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

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):

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

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:

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

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):

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

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

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

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 UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA 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.

5.2.3.10Improvement 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).

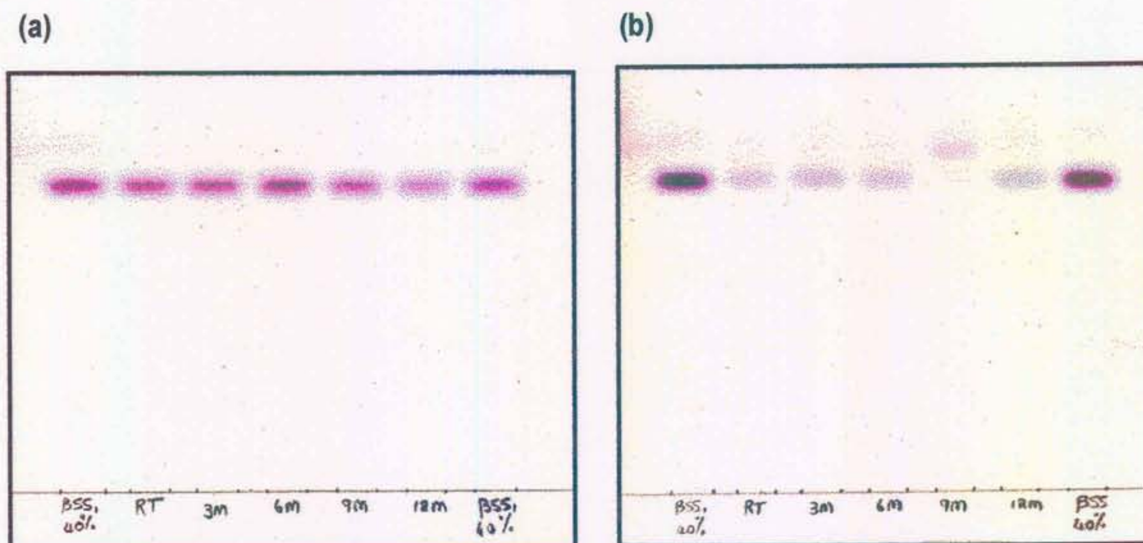


Figure 5.1: TLC on aluminium plates of chloroform extracts of **(a)** *P. africana* bark extract (5:1) and **(b)** *S. repens* berry extract (4:1) stored at 40°C for up to 12 months. On the sides are 40% β-sitosterol standard.

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).

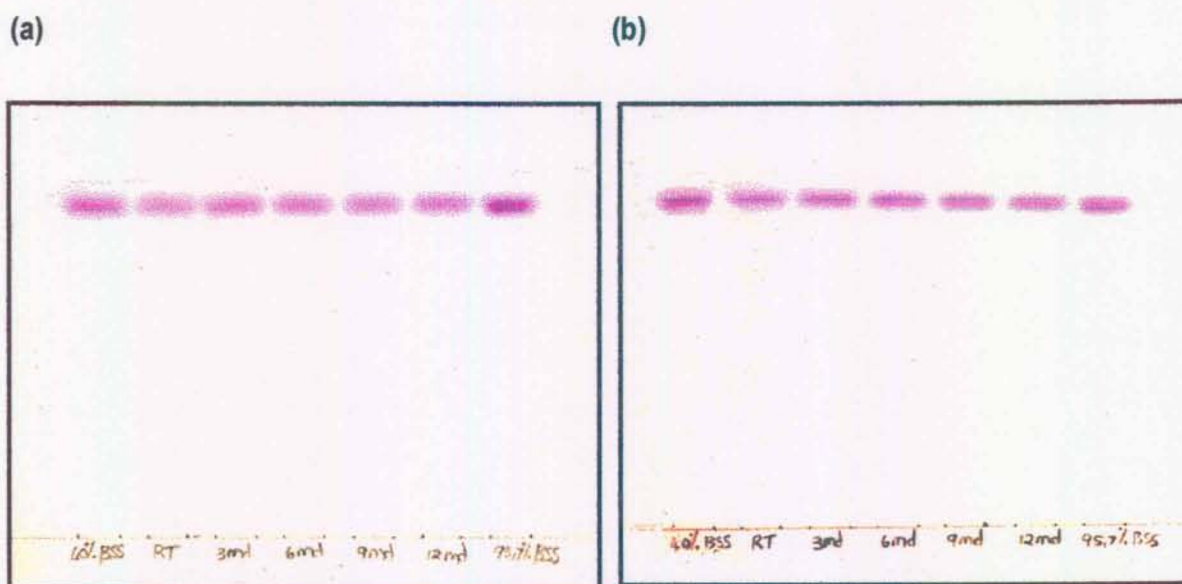


Figure 5.2: TLC on aluminium plates of chloroform extracts of **(a)** Immunochoice® and **(b)** Moducare® stored at 40°C for up to 12 months. On the left is 40% β-sitosterol and on the right is 95.7% β-sitosterol standard.

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.

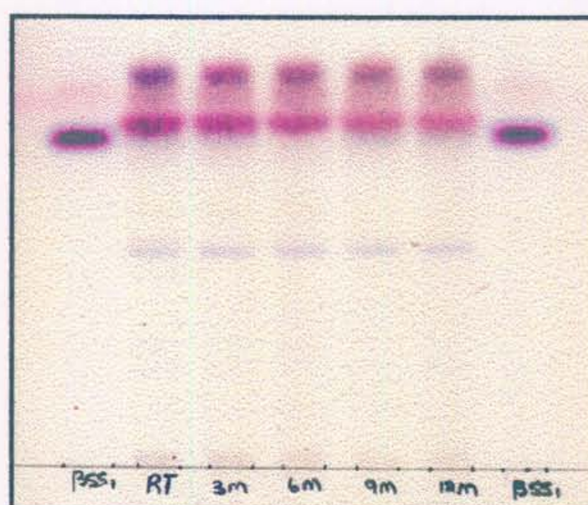


Figure 5.3: TLC on an aluminium plate of chloroform extracts of Nutricare® stored at 40°C for up to 12 months. On the sides are 40% β-sitosterol standard.

