ANALYSIS OF STEROLS AND STEROLINS IN
*Hypoxis hemerocallidea* AND RELATED HERBAL MEDICINE

by

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CONTENTS

ACKNOWLEDGEMENTS ii

LIST OF TABLES xiii

LIST OF FIGURES xvi

LIST OF ABBREVIATIONS xx

PUBLICATIONS / PRESENTATIONS EMANATING FROM THIS STUDY xxiii

CHAPTER 1 - PHYTOTHERAPY AND BENIGN PROSTATIC HYPERPLASIA 1

1.1 INTRODUCTION 1

1.1.1 History 1

1.1.2 Aim of chapter 1

1.2 STEROLS: PHYTOSTEROLS VERSUS CHOLESTEROL 2

1.3 THERAPEUTIC INDICATIONS OF PHYTOSTEROLS 4

1.3.1 Immunomodulation – Reinstating the balance in the immune system 4

1.3.1.1 The immune response 4

1.3.1.2 Immune stimulation and suppression by sterols/sterolins 5

1.3.2 Hypercholesterolaemia 6

1.3.3 Benign prostatic hyperplasia 7

1.3.3.1 Allopathic treatment 7

1.3.3.2 Phytosterols and BPH 8

1.3.3.3 Phytosterols' mechanism of action in BPH 9

1.3.3.4 β-Sitosterol, β-sitosterolin or combination? 9

iii
1.4 PHYTOSTEROL CONTAINING BPH PHYTOTHERAPEUTICS

1.4.1 *Hypoxis hemerocallidea* (African potato)

1.4.1.1 General information

1.4.1.2 General composition

1.4.1.3 Mechanism of action

1.4.1.4 Extraction

1.4.1.5 Presentation and dosage

1.4.1.6 Efficacy

1.4.1.7 Side effects

1.4.2 *Prunus africana* (African plum)

1.4.2.1 General information

1.4.2.2 General composition

1.4.2.3 Mechanism of action

1.4.2.4 Extraction

1.4.2.5 Dosage

1.4.2.6 Efficacy

1.4.2.7 Side Effects

1.4.3 *Serenoa repens* (saw palmetto)

