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Determination of stigmasterol, β-sitosterol and stigmastanol in oral dosage forms using high performance liquid chromatography with evaporative light scattering detection

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Abstract

A validated and repeatable high performance liquid chromatography (HPLC) method with online evaporative light scattering (ELSD) was developed for the analysis of two sterols, stigmasterol, β-sitosterol and a stanol, stigmastanol, found to be common in many herbal formulations and health care supplements. The method is based on the separation of the three marker compounds on a C_8 column (Phenomenex Luna, 5 μm, 150 mm × 4.6 mm i.d.) using methanol:water (95:5 v/v) as the mobile phase, and a flow rate of 1 ml/min to separate all the marker compounds within 12 min. Cholesterol (50 μg/ml) was used as internal standard and methanol as the extraction solvent. The ELSD response parameters were optimised and the limits of detection (LOD) and quantification (LOQ) were calculated to be 2 and 5 μg/ml, respectively, which is more sensitive than obtained by photo diode array detection (5 and 7 μg/ml). Using ELSD, the percentage relative standard deviation (%R.S.D.) of intra-day and inter-day (3 days) precision for each marker was better than 3%, the accuracy data were within 97–103% and the recovery data were found to be within 95–107% for the five commercially available products examined. This method was used to assay commercially available products formulated as oral dosage forms purported to contain African Potato and associated sterols and stanol and proved to be suitable for the routine analysis and quality control of such products.

Keywords: African Potato; Stigmasterol; β-Sitosterol; Stigmastanol; HPLC-ELSD; Quality control

1. Introduction

Herbal medicines are often considered foods as well as medicines and are used in preventative and curative treatments throughout the world. Sterols and stanols have secured an important place in the realm of health supplements with extensive scientific support for their prophylactic and therapeutic use for various physical ailments like atherosclerosis [1,2] benign prostatic cancer [3] and colon cancer [4,5]. Phytosterols (plant sterols) are members of the 'terpene' family of natural products which includes more than 100 different phytosterols and more than 4000 other types of triterpenes [6,7]. Plant membranes contain several types of phytosterols that are similar in structure to cholesterol but include a methyl or ethyl group at C-24 (Fig. 1). Similar to cholesterol in animals, phytosterols are thought to stabilize plant membranes. An increase in the sterol/phospholipid ratio leads to membrane rigidification [8]. Even though sterols and their glycosides have been evaluated for a variety of biological activities [9–21], it has also been stated that the ubiquitous presence of plant sterols and their glycosides in all vegetables and fruits makes it highly unlikely that they have significant toxicity-related properties. Many reports of their medicinal properties are based on in vitro data or unrealistic high in vivo doses, making the therapeutic application of these compounds highly questionable [22–24].

Average consumption of phytosterols is approximately 250 mg/day, which are mostly derived from vegetable oils, cereals, fruits and vegetables [25–28]. For vegetarians, dietary phytosterols have been estimated to be almost twice this level [29,30]. Phytostanols are much less abundant in nature than phy-