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

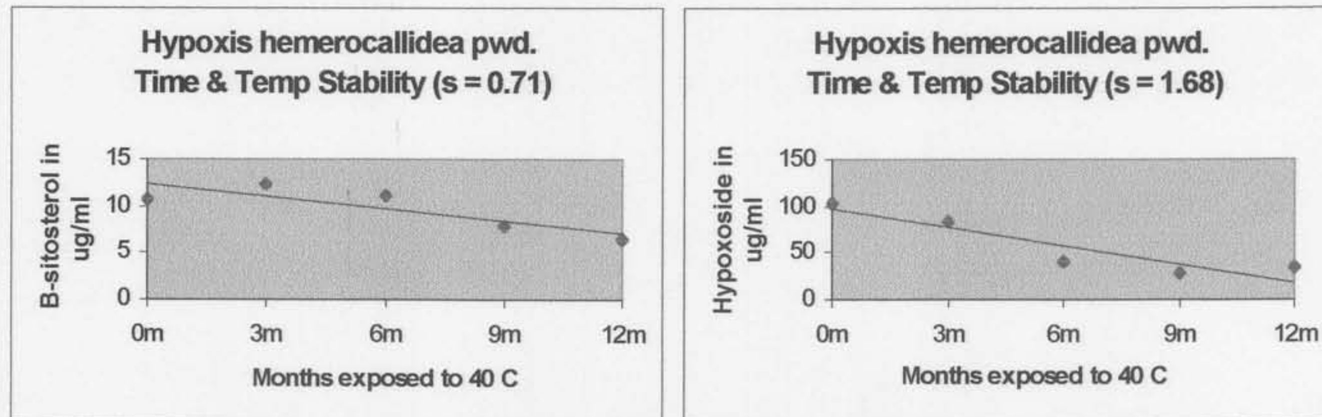


Figure 5.4: Graphs of β-sitosterol and hypoxoside (isolated with solid phase extraction and analysed with HPLC) in *H. hemerocallidea* powder stored at 40 °C for up to 12 months.

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).

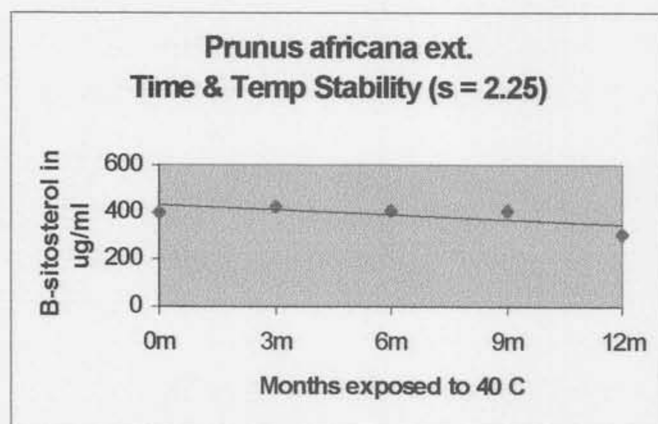
Hypoxoside decreased quickly up to 9 months (73.3% decrease) and then remained relatively stable up to 12 months. The standard deviation of the HPLC method (s) with FLD for hypoxoside was 1.68 μg/ml (4.0%).

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



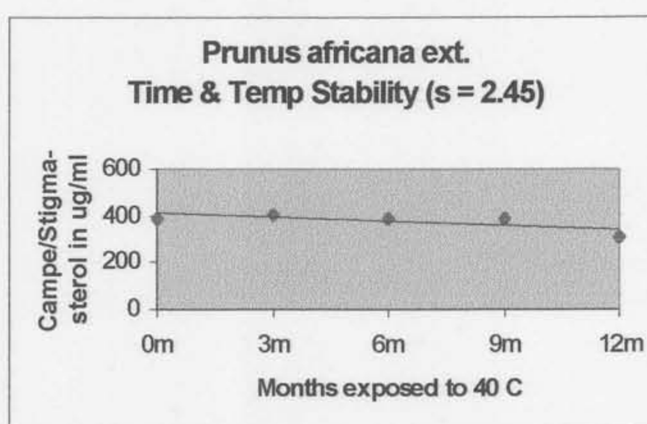


Figure 5.5: Graphs of β-sitosterol, campesterol and/or stigmasterol in *P. africana* extract (5:1) stored at 40 °C for up to 12 months.

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.

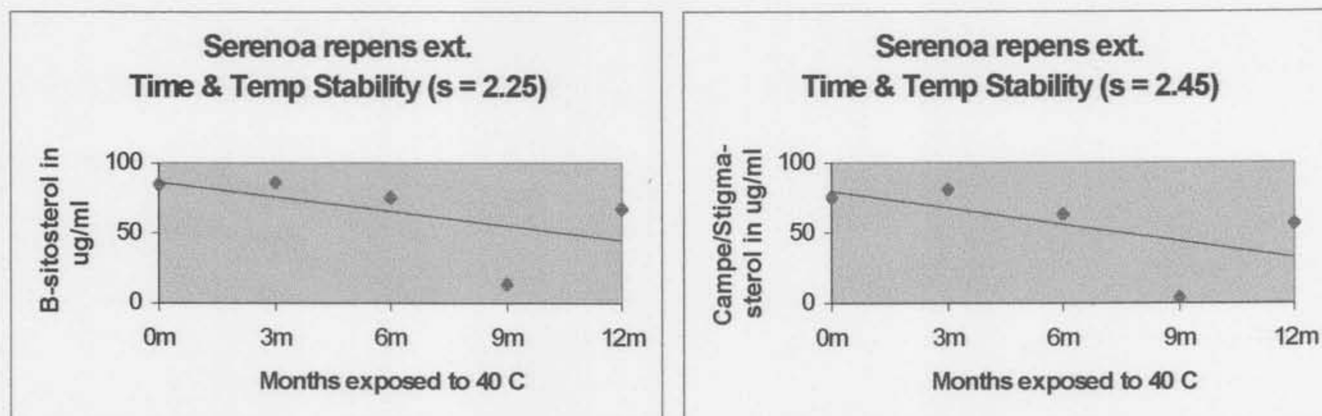


Figure 5.6: Graphs of β-sitosterol, campesterol and/or stigmasterol in *S. repens* extract (4:1) stored at 40 °C for up to 12 months.

