, probably using different extraction procedures. As noticed in the different compounds extracted e.g. with acetone versus chloroform from *H. hemerocallidea*, the extraction procedure largely determines the composition of the product and the compounds that it contains. Therefore, the botanical product of one company may differ dramatically from that of another regarding specific components present and, thus also effectiveness, as we often do not know what the active components of these products are. This holds true, even if they use material that originates from the same plant. Then there is also natural variability among the plants themselves (Lowe & Fagelman, 1999).

The implication is that the results of basic or general research and clinical trials cannot be automatically accepted and/or transferred from one manufacturer's product to another. The different preparations from each individual manufacturer need to be evaluated individually. The necessity of this principle was emphasised by the Committee on Other Medical Therapies of the Fourth International Consultation on Benign Prostatic Hyperplasia (Lowe & Fagelman, 1999). If this is not possible, the products should at least be qualitatively compared. This is where a well-developed and reliable technique, such as the TLC method developed in this study, could play an important role.

CHAPTER 4

HPLC METHOD EVALUATION AND APPLICATION

4.1 INTRODUCTION

4.1.1 History

In the early days of liquid chromatography, Tsvett and his successors allowed the mobile phase to pass through the stationary phase, under the influence of gravity, arranged in a glass column (Von Zahn & Rheinholz, 1980). The flow rates were extremely slow, but attempts to speed up this classic procedure by application of vacuum or pressure were, however, not effective because increase in flow rates were accompanied by decrease in column efficiency. It was not until the late 1960's that the technology for producing and using packing material, with particle diameters as small as 5 to 10 μm, was developed. This technology required sophisticated instruments that contrasted markedly with the simple devices that preceded them. The name *high performance liquid chromatography* (HPLC) usually refers to these newer procedures to distinguish them from their predecessors (like TLC), which still find considerable use for preparative purposes (Skoog et al., 1996). Analyses that previously took days are now performed by HPLC in minutes.

Thin layer chromatography is a good technique for quick, qualitative purposes, but for quantitative measurements, it is limited. High performance liquid chromatography (HPLC) is required to do exact measurements of phytosterols. HPLC quantification of phytosterols can be applied to determine β-sitosterol levels in plant extracts e.g. for product comparisons or stability determinations, and in serum to determine bioavailability and bio-equivalency.

4.1.2 Aim of chapter

In this chapter, the published method of Emara et al (1999), used to determine the "bioavailability of β-sitosterol from *Pygeum africanum* extract in humans", is evaluated and applied as a quantification method for phytosterols in plant extracts and related products. The method of Emara et al. (1999) is also adjusted to determine hypoxoside and solid phase extraction (SPE) is applied to isolate compounds and prepare crude plant extracts suitable for HPLC injection.

4.1   MATERIAL AND METHODS

4.2.1   Material

4.2.1.1 Plant material

Plant material, products and standards as described in Chapter 2, 2.1 & 2.2 were used.

4.2.2   Apparatus

4.2.2.1 SPE-equipment

Waters Sep-Pak Plus C_{18} cartridges for solid phase extraction.

4.2.2.2 HPLC-equipment

Hewlett Packard 1050 HPLC, equipped with an HP 1050 multiple wavelength (MWD) and HP 1046A programmable fluorescence detector (FLD). For determination of the optimal wavelengths for the MWD and FLD, the University of Pretoria Dept. Chemical Pathology's diode array detector HP 1050 and fluorescence detector HP 1100 were used as well.

4.2.3   Methods

4.2.3.1 Extraction

4.2.3.1 (a) Simple extraction

Simple extraction was performed on plant material as described in Chapter 3, 3.2.2.1 (b) (i). The extractant used, depended on the compound of interest. For hypoxoside, methanol was used, for phytosterols, chloroform and for sterolins or the *red spot compound,* acetone or methanol. See Chapter 3, 3.3.1.3. Water extraction was also performed as described in Chapter 3, 3.2.2.1 (b) (iii).

For comparison purposes of phytosterols in different plants and products, simple extraction with a dense solvent, chloroform [as described in Chapter 3, 3.2.2.1 (b) (i)] was performed.
In the case of powders, 500 mg was extracted with 5 ml chloroform, and in the case of products, the contents of one capsule was extracted with 5 ml chloroform. As capsule weight varies, the powder mass removed from the capsules was standardised: DISCOVERIES OF NATURE's Hypoxis – African potato 5 (600 mg), PLANTANICAL MEDICINE's Hypoxis – African potato 6 (500 mg),

Immunochoice® (300 mg), Moducare® (200 mg), Nutricare® (400 mg), Phytogard® (400 mg) and Prostol Herbal® (500 mg) extracted with 5 ml chloroform. The dried extracts were redissolved in HPLC methanol to yield the desired concentration.

To improve the sensitivity of analysis, the herb powders (*H. hemerocallidea* powder - African potato 1, 4, 5 and 6, *P. africana* bark and leaf powder, *S. repens* berry powder) and products containing herb powders (Nutricare®) were prepared as 10 mg/ml concentrations, but to avoid overloading, the powder extracts (*P. africana* extract and *S. repens* extract) and products containing extracts (Immunochoice®, Moducare®, Phytogard® and Prostol Herbal®) were prepared as 1 mg/ml concentrations before HPLC injection. Harzol® (German equivalent of Moducare®) was analysed previously – 203 mg was extracted and the concentration injected into HPLC was 0.13 mg/ml.

If the extracts were to be used for quantification with HPLC, HPLC grade solvents were used from extraction to injection. This was done as a contaminant was found in some analytical grade extractants (stored in plastic containers) with exactly the same retention time as β-sitosterol in the HPLC system used, and accumulation of this contaminant was feared. This is discussed at a later stage.

4.2.3.1 (b) Solid phase extraction (SPE)

Solid phase extraction (SPE) can be used to clean and concentrate analytical samples. SPE can be used in either of two ways: In the first approach, the analytes of interest are retained on the packing material, and the sample and most contaminants pass through the packing unretained. In the second approach, the sample is applied to the SPE cartridge, and the analytes of interest are collected as they pass through the adsorbent, unretained. Contaminants are held on the packing and can be disposed with the cartridge.

For hypoxoside analysis *H. hemerocallidea* was extracted with methanol, but methanol extracts to many highly polar compounds, that effect the subsequent separation and makes the extract unsuitable for HPLC injection. When a methanol extract is injected into the HPLC, neither hypoxoside nor phytosterols are detected, therefore, SPE was applied to separate hypoxoside and β-sitosterol from the other compounds in the methanol extract.

4.2.3.1 (b) (i) Preparation of plant samples for HPLC injection with Solid Phase Extraction (SPE)

Powdered plant material (*H. hemerocallidea* powder) was extracted with simple extraction (methanol) as described in Chapter 3, 3.2.2.1(b)(i), dried and redissolved in a proper solvent (50% aqueous methanol) to yield a particular concentration (10 mg/ml).

After several trial runs the following SPE method was developed and applied:

1. Condition/saturate the SPE sorbent (C_{18} cartridge): Pass 5 ml of 50% aqueous methanol through the column to wet it and to ensure reproducible retention of the compound of interest. Collect the liquid that elutes (saturation solution).

2. Retention: Apply 2 ml of the 10 mg/ml sample (20 mg), dissolved in 50% aqueous methanol to the sorbent. Selective retention of the analyte is allowed, while the other materials are removed. Collect the liquid that elutes (sample elute).

3. Rinse/Elution: (a) Polar eluent: wash the column with 10 ml 50% aqueous methanol to remove the polar compound hypoxoside. Collect the eluting liquid (water-methanol fraction 1). Repeat twice with 5 ml 50% aqueous methanol, but collect the fractions separately (water-methanol fraction 2 and 3), to ascertain that all the hypoxoside is recovered.

    (b) Intermediate polarity eluent: Wash the column with 10 ml acetone to mediate mixing of the water-methanol and hexane fractions. Collect the eluting liquid (acetone fraction).

    (c) Non-polar eluent: wash the column with 10 ml hexane to remove the non-polar phytosterols (mainly β-sitosterol). Collect the eluting liquid (hexane fraction 1). Repeat twice with 5 ml hexane, but collect the fractions separately (hexane fraction 2 and 3), to ascertain that all the phytosterols are recovered.

4. Dry the collected fractions and redissolve them in 1.0 ml HPLC grade methanol.

5. Inject 100 µl samples into the HPLC. Use FLD detection for hypoxoside and MWD for sterols.

*The *modified SPE method* is the same as above, but only two 10 ml 50% water-methanol fractions are eluted, no acetone and only one 10 ml hexane fraction.

4.2.3.1 (c) Water extraction of *H. hemerocallidea*

According to African potato 1's promotional pamphlet, one tablespoon (6.4 g) dried, grated corm of *H. hemerocallidea* must be simmered in six cups (1200 ml) water for 15 to 20 minutes and refrigerated until use.

4.2.3.2 Preparation of standards

All the standards, sterols, β-sitosterolin and hypoxoside were dissolved in methanol, as 96.5% methanol was used as HPLC mobile phase and injection of another solvent would cause changes in retention times.

4.2.3.3 HPLC for quantitative measurements

The method as described by Emara et al. (1999) for determining the "bioavailability of β-sitosterol from *Pygeum africanum* extract in humans", was applied for quantitative determinations of phytosterols in plant extracts.

Chromatographic parameters: Phenomenex LUNA C_{18} column, 250 mm x 4.6 mm and 5 μm particle size, and a Phenomenex Security Guard C_{18} pre-column. The temperature was controlled at 50 °C and the mobile phase was isocratic 96.5% methanol, with a flow rate of 2 ml/min. The MWD was set at 205 nm and the FLD was set with an excitation wavelength of 230 nm and an emission wavelength of 245 nm (FLD for hypoxoside measurement). The injection volume was usually 100 μl, but when the sample volume was very limited, 10 μl loops were used; in these cases, a correction factor was applied in the calibration and quantification.

4.2.3.3 (a) Calibration curve from composite sample for multiple determinations

The β-sitosterol (c. 95.7%), campesterol (c. 65%), stigmasterol (c. 95%) standards as well as isolated β-sitosterolin and hypoxoside were simultaneously dissolved in HPLC grade methanol to give a 1 mg/ml stock solution, which was serially diluted to give concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56 & 0.78 μg/ml. This series was injected as 100 μl volumes into the HPLC with MWD, and recorded to set up calibration curves by calibration of peak area. As the purity of the standards was not the same, the concentrations were adjusted accordingly, e.g. β-sitosterol's (c. 95.7% pure) concentrations were adjusted with a factor 0.957 giving 95.7 μg/ml instead of 100 μg/ml *etc.* The purity of the isolated standards, β-sitosterolin and hypoxoside, was unknown, therefore, these were regarded as 100% pure. From the composite sample, four calibration curves were constituted, and they could be used to determine simultaneously β-sitosterol, campesterol and/or stigmasterol (these last two compounds could not be separated in an isocratic system with 96.5% methanol), β-sitosterolin and hypoxoside and report the quantities in μg/ml.

4.2.3.3 (b) Calibration curves for single determinations

Individual samples of 100 μg/ml of each standard and isolated compound, were also prepared for cases in which one of these compounds was measured on its own. Only β-sitosterol was determined in serum, but in herbal powders and products, β-sitosterol, campesterol and/or stigmasterol and hypoxoside could be measured. However, β-sitosterolin could not be detected, but it was included in the calibration curves of the composite sample.

4.3   RESULTS AND DISCUSSION

4.3.1   Evaluation of the HPLC method of Emara et al. (1999)

Emara et al (1999)'s HPLC method to determine the "bioavailability of β-sitosterol from *Pygeum africanum* extract in humans", was evaluated by using the same chromatographic parameters that they used, and injecting sterol standards e.g. 100 μg/ml stigmasterol (dissolved in HPLC grade methanol) into the HPLC.

The mobile phase composition was kept isocratic at 96.5 % methanol, but the MWD's wavelength was adjusted, to determine the wavelength of optimal absorption. The chosen wavelength 205 nm of Emara et al (1999) was applied as well as shorter wavelengths 203 nm and 200 nm, and longer wavelengths 208 nm and 210 nm. The large number of compounds that absorbed at 200 and 203 nm, makes it clear why these wavelengths were not used. At 208 nm and 210 nm, the spectra were less crowded, but 205 nm was clearly the best choice – only five compounds in the stigmasterol solution absorbed at 205 nm. The same results were found for β-sitosterol. See Figure 4.1
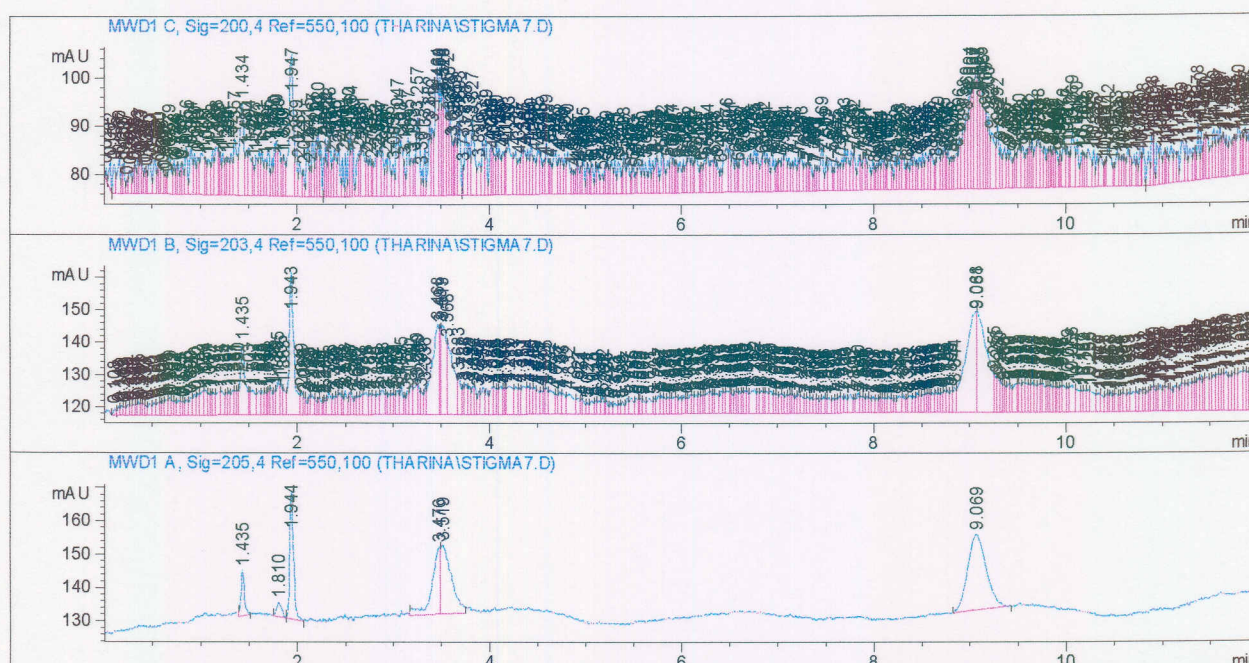
Figure 4.1: Absorbance spectra of 100 μg/ml 95% stigmasterol standard dissolved in HPLC grade methanol and measured with MWD at wavelengths 200.4, 203.4 and 205.4 nm.

To confirm that 205 nm is indeed the wavelength of optimal absorbance, the absorbance spectra of β-sitosterol, campesterol, stigmasterol, β-sitosterolin and hypoxoside were determined, with a diode array detector. The maximum absorption, with minimal interference of other compounds was confirmed to be 205 nm.

The next step was to change the mobile phase composition: A 1:1 mixture of β-sitosterol and campesterol standards, separated well with 96.5% methanol as mobile phase. β-sitosterol had a retention time of 12.139 and campesterol 10.719 minutes. When the mobile phase was changed to 100% methanol, the baselines of the two peaks were barely separated and it was feared that with complex samples the peaks might overlap. The advantage of 100% methanol, however, was shorter retention times. β-sitosterol had a retention time of 6.221 and campesterol 5.702 minutes. A lower percentage methanol (lower than 96.5%) as mobile phase, would result in good resolution, but even longer retention times than c. 12 minutes. Therefore, the mobile phase was kept the same as in the method of Emara et al. (1999): 96.5% methanol in an isocratic system for separation of phytosterols.

4.3.2 Calibration curves

Three types of calibration curves were constituted:

(a) Calibration curves from a composite sample of five standards to simultaneously determine β-sitosterol, campesterol and/or stigmasterol, β-sitosterolin and hypoxoside.

See this chapter 4.2.3.3 (a) for the preparation method and Figure 4.2 and Figure 4.3.

(b) Calibration curves for single determinations. See this chapter, 4.2.3.3 (b).

(c) A calibration curve to determine β-sitosterol in serum. Discussed at a later stage.

Although the retention times varied with temperature and changes in mobile phase composition (as result of evaporation etc.), the retention times of the five standards in the composite sample, with 96.5% methanol as mobile phase, were as follows: β-sitosterol (t_R=10.020), campesterol (t_R=8.933) stigmasterol (t_R=8.933), β-sitosterolin (t_R=5.534) and hypoxoside (t_R=1.195). Note that campesterol and stigmasterol had exactly the same retention time, as a 96.5% mobile phase could not separate them. This means that phytosterol peaks with a retention time of c. 8.933 can either be campesterol, stigmasterol or a combination of the two and quantities were reported as such.
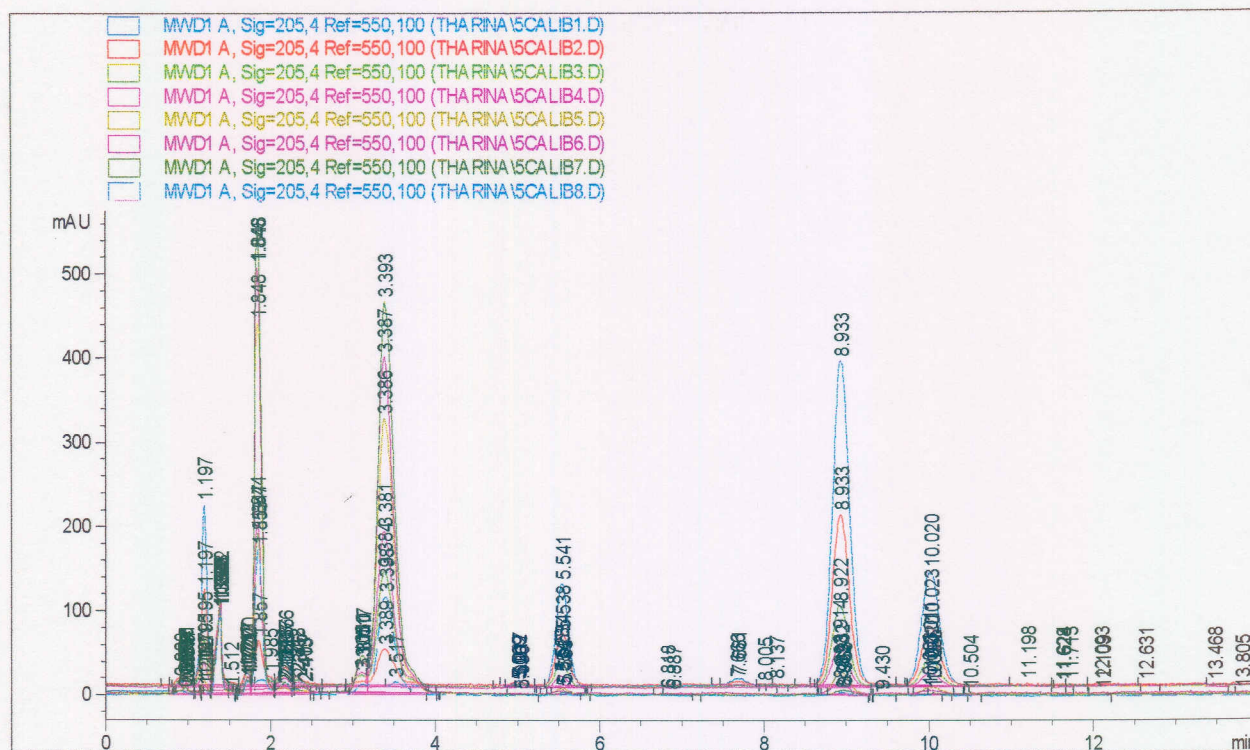
Figure 4.2: Overlayed absorbance spectra of eight different concentrations of 95.7% β-sitosterol (t_R=10.020), 65% campesterol (t_R=8.933) 95% stigmasterol (t_R=8.933), β-sitosterolin (t_R=5.534) and hypoxoside (t_R=1.195) in HPLC grade methanol, measured with MWD at a wavelength of 205.4 nm with 96.5% methanol as mobile phase. These absorbance spectra were used to calculate calibration curves for each compound.

The chromatogram in Figure 4.2 was used to constitute calibration curves for the five compounds. The correlation coefficients were as follows: β-sitosterol (0.9997), campesterol and stimasterol (0.9996), β-sitosterolin (0.9996) and hypoxoside (0.9997).

The MWD's limit of detection for β-sitosterol was c. 2.0 μg/ml with a sample volume of 100 μl.
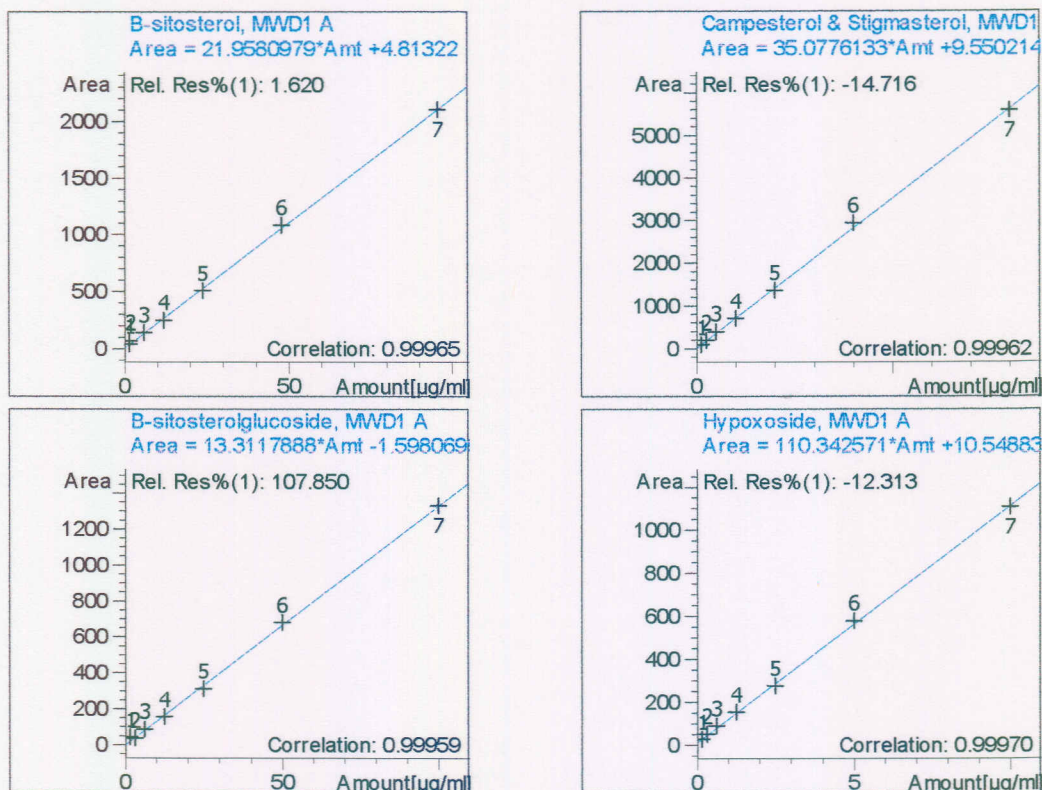
Figure 4.3: Calibration curves of β-sitosterol, campesterol and stigmasterol, β-sitosterolin and hypoxoside, obtained from MWD absorbance spectra, with their correlation coefficients.

As the FLD is much more sensitive and selective than the MWD, the fluorescence spectra of β-sitosterol, campesterol, stigmasterol, β-sitosterolin and hypoxoside were determined. Unfortunately none of the sterols or β-sitosterolin fluoresce, but at an excitation wavelength of 230 nm hypoxoside fluoresces and emits UV light of 345 nm. See Figure 4.4

Hypoxoside's retention time is very short (t_R=1 – 2 min) with 96.5% methanol as mobile phase, and it usually elutes with other polar compounds that overlap with the hypoxoside peak when measured with MWD. However, the number of compounds that fluoresce at 230 nm and emit UV light at 345 nm are few. Thus, FLD was used for sensitive and selective determination of hypoxoside and a new calibration curve for hypoxoside was constituted. This new calibration curve from fluorescence spectra had a correlation coefficient of 0.9996.
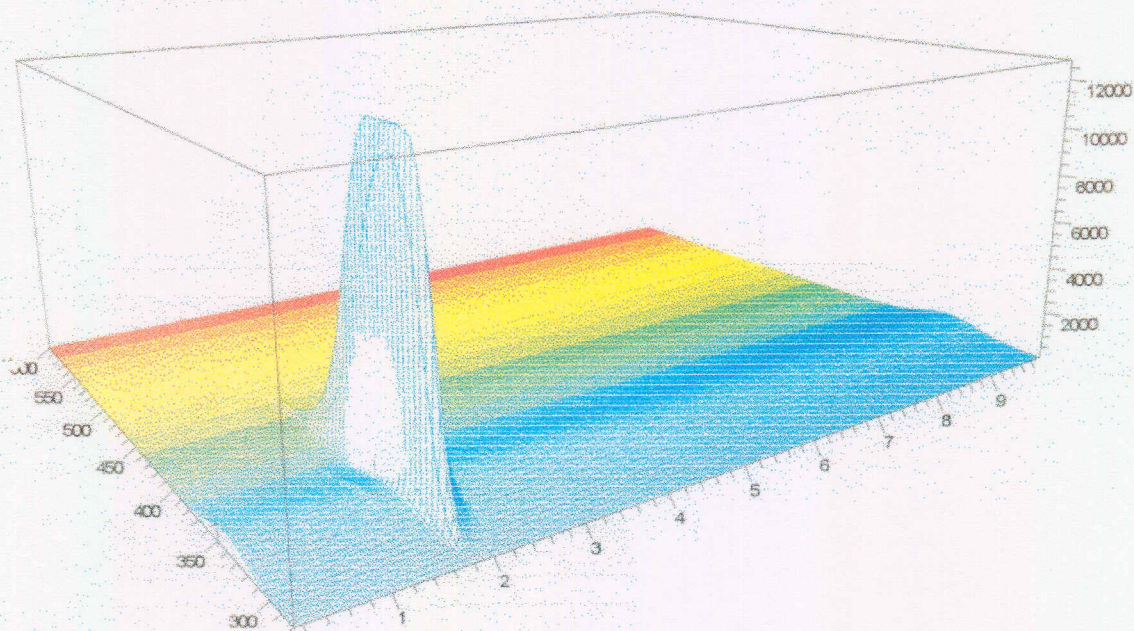
Figure 4.4: Three dimensional fluorescence spectra of hypoxoside. At an excitation wavelength of 230 nm, hypoxoside emits UV light of 345 nm.

4.3.3 Solid phase extraction (SPE)

To clean up the crude methanol extract for hypoxoside analysis, the developed SPE method was applied. See the SPE method in 4.2.3.1 (b).

After SPE, the sample was analysed for hypoxoside and β-sitosterol with HPLC. Chloroform extraction of phytosterols is much easier than methanol extraction followed by SPE; but it is not necessarily more effective. See this chapter, 4.3.4. Three fractions of 50% water-methanol (10 ml +5 ml + 5 ml), one fraction of acetone (10 ml) and three fractions of hexane (10 ml + 5 ml + 5 ml) were eluted through the C_{18} cartridge, to ascertain that all the hypoxoside and β-sitosterol were removed from the cartridge. The water-methanol fractions were supposed to extract the hypoxoside, the acetone should mediate the mixing of the water-methanol and hexane, and the hexane should extract the β-sitosterol. See Figure 4.5 for TLC of the different eluants tested in the development of the SPE process to isolate hypoxoside and β-sitosterol from *H. hemerocallidea* methanol extract.

1   2   3   4   5   6   7   8   9   10
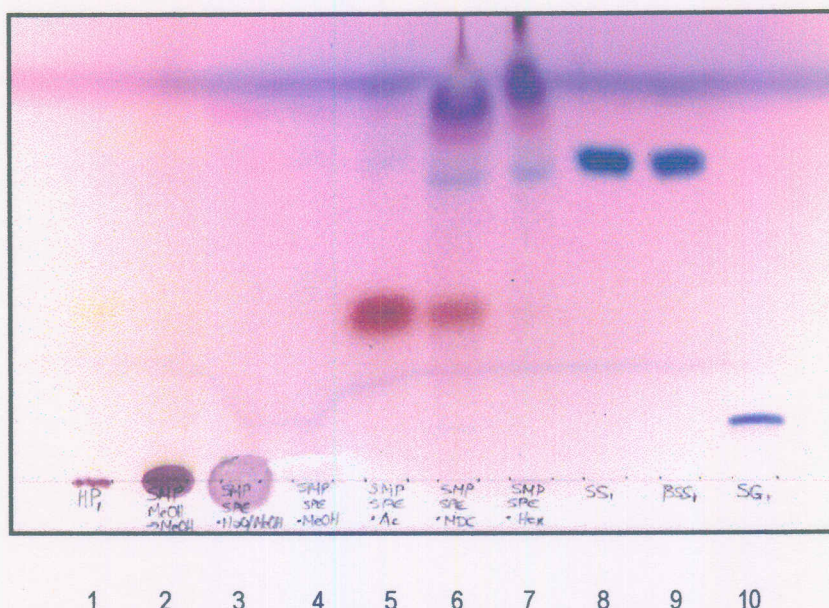
Figure 4.5: TLC on a glass plate of different SPE eluants tested in the development of a SPE process to isolate hypoxoside and β-sitosterol from *H. hemerocallidea* methanol extract. From left to right: Isolated hypoxoside (1), 10 mg/ml African potato 1 methanol extract, placed on the SPE C_{18} cartridge (2) and the following eluants in the order as a applied to the SPE cartridge: 50% water-methanol (3), 100% methanol (4), acetone (5), methylene dichloride (6) and hexane (7). On the right are 95% stigmasterol standard (8), 97.5% β-sitosterol standard (9) and isolated β-sitosterolin (10). The plate was developed in CEF and sprayed with *p*-anisaldehyde.

4.3.3.1 Detection of a contaminant in analytical grade solvents

To determine how complete the extraction process was from the SPE C_{18} cartridge, 2 ml of a standard mixture containing 25 μg/ml β-sitosterol and 25 μg/ml hypoxoside in 50% water-methanol, was applied to a conditioned cartridge. The method as described in 4.2.3.1 (b) was applied and the following results obtained from HPLC with FLD and MWD respectively. See Table 4.1.

According to the results from 50 μg hypoxoside, 41.04 μg (82.12%) was extracted; and from 50 μg β-sitosterol, 155.6 μg (311.2%) was extracted. As this is clearly impossible, the possibility of a contaminant was investigated.

Table 4.1: HPLC quantities of hypoxoside and β-sitosterol from a standard mixture containing 50 μg of both, applied to a C_{18} SPE cartridge

SPE Fraction	Hypoxoside (μg/ml)	β-sitosterol (μg/ml)
Sample eluate	14.3	3.1
50% Water-methanol 1	25.5	0
50% Water-methanol 2	0.45	0
50% Water-methanol 3	0.22	0
Acetone	0.27	112.8
Hexane 1	0.13	21
Hexane 2	0.17	9.3
Hexane 3	0	9.4
Total	**41.04**	**155.6**

To determine whether the contaminant was in the SPE cartridge or in the eluants, 10 ml of all the solvents used (water, methanol, acetone and hexane) were dried and redissolved in 1.0 ml HPLC grade methanol and injected into the HPLC to scan for a contaminant. Some of the solvents presented a peak at the retention time of β-sitosterol.

Table 4.2: HPLC quantities per 10 ml (calibrated according to β-sitosterol's calibration curve) analytical grade solvent of a contaminant with exactly the same retention time as β-sitosterol.

