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Determination of Campesterol, Stigmasterol, and beta-Sitosterol in Saw Palmetto Raw Materials and Dietary Supplements by Gas Chromatography: Single-Laboratory Validation

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Abstract

In conjunction with an AOAC Presidential Task Force on Dietary Supplements, a method was validated for measurement of 3 plant sterols (phytosterols) in saw palmetto raw materials, extracts, and dietary supplements. AOAC Official Method 994.10, “Cholesterol in Foods,” was modified for purposes of this validation. Test samples were saponified at high temperature with ethanolic potassium hydroxide solution. The unsaponifiable fraction containing phytosterols (campesterol, stigmasterol, and beta-sitosterol) was extracted with toluene. Phytosterols were derivatized to trimethylsilyl ethers and then quantified by gas Chromatography with a hydrogen flame ionization detector. The presence of the phytosterols was detected at concentrations greater than or equal to 1.00 mg/100 g based on 2–3 g of sample. The standard curve range for this assay was 0.00250 to 0.200 mg/mL. The calibration curves for all phytosterols had correlation coefficients greater than or equal to 0.995. Precision studies produced relative standard deviation values of 1.52 to 7.27% for campesterol, 1.62 to 6.48% for stigmasterol, and 1.39 to 10.5% for beta-sitosterol. Recoveries for samples fortified at 100% of the inherent values averaged 98.5 to 105% for campesterol, 95.0 to 108% for stigmasterol, and 85.0 to 103% for beta-sitosterol.

Saw palmetto (*Serenoa repens*) is a native plant of North America, most commonly found in Florida. The berries of the saw palmetto plant have been used for medicinal purposes for centuries. Both berry raw material and extract are found in dietary supplements and are most commonly used to treat symptoms related to benign prostatic hyperplasia (BPH). In conjunction with an AOAC Task Force on Dietary Supplements, a method was validated for measurement of 3 plant sterols (phytosterols) in saw palmetto raw materials, extracts, and dietary supplements. AOAC Official Method **994.10**, “Cholesterol in Foods,” was modified for purposes of this validation. The phytosterols campesterol, stigmasterol, and beta-sitosterol are chemically very similar to cholesterol. They extract and chromatograph very comparably to cholesterol. Due to the similarities of these compounds, a number of laboratories have successfully extended AOAC Official Method **994.10** to measure not only cholesterol, but these phytosterols as well. The following test materials were used in this validation: powdered saw palmetto berry, saw palmetto (*Serenoa repens*) dried fruit CO_2 extracts, saw palmetto 45% powdered extract, ProstActive® Once Daily, Prostasan™ Prostrate Capsules, Liquid Herbal Extract: saw palmetto, alcohol-free saw palmetto, ProstActive Plus saw palmetto combined with nettle root extract, pygeum and saw palmetto standardized herbal extracts, and saw palmetto pygeum lycopene complex tablets.

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Experimental

Apparatus

a. *Centrifuge tubes.*—Pyrex No. 13, 15 mL, silanized. Tubes were silanized using a commercial silinizing reagent (Surfasil™ Siliconizing Fluid, No. 42801, Pierce Chemical Co., Rockford, IL). Manufacturer's instructions were followed for silanization. Before each reuse, tubes were cleaned with water, ethanol, hexane, and acetone and oven-dried at 100°C.

b. *Plastic stoppers*.

c. *Gas Chromatography (GC).*—With 2-ramp oven temperature programming (Hewlett Packard Model 5890A is suitable) and hydrogen flame ionization detector (FID; Agilent Technologies, Palo Alto, CA).

d. *Column.*—Capillary, split-mode, 25 m × 0.32 mm × 0.17 μm film thickness, cross-linked 5% phenyl-methyl silicone or methyl silicone gum (e.g., Hewlett Packard No. HP-5, Ultra 2, or HP-1; Agilent Technologies).

e. *Deactivated split inlet liner filled with glass wool*.

f. *Rotary evaporator*.—With glass condenser flask between the concentration flask and metal shaft.

g. *Magnetic stirrer-hot plate.*—With variable speed and heat controls; Model PC320, Corning (Corning, NY).

h. *Micropipets.*—Delivering 100 and 200 μL.

i. *Vortex mixer*.—Model M37615, Type 37600 Mixer: Thermolyne (Dubuque, IA).

j. *Balance*.—Analytical, weighing to 0.0001 g.

k. *Centrifuge*.—Model K; International Equipment Co. (Needham Heights, MA).