1.4.3.1 General information

1.4.3.2 General composition

1.4.3.3 Mechanism of action

1.4.3.4 Extraction

1.4.3.5 Dosage

1.4.3.6 Efficacy

1.4.3.7 Side effects

1.5 SIDE EFFECTS OF PHYTOSTEROLS

1.6 CONCLUSION

1.7 AIM OF THE STUDY

---

CHAPTER 2 - MATERIAL, STANDARDS AND METHODS

2.1 MATERIAL

2.1.1 Plant material
### 2.1.2 Products  

### 2.2 STANDARDS  

### 2.3 METHODS  

---

#### CHAPTER 3 - TLC METHOD DEVELOPMENT AND APPLICATION  

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 INTRODUCTION</td>
<td>29</td>
</tr>
<tr>
<td>3.1.1 History</td>
<td>29</td>
</tr>
<tr>
<td>3.1.2 Aim of chapter</td>
<td>29</td>
</tr>
<tr>
<td>3.2 MATERIAL AND METHODS</td>
<td>30</td>
</tr>
<tr>
<td>3.2.1 Material</td>
<td>30</td>
</tr>
<tr>
<td>3.2.2 Methods</td>
<td>30</td>
</tr>
<tr>
<td>3.2.2.1 Extraction</td>
<td>30</td>
</tr>
<tr>
<td>3.2.2.1 (a) Selecting extractants according to polarity and solvent strength</td>
<td>31</td>
</tr>
<tr>
<td>3.2.2.1 (b) Types of extraction used</td>
<td>31</td>
</tr>
<tr>
<td>3.2.2.1 (b)(i) Simple extraction</td>
<td>31</td>
</tr>
<tr>
<td>3.2.2.1 (b)(ii) Soxhlet extraction</td>
<td>32</td>
</tr>
<tr>
<td>3.2.2.1 (b)(iii) Water extraction of <em>H. hemerocalleida</em></td>
<td>32</td>
</tr>
<tr>
<td>3.2.2.2 Preparation of standards</td>
<td>32</td>
</tr>
<tr>
<td>3.2.2.3 Thin layer chromatography (TLC)</td>
<td>33</td>
</tr>
<tr>
<td>3.2.2.3 (a) Visualisation of separated components</td>
<td>33</td>
</tr>
<tr>
<td>3.2.2.3 (b) The retardation factor (Ri)</td>
<td>33</td>
</tr>
<tr>
<td>3.2.2.4 Fluorescence</td>
<td>34</td>
</tr>
<tr>
<td>3.2.2.5 Hydrolysis of &quot;stigmasterol&quot;</td>
<td>34</td>
</tr>
<tr>
<td>3.2.2.6 Isolation of the red spot compound with preparative thin layer chromatography (PTLC)</td>
<td>35</td>
</tr>
<tr>
<td>3.2.2.7 Structure elucidation of the red spot compound with Nuclear Magnetic Resonance Spectroscopy (NMR)</td>
<td>35</td>
</tr>
<tr>
<td>3.3 RESULTS AND DISCUSSION</td>
<td>36</td>
</tr>
<tr>
<td>A: METHOD DEVELOPMENT</td>
<td>36</td>
</tr>
<tr>
<td>3.3.1 Extractant evaluation</td>
<td>36</td>
</tr>
<tr>
<td>3.3.1.1 The solubility of phytosterols in acetone, methanol and chloroform</td>
<td>36</td>
</tr>
<tr>
<td>3.3.1.2 Extraction</td>
<td>36</td>
</tr>
</tbody>
</table>
3.3.1.2 (a)(i) Acetone extraction
3.3.1.2 (a)(ii) Acetone extract redissolved in methanol, diethylether and water
3.3.1.2 (b) Methanol, ethanol and chloroform extraction
3.3.1.2 (c) Dichloromethane and diethylether extraction
3.3.1.2 (d) Hexane extraction
3.3.1.3 Best extractant evaluation
3.3.2 Mobile phase evaluation
3.3.3 Spray reagent evaluation
3.3.3.1 P-Anisaldehyde spray reagent
3.3.3.2 Vanillin spray reagent
3.3.3.3 Perchloric acid spray reagent
3.3.3.4 50% Sulphuric acid in methanol spray reagent
3.3.3.5 Phosphoric acid spray reagent
3.3.3.6 P-Toluene sulphonic acid spray reagent
3.3.3.7 Trichloroacetic acid spray reagent
3.3.3.8 Best spray reagent evaluation

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. hemerocallea* from three different sources – extracts compared
3.3.4.1 (b) Comparison of sterols/sterolins in BPH phytotherapeutics: *H. hemerocallea* powder, *P. africana* extract and *S. repens* powder/extract
3.3.4.2 Analysis of different phytosterol products
3.3.4.3 Analysis of herb powder versus the herbal extract of *P. africana* and *S. repens*
3.3.4.4 Hydrolysis of “stigmastrolin” and the *red spot compound*
3.3.4.5 Isolation of the *red spot compound* with preparative thin layer chromatography (PTLC)
3.3.4.6 Structure elucidation of the *red spot compound* with Nuclear Magnetic Resonance Spectroscopy (NMR)
3.3.4.7 Water extraction of African potato

3.4 CONCLUSION
CHAPTER 4 - HPLC METHOD EVALUATION AND APPLICATION

4.1 INTRODUCTION

4.1.1 History

4.1.2 Aim of chapter

4.2 MATERIAL AND METHODS

4.2.1 Material

4.2.1.1 Plant material

4.2.2 Apparatus

4.2.2.1 SPE-equipment

4.2.2.2 HPLC-equipment

4.2.3 Methods

4.2.3.1 Extraction

4.2.3.1 (a) Simple extraction

4.2.3.1 (b) Solid phase extraction (SPE)