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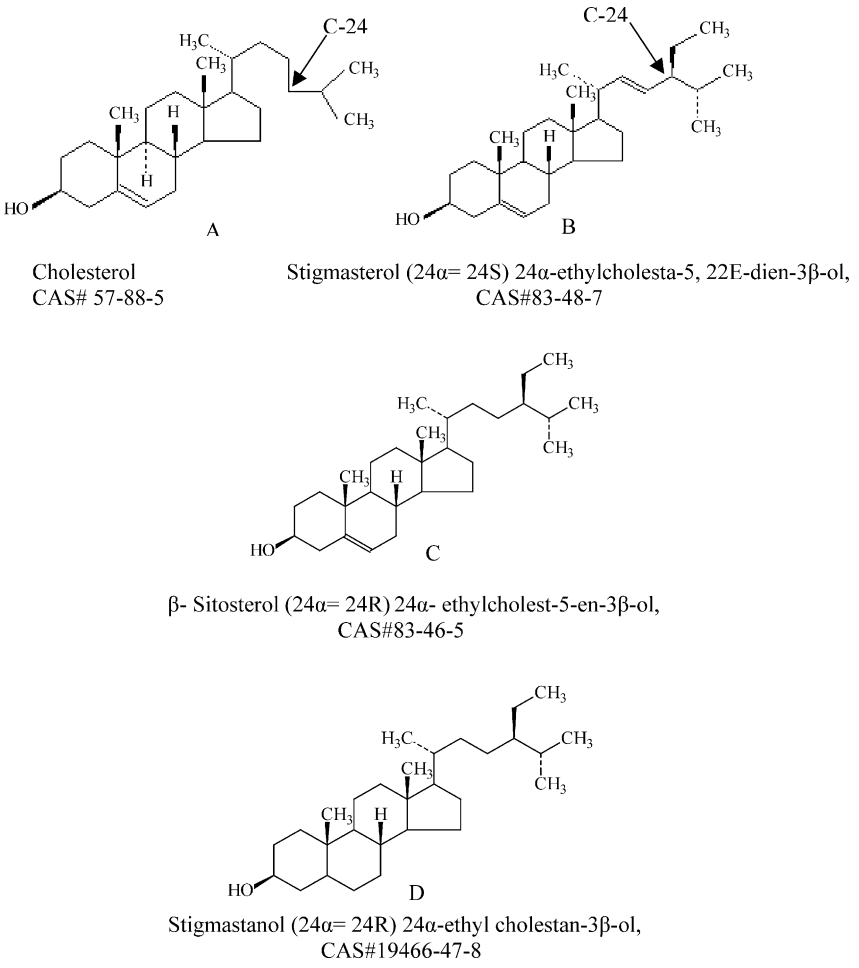

Fig. 1. The structures of: cholesterol (A), stigmasterol (B), β-sitosterol (C) and stigmastanol (D).

tosterols and consequently we typically consume much lower amounts (∼25 mg/day) in our diets [26,30]. Sterol mixtures have also been reported to be an effective adjuvant in the treatment of pulmonary tuberculosis [31,32] and were also found to prevent immune suppression in marathon runners [33]. In HIV positive patients, β-sitosterol–β-sitosterol glycoside (BSS–BSSG) mixture showed a significant decrease in the plasma viral loads and stable CD4 cell counts over a period of 40 months [34]. Feline immune virus (FIV) infected cats maintained stable CD4 cell counts over extended periods of time on treatment with sterol mixtures [35]. Sterols have also been found to be effective in rheumatoid arthritis [36], allergic rhinitis and sinusitis [37].

Since the mid-1990s phytosterols have also been used in strategies for lowering cholesterol and for reducing the risk of cardiovascular diseases (CVD). Due to poor solubility and bioavailability of free phytosterols, serum cholesterol lowering effects were not always consistent and very high doses (up to 25–50 g/day) appear to be required for efficacy. The problems of solubility and bioavailability have led to many confounding results in early clinical studies [29]. The advent of more predictable and effective 'statin' drugs has resulted in a rapidly diminished use of phytosterol products. In recent years, the increasing interest in functional foods and the use of phytosterols for reducing serum cholesterol and increasing immunity has regained considerable momentum. For this reason there are many herbal formulations containing a natural content of sterols amongst which, many have been fortified with additional amounts of free sterols and stanols. Several formulations of *Hypoxis hemerocallidea* (Family: Hypoxidaceae) commonly known as 'African Potato' (AP) are promoted for their sterol content [38].

In a recent review, Abidi discussed various methods for the analysis of sterols and structurally related compounds [39]. Most of those methods are tedious and mainly involve gas chromatography–mass spectrometry (GC–MS). Other chromatographic methods such as HPLC using UV or evaporative light scattering detection (ELSD) detection involved the use of relatively complex multi-component solvent mixtures as mobile phase [40–42] to determine various sterols and related compounds. However, none of those methods have been used for the simultaneous quantitative analysis of the combination, β-sitosterol (BSS), stigmasterol (STG) and stigmastanol (STN), purported to be present in preparations containing AP.