(*Note*: Equivalent apparatus may be substituted. All glassware is class A.)

Reagents

a. *Dimethylformamide (DMF).*—Spectrophotometry grade; J.T. Baker (Phillipsburg, NJ).

b. *Hexamethyldisilane (HMDS).*—Derivatization grade, No. 3-3011; Supelco (Bellefonte, PA).

c. *Potassium hydroxide solutions.*—50% KOH (w/w), 1 M KOH, and 0.5 M KOH. (*1*) *50% KOH (w/w).*—500 g KOH (Certified A.C.S. Pellets; Fisher Scientific, Fairlawn, NJ) was dissolved in 500 g water; (*2*) *1 M KOH.*—56 g KOH was dissolved in ca 800 mL water with cooling and diluted to the mark in a 1 L volumetric flask; (*3*) *0.5 M KOH.*—One part of 1 M KOH solution was diluted with 1 part water.

d. *Trimethylchlorosilane (TMCS).*—No. 88530; Pierce Chemical Co.

e. *Toluene.*—Distilled in glass, HPLC grade (Fisher Scientific).

f. *Sodium sulfate.*—Anhydrous, certified A.C.S. grade (Fisher Scientific).

g. *Glass wool.*—Fiberglass, 8 micron (Corning).

h. *Acetone.*—HPLC grade (Fisher Scientific).

i. *95% Ethanol.*—Remet Alcohol Division (La Mirada, CA).

j. *n-Heptane.*—HPLC grade, No. 34873-4L: Sigma-Aldrich Co. (St. Louis, MO).

k. *Water.*—Prepared with a Milli-Q® purification system; Millipore Corp. (Bedford, MA).

(*Note:* Equivalent reagents may be substituted.)

Reference Standards

a. 5α-Cholestane internal standard (IS) solution.—No. C-8003; Sigma-Aldrich Co.

b. Campesterol (CAS No. 474-62-4).—No. 03072-641, >95% pure; Chromadex (Santa Ana, CA).

c. Stigmasterol (CASNo. 83-48-7).—No. S-2424, >95% pure; Sigma-Aldrich Co.

d. beta-Sitosterol (CAS No. 83-46-5).—No. S-9889, >98% pure; Sigma-Aldrich Co.

(*Note*: Equivalent reference materials may be substituted.)

Preparation of Standards

(a) IS—Ca 0.0100 g 5α-cholestane was weighed into a 100 mL volumetric flask and diluted to mark with *n*-heptane to make a solution with a concentration of 0.100 mg/mL. Solutions were stored protected from light at room temperature when not in use and prepared fresh as needed.

(b) GC calibration standards—In a 100 mL volumetric flask, campesterol, Stigmasterol, and beta-sitosterol were accurately weighed to the nearest 0.1 mg so that the concentration was ca 0.200 mg/mL after dilution to volume with DMF. The solution was mixed by inverting the flask numerous times. Then the standard was diluted as necessary to obtain 5 additional standards ranging in concentration from ca 0.00250 to 0.200 mg/mL. Solutions were stored protected from light at room temperature when not in use. Standards were shown to be stable for at least 1 year.

(c) Mixed standards—Prepared by appropriate dilution to the concentrations listed in Table 1.

Preparation of Samples

(a) Saponification—An appropriate amount of homogenous product was weighed (2.00 to 3.00 g to nearest 0.01 g) into a 250 or 300 mL Erlenmeyer flask. Amagnetic stir bar was placed into the flask, and 40 mL of 95% ethanol and 8 mL of 50% KOH solution were added to the flask.

The flask was placed on a magnetic stirrer hot plate with a condenser attached, and the contents were refluxed for 80 ± 10 min. To ensure complete saponification, test portions were occasionally visually checked, and any clumps were dispersed with a glass rod or by adding KOH solution to the test portion while stirring.

