CHAPTER 4

HPLC METHOD EVALUATION AND APPLICATION

4.1 INTRODUCTION

4.1.1 History

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

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

4.1.2 Aim of chapter

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

4.1 MATERIAL AND METHODS

4.2.1 Material

4.2.1.1 Plant material

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

4.2.2 Apparatus

4.2.2.1 SPE-equipment

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

4.2.2.2 HPLC-equipment

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

4.2.3 Methods

4.2.3.1 Extraction

4.2.3.1 (a) Simple extraction

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

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

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

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

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

4.2.3.1 (b) Solid phase extraction (SPE)

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

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

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

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

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

1. Condition/saturate the SPE sorbent (C_{18} cartridge): Pass 5 ml of 50% aqueous methanol through the column to wet it and to ensure reproducible retention of the compound of interest. Collect the liquid that elutes (saturation solution).
2. Retention: Apply 2 ml of the 10 mg/ml sample (20 mg), dissolved in 50% aqueous methanol to the sorbent. Selective retention of the analyte is allowed, while the other materials are removed. Collect the liquid that elutes (sample elute).
3. Rinse/Elution: (a) Polar eluent: wash the column with 10 ml 50% aqueous methanol to remove the polar compound hypoxoside. Collect the eluting liquid (water-methanol fraction 1). Repeat twice with 5 ml 50% aqueous methanol, but collect the fractions separately (water-methanol fraction 2 and 3), to ascertain that all the hypoxoside is recovered.
   (b) Intermediate polarity eluent: Wash the column with 10 ml acetone to mediate mixing of the water-methanol and hexane fractions. Collect the eluting liquid (acetone fraction).
   (c) Non-polar eluent: wash the column with 10 ml hexane to remove the non-polar phytosterols (mainly β-sitosterol). Collect the eluting liquid (hexane fraction 1). Repeat twice with 5 ml hexane, but collect the fractions separately (hexane fraction 2 and 3), to ascertain that all the phytosterols are recovered.
4. Dry the collected fractions and redissolve them in 1.0 ml HPLC grade methanol.
5. Inject 100 µl samples into the HPLC. Use FLD detection for hypoxoside and MWD for sterols.

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

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

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

4.2.3.2 Preparation of standards

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

4.2.3.3 HPLC for quantitative measurements

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

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

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

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

4.2.3.3 (b) Calibration curves for single determinations

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

4.3 RESULTS AND DISCUSSION

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

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

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

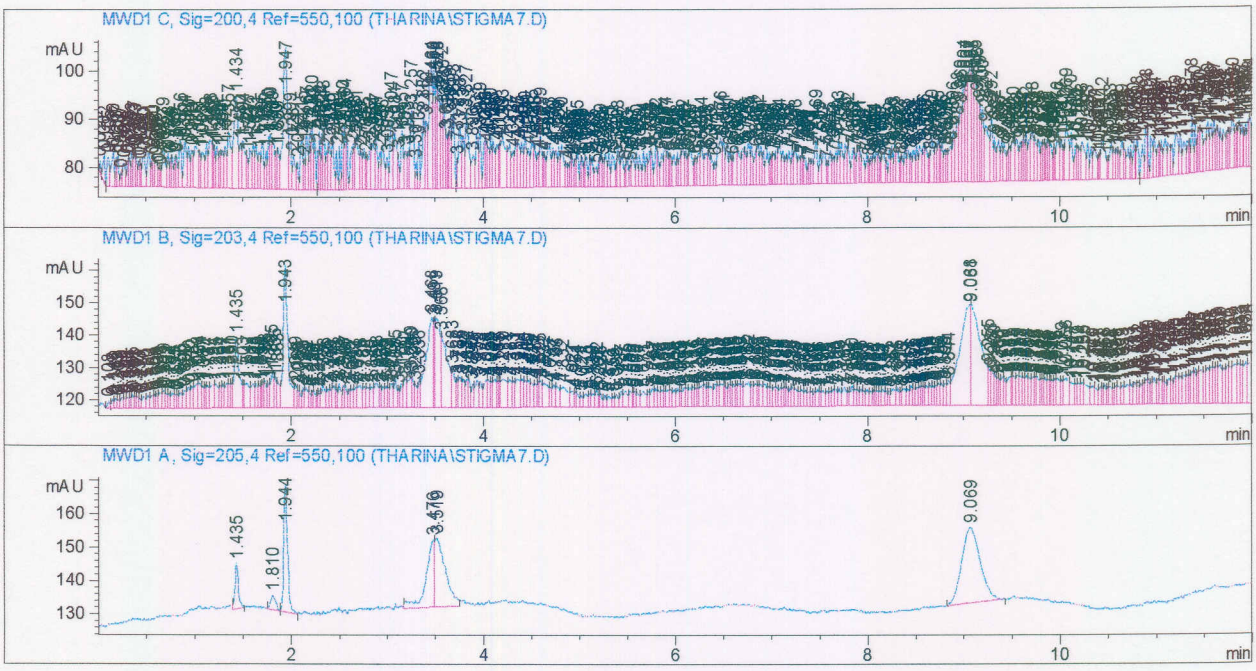

Figure 4.1: Absorbance spectra of 100 µg/ml 95% stigmasterol standard dissolved in HPLC grade methanol and measured with MWD at wavelengths 200.4, 203.4 and 205.4 nm.