Solvent	"β-sitosterol" contents (μg/ml)
Water	0
Methanol	3.21
Acetone	68.19
Hexane	19.15

When the dried acetone, dissolved in HPLC grade methanol, was spiked with β-sitosterol, it gave a symmetric peak on the HPLC. The contaminant had exactly the same retention time as β-sitosterol! See Figure 4.6.
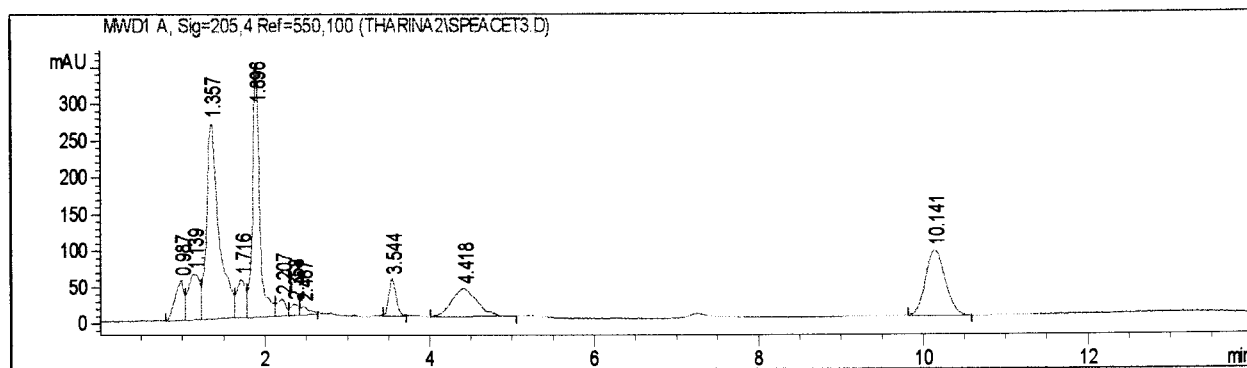
Figure 4.6: HPLC chromatogram of analytical grade acetone, stored in a plastic bottle (dried and redissolved in HPLC grade methanol – 10 mg/ml) with a contaminant with exactly the same retention time as β-sitosterol (t_R = 10.141) and calibrated as 68.19 μg/ml β-sitosterol. The mobile phase was 96.5% methanol.

All contaminated eluants were analytical grade solvents stored in plastic bottles. Further investigation found that the plasticizer (phthalic acid), dissolved in HPLC methanol and injected into the HPLC had a retention time of 10.282. It was, therefore, calibrated by the HPLC software as β-sitosterol.

All the HPLC grade and analytical grade solvents were evaluated for the contaminant and impure solvents were avoided in future work.

A standard mixture of 25 μg/ml β-sitosterol and 25 μg/ml hypoxoside in 50% water-methanol, was put through the SPE process again. However, this time pure solvents were used. The yield was found to be 100.3 % for hypoxoside and 107.7% for β-sitosterol. From the results it was clear that the second hexane fraction contained so little β-sitosterol that only one fraction was needed for elution. On the other hand, two 10 ml fractions water-methanol were necessary to elute hypoxoside. Acetone was not needed at all. The SPE method in 4.2.3.1 (b) was modified accordingly – *Modified SPE method.*

After a number of *H. hemerocallidea* powder SPE analyses with the same pattern, it was accepted that hypoxoside elutes in the second water-methanol fraction although this was not the case with the isolated hypoxoside.

4.3.4   Methanol versus chloroform extraction of phytosterols

As methanol and chloroform both extract phytosterols, it was important to quantitatively confirm the conclusion from TLC in Chapter 3, 3.3.1.3, that chloroform is the best extractant for phytosterols.

The methanol extract had to be cleaned with SPE. The standard mixture with uncontaminated solvents (See section 4.3.3) gave a yield of 107.7% for β-sitosterol, which indicates complete extraction from the C_{18} cartridge. Therefore, the HPLC quantities from the SPE sample could directly be compared with those from the simple chloroform extraction.

A 10 mg/ml methanol extract (2 ml x 10 mg/ml = 20 mg), extracted from 500 mg of *H. hemerocallidea* powder (African potato 1), was placed through the *modified SPE process* as described in 4.2.3.1 (b). The hexane fraction was dried, redissolved in 1.0 ml HPLC grade methanol and injected into the HPLC:

Dry mass applied to SPE = 20 mg.

Hexane fraction, dried and redissolved in 1.0 ml MeOH = 10.83 μg/ml β-sitosterol.

Thus, 20 mg extract = 10.83 μg β-sitosterol.

But, total volume of 10 mg/ml extract = 11.5 ml and that is 115 mg dry mass extract.

Thus, 115 mg extract = 62.3 μg β-sitosterol.

$$\frac{0.0623 \text{ mg}}{500 \text{ mg}} \times 100 = \textbf{0.0125\%} \ \beta\text{-sitosterol in } H. \text{ hemerocallidea powder with MeOH}$$

A chloroform extract of *H. hemerocallidea* powder, dried and redissolved in HPLC grade methanol (10 mg/ml) was also injected into HPLC:

10 mg/ml chloroform extract = 187.5 μg/ml β-sitosterol

187.5 μg β-sitosterol per 1.0 ml chloroform extract

But, total volume of 10 mg/ml extract = 0.13 ml

Thus, 0.13 ml = 24.4 μg β-sitosterol

$$\frac{0.0244 \text{ mg}}{500 \text{ mg}} \times 100 = \textbf{0.0049\%} \ \beta\text{-sitosterol in } H.hemerocallidea \text{ powder with } CHCl_3$$

4.3.5 HPLC Application

Chloroform extracts of *H. hemerocallidea, P. africana, S. repens* and related phytosterol containing products were analysed with HPLC. The mass of the raw material, dry mass extracted with chloroform and HPLC quantities were taken into consideration to calculate the percentage β-sitosterol, campesterol and/or stigmasterol in the particular raw material. Chloroform, however, does not extract all the phytosterols present in the plant material/product. Methanol extracts more (but not necessarily all) phytosterols per gram material, but time consuming SPE is necessary. See section 4.3.4. Therefore, the calculated percentages are not absolute and were used for comparison purposes only.

4.3.5.1 Phytosterol analysis of different plants

4.3.5.1 (a) *H. hemerocallidea*

H. hemerocallidea powder from four different sources was extracted with chloroform (5 ml), dried, redissolved in HPLC methanol and analysed with HPLC. The percentage β-sitosterol was calculated from 500 mg *H. hemerocallidea* powder for African potato 1 and 4, 597 mg for African potato 5 (African potato 5 contains 627 mg *H. hemerocallidea* per 630 mg capsule, but 600 mg powder was used thus 597 mg *H. hemerocallidea*), and 326 mg for African potato 6 (African potato 6 contains 300 mg *H. hemerocallidea* per 460 mg capsule, but 500 mg powder was used; thus 326 mg *H. hemerocallidea*).

Table 4.3: β-Sitosterol contents of *H. hemerocallidea* powder from four different sources.

H. hemerocallidea	[Extract] (mg/ml)	β-sitosterol (μg/ml)	Extract Volume (ml)	μg βSS extracted	Percentage βSS in plant
African potato 1	10	187.5	0.13	24.4	0.00488 %
African potato 4	10	118.9	0.16	19.02	0.00380 %
African potato 5	10	144.1	0.16	23.06	0.00386 %
African potato 6	10	158.7	0.12	19.04	0.00584 %

African potato 6 was the only *H. hemerocallidea* powder to contain 0.0014% campesterol and/or stigmasterol. See Figure 4.7. Hypoxoside was not measured as chloroform extraction was performed and hypoxoside is not extracted by chloroform.
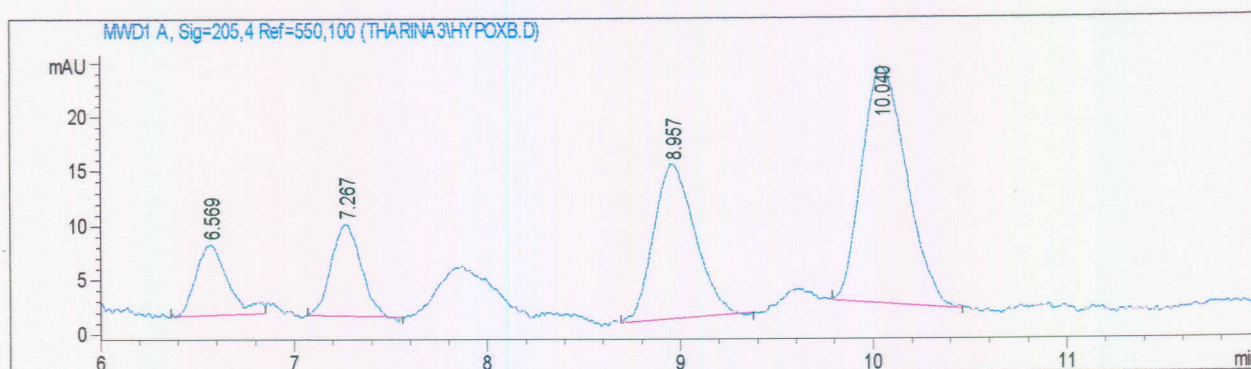
Figure 4.7: HPLC chromatogram of a chloroform extract of *H. hemerocallidea* powder from PLANTANICAL MEDICINE (African potato 6), which contains campesterol and/or stigmasterol (t_R=8.957) and β-sitosterol (t_R= 10.040). The mobile phase was 96.5% methanol.

4.3.5.1 (b) *P. africana*

P. africana bark extract (5:1), bark powder and leaf powder (500 mg of each) was extracted with chloroform (5 ml)), dried, dissolved in HPLC methanol and analysed with HPLC.

Table 4.3: β-Sitosterol (βSS) contents of *P. africana* bark extract (5:1), bark powder and leaf powder.

P. africana	[Extract] (mg/ml)	β-sitosterol (µg/ml)	Extract Volume (ml)	µg βSS from 500mg	Percentage βSS in plant
Bark extract (5:1)	1	401.14	28	11 231.9	**2.25 %**
Bark powder	10	239.71	0.49	117.5	**0.023 %**
Leaf powder	10	72.86	1.51	110.0	**0.022 %**

Table 4.4: Campesterol (CS) and/or stigmasterol (SS) contents of *P. africana* bark extract (5:1), bark powder and leaf powder.

P. africana	[Extract] (mg/ml)	Campe- &/or Stigmasterol (µg/ml)	Extract Volume (ml)	µg CS &/or SS from 500 mg	Percentage CS &/or SS in plant
Bark extract (5:1)	1	386.08	28	10 810.2	**2.16 %**
Bark powder	10	50.08	0.49	24.5	**0.0049 %**
Leaf powder	10	0	0	0	0

P. africana leaf powder apparently does not contain any campesterol and/or stigmasterol, but the β-sitosterol content is very similar to that of the bark powder. See Figure 4.8.
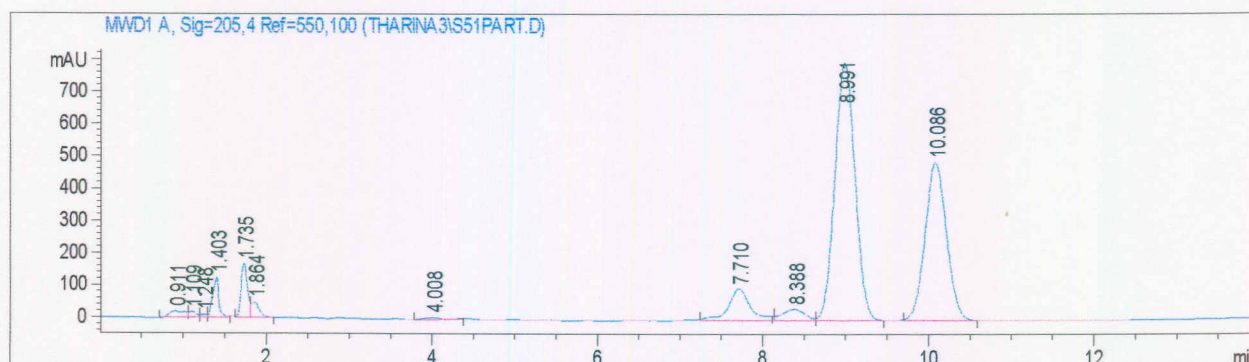
Figure 4.8: HPLC chromatogram of a chloroform extract of *P. africana* bark extract (5:1) with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=8.991) and β-sitosterol (t_R= 10.086).

4.3.5.1 (c) *S. repens*

S. repens berry extract (4:1) and powder (500 mg of each), was extracted with chloroform (5 ml), dried, redissolved in HPLC methanol and analysed with HPLC.

Table 4.5: β-Sitosterol (βSS) contents of *S. repens* berry extract (4:1) and powder.

S. repens	[Extract] (mg/ml)	β-sitosterol (µg/ml)	Extract Volume (ml)	µg βSS from 500mg	Percentage βSS in plant
Berry extract (4:1)	1	84.68	0.4	33.9	0.0068 %
Berry powder	3.75	18.6	0.08	1.49	0.00030 %

Table 4.6: Campesterol (CS) and/or stigmasterol (SS) contents of *S. repens* berry extract (4:1) and powder.

P. africana	[Extract] (mg/ml)	Campe- &/or Stigmasterol (µg/ml)	Extract Volume (ml)	µg CS &/or SS from 500 mg	Percentage CS &/or SS in plant
Berry extract (4:1)	1	76.09	0.4	30.4	0.0061 %
Berry powder	3.75	10.3	0.08	0.82	0.00016 %
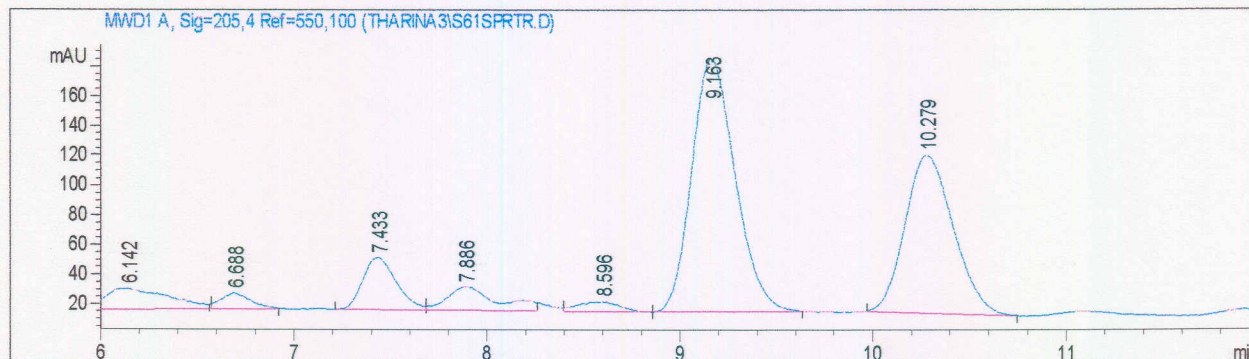
Figure 4.9: HPLC chromatogram of a chloroform extract of *S. repens* berry extract (4:1) with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=9.163) and β-sitosterol (t_R= 10.279).

4.3.5.2 Phytosterol analysis of different products

4.3.5.2 (a) Immunochoice®

Immunochoice®; label claim - 20 mg plant sterols and sterolins and 50 mg absorption enzymes per capsule, but only 7.4 mg phytosterols could be determined.

Table 4.7: β-Sitosterol (βSS), campesterol (CS) and/or stigmasterol (SS) contents of Immunochoice®.

[Extract] (mg/ml)	βSS (μg/ml)	CS &/or SS (μg/ml)	Extract Volume (ml)	βSS + CS &/or SS (μg)	Percentage of 20 mg
1	524.96	217.92	10	7428.9	37.14 %
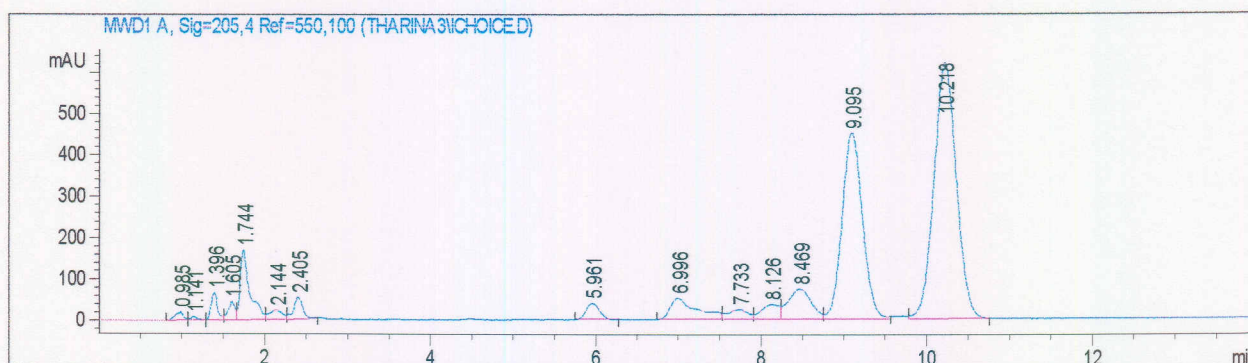
Figure 4.10: HPLC chromatogram of a chloroform extract of Immunochoice® with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=9.095) and β-sitosterol (t_R= 10.218).

4.3.5.2 (b) Harzol®

Harzol®; label claim - 10 mg β-sitosterol and 0.1 mg β-sitosterolin per capsule, but 6.7 mg phytosterols could be determined. It is the German equivalent of Moducare® and was originally associated with *H. hemerocallidea*. It is important to note that the term β-sitosterol on a product represents a defined extract of phytosterols with β-sitosterol as the main component, therefore, even though Harzol®'s container states that every capsule contains 10 mg β-sitosterol and 0.1 mg β-sitosterolin, it in fact contains mainly β-sitosterol and smaller amounts of campesterol, stigmasterol and other sterols along with their glucosides (Berges et al., 1995).

Table 4.8: β-Sitosterol (βSS), campesterol (CS) and/or stigmasterol (SS) contents of Harzol®.

[Extract] (mg/ml)	βSS (µg/ml)	CS &/or SS (µg/ml)	Extract Volume (ml)	βSS + CS &/or SS (µg)	Percentage of 10 mg
0.13	62.5	4.1	100	6660	66.6 %
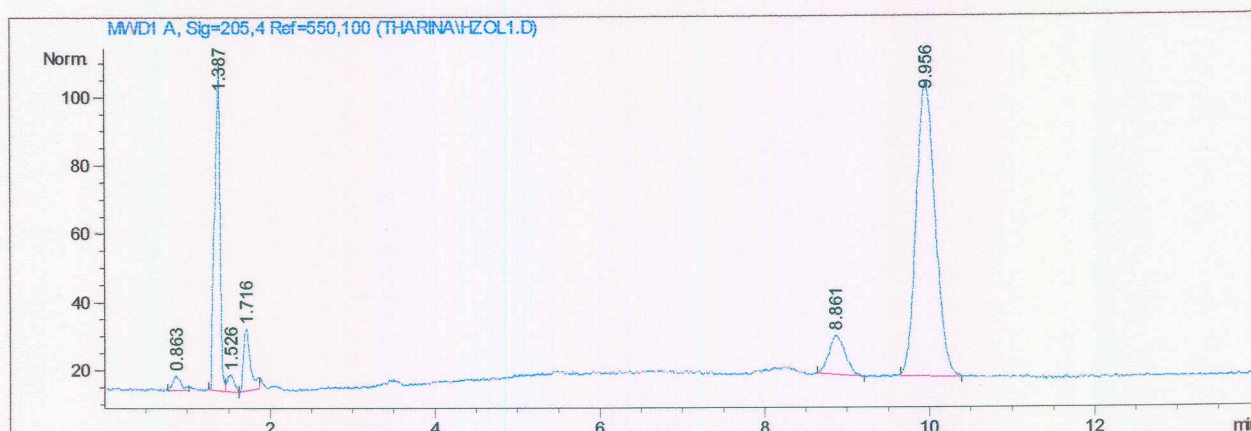
Figure 4.11: HPLC chromatogram of a chloroform extract of Harzol® with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=8.861) and β-sitosterol (t_R= 9.958).

4.3.5.2 (c) Moducare®

Moducare®; label claim - 20 mg β-sitosterol and 0.2 mg β-sitosterolin per capsule, but 12.4 mg phytosterols could be determined. It was originally associated with *H. hemerocallidea*.

Table 4.9: β-Sitosterol (βSS), campesterol (CS) and/or stigmasterol (SS) contents of Moducare®.

[Extract] (mg/ml)	βSS (µg/ml)	CS &/or SS (µg/ml)	Extract Volume (ml)	βSS + CS &/or SS (µg)	Percentage of 20 mg
1	529.88	355.42	14	12 394.2	61.97 %
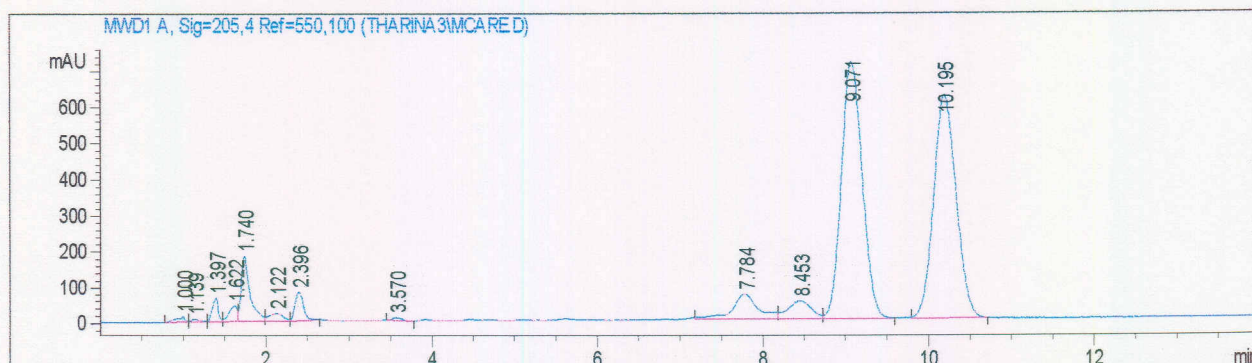
Figure 4.12: HPLC chromatogram of a chloroform extract of Moducare® with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=9.071) and β-sitosterol (t_R=10.195).

4.3.5.2 (d) Nutricare®

Nutricare®; label claim - contains 400 mg freeze-dried enzymes and sterol compound per capsule.

Table 4.10: β-Sitosterol (βSS), campesterol (CS) and/or stigmasterol (SS) contents of Nutricare®.

[Extract] (mg/ml)	βSS (μg/ml)	CS &/or SS (μg/ml)	Extract Volume (ml)	βSS + CS &/or SS (μg)	Percentage of 400 mg
10	192.10	-	2.7	518.67	0.13 %

Note that there is uncertainty regarding the β-sitosterol peak in Nutricare® and spiking with β-sitosterol did not resolve the question. See Chapter 5, 5.4. The same problem was experienced with campesterol and/or stigmasterol and values were, therefore, not calculated from the HPLC data.

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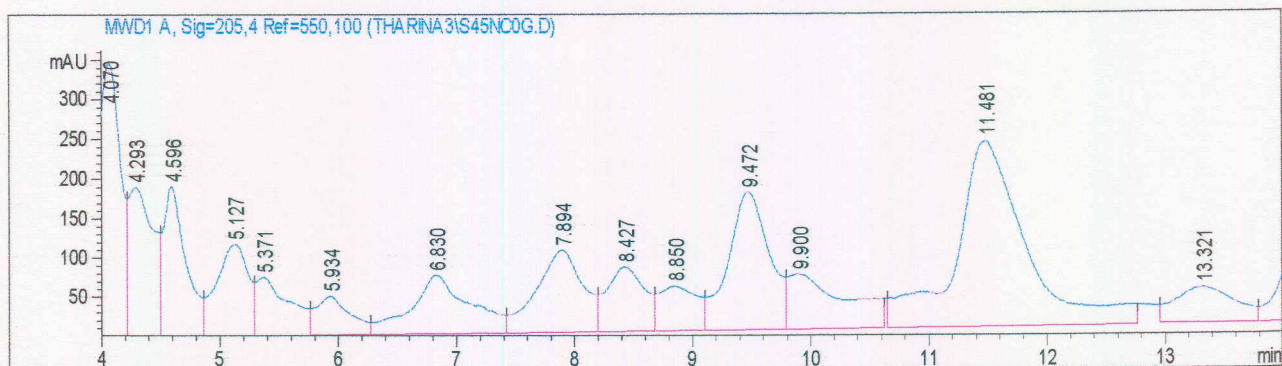
Figure 4.13: HPLC chromatogram of a chloroform extract of Nutricare® with 96.5% methanol as mobile phase. The peak at t_R=9.472 minutes was assumed to be β-sitosterol, although there is some uncertainty.

4.3.5.2 (e) Phytogard®

Phytogard®; label claim - 10 mg glutathione, 75 mg food state selenium and 60 mg β-sitosterol per capsule, but 31.0 mg phytosterols could be determined.

Table 4.11: β-Sitosterol (βSS), campesterol (CS) and/or stigmasterol (SS) contents of Phytogard®.

[Extract] (mg/ml)	βSS (μg/ml)	CS &/or SS (μg/ml)	Extract Volume (ml)	βSS + CS &/or SS (μg)	Percentage of 60mg
1	738.16	61.86	38.8	31 040.4	51.7 %
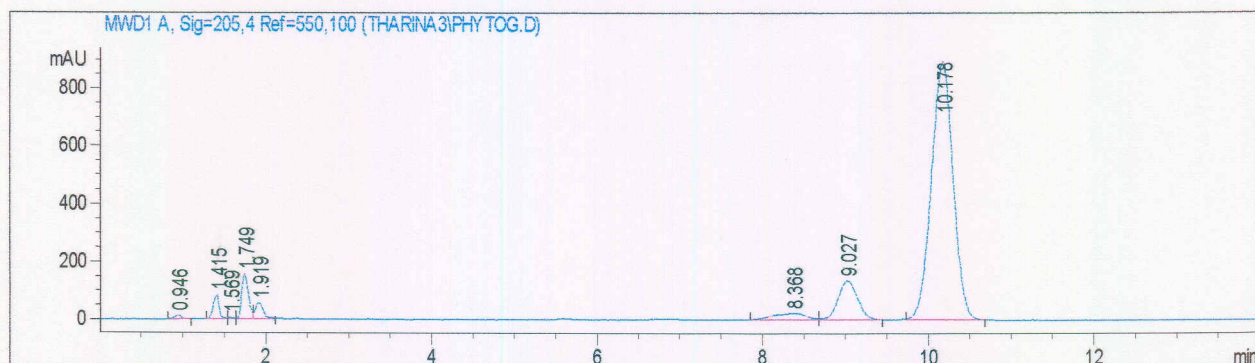
Figure 4.14: HPLC chromatogram of a chloroform extract of Phytogard® with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=9.027) and β-sitosterol (t_R=10.178).

4.3.5.2 (f) Prostol Herbal®

Prostol Herbal®; label claim - 200 mg of of *S. repens* powder, 200 mg of *P. africana* extract (5:1) and 100 mg pumpkin seed extract (4:1) per capsule.

Table 4.12: β-Sitosterol (βSS), campesterol (CS) and/or stigmasterol (SS) contents of Prostol®.

| [Extract] (mg/ml)	βSS (μg/ml)	CS &/or SS (μg/ml)	Extract Volume (ml)	βSS + CS &/or SS (μg)	Percentage of 500mg
1	267.8	262.4	17.5	9 278.5	1.86 %
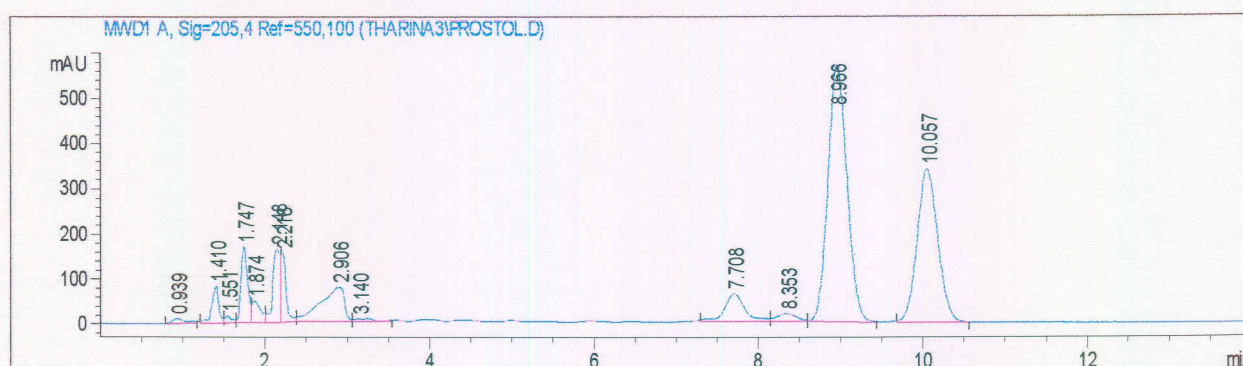
Figure 4.15: HPLC chromatogram of a chloroform extract of Prostol Herbal® with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=8.966) and β-sitosterol (t_R=10.057).

4.3.6   Water extraction of *H. hemerocallidea* powder

In Chapter 3, 3.3.4.7, according to TLC, water extracts hypoxoside and the *red spot compound* from *H. hemerocallidea* powder, but not phytosterols or β-sitosterolin, therefore, the therapeutic effect of African potato 1 tea was ascribed possibly to hypoxoside or the *red spot compound*, or other unknown compounds.

To confirm the TLC conclusion, *H. hemerocallidea* (African potato 1) powder was extracted with water and simmered for 20 minutes as described in 4.2.3.1 (c). The tea was dried, redissolved in methanol (17.2 mg/ml) and injected into HPLC. The MWD did not detect β-sitosterol, nor did the FLD detect hypoxoside in the water extract. When a methanol extract is injected into the HPLC, neither

hypoxoside nor phytosterols is measured. See this Chapter, 4.2.3.1 (b). Therefore, it was decided to do SPE on the water extract of *H. hemerocallidea* powder.

In this experiment the tea was dried and redissolved in 50% water-methanol for a 10 mg/ml concentration. A 2.0 ml volume (20 mg) was applied to the C_{18} cartridge and the *modified SPE method* was used. See 4.2.3.1 (b) (i). The sample elute and water-methanol fraction 1 did not contain hypoxoside, but the second water-methanol fraction contained 29.006 µg/ml hypoxoside:

Mass of grated corms extracted = 1.28 g with 240 ml distilled water.

2 ml 10 mg/ml Tea applied to SPE = 20 mg

Dry mass applied to SPE = 20 mg.

Water-methanol fraction 2, dried and redissolved in 1ml MeOH = 29 µg/ml hypoxoside.

Thus, 20 mg extract = 29 µg hypoxoside.

But, total volume of 10 mg/ml extract = 56.9 ml and that is 569 mg dry mass extract.

Thus, 569 mg extract = 825.05 µg hypoxoside.

$$\frac{0.825 \text{ mg} \times 100}{1280 \text{ mg}} = \textbf{0.064\%} \text{ hypoxoside extracted from } \textit{H. hemerocallidea} \text{ with water.}$$

None of the fractions contained β-sitosterol, and β-sitosterolin was also not detected.

4.4   CONCLUSION

The HPLC method of Emara et al (1999) to determine β-sitosterol in serum, could also be applied to determine β-sitosterol in plant material and products. At a wavelength of 205 nm and 96.5% methanol as mobile phase(isocratic system), the conditions were optimal to quantify β-sitosterol, but the detection limit of the MWD was 2 μg/ml. As none of the phytosterols or β-sitosterolin fluoresce, the more sensitive FLD could not be used.

Hypoxoside, on the other hand, does fluoresce and the FLD was used at an excitation wavelength of 230 nm and an emission wavelength of 345 nm. Hypoxoside is usually analysed with UV spectroscopy at 294 nm (Koch & Brosch-Starzengruber, 1991) or with HPLC with a UV detector at 260 nm (Koch & Brosch-Starzengruber, 1991; Kruger et al., 1994; Albrecht et al., 1995). None of the published methods on hypoxoside quantification used fluorescence detection.