After refluxing, the heat was turned off and 60 mL of 95% ethanol was added through the top of the condenser while stirring the solution. The 95% ethanol was added carefully to avoid splashing of alcohol from the top of the condenser. After being stirred for ca 15 min, the flask was removed from the condenser and closed with a stopper, and the solution was cooled to room temperature. Test solution was stable for up to 1 week if tightly sealed.

(b) Extraction—100 mL toluene (V1) was added to the saponified test portion while stirring on a magnetic stirrer. The solution was poured into a 500 mL separatory funnel without rinsing,

and 110 mL of 1 M KOH solution was added and shaken vigorously for at least 20 s. The layers were allowed to separate and the aqueous lower layer, which was turbid, was discarded. KOH solution (40 mL of 0.5 M) was added to the separatory funnel. The funnel was inverted, and the contents were gently swirled for at least 10 s. The aqueous lower layer was discarded.

The toluene layer was washed with 40 mL water by gently rotating the separatory funnel. The layers were allowed to separate, and the aqueous phase was discarded. The water wash was repeated at least 3 times, shaken more vigorously each time. If emulsification occurred, a small amount of 95% ethanol was added, the contents of funnel were swirled, the layers were allowed to separate, and the water washes were continued. After the final wash, the toluene layer was crystal clear.

The toluene layer was poured from the top of the separatory funnel through a glass funnel containing a plug of glass wool and ca 20 g Na_2SO_4 into a 125 mL Erlenmeyer flask containing ca 2 g Na_2SO_4. The flask was stoppered, and the contents were swirled. The mixture was allowed to stand for at least 15 min. Test solutions were held up to 24 h if tightly sealed.

The toluene extract (V2; 25 mL) was pipetted into a 125 mL flat-bottom boiling flask, and the contents were evaporated to dryness on a rotary evaporator set at 55°C. Ca 3 mL acetone was added, and the contents were evaporated to dryness again. The residue was dissolved in 3.0 mL DMF (V3). The final concentration of campesterol, stigmasterol, and beta-sitosterol in DMF needed to be within range of working standard solutions. If, after quantification by GC, the test portion concentration fell outside of the standard curve, the amount of V2 evaporated was changed so that the final concentration of campesterol, stigmasterol, and beta-sitosterol in DMF fell within the range of the standards.

(c) Derivatization—Aliquots (1.0 mL) of working standard solutions and test solution were pipetted into separate 15 mL centrifuge tubes. To each tube, 0.2 mL HMDS and 0.1 mL TMCS were added. The tubes were stoppered and shaken vigorously on a Vortex mixer for 30 s. The solution was allowed to stand undisturbed for 15 min. To each tube, 1.0 mL 5α-cholestane IS solution and 10 mL water were added. The tubes were stoppered, shaken vigorously for 30 s, and centrifuged for ca 2 min.

A sufficient portion of heptane (upper) layer was transferred to an injection vial, with ensurance that no aqueous layer was transferred. Derivatized standards and test solutions were analyzed within 24 h.

Determination

Standards and samples were analyzed using the instrumental conditions shown in Table 2. At least one set of GC calibration standards was injected at the beginning of the run and one at the end of the run. A standard was run in between each sample to avoid possible analyte carryover. When using standards that had carryover in the standard curve, standard peaks were integrated from valley to valley.

Calculations

A standard calibration curve was generated by using the ratio of the analyte peak area versus the area of the internal standard peak for each concentration level. A calibration curve was produced for each analyte. High-level test solutions were diluted to fall within the standard range. Weighting (1/x) was necessary to obtain acceptable linearity at lower standard concentrations:

$$y=mx+b$$

where *y* = relative peak area (area of analyte/area of IS), m = slope of the line generated by a standard curve, x = concentration of analyte found (mg/mL), and b = *y*-intercept of the line generated by the standard curve.

Grams of test portion/mL derivatized was calculated as follows:

$$\text{Test portion derivatized, g/mL} = (W1/V1) \times (V2/V3)$$

where W1 = weight of test portion (g), V1 = volume of toluene used in extraction (mL), V2 = aliquot of extract taken to dryness (mL), and V3 = volume of DMF used to dissolve residue (mL).