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

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

4.3.2 Calibration curves

Three types of calibration curves were constituted:

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

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

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

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

Although the retention times varied with temperature and changes in mobile phase composition (as result of evaporation *etc.*), the retention times of the five standards in the composite sample, with 96.5% methanol as mobile phase, were as follows: β-sitosterol (t_R=10.020), campesterol (t_R=8.933) stigmasterol (t_R=8.933), β-sitosterolin (t_R=5.534) and hypoxoside (t_R=1.195). Note that campesterol and stigmasterol had exactly the same retention time, as a 96.5% mobile phase could not separate them. This means that phytosterol peaks with a retention time of c. 8.933 can either be campesterol, stigmasterol or a combination of the two and quantities were reported as such.

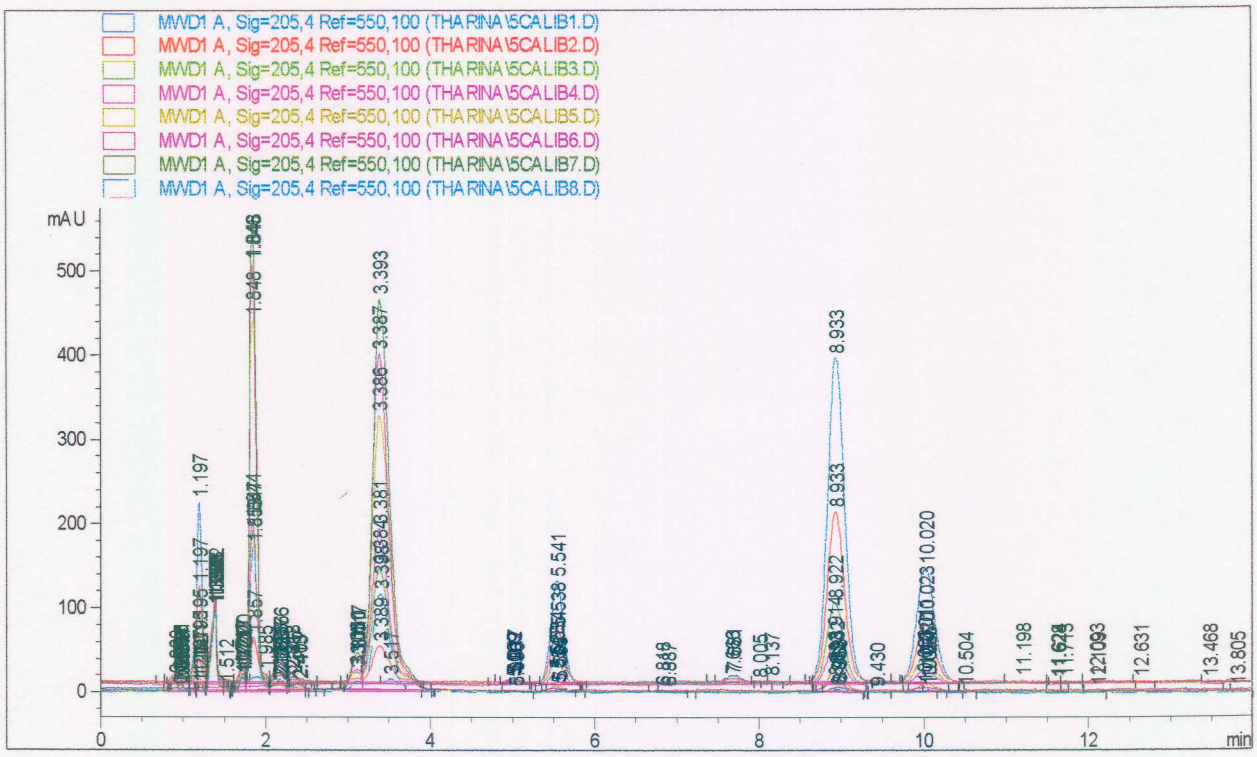

Figure 4.2: Overlayed absorbance spectra of eight different concentrations of 95.7% β-sitosterol (t_R=10.020), 65% campesterol (t_R=8.933) 95% stigmasterol (t_R=8.933), β-sitosterolin (t_R=5.534) and hypoxoside (t_R=1.195) in HPLC grade methanol, measured with MWD at a wavelength of 205.4 nm with 96.5% methanol as mobile phase. These absorbance spectra were used to calculate calibration curves for each compound.

