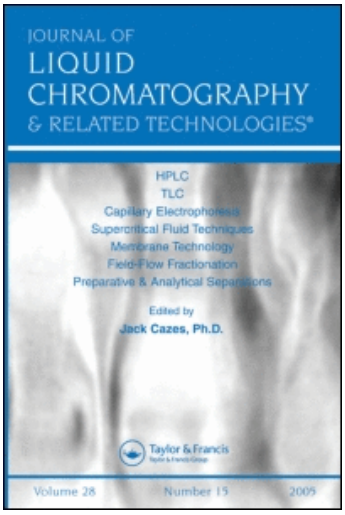

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Determination of Sterols and Fatty Acids in Prostate Health Dietary Supplements by Silica Gel High Performance Thin Layer Chromatography with Visible Mode Densitometry

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Abstract: A quantitative method using silica gel high performance thin layer chromatography plates, semiautomated bandwise sample application, and automated visible mode densitometry has been developed for the determination of the steroids and fatty acids in two nutritional supplements consumed to aid prostate and urinary health. The mobile phase was petroleum ether-diethyl ether-glacial acetic acid (80:20:1), and lipids were detected with phosphomolybdic acid reagent. A softgel containing 320 mg of saw palmetto extract with 85–95% of sterols plus fatty acids and a caplet product containing 300 mg of mixed sterols were analyzed. By comparison to a mixed neutral lipids standard, results averaged 32.1% of free fatty acids plus free sterols for the softgels and 141% recovery of mixed sterols for the caplets relative to these stated label values. Other neutral lipids were found in the samples, including triacylglycerols, steryl esters, and two unidentified fractions.

Keywords: Thin layer chromatography, Sterols, Fatty acids, Nutraceuticals, Prostate medications

INTRODUCTION

Nutritional supplements (nutraceuticals) are being consumed in increasing amounts worldwide to improve prostate and urinary health. Standardized commercial supplements contain sterols and fatty acids as the major active

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ingredients. Silica gel high performance thin layer chromatography (HPTLC) has been used repeatedly for the quantitative determination of these neutral lipids plus triacylglycerols, methyl esters, and cholesteryl esters in a variety of biological samples,[1,2] but no TLC or HPTLC method has been reported for the for the assay of nutritional supplements containing neutral lipids. The purpose of this research was to modify the HPTLC-densitometry methods used previously for biological samples[3] to analyze two nutritional supplements in order to verify the accuracy of the label declarations for sterols and free fatty acids and to detect any other neutral lipids present in the formulations. The new method uses semiautomated sample application in place of manual application and quantification with a flatbed scanner densitometer rather than a slit scanning densitometer. The method was found to be rapid and precise and to have high sample throughput for the assay of this type of nutritional supplement.

EXPERIMENTAL

Preparation of Standard and Sample Solutions

The standard for HPTLC was Nonpolar Lipid Mix B (Matreya, Inc., Pleasant Gap, PA, USA), which contained a mixture of 5.00 mg each of cholesterol (CH), oleic acid (OA), triolein (TO), methyl oleate (MO), and cholesteryl oleate (CO) in 1.00 mL of chloroform to serve as marker compounds for free sterols (FS), free fatty acids (FFA), triacylglycerols (TG), methyl esters (ME), and steryl esters (SE), respectively. The standard was diluted in a 25 mL volumetric flask with chloroform-methanol (2:1) to prepare a TLC standard that contained 0.200 mg mL^{-1} of each compound.

Prostate health dietary supplements were purchased from local pharmacies, one a caplet and one a soft gelatin capsule (softgel). The caplet was labeled "300 mg β-sitosterol (pure mixed sterols from natural plant sources)" and the softgel "320 mg saw palmetto extract (standardized to contain 85–95% fatty acids and sterols)". A sample solution of the softgel was prepared for determination of sterols by cutting open the shell with a knife, transferring the contents and shell pieces to 25 mL volumetric flask, adding about 20 mL of chloroform-methanol (2:1), and stirring magnetically for 20 min to completely dissolve the entire contents except for the shell. The solution was diluted to the line with chloroform-methanol (2:1) and shaken to make homogeneous. For determination of fatty acids, the solution was diluted 1 + 15 by mixing 0.500 mL of sample and 7.50 mL of chloroform-methanol (2:1). The caplets were ground to a fine powder in a mortar and pestle, transferred to a 100 mL volumetric flask by washing with about 70 mL of chloroform-methanol (2:1), and magnetically stirred for 1 hour. The stir bar was removed with a magnetic rod, and the solution was diluted to volume with chloroform-methanol (2:1) and mixed by shaking. Undissolved excipients

were removed by filtering through a Pall Gelman (Ann Arbor, MI, USA) Acrodisc LC 13 mm syringe filter with 0.45 μm PVDF membrane into a capped vial. To prepare the sample TLC test solution, the clear sample stock solution was diluted 1 + 19 by mixing 100 μL of sample stock solution with 1900 μL of chloroform-methanol (2:1). Digital Drummond (Broomall, PA, USA) microdispensers (100 and 1000 μL) were used to measure volumes.