The content of each phytosterol component in test portions was calculated as follows:

$$\text{mg Phytosterol}/100\text{ g test portion} = \frac{\text{concentration of analyte found, mg/mL}}{\text{test portion derivatized, g/mL}} \times 100$$

Results and Discussion

The limit of quantification (LOQ) for this method was found to be 1.00 mg/100 g. Calibration curves were generated with each data set, on each day during the course of this validation. The calibration range encompassed the expected concentration of each extracted and diluted test material range. Calibration standards (consisting of 6 concentration levels ranging from 0.00250 to 0.200 mg/mL) were analyzed at a minimum before and after each sample set with another set interspersed throughout the run. Each test sample was placed between the interspersed standards to avoid possible carryover. The response ratio of the analyte (peak area of analyte/peak area of IS) versus concentration was used to construct the calibration curve using a 1/x weighted linear regression method. The calibration curves had correlation coefficients (r) of greater than or equal to 0.995.

Precision of the method was evaluated by having 6 replicates of each test material analyzed by the same analyst on 2 separate days. If the initial sample extract fell above the highest standard of the curve, as indicated above, the extract was diluted to fit on the curve. For the majority of the precision results, the RSD_rS were either below or within the target RSD_r ranges with a few exceeding this range. The HorRat values were calculated, and all precision results (Day 1, Day 2, and Days 1 and 2 combined) for all test materials had values below 0.3 or between 0.3 and 1.3. Based on the acceptable HorRat values for this single laboratory validation (SLV), the collaborative study should be successful. The target RSD_r and HorRat values were calculated as follows (1):

$$\text{Target } RSD_r = \text{acceptable intralaboratory precision} \quad (0.500 \text{ to } 0.667)\, PRSD_R$$

$$PRSD_R = 2\,C^{-0.15}$$

C=Mean concentration expressed as a mass fraction

$$\text{HorRat value} = RSD_r/PRSD_R$$

Precision results are presented in Table 3. Example chromatograms of a mixed standard and test materials with varying amounts of campesterol, stigmasterol, and beta-sitosterol are presented in Figures 1 through 4.

For this SLV, a negative control was not used. In order to determine accuracy, the powdered saw palmetto sample was fortified 7 times with the mixed reference standard at 0.5, 1, and 2

times the amount detected, or at 3 levels that bracketed the amounts detected. In addition, a single midpoint fortification was conducted on the remaining 9 test materials in replicates of 6. Recovery was conducted on 2 separate days. The Days 1 and 2 combined averages recovery for each phytosterol was within 80–120%. On each individual day, average recoveries also met this criteria except for beta-sitosterol in 2 test materials. Furthermore, beta-sitosterol appears to have a lower recovery than the other phytosterol components. Accuracy results are shown in Table 4.

To demonstrate ruggedness relative to the reference method, the following were investigated as part of the SLV: change in operating conditions, second analyst, and expansion of saponified solution stability. The operating conditions in the reference method were modified in this SLV; however, it appeared that other conditions caused more chromatographic carryover between sample injections in certain test materials. Additional method ruggedness tests included using a different analyst to conduct precision over 2 days for at least 2 test materials. Furthermore, a second analyst conducted some of the fortification recovery accuracy results. Table 5 presents the second analyst's precision data. These data showed good precision (the RSD_rs are mostly within or below the target RSD_r ranges), and the HorRat values were all below 0.3 or between 0.3 and 1.3. The saponified solution stability was tested and proved to be stable at room temperature up to 7 days. This was achieved by comparison to freshly saponified sample solutions. Table 6 presents the saponified solution stability results.

Conclusions

The validation process showed the precision and accuracy required for determination of campesterol, stigmasterol, and beta-sitosterol in saw palmetto raw materials and dietary supplements. The collaborative study protocol for the method has been approved by AOAC INTERNATIONAL, and a collaborative study is currently in progress.