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

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

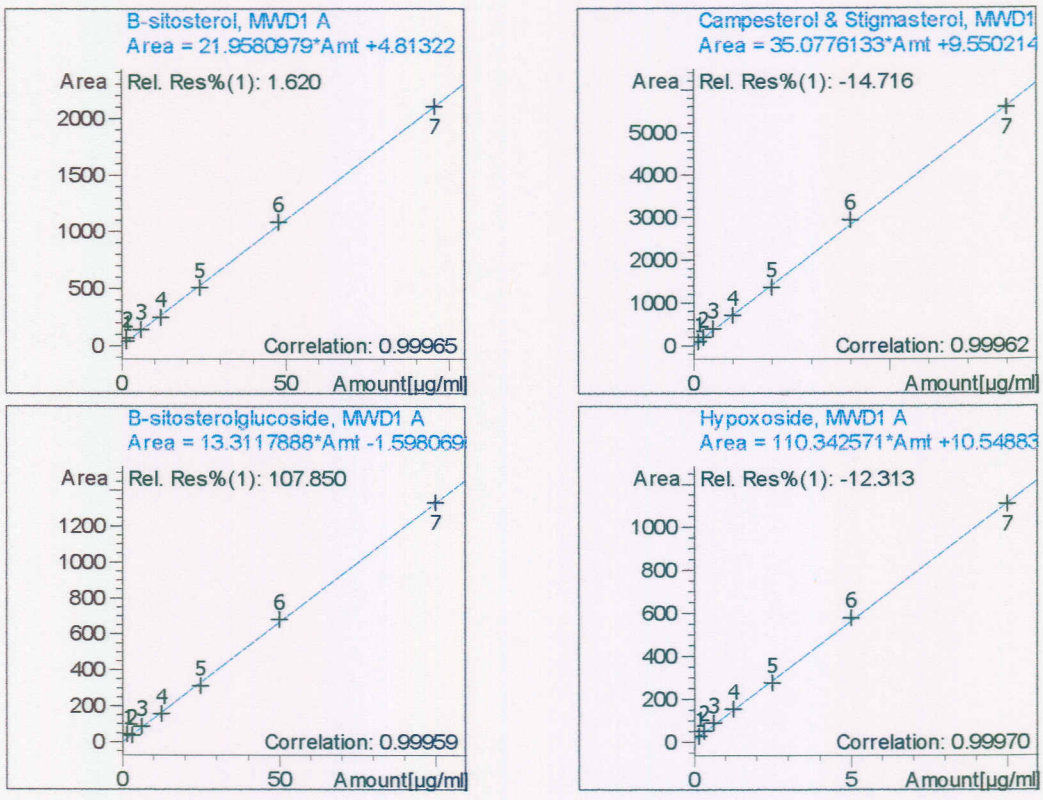

Figure 4.3: Calibration curves of β-sitosterol, campesterol and stigmasterol, β-sitosterolin and hypoxoside, obtained from MWD absorbance spectra, with their correlation coefficients.

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

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

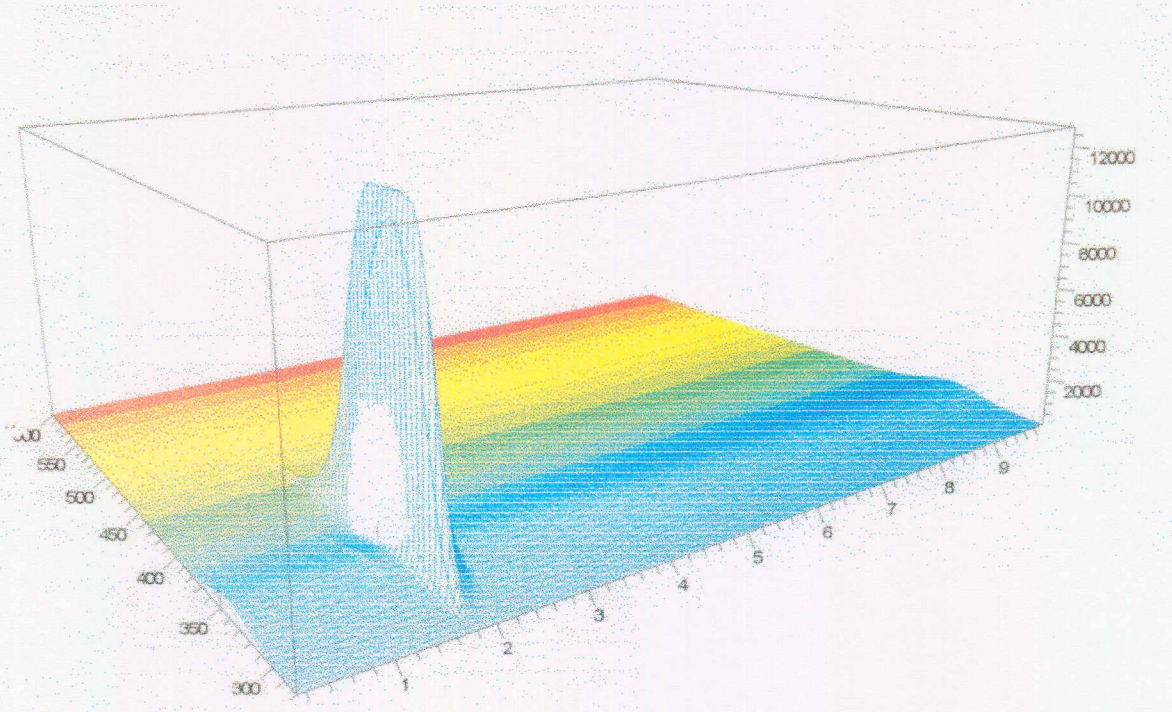

Figure 4.4: Three dimensional fluorescence spectra of hypoxoside. At an excitation wavelength of 230 nm, hypoxoside emits UV light of 345 nm.