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

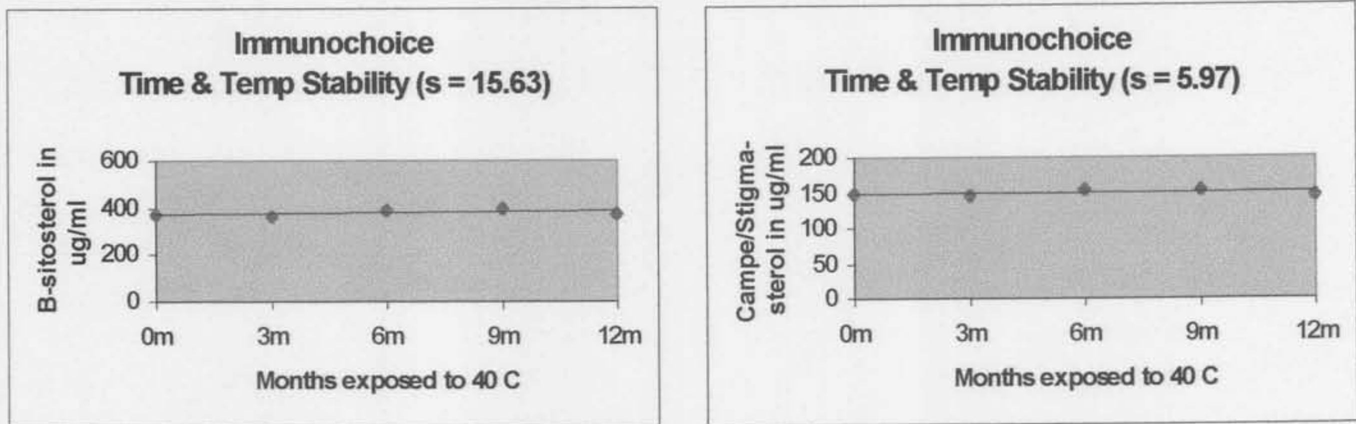


Figure 5.7: Graphs of β-sitosterol, campesterol and/or stigmasterol in Immunochoice® stored at 40 °C for up to 12 months.

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

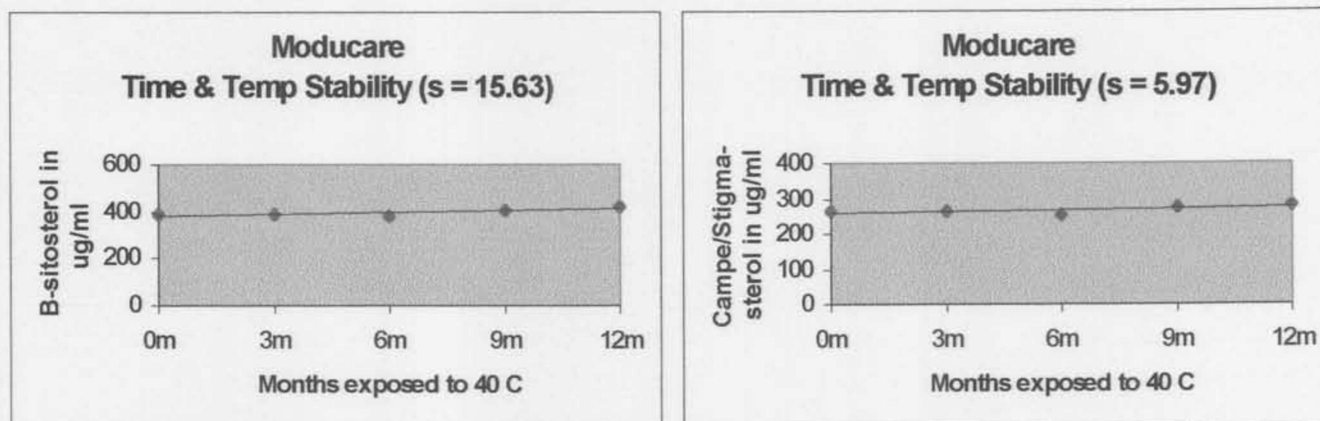


Figure 5.8: Graphs of β-sitosterol, campesterol and/or stigmasterol in Moducare® stored at 40 °C for up to 12 months.

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

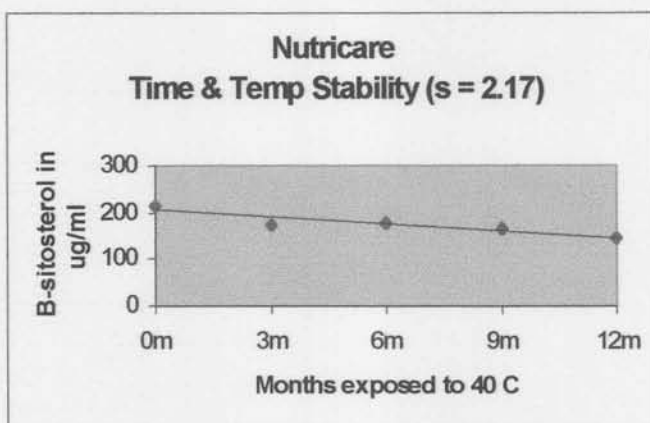


Figure 5.9: A graph of β-sitosterol in Nutricare® stored at 40 °C for up to 12 months.

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

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).

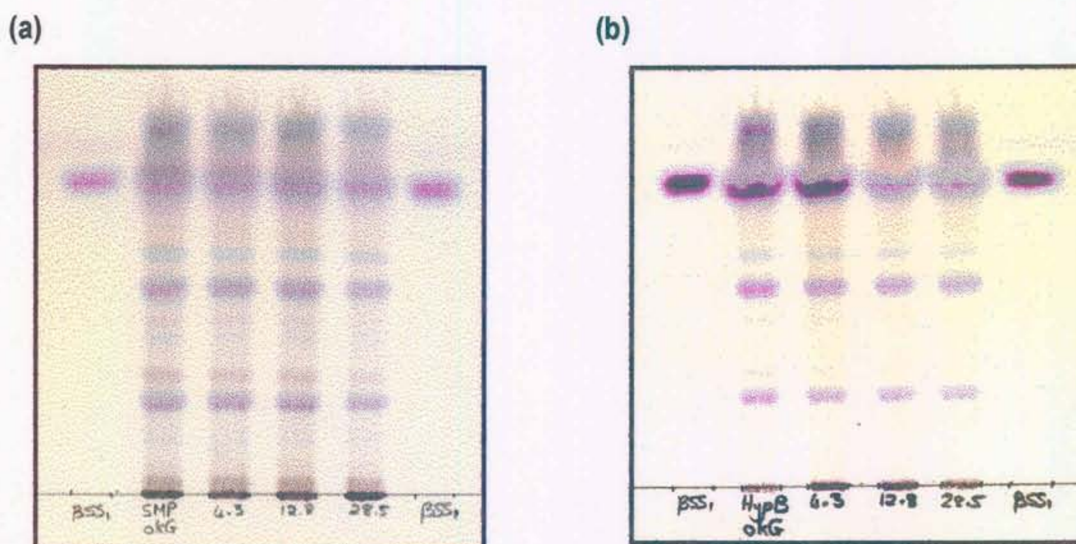


Figure 5.10: TLC on aluminium plates of chloroform extracts of **(a)** *H. hemerocallidea* powder (African potato 1) and **(b)** PLANTANICAL MEDICINE's Hypoxis (African potato 6), gamma irradiated at 0, 4.3, 12.8 and 28.5 kGray. On the sides are 40% β-sitosterol standard.