References

1. Horwitz W. AOAC Requirements for Single Laboratory Validation of Chemical Methods for Dietary Supplements. 2002Draft §3.4.1

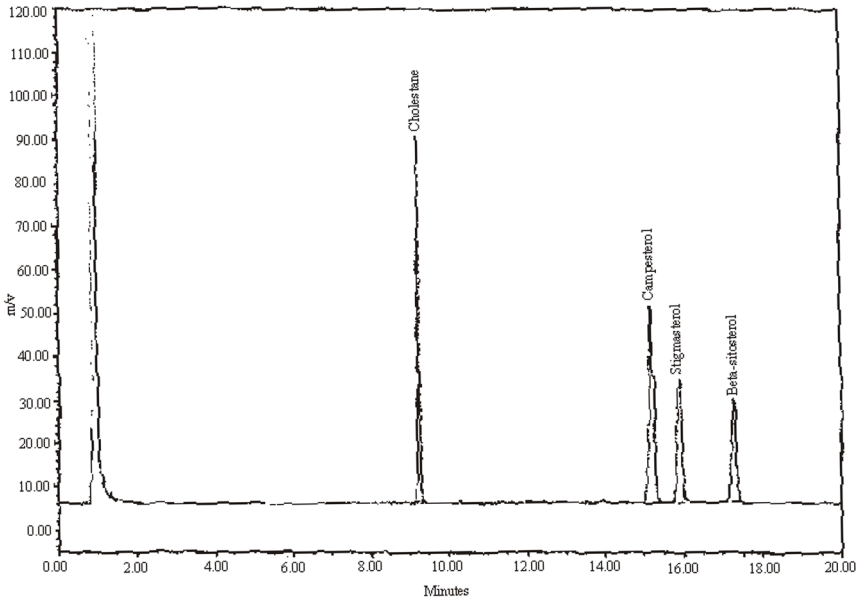

Figure 1.
An example chromatogram of a mixed standard, 0.100 mg/mL.

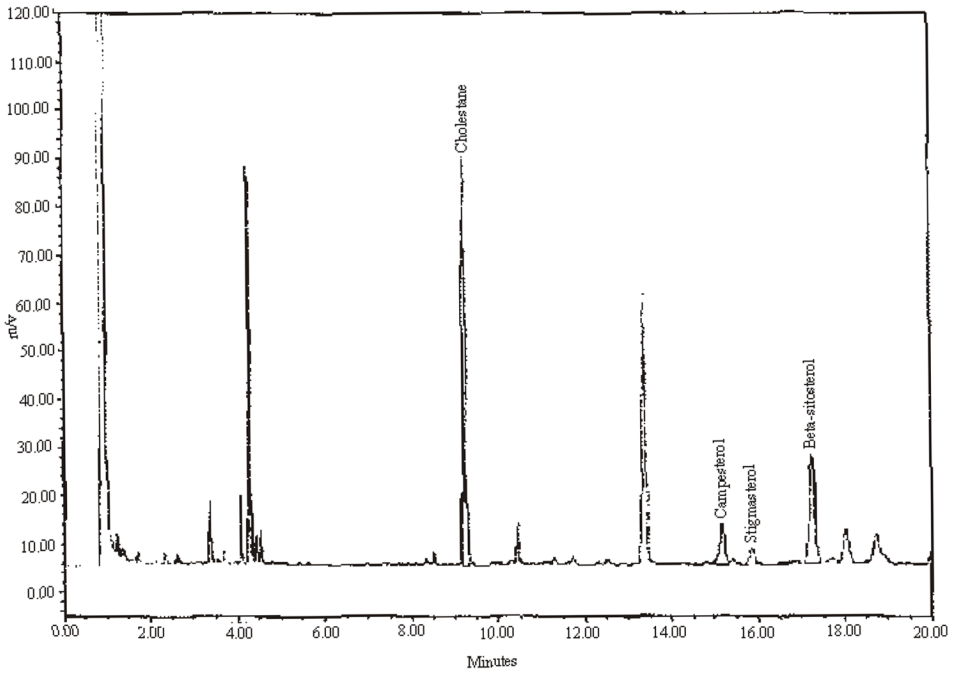

Figure 2.
An example chromatogram for saw palmetto 45% powered extract.