A review article of Nicoletti et al. (1992) referred to the methanolic extracts of *Hypoxis* rhizomes that are usually complex mixtures of glycosides, whose separation is very difficult to achieve by column chromatography. The use of countercurrent distribution (Craig-Post apparatus), with the bi-phase solvent system n-BuOH/EtOAc/H2O in suitable compositions, was suggested.
In our study an SPE method was developed and applied to clean up the crude methanol extract for hypoxoside analysis. β-sitosterol was determined simultaneously.

The mobile phase of 96.5% methanol (isocratic system) could not separate campesterol ($C_{28}H_{48}O$; mol wt 400.66 g/mol) and stigmasterol ($C_{29}H_{48}O$; 412.67 g/mol) as they had exactly the same retention time (t_R= 8.9 minutes). Their chemical structures (See Chapter 1, Figure 1.1) are very similar to β-sitosterol ($C_{29}H_{50}O$; mol wt 414.69 g/mol).

Masohan & Bhatia (1996) analysed bio-crudes for steroids and terpenoids. They separated steroids by using reverse phase HPLC with a C_{18} column, 4% isopropanol mixed with acetonitrile as the mobile phase and an ultraviolet detector set at 210 nm. They managed to separate campesterol and stigmasterol, but the sterols had very long retention times. Stigmasterol had a retention time of 67.4 minutes, cholesterol (t_R=70.0), campesterol (t_R=78.4) and β-sitosterol (t_R=93.6). They concluded that steroids have a regular elution pattern according to the polarity and position of the functional groups attached.

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In the study performed here, the main focus was on the analysis of β-sitosterol as major phytosterol. If in future it is required to focus on other phytosterols e.g. campesterol or stigmasterol, it might be considered to change the mobile phase e.g. to 4% isopropanol mixed with acetonitrile, but that would mean very long retention times and routine analyses would be time consuming.

Although β-sitosterolin was included in the calibration curve for multiple determinations and the retention time was determined, it was never detected in any of the samples analysed with the HPLC method of Emara et al. (1999). This is in contrast with the developed TLC method with which β-sitosterolin could be detected. See Chapter 3, Figure 3.2 for β-sitosterolin (R_f = 0.16) on TLC. The method of Emara et al. (1999) is probably not sensitive enough to detect the low quantities of β-sitosterolin present in plants.

Before the standard mixture of 25 μg/ml β-sitosterol and hypoxoside was subjected to SPE to evaluate the process, strange results were obtained. Usually during any extraction process, there is a decrease with a constant factor of the mass extracted, from the first to the last fraction. This is usually an indication of the completeness of extraction. However, in the SPE results obtained, the second and the third fraction often extracted the same quantity. See Table 4.1, hexane fraction 2 and 3. This was an indication of the contaminant.

The importance of detecting method errors was experienced first-hand. Analyses of standard samples to evaluate new methods and blank determinations (e.g. collecting the saturation solvent of the C_{18} SPE cartridge and analysing it with HPLC) to control existing methods are essential. This was demonstrated by detecting the contaminant in the standard mixture. This contaminant, found in the analytical grade solvents, may have been the plasticizer, phthalic acid or one of its derivatives.

Methanol extracts more β-sitosterol per gram *H. hemerocallidea* powder than chloroform, but the analysis involves SPE which is time consuming. However, chloroform is a more selective extractant for phytosterols and after drying the extract and redissolving it in methanol, it can be directly injected into HPLC. Therefore, for comparative purposes e.g. product comparison or stability testing, simple extraction with chloroform was applied.

The HPLC quantities obtained (after chloroform extraction or methanol SPE) should always be interpreted with regards to the method applied, and should not be seen as the absolute quantities in

the plant material or product. However, if the same method is applied for analyses of different plant material or different products, they can be compared.

The HPLC results after chloroform extraction of *H. hemerocallidea* from 4 sources indicated that African potato 6 from PLANTANICAL MEDICINE had the highest content of β-sitosterol.

The *P. africana* bark and leaf powder analysed, were similar in their contents of β-sitosterol, but the leaf powder sample analysed, did not contain campesterol &/or stigmasterol. The bark and leaves were not from the same tree, and a number of samples must be analysed for a general conclusion. However, it is important to investigate whether *P. africana* leaves are a good source of β-sitosterol, and whether the leaves cannot perhaps replace the bark in prostate remedies and products. *P. africana* is on the list of endangered species, and a less destructive harvesting method (e.g. leaf collection) might prevent it from becoming extinct.

The β-sitosterol contents of herbal extracts appeared higher than expected, when compared to the herb powders e.g. *S. repens* berry extract (4:1) contained 22.8 times more β-sitosterol than the berry powder, and not only 4 times more as expected from a (4:1) extract. In the case of *S. repens* the (4:1) extract and powder were from the same source. It is possible that the extraction method, used by the company producing the extract, was relatively selective for phytosterols.

The TLC results of water extraction of *H. hemerocallidea* (See Chapter 3, 3.3.4.6) were confirmed with HPLC. The water extract contains hypoxoside, but not phytosterols or β-sitosterolin. Unfortunately the isolated *red spot compound* decomposed (See Chapter 6, 6.4) before its retention time (t_R) on HPLC could be determined and, therefore, there can be no comment on its presence or absence in the water extract with HPLC.

The number of positive reports of the therapeutic effects of the African potato tea, received by distributors of the corms, makes the placebo effect as only mechanism unlikely. The anti-cancer effects of hypoxoside, were discussed in Chapter 1 (See section 1.4.1.3), but it is also possible that other compounds extracted by the tea (even in minute quantities) may have a therapeutic action, perhaps even the *red spot compound*.

CHAPTER 5

APPLICATION OF TLC AND HPLC FOR STABILITY AND BIOAVAILABILITY

5.1. INTRODUCTION

5.1.1 Industrial application of TLC and HPLC

From chlorophyll to plant glandular hairs (Von Zahn & Rheinholz, 1980), plants were important in the development of thin layer chromatography and although an old method, it is one of the most commonly used techniques in natural product research (Houghton & Raman, 1998). In the natural pharmaceutical industry, it is used as a quick, reproducable and cost-effective analysis method to qualitatively identify and approve plant material by comparison with a standard sample. Differences in important band intensities can indicate low/high levels of the active compounds and, therefore, low/high activity, as well as whether more quantitative testing is needed. In isolating compounds with column chromatography (See Chapter 6), TLC can be used to determine which fractions contain the active compounds and the purity thereof. It can also be used to determine optimal extraction by comparing the product of different extractants (personal experience).

TLC is applied in the pharmaceutical industry; clinical chemistry; forensic chemistry; biochemistry; cosmetology; food analysis; environmental analysis; analysis of inorganic substances *etc.* Currently, the majority of TLC users work in the area of pharmaceutical investigation. In the pharmaceutical industry, TLC is used for the identification, purity assays and determination of the concentration of active ingredients, auxiliary substances and preservatives in drugs and drug preparations (Hahn-Deinstrop, 2000).

However, HPLC is currently the most widely used of all separation techniques, with annual equipment sales approaching the billion dollar mark, because of its versatility and wide applicability (Skoog et al., 1996).

The five most widely used types of HPLC are partition, adsorption, ion-exchange and two types of size-exclusion chromatography (gel-permeation chromatography and gel-filtration chromatography). High performance partition chromatography has typical applications in analysis of pharmaceuticals,

biochemicals, food products, industrial chemicals, pollutants, forensic chemistry and clinical medicine (Skoog et al., 1996) and was also the technique applied in this study

5.1.2 Aim of chapter

In this chapter TLC and HPLC will be compared for industrial and clinical applications with respect to phytosterols. Little data is available on the shelf-life of sterols and their stability against gamma irradiation which is used to prevent microbial contamination of raw material and products. Therefore samples, subjected to accelerated stability testing (storage at 40 °C for up to 12 months) and different levels of gamma irradiation, are analysed.

Information on the bioavailability of β-sitosterol from certain products is known, but no bio-equivalence study on the bioavailability of β-sitosterol from South African products has been published and some companies claim better bioavailability of their products due to added enzymes and specialised growth and harvesting methods. A bio-equivalence study to compare the serum β-sitosterol levels reached with four South African and three European sterol containing products, was planned and ethical authorisation was obtained. The bio-equivalence study will only be performed once the sensitivity and efficacy of the HPLC method has been established.

5.2 MATERIAL AND METHODS

5.2.1 Material

5.2.1 (a) Plant material

Plant material, products and standards as described in Chapter 2, 2.1 and 2.2 were used.

5.2.1 (b) Human serum

Volunteers were recruited from the Phytopharmacology Laboratory. The volunteers gave their written consent to take part in the pilot study. Blood was drawn from the brachial vein of the volunteers after 12 hours of fasting. The blood samples were centrifuged and the serum decanted into test tubes. The serum was immediately frozen at -20°C until assayed. See detail of ethical approval and serum preparation in 5.2.3.8 and 5.2.3.9.

5.2.2 Apparatus

5.2.2.1 SPE-equipment

See Chapter 4, 4.2.2.1

5.2.2.2 HPLC-equipment

See Chapter 4, 4.2.2.2 for equipment specifications and 4.2.3.3 for chromatographic parameters.

5.2.3 Methods

5.2.3.1 Preliminary preparations for stability analysis

5.2.3.1 (a) Accelerated stability testing

H. hemerocallidea powder (African potato 1), *P. africana* bark extract (5:1), *S. repens* berry extract (4:1), Immunochoice®, Moducare® and Nutricare® capsules, were stored at 40 °C and 70% relative humidity in a Labotec Incoterm oven for up to 12 months. A sample of each was kept at room temperature. After 3, 6, 9 and 12 months, samples were removed and frozen at -20 °C until assayed. All the samples were analysed at the same time after 12 months.

5.2.3.1 (b) Stability against gamma irradiation

H. hemerocallidea powder from 2 sources (African potato 1 and 6), *P. africana* bark extract (5:1), *S. repens* berry extract (4:1), Immunochoice®, Moducare® and Nutricare® capsules were exposed to different intensities of irradiation in the order of 4, 13 and 28 kGray. See Table 5.1 for the specified intensities for each sample, as all were not irradiated at the same time.

Table 5.1: Irradiation intensities to which selected samples were exposed.

Product	Irradiation levels in kGray
African potato 1	4.3; 12.8; 28.5
African potato 6	4.3; 12.8; 28.5
S. repens berry extract (4:1)	4.4; 13.8; 27.9
P. africana bark extract (5:1)	4.4; 13.8; 27.9
Moducare®,	4.4; 13.8; 27.9
Immunochoice®	4.4; 13.8; 27.9
Nutricare®	2.3; 4.1; 11.8; 27.9;

5.2.3.2 Preliminary preparation for serum analysis

5.2.3.2 (a) Calibration curve for β-sitosterol in serum

To compensate for variables and substances in serum that could interfere with the quantification of β-sitosterol, a new calibration curve was constituted.

1. A stock solution of 100 μg/ml β-sitosterol in serum was prepared by spiking 10 ml serum with 100 μl 10 mg/ml 95.7% β-sitosterol standard in chloroform and mixing it for 1 minute on a VM-300 vortex mixer.

2. This 100 μg/ml β-sitosterol in serum mixture was serially diluted with serum to give concentrations of 50, 25, 12.5, 6.25, 3.125, 1.56 μg/ml β-sitosterol in serum.

3. The spiked serum series was extracted with chloroform as described in 5.2.3.9 (c) (i). After centrifugation, 3 ml organic phase was removed, dried and redissolved in 0.2 ml (100 μg/ml and 50 μg/ml samples) or 1.0 ml (25, 12.5, 6.25, 3.125, 1.56 μg/ml) methanol.

4. This means that in the case of the 100 μg/ml β-sitosterol in serum sample:

5 ml x 100 μg/ml β-sitosterol = 500 μg

If 100% extracted, then 500 μg in 3.0 ml chloroform dried.

500 μg in dissolved in 0.2 ml HPLC methanol = 2500 μg/ml

and 1250 μg/ml from the 50 μg/ml β-sitosterol in serum sample.

For the 25 μg/ml and other samples: 5 ml x 25 μg/ml β-sitosterol = 125 μg/1.0 ml = 125 μg/ml *etc.*

5. According to the example, theoretically with 100% extraction, the final concentrations of the series should be: 2500, 1250,125, 62.5, 31.25, 15.625 and 7.8 μg/ml β-sitosterol .

6. The samples were injected into the HPLC with MWD, and β-sitosterol was recorded to set up a new serum-derived calibration curve by calibration of peak area.

5.2.3.3 Extraction

5.2.3.3 (a) Simple extraction

Simple extraction with a dense solvent, chloroform [as described in Chapter 3, 3.2.2.1 (b) (i)], was used to prepare plant extracts for TLC and HPLC. The same chloroform extracts were used for TLC and HPLC. However, in HPLC determination of β-sitosterol and hypoxoside in *H. hemerocallidea*, simple extraction with methanol followed by solid phase extraction was performed, while chloroform extraction was applied for TLC.

As discussed in 4.2.3.1(a), 500 mg of the herb powders was extracted with 5 ml chloroform, and in the case of products, the contents of one capsule were extracted with 5 ml chloroform. As capsule weight varies, the powder mass removed from the capsules of a particular product was standardised for that product: PLANTANICAL MEDICINE's Hypoxis – African potato 6 (500 mg), Immunochoice® (300 mg), Moducare® (200 mg) and Nutricare® (400 mg) extracted with 5 ml chloroform. The dried extracts were redissolved in HPLC methanol to yield the desired concentration.

Again to improve the sensitivity of analysis, the herb powders (*H. hemerocallidea* powder - African potato 1 and 6 for TLC) and products containing herb powders (Nutricare®) were prepared as 10 mg/ml concentrations; but to avoid overloading, the herbal extracts (*P. africana* extract and *S. repens* extract) and products containing extracts (Immunochoice® and Moducare®) were prepared as 1 mg/ml concentrations before TLC application and HPLC injection.

The TLC band intensities and HPLC quantities of β-sitosterol, campesterol and/or stigmasterol and hypoxoside reported, are, therefore, only for comparison purposes within a certain product or plant material and do not reflect absolute quantities for inter-product or plant material comparisons. See

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Chapter 4, 4.3.5.1 for comparison of phytosterols extracted from different plants and 4.3.5.2 for comparison of phytosterols extracted from different products.

5.2.3.3 (b) Solid phase extraction

To determine the stability of β-sitosterol and hypoxoside in *H. hemerocallidea* powder (African potato 1), methanol extraction was performed and the two compounds were isolated with the *modified SPE method*. See 4.2.3.1 (b) (i). After a number of *H. hemerocallidea* powder SPE analyses with the same pattern, it was accepted that hypoxoside elutes in the second water/methanol fraction. Therefore, only the second water/methanol fraction (hypoxoside) and hexane fraction (β-sitosterol) were collected, dried, redissolved in methanol and injected into the HPLC.

5.2.3.3 (c) Serum extraction

See this chapter, 5.2.3.9 (c) (i).

5.2.3.4 Thin layer chromatography (TLC)

TLC was performed as described in Chapter 3, 3.2.2.3. As phytosterols were investigated, aluminium plates could be used instead of the more expensive glass plates used for the *red spot compound*. TLC does not separate β-sitosterol, campesterol, stigmasterol *etc*. Only one band, a blue-purple, "phytosterol-complex", is visible. See Chapter 3, 3.3.1.2 (a) (i). The plates were developed in chloroform:ethylacetate:formic acid in the ratios 5:4:1 (CEF), for optimal separation of phytosterols, and in ethylacetate:methanol:water in the ratios 10:1.35:1 (EMW) for hypoxoside examinations. The plates were sprayed with *p*-anisaldehyde and heated in a Gallenkamp Hotbox oven at c. 110 °C until the colour bands were optimally visible.

5.2.3.4 (a) Sensitivity determination of TLC

To determine the detection limit or minimum quantity β-sitosterol visible on TLC, a stock solution of 1 mg/ml (1 μg/μl) 95.7% β-sitosterol in chloroform was prepared and serially diluted to give concentrations of 0.2, 0.1, 0.04 and 0.02 mg/ml (μg/μl). These concentrations were applied (5 or 10 μl) on a TLC plate for a total mass of 10, 5, 1.0, 0.5, 0.2 and 0.1 μg β-sitosterol. The plates were developed in CEF and sprayed with *p*-anisaldehyde as described above.

5.2.3.4 (b) Detectability of β-sitosterol in serum with TLC

Serum spiked with 20 μl 1 mg/ml 95.7% β-sitosterol standard and an unspiked serum sample (control) were extracted with chloroform as described in 5.2.3.9 (c) (i). The samples were applied (10 μl) to a TLC plate with 200 μg/ml 95.7% β-sitosterol and 99+% cholesterol in chloroform, developed in CEF and sprayed with *p*-anisaldehyde as described in above.

5.2.3.5 High performance liquid chromatography (HPLC)

The method used by Emara et al. (1999) for determining the "bioavailability of β-sitosterol from *Pygeum africanum* extract in humans", was applied for quantitative determinations of phytosterols for plant extracts and serum. See Chapter 4, 4.2.3.3.

Note that with the particular mobile phase (isocratic 96.5% methanol) used, the HPLC system could not distinguish between campesterol and stigmasterol as they had exactly the same retention time. Therefore, the quantities reported are either campesterol or stigmasterol or a combination of the two compounds.

5.2.3.6 Calculation of standard deviation

The repeatability of the HPLC method was determined by injecting the same sample 5 times (n = 5) and calculating the standard deviation (s) according to the following equation (Skoog et al., 1996):

$$s = \sqrt{\frac{\sum\limits_{i=1}^{N}(x_i - \bar{x})^2}{N-1}} \qquad \text{.....equation 5.1}$$

where s = sample standard deviation

N = number of replicate samples

x_i = individual samples

$\bar{x}$ = sample mean

For stability testing the standard deviation (s) of the HPLC method was calculated by injecting the same sample of the particular plant material or product five times.

For the bioavailability assay the standard deviation (s) of the HPLC method was calculated by injecting the same standard five times. The standard deviation (s) of sterol extraction from serum with chloroform, was also determined with the equation above and n = 5. The areas of the cholesterol peak extracted from the same serum were used. As the cholesterol peak was not calibrated, the standard deviation in area units was expressed as a percentage of the area mean.

This was done to give an indication of the variation in the method, for a more accurate standard deviation, the sample number (n) should be larger.

5.2.3.7 Determination of the degradation rate constant and shelf-life

The order of a reaction is dependant on the amount of concentration terms that influence the reaction rate (or the amount of reactants that take part in the reaction). In first-order reactions the rate is proportional to the concentration of one of the reactants. In second-order, the rate is proportional to the concentration of two of the reactants etc. In zero-order reactions, the rate is constant and independent of the concentration of any of the reactants. The rate of reaction may depend on a smaller number of concentration terms than that predicted by the overall stoichiometric equation. The rate may be determined by the slowest or rate-determining step. The order of a particular reaction should, therefore, be determined by experiment (Lund, 1994).

The degradation of β-sitosterol at 40 °C was assumed to be a zero-order reaction and the rate constant k_0 was calculated from the regression equation of the straight line:

$$m = -k_0 \quad \ldots\ldots\ldots \text{equation 5.2}$$

with m = gradient of straight line

The shelf-life of a medicinal product kept in its closed container or under specified conditions is defined as the time from manufacture or preparation until the content of the active constituent has been reduced by 10%. This time is known as the $t_{10\%}$ and the following equation from the Pharmaceutical Codex is used to calculate the $t_{10\%}$ of a zero order reaction (Lund, 1994):

$$t_{10\%} = \frac{0.1a}{k} \quad \ldots\ldots\ldots \text{equation 5.3}$$

where $t_{10\%}$ = the shelf-life

a = the initial concentration

k = the rate constant

Note that in some publications the $t_{90\%}$ is used in the place of $t_{10\%}$ to indicate shelf-life.

The shelf-life at 25 °C can be calculated from the shelf-life at 40°C with the Q_{10}-value. The Q_{10}-value is the factor with which a rate constant increases for a 10 °C rise in temperature. The Q_{10}-value is used to estimate stability when the activation energy is unknown and is an estimation of activation energy. The following equation from the Pharmaceutical Codex is used (Lund, 1994):

Q_{10} = 2 calculates the minimum estimated value

Q_{10} = 3 calculates the most probable estimated value

Q_{10} = 4 calculates the maximum estimated value

$$t_{s\,(T2)} = \frac{t_{s\,(T1)}}{Q_{10}^{\,(T2-T1\,/\,10)}}$$ .... equation 5.4

where $t_{s\,(T1)}$ and $t_{s\,(T2)}$ are the shelf-lives at temperatures T1 and T2.

The Q_{10} approach is not suitable for the more precise predictions of shelf-life required in the development of a new product (Lund, 1994), but in this study Q_{10} = 3 was used to estimate the most probable shelf-life of the herbal material and products.

5.2.3.8 Ethical approval for clinical trial

A clinical trial was planned to perform the bio-equivalence of four South African and three European phytosterol containing products.

The clinical trial was divided in three parts:

Part 1: Pilot study to determine the efficacy and sensitivity of the HPLC method to analyse β-sitosterol in serum.

Part 2: Bio-equivalence study on four South African products (African potato 1 tea, Immunochoice®, Moducare® and Nutricare®).

Part 3: Bio-equivalence study on three European products (Harzol®, Permixon® and Tadenan®).

Part 2 and 3 could only be performed once the sensitivity and efficacy of the HPLC method was confirmed (pilot study).

The protocol for the trial (S83/200... s Committee, Faculty of Medicine, University of Pretoria and Pretoria Academic Hospital. See Appendix B.

5.2.3.9 Preparation of blood samples for HPLC injection

5.2.3.9 (a) Time schedule for blood sampling in part 1 of clinical trial

1. Draw c. 20 ml of blood from the brachial vein of volunteers after 12 hours of fasting to determine their base-line sterol contents of the serum (time $t = 0$).

2.1 Pharmacokinetic pilot study with Moducare[®]:

Administer 9 capsules of Moducare[®] (180 mg β-sitosterol; three times the daily dosage) to three volunteers, as a single dosage. The capsules should be weighed in advance to determine the exact contents (and quantity of β-sitosterol) ingested, as capsule weight usually varies.

2.2 Study no. 2 with Moducare[®]:

Administer 20 capsules of Moducare[®] (400 mg β-sitosterol; 6.7 times the daily dosage) to one volunteer. Again the capsules were weighed in advance to determine the exact contents ingested.

2.3 Study with Tadenan[®]:

Administer 3 capsules of Tadenan[®] (equivalent to 18 mg β-sitosterol in 150 mg *P. africana* extract; as used by Emara et al., 1999) to one volunteer and 9 capsules Tadenan[®] (equivalent to 54 mg β-sitosterol in 450 mg *P. africana* extract) to another. The capsules were weighed in advance to determine the exact contents ingested, as capsule weight usually varies.

3.1 Pharmacokinetic pilot study with Moducare[®]:

Draw venous blood after 0.17, 0.67, 1.0; 1.5; 2; 2.5, 3; 4; 5; 7 and 9 hours from the three volunteers.

3.2 Study no. 2 with Moducare[®]:

Draw venous blood after 3 hours [In Emara et al.'s study, the time of maximum absorption (t_{max}) was 2.8 hours].

3.3 Study with Tadenan[®]:

Draw venous blood after 3 hours from both volunteers.

5.2.3.9 (b) Serum preparation

1. Centrifuge the test tubes at 3000 g in a Heraeus Labofuge 200 centrifuge for 15 minutes.
2. Decant the supernatant plasma of all six tubes into one test tube.

3. Immediately after centrifugation, freeze the serum samples and store at -20°C until assayed.

5.2.3.9 (c) Analysis - According to the method of Emara et al. (1999):

5.2.3.9 (c) (i) Sample treatment

1. Extract 5 ml of the serum samples with 5 ml chloroform by mixing for 1 minute in a vortex mixer VM-300. (Use 40 ml test tubes for the extraction and mixing, as the contact area is to small in 10 ml tubes).

2. Centrifuge at 1700 g (glass tubes used) in a Hettich EBA 12 for 20 minutes for phase separation.

3. Transfer 3 ml from the bottom organic layer to glass vials.

4. Remove the chloroform from the samples by evaporation with a stream of air in a fume cupboard.

5. Dissolve each sample residue in 0.2 ml HPLC grade methanol, mix for 1 minute in a vortex mixer and sonicate for 5 minutes in a Bransonic 52 ultrasonic bath before injecting it into the HPLC.

5.2.3.10 Improvement of β-sitosterol extraction from serum

5.2.3.10(a) Mixing by turning instead of vortex mixing

The same method as in 5.2.3.9 (c) (i), but instead of vigorously mixing the serum-chloroform mixture for 1 minute on a vortex mixer (and emulsion formation), the 40 ml tube containing the serum/chloroform was gently turned for 20 revolutions.

To determine which mixing method was more effective, serum was spiked with 60 μg/ml β-sitosterol and subjected to vortex mixing or gentle turning. After extraction the samples were treated as described in 5.2.3.9 (c) (i)

5.2.3.10(b) Changing the extraction ratio

Emara et al. (1999) used an extraction ratio of serum to chloroform of 1:5, but this was changed to a 1:1 ratio. Thus, instead of extracting 1 ml serum with 5 ml chloroform, 5 ml serum was extracted with 5 ml chloroform, as discussed in 5.2.3.9 (c) (i).

5.2.3.11 Stability of β-sitosterol in serum

During the study with Tadenan®, serum, sampled after 3 hours from the volunteer who ingested 9 capsules Tadenan®, was used to test the stability or instability of β-sitosterol in serum. The serum sampled after 3 hours was divided and one part was frozen immediately and the other was left at room temperature for 2 hours before freezing and storage at –20 °C.

5.2.3.12 Drying the organic phase with heat (90 °C) as in the method of Emara et al. (1999)

After centrifugation of the serum-chloroform mixture, Emara et al. (1999) transferred the organic layer to a test tube and removed the solvent by evaporation using a temperature regulated sand bath adjusted to 90°C. As discussed in 5.2.3.9 (c) (i) the chloroform was usually removed from the samples by evaporation with a stream of air in a fume cupboard. However, to determine the influence of high temperature as used by Emara et al. (1999), the organic phase from serum of the Tadenan® study (sampled after 3 hours) was dried in a Labotec Incoterm oven set at 90 °C.

5.3 RESULTS AND DISCUSSION

5.3.1 Stability analysis of phytosterols

5.3.1.1 Accelerated stability testing of phytosterols

5.3.1.1 (a) TLC

The colour intensity of the blue-purple, "phytosterol-complex" band [See Chapter 3, 3.3.1.2 (a) (i)] was used as an indication of concentration – a decrease in colour intensity was assumed to indicate a decrease in the concentration of phytosterols.

5.3.1.1 (a) (i) *H. hemerocallidea* powder (African potato1)

As only 10 µl could be applied to TLC plates, TLC was not sensitive enough for the low β-sitosterol and hypoxoside levels in the hexane and second water-methanol fractions. Unfortunately, there was not enough sample left for fraction concentration or re-extraction. Hypoxoside was visible in the methanol extracts developed in EMW, but the extracts were complex and no decrease in intensity of the hypoxoside bands was visible. The "phytosterol-complex" did not separate in EMW. However, in the chloroform extracts (redissolved in methanol) of irradiated African potato 1 and 6, a decrease in the band intensity of the "phytosterol-complex" was visible. See Figure 5.10 and section 5.3.1.2 (a) (i).

5.3.1.1 (a) (ii) *P. africana* extract (5:1)

The colour intensity of the phytosterol band in the chloroform extracts of *P. africana* extract powder (5:1), remained the same up to the sample which was stored for 9 months, however, the band of the 12-month sample had lower intensity. See Figure 5.1 (a).

5.3.1.1 (a) (iii) *S. repens* extract (4:1)

The colour intensity of the phytosterol band in the chloroform extracts of *S. repens* extract powder (4:1), remained the same up to 12 months at 40 °C. However, something clearly went wrong with the 9-month sample – the "phytosterol-complex" was not visible, but a purple spot ($R_f = 0.8$) appeared above the position where the "phytosterol-complex" ($R_f = 0.73$) was expected. This purple band was not present in any of the other samples' lanes, which indicated that it was probably a degradation product. As the 12-month sample was taken from 9-month sample, but stored three months longer at

40 °C, did not contain the mentioned band at R_f = 0.8, the origin was probably due to problems during the extraction process, rather than storage at 40 °C. See Figure 5.1 (b).

(a)   **(b)**
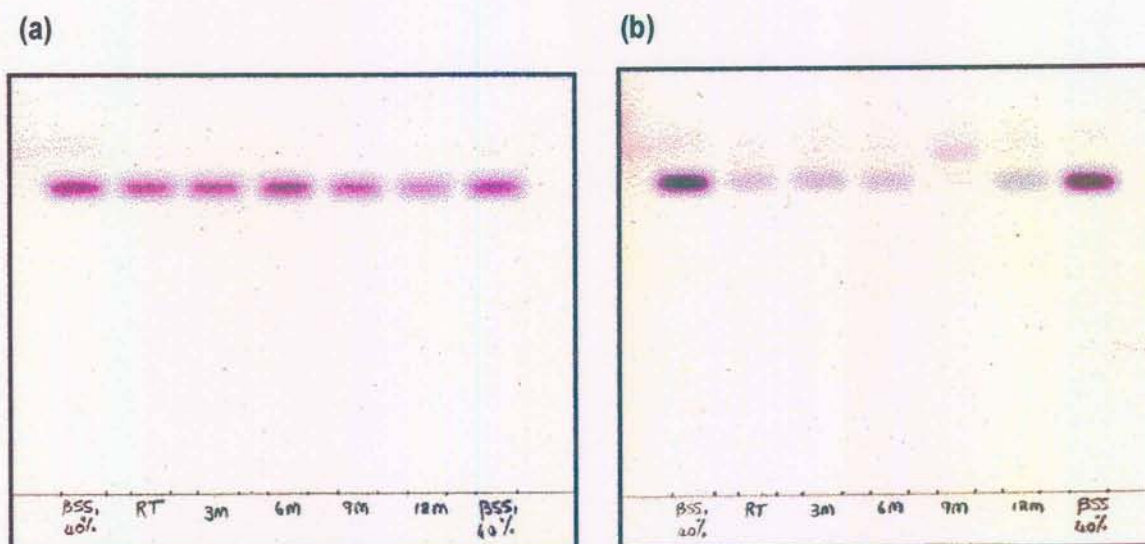
Figure 5.1: TLC on aluminium plates of chloroform extracts of **(a)** *P. africana* bark extract (5:1) and **(b)** *S. repens* berry extract (4:1) stored at 40°C for up to 12 months. On the sides are 40% β-sitosterol standard.

5.3.1.1 (a) (iv) Immunochoice®

The colour intensity of the phytosterol band in the chloroform extracts of Immunochoice® remained the same up to the 12 months at 40°C sample, but the concentration of the extracts was too high and comparison on TLC is difficult. The 1 mg/ml concentration should have been diluted to a concentration where the compounds were just visible on TLC. See Figure 5.2 (a).

5.3.1.1 (a) (v) Moducare®

Similar to the related product Immunochoice®, the Moducare® chloroform extracts' phytosterol bands remained the same up to the 12-month sample. However, the extracts should have been diluted to a concentration where the compounds were just visible. See Figure 5.2 (b).