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).

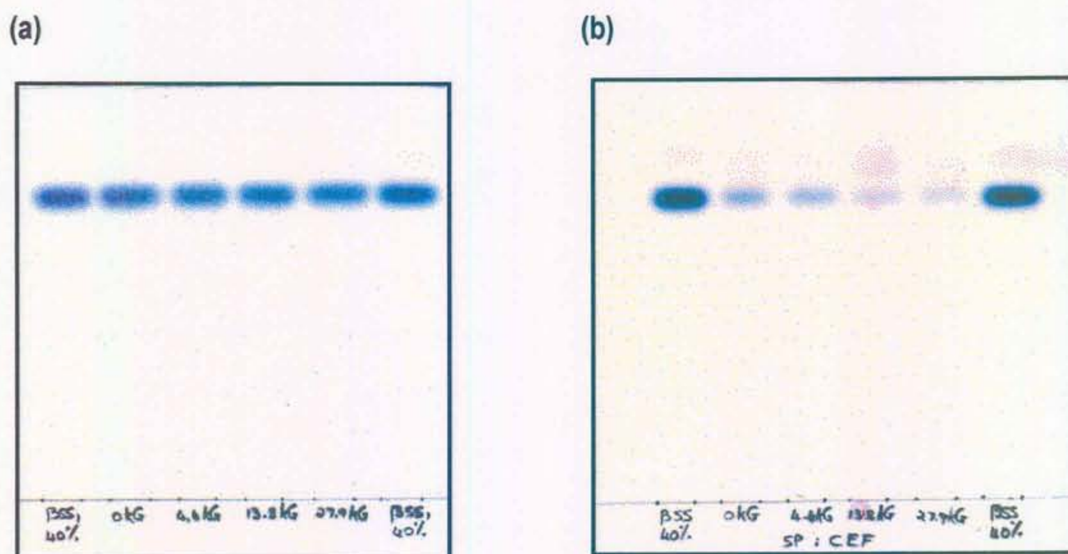


Figure 5.11: TLC on aluminium plates of chloroform extracts of **(a)** *P. africana* bark extract (5:1) and **(b)** *S. repens* berry extract (4:1), gamma irradiated at 0, 4.4, 13.8 and 27.9 kGray. On the sides are 40% β-sitosterol standard.

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).

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

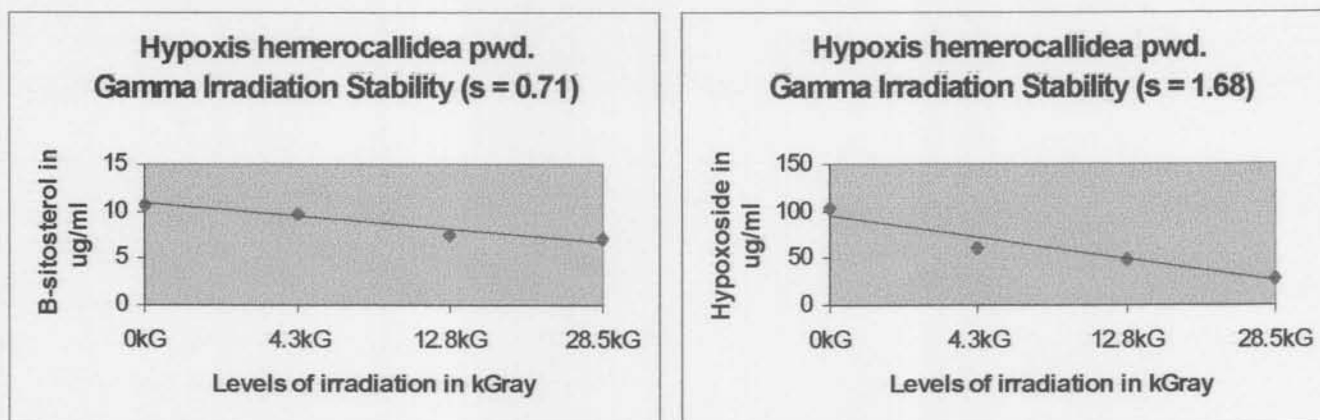


Figure 5.13: Graphs of β-sitosterol and hypoxoside (isolated with solid phase extraction and analysed with HPLC) in *H. hemerocallidea* powder, gamma irradiated at different intensities.

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

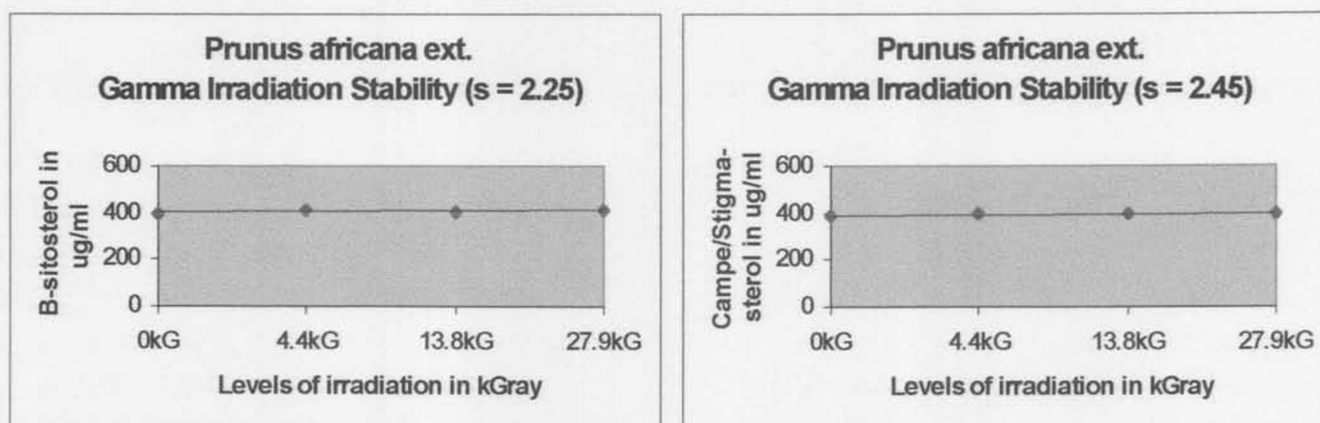


Figure 5.14: Graphs of β-sitosterol, campesterol and/or stigmasterol in *P. africana* extract (5:1), gamma irradiated at different intensities.