Thin Layer Chromatographic Analysis

Analyses were performed on 10 × 20 cm high performance silica gel GLP plates (Art. 5613/6, EMD Chemicals, Inc., Gibbstown, NJ, USA, an affiliate of Merck KGaA, Darmstadt, Germany). The plates were precleaned by development to the top with methanol and dried for 30 min at 120°C on a Camag (Wilmington, NC, USA) plate heater before use. Standard and sample solutions were applied to the plates by means of a Camag Linomat 4 spray-on band applicator equipped with a 100 μL syringe and operated with the following settings: band length 6 mm, application rate 4 s μL^{-1}, table speed 10 mm s^{-1}, distance between bands 4 mm, distance from the side edge 0.7 mm, distance from the bottom of the plate 1.0 cm. The standard solution volumes applied for each analysis were 2.00, 4.00, 6.00, 8.00, and 10.0 μL. Duplicate aliquots of 2.00 μL for sterols and 4.00 μL for fatty acids in the softgels, and 6.00 μL for sterols in the caplets were applied for samples, the volumes being chosen to obtain scan areas between the areas of the highest and lowest standards.

Plates were developed for a distance of 8.2 cm beyond the origin with the mobile phase petroleum ether (36–60°C)-diethyl ether-glacial acetic acid (80:20:1) in a vapor-equilibrated Camag twin-trough chamber containing a saturation pad (Analtech, Newark, DE, USA). The development time was 12–14 min.

After development, the mobile phase was evaporated from the plates by drying them for 2 min under a stream of cold air from a hair-dryer, and the plates were sprayed with phosphomolybdic acid (PMA) solution (5% in absolute ethanol) using a Knotes (Vineland, NJ) Chromaflex sprayer. The plates were heated for 10 min at 110°C on the plate heater, and the neutral lipid fractions were detected as blue zones on a yellow background.

The plates containing the chromatograms were scanned using a Chrom-Image flatbed scanner densitometer (AR2i Company, LePlessis Robinson, France) with the visible light source, and areas of standard and sample zones were measured using the Galaxie-TLC software as previously described.[4] The software determined the calibration curve on each plate by second order polynomial regression of the weights and areas of the standard zone scans and automatically interpolated the weights (μg) for the sample zones from their scan areas. Calibration curve correlation coefficients were

at least 0.995 in all analyses. Sterols were quantified relative to a CH calibration curve and fatty acids relative to an OA calibration curve. For the softgel analysis, the mean interpolated μg sterol or fatty acid per μL of sample aliquot was converted by calculation to mg in the softgel and then to percent of the 320 mg saw palmetto extract label value; sterol and fatty acid percentages were added and compared to the "85–95%" label declaration. For the caplet, μg mixed sterols per μL applied was converted to mg in the caplet, and the % recovery compared to the 300 mg mixed sterols label value was calculated. Reproducibility was evaluated by calculating the percent difference between the duplicate experimental neutral lipid weights for each sample and spotting one sample 4 times and calculating the relative standard deviation (RSD).

RESULTS AND DISCUSSION

Quantitative recovery of neutral lipids from biological samples is obtained by grinding with chloroform-methanol (2:1),[1,2] and the same solvent with magnetic stirring for 20 or 60 min was found to completely dissolve the neutral lipids from the supplements. Test solutions of softgels were clear and used directly, but excipients of caplets did not dissolve and filtration was necessary.

Lipids were qualitatively identified by comparison of the migration of sample and standard zones. R_F values of the neutral lipid standards were CH, 0.12; OA, 0.21; TO, 0.54; MO, 0.64; and CO, 0.78. The lipid zones were flat, well resolved bands, as shown in Figure 1. The detection and quantification limits were approximately 0.200 μg for CH and 0.400 μg for OA; 0.400 μg was the weight of the lowest standard for the calibration curves of both compounds. The mixed sterol zone was detected in sample chromatograms of the caplets, along with two additional light zones; one comigrated with the CO standard zone and presumably contained SE, and the other (R_F 0.091) was not identified because it migrated below the CH zone. In the softgel chromatograms, sterol and free fatty acid zones were detected with four additional zones: two (R_F 0.54 and 0.78) comigrated with the TO and CO standard zones and presumably contained TG and SE, respectively. The third (R_F 0.66) migrated above the ME zone and the fourth (R_F 0.091) migrated below the CH zone; these two additional zones could not be identified with the standards used in this study. Inert ingredients listed as being present in the formulations, including gelatin, glycerin, zinc, dicalcium phosphate, cellulose, stearic acid, magnesium silicate, and silica, were not detected by the PMA reagent.