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

4.2.3.1 (c) Water extraction of H. hemerocallisidea

4.2.3.2 Preparation of standards

4.2.3.3 HPLC for quantitative measurements

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

4.2.3.3 (b) Calibration curves for single determinations

4.3 RESULTS AND DISCUSSION

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

4.3.2 Calibration curves

4.3.3 Solid phase extraction (SPE)

4.3.3.1 Detection of a contaminant in analytical grade solvents

4.3.4 Methanol versus chloroform extraction of phytosterols

4.3.5 HPLC Application

4.3.5.1 Phytosterol analysis of different plants

4.3.5.1 (a) H. hemerocallisidea

4.3.5.1 (b) P. Africana
4.3.5.1 (c) *S. repens*

4.3.5.2 Phytosterol analysis of different products

4.3.5.2 (a) Immunochoice®

4.3.5.2 (b) Harzol®

4.3.5.2 (c) Moducare®

4.3.5.2 (d) Nutricare®

4.3.5.2 (e) Phytofend®

4.3.5.2 (f) Prostol Herbal®

4.3.6 Water extraction of *H. hemerocallis* powder

4.4 CONCLUSION

CHAPTER 5 - APPLICATION OF TLC AND HPLC FOR STABILITY AND BIOAVAILABILITY

5.1 INTRODUCTION

5.1.1 Industrial application of TLC and HPLC

5.1.2 Aim of chapter

5.2 MATERIAL AND METHODS

5.2.1 Material

5.2.1 (a) Plant material

5.2.1 (b) Human serum

5.2.2 Apparatus

5.2.2.1 SPE-equipment

5.2.2.2 HPLC-equipment

5.2.3 Methods

5.2.3.1 Preliminary preparations for stability analysis

5.2.3.1 (a) Accelerated stability testing

5.2.3.1 (b) Stability against gamma irradiation

5.2.3.2 Preliminary preparation for serum analysis:

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

5.2.3.3 Extraction

5.2.3.3 (a) Simple extraction

5.2.3.3 (b) Solid phase extraction
5.2.3.3 (c) Serum extraction
5.2.3.4 Thin layer chromatography (TLC)
5.2.3.4 (a) Sensitivity determination of TLC
5.2.3.4 (b) Detectability of β-sitosterol in serum with TLC
5.2.3.5 High performance liquid chromatography (HPLC)
5.2.3.6 Calculation of standard deviation
5.2.3.7 Determination of the degradation rate constant and shelf-life
5.2.3.8 Ethical approval for clinical trial
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
5.2.3.9 (b) Serum preparation
5.2.3.9 (c) Analysis - According to the method of Emara et al. (1999):
5.2.3.9 (c)(i) Sample treatment
5.2.3.10 Improvement of β-sitosterol extraction from serum
5.2.3.10(a) Mixing by turning instead of vortex mixing
5.2.3.10(b) Changing the extraction ratio
5.2.3.11 Stability of β-sitosterol in serum
5.2.3.12 Drying the organic phase with heat (90 °C) as in the method of Emara et al. (1999)

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
5.3.1.1 (a)(i) H. hemerocalidea powder (African potato1)
5.3.1.1 (a)(ii) P. africana extract (5:1)
5.3.1.1 (a)(iii) S. repens extract (4:1)
5.3.1.1 (a)(iv) Immunochoice®
5.3.1.1 (a)(v) Moducare®
5.3.1.1 (a)(vi) Nutricare®
5.3.1.1 (b) HPLC
5.3.1.1 (b)(i) H. hemerocalidea powder (African potato1)
5.3.1.1 (b)(ii) P. africana extract (5:1)
5.3.1.1 (b)(iii) S. repens extract (4:1)
5.3.1.1  (b)(iv) Immunochoice®

5.3.1.1  (b)(v) Moducare®

5.3.1.1  (b)(vi) Nutricare®

5.3.1.2  Stability of phytosterols against gamma irradiation

5.3.1.2  (a) TLC

5.3.1.2  (a)(i) H. hemerocallidea powder (African potato 1 & 6)

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

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

5.3.1.2  (a)(iv) Immunochoice®

5.3.1.2  (a)(v) Moducare®

5.3.1.2  (a)(vi) Nutricare®

5.3.1.2  (b) HPLC

5.3.1.2  (b)(i) H. hemerocallidea powder (African potato 1)