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

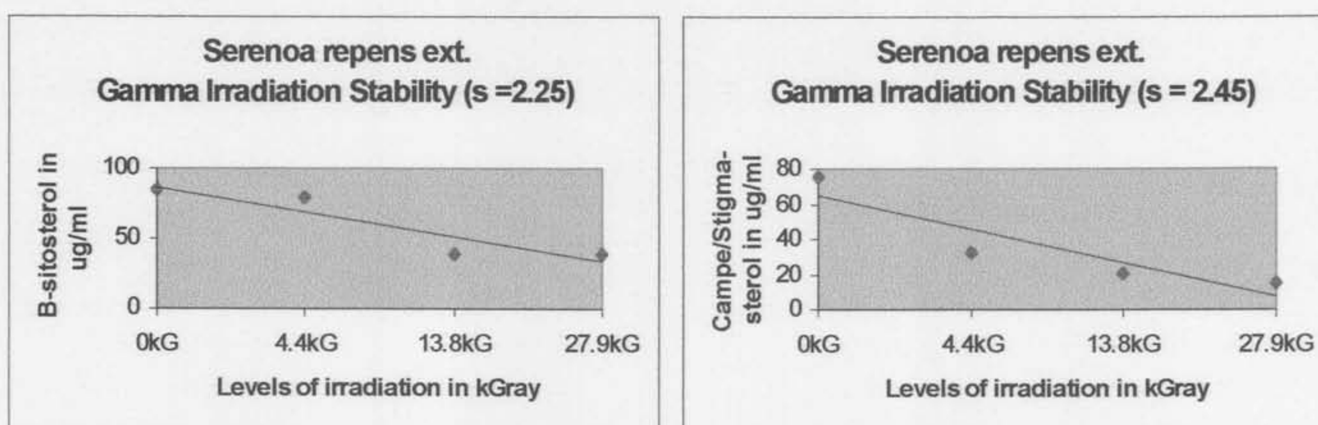


Figure 5.15: Graphs of β-sitosterol, campesterol and/or stigmasterol in *S. repens* extract (5:1), gamma irradiated at different intensities.

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

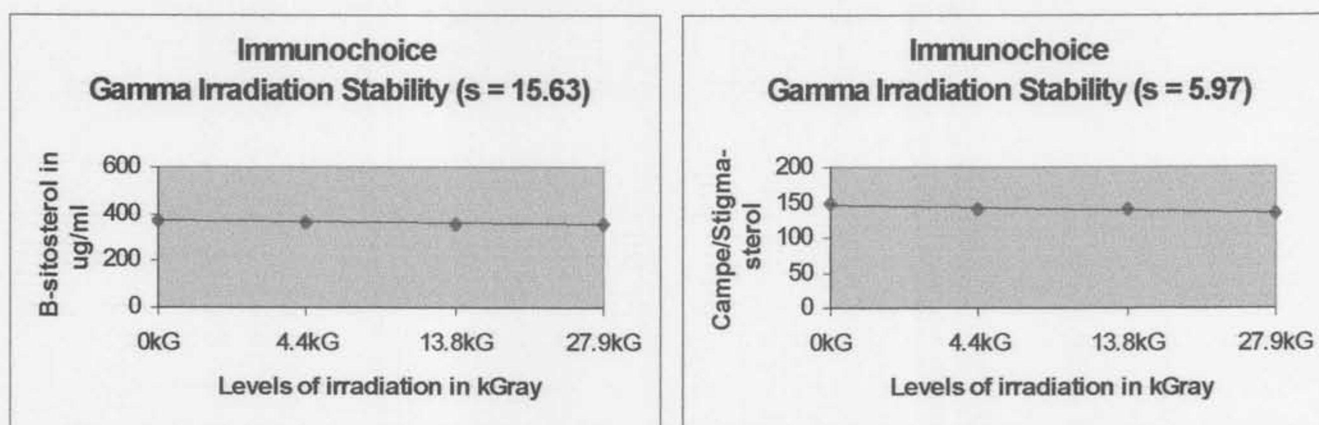


Figure 5.16: Graphs of β-sitosterol, campesterol and/or stigmasterol in Immunochoice®, gamma irradiated at different intensities.

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

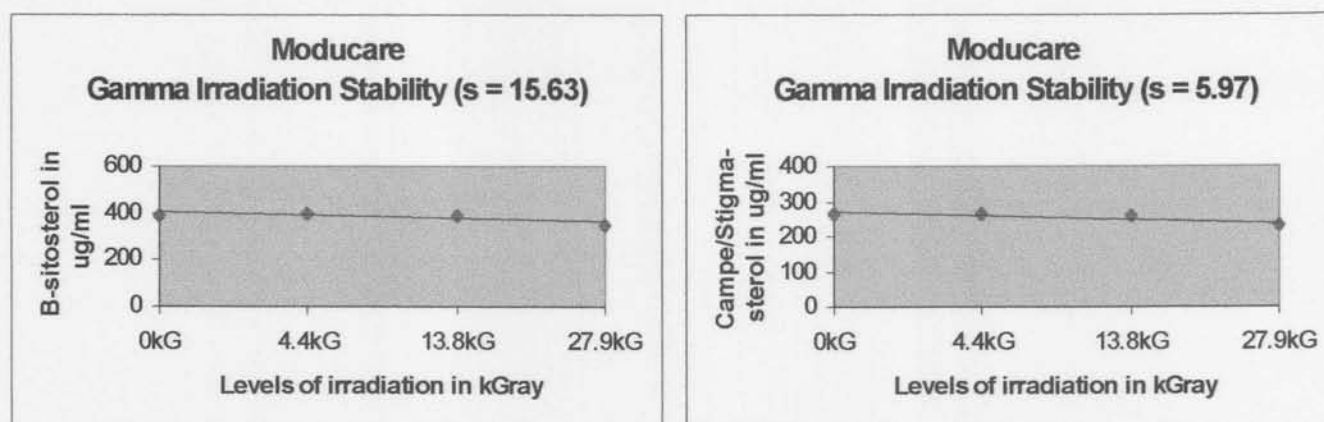


Figure 5.17: Graphs of β-sitosterol, campesterol and/or stigmasterol in Moducare®, gamma irradiated at different intensities.