(a)

(b)
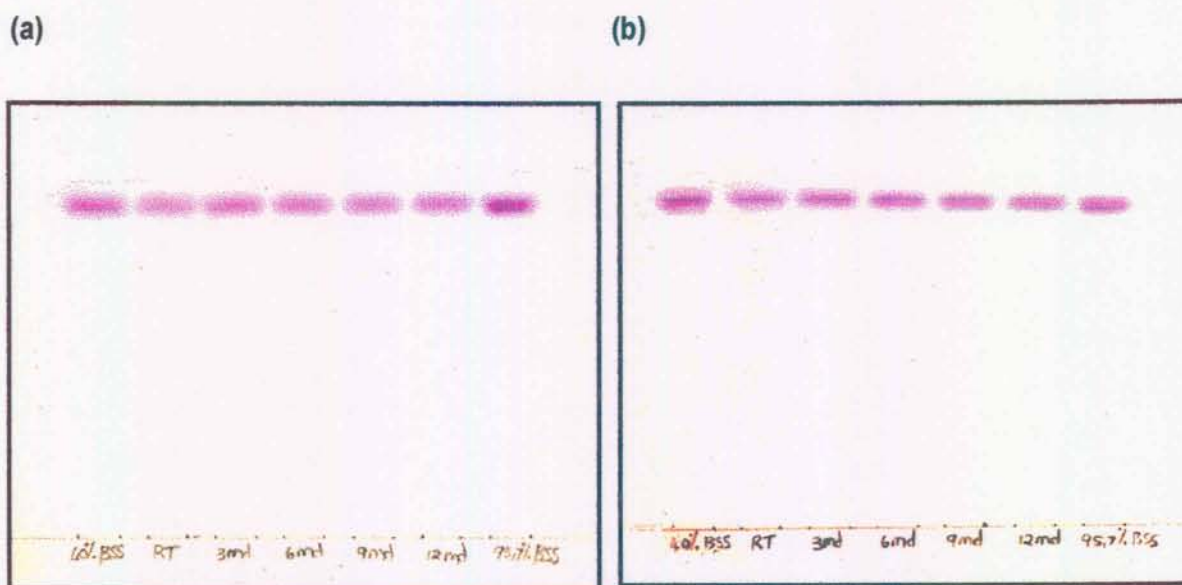
Figure 5.2: TLC on aluminium plates of chloroform extracts of **(a)** Immunochoice® and **(b)** Moducare® stored at 40°C for up to 12 months. On the left is 40% β-sitosterol and on the right is 95.7% β-sitosterol standard.

5.3.1.1 (a) (vi) Nutricare®

The colour intensity of the phytosterol band in the chloroform extracts of Nutricare® decreased from the control to the 3-month sample, but the intensities of the 3-month and 6-month samples appear similar. From 6 months to 12 months the band intensities decreased. See Figure 5.3.
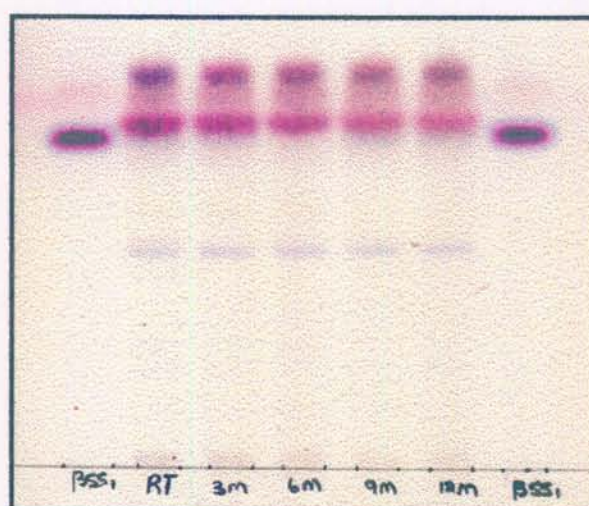
Figure 5.3: TLC on an aluminium plate of chloroform extracts of Nutricare® stored at 40°C for up to 12 months. On the sides are 40% β-sitosterol standard.

Note that the "phytosterol-complex" in this case was slightly different from that of the previously discussed plant material and products. The R_f value was higher (R_f = 0.76) than that of the standards (R_f = 0.72) and the colour of the "phytosterol-complex" band was maroon and not blue-purple as with the standards. See Figure 5.3.

5.3.1.1 (b) HPLC

5.3.1.1 (b) (i) *H. hemerocallidea* powder (African potato1)

Table 5.2: Levels of β-sitosterol and hypoxoside (isolated with solid phase extraction and analysed with HPLC) in *H. hemerocallidea* powder stored at 40 °C for up to 12 months.

Compound	0 months	3 months	6 months	9 months	12 months
β-sitosterol in μg/ml	10.83	12.38	11.14	7.86	6.48
Hypoxoside in μg/ml	105.8	82.3	41.5	28.2	33.9
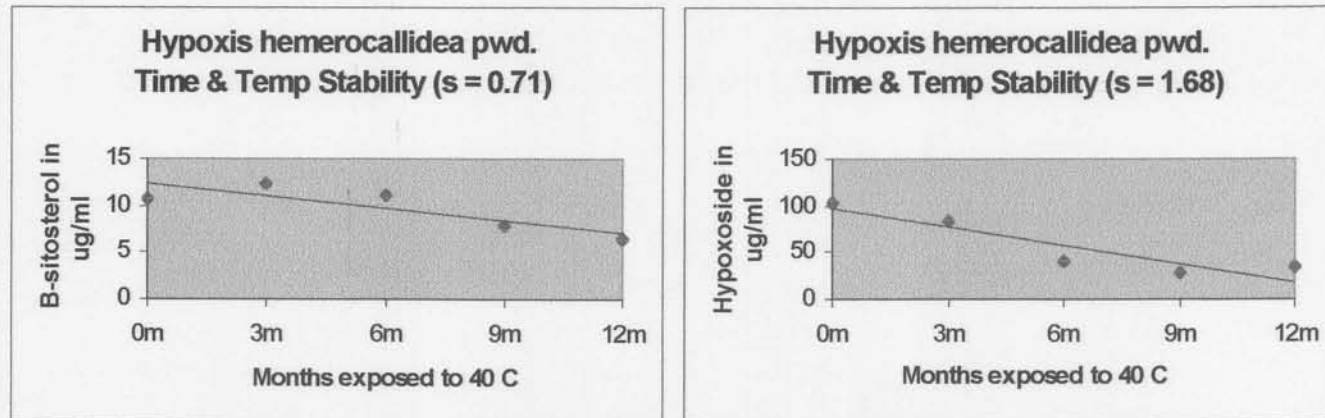
Figure 5.4: Graphs of β-sitosterol and hypoxoside (isolated with solid phase extraction and analysed with HPLC) in *H. hemerocallidea* powder stored at 40 °C for up to 12 months.

β-Sitosterol in *H. hemerocallidea* powder remained relatively stable at 40 °C for 6 months and then decreased with 40.2% up to 12 months. The slight increase in the 3 and 6-month samples can be attributed to normal variation of extraction. The standard deviation of the HPLC method (s) with MWD for β-sitosterol, was 0.71 μg/ml (6.4%). Note that no campesterol and/or stigmasterol were detected in *H. hemerocallidea* powder and were, therefore, not reported. Even chloroform extraction

of African potato 1 did not reveal any campesterol and/or stigmasterol, in contrast to results found with PLANTANICAL MEDICINE's African potato 6. See Chapter 4, 4.3.5.1 (a).

Hypoxoside decreased quickly up to 9 months (73.3% decrease) and then remained relatively stable up to 12 months. The standard deviation of the HPLC method (s) with FLD for hypoxoside was 1.68 μg/ml (4.0%).

The zero-order rate constant (k_0) for the degradation of β-sitosterol at 40 °C was 0.46 μg/ml.month^{-1}, the shelf-life ($t_{10\%}$) at 40 °C was 2.35 months and the shelf-life at 25 °C was 12.21 months (with Q_{10} = 3).

5.3.1.1 (b) (ii) *P. africana* extract (5:1)

Table 5.3: Levels of β-sitosterol, campesterol and/or stigmasterol in *P. africana* extract (5:1) stored at 40 °C for up to 12 months.

Phytosterol	0 months	3 months	6 months	9 months	12 months
β-sitosterol (μg/ml)	401.14	422.06	405.05	401.69	311.62
Campesterol &/or Stigmasterol (μg/ml)	386.08	405.76	389.24	386.72	304.46
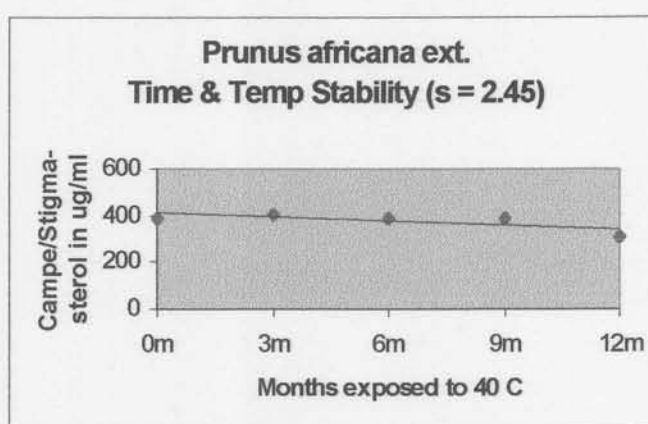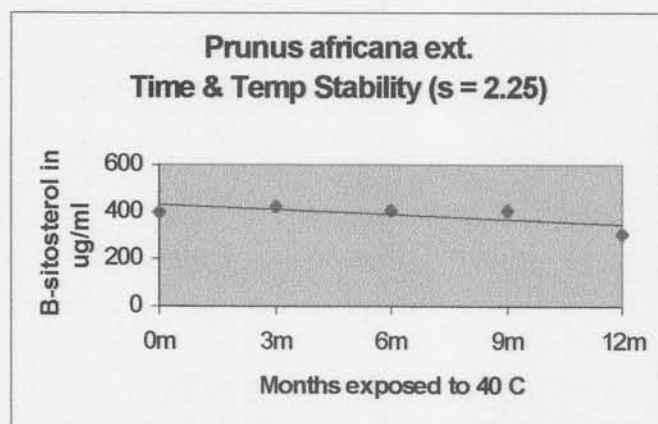
Figure 5.5: Graphs of β-sitosterol, campesterol and/or stigmasterol in *P. africana* extract (5:1) stored at 40 °C for up to 12 months.

β-Sitosterol in *P. africana* extract (5:1) remained relatively stable up to 9 months at 40 °C, and then decreased with 22.3% up to 12 months. The increase with the sample stored for 3 months is again attributed to variation during extraction. Note that campesterol and/or stigmasterol levels also increased in the sample stored for 3 months at 40 °C. The standard deviation for the HPLC method for β-sitosterol, was 2.25 μg/ml (0.7%).

Campesterol and/or stigmasterol were also stable up to 9 months at 40 °C and then decreased with 21.14% up to 12 months. The HPLC standard deviation for campesterol/stigmasterol was 2.45 μg/ml (0.8%).

The zero-order rate constant (k_0) for the degradation of β-sitosterol at 40 °C was 5.67 μg/ml.month^{-1}, the shelf-life ($t_{10\%}$) at 40 °C was 7.07 months and the shelf-life at 25 °C would be 36.74 months (with $Q_{10} = 3$).

5.3.1.1 (b) (iii) *S. repens* extract (4:1)

Table 5.4: Levels of β-sitosterol, campesterol and/or stigmasterol in *S. repens* extract (4:1) stored at 40 °C for up to 12 months.

Phytosterol	0 months	3 months	6 months	9 months	12 months
β-sitosterol (μg/ml)	84.68	85.87	75.27	14.14	67.73
Campesterol &/or Stigmasterol (μg/ml)	76.09	80.93	63.12	3.64	56.58

β-Sitosterol remained relatively stable in *S. repens* extract up to 3 months at 40 °C and then decreased. Unfortunately something went wrong during the extraction of the sample stored for 9 months [as seen in Figure 5.1 (b) with TLC, but there was not enough material left to repeat the assay. After 12 months at 40 °C β-sitosterol decreased with 20%. The standard deviation of the HPLC method for β-sitosterol was 2.25 μg/ml (as with *P. africana*).

Campesterol and/or stigmasterol behaved the same as β-sitosterol – remained stable up to 3 months at 40 °C and then decreased. After 12 months at 40 °C campesterol and/or stigmasterol

decreased with 25.6%. The standard deviation for the HPLC method for campesterol/stigmasterol was 2.45 µg/ml (as with *P. africana*). See Figure 5.6.
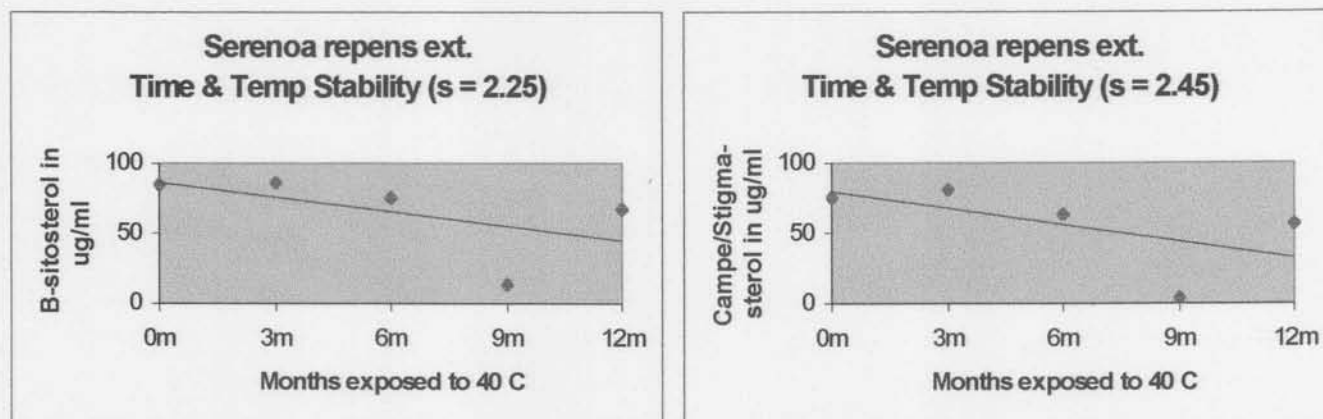
Figure 5.6: Graphs of β-sitosterol, campesterol and/or stigmasterol in *S. repens* extract (4:1) stored at 40 °C for up to 12 months.

The zero-order rate constant (k_0) for the degradation of β-sitosterol at 40 °C was 1.57 µg/ml.month^{-1}, the shelf-life ($t_{10\%}$) at 40 °C was 5.39 months and the shelf-life at 25 °C would be 28.01 months (with Q_{10} = 3). Note that the sample stored for 9 months was excluded in the calculation of the gradient and the zero-order rate constant.

5.3.1.1 (b) (iv) Immunochoice®

Table 5.5: Levels of β-sitosterol, campesterol and/or stigmasterol in Immunochoice® stored at 40 °C for up to 12 months.

| Phytosterol	0 months	3 months	6 months	9 months	12 months
β-sitosterol (µg/ml)	374.4	367.4	389.3	396.6	373.9
Campesterol &/or Stigmasterol (µg/ml)	147.5	146.3	154.6	154.8	146.2
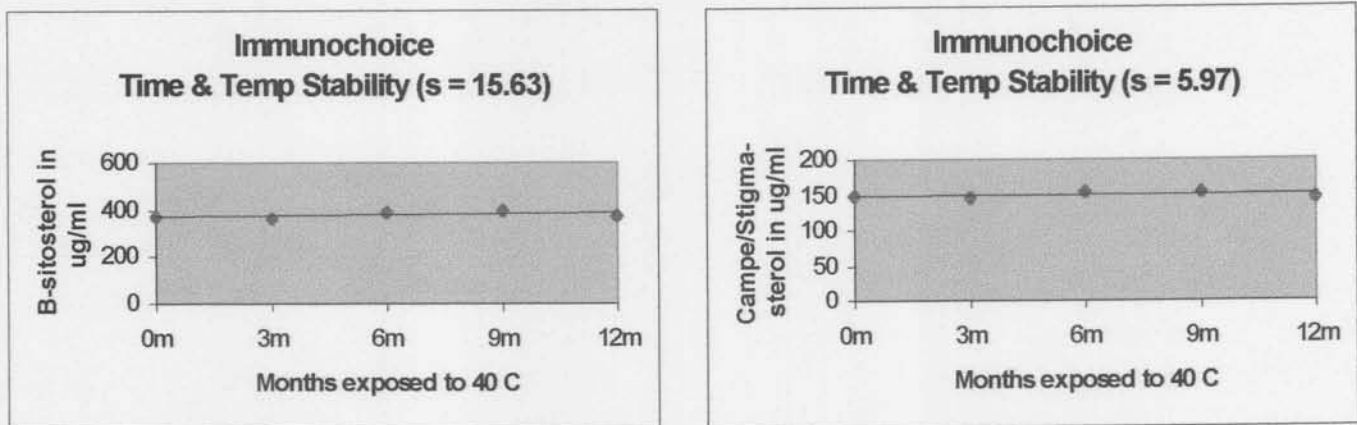
Figure 5.7: Graphs of β-sitosterol, campesterol and/or stigmasterol in Immunochoice® stored at 40 °C for up to 12 months.

β-Sitosterol remained stable in Immunochoice® stored at 40 °C for up to 12 months. The concentration fluctuations were attributed to standard deviation of the HPLC method (s = 15.63 μg/ml, 4.0%) for β-sitosterol and normal variation of extraction.

The same can be said for campesterol and/or stigmasterol. They remained stable in Immunochoice® stored at 40 °C for up to 12 months. The standard deviation of the HPLC method for campesterol and/or stigmasterol, was 5.97 μg/ml (3.9%).

The high standard deviation of the HPLC method (s) may have led to a positive gradient and, therefore, the zero-order rate constant (k_0) for the degradation of β-sitosterol at 40 °C and the shelf-life ($t_{10\%}$) of Immunochoice® could not be calculated. In any case Immunochoice® appears to be extra ordinarily stable.

5.3.1.1 (b) (v) Moducare®

Table 5.6: Levels of β-sitosterol, campesterol and/or stigmasterol in Moducare® stored at 40 °C for up to 12 months.

Phytosterol	0 months	3 months	6 months	9 months	12 months
β-sitosterol (μg/ml)	390.8	389.2	377.1	407.0	421.2
Campesterol &/or Stigmasterol (μg/ml)	263.8	262.3	251.9	278.2	279.9

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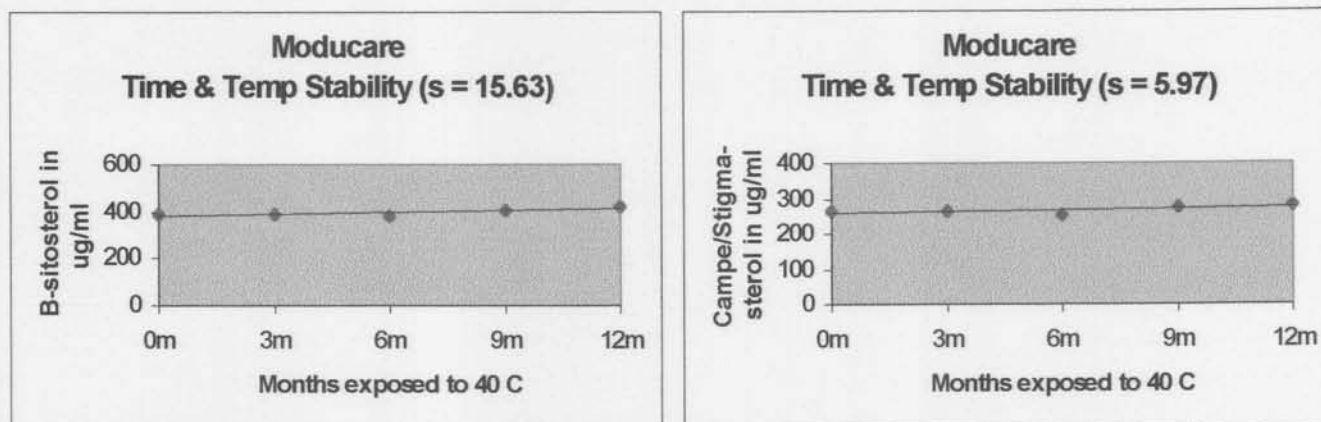
Figure 5.8: Graphs of β-sitosterol, campesterol and/or stigmasterol in Moducare® stored at 40 °C for up to 12 months.

β-Sitosterol remained stable in Moducare®stored at 40 °C for up to 12 months. The slight increase from 6 to 9 to 12 months is attributed to variation of extraction and the standard deviation of the HPLC method (s = 15.63 µg/ml, as with Immunochoice®).

Campesterol and/or stigmasterol also remained stable in Moducare® stored at 40 °C for up to 12 months. The increase from 6 to 9 months is also attributed to variation of extraction and the standard deviation of the HPLC method (s = 5.97 µg/ml, as with Immunochoice®).

As with Immunochoice the standard deviation of the HPLC method (s) was high and it may have caused a positive gradient. Therefore, the zero-order rate constant (k_0) for the degradation of β-sitosterol at 40 °C and the shelf-life ($t_{10\%}$) of Moducare® could unfortunately not be calculated.

5.3.1.1 (b) (vi) Nutricare®

Table 5.7: Levels of β-sitosterol in Nutricare® stored at 40 °C for up to 12 months.

Phytosterol	0 months	3 months	6 months	9 months	12 months
β-sitosterol (µg/ml)	216.3	172.6	177.5	167.68	146.57
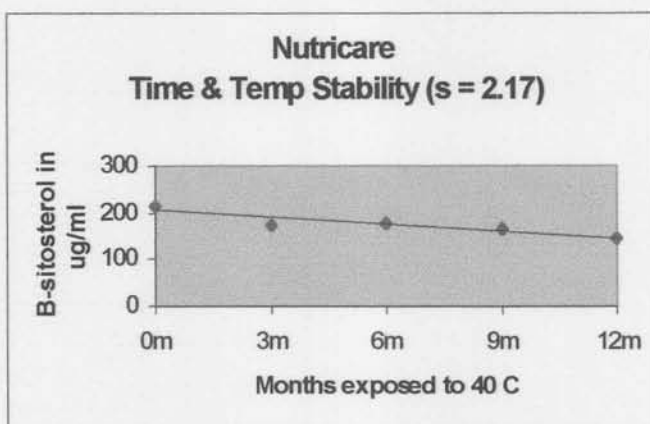
Figure 5.9: A graph of β-sitosterol in Nutricare® stored at 40 °C for up to 12 months.

β-Sitosterol decreased with 32.2% up to 12 months exposed to 40 °C. Again the slight increase from 3 to 6 months was attributed to normal variation of extraction and the standard deviation of the HPLC method (s = 2.17 μg/ml, 1.5%) for β-sitosterol.

The uncertainty regarding the peak, measured as β-sitosterol, is discussed in section 5.4 Conclusion.

The zero-order rate constant (k_0) for the degradation of β-sitosterol at 40 °C was 5.16 μg/ml.month^{-1}, the shelf-life ($t_{10\%}$) at 40 °C was 4.20 months and the shelf-life at 25 °C would be 21.80 months (with Q_{10} = 3).

5.3.1.2 Stability of phytosterols against gamma irradiation

5.3.1.2 (a) TLC

The colour intensity of the blue-purple phytosterol band was used as an indication of concentration – a decrease in colour intensity was assumed to indicate a decrease in the concentration of phytosterols.

5.3.1.2 (a) (i) *H. hemerocallidea* powder (African potato1 & 6)

Note that chloroform extraction was performed on African potato 1 powder for TLC. Therefore, although the same powder was extracted, the HPLC values obtained in 5.3.1.2 (b) (i) were from

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different extracts (methanol extraction with solid phase extraction and fractionation) than those used for TLC. The HPLC results of *H. hemerocallidea* powder, therefore, do not necessarily correspond with the TLC results, as could be expected when the same samples were used for HPLC and for TLC. African potato 6, exposed to the different levels of irradiation, was also extracted with chloroform (dried and redissolved in methanol) and chromatographed.

Differences in the colour intensity of the phytosterol band in the chloroform extracts of African potato 1 powder could not be detected, although the control sample seemed a little darker than the 4.3 kGray sample. See Figure 5.10 (a). However, the phytosterol band in the chloroform extracts of African potato 6 (PLANTANICAL MEDICINE's Hypoxis) exposed to 12.8 and 28.5 kGray clearly had a lower colour intensity than that of the control and the sample exposed to 4.3 kGray. See Figure 5.10 (b).

(a)

(b)
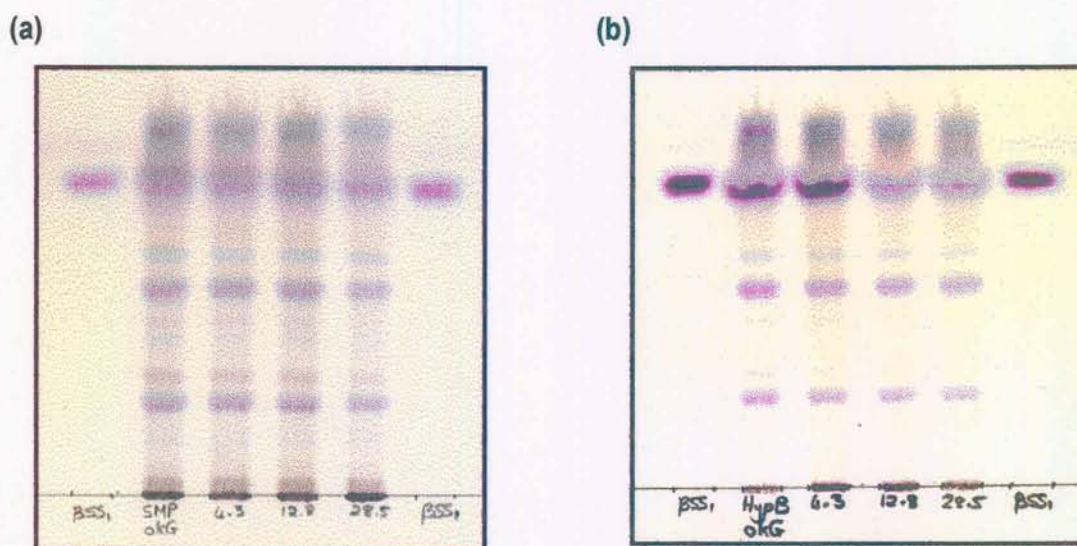
Figure 5.10: TLC on aluminium plates of chloroform extracts of **(a)** *H. hemerocallidea* powder (African potato 1) and **(b)** PLANTANICAL MEDICINE's Hypoxis (African potato 6), gamma irradiated at 0, 4.3, 12.8 and 28.5 kGray. On the sides are 40% β-sitosterol standard.

5.3.1.2 (a) (ii) *P. africana* extract (5:1)

The colour intensity of the phytosterol band in the chloroform extracts of *P. africana* extract (5:1), remained the same – even in the sample exposed to 27.9 kGray. For comparison purposes the 1

mg/ml extracts should have been diluted until the compounds were just visible on TLC. See Figure 5.11 (a).

5.3.1.2 (a) (iii) *S. repens* extract (4:1)

The colour intensity of the phytosterol band in the chloroform extracts of *S. repens* extract powder (4:1), decreased from the control sample to 4.4 kGray *etc.*, with the lowest intensity in the sample exposed to 27.9 kGray. See Figure 5.11 (b).

(a)   **(b)**
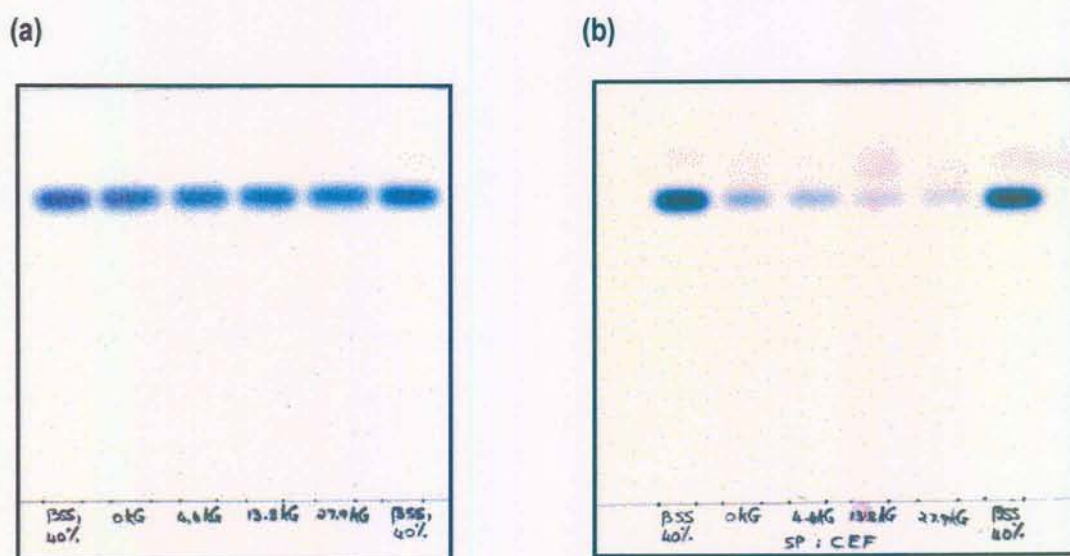
Figure 5.11: TLC on aluminium plates of chloroform extracts of **(a)** *P. africana* bark extract (5:1) and **(b)** *S. repens* berry extract (4:1), gamma irradiated at 0, 4.4, 13.8 and 27.9 kGray. On the sides are 40% β-sitosterol standard.

5.3.1.2 (a) (iv) Immunochoice®

The colour intensity of the phytosterol band in the chloroform extracts of Immunochoice® remained the same, despite gamma irradiation of as high as 27.9 kGray. See Figure 5.12 (a).

5.3.1.2 (a) (v) Moducare®

Again, similar to Immunochoice®, the colour intensity of the phytosterol band in the Moducare® chloroform extracts remained the same up exposure of 27.9 kGray gamma irradiation. See Figure 5.12 (b).

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(a)

(b)

Figure 5.12: TLC on aluminium plates of chloroform extracts of **(a)** Immunochoice® and **(b)** Moducare®, gamma irradiated at 0, 4.4, 13.8 and 27.9 kGray. On the sides are 40% β-sitosterol standard.

5.3.1.2 (a) (vi) Nutricare®

The phytosterol band intensity decreased from the control to the 2.3 kGray Nutricare® sample, but, thereafter, the intensity remained the same. The Rf-value of the "phytosterol-complex" seemed slightly higher (R_f = 0.76) than those of the standards (R_f = 0.72), and the colour of the band was maroon and not blue-purple as in the case of the standards. In previous work done on Nutricare® extracted with acetone, the intensity of the *red spot compound* decreased on TLC in samples irradiated at 2.3, 4.1 and 11.8 kGray.

5.3.1.2 (b) HPLC

5.3.1.2 (b) (i) *H. hemerocallidea* powder (African potato1)

Table 5.8: Levels of β-sitosterol and hypoxoside (isolated with solid phase extraction and analysed with HPLC) in *H. hemerocallidea* powder, gamma irradiated at different intensities.

Compound	0 kGray	4.3 kGray	12.8 kGray	28.5 kGray
β-sitosterol (µg/ml)	10.83	9.82	7.45	7.04
Hypoxoside (µg/ml)	105.8	60.5	49.0	29.3
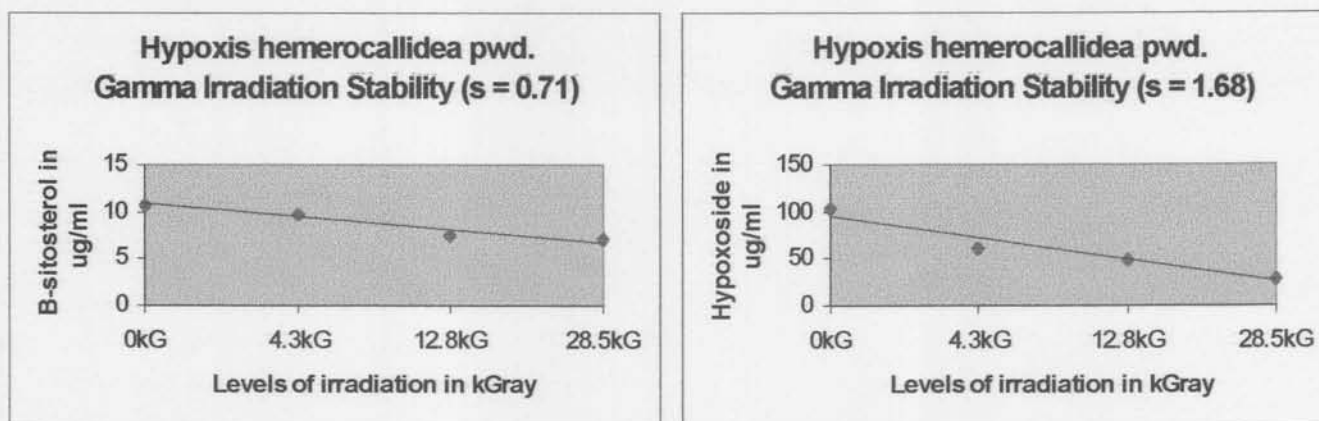
Figure 5.13: Graphs of β-sitosterol and hypoxoside (isolated with solid phase extraction and analysed with HPLC) in *H. hemerocallidea* powder, gamma irradiated at different intensities.