The main objective of this study was, therefore, to develop and validate a rapid and efficient analytical method for the simultaneous determination of BSS, STG and STN (Fig. 1) as marker compounds, which are found to be common in commercial formulations containing such compounds.

Unlike BSS and STG, STN does not have the necessary chromophores to enable detection by UV. Hence an ELSD detector [43] was coupled to the UV detector to monitor this compound and permit the simultaneous determination of BSS and STG together with STN. Hence, in the present study, an HPLC–ELSD method was developed and validated for the analysis of BSS, STG and STN in commercially available oral dosage forms purported to contain African Potato (AP) material or extracts thereof. Cholesterol (CHOL) was used as an internal standard. The ELSD results were compared with the UV results.

2. Experimental

2.1. Reagents and chemicals

Methanol (HPLC Grade) was purchased from Romil Ltd. (The Source, Waterbeach, Cambridge, UK). Stigmasterol (95%), stigmastanol (95%), β-sitosterol (97%) were purchased from Sigma (St. Louis, MO, USA) and cholesterol from Croda Chemicals Ltd. (North Humberside, UK). Water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA) and all samples were filtered using durapore (PVDF) filters purchased from the same source. Five commercial products (A–E) were purchased from a local pharmacy in Grahamstown, South Africa. The labeled weight/unit of each of the formulations was 400 mg (A), 500 mg (B), 400 mg (C), 200 mg (D) and 1000 mg (E), respectively. Four of the products (products A–D) were capsules, containing pulverized AP fortified with additional quantities of sterols and stanols, and one product (product E) was formulated as a tablet.

2.2. Instrumentation and chromatographic conditions

All experiments were performed using a Waters Alliance HPLC system equipped with a separation module (model 2690), a PDA detector (model 2996), an online degasser, an autosampler (Waters corporation, Milford, MA, USA) and a Phenomenex Luna C_8 Column (5 μm, 50 mm × 4.6 mm i.d.). The chromatographic elution was accomplished isocratically with methanol–water (95:5 v/v) at a flow rate of 1 ml/min. The temperature was maintained at 23 ± 1 °C and the injection volume was 10 μl. PDA detection was achieved in the range of 200–400 nm, 210 nm results were used for quantitative purposes. After the UV detection, the chromatographic column effluent was subjected to detection by ELSD (Alltech 2000, Alltech Associates Inc., Deerfield, USA). Nebulisation of the effluent in the ELSD was provided by a stream of pressurised air (0.7 l/min) and the nebulised effluent was evaporated at 100 °C. The detector was set at a gain of 16, with output interfaced, via a SATIN box, to a Waters Empower® Chromatographic Manager.

2.3. Preparation of standard solutions

Separate stock solutions of the reference standards were made by weighing out appropriate amounts into volumetric flasks and filling to volume with methanol. Serial dilutions of the relevant stock solutions were made to prepare five concentrations of each standard solution over the concentration range of 10–100 μg/ml. These solutions were prepared on three different days and were used for the linearity experiments. On each of these 3 days, two concentrations (30 and 60 μg/ml) of the standard solutions were separately prepared for use in the accuracy experiments. CHOL solution (500 μg/ml in methanol) was added to each final dilution as the internal standard (IS) in order to obtain a final concentration of 50 μg/ml.

2.4. Sample preparation and extraction

2.4.1. Hard gelatin capsules

Fifteen capsules of each product (A–D) were emptied and mixed well separately. Powder (50 mg) was weighed into a 10 ml volumetric flask and 8 ml of methanol was added. The mixture was ultrasonicated for 20 min and was allowed to cool for 5 min, thereafter the volume was made up with methanol. One hundred milliliters of this extract was brought into a 10 ml volumetric flask, followed by addition of 1.0 ml of a 500 μg/ml CHOL solution (internal standard). The volume was made up to 10 ml with methanol and the solution was vortexed for 1 min before filtering through a 0.45 μm PVDF membrane and analyzed.

2.4.2. Solid oral dosage forms (tablets)

Fifteen tablets (product E) were weighed and powdered using a mortar and pestle. The powder (50 mg) was extracted as mentioned above for capsules.