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

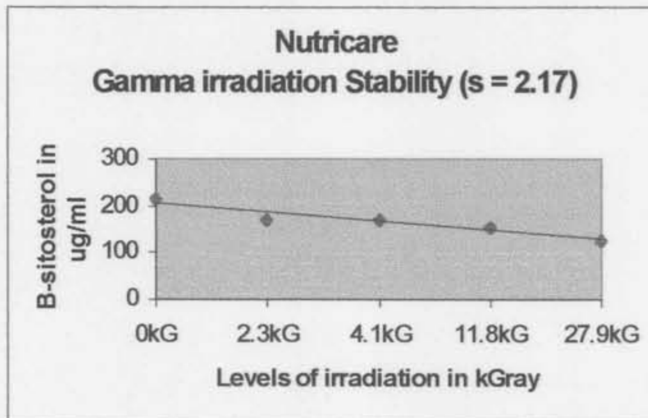


Figure 5.18: A graph of β-sitosterol in Nutricare®, gamma irradiated at different intensities.

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.

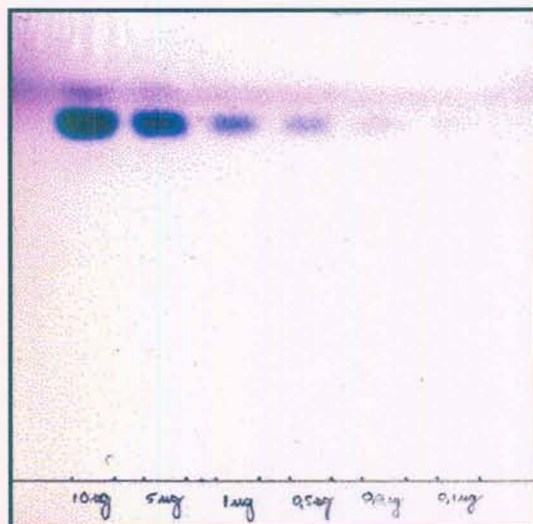


Figure 5.19: TLC on an aluminium plate of a dilution series of 95.7% β-sitosterol standard in chloroform with total mass 10, 5, 1.0, 0.5, 0.2 and 0.1μg applied.

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.

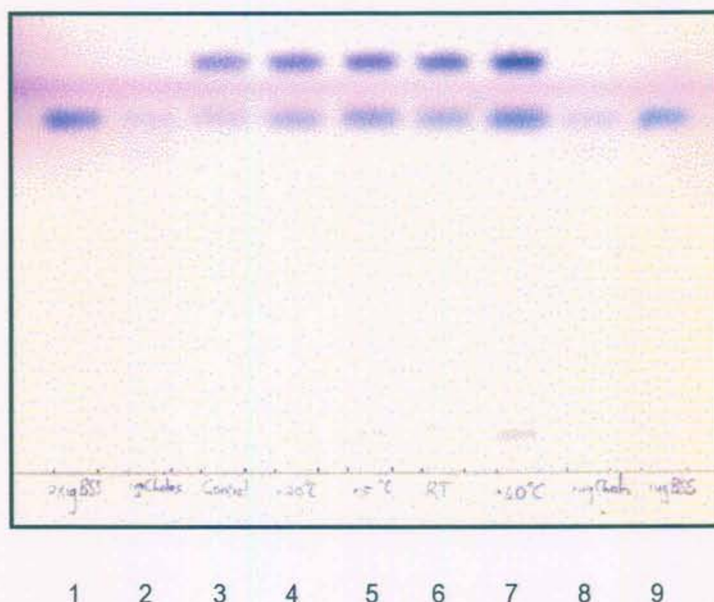


Figure 5.20: TLC on an aluminium plate of β-sitosterol-spiked and unspiked serum samples extracted with chloroform. From the left: 95.7% β-sitosterol standard (1), 99+% cholesterol standard (2), unspiked serum control (3) and β-sitosterol-spiked serum (4). Applications 5 - 7 were β-sitosterol-spiked 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.

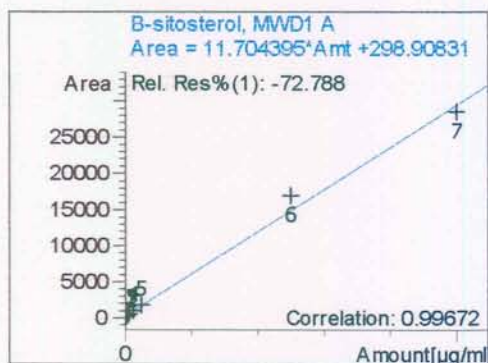


Figure 5.21: Calibration curve to determine β-sitosterol in serum, obtained from MWD absorbance spectra, with a correlation coefficient of 0.9967.

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.

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.

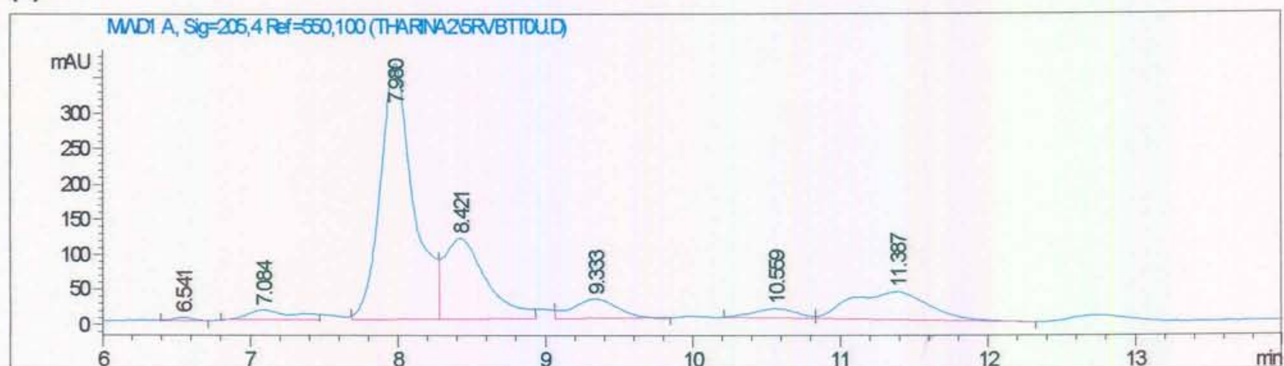
The high peak at t_R = c. 8.0 minutes was identified as cholesterol with the means of 99+% cholesterol standard.