4.3.3 Solid phase extraction (SPE)

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

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

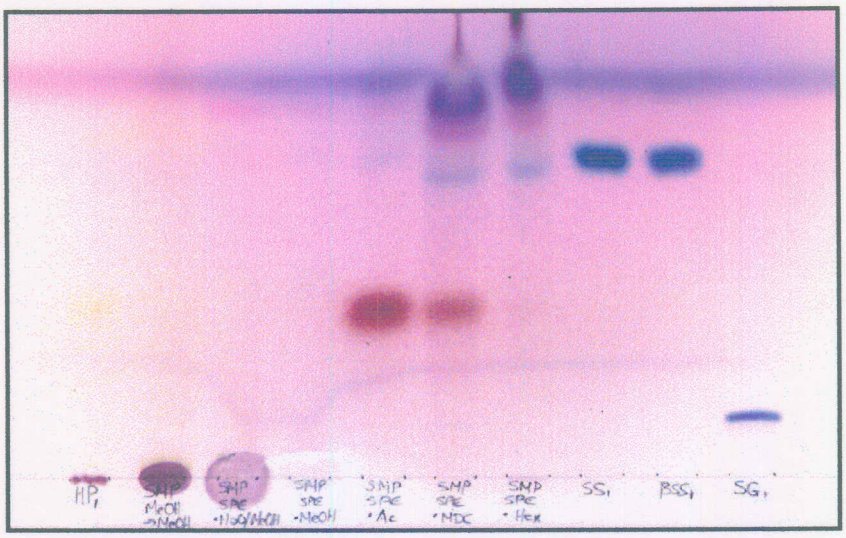

Figure 4.5: TLC on a glass plate of different SPE eluants tested in the development of a SPE process to isolate hypoxoside and β-sitosterol from *H. hemerocallidea* methanol extract. From left to right: Isolated hypoxoside (1), 10 mg/ml African potato 1 methanol extract, placed on the SPE C_{18} cartridge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

4.3.3.1 Detection of a contaminant in analytical grade solvents

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

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

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

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

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

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

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

When the dried acetone, dissolved in HPLC grade methanol, was spiked with β-sitosterol, it gave a symmetric peak on the HPLC. The contaminant had exactly the same retention time as β-sitosterol! See Figure 4.6.

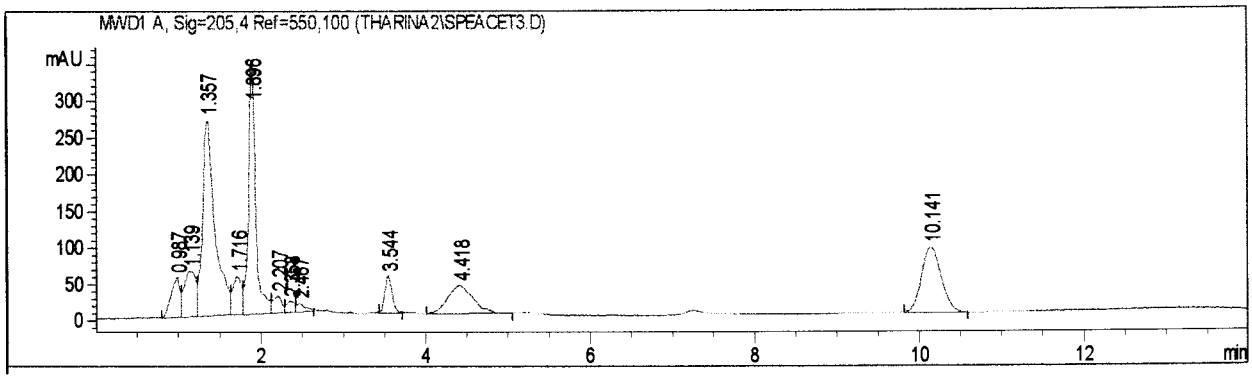

Figure 4.6: HPLC chromatogram of analytical grade acetone, stored in a plastic bottle (dried and redissolved in HPLC grade methanol – 10 mg/ml) with a contaminant with exactly the same retention time as β-sitosterol (t_R = 10.141) and calibrated as 68.19 μg/ml β-sitosterol. The mobile phase was 96.5%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

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

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

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

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

4.3.4 Methanol versus chloroform extraction of phytosterols

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

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

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

Dry mass applied to SPE = 20 mg.
Hexane fraction, dried and redissolved in 1.0 ml MeOH = 10.83 μg/ml β-sitosterol.
Thus, 20 mg extract = 10.83 μg β-sitosterol.
But, total volume of 10 mg/ml extract = 11.5 ml and that is 115 mg dry mass extract.
Thus, 115 mg extract = 62.3 μg β-sitosterol.

