



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

by

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LIST OF ABBREVIATIONS

- $[\alpha]_D^{25}$ - specific rotation of plane polarized light at 25 °C
- ϵ° - 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 - dehydroepiandrosterone
- 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
- ^1H - proton
- HDL - high density lipoprotein
- HIV - human immuno-deficiency virus
- HPLC - high performance/pressure liquid chromatography



IC₅₀ - 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

PGD₂ - prostaglandin D₂

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_{H1} - T-helper cell type 1

T_{H2} - T-helper cell type 2

TLC - thin layer chromatography

t_{max} - time of maximum absorption

TNF- α - tumor necrosis factor alpha



TXB₂ - tromboxane B₂

UV - Ultra violet

w/w - weight/weight

PUBLICATIONS / PRESENTATIONS EMANATING FROM THIS STUDY

Scientific conferences:

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 Conference. 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 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 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 benige 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 plantsterole 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.

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 definitief 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.