β-Sitosterol levels in *H. hemerocallidea* powder decreased with gamma irradiation exposure. Even at low intensities such as 4.3 kGray there was a decrease of 9.3%. Up to 28.5 kGray β-sitosterol decreased with 35%. The standard deviation of the HPLC method with multiple wavelenth detection for β-sitosterol, was 0.71 µg/ml (6.4%).

Hypoxoside levels decreased rapidly when exposed to gamma irradiation. Again low intensities such as 4.3 kGray caused hypoxoside to break down with as much as 42.8%. Up to 28.5 kGray hypoxoside in *H. hemerocallidea* powder decreased with 72.3%. The standard deviation for HPLC with fluorescence detection for hypoxoside was 1.68 µg/ml (4.0%).

5.3.1.2 (b) (ii) *P. africana* extract (5:1)

Table 5.9: Levels of β-sitosterol, campesterol and/or stigmasterol in *P. africana* extract (5:1), gamma irradiated at different intensities.

Phytosterol	0 kGray	4.4 kGray	13.8 kGray	27.9 kGray
β-sitosterol (μg/ml)	401.14	416.67	408.61	415.58
Campesterol &/or Stigmasterol (μg/ml)	386.08	399.97	393.63	398.5

Figure 5.14: Graphs of β-sitosterol, campesterol and/or stigmasterol in *P. africana* extract (5:1), gamma irradiated at different intensities.

β-Sitosterol remained stable in *P. africana* extract when exposed to gamma irradiation of as high as 27.9 kGray. The slight increase of β-sitosterol in the exposed samples was attributed to normal variation during the extraction process and the standard deviation of the HPLC method ($s = 2.25$ μg/ml, 0.7% for β-sitosterol determination).

Campesterol and/or stigmasterol also remained stable in *P. africana* extract exposed to high intensity gamma irradiation. The standard deviation of the HPLC method to determine campesterol and/or stigmasterol was 2.45 μg/ml.

5.3.1.2 (b) (iii) *S. repens* extract (4:1)

Table 5.10: Levels of β-sitosterol, campesterol and/or stigmasterol in *S. repens* extract (4:1), gamma irradiated at different intensities.

Phytosterol	0 kGray	4.4 kGray	13.8 kGray	27.9 kGray
β-sitosterol (µg/ml)	84.68	79.36	39.32	39.26
Campesterol &/or Stigmasterol (µg/ml)	76.09	32.48	20.35	15.67
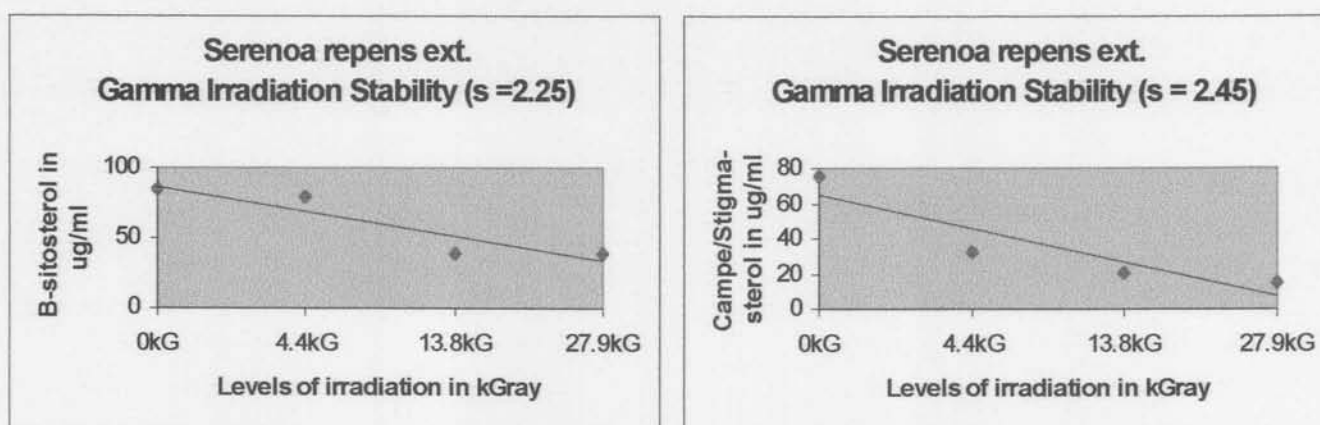
Figure 5.15: Graphs of β-sitosterol, campesterol and/or stigmasterol in *S. repens* extract (5:1), gamma irradiated at different intensities.

β-Sitosterol remained relatively stable after gamma irradiation of 4.4 kGray, but after irradiation up to 27.9 kGray, there was a decrease of 53.6%. The standard deviation for the HPLC method for β-sitosterol was 2.25 µg/ml (as with *P. africana*).

Surprisingly campesterol and/or stigmasterol decreased by 57.3% after mild gamma irradiation exposure of 4.4 kGray. After irradiation up to 27.9 kGray there was a decrease of campesterol and/or stigmasterol of 79.4%. The standard deviation for the HPLC method for campesterol/stigmasterol was 2.45 µg/ml (as with *P. africana*).

5.3.1.2 (b) (iv) Immunochoice®

Table 5.11: Levels of β-sitosterol, campesterol and/or stigmasterol in Immunochoice®, gamma irradiated at different intensities

Phytosterol	0 kGray	4.4 kGray	13.8 kGray	27.9 kGray
β-sitosterol (μ/gml)	374.4	363.3	359.0	358.4
Campesterol &/or Stigmasterol (μg/ml)	147.5	139.3	140.8	136.4
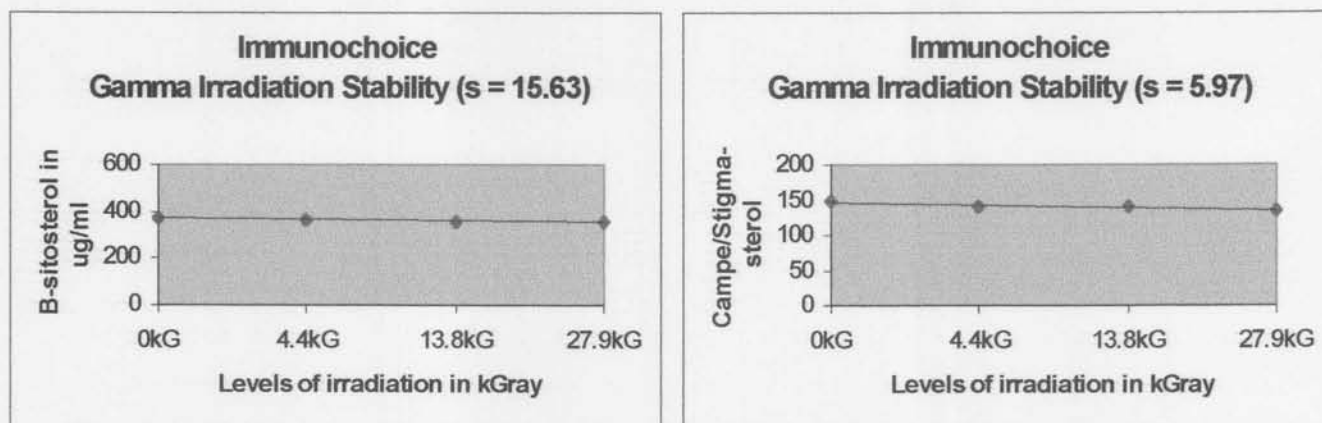
Figure 5.16: Graphs of β-sitosterol, campesterol and/or stigmasterol in Immunochoice®, gamma irradiated at different intensities.

β-Sitosterol in Immunochoice® remained relatively stable after gamma irradiation of up to 27.9 kGray. The slight decrease of β-sitosterol in the irradiation-exposed samples may be due to normal variation of extraction and standard deviation of the HPLC method (s = 15.63 μg/ml, 4.0%).

In general campesterol and/or stigmasterol in Immunochoice® reacted similarly to β-sitosterol. They remained relatively stable after gamma irradiation of as high as 27.9 kGray. The standard deviation of the HPLC method for the determination of campesterol and/or stigmasterol, was 5.97 μg/ml (3.9%).

5.3.1.2 (b) (v) Moducare®

Table 5.12: Levels of β-sitosterol, campesterol and/or stigmasterol in Moducare®, gamma irradiated at different intensities.

Phytosterol	0 kGray	4.4 kGray	13.8 kGray	27.9 kGray
β-sitosterol (μg/ml)	390.8	399.8	385.3	350.8
Campesterol&/or Stigmasterol (μg/ml)	263.8	265.4	258.4	233.4

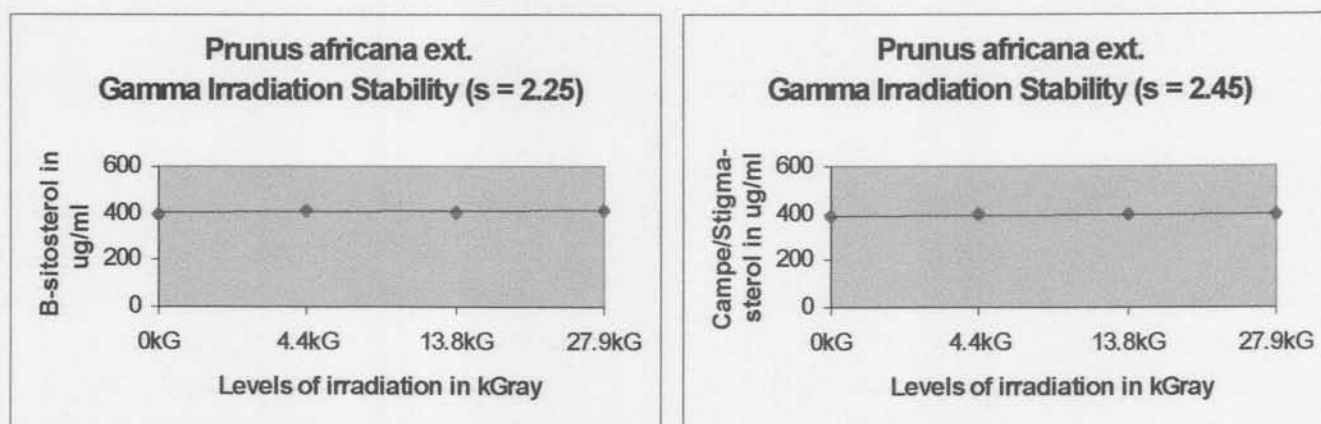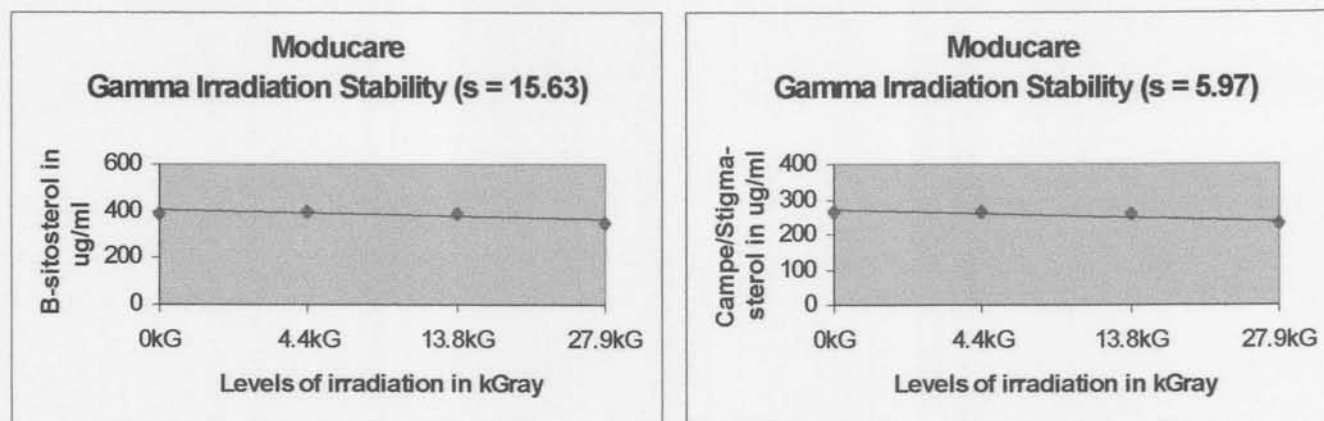**Figure 5.17:** Graphs of β-sitosterol, campesterol and/or stigmasterol in Moducare®, gamma irradiated at different intensities.

β-Sitosterol in Moducare® remained relatively stable after gamma irradiation exposure of up to 13.8 kGray. A further increase in irradiation intensity up to 27.9 kGray, caused 10.2% degradation of β-sitosterol. Again the slight increase in the 4.4 kGray sample is attributed to variation of extraction and the standard deviation of the HPLC method (s = 15.63 μg/ml, as with Immunochoice®) to determine β-sitosterol.

Campesterol and/or stigmasterol reacted in the same way. They remained relatively stable in Moducare® up to 13.8 kGray and then deceased slightly by 11.5%. The standard deviation of the HPLC method to determine campesterol and/or stigmasterol was 5.97 μg/ml (as with Immunochoice®).

5.3.1.2 (b) (vi) Nutricare®

Table 5.13: Levels of β-sitosterol in Nutricare®, gamma irradiated at different intensities.

Phytosterol	0 kGray	2.3 kGray	4.1 kGray	11.8 kGray	27.9 kGray
β-sitosterol (μg/ml)	216.3	170.2	169.16	153.46	126.71
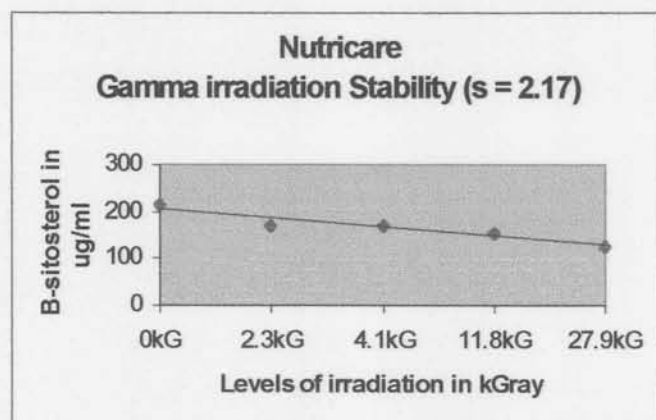
Figure 5.18: A graph of β-sitosterol in Nutricare®, gamma irradiated at different intensities.

β-Sitosterol levels in Nutricare® decreased by 21.3% after very mild gamma irradiation of 2.3 kGray. Up to irradiation of 27.9 kGray, β-sitosterol decreased by 41.4%. The standard deviation of the HPLC method to determine β-sitosterol was 2.17 μg/ml (1.5%).

The uncertainty regarding the peak, measured as β-sitosterol, is discussed in section 5.4 Conclusion.

5.3.2 Bioavailability analysis of phytosterols

5.3.2.1 TLC

5.3.2.1 (a) Sensitivity of TLC for visualising β-sitosterol

The plates were developed as discussed in this chapter, 5.2.3.4 (a). See Figure 5.19.
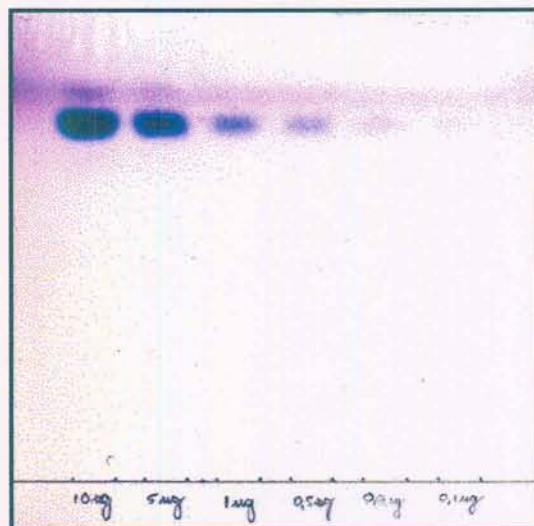
Figure 5.19: TLC on an aluminium plate of a dilution series of 95.7% β-sitosterol standard in chloroform with total mass 10, 5, 1.0, 0.5, 0.2 and 0.1μg applied.

The visual detection limit of β-sitosterol with TLC, sprayed with *p*-anisaldehyde, is between 0.2 and 0.5 μg.

5.3.2.1 (b) Detectability of β-sitosterol in serum with TLC

To determine whether β-sitosterol in serum was detectable with TLC, a serum sample spiked with β-sitosterol and an unspiked control were extracted with chloroform and applied on TLC. See the preparation method in 5.2.3.4 (b) and Figure 5.20.
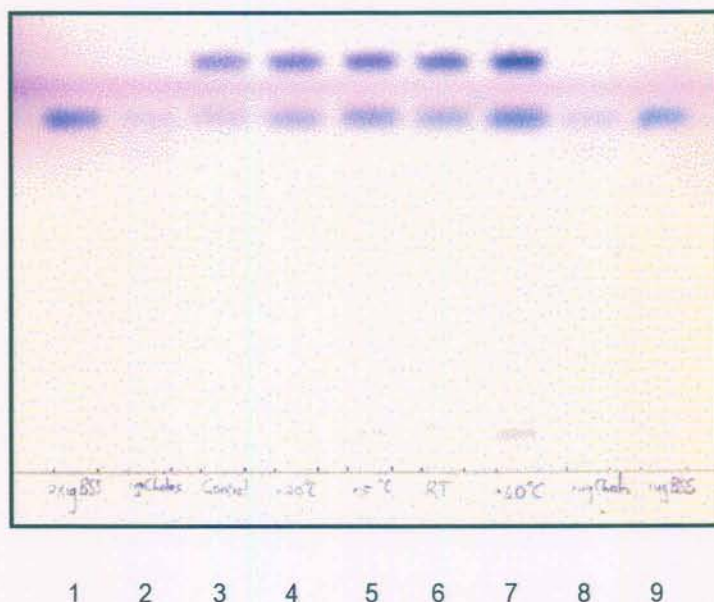
Figure 5.20: TLC on an aluminium plate of β-sitosterol-spiked and unspiked serum samples extracted with chloroform. From the left: 95.7% β-sitosterol standard (1), 99+% cholesterol standard (2), unspiked serum control (3) and β-sitosterol-spiked serum (4). Applications 5 - 7 were β-sitosterol-spiked serum stored for three hours at different temperatures for a stability study. On the right are 99+% cholesterol standard (8) and 95.7% β-sitosterol standard (9).

The "sterol-complex" band ($R_f = 0.8$), including cholesterol, was visible in the unspiked serum control (lane 3) and in the β-sitosterol-spiked sample (lane 4). The "sterol complex" band had a higher intensity in the β-sitosterol spiked serum (cholesterol + β-sitosterol) than in the unspiked serum (cholesterol alone). An unknown band ($R_f = 0.95$) was noticed above the "sterol-complex" in the serum samples (spiked and unspiked).

The β-sitosterol and cholesterol standards had the same R_f – value ($R_f = 0.8$).

5.3.2.2 HPLC

5.3.2.2 (a) Calibration curve to determine β-sitosterol in serum

To compensate for variables and substances in serum that could interfere with the quantification of β-sitosterol, a new calibration curve was constituted from different concentrations β-sitosterol-spiked serum, extracted with chloroform. See the method in this chapter, 5.2.3.2 and Figure 5.21.
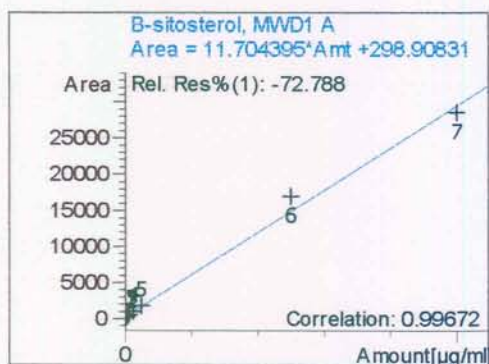
Figure 5.21: Calibration curve to determine β-sitosterol in serum, obtained from MWD absorbance spectra, with a correlation coefficient of 0.9967.

The peaks were recorded by the MWD, but the quantities of β-sitosterol in the standard-spiked serum samples were also calibrated according to β-sitosterol's calibration curve in Chapter 4, 4.3.2 (See Figure 4.3). By comparing these HPLC measured quantities with the theoretical β-sitosterol concentrations in the case of 100% extraction, the completeness of chloroform extraction from the serum could be determined.

See Table 5.14 for the HPLC measured β-sitosterol concentrations and the percentages extracted by chloroform.

Table 5.14: The theoretical β-sitosterol concentrations in serum used to constitute the calibration curve, versus the concentrations after chloroform extraction.

Theoretical concentration β-sitosterol (μg/ml)	β-sitosterol concentration via HPLC (μg/ml)	Percentage extracted
2500	1300.37	52.01%
1250	771.04	61.68%
125	80.63	64.50%
62.5	44.38	71.01%
31.25	23.22	74.30%
15.625	10.92	69.89%
7.8	4.62	59.23%
Mean percentage extracted		64.66%

5.3.2.2 (b) Clinical trial: Part 1

Part 1 of the clinical trial, was a pilot study to determine the efficacy and sensitivity of the HPLC method of Emara et al. (1999). At the same time, it was attempted to evaluate the pharmacokinetics of β-sitosterol [time of maximum absorption (t_{max}) measurement *etc.*) in order to plan the time schedule for drawing blood samples in part 2 and 3 of the clinical trial.

5.3.2.2 (b) (i) Pharmacokinetic pilot study with Moducare®

The blood of three volunteers was sampled as discussed in 5.2.3.9 (a) and prepared and analysed as discussed 5.2.3.9 (b) and (c).

In none of the serum samples collected up to 9 hours after ingesting 180 mg β-sitosterol (9 capsules Moducare®), could any trace of β-sitosterol be detected.

An amendment to the approved protocol (S83/2000) of the clinical trial was submitted to the Ethics Committee, Faculty of Medicine, University of Pretoria and Pretoria Academic Hospital, to increase the dosage of β-sitosterol to a maximum of 400 mg (20 capsules Moducare®) daily. The amendment was accepted. See Appendix B.

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5.3.2.2 (b) (ii) Study no. 2 with Moducare®

The volunteer's blood was sampled as discussed in 5.2.3.7 (a), prepared and analysed as discussed 5.2.3.7 (b) and (c).

Even at as high a dosage as 400 mg β-sitosterol (20 capsules Moducare®), no β-sitosterol was detected in the serum.

5.3.2.2 (b) (iii) Study with Tadenan®

Tadenan® was one of the dosage forms used by Emara et al. (1999) to determine the "bioavailability of β-sitosterol from *Pygeum africanum* extract in humans". They administered three capsules of Tadenan® (equivalent to 18 mg β-sitosterol, in 150 mg *P. africana* extract).

The volunteers' blood was sampled as discussed in 5.2.3.7 (a), and prepared and analysed as discussed 5.2.3.7 (b) and (c).

β-Sitosterol was not detected in any of the serum of the two volunteers, neither after 3 (equivalent to 18 mg β-sitosterol) nor after 9 capsules of Tadenan® (equivalent to 54 mg β-sitosterol). This was confirmed by spiking the samples with 95.7% β-sitosterol. See Figure 5.22.

The high peak at t_R = c. 8.0 minutes was identified as cholesterol with the means of 99+% cholesterol standard.

5.3.2.2 (c) Improved β-sitosterol extraction from serum

5.3.2.2 (c) (i) Mixing by turning instead of vortex mixing

Emara et al. (1999) extracted β-sitosterol from serum by mixing the serum and chloroform on a vortex mixer for 1 minute. However, vortex mixing lead to the formation of an emulsion, which had to be centrifuged at high speed to separate the phases again for removal of the organic phase. It was feared that the vigorous mixing might lead to complex formation between β-sitosterol and serum proteins, and that the complexed β-sitosterol might, therefore, not be available for extraction.

Therefore, a more gentle approach was attempted in which the serum and chloroform was mixed by turning the 40ml tube sideways for 20 revolutions. The result was 83.3 % extraction of β-sitosterol via turning and 60% extraction via vortex mixing. The results of vortex mixing was confirmed when

(a)
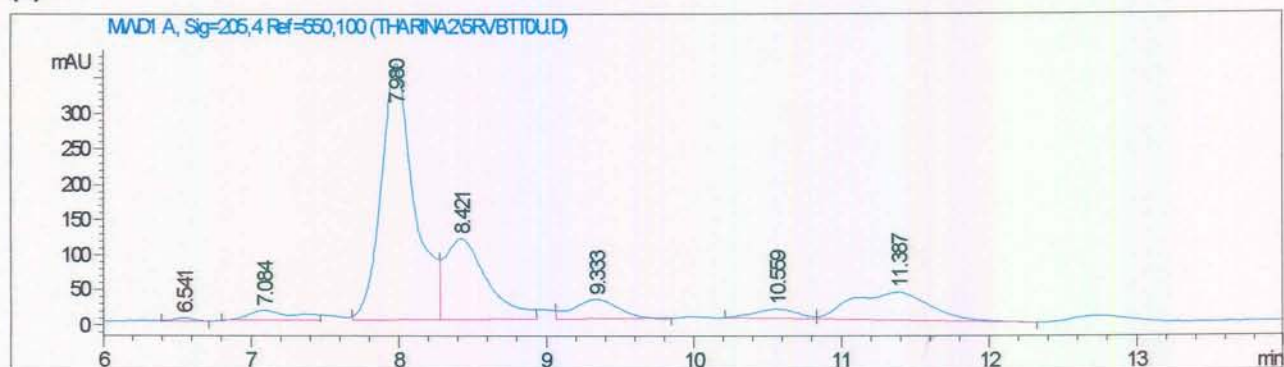
(b)
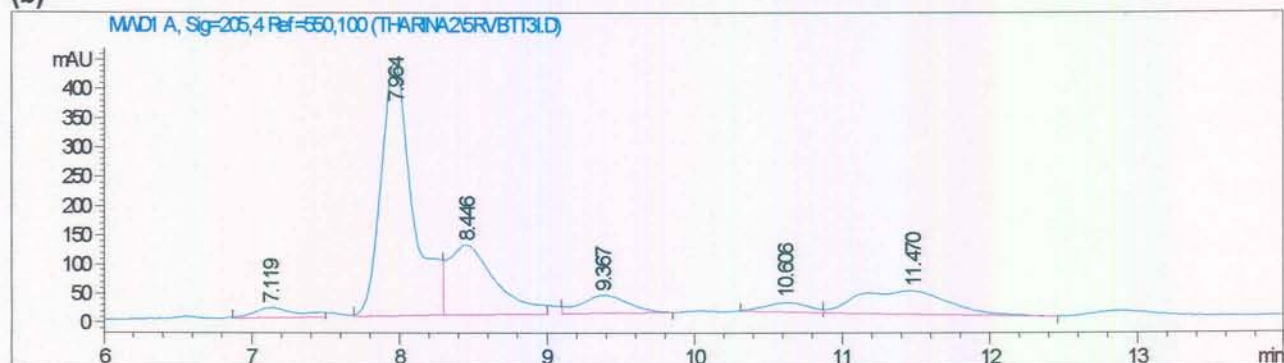
(c)
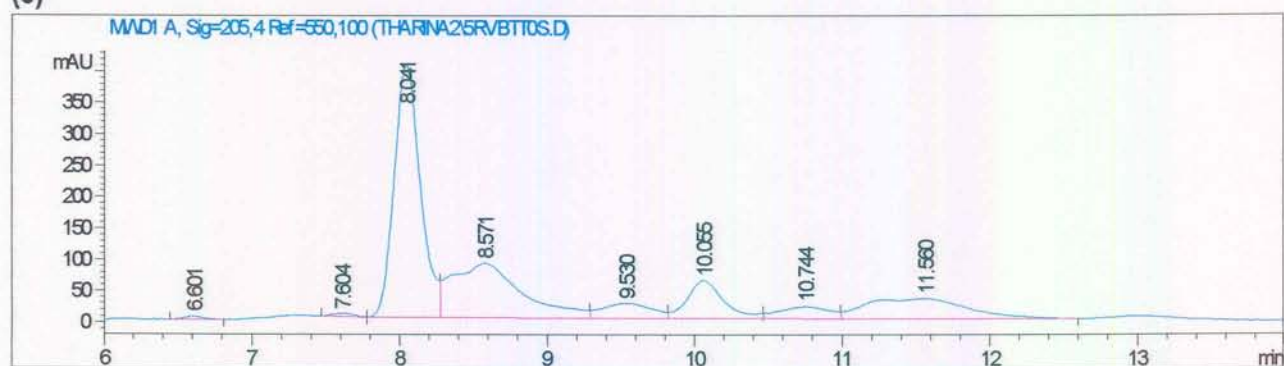
Figure 5.22: HPLC chromatogram of chloroform extracted serum sampled at (a) time = 0, (b) time = 3 hours after ingesting 9 capsules of Tadenan® (equivalent to 54 mg β-sitosterol) and (c) time = 0 sample spiked with 95.7% β-sitosterol standard after extraction. See the β-sitosterol peak at t_R = 10.055 in (c) is absent in (a) and (b). See the cholesterol peak at t_R = c. 8.0 minutes [t_R = 7.980 in (a), 7.964 in (b) and 8.041 in (c)].

preparing the calibration curve for determination of β-sitosterol in serum. See this chapter 5.3.2.2 (a). The results of "turning" were not reproducible and, therefore, vortex mixing was preferred. When comparing the chromatograms of the vortex mixed and gently turned samples, the cholesterol peak

was approximately five times higher in the case of the vortex mixed samples compared to the turned samples. This indicated better extraction of cholesterol (and probably β-sitosterol) with vortex mixing compared to turning.

5.3.2.2 (c) (ii) Changing the extraction ratio

Emara et al. (1999) used an extraction ratio of serum to chloroform of 1:5, but when 1.0 ml serum was extracted with 5 ml chloroform the peaks were poorly defined and not very clear (except the cholesterol peak). See Figure 5.23 and compare with Figure 5.22 (b) in which the same serum sample was extracted in a 1:1 ratio.
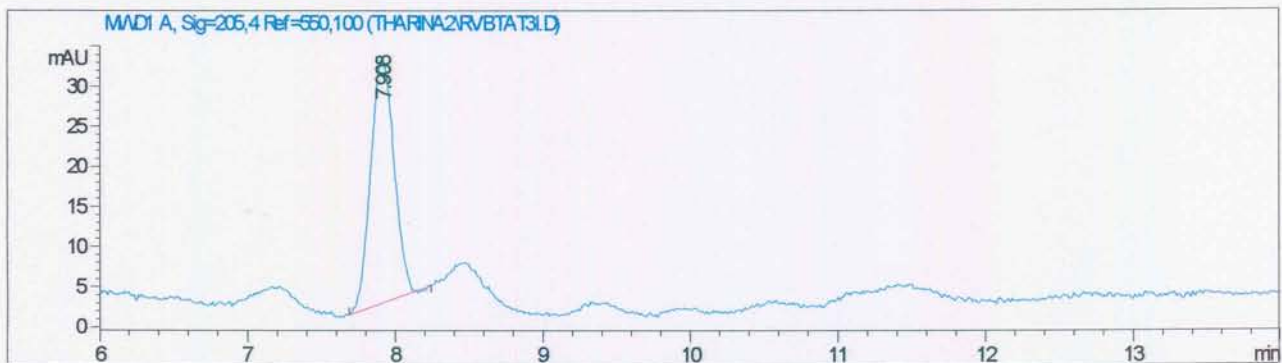
Figure 5.23: HPLC chromatogram of serum extracted 1:5 with chloroform. The serum was sampled 3 hours after ingesting 9 capsules of Tadenan® (equivalent to 54 mg β-sitosterol). The peaks are poorly defined at this extraction ratio.

5.3.2.2 (d) Stability of β-sitosterol in plasma

Serum collected during the study with Tadenan® (at t=3 hours) was used to determine whether the instability of β-sitosterol was not the reason for the lack of results. However, freezing the serum immediately or exposing it to room temperature for 2 hours before freezing, did not seem to make any difference. β-Sitosterol was not detected in any of the samples.