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

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

10 mg/ml chloroform extract = 187.5 μg/ml β-sitosterol
187.5 μg β-sitosterol per 1.0 ml chloroform extract
But, total volume of 10 mg/ml extract = 0.13 ml
Thus, 0.13 ml = 24.4 μg β-sitosterol

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

4.3.5 HPLC Application

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

4.3.5.1 Phytosterol analysis of different plants

4.3.5.1 (a) *H. hemerocallidea*

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

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

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

African potato 6 was the only *H. hemerocallidea* powder to contain 0.0014% campesterol and/or stigmasterol. See Figure 4.7. Hypoxoside was not measured as chloroform extraction was performed and hypoxoside is not extracted by chloroform.

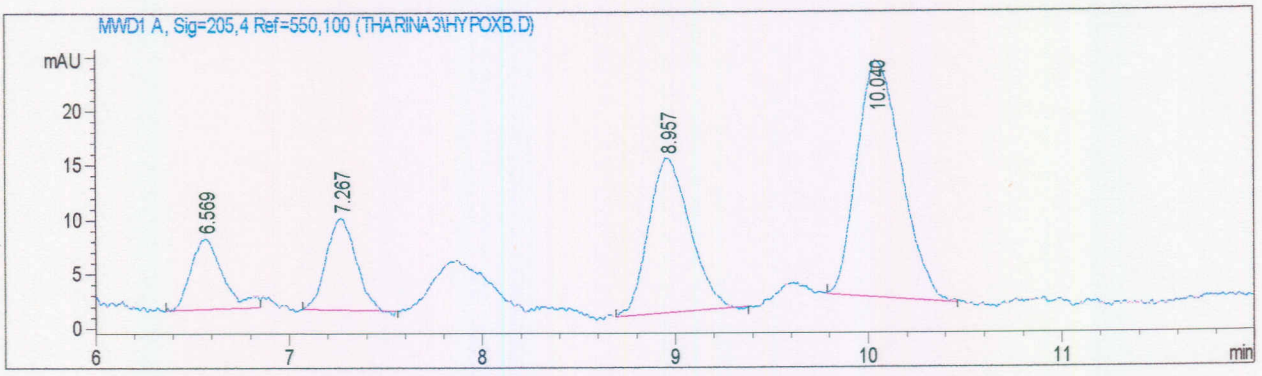

Figure 4.7: HPLC chromatogram of a chloroform extract of *H. hemerocallidea* powder from PLANTANICAL MEDICINE (African potato 6), which contains campesterol and/or stigmasterol (t_R=8.957) and β-sitosterol (t_R= 10.040). The mobile phase was 96.5% methanol.

4.3.5.1 (b) *P. africana*

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

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

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

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

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

P. africana leaf powder apparently does not contain any campesterol and/or stigmasterol, but the β-sitosterol content is very similar to that of the bark powder. See Figure 4.8.

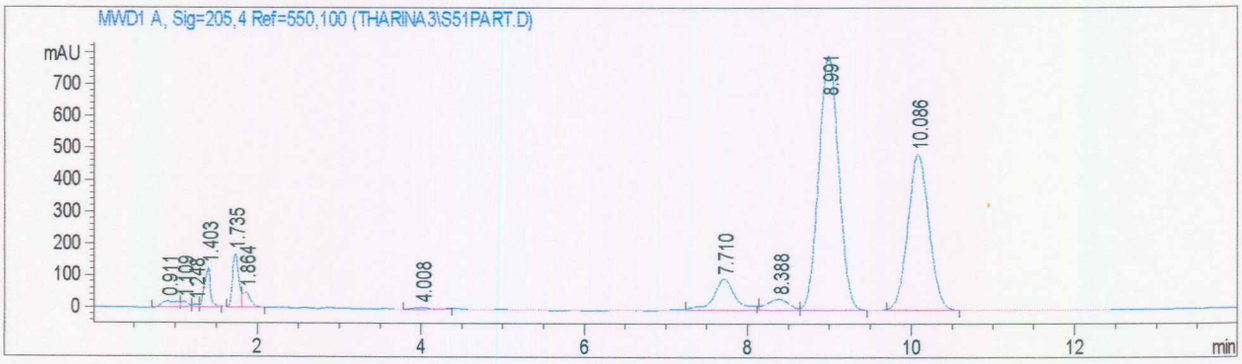

Figure 4.8: HPLC chromatogram of a chloroform extract of *P. africana* bark extract (5:1) with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=8.991) and β-sitosterol (t_R= 10.086).

4.3.5.1 (c) *S. repens*

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

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

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

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

P. africana	[Extract] (mg/ml)	Campe- &/or Stigmasterol (µg/ml)	Extract Volume (ml)	µg CS &/or SS from 500 mg	Percentage CS &/or SS in plant
Berry extract (4:1)	1	76.09	0.4	30.4	**0.0061 %**
Berry powder	3.75	10.3	0.08	0.82	**0.00016 %**