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

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

5.3.1.2  (b)(iv) Immunochoice®

5.3.1.2  (b)(v) Moducare®

5.3.1.2  (b)(vi) Nutricare®

5.3.2  Bioavailability analysis of phytosterols

5.3.2.1  TLC

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

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

5.3.2.2  HPLC

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

5.3.2.2  (b) Clinical trial: Part 1

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

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

5.3.2.2  (b)(iii) Study with Tadenan®

5.3.2.2  (c) Improved β-sitosterol extraction from serum

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

5.3.2.2  (c)(ii) Changing the extraction ratio

5.3.2.2  (d) Stability of β-sitosterol in plasma
5.3.2.2 (e) Drying the organic phase with heat 90 °C [according to the method of Emara et al. (1999)]

5.3.2.2 (f) Standard deviation

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

5.3.2.2 (f)(ii) Standard deviation of extraction

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

5.4 CONCLUSION

5.4.1 Conclusions from the stability analysis of phytosterols

5.4.2 Conclusions from the bioavailability analysis of phytosterols

5.4.3 General conclusions on TLC and HPLC

CHAPTER 6 - COLUMN CHROMATOGRAPHY FOR ISOLATION

6.1 INTRODUCTION

6.1.1 Origin and interest in the red spot compound

6.1.2 Aim of chapter

6.2 MATERIAL AND METHODS

6.2.1 Material

6.2.2 Methods

6.2.2.1 Extraction

6.2.2.1 (a) Simple extraction

6.2.2.2 Preparation of standards

6.2.2.3 Isolation of the red spot compound

6.2.2.3 (a) Extract fractionation with column chromatography

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

6.2.2.3 (c) Second fractionation with column chromatography

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

6.2.2.4 Structure elucidation with Nuclear Magnetic Resonance Spectroscopy (NMR)

6.3 RESULTS

6.3.1 Extraction

6.3.2 Isolation of the red spot compound

6.3.3 NMR report by Dr. Coetzee, SASOL

6.4 CONCLUSION
LIST OF TABLES

Table 1.1: The dichotomy of T-helper cells based on their defining cytokine profiles and functions (Bouic et al., 1999)  
Table 1.2: Different names and tradenames of some important BPH phytotherapeutic agents (composed from data by Lowe et al., 1998)  
Table 1.3: Composition and limits of a standardized Hypoxis plant extract detected by HPLC analyses (composed from data by Albrecht et al., 1995a)  
Table 3.1: Solvent strengths, polarities and boiling points of the extractants used (from Snyder & Kirkland, 1979)  
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  
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  
Table 3.4: Relative amounts of sterols, sterolins and hypoxoside extracted by methanol and chloroform respectively from H. hemerocallidea powder, P. africana extract (5:1), S. repens powder and extract (4:1)  
Table 4.1: HPLC quantities of hypoxoside and β-sitosterol from a standard mixture containing 50 µg of both, applied to a C18 SPE cartridge  
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  
Table 4.3: β-Sitosterol contents of H. hemerocallidea powder from four different sources  
Table 4.3: β-Sitosterol (BSS) contents of P. africana bark extract (5:1), bark powder and leaf powder  
Table 4.4: Campesterol (CS) and/or stigmasterol (SS) contents of P. africana bark extract (5:1), bark powder and leaf powder  
Table 4.5: β-Sitosterol (BSS) contents of S. repens berry extract (4:1) and powder  
Table 4.6: Campesterol (CS) and/or stigmasterol (SS) contents of S. repens berry extract (4:1) and powder
Table 4.7: β-Sitosterol (βSS), campesterol (CS) and/or stigmasterol (SS) contents of Immunochoice®

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

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

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

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

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

Table 5.1: Irradiation intensities to which selected samples were exposed

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

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

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

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

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

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

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

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

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

Table 5.11: Levels of β-sitosterol, campesterol and/or stigmasterol in Immunochoice®, gamma irradiated at different intensities
Table 5.12: Levels of β-sitosterol, campesterol and/or stigmasterol in Moducare®, gamma irradiated at different intensities