5.3.2.2 (e) Drying the organic phase with heat 90 °C [according to the method of Emara et al. (1999)]

After centrifugation of the serum/chloroform mixture, Emara et al. (1999) transferred the organic layer to a test tube and removed the solvent by evaporation using a temperature-regulated sand-bath adjusted to 90°C.

Serum collected after three hours in the study with Tadenan® was dried at 90 °C. The drying process took 30 minutes. However, no new peaks appeared that could be calibrated as β-sitosterol.

5.3.2.2 (f) Standard deviation

See this chapter, 5.2.3.6.

5.3.2.2 (f) (i) Standard deviation of the HPLC method

The standard deviation (s) of the HPLC method for β-sitosterol determination was 3.31 µg/ml (2.3%).

5.3.2.2 (f) (ii) Standard deviation of extraction

The standard deviation (s) of sterol extraction from serum with chloroform was 30.41%.

5.3.2.2 (g) Sensitivity of the HPLC's MWD for detecting β-sitosterol

The MWD's limit of detection for β-sitosterol was c. 2.0 µg/ml with a sample volume of 100 µl.

5.4 CONCLUSION

5.4.1 Conclusions from the stability analysis of phytosterols

The stability data provided by TLC and HPLC, regarding the stability of phytosterols against increased temperature and gamma irradiation of different intensities, was surprisingly similar. See Table 5.15 (a) and (b).

Table 5.15 (a): Similarities and differences between HPLC and TLC results of β-sitosterol (βSS), campesterol (CS) and/or stigmasterol (SS) in samples stored at 40 °C for up to 12 months.

Accelerated stability testing of phytosterols			
Plant / Product	**HPLC: βSS**	**HPLC: CS &/ or SS**	**TLC: Phytosterols incl. βSS, CS &/or SS**
P. africana ext.	Stable up to 9 months, then 22% decrease.	Stable up to 9 months, then 21% decrease.	Stable up to 9 months, then decrease.
S. repens ext	Stable up to 3 months, then gradual decrease of 20% to 12 months. 9 months: low value.	Stable up to 3 months, then gradual decrease of 26% to 12 months. 9 months?	Stable up to 12 months, but something wrong with 9-month sample.
Immunochoice®	Stable up to 12 months.	Stable up to 12 months.	Stable up to 12 months.
Moducare®	Stable up to 12 months.	Stable up to 12 months.	Stable up to 12 months.
Nutricare®	Decrease of 32% up to 12 months. Uncertainty around the β-sitosterol-peak.	_	Gradual decrease up to 12 months. "phytosterol-complex"s R_f-value different from standards.

As the same samples of *H. hemerocallidea* powder could not be used for HPLC and TLC (simultaneous determination of β-sitosterol and hypoxoside, methanol extraction, solid phase extraction *etc.*), *H. hemerocallidea* data was not included in Table 5.15. However, it was clear that

degradation of β-sitosterol and hypoxoside took place during exposure of *H. hemerocallidea* powder to increased temperature and gamma irradiation.

Table 5.15 (b): Similarities and differences between HPLC and TLC results of β-sitosterol (βSS), campesterol (CS) and/or stigmasterol (SS) in samples exposed to gamma irradiation.

Stability of phytosterols against gamma irradiation			
Plant / Product	HPLC: βSS	HPLC: CS &/ or SS	TLC: Phytosterols incl. βSS, CS &/or SS
P. africana ext.	Stable up to 27.9 kGray.	Stable up to 27.9 kGray.	Stable up to 27.9 kGray.
S. repens ext	Stable at 4.4kGray, but 53.6% decrease up to 27.9 kGay.	57.3 % Decrease at 4.4 kGray, then gradual decrease up to 79.4% at 27.9 kGray.	Gradual decrease from 0 to 27.9 kGray.
Immunochoice®	Stable up to 27.9 kGray.	Stable up to 27.9 kGray.	Stable up to 27.9 kGray.
Moducare®	Stable up to 27.9 kGray.	Stable up to 27.9 kGray.	Stable up to 27.9 kGray.
Nutricare®	21.3% Decrease after 2.3 kGray and 41.4% decrease up to 27.9kG.	–	Decrease at 2.3 kGray, but then stable.

It seems that phytosterols are stable in isolated form eg. Moducare® and Immunochoice. See chapter 2, 2.1.2 for their composition. HPLC and TLC of *P. africana* extract (5:1) indicated that it mainly contained phytosterols, and they too remained stable up to 9 months at 40 °C. On the other hand, the phytosterols in plant material (*H. hemerocallidea* powder) and products containing plant material (Nutricare®, which contains barley, lupins and fenugreek sprouts blended with phytosterol containing oilseed sprouts) decreased when exposed to 40 °C and gamma irradiation. Furthermore, the phytosterols in *S. repens* extract (4:1), which according to TLC and HPLC, also contains other compounds (fatty acids!), decreased when exposed to 40 °C and gamma irradiation. It is, therefore, possible that other compounds present in plant material catalyses the degradation of phytosterols when exposed to 40 °C or gamma irradiation. In the absence of these compounds (isolated form), the phytosterols remain relatively stable despite increased temperature or gamma irradiation. It is also possible that the stability of β-sitosterol is related to concentration, thus that a higher stability is achieved at higher concentrations. However, these conclusions should be confirmed by exposing the

herbal extract and whole herb powder of the same plant to increased temperature and gamma irradiation.

The shelf-life of *H. hemerocallidea* powder was 1.02 years at 25 °C, *P. africana* extract (5;1)'s was 3.06 years, *S. repens* extract (4:1)'s was 2.33 years and Nutricare® had a shelf-life of 1.82 years at 25 °C. The shelf-lives of Immunochoice® and Moducare® could not be calculated, because their zero order rate constants (k_0) was negative (the gradients of the graphs of β-sitosterol concentration against time, were positive). However, both remained more stable than *P. africana* extract (5:1) and their shelf-lives would probably have been longer than 3 years.

It seems that herbal extracts, and products with phytosterols in isolated form, have longer shelf-lives than herb powders. However, these shelf-lives were calculated according to the stability of β-sitosterol. Campesterol, stigmasterol, hypoxoside and the *red spot compound* stabilities were not taken into consideration. The mixture of potentially active constituents in herbal products, may have different degradation rates and stabilities. Calculating the shelf-life and expiry date can become very complex.

The stability studies were an attempt to use the developed TLC technique and the adapted HPLC method to illustrate the potential use. In an exhaustive study more determinations should be made to determine the variation (HPLC method and extraction variation) more accurately and to determine whether the differences are statistically significant.

The advantage of gamma irradiation for microbial decontamination and sterilisation of pharmaceutical raw materials is, that there is no heat transfer to the product as with heat sterilisation or pasteurisation. For sterilisation of medical and surgical equipment, a dose of 25 kGray is usually applied, but for pharmaceutical raw material a dose of 8 kGray is usually used. However, each product should be evaluated at different irradiation levels to determine the ideal dose which kills the micro-organisms without causing degradation of the actives.

For stability comparison, it is important that all the samples of the particular material are analysed at the same time, as differences in the extraction process (e.g. longer time on the horizontal shaking apparatus [See Chapter 3, 3.2.2.1 (b) (i)] *etc.* could influence the completeness of extraction and

increase the normal variation of extraction. Furthermore, the HPLC method's standard deviation is also increased if the analyses are not perfomed on the same day.

In the analysis of Nutricare®, there is uncertainty regarding the peak measured as β-sitosterol. With freshly prepared 96.5% mobile phase, a shoulder was seen to the right of the measured peak and spiking with β-sitosterol standard confirmed this shoulder's identity as β-sitosterol. However soon after the first run with freshly prepared mobile phase, this shoulder vanished under the bigger (measured) peak to the left as result of poorer resolution. In the TLC plates of Nutricare®, the "phytosterol-complex" band also had a slightly higher R_f value than that of the standards and the colour of the band was maroon and not blue-purple as with the standards. As the manufacturers claim that Nutricare® contains phytosterols, the peak/band was accepted to be β-sitosterol for the aim of this study. It is possible that the peak measured on HPLC as β-sitosterol, and identified on TLC as the "phytosterol-complex", could be a complex of modified phytosterols other than β-sitosterol, campesterol and stigmasterol. This product should be examined further.

TLC on Nutricare® extracted with acetone, indicated the *red spot compound* to be unstable when exposed to irradiation. A decrease in the intensity of the red spot on TLC was noticed in samples irradiated even at mild intensities such as 2.3kGray. This corresponds with results regarding the instability of the *red spot compound*'s after isolation, as discussed at a later stage.

5.4.2 Conclusions from the bioavailability analysis of phytosterols

The sensitivity of TLC was remarkably similar to that of HPLC. The detection limit of TLC to visualise β-sitosterol was between 0.2 and 0.5 μg and the detection limit of the HPLC's MWD for β-sitosterol was 2.0 μg/ml with a sample volume of 100 μl. Thus, the detection limit of the HPLC for β-sitosterol in mass quantity was 0.2 μg.

Unfortunately, TLC could not separate the phytosterols from cholesterol. Chloroform extracts phytosterols and cholesterol, but the cholesterol contents of serum is much higher than the β-sitosterol contents (50% absorption vs. 5% absorption, See Chapter 1, 1.2) Therefore, chloroform extracts much more cholesterol from serum than β-sitosterol. The "phytosterol-complex" band seen in the unspiked control on the TLC plate in Figure 5.20 is cholesterol and not phytosterols, as

confirmed with HPLC. Thus, as TLC cannot distinguish between cholesterol and β-sitosterol, β-sitosterol in serum cannot be determined with TLC.

Using the method of Emara et al (1999) with MWD, did not detect any β-sitosterol in serum. Nothing was measured over the 9-hour period in the serum of any of the three volunteers in the pharmacokinetic study with Moducare® (180 mg β-sitosterol ingested). Not even after a dosage of 400mg β-sitosterol in the form of 20 capsules of Moducare®, did a β-sitosterol peak appear. To exclude the possibility of poor bioavailability from Moducare®, Tadenan® as used by Emara et al. (1999), was investigated. However, even 9 capsules of Tadenan®, which was three times more than Emara et al. (1999) applied, did not provide a β-sitosterol peak on HPLC.

Attempts to improve the method of extraction of Emara et al. (1999)'s of β-sitosterol from serum proved unsuccessful in detecting β-sitosterol. However, an extraction ratio of serum to chloroform of 1:1 instead of 1:5, definitely improved the appearance of other peaks on the HPLC chromatogram.

To ascertain whether Emara et al. (1999) did not perhaps measure a degradation product from a serum compound, which originated from drying the organic phase at 90 °C, serum samples were dried in an oven at 90 °C, but still no peak at the β-sitosterol retention time could be detected.

Emara et al (1999) reported that their HPLC method was sensitive enough to detect with preciseness, a lower limit of 1.0 μg β-sitosterol/ml serum. In their study the serum β-sitosterol concentrations ranged from 3.81 to 16.20 μg/ml with Tadenan® and the mean maximum β-sitosterol serum concentration (C_{max}) was 9.80 μg/ml. Tadenan®'s time of maximum absorption (t_{max}) was 2.8 hours and the half-life ($t_{1/2}$) was 2.53 hours. They reported the relative bioavailability of β-sitosterol based on the C_{max} as 88.16%, and the overall bioavailability judged from the area under the curve (AUC_{0-8}), as 102.02%. However, Salen et al. (1970) found that the human absorption of β-sitosterol was 5% or less of the daily intake. In their study, plasma concentrations of β-sitosterol ranged from 3 to 10 μg/ml after a daily fat intake typical of the American diet.

Ritschel et al. (1990) found the absolute bioavailability of β-sitosterol in beagle dogs after oral administration to be 9%.

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Apart from the article by Emara et al. (1999), none of the other publications (Gould et al., 1969; Salen et al., 1970; Lees et al., 1977; Salen et al., 1989; Ritschel et al., 1990) on the quantification of phytosterols *in vivo*, applied HPLC (with MWD) as analysis method. The reason is probably because the phytosterols do not contain a chromophore ensuring sufficient sensitivity of the MWD. In these studies, the phytosterols were radio-labeled and quantified with a liquid scintillation system. In two other studies in which phytosterols, stanols and cholesterol metabolites (Phillips et al., 1999) and phytosterols and 7-ketocholesterol (Dyer et al., 1995) were determined in serum, capillary gas chromatography was used for analysis. The possibility exists that Emara et al. (1999) quantified a contaminant similar to the one encountered in the solvents stored in plastic containers. See Chapter 4, 4.3.3.1.

As part 1 of the planned clinical trial was unsuccessful, due to method insensitivity, part 2 and 3 could not be performed.

5.4.3 General conclusions on TLC and HPLC

From Table 5.15 one can conclude that TLC can be used as a simple, reproducible and cost-effective method to qualitatively indicate the stability of phytosterols in *H. hemerocallidea* and related herbal medicine. However, TLC can only be used to indicate the general stability of the phytosterols, as it cannot differentiate between β-sitosterol, campesterol, stigmasterol (or cholesterol). Therefore, TLC cannot be used to determine β-sitosterol in serum for bioavailability determination, but as discussed, HPLC-MWD is also not effective.

However, a number of reasons exist to use TLC to indicate qualitatively the stability of phytosterols.

1. TLC is a cheap and simple method and approximately fifteen samples can be analysed at the same time and results of different samples on the same plate can easily be compared.

2. All the compounds that separate are visible with TLC, whereas in HPLC compounds with retention times longer than the run time, are not detected. See for example Figure 5.1 (b). The "phytosterol-complex" in the *S. repens* sample exposed to 40 °C for 9 months, decomposed, and the degradation product is visible on TLC, but its retention time was longer than the HPLC run time of 14 minutes and was, therefore, not detected.

3. Crude plant extracts can be applied on TLC without the risk of blocked columns and time-consuming clean up procedures such as SPE are unnecessary. However, these complex extracts do not always separate completely.

4. The apparatus and solvents (analytical grade) used for TLC are cheap in comparison with the expensive HPLC systems and HPLC grade solvents.

5. TLC is technologist-friendly and does not need supervision from a superior.

However, HPLC also has advantages.

1. TLC is not quantitative, whereas HPLC is highly quantitative.

2. TLC does not separate compounds with very similar structures e.g. phytosterols, whereas HPLC can separate them (depending on the mobile phase).

3. HPLC systems equipped with an auto-sampler can analyse many samples overnight, without supervision, whereas ordinary TLC has to be performed manually.

CHAPTER 6

COLUMN CHROMATOGRAPHY FOR ISOLATION

6.1   INTRODUCTION

6.1.1   Origin and interest in the *red spot compound*

When TLC plates with acetone and methanol extracts of *H. hemerocallidea* and sprayed with *p*-anisaldehyde were overheated by accident, a red spot with a R_f-value of c. 0.58 in mobile phase CEF, was noticed. The same red band was also seen in the acetone extracts of *P. africana, S. repens,* Moducare®, Harzol®, Immunochoice® and Nutricare®, when the plates were overheated. As the 95% pure stigmasterol standard at high concentrations (10 mg/ml) had a similar red band (below the stigmasterol band) with the same R_f-value, it was suspected that the *red spot compound*, could be stigmasterol glucoside (stigmasterolin). It was also attempted to hydrolyse the "glucoside" with hydrochloric acid to the sterol. Although "stigmasterolin" in stigmasterol standard disappeared after acid hydrolysis, the *red spot compound* in *H. hemerocallidea* remained unchanged. See Chapter 3, 3.3.4.4.

African potato tea has substantial positive feed-back according to the distributors, but phytosterols and sterolins were absent in the *H. hemerocallidea* water extract. The *red spot compound* however, was extracted from *H. hemerocallidea* with water. The *red spot compound* was also present in relatively high concentrations in *S. repens,* whereas the phytosterol concentration of this potent BPH phytotherapeutic, was very low. See Chapter 3, 3.3.4.1 (b). The possibility exists that the *red spot compound* may be an active compound and, therefore, it was decided to isolate and attempt structure elucidation of this compound.

6.1.2   Aim of chapter

To isolate and elucidate the chemical structure of the *red spot compound.*

6.2 MATERIAL AND METHODS

6.2.1 Material

H. hemerocallidea powder (African potato 1), stigmasterol standard and β-sitosterolin, as described in Chapter 2, 2.1 and 2.2, were used.

6.2.2 Methods

6.2.2.1 Extraction

6.2.2.1 (a) Simple extraction

Simple extraction, as described in Chapter 3, 3 2.2.1 (b) (i), was modified for a large quantity. *H. hemerocallidea* powder (100 g) was extracted twice with 1.0 liter acetone by shaking the flask on a horizontal shaking machine for 30 minutes at a time. The supernatant of the two fractions was filtered with a Buchner funnel under vacuum and combined. The acetone was removed from the filtrate under vacuum in a Büchi Rotavapor R-114 with a KNF Neuberger Laboport vacuum pump. The precipitate (c. 2.7 g) was dissolved in c. 10 ml ethylacetate:methanol:water (EMW) in the ratios 10:1.35:1, for a concentrated solution. The presence of the *red spot compound* in the sample was confirmed by TLC (on glass plates), with CEF mobile phase, *p*-anisaldehyde spray reagent and overheated at 110 °C as described in Chapter 3, 3.2.2.3.

6.2.2.2 Preparation of standards

The standards were dissolved in acetone.

6.2.2.3 Isolation of the *red spot compound*

6.2.2.3 (a) Extract fractionation with column chromatography

For the first fractionation, a column (90 cm x 2,5 cm, packed height = c. 86 cm) was packed with 200 g Macherey-Nagel silica gel 60 (0.063 – 0.2 mm, density = 0.56 g/cm^3) suspended in EMW. The *H. hemerocallidea* sample from 6.2.2.1 (a) was applied and eluted with EMW as mobile phase. Fractions of 18 ml (c. 150 fractions) were collected by a Foxy® Junior fraction collector.

6.2.2.3 (b) Examination of fractions with thin layer chromatography (TLC)

TLC was performed as described in Chapter 3, 3.2.2.3. As the *red spot compound* was investigated, glass plates were used. Every fourth fraction was applied (10 μl volumes) on a glass plate, developed in chloroform:ethylacetate:formic acid (CEF) in the ratios 5:4:1, sprayed with *p*-anisaldehyde and overheated. The fractions containing the *red spot compound* was identified and chromatographed again, but this time the three fractions in between were applied on the plate as well. Three fractions that contained the *red spot compound* were identified, but these fractions were not pure. The fraction containing the highest concentration of *red spot compound* was dried under vacuum in a Büchi Rotavapor R-114 with a KNF Neuberger Laboport vacuum pump. The residue (21 mg) was redissolved in c. 2 ml CEF and refractionated.

6.2.2.3 (c) Second fractionation with column chromatography

For the second fractionation a short column (13 cm x 2 cm, packed height = c. 8 cm) was packed with Merck silica gel 60 (0.040 – 0.063 mm, density = 0.67 g/cm^3) suspended in CEF. The *H. hemerocaliidea* sample from 6.2.2.3 (b) was applied and eluted with CEF. Fractions of 6 ml (c. 50 fractions) were collected by a Foxy® Junior fraction collector.

6.2.2.3 (d) Examination of fractions with thin layer chromatography (TLC)

All the fractions were separated by TLC and the fractions containing the *red spot compound* were identified and combined. The combined fractions were dried under vacuum in a Büchi Rotavapor R-114 with KNF Neuberger Laboport vacuum pump and sent for NMR analysis.

6.2.2.4 Structure elucidation with Nuclear Magnetic Resonance Spectroscopy (NMR)

The isolated compound, 95% stigmasterol standard and β-sitosterolin were analysed by Dr. J. Coetzee, SASOL Technology, Research and Development.

A 500 MHz Bruker Avance NMR spectrometer was used for proton (^{1}H) and carbon (^{13}C) analyses of the isolated *red spot compound*, and ^{1}H analysis of the standards. The sample and standards were dissolved in deuterated acetone for NMR analyses.

6.3 RESULTS

6.3.1 Extraction

The acetone extract of *H. hemerocallidea* powder (African potato 1) was dried and redissolved in ethylacetate:methanol:water (10:1.35:1) as this was the mobile phase for the first column. The precipitate did not dissolve completely in EMW, but the final concentration was c. 200 mg/ml. TLC confirmed the presence of the *red spot compound* in the sample. See Figure 6.1 for the *red spot compound* in African potato 1 acetone extract, as well as other important phytosterol containing products, that also contain the *red spot compound*.

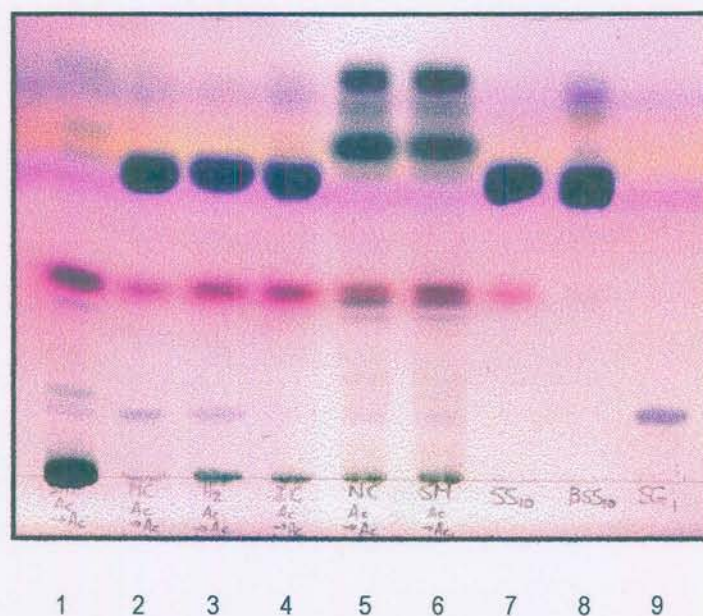**Figure 6.1:** TLC on a glass plate of acetone extracts of African potato 1 (1), Moducare®(2), Harzol®(3), Immunochoice®(4), Nutricare®(5) and Nutricare®'s sterol mixture without inactives (6). On the right are 10 mg/ml 95% stigmasterol standard (7), 10 mg/ml 40% β-sitosterol standard (8) and 1 mg/ml β-sitosterolin (9). The plate was developed in CEF and sprayed with *p*-anisaldehyde.

6.3.2 Isolation of the *red spot compound*

The *red spot compound* eluted early from the first column and when every fourth fraction was chromatographed on TLC, it was noticed in fraction 11. At very high concentrations the colour became more brown than red. See Figure 6.2.

Figure 6.2: TLC on a glass plate of every fourth fraction (from 1 to 67) of the acetone extract of *H. hemerocallidea* that eluted from the first column with ethylacetate:methanol:water (10:1.35:1) as mobile phase. The plate was developed in CEF and sprayed with *p*-anisaldehyde. See the *red spot compound* in fraction 11.

Fractions 1 to 15 were chromatographed on TLC and the *red spot compound* was present in fractions 10 to 14. However, these fractions were not pure and contained another blue band (R_f = 0.18) together with the *red spot compound* (R_f = 0.43). See Figure 6.3.

Figure 6.3: TLC on a glass plate of the first fifteen fractions of the acetone extract of *H. hemerocallidea* that eluted from the first column with ethylacetate:methanol:water (10:1.35:1) as mobile phase. See the *red spot compound* ($R_f = 0.43$) in fractions 10 to 14 along with a blue band ($R_f = 0.18$). The plate was developed in CEF and sprayed with *p*-anisaldehyde.

By means of the isolated β-sitosterolin, the blue band in fraction 12 was identified. See Figure 6.4.

1   2   3

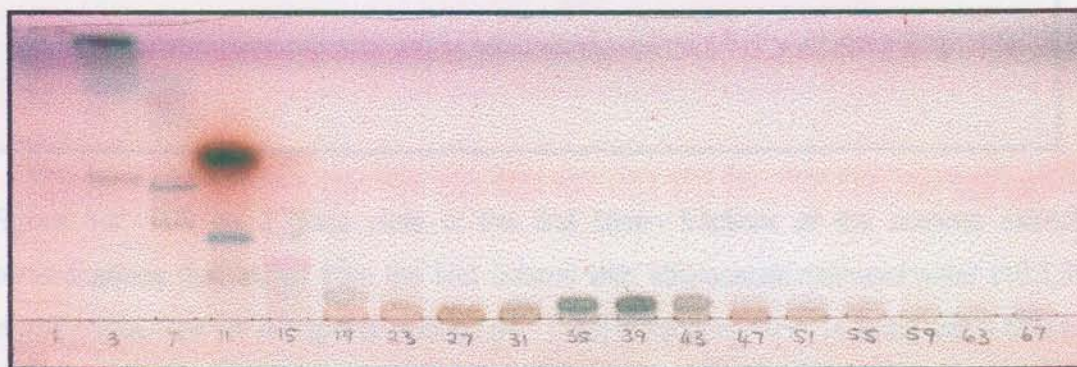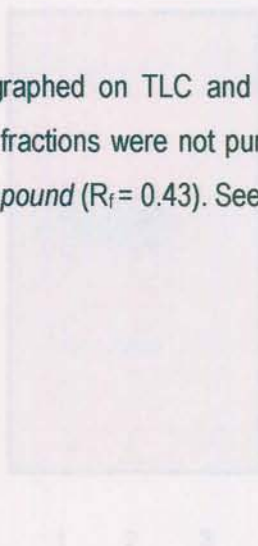**Figure 6.4:** TLC on a glass plate of fraction 12 (1) of the acetone extract of *H. hemerocallidea* that eluted from the first column with ethylacetate:methanol:water (10:1.35:1) as mobile phase. Co-chromatographed is fraction 12 spiked with β-sitosterolin (2), and isolated β-sitosterolin (3). The plate was developed in CEF and sprayed with *p*-anisaldehyde.
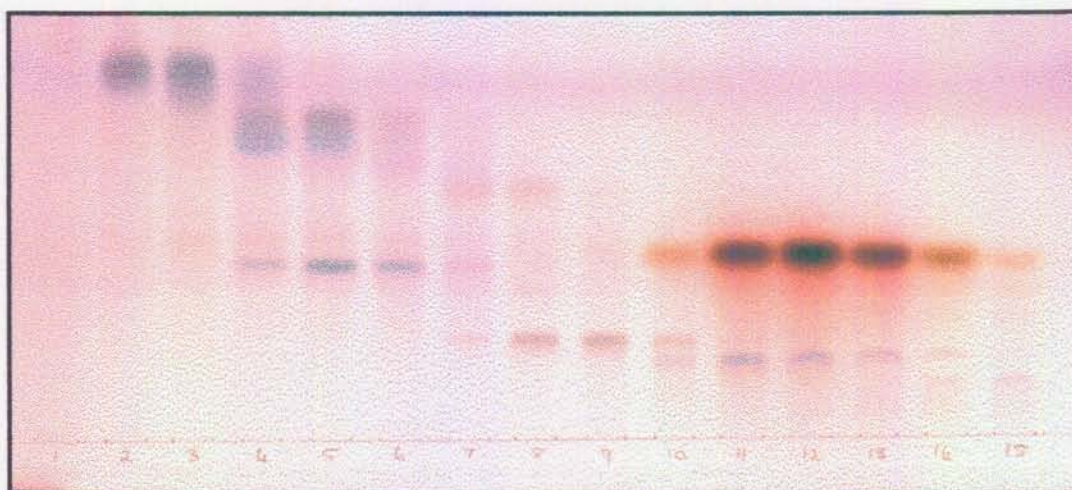
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In fraction 12, the intensity of the *red spot compound* was the highest, and therefore it was fractionated again to separate the *red spot compound* from β-sitosterolin. Fraction 11 and 13 were refrigerated and kept as a back-up. Again the *red spot compound* eluted early from the second column. All the fractions were chromatographed on TLC and the *red spot compound* was visible in fractions 2 and 3. β-sitosterolin was separated and present in fractions 7 and 8. See Figure 6.5.

Fractions 2 and 3 from the second column were combined, dried and sent for structure elucidation at SASOL.
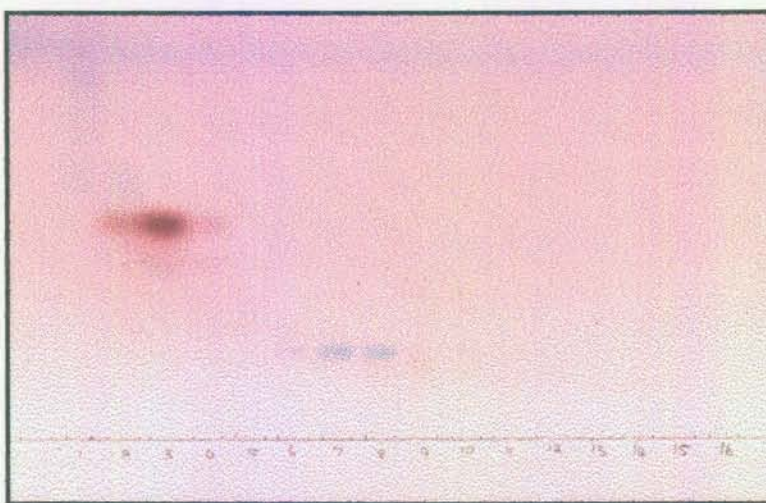
Figure 6.5: TLC on a glass plate of the first sixteen fractions of the *H. hemerocallidea* extract that eluted from the second column with chloroform:ethylacetate:formic acid (5:4:1) as mobile phase. See the *red spot compound* in fractions 2 and 3 and β-sitosterolin in fractions 7 and 8. The plate was developed in CEF and sprayed with *p*-anisaldehyde.

6.3.3 NMR report by Dr. Coetzee, SASOL

The ^{1}H NMR spectrum of this compound displays signals in the aromatic region between δ 8.10 and 8.25 together with several signals between 3.5 and 5.0 ppm, which is typical of protons from a sugar. The above observations are confirmed by the appearance of carbinol signals around 70 ppm and phenolic signals between 110 and 140 ppm in the ^{13}C NMR spectrum. The ^{13}C NMR spectrum

displays further the presence of a coumarin type lactone carbonyl (typical value 160.6 ppm) at 161.4 ppm.

The compound unfortunately decomposed in solution (deuterated acetone) before further NMR work could be done to elucidate the structure. The compound is accompanied by other glycosides from the same column fraction and displays a very distinct red colour on TLC when sprayed with *p*-anisaldehyde. Coumarins represent the fully oxidised state of the isoflavanoids and both classes of compounds display characteristic fluorescence under UV irradiation. Collective consideration of the above data led to the conclusion that this compound can be either a coumarin or an isoflavanoid with a sugar unit attached to it.

See Appendix A for the ^{1}H and ^{13}C NMR spectra of the *red spot compound* and for the reference ^{1}H spectra of β-sitosterol, campesterol and stigmasterol, as obtained from SASOL's ACD/HNMR Dictionary.

6.4 CONCLUSION

The developed separation method with column chromatography was successful in isolating the *red spot compound*. According to the NMR spectra, impurities were present, but the sample was pure enough for NMR analysis.

The fact that the three fractions obtained from the first column contained the *red spot compound* and β-sitosterolin (β-sitosterol glucoside), supported the suspicion that the *red spot compound* could be stigmasterolin (stigmasterol glucoside), as similar structures will have similar elution patterns. Stigmasterol standard and the isolated β-sitosterolin were supplied to the chemists at SASOL for comparison of the standards' NMR spectra with that of the sample. However, there was no match between the NMR spectra of stigmasterol glucoside and the isolated *red spot compound*.

Dr. Coetzee concluded that the compound could be either a coumarin or an isoflavanoid with a sugar unit attached to it. Dr. David Katerere (Phytopharmacology Laboratory, University of Pretoria), consulted for a second opinion, was not convinced that the compound was a coumarin and felt that two-dimensional experiments (COSY *etc.*) were required. He thought that the sugar moiety could be a rhamnose due to the sharp peak at 1.2 ppm (^{1}H spectra), which represents a methyl group.

The back-up fractions from the first column, fractions 11 and 13 (that contained β-sitosterolin as well) were purified through another column, packed as described in 6.2.2.2 (c). Unfortunately, the *red spot compound* in these samples decomposed as well. After fractionation of the two back-up fractions, TLC was performed and the band intensity of the *red spot compound* was very poor in both. A new band, which could have been decomposition metabolites of the *red spot compound,* appeared in the two following fractions.