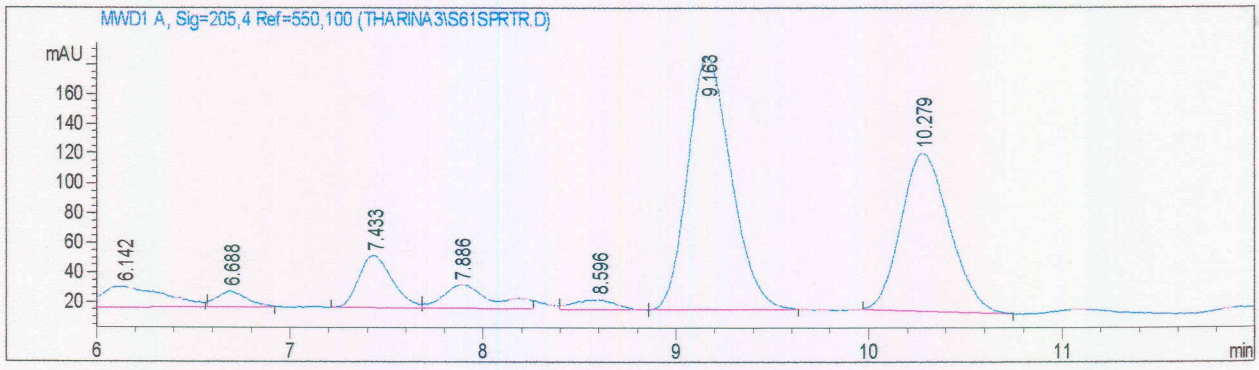

Figure 4.9: HPLC chromatogram of a chloroform extract of *S. repens* berry extract (4:1) with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=9.163) and β-sitosterol (t_R= 10.279).

4.3.5.2 Phytosterol analysis of different products

4.3.5.2 (a) Immunochoice®

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

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

[Extract] (mg/ml)	βSS (µg/ml)	CS &/or SS (µg/ml)	Extract Volume (ml)	βSS + CS &/or SS (µg)	Percentage of 20 mg
1	524.96	217.92	10	7428.9	**37.14 %**

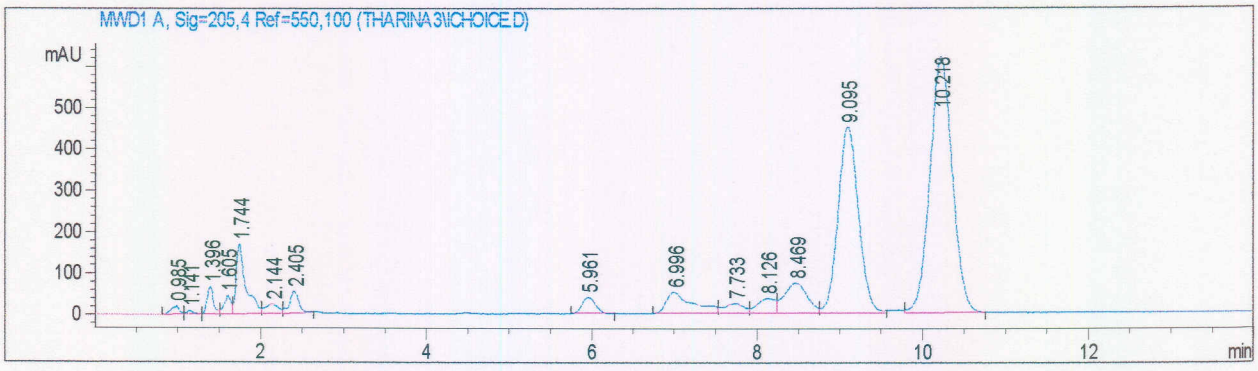

Figure 4.10: HPLC chromatogram of a chloroform extract of Immunochoice® with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=9.095) and β-sitosterol (t_R= 10.218).

4.3.5.2 (b) Harzol®

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

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

[Extract] (mg/ml)	βSS (µg/ml)	CS &/or SS (µg/ml)	Extract Volume (ml)	βSS + CS &/or SS (µg)	Percentage of 10 mg
0.13	62.5	4.1	100	6660	**66.6 %**

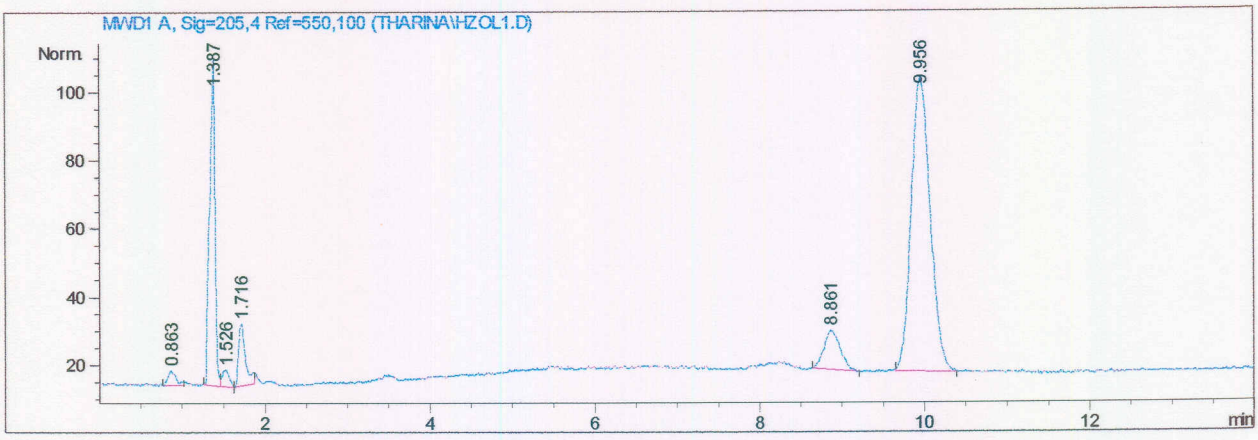

Figure 4.11: HPLC chromatogram of a chloroform extract of Harzol® with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=8.861) and β-sitosterol (t_R= 9.958).