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

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

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

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
LIST OF FIGURES

Figure 1.1: The chemical structures, molecular formulae and weights of cholesterol, β-sitosterol, campesterol and stigmasterol

Figure 1.2: Chemical structure of β-sitosterolin

Figure 1.3: Transformation of testosterone into 5α-dihydrotestosterone by 5α-reductase

Figure 1.4: Chemical structure of hypoxoside

Figure 1.5: The biotransformation of hypoxoside to cytotoxic rooperol

Figure 1.6: Structural differences between betamethasone and β-sitosterol

Figure 3.1: TLC on aluminium plates of acetone extracts of African potato 1 and 2, S. repens berry powder, P. africana bark extract (5:1) and Warrenchem Betasitosterol

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

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)

Figure 3.4: TLC on a glass plate, of different extracts of H. hemerocallidea and P. africana extract (5:1), developed in CEF and sprayed with vanillin

Figure 3.5: Overheated TLC on a glass plate of methanol and chloroform extracts of African potato 1, P. africana extract (5:1), Tadenan® (P. africana extract), S. repens powder and extract (4:1) and Permixon® (S. repens extract)

Figure 3.6: TLC on an aluminium plate of chloroform extracts of different phytosterol products

Figure 3.7: TLC on an aluminium plate of chloroform extracts of P. africana leaf powder, bark powder and bark extract (5:1), S. repens berry powder and berry extract (4:1)

Figure 3.8: Overheated TLC on a glass plate of unhydrolysed and hydrolysed 10 mg/ml and 5 mg/ml 95% stigmasterol standard

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 CEF and sprayed with p-anisaldehyde

Figure 4.1: Absorbance spectra of 100 μg/ml 95% stigmasterol standard dissolved in HPLC grade methanol, measured with MWD at wavelengths 200.4, 203.4 and 205.4 nm
Figure 4.2: Overlayed absorbance spectra of eight different concentrations of 95.7% β-sitosterol (tR=10.020), 65% campesterol (tR=8.933) 95% stigmasterol (tR=8.933), β-sitosterol (tR=5.534) and hypoxoside (tR=1.195) in HPLC grade methanol, measured with MWD at a wavelength of 205.4 nm, with 96.5% methanol as mobile phase.

Figure 4.3: Calibration curves of β-sitosterol, campesterol and stigmasterol, β-sitosterol and hypoxoside obtained from MWD absorbance spectra, with their correlation coefficients.

Figure 4.4: Three dimensional fluorescence spectra of hypoxoside.

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. hemerocalidea methanol extract.

Figure 4.6: HPLC chromatogram of analytical grade acetone, stored in a plastic bottle, with a contaminant with exactly the same retention time as β-sitosterol (tR = 10.141) and calibrated as 68.19 µg/ml β-sitosterol.

Figure 4.7: HPLC chromatogram of a chloroform extract of H. hemerocalidea powder from PLANTANICAL MEDICINE (African potato 6).

Figure 4.8: HPLC chromatogram of a chloroform extract of P. africana bark extract (5:1).

Figure 4.9: HPLC chromatogram of a chloroform extract of S. repens berry extract (4:1).

Figure 4.10: HPLC chromatogram of a chloroform extract of Immunochoice®.

Figure 4.11: HPLC chromatogram of a chloroform extract of Harzol®.

Figure 4.12: HPLC chromatogram of a chloroform extract of Moducare®.

Figure 4.13: HPLC chromatogram of a chloroform extract of Nutricare®.

Figure 4.14: HPLC chromatogram of a chloroform extract of Phytogard®.

Figure 4.15: HPLC chromatogram of a chloroform extract of Prostol Herbal®.

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.

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.

Figure 5.3: TLC on an aluminium plate of chloroform extracts of Nutricare®, stored at 40°C for up to 12 months.