3. Results and discussion

3.1. Method development

The two sterols and the stanol as well as the internal standard cholesterol were well separated within 12 min using isocratic elution with methanol:water (95:5 v/v) at a room temperature of 23 ± 1 °C and a flow rate of 1 ml/min. The retention times of the analytes at this optimised condition were CHOL (7.63 min), BSS (8.92 min), STG (9.85 min), STN (11.61 min). An increase in methanol content in the mobile phase reduced the resolution of all the above compounds. Substitution of the mobile phase with acetonitrile–methanol (85:15 v/v) gave a similar elution pattern, but the previous mobile phase was used for further analysis considering its low cost. The most important parameters affecting the ELSD response are the nebuliser gas flow rate and the drift tube temperature. The gas flow rate influences the droplet size of the column effluent before evaporation occurs. Higher flow rates result in the formation of smaller aerosol droplets and less scattering of light with subsequent lower sensitivity but an increased stability. On the other hand, lower gas flow rates are associated with larger droplet formation, augmented light scattering and therefore a higher response but baseline stability is compromised. It is therefore pertinent to optimise this parameter to ensure that the optimal signal to noise ratio (S/N) is achieved [44]. The gas flow rate was therefore investigated over the range of 0.5–2.5 l/min in increments of 0.2 l/min. The sensitivity was highest at 0.7 l/min and nebuliser gas flow rates higher than 2 l/min decreased the sensitivity. As a result,