4.3.5.2 (c) Moducare®

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

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

[Extract] (mg/ml)	βSS (µg/ml)	CS &/or SS (µg/ml)	Extract Volume (ml)	βSS + CS &/or SS (µg)	Percentage of 20 mg
1	529.88	355.42	14	12 394.2	**61.97 %**

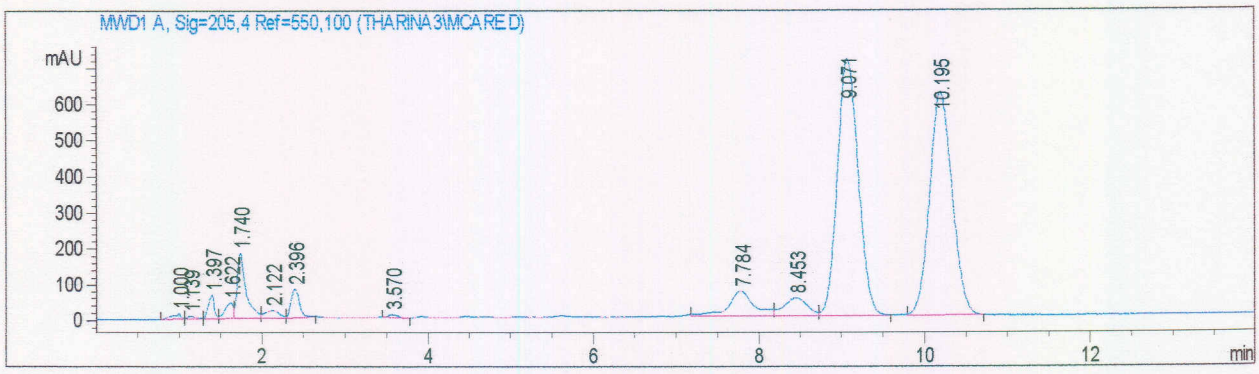

Figure 4.12: HPLC chromatogram of a chloroform extract of Moducare® with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=9.071) and β-sitosterol (t_R=10.195).

4.3.5.2 (d) Nutricare®

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

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

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

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

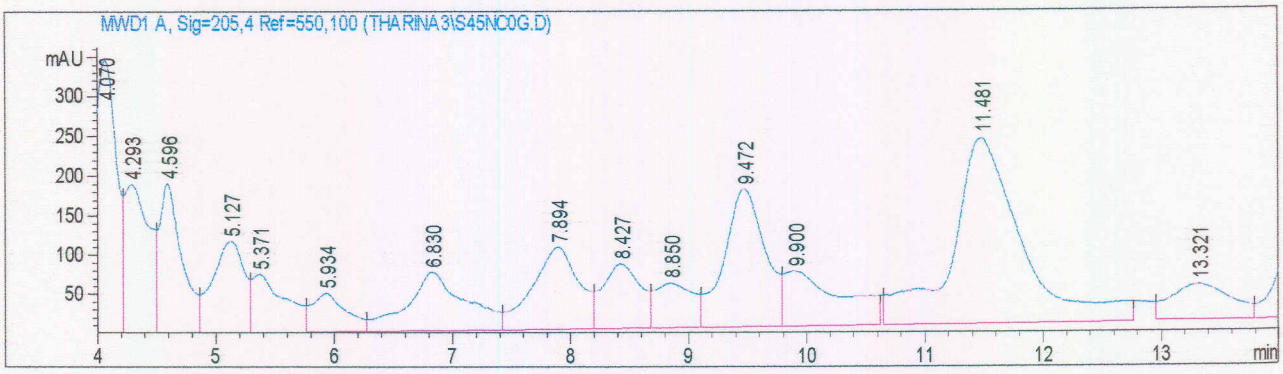

Figure 4.13: HPLC chromatogram of a chloroform extract of Nutricare® with 96.5% methanol as mobile phase. The peak at t_R=9.472 minutes was assumed to be β-sitosterol, although there is some uncertainty.