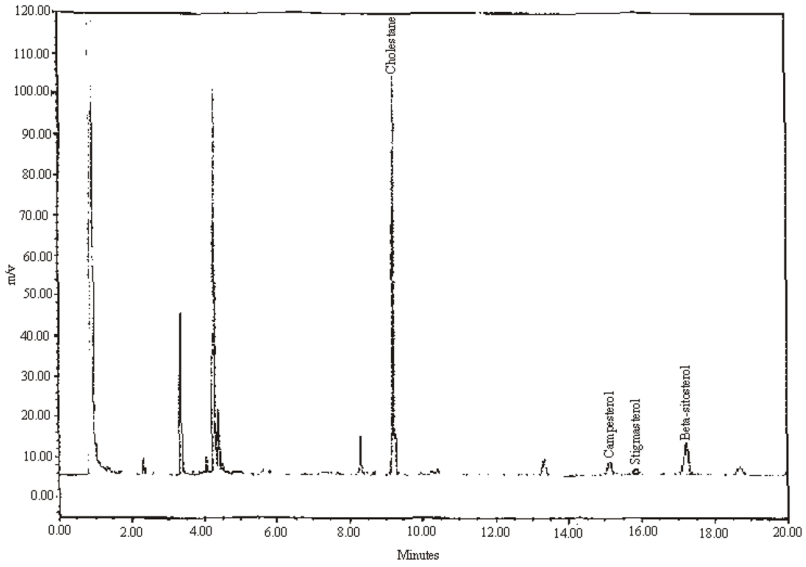

Figure 3.
An example chromatogram for alcohol-free saw palmetto.

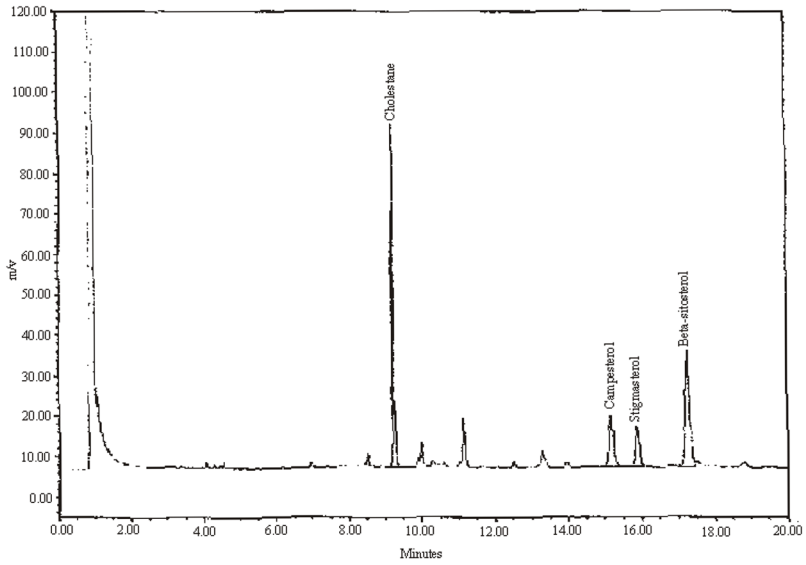

Figure 4.
An example chromatogram for saw palmetto pygeum lycopene complex tablets.

Table 1

Preparation of mixed standards

Standard No.	Intermediate volume, mL	Final volume, mL	Concentration, mg/mL
1	NA[a]	50	0.200
2	25	50	0.100
3	25	50	0.0500
4	10	50	0.0100
5	25	50	0.00500
6	25	50	0.00250

[a] NA = Not applicable.