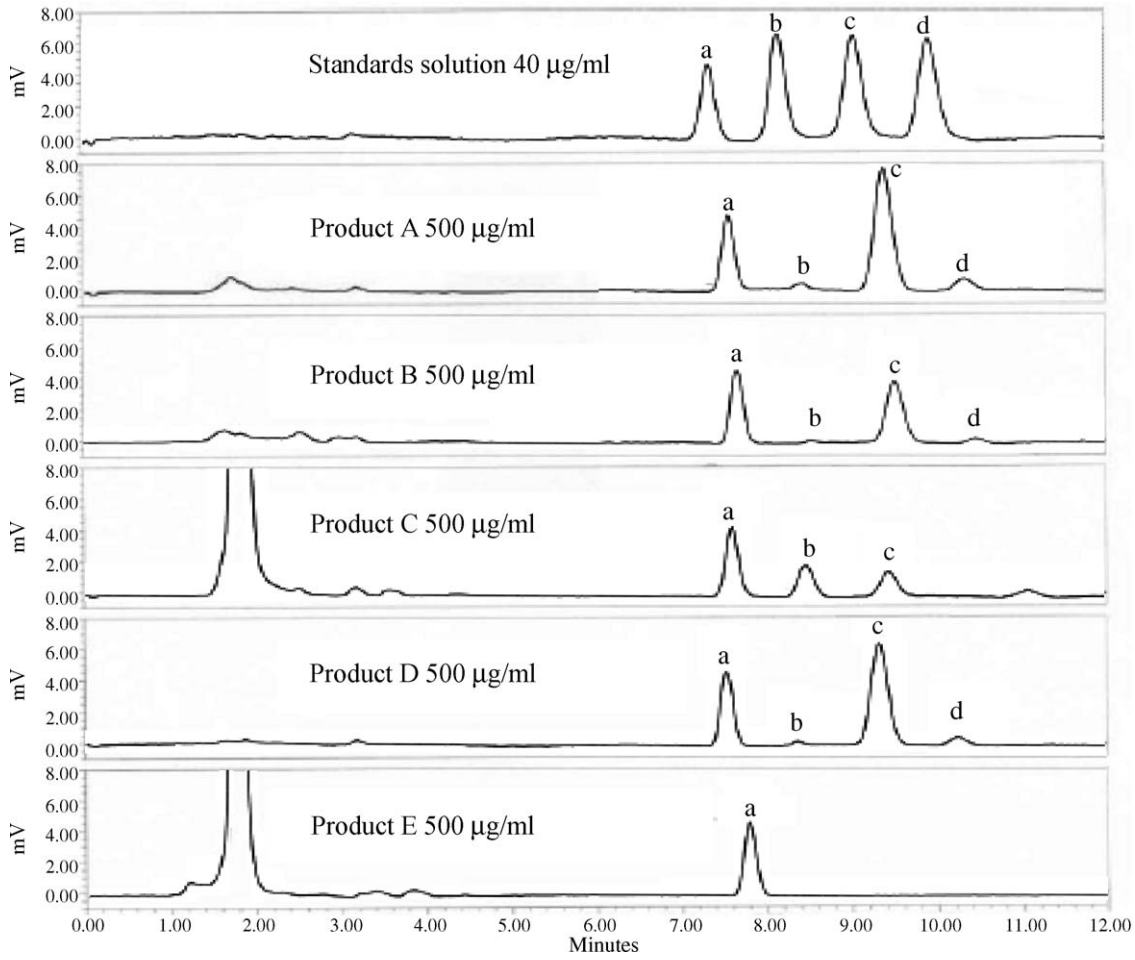

Fig. 2. ELSD chromatograms of reference standards solution and commercial products: (a) cholestrol, (b) stigmasterol, (c) β-sitosterol and (d) stigmastanol.

0.7 l/min was chosen for an optimal S/N ratio. Mobile phases with high polarity solvents require higher drift tube temperatures than those consisting of predominantly organic non-polar components. Similarly, optimal sensitivity of non-volatile solutes requires higher drift tube temperatures than semi-volatile solutes [20]. It was therefore predicted that a relatively high drift tube temperature would be required for the adequate evaporation since the sterols are not highly volatile and the mobile phase consisted of polar solvents. The effect of temperature on sensitivity was determined over the range of 50–110 °C in increments

Table 1
Linearity

Constituents	$y = ax + b$ [linear model (PDA detector)]	Determination coefficient, r^2 ($n = 3$)	$Y = AX + B$ [log linear plot (ELSD detector)]	Determination coefficient, r^2 ($n = 3$)
Day-I				
Stigmasterol	$y = 0.0245x + 0.0524$	0.9991	$Y = 1.13103X - 1.9355$	0.9987
β-Sitosterol	$y = 0.213x + 0.0629$	0.9987	$Y = 1.2254X - 1.7701$	0.9947
Stigmastanol	–	–	$Y = 1.3276X - 1.9183$	0.9989
Day-II				
Stigmasterol	$y = 0.025x + 0.0673$	0.9995	$Y = 1.3296X - 1.9112$	0.9915
β-Sitosterol	$y = 0.0218x + 0.0504$	0.9994	$Y = 1.3364X - 1.9129$	0.9940
Stigmastanol	–	–	$Y = 1.3069X - 1.8270$	0.9963
Day-III				
Stigmasterol	$y = 0.255x + 0.0310$	0.9995	$Y = 1.3398X - 1.9603$	0.9941
β-Sitosterol	$y = 0.222x + 0.0375$	0.9993	$Y = 1.3318X - 1.9275$	0.9975
Stigmastanol	–	–	$Y = 1.3529X - 1.9323$	0.9965

PDA: y is the peak area ratio, x is the concentration; ELSD: Y is the log peak area ratio, X is the log concentration; n is the number of injections. Each concentration (5, 10, 20, 40, 80, 100 µg/ml) was injected three times.

of 10 °C and thereafter by increments of 2.5 °C for fine tuning. A drift tube temperature of 100 °C was finally selected. Baseline separation of all the marker compounds was obtained by ELSD detection as shown in Fig. 2. Due to their poor chromophores, the sterol markers gave weak UV signals at 210 nm, when compared to detection by ELSD. Moreover, STN could only be analysed using ELSD. This confirms the usefulness of the ELSD over UV detection for poor absorbing or non-absorbing compounds.

The extraction efficiency with methanol was investigated as follows. About 50 mg of product D, which contained sufficient quantities of the relevant marker compounds, was sonicated over a period of 1 h and samples were withdrawn and analysed at 10 min intervals. It was found that 20 min ultrasonication was the minimum time required for the maximum extraction of the marker compounds. The same procedure was repeated with ethyl acetate but due to compatibility with the mobile phase used, methanol proved to be the solvent of choice.