5.3.2.2 (c) Improved β-sitosterol extraction from serum

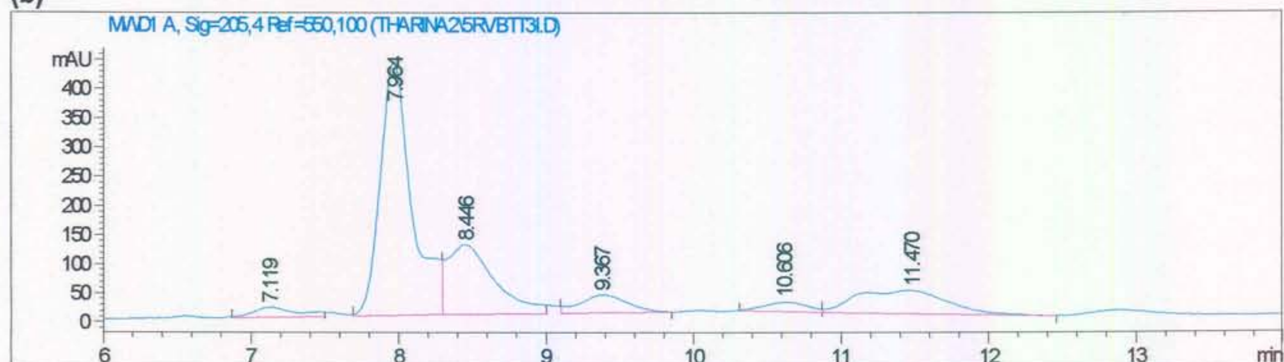
5.3.2.2 (c) (i) Mixing by turning instead of vortex mixing

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)

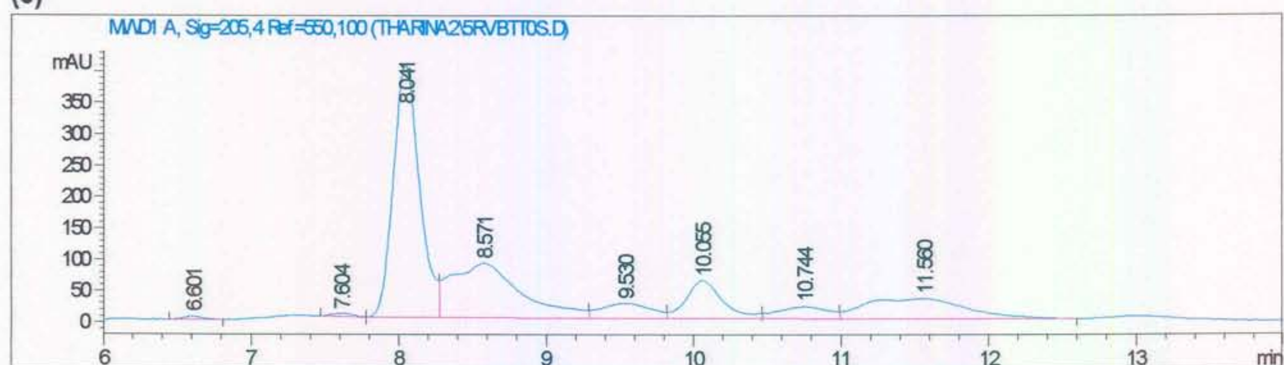


Figure 5.22: HPLC chromatogram of chloroform extracted serum sampled at (a) time = 0, (b) time = 3 hours after ingesting 9 capsules of Tadenan® (equivalent to 54 mg β-sitosterol) and (c) time = 0 sample spiked with 95.7% β-sitosterol standard after extraction. See the β-sitosterol peak at t_R = 10.055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

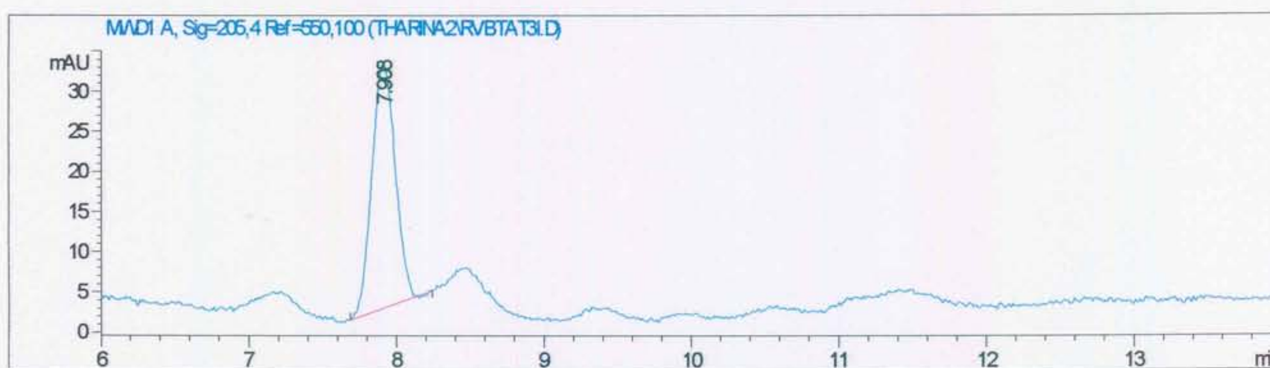


Figure 5.23: HPLC chromatogram of serum extracted 1:5 with chloroform. The serum was sampled 3 hours after ingesting 9 capsules of Tadenan® (equivalent to 54 mg β-sitosterol). The peaks are poorly defined at this extraction ratio.

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 ($AUC_{0\text{-}8}$), 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%.

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.

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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*.

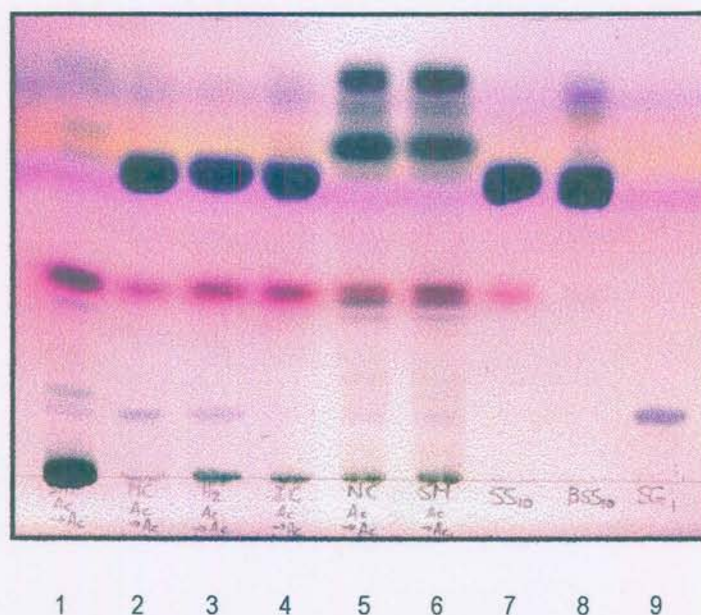


Figure 6.1: TLC on a glass plate of acetone extracts of African potato 1 (1), Moducare®(2), Harzol®(3), Immunochoice®(4), Nutricare®(5) and Nutricare®'s sterol mixture without inactives (6). On the right are 10 mg/ml 95% stigmasterol standard (7), 10 mg/ml 40% β-sitosterol standard (8) and 1 mg/ml β-sitosterolin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