Table 1 shows the results of a survey of four samples of the softgel supplement. The percentages of FFA and FS in saw palmetto extract, when added together, were much less than the value declared on the label; the average sum was 32.1% instead of 85–95%. It was obvious from visual semiquantitative

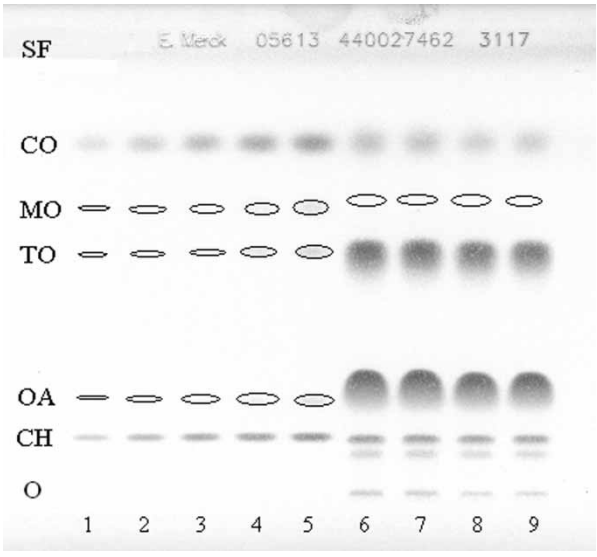

Figure 1. Image of typical HPTLC chromatograms of the neutral lipid fractions in saw palmetto extract: 2.00, 3.00, 4.00, 5.00, and 6.00 μL, respectively, of the neutral lipid standard (lanes 1–5), and duplicate 4.00 μL of saw palmetto sample solution 1 (lanes 6–7) and sample solution 2 (lanes 8–9). Abbreviations: O, origin; CH, cholesterol; OA, oleic acid; TO, triolein; MO, methyl oleate; CO, cholesteryl oleate; SF, solvent front. The plate was scanned using the white light source with the ChromImage scanner. The lightest zones were outlined so they would be readily visible. This plate was chosen to illustrate standard and sample chromatograms but was not used for quantification.

Table 1. Data for the determination of free sterols and free fatty acids in the softgel nutritional supplement

Sample number	μg μL^{-1}	mg $softgel^{-1}$	% of 320 mg label value
Free fatty acids			
1	0.235	94.2	29.4
2	0.233	93.3	29.2
3	0.239	95.8	29.9
4	0.241	96.3	30.1
Free sterols			
1	0.280	7.01	2.19
2	0.274	6.86	2.14
3	0.287	7.17	2.24
5	0.402	10.0	3.14

Table 2. Data for the determination of mixed sterols in the caplet nutritional supplement

Sample number	Mg μL^{-1}	mg $caplet^{-1}$	Recovery (%)
1	0.232	463	154
2	0.202	404	135
3	0.209	419	140
4	0.200	400	133

comparison of the intensities of the extra zones in the extract chromatograms (Figure 1) that the amounts of sterols and fatty acids must be much below the 85–95% level stated on the label: the TG zone is very intense, and the intensities of SE zone and the unidentified zone below the FS together might equal, or even exceed, the FS. For the caplet (Table 2), percent recoveries of mixed sterols were much higher than 100% (141%). High results compared to label values (112–247%) were also obtained for the HPTLC-densitometry analysis of lutein dietary supplements.[5] The degree of variation in ingredient content among samples seen in Table 1 is typical for drugs and nutraceuticals, except for saw palmetto sample 5.

Reproducibility was determined by spotting four 6.00 μL aliquots of a test solution prepared from a caplet. The average recovery relative to the label value was 141%, and the RSD was 1.23%. As another measure of precision, percent difference values of the areas of duplicate samples spotted in the analyses of the other samples averaged 1.41% with a range of 0.312–3.66%. These values confirm the good repeatability of the method.

A new method has been demonstrated for assay of two prostate health nutraceuticals containing neutral lipid active ingredients using a widely recognized neutral lipid reference standard . The method has high throughput and is precise, and it is suitable for quality control in the manufacture of these products. Sterols and fatty acids are separated as fractions on silica gel; it is required to use C-18 bonded silica gel[6] or silver-impregnated silica gel[7] layers to resolve individual compounds within these fractions. The method can be used to analyze other formulations if the zones of the additional active and inactive ingredients separate from the sterol and fatty acid zones or are not detected by PMA.

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