3.2. Linearity

Data obtained from triplicate injections of the mixture of standard solutions over 3 days of analysis were processed for linearity. The UV mode resulted in a determination coefficient of $r^2 > 0.997 \pm 0.002$ at a concentration range of 10–100 μg/ml. Using the same concentration range in the ELSD mode, a second order polynomial calibration (peak area ratio against concentration) was observed. After log transformation, the data provided a linear function for the reference standards as shown in Table 1.

3.3. Limits of detection and quantification

The limits of detection (LOD) (signal/noise > 3) and the limits of quantification (LOQ) (signal/noise > 10) were determined by analysing dilutions of a solution containing all the marker compounds. With ELSD detection, the LOD and LOQ were 2 and 5 μg/ml, respectively and with the UV detection, they were 5 and 7 μg/ml for all the compounds. The uniformity in response in ELSD is due to the uniform response factor shown towards all structurally similar compounds and in UV detection to the similarity of the spectrum at the wavelength used for quantification (210 nm).

3.4. Accuracy and precision

The accuracy and precision of the method were determined by analysing three sets of samples at two different concentrations (30 and 60 μg/ml), which were prepared as described earlier in Section 2.3. The percentage accuracy was calculated using the calibration curves and was found to be between 97.92 and 104.75% for UV detection and between 97.84 and 102.67% for ELSD detection. The intra-day precision (repeatability) was between R.S.D. 0.83 and 2.98% for UV detection and between R.S.D. 1.29 and 1.89% for ELSD detection. The inter-day precision ($n = 3$) was at most R.S.D. 2.47% for UV detection and at most R.S.D. 2.03% for ELSD detection (Table 2). It can be concluded that the ELSD results are similar to those obtained by UV. For further experiments, ELSD results only are reported.

Table 2
Accuracy and precision

Compound and detector used	Day-I				Day-II				Day-III				Inter-day precision R.S.D. (%, $n = 3$)
	Actual weight (μg/ml)	Calculated weight (μg/ml)	Accuracy (%)	R.S.D. (%, $n = 3$)	Actual weight (μg/ml)	Calculated weight (μg/ml)	Accuracy (%)	R.S.D. (%, $n = 3$)	Actual weight (μg/ml)	Calculated weight (μg/ml)	Accuracy (%)	R.S.D. (%, $n = 3$)	
Stigmasterol (PDA)	30.01	30.28	100.90	1.52	30.12	29.74	98.77	2.98	31.26	30.61	97.92	1.66	1.54
	60.86	61.29	100.72	0.83	61.22	61.11	99.82	2.86	61.32	60.60	98.84	1.24	0.94
Stigmasterol (ELSD)	30.01	30.10	100.29	1.71	30.12	30.85	102.45	1.48	31.26	31.69	101.40	1.50	1.06
	60.86	61.75	101.46	1.58	61.22	62.42	101.96	1.79	61.32	62.96	102.67	1.53	0.59
β-Sitosterol (PDA)	30.23	30.22	99.96	1.32	30.47	30.80	101.11	1.62	30.46	31.15	102.29	0.92	1.15
	60.46	61.22	101.25	2.58	60.94	60.85	99.85	1.51	60.92	63.81	104.75	1.93	2.47
β-Sitosterol (ELSD)	30.23	29.58	97.84	1.47	30.47	29.93	98.23	1.50	30.46	30.92	101.52	1.65	2.03
	60.46	60.60	100.23	1.77	60.94	61.70	101.24	1.79	60.92	60.86	99.91	1.79	0.69
Stigmasterol (ELSD)	30.96	31.14	100.58	1.29	30.56	30.92	101.18	1.68	30.40	30.27	99.60	1.50	0.79
	61.25	62.86	102.62	1.89	62.34	63.86	102.44	1.80	60.52	60.97	100.75	1.85	1.01