Figure 5.4: Graphs of β-sitosterol and hypoxoside (isolated with solid phase extraction and analysed with HPLC) in H. hemerocalidea powder, stored at 40 °C for up to 12 months.
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

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

Figure 5.7: Graphs of β-sitosterol, campesterol and/or stigmasterol in Immunochoice®, stored at 40 °C for up to 12 months

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

Figure 5.9: A graph of β-sitosterol in Nutricare®, stored at 40 °C for up to 12 months

Figure 5.10: TLC on aluminium plates of chloroform extracts of (a) *H. hemerocallidea* powder (African potato 1) and (b) PLANANTICAL MEDICINE's Hypoxis (African potato 6), gamma irradiated at 0, 4.3, 12.8 and 28.5 kGray

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

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

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

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

Figure 5.15: Graphs of β-sitosterol, campesterol and/or stigmasterol in *S. repens* extract (5:1), gamma irradiated at different intensities

Figure 5.16: Graphs of β-sitosterol, campesterol and/or stigmasterol in Immunochoice®, gamma irradiated at different intensities

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

Figure 5.18: A graph of β-sitosterol in Nutricare®, gamma irradiated at different intensities

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 0.1 μg applied

Figure 5.20: TLC on an aluminium plate of β-sitosterol-spiked and unspiked serum samples extracted with chloroform
Figure 5.21: Calibration curve to determine β-sitosterol in serum, obtained from MWD absorbance spectra, with a correlation coefficient of 0.99672

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

Figure 5.23: HPLC chromatogram of serum extracted 1:5 with chloroform

Figure 6.1: TLC on a glass plate of acetone extracts of African potato 1, Moducare®, Harzol®, Immunochoice®, Nutricare® and Nutricare®'s sterol mixture without inactives

Figure 6.2: TLC on a glass plate of every fourth fraction (from 1 to 67) of the acetone extract of H. hemerocallis, that eluted from the first column with ethylacetate:methanol:water (10:1.35:1) as mobile phase

Figure 6.3: TLC on a glass plate of the first fifteen fractions of the acetone extract of H. hemerocallis, that eluted from the first column with ethylacetate:methanol:water (10:1.35:1) as mobile phase

Figure 6.4: TLC on a glass plate of fraction 12 of the acetone extract of H. hemerocallis, that eluted from the first column with ethylacetate:methanol:water (10:1.35:1) as mobile phase

Figure 6.5: TLC on a glass plate of the first sixteen fractions of the H. hemerocallis extract, that eluted from the second column with chloroform:ethylacetate:formic acid (5:4:1) as mobile phase
LIST OF ABBREVIATIONS

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

$\varepsilon^{\circ}$ - 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 - $\beta$-sitosterol

BSSG - $\beta$-sitosterol glucoside ($\beta$-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 - dehydroepiandrosterone

DHT - 5$\alpha$-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_{i} - 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_{h1} - T-helper cell type 1
T_{h2} - T-helper cell type 2
TLC - thin layer chromatography
t_{max} - time of maximum absorption
TNF-\alpha - tumor necrosis factor alpha
TXB$_2$ - tromboxane B$_2$
UV - Ultra violet
w/w - weight/weight
PUBLICATIONS / PRESENTATIONS EMANATING FROM THIS STUDY

Scientific conferences:


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.


Other:

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®, Harzo®, 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 chromatography 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 isoflavonoid, 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. hemerocallisidea 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 *Serenova repens*) en verwante produkte ondersoek.

Die doel van die studie was om dunlaag- (TLC) en hoë-druk vloeistof chromatografiese metodes (HPLC) te ontwikkel, te evaluateer 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 kwantifieer. ‘n Sensitiewe metode om hipoksosied (norlignaan diglukosied uniek aan Hypoxidaceae) te bepaal is ook ontwikkel. Hipoksosied is uit die methanolstrak van *H. hemerocallidea* geïsoleer met soliede-fase ekstraksiie en met fluoressensie meting (eksitasie golflengte van 230 nm en emissie golflengte van 345 nm) gekwantifieer.

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 vervalsdatums 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.
TLC en HPLC analise het aangetoon dat medisinale Afrika-aartappeltjie 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 aartappeltjie 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 definitief nie 'n steroïd is nie, maar moontlik 'n koumarien of isoflavonoid 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 steroïe 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.