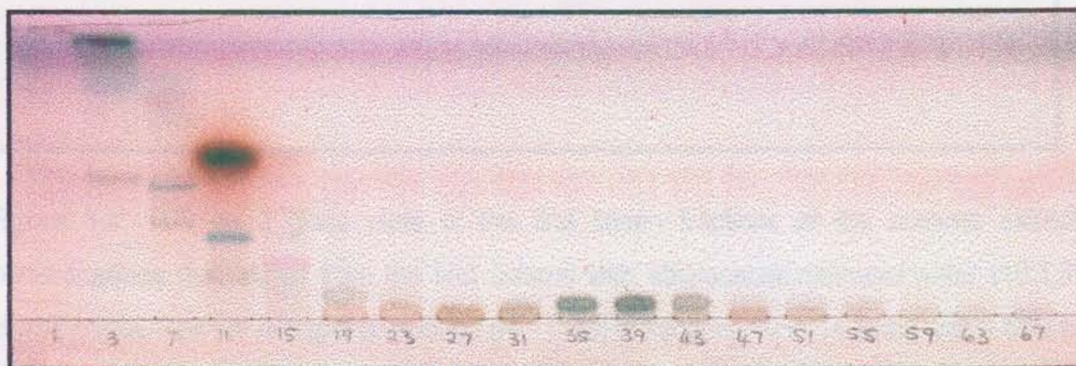
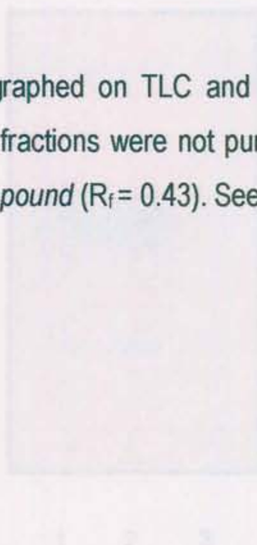


Figure 6.2: TLC on a glass plate of every fourth fraction (from 1 to 67) of the acetone extract of *H. hemerocallidea* that eluted from the first column with ethylacetate:methanol:water (10:1.35:1) as mobile phase. The plate was developed in CEF and sprayed with *p*-anisaldehyde. See the *red spot compound*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

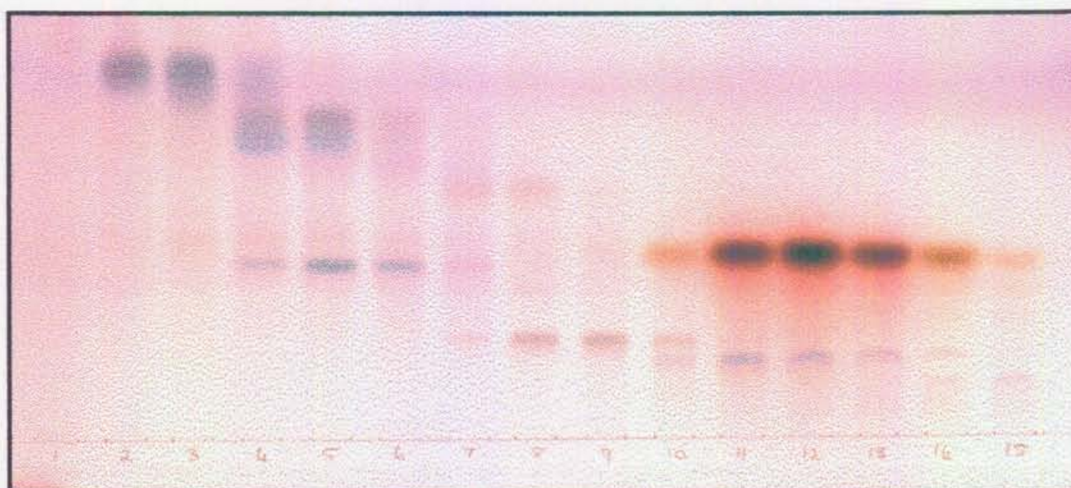
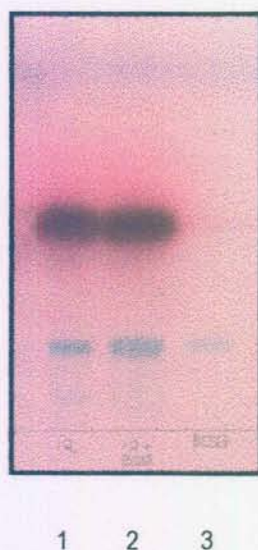


Figure 6.3: TLC on a glass plate of the first fifteen fractions of the acetone extract of *H. hemerocallidea* that eluted from the first column with ethylacetate:methanol:water (10:1.35:1) as mobile phase. See the *red spot compound* (R_f = 0.43) in fractions 10 to 14 along with a blue band (R_f = 0.18).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

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.

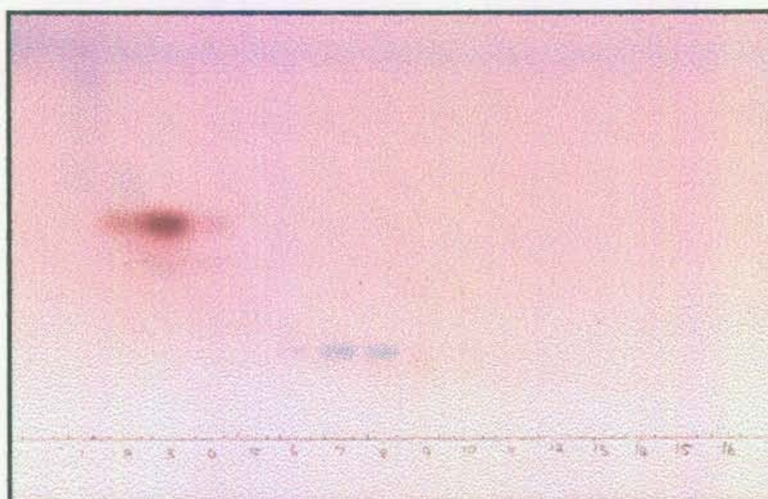


Figure 6.5: TLC on a glass plate of the first sixteen fractions of the *H. hemerocallidea* extract that eluted from the second column with chloroform:ethylacetate:formic acid (5:4:1) as mobile phase. See the *red spot compound* in fractions 2 and 3 and β-sitosterolin in fractions 7 and 8. The plate was 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

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 ($AUC_{0\text{-}8}$), 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).