The decomposition may have been caused by traces of formic acid, left in the dried residue from the chloroform:ethylacetate:formic acid (5:4:1) eluant of the second column. In future work to elucidate the structure, another solvent system may have to be used. The fraction containing the *red spot compound* may have to be neutralised with ammonia to form the volatile ammonium formiate before drying. Alternatively freeze drying followed by two or three steps of redissolving of the extract in water and freeze drying, should be attempted.

In any case the *red spot compound* does not appear to be a steroid compound or related to the sterols or sterolins.

CHAPTER 7

CONCLUSION

The aim of this study was to develop and apply TLC and HPLC methods for the qualitative and quantitative analyses of sterols and sterolins in three BPH phytotherapeutics (*Hypoxis hemerocallidea, Prunus africana* and *Serenoa repens*) and related products.

A TLC analysis method was developed for good visibility and separation of phytosterols and sterolins. This method was used to qualitatively compare the phytosterol and sterolin contents of three BPH phytotherapeutics and a variety of phytosterol-containing commercial products. By using different extractants, other important actives e.g. hypoxoside, could also be investigated with the same TLC method (other mobile phase) thus saving cost. The composition of the phytotherapeutics, revealed by TLC, corresponded with literature data and confirmed previous reported results. The developed TLC method could unfortunately not separate any of the sterols (β-sitosterol, campesterol and stigmasterol) tested and only general phytosterol content, was indicated. This technique presents the natural medicine industry with a fast, affordable and reliable method of qualitatively controlling phytosterol levels in herbal products.

Our results indicated that *H. hemerocallidea* powder contained sterols, sterolins (including the *red spot compound*) and hypoxoside; *P. africana* extract (5:1) contained a very high concentration of sterols and a low concentration of β-sitosterolin and the *red spot compound*, but *S. repens* extract (4:1) contained hardly any sterols, a low concentration β-sitosterolin and a relatively high concentration of the *red spot compound* (lower than *H. hemerocallidea* powder).

Although *p*-anisaldehyde was preferred as spray reagent, vanillin was also shown to indicate sterolins if present in high levels whereas Eloff (1999) found, in his study, that vanillin did not reveal the sterolins. It was also demonstrated that the type of TLC plate could influence the visibility of compounds and that overheating the plate is not always negative. An interesting compound, the *red spot compound*, was noticed when glass plates, were overheated. This compound is present in acetone, methanol and water extracts of *H. hemerocallidea* and in the acetone extracts of *P. africana, S. repens*, Moducare®, Harzol®, Immunochoice® and Nutricare®. This general presence of

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the *red spot compound* in almost all important phytosterol containing products/herbs, could point to a possible important role/function. An attempt was made to isolate the *red spot compound* with PTLC using the developed TLC method. This was unsuccessful as the separation on the thicker preparative plate (2 mm) was not as good as on the ordinary glass TLC plates (0.25 mm). A column chromagraphy method was successfully developed and applied to isolate the compound. From the proton and carbon NMR spectra, it was concluded, that the compound was definitely not a steroid and could either be a coumarin or an isoflavanoid, with a sugar unit (possibly a rhamose) attached to it. Further analyses to elucidate the structure failed due to decomposition of the compound. Further work on the structure elucidation is required, possibly using different eluants for column chromatography or drying the relative fractions. The possible therapeutic activity of the *red spot compound,* with regards to the immune system and BPH, should also be investigated, especially if this is a novel compound.

The HPLC method of Emara et al. (1999) to determine the "bioavailability of β-sitosterol from *Pygeum africanum* extract in humans", was used in a new application to quantitatively determine phytosterols in plant extracts. The column, wavelength and mobile phase used by Emara et al. (1999) were confirmed to be acceptable for separation and detection of sterols with HPLC. Unfortunately, it was not possible to separate campesterol and stigmasterol with this method. Although the retention time of β-sitosterolin could be determined, it was never detected in any of the samples analysed with Emara et al. (1999)'s HPLC method. This is in contrast with the developed TLC method with which β-sitosterolin could be detected in acetone and chloroform extracts of *H. hemerocallidea etc.* The method of Emara et al. (1999) is probably not sensitive enough to detect the low quantities of β-sitosterolin present in plants.

Hypoxoside is usually analysed by HPLC with a UV detector at 260 nm. Hypoxoside, however, fluoresces, and as quantification with the multiple wavelength ultraviolet detector was not selective (early retention time and peak overlap with solvent peaks *etc.*), the more selective and more sensitive fluorescence detector was applied. At an excitation wavelength of 230 nm, hypoxoside emits UV light of 345 nm.

A SPE method was developed for isolation of hypoxoside from the crude methanol extract for its determination by HPLC. This SPE method could be used to clean up methanol extracts for the simultaneous determination of hypoxoside and β-sitosterol. This adapted HPLC method, using

fluorescence to determine hypoxoside, is a new and sensitive method for determining this compound in plant and related products.

A complication of using extractants, stored in plastic containers, was experienced and analysts should take caution when analysing phytosterols with the HPLC method of Emara et al. (1999), as a contaminant with exactly the same retention time as β-sitosterol was detected in these solvents.

The sensitivity of the developed TLC method and the HPLC method (with MWD) of Emara et al. (1999) for β-sitosterol were similar. However, during the pilot study of the planned clinical trial, the method of Emara et al. (1999) was found not sensitive enough to determine β-sitosterol in serum. Notwithstanding improvements made to the method i.e. changing the extraction ratio and experimenting with higher dosages and different products, the method of Emara et al. (1999) for β-sitosterol determinations in serum, could not be confirmed. Emara et al. (1999) used the method to determine bioavailability of β-sitosterol from *Pygeum africanum* extract in humans, but none of the other published studies on quantification of β-sitosterol in serum, used HPLC (with MWD). The reason is probably because the phytosterols do not contain a chromophore ensuring sufficient sensitivity of the MWD. Emara et al. (1999) reported the overall bioavailability of β-sitosterol, judged from the area under the curve (AUC_{0-8}), as 102.02%. However, Salen et al. (1970) found that the human absorption of β-sitosterol was 5% or less of the daily intake and Ritschel et al. (1990) found the absolute bioavailability of β-sitosterol in beagle dogs after oral administration to be about 9%.

As result, the proposed clinical trial to compare the bioavailability of β-sitosterol from phytosterol containing South African and European products could not be performed. In a future study this bio-equivalence study should still be performed - serum could be analysed by means of gas chromatography. Either of two methods, in which phytosterols, stanols and cholesterol metabolites (Phillips et al., 1999) or phytosterols and 7-ketocholesterol (Dyer et al., 1995) were determined in serum with capillary gas chromatography, could be used.

TLC and HPLC analysis of the water extract of *H. hemerocallidea*, prepared in the same way as African potato tea (commonly marketed and sold for medicinal uses), indicated that it contained hypoxoside, but no β-sitosterol or β-sitosterolin, could be detected. This confirms the finding by Eloff (1999) that water does not extract sterols or sterolins. The implication of this finding is that β-sitosterol, which is accepted to be the active of *H. hemerocallidea*, might not be the main active in African potato tea. The anti-cancer effects of rooperol (*in vivo* metabolite of hypoxoside, which could

be shown to be present in the water extract) is known, but the possibility that the *red spot compound* (which is also extracted by water, according to TLC) might have medicinal value, should also be further investigated.

Little is known regarding the stability of phytosterols subjected to increased temperature and gamma irradiation. Up to now, the lack of good or reliable methods to do the necessary quality control has restricted the natural medicine industry in this regard, with the patient at the end of the day possibly being the victim of inferior quality products. The developed TLC method was applied to indicate the stability of phytosterols and the results found, were confirmed with the adapted HPLC method. Phytosterols in isolated form were more stable when subjected to increased temperature and gamma irradiation than the phytosterols in plant material. Possible reasons for this are that other compounds present in the plant material might catalyse the degradation of the phytosterols when exposed to increased temperature or gamma irradiation and that in the absence of these compounds (isolated form), the phytosterols remain relatively stable despite increased temperature or gamma irradiation. It is also possible that the stability of β-sitosterol is related to concentration, thus that a higher stability is achieved at higher concentrations. However, these conclusions should be confirmed by analyses of the herbal extract and whole herb powder of the same plant, exposed to increased temperature and gamma irradiation. The data on the stability of β-sitosterol from the accelerated stability testing, were used to determine the shelf-lives of the phytosterol products and BPH phytotherapeutic plants. It appears that phytosterols in isolated form, have longer shelf-lives than phytosterols in herbal powders. However, as with all herbal medicine, difficulty remains in determining the expiry date of these materials, as β-sitosterol might not necessarily be the only active.

The sterols and sterolins in *H. hemerocallidea* and related herbal medicine can be qualitatively and quantitatively analysed with the developed TLC and adapted HPLC methods. This provides natural medicine industry with necessary procedures to ensure proper quality, safety and stability and opens the door for future research in this very important field.

"For now we are looking in a mirror that gives only a dim reflection, but then we shall see in reality and face to face! Now I know in part; and then I shall know and understand fully and clearly…"
- 1 Corinthians 13:12 (Amplified New Testament).

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REFERENCES

ALBRECT, C.F., KRUGER, P.B., SMIT, B.J., FREESTONE, M., GOUWS, L., MILLER, R. & VAN JAARSVELD, P.P. 1995. The pharmacokinetic behaviour of hypoxoside taken orally by patients with lung cancer in a phase I trial. *South African Medical Journal*, 85, 861-865.

ALBRECHT, C.F., THERON, E.J. & KRUGER, P.B. 1995. Morphological characterisation of the cell-growth inhibitory activity of rooperol and pharmacokinetic aspects of hypoxoside as an oral prodrug for cancer therapy. *South African Medical Journal*, 85, 853-860.

ANON. 2000. Plant fats proven to bolster the immune system. *Internet:* http://www.naturaltrends.com./prod03.htm. Access: 31 Jan 2000.

ARNOLD, T.H. & DE WET, B.C. 1993. *Plants of Southern Africa: names and distribution*. Pretoria: National Botanical Institute.

BAYNE, C.W., DONNELLY, F., ROSS, M. & HABIB, F.K. 1999. *Serenoa repens* (Permixon®): A 5α-reductase types I and II inhibitor – New evidence in a coculture model of BPH. *The Prostate*, 40, 232-241.

BERGES, R.R., WINDELER, J. & TRAMPISCH, H. 1995. Randomised placebo-controlled double blind clinical trial of beta-sitosterol in patients with benign prostatic hyperplasia. *Lancet*, 345, 1529-1532.

BOUIC, P.J.D. 1997. Immunomodulation in HIV/AIDS: The Tygerberg /Stellenbosch University Experience. *AIDS Bulletin*, 6 (3), 18-20.

BOUIC, P.J.D. & LAMPRECHT, J.H. 1999. Plant sterols and sterolins: A review of their immune-modulating properties. *Alternative Medicine Review*, 4 (3), 170-177.

BOUIC, P.J.D., ETSEBETH, S., LIEBENBERG, R.W., ALBRECHT, C.F., PEGEL, K. & VAN JAARSVELD, P.P. 1997. Beta-sitosterol and beta-sitosterol glucoside stimulate human peripheral blood lymphocyte proliferation: implications for their use as an immunomodulatory vitamin combination. *International Journal of Immunopharmacology*, 18 (12), 693-700.

CANNELL, R.J.P. 1998. *Natural products isolation*. Totowa: Humana Press Inc.

CARRARO, J.C., RAYNAUD, J.P., KOCH, G., CHISHOLM, G.D., DI SILVERIO, F., TEILLAC, P., DA SILVA, F.C., CAUQUIL, J., CHOPIN, D.K., HAMDY, F.C., HANUS, M., HAURI, D., KALINTERIS, A., MARENCAK, J., PERIER, A. & PERRIN, P. 1996. Comparison of phytotherapy (Permixon®) with finasteride in the treatment of benign prostate hyperplasia: a randomized international study of 1098 patients. *Prostate*, 29 (4), 231-240.

CHATELAIN, C., AUTET, W. & BACKMAN, F.1999. Comparison of once and twice daily dosage forms of *Pygeum africanum* extract in patients with benign prostatic hyperplasia: a randomized, double-blind study, with long-term open label extension. 1999. *Urology,* 54 (3), 473-478.

DE SILVERIO, F., MONTI, S., SCIARRA, A., VARASANO, P.A., MARTINI, C., LANZARA, S., D'ERAMO, G., DI NICOLA, S. & TOSCANO, V. 1998. Effects of long-term treatment with *Serenoa repens* (Permixon®) on the concentrations and regional distribution of androgens and epidermal growth factor in benign prostatic hyperplasia. *The Prostate,* 37, 77-83.

DONALD, P.R., LAMPRECHT, J.H., FREESTONE, M., ALBRECHT, C.F., BOUIC, P.J.D., KOTZE, D. & VAN JAARSVELD, P.P. 1997. A randomised placebo-controlled trial of the efficacy of beta-sitosterol and its glucoside as adjuvants in the treatment of pulmonary tuberculosis. *The International Journal of Tuberculosis and Lung Disease,* 1 (5), 518-522.

DYER, R.G., HETHERINGTON, C.S., ALBERTI, K.G.M.M. & LAKER, M.F. 1995. Simultaneous measurement phytosterols (campesterol and β-sitosterol) and 7-ketocholesterol in human lipoproteins by capillary column gas chromatography. *Journal of Chromatography B,* 663, 1-7.

ELOFF, J.N. 1998. The presence of antibacterial compounds in *Anthocleista grandiflora* (Loganiaceae). *South African Journal of Botany,* 64, 209-212.

ELOFF, J.N. 1999. A preliminary investigation into the composition of the compounds present in Immunochoice® and Moducare®. Confidential report to BIOMOX PHARMACEUTICALS (Pty) Ltd.

EMARA, L.H., EL-MENSHAWI, B.S. & ESTEFAN, M.Y. 1999. Bioavailability of β-sitosterol from *Pygeum africanum* extract in humans. *Pharmaceutical and Pharmacological Letters,* 9 (2), 80-83.

FITZPATRICK, J.M. 1999. Phytotherapy for treatment of benign prostatic hyperplasia: case not proven. *Urology,* 53, 462-464.

GERBER, G.S., ZAGAJA, G.P., BALES, G.T., CHODAK, G.W. & CONTRERAS, B.A. 1998. Saw palmetto (*Serenoa repens*) in men with lower urinary tract symptoms: effects on urodinamic parameters and voiding symptoms. *Urology,* 51 (6), 1003-1007.

GOEPEL, M., HECKER, U., KREGE, S., RUBBEN, H. & MICHEL, M.C. 1999. Saw palmetto extracts potently and noncompetitively inhibit human α_1-adrenoceptors *in vitro. The Prostate,* 38, 208-215.

GOULD, R.G., JONES, R. J., LEROY, G.V., WISSLER, R.W. & TAYLOR, C.B. 1969. Absorbability of β-sitosterol in humans. *Metabolism,* 18 (8), 652- 662.

GUPTA, M.B., NATH, R., SRIVASTAVA, N., SHANKER, K., KISHOR, K. & BHARGAVA, K.P. 1988. Anti-inflammatory and antipyretic activities of β-sitosterol. *Journal of Medicinal Plant Research,* 39, 157-163.

HAHN-DEINSTROP, E. 2000. *Applied thin layer chromatography.* Weinheim: Wiley-VCH.

HARDMAN, J.G. & LIMBIRD, L.E. 1996. *Goodman and Gilman's pharmacological basis of therapeutics.* 9th ed. New York: The McGraw-Hill Companies, Inc.

HOUGHTON, P.J. & RAMAN, A. 1998. *Laboratory handbook for the fractionation of natural extracts.* London: Chapman & Hall.

INGRAHAM, J.L. & INGRAHAM, C.A. 1995. *Introduction to microbiology.* Belmont: Wadsworth Publishing Company.

IVORRA, M.D., D'OCON, M.P., PAYA, M. & VILLAR, A. 1988. Antihyperglycemic and insulin-releasing effects of β-sitosterol 3-β-D-glucoside and its aglycone, β-sitosterol. *Archives Internationales de Pharmacodynamie et de Therapie,* 296, 224-231.

KADOW, C. & ABRAMS, P.H. 1986. A double-blind trial of the effect of beta-sitosteryl glucoside (WA184) in the treatment of benign prostatic hyperplasia. *European urology,* 12, 187-189.

KLIPPEL, K.F., HILTL, D.M. & SCHIPP, B. 1997. A multicentric, placebo-controlled, double-blind clinical trial of β-sitosterol (phytosterol) for the treatment of benign prostatic hyperplasia. *British Journal of Urology,* 80, 427-432.

KOCH, H.P. & BROSCH-STARZENGRUBER, N.B. 1991. Biopharmaceutical investigation of hypoxoside, a natural antitumor compound from plant origin. *Journal of Biopharmaceutical Sciences,* 2 (4), 305-318.

KROSCHWITZ, J.I. & HOWE-GRANT, M. 1997. *Kirk-Othmer encyclopedia of chemical technology.* 4th ed. New York: John Wiley & Sons, Inc.

KRUGER, P.B., ALBRECHT, C.F., LIEBENBERG. R.W., VAN JAARSVELD, P.P. 1994. Studies on hypoxoside and rooperol analogues form *Hypoxis rooperi* and *Hypoxis latifolia* and their biotransformation in man by using high-performance liquid chromatography with in-line sorption enrichment and diode-array detection. *Journal of Chromatography B,* 662, 71-78.

LEES, A.M., MOK, H.Y.I., LEES, R.S., MCCLUSKEY, M.A. & GRUNDY, S.M. 1977. Plant sterols as cholesterol-lowering agents: clinical trials in patients with hypercholesterolemia and studies of sterol balance. *Atherosclerosis,* 28, 325-338.

LEVIN, R.M., LEVIN, S.S., ZHAO, Y. & BUTTYAN, R. 1997. Cellular and molecular aspects of bladder hypertrophy. *European Urology,* 32 (1), 15-21.

LEVIN, R.M., RIFFAUD, J.P., BELLAMY, F., ROHRMANN, D., HABIB, M., KRANOPOLSKY, L., ZHAO, Y. & WEIN, A.J. 1996. Protective effect of Tadenan® of bladder function secondary to partial outlet obstruction. *The Journal of Urology,* 155 (4), 1466-1470.

LOWE, F.C. & FAGELMAN, E. 1999. Phytotherapy in the treatment of benign prostatic hyperplasia: an update. *Urology,* 53, 671-678.

LOWE, F.C., DREIKORN, K., BORKOWSKI, A., BRAECKMAN, J., DENIS, L., FERRARI, P., GERBER, G., LEVIN, R., PERRIN, P. & SENGE, T. 1998. Review of recent placebo-controled trials utilizing phytotherapeutic agents for treatment of BPH. *The Prostate,* 37, 187-193.

LUND, W. 1994. *The pharmaceutical codex.* 12th ed. London: Pharmaceutical Press.

MARKS, L.S. & TYLER, V.E. 1999. Saw palmetto extract: newest (and oldest) treatment alternative for men with symptomatic benign prostatic hyperplasia. *Urology,* 53, 457-461.

MASOHAN, A. & BHATIA, V.K. 1996. HPLC in the analysis of steroidal compound mixtures as applied to biocrudes. *Journal of Chromatographic Science,* 34, 550-555.

MCALISTER, B.G. & VAN STADEN, J. 1995. Effect of artificially induced stress conditions on the growth of the medicinal plant *H. hemerocallidea. South African Journal of Botany,* 61 (2), 85-89.

NGUYEN, T.T. 1999. The cholesterol-lowering action of plant stanol esters. *Journal of nutrition,* 129 (12), 2109-2112.

NICOLETTI, M., PIERETTI, S., CAPASSO, A. & GALEFFI, C. 1996. Analgesic effects induced by hypoxoside, a norlignan glucoside from *Hypoxis* spp. *Phytotherapy Research,* 10, 398-401.

PEGEL, K.H., 1997. The importance of sitosterol and sitosterolin in human and animal nutrition. *South African Journal of Science,* 93, 263-267.

PHILLIPS, K.M., RUGGIO, D.M. & BAILEY, J.A. 1999. Precise quantitative determination of phytosterols, stanols, and cholesterol metabolites in human serum by capillary gas-liquid chromatography. *Journal of Chromatography B,* 732, 17-29.

PLATEL, S.B., HONDA, A. & SALEN, G. 1998. Sitosterolemia: exclusion of genes involved in reduced cholesterol biosynthesis. *Journal of Lipid Research,* 39, 1055-1061.

QUIRKE, J.M.E., TIGANI, R.A. & DILLEY, A.S. 1998. Derivitization for electrospray ionization mass spectrometry. Three electrochemically ionizable derivatives. *Analytical Chemistry,* 70, 1544-1554.

RHODES, L., PRIMKA, R.L., BERMAN, C., VERGULT, G., GABRIEL, M., MALICE, M.P. & GIBELIN, B. 1993. Comparison of finasteride (Proscar®), a 5α-reductase inhibitor, and various commercial plant extracts in in vitro and in vivo 5α-reductase inhibition. *The Prostate,* 22, 43-51.

RITSCHEL, W.A., KASTNER, U. HUSSAIN, A.S. & KOCH, H.P. 1990. Pharmacokinetics and bioavailability of β-sitosterol in the beagle dog. *Arzneimittel-Forschung/Drug Resistance,* 40 (4), 463-468.

SALEN, G., AHRENS, E.H. & GRUNDY, S.M. 1970. Metabolism of β-sitosterol in man. *The Journal of Clinical Investigation,* 49, 952-967.

SALEN, G., SHORE, V., TINT, G.S., FORTE, T., SHEFER, S., HORAK, I., HORAK, E., DAYAL, B., NGUYEN, L., BATTA, A.K., LINDGREN, F.T. & KWITEROVICH, P.O. 1989. Increased sitosterol absorption, decreased removal, and expanded body pools compensate for reduced cholesterol synthesis in sitosterolemia with xanthomatosis. *Journal of Lipid Research,* 30, 1319-1330.

SCHULZ, V., HANSEL, R. & TYLER, V.E. 1998. *Rational Phytotherapy. A physician's guide to herbal medicine.* Berlin: Springer-Verlag.

SEGARS, L.W., 1999. Saw palmetto extracts for benign prostatic hyperplasia. *Journal of Family Practice,* 48 (2), 88.

SKOOG, D.A., WEST, D.M. & HOLLER, F.J. 1996. *Fundamentals of analytical chemistry.* 7th ed. Forth Worth: Saunders College Publishing.

SMIT, B.J., ALBRECHT, C.F., LIEBENBERG, R.W., KRUGER, P.B., FREESTONE, M., GOUWS, L., THERON, E., BOUIC, P.J.D., ETSEBETH, S. & VAN JAARSVELD, P.P. 1995. A phase I trial of hypoxoside as an oral prodrug for cancer therapy - absence of toxicity. *South African Medical Journal,* 85, 865-870.

SNYDER, L.R. & KIRKLAND, J.J. 1979. *Introduction to modern liquid chromatography.* 2nd ed. New York: John Wiley & Sons, Inc.

STAHL, E. 1969. *Thin-layer chromatography.* 2nd ed. Berlin: Springer-Verlag.

STECHER, P.G., WINDHOLZ, M. & LEAHY, D.S. 1968. *The Merck Index.* 8th ed. Rahway: Merck & Co., Inc.

TOSCANO, V. & HORTON, R. 1987. Circulating dihydrotestosterone may not reflect peripheral formation. *Journal of Clinical Investigations,* 79, 1653-1658.

TOTH, I., SZECHI, M., JULESZ, J., FAREDIN, I. & BEHNKE, B. 1996. *In vitro* inhibition of testicular Δ^5-3β-hydroxysteroid dehydrogenase and prostatic 5α-reductase in rats and humans by Strogen forte extract. *International Urology and Nephrology,* 8 (3), 337-348.

VAN WYK, B.E., VAN OUDTSHOORN, B. & GERICKE, N. 1997. *Medicinal plants of South Africa.* Pretoria: Briza Publications.

VANDERHAEGHE, L.R. & BOUIC, P.J.D. 1999. *The Immune System Cure.* Hermanus: Pyramid Publishers.

VON ZAHN, P. & RHEINHOLZ, I. 1980. *The many faces of research*. Vienna: Econ Verlag.

WEISSER, H., TUNN, S., BEHNKE, B. & KRIEG, M. 1996. Effects of the *Sabal serrulata* extract IDS 89 and its subfractions on 5α-reductase activity in human benign prostatic hyperplasia. *The Prostate*, 28, 300-306.

WILT, T.J., ISHANI, A., STARK, G., MACDONALD, R., LAU, J. & MULROW, C. 1998. Saw palmetto extracts for treatment of benign prostatic hyperplasia. *The Journal of the American Medical Association*, 280 (18), 1604-1609.

YABLONSKY, F., NICOLAS, V., RIFFAUD, J.P. & BELLAMY, F. 1997. Antiprolliferative effect of *Pygeum africanum* extract on rat prostatic fibroblasts. *The Journal of Urology*, 157 (6), 2381-2387.