Table 3
Content in dosage forms using ELSD

Name of product	Average weight (mg) per unit	Labeled weight (mg) per unit	Labeled claim of sterols (mg) per unit	Amount in mg ± S.D./500 mg dosage form ($n = 3$)			Total sterols compared to label claim (%)
				Stigmasterol	β-Sitosterol	Stigmastanol	
A	520	400	40	+	48.1 ± 0.4	7.4 ± 0.7	105.9 ± 0.7
B	625	500	30	−	22.5 ± 0.8	+	72.8 ± 2.5
C	516	400	25	15.2 ± 0.1	13.1 ± 0.7	−	94.4 ± 4.3
D	278	200	20	+	38.1 ± 0.6	+	86.7 ± 1.3
E	1073	1000	100	−	−	−	−

(+): The amount is between LOD and LOQ (5/500 mg); (−): the amount is below LOD (2/500 mg).

Table 4
Recovery of sterols and stanol

Constituent	Spiking level (mg/500 mg dosage form)	Intra-day recovery S.D. (%, $n = 3$)						Inter-day R.S.D. (%, $n = 3$)
		Day-I		Day-II		Day-III		
		Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	
Stigmasterol (ELSD)	15.04	95.29	3.85	96.86	2.66	96.53	0.44	0.86
	30.08	100.13	3.37	97.93	1.03	100.31	0.68	1.33
	45.12	98.24	4.25	98.12	2.29	98.4	3.03	0.14
β-Sitosterol (ELSD)	15.06	97.34	2.63	95.64	0.39	105.4	2.59	5.21
	30.12	99.52	3.70	95.72	0.87	105.03	3.60	4.67
	45.24	102.23	2.81	97.31	2.31	98.83	1.31	2.56
Stigmastanol (ELSD)	15.11	102.93	4.00	102.26	2.12	96.12	2.11	3.73
	30.22	103.66	2.28	102.81	1.61	100.83	0.44	1.41
	45.33	106.88	1.83	99.35	3.66	97.78	1.85	4.80

3.5. Sample analysis

Standard and sample solutions were found to be stable on storage for 10 days at room temperature. Fig. 2 shows the HPLC–ELSD chromatograms of five commercial AP products. PDA analysis showed the peaks to be homogenous. The assay values and the individual sterol content along with the labeled claim of each selected commercial product as obtained with ELSD detection are given in Table 3. The results for the sterols were confirmed by the UV results (not shown). Product A was found to have the highest content of sterols, whereas product E performed the worst with sterols and stanol below LOD although the label claim was the highest. There could be a slight understatement in the reported quantities of total sterols, because compounds present below the LOQ (5/500 mg) were not taken up in the total content.

3.6. Recovery

Product D was chosen for intra-day and inter-day recovery studies, since it contained all three marker compounds above the LOD. This formulation was spiked with low, medium and high concentrations, i.e., 15, 30, 45 μg/ml, respectively of the three marker compounds. This was done on three different days and the analyses were performed in triplicate (Table 4). Recovery values were between 95.29 and 106.88%. This range is only slightly larger than the range found for accuracy. It can therefore be concluded that the influence of the product components on the recovery is small. The intra-day precision was at most R.S.D. 4.25% and the inter-day precision ($n = 3$) at most R.S.D. 4.80%. Both these values are higher than the corresponding values in the accuracy experiments which indicate that the presence of product components may have an influence on the precision.

4. Conclusions

This paper describes a simple HPLC/ELSD method, to quantitate stigmasterol, β-sitosterol and stigmastanol. Baseline separation was achieved within a short analysis time using a Phenomenex Luna C_8 5 μm, 150 mm × 4.6 mm i.d. column. ELSD is needed for detection of stigmastanol, which is transparent in the UV range. ELSD detection also provides advantages over UV detection in terms of sensitivity; however the LOQ was limited to 5/500 mg product. ELSD is relatively inexpensive and easily operable compared to GC and MS detection. This simple, rapid, precise and accurate method was successfully applied in the analysis of commercially available solid dosage forms. The analyses showed the quantitative compositions of the products to be quite different. These results therefore indicate that suitable quality control measures need to be implemented to ensure consistent quality and efficacy of marketed products.

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