4.3.5.2 (e) Phytogard®

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

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

[Extract] (mg/ml)	βSS (µg/ml)	CS &/or SS (µg/ml)	Extract Volume (ml)	βSS + CS &/or SS (µg)	Percentage of 60mg
1	738.16	61.86	38.8	31 040.4	**51.7 %**

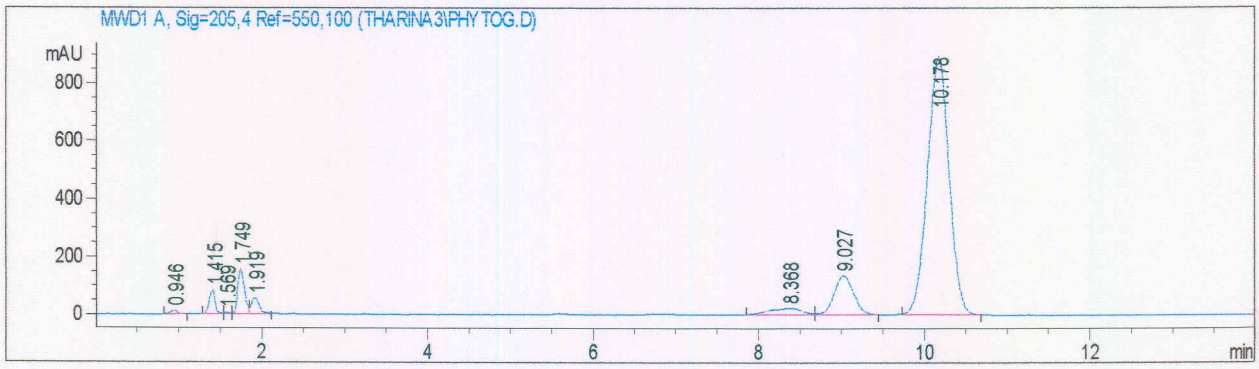

Figure 4.14: HPLC chromatogram of a chloroform extract of Phytogard® with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=9.027) and β-sitosterol (t_R=10.178).

4.3.5.2 (f) Prostol Herbal®

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

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

[Extract] (mg/ml)	βSS (µg/ml)	CS &/or SS (µg/ml)	Extract Volume (ml)	βSS + CS &/or SS (µg)	Percentage of 500mg
1	267.8	262.4	17.5	9 278.5	**1.86 %**

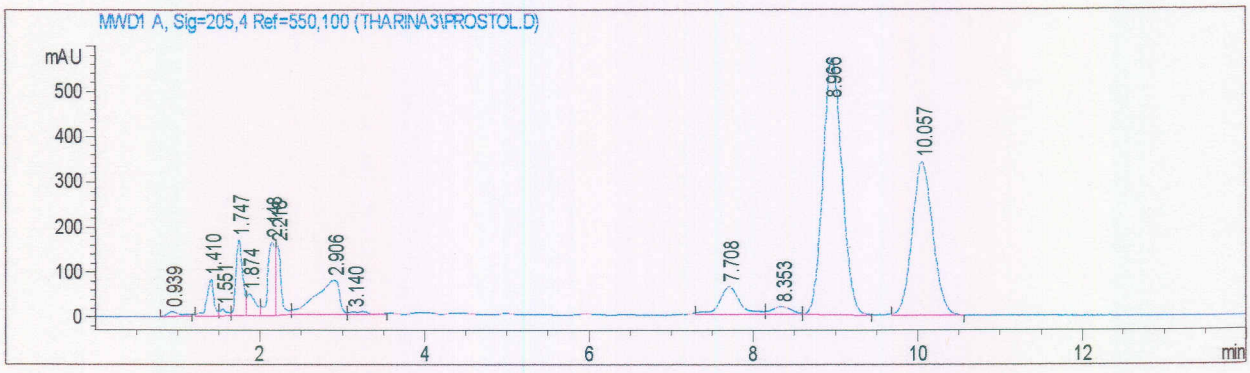

Figure 4.15: HPLC chromatogram of a chloroform extract of Prostol Herbal® with 96.5% methanol as mobile phase. See campesterol and/or stigmasterol (t_R=8.966) and β-sitosterol (t_R=10.057).

4.3.6 Water extraction of *H. hemerocallidea* powder

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

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

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

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

Mass of grated corms extracted = 1.28 g with 240 ml distilled water.
2 ml 10 mg/ml Tea applied to SPE = 20 mg
Dry mass applied to SPE = 20 mg.
Water-methanol fraction 2, dried and redissolved in 1ml MeOH = 29 μg/ml hypoxoside.
Thus, 20 mg extract = 29 μg hypoxoside.
But, total volume of 10 mg/ml extract = 56.9 ml and that is 569 mg dry mass extract.
Thus, 569 mg extract = 825.05 μg hypoxoside.

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

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

4.4 CONCLUSION

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

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

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

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

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

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

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

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

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

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

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

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

The HPLC results after chloroform extraction of *H. hemerocallidea* from 4 sources indicated that African potato 6 from PLANTANICAL MEDICINE had the highest content of β-sitosterol.
The *P. africana* bark and leaf powder analysed, were similar in their contents of β-sitosterol, but the leaf powder sample analysed, did not contain campesterol &/or stigmasterol. The bark and leaves were not from the same tree, and a number of samples must be analysed for a general conclusion. However, it is important to investigate whether *P. africana* leaves are a good source of β-sitosterol, and whether the leaves cannot perhaps replace the bark in prostate remedies and products. *P. africana* is on the list of endangered species, and a less destructive harvesting method (e.g. leaf collection) might prevent it from becoming extinct.

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

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

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