APPENDIX A

CONTENTS

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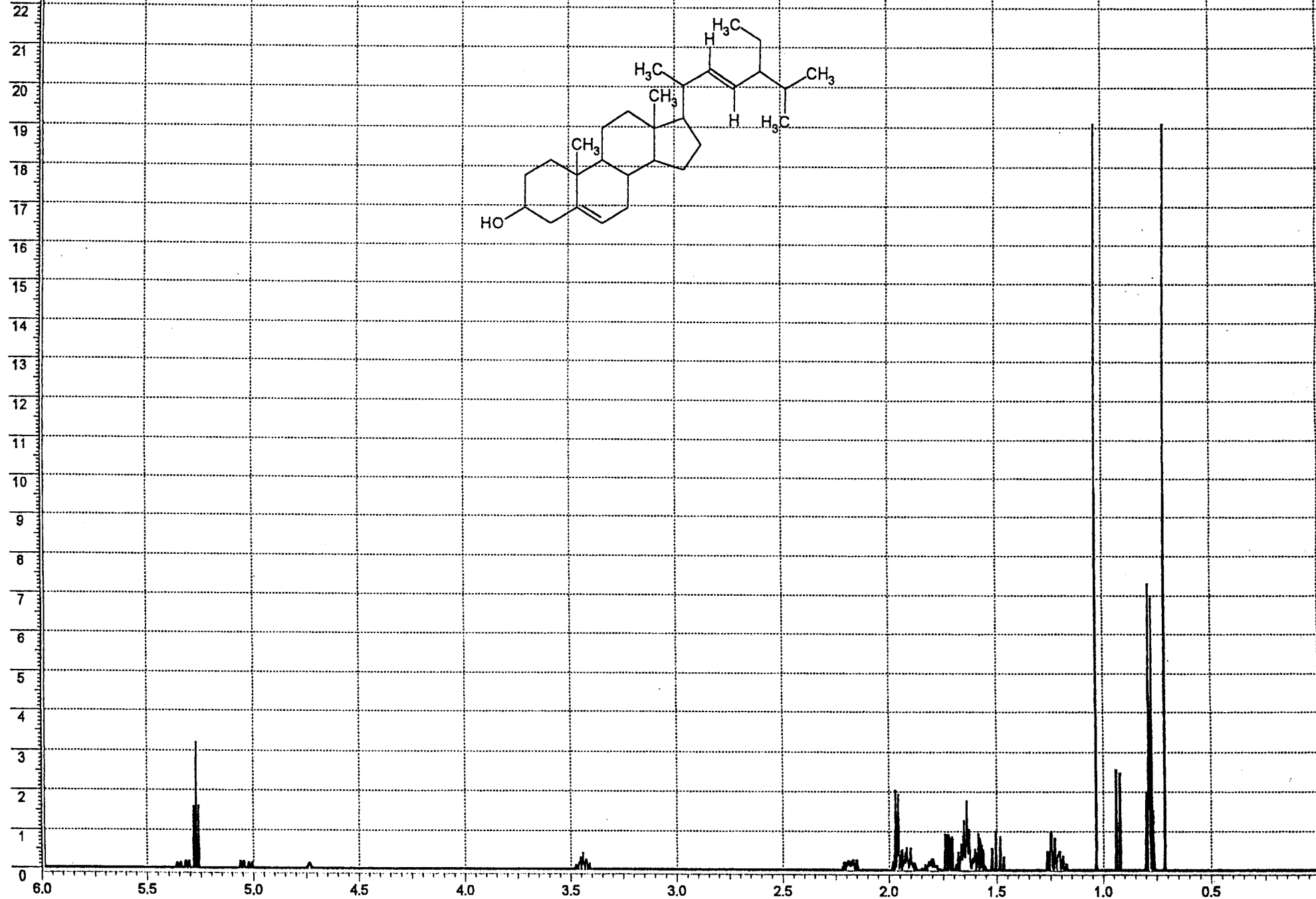Line Width: 0.10 Hz
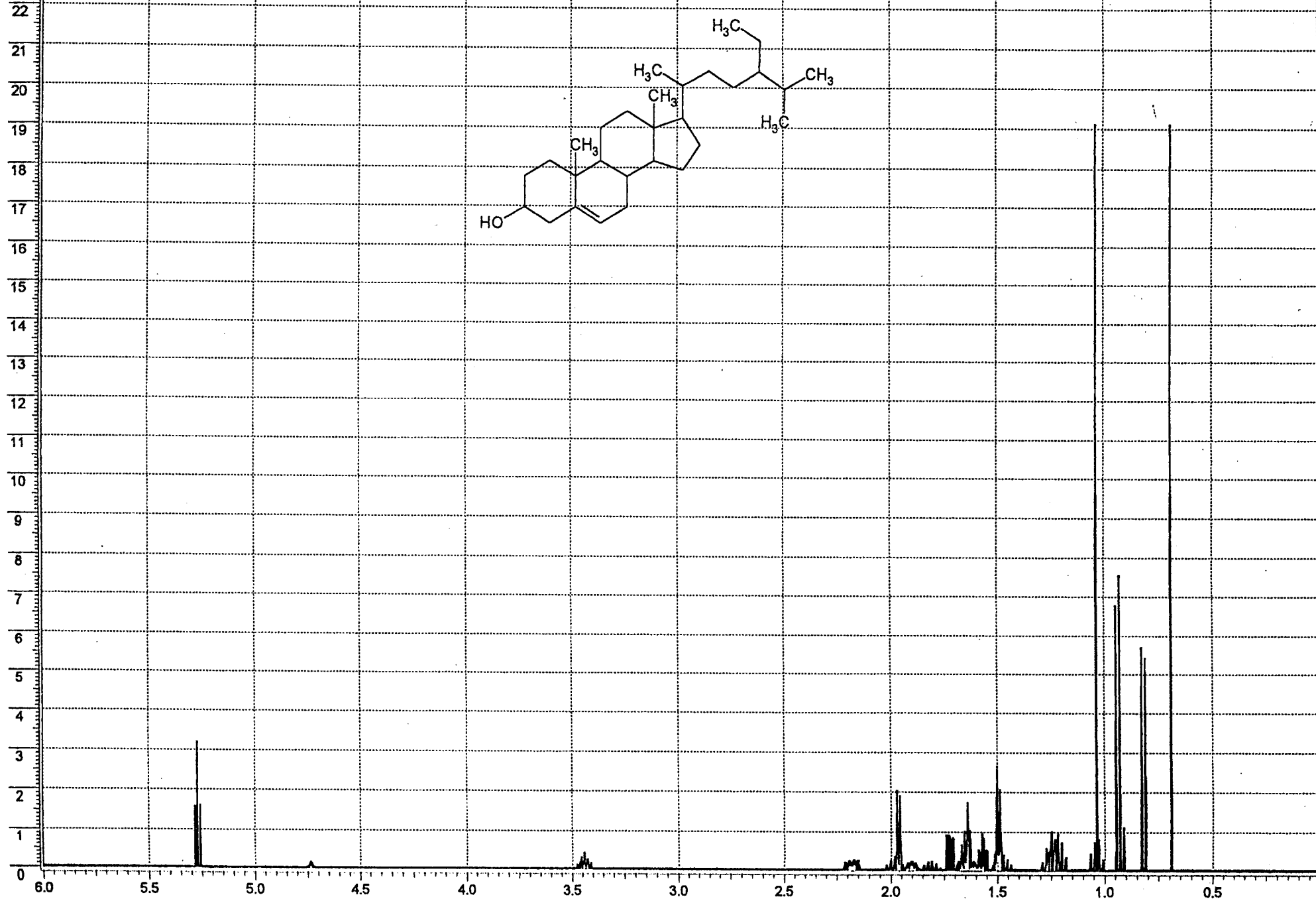
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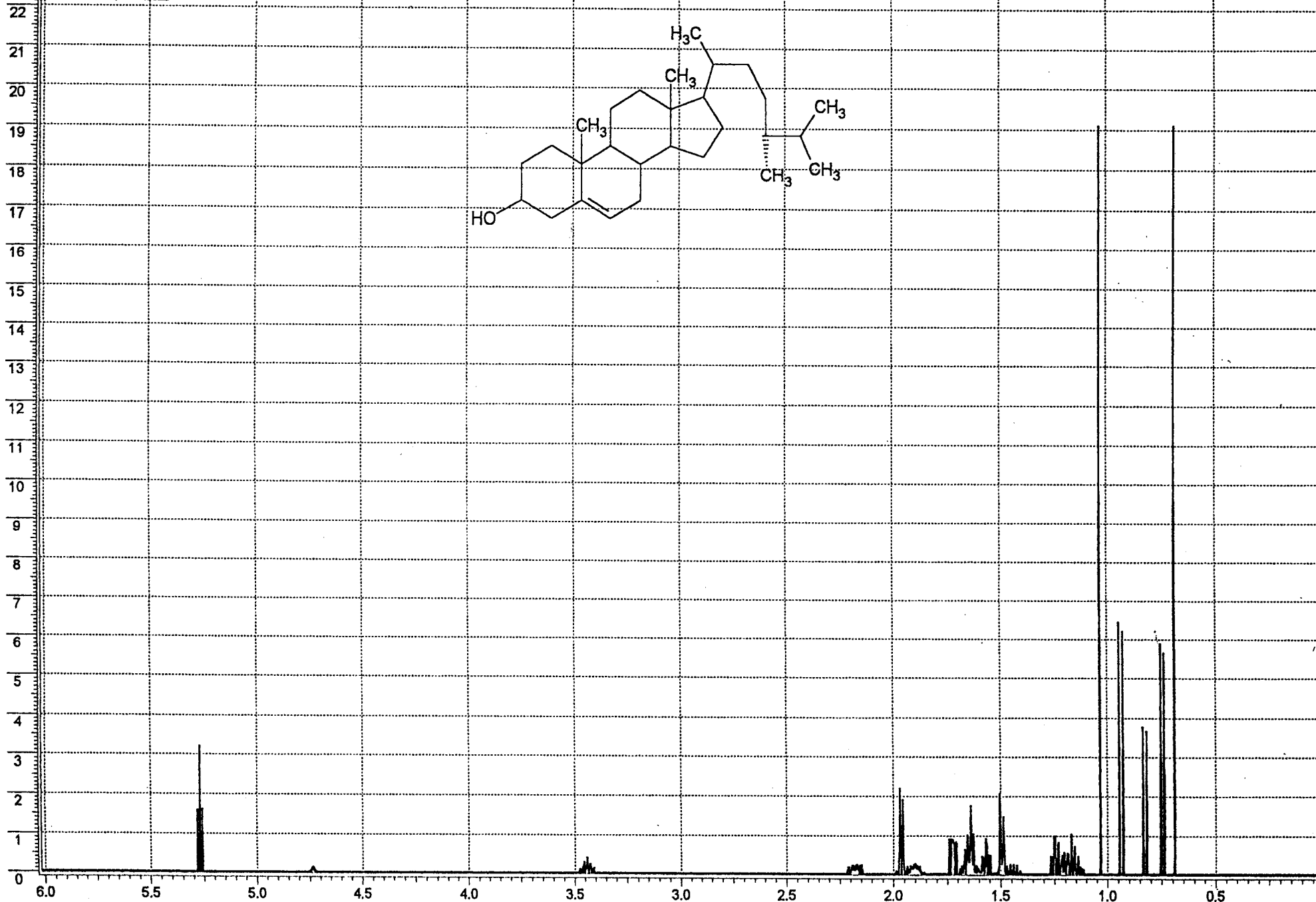
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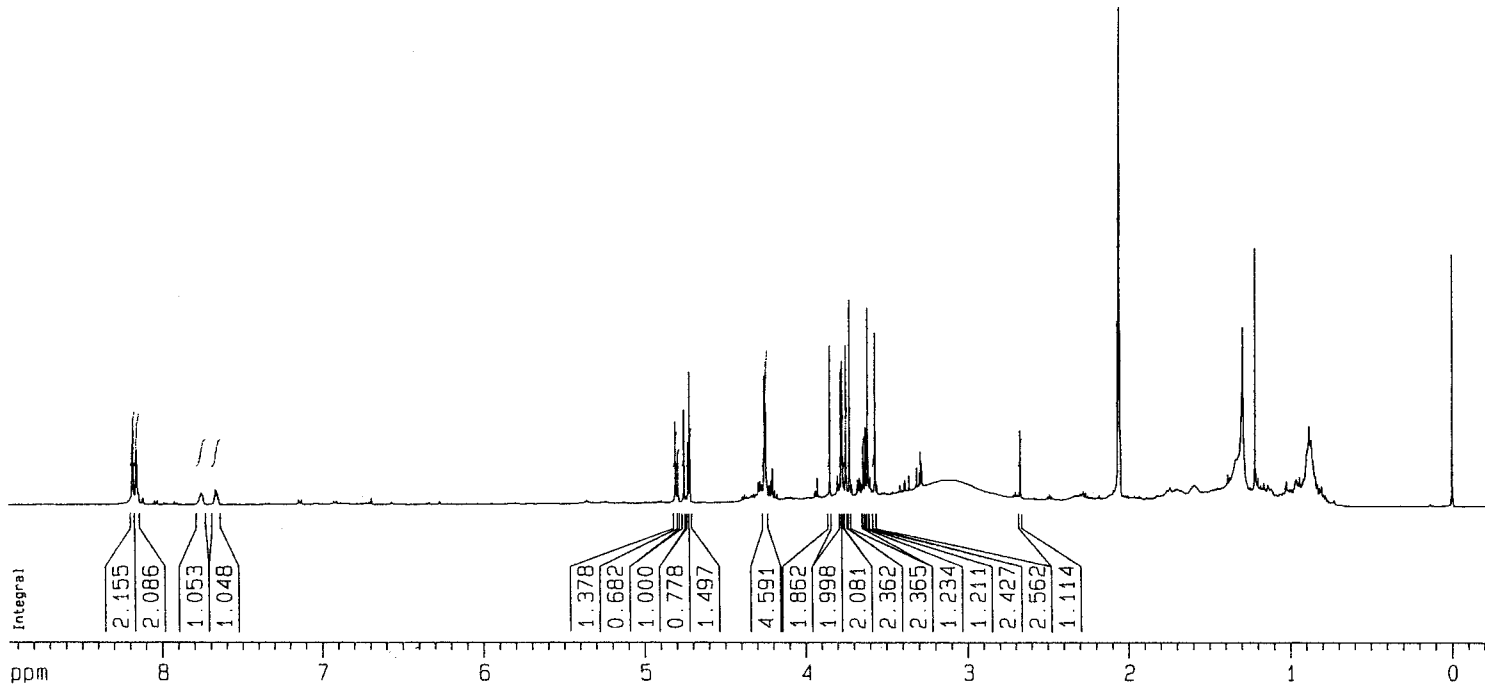Line Width: 0.10 Hz

Rooi Kol/Acetone-D6/1H

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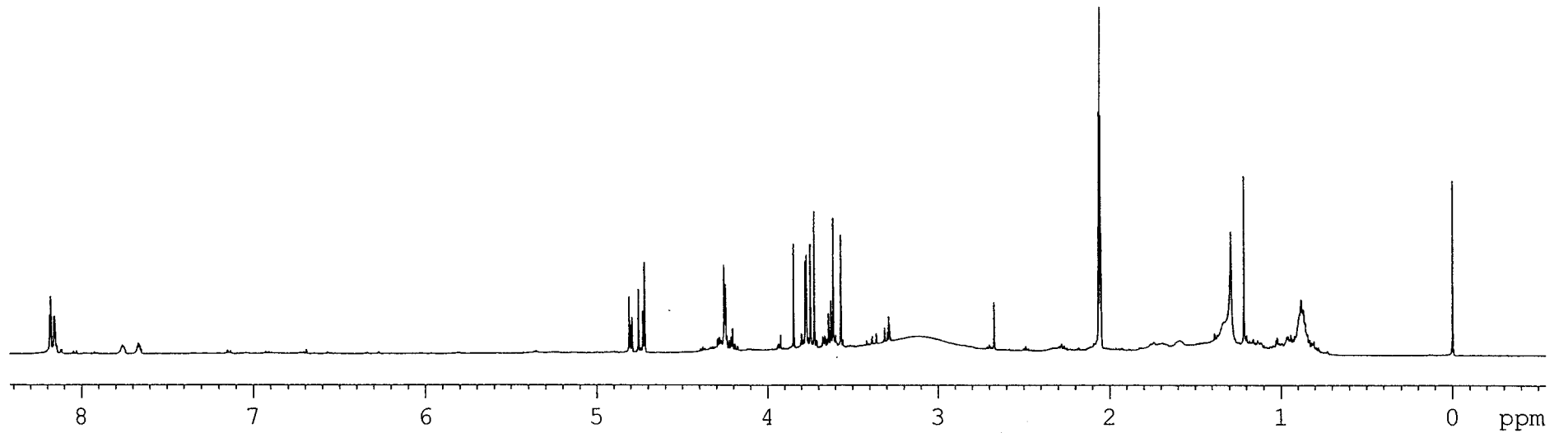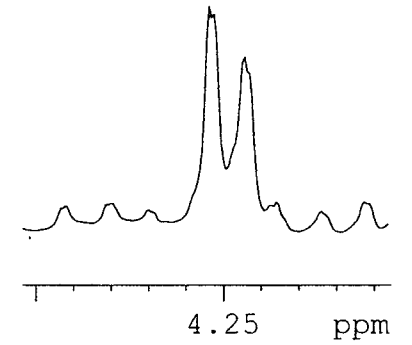EXPNO   1
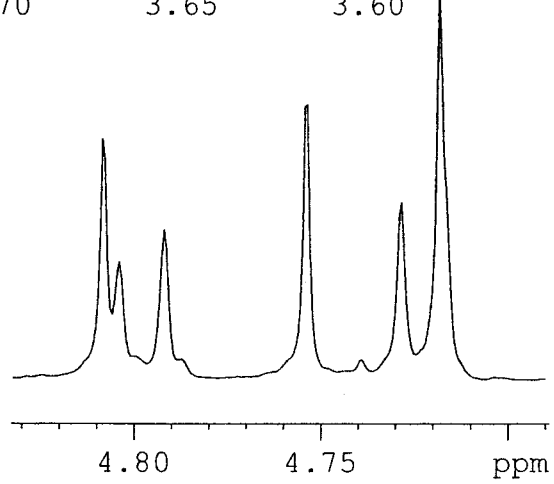PROCNO   1

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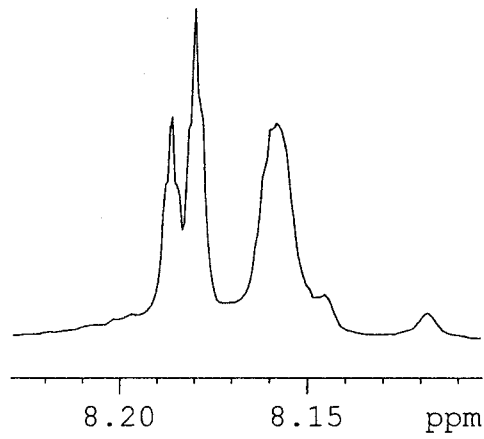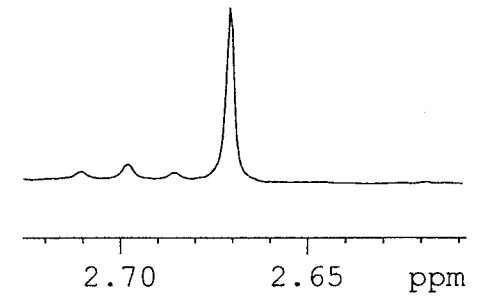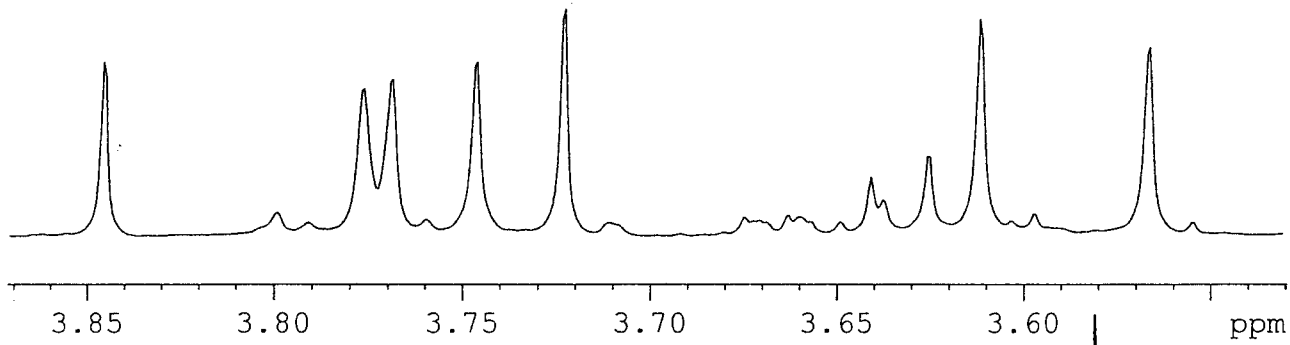Date_   20010603
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NS   87
DS   2
SWH   10330.578 Hz
FIDRES   0.157632 Hz
AQ   3.1720407 sec
RG   362
DW   48.400 usec
DE   6.00 usec
TE   300.0 K
D1   1.00000000 sec

======== CHANNEL f1 ========
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PL1   -3.00 dB
SFO1   500.1330885 MHz

F2 - Processing parameters
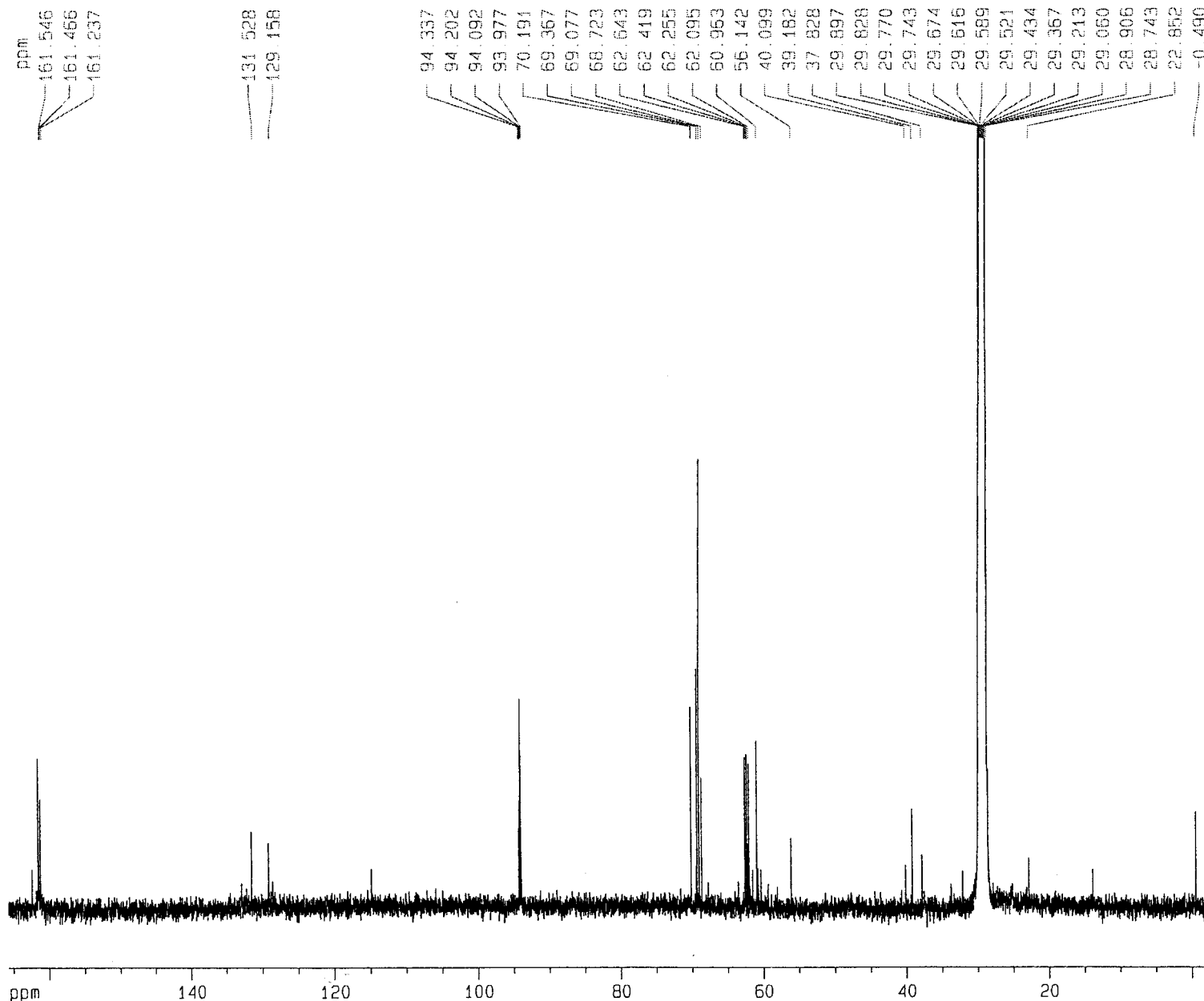SI   32768
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CY   0.00 cm
F1P   8.953 ppm
F1   4477.57 Hz
F2P   -0.307 ppm
F2   -153.37 Hz
PPMCM   0.46297 ppm/cm
HZCM   231.54744 Hz/cm

Integral

2.155 2.086 1.053 1.048   1.378 0.682 1.000 0.778 1.497 4.591 1.862 1.998 2.081 2.362 2.365 1.234 1.211 2.427 2.562 1.114

ppm   8   7   6   5   4   3   2   1   0

Rooi Kol/Acetone-D6/13C

Current Data Parameters
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EXPNO   2
PROCNO   1

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PULPROG   zgpg30
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NS   56974
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FIDRES   0.458222 Hz
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RG   1448.2
DW   16.650 usec
DE   6.00 usec
TE   300.0 K
D1   2.00000000 sec
d11   0.03000000 sec
d12   0.00002000 sec

======== CHANNEL f1 ========
NUC1   13C
P1   10.00 usec
PL1   8.00 dB
SFO1   125.7703143 MHz

======== CHANNEL f2 ========
CPDPRG2   waltz16
NUC2   1H
PCPD2   100.00 usec
PL2   -3.00 dB
PL12   17.50 dB
PL13   17.50 dB
SFO2   500.1320005 MHz

F2 - Processing parameters
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GB   0
PC   1.40

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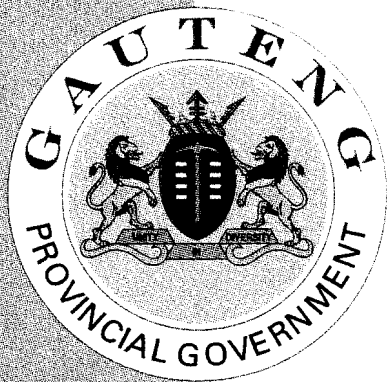PPMCM   8.43345 ppm/cm
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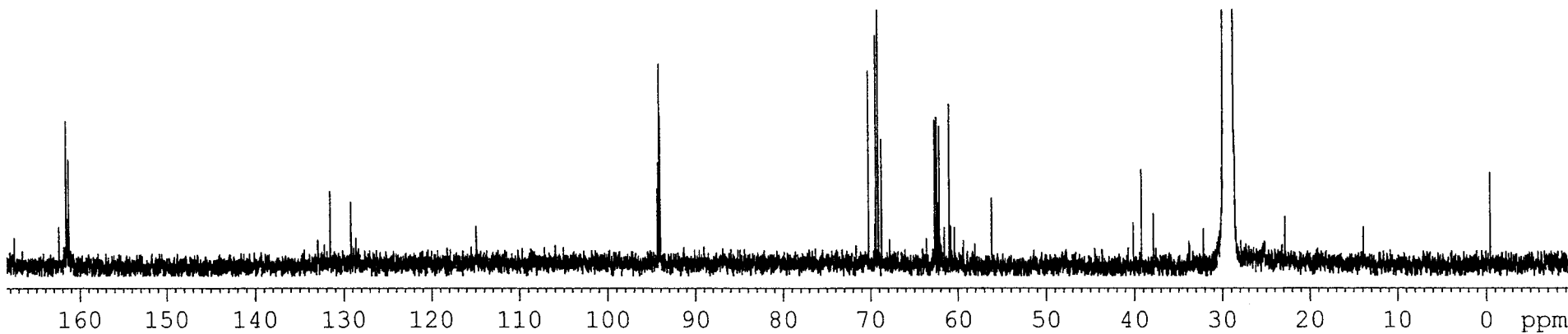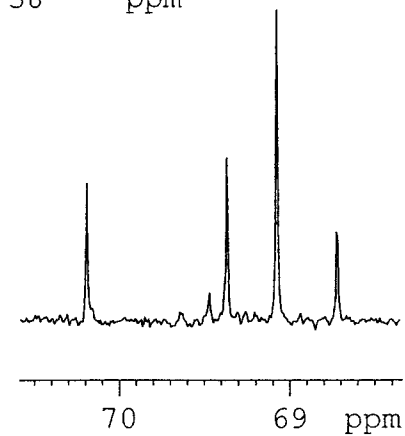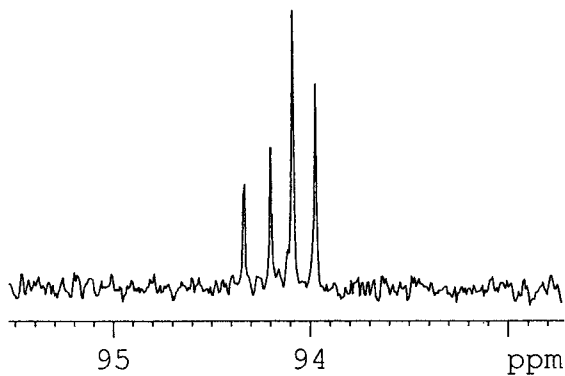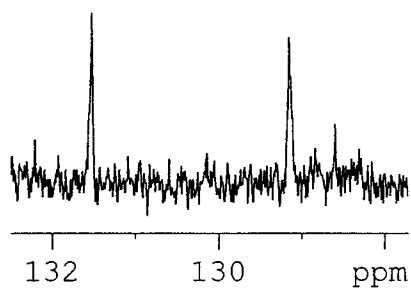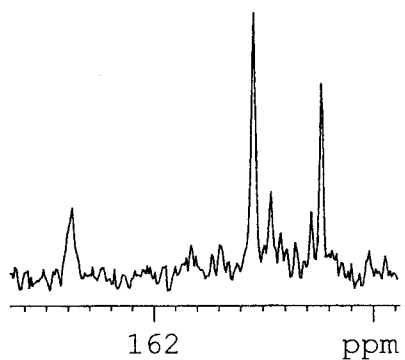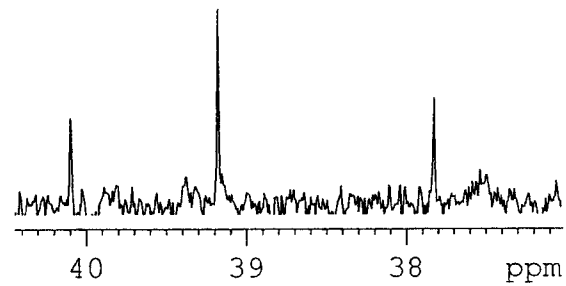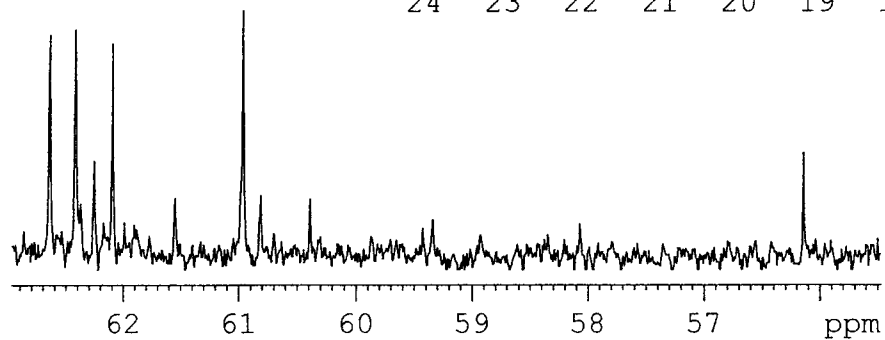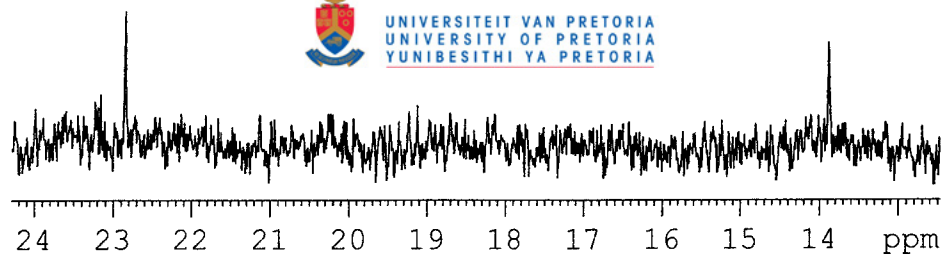
APPENDIX B

CONTENTS

Ethics Committee approval of the protocol of the proposed clinical trial (S83/2000)

Ethics Committee approval of amendment to the protocol (Amendment to S83/2000)

DEPARTMENT DEPARTEMENT
OF VAN
HEALTH GESONDHEID

Tel:	(012) 354 1560
Fax/Faks:	(012) 354 1831
Ref/Verw:	Ethics Committee
Enquiries/Navrae:	**Dr R Sommers**
	Ward 4 Room 19
Date :	**20/07/2000**

Number : **S83/2000**

Title : Protocol of proposed clinical trial, including pilot study, to determine the absorption of sterols and steroplins in human volunteers.

Investigator : A C Retief; Department of Pharmacology;
Prof J R Snyman; Dept Pharmacology
Pretoria Academic Hospital; Pretoria.

This Protocol and Informed Consent has been considered by the Ethics Committee, Faculty of Medicine, Univ.of Pretoria and Pretoria Academic Hospitals on 19/07/2000 and found to be acceptable.

Dr J.E.Davel	(female) MBChB:Hospital Superintendent
Prof A.P.du Toit	BA;DiplTheo; BA (Hons);MA;DPhil:Philosopher
Prof C.I. Falkson	(female)MBChB;M.Med(Int);MD;Med. Oncologist
Prof G. Falkson	CHAIRPERSON; MBChB;M.Med(Int);MD;OSG: Medical Oncologist
Mrs C Gerber	(female)BA(FineArts);Architectural Draughting (Boston House College Pta)
Prof S.V. Grey	(female)BSc(Hons);MSc; DSc :Deputy Dean
Mrs R Jooste	(female) Dip. Pharm; M Pharm; Pharmacist.
Dr V.O.L. Karusseit	MBChB;MFGP(SA);M.Med(Chir); FCS (SA): Surgeon
Dr S.Khan	(female)MB.BCh.; Med.Adviser (Gauteng Dept.of Health).
Ms B.C.F.Magardie	(female) BCur:Matron/Senior Nursing-Sister
Miss B Mullins	(female) BscHons;Teachers Diploma;
Dr P.Z Njongwe	(female)MBChB ;D.P.H;DTMtH; DOH ;F.F.C.H(CM) S.A.Chief Med.Super of Pretoria Academic Hospital.
Snr Sr J. Phatoli	(female) BCur(Et.Al)Senior Nursing-Sister
Prof H.W. Pretorius	MBChB;M.Med (Psych) MD: Psychiatrist
Prof P. Rheeder	MBChB;MMed(Int);LKI(SA);MSc (KLIN.EPI): Specialist Physician
Prof M.M.S.Smuts	(female) BVSc; DVSc
Prof De K.Sommers	BChB; HDD; MBChB; MD: Pharmacologist
Dr R Sommers	SECRETARIAT (female)MBChB; M.med (Int);MPhar.Med;
Prof FFW van Oosten	BA; LLB ; LLD ;LLD ;Head of Department of Public Law and Prof in Criminal Law and Medical Law

Student Ethics Sub-Committee

Mrs E Ahrens	(female)B.Cur;
Prof S.V. Grey	(female)BSc(Hons);MSc ;DSc :Deputy Dean
Prof P. Rheeder	MBChB;MMed(Int);LKI(SA);MSc (KLIN.EPI): Specialist Physician
Dr R Sommers	SECRETARIAT (female)MBChB; M.med (Int); MPharMed;
Dr C van der Westhuizen	(female) D.Cur; M.Ed.

PROF G FALKSON;
MBChB;M.Med(Int);MD;OSG: Medical Oncologist
CHAIRPERSON of the Ethics Committee at Pretoria Academic Hospital;

PROF P RHEEDER;
BChB;MMed(Int);LKI(SA);MSc (KLIN.EPI):Specialist Physician
CHAIRPERSON of the Student Ethics Committee at P.A. H

DEPARTMENT DEPARTEMENT
OF VAN
HEALTH GESONDHEID

Tel:	(012) 354 1560
Fax/Faks:	(012) 354 1831
Ref/Verw:	Ethics Committee
Enquiries/Navrae:	**Dr R Sommers** Ward 4 Room 19

Date : **1/03/2001**

Number	:	Amendment to **S83/2000**

Title	:	Protocol of proposed clinical trial, including pilot study, to determine the absorption of sterols and steroplins in human volunteers.

Investigator : A C Retief; Department of Pharmacology;
Prof J R Snyman; Dept Pharmacology
Pretoria Academic Hospital; Pretoria.

This Addendum has been considered by the Ethics Committee, Faculty of Medicine, Univ.of Pretoria and Pretoria Academic Hospitals on 28/02/2001 and found to be acceptable.

Dr J.E.Davel	(female) MBChB:Hospital Superintendent
Prof A.P.du Toit	BA;DiplTheo; BA (Hons);MA;DPhil:Philosopher
Prof S.V. Grey	(female)BSc(Hons);MSc; DSc :Deputy Dean
Mrs R Jooste	(female) Dip. Pharm; M Pharm; Pharmacist.
Dr V.O.L. Karusseit	MBChB;MFGP(SA);M.Med(Chir); FCS (SA): Surgeon
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Miss B Mullins	(female) BscHons;Teachers Diploma;
Snr Sr J. Phatoli	(female) BCur(Et.Al)Senior Nursing-Sister
Prof H.W. Pretorius	MBChB;M.Med (Psych) MD: Psychiatrist
Prof P. Rheeder	MBChB;MMed(Int);LKI(SA);MSc (KLIN.EPI): Specialist Physician
Dr C F Slabber	MB BCh, FCP (SA) Acting Head; Dept Medical Oncology
Prof De K.Sommers	BChB; HDD; MBChB; MD: Pharmacologist
Dr R Sommers	SECRETARIAT (female)MBChB; M.med (Int);MPhar.Med;

Student Ethics Sub-Committee

Mrs E Ahrens	(female)B.Cur;
Prof S.V. Grey	(female)BSc(Hons);MSc ;DSc :Deputy Dean
Prof P. Rheeder	MBChB;MMed(Int);LKI(SA);MSc (KLIN.EPI): Specialist Physician
Dr R Sommers	SECRETARIAT (female)MBChB; M.med (Int); MPharMed;
Dr C van der Westhuizen	(female) D.Cur; M.Ed.

PROF. P. RHEEDER;
CHAIRPERSON of the Student Ethics Committee at P.A. H.
BChB;MMed(Int);LKI(SA);MSc (KLIN.EPI):Specialist Physician