Table 2

Gas chromatography conditions

Column	Capillary column, split mode (25 m × 0.32 mm) cross-linked 5% phenyl-methyl silicone or methyl silicone gum
Film thickness	0.17 μm
Detector	Hydrogen flame ionization detector (FID)
	Temperatures
Column	190°C, hold 2 min; increase 20°/min to 230°C, hold 3 min; increase 40°/min to 255°C, hold 25 min
Injector	250°C
Detector	300°C
	Flow rates
Carrier	2 mL/min, split vent ca 30 mL/min, purge vent ca 3 mL/min helium
Makeup	20 mL/min helium
Hydrogen	35 mL/min
Air	280 mL/min
Injection volume	1 μL

Table 3

Precision data

Test material identification/replicates	Day 1 precision mg/100 g			Day 2 precision mg/100 g			Days 1 and 2 precision combined, mg/100 g		
	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol
Saw palmetto 45% powdered extract									
1	34.9	16.8	114	34.8	17.4	105			
2	35.2	16.7	116	34.5	16.9	101			
3	34.5	16.8	115	35.2	17.5	103			
4	36.7	17.2	116	34.7	17.6	102			
5	34.8	16.5	115	35.3	17.4	103			
6	34.6	16.7	114	34.7	16.8	102			
Mean	35.1	16.8	115	34.9	17.3	103	35.0	17.0	109
SD[a]	0.813	0.232	0.894	0.314	0.333	1.37	0.602	0.372	6.53
RSD_r, %[b]	2.32	1.38	0.777	0.900	1.92	1.33	1.72	2.19	5.99
Target RSD_r low[b]	3	4	3	3	4	3	3	4	3
Target RSD_r high[c]	4	5	4	4	5	4	4	5	4
$PRSD_R$[c]	6.60	7.37	5.52	6.60	7.33	5.61	6.60	7.35	5.56
HorRat[d]	0.4	0.2	0.1	0.1	0.3	0.2	0.3	0.3	1.0
Alcohol-free saw palmetto									
1	2.50	1.13	7.34	2.37	1.27	6.06			
2	2.52	1.19	7.67	2.50	1.35	6.37			
3	2.57	1.23	7.61	2.51	1.35	6.39			
4	2.61	1.24	7.81	2.44	1.34	6.28			
5	2.60	1.22	7.70	2.37	1.26	6.10			
6	2.52	1.20	7.53	2.50	1.33	6.26			
Mean	2.55	1.20	7.61	2.45	1.32	6.24	2.50	1.26	6.93
SD	0.0463	0.0397	0.162	0.0655	0.0408	0.137	0.0770	0.0713	0.728
RSD_r, %	1.82	3.31	2.13	2.67	3.09	2.20	3.08	5.66	10.5
Target RSD_r low	5	5	4	5	5	4	5	5	4
Target RSD_r high	7	7	6	7	7	6	7	7	6
$PRSD_R$	9.77	10.9	8.30	9.83	10.8	8.55	9.80	10.9	8.41
HorRat	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.5	1.0
Liquid Herbal Extract: saw palmetto									
1	1.98	1.34	7.17	1.85	1.47	7.50			
2	2.04	1.34	7.37	1.83	1.52	7.84			
3	1.98	1.35	7.23	1.82	1.57	8.00			
4	1.95	1.33	7.11	1.88	1.44	7.77			
5	1.97	1.40	7.33	1.87	1.50	7.80			
6	2.29	1.54	7.78	1.84	1.49	7.90			
Mean	2.04	1.38	7.33	1.85	1.50	7.80	1.94	1.44	7.57
SD	0.128	0.0807	0.240	0.0232	0.0445	0.169	0.131	0.0864	0.315
RSD_r, %	6.27	5.85	3.27	1.25	2.97	2.17	6.75	6.00	4.16
Target RSD_r low	5	5	4	5	5	4	5	5	4
Target RSD_r high	7	7	6	7	7	6	7	7	6
$PRSD_R$	10.1	10.7	8.34	10.3	10.6	8.26	10.2	10.6	8.30
HorRat	0.6	0.5	0.4	0.1	0.3	0.03	0.7	0.6	0.5
Powdered saw palmetto berry									

Test material identification/replicates	Day 1 precision mg/100 g			Day 2 precision mg/100 g			Days 1 and 2 precision combined, mg/100 g		
	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol
1	5.28	3.93	30.9	5.01	3.95	33.4			
2	6.12	3.90	30.3	4.99	3.91	33.6			
3	5.30	3.82	30.4	5.01	4.05	33.9			
4	5.89	4.50	31.8	4.97	3.82	33.3			
5	5.67	4.28	30.4	5.33	4.18	34.3			
6	5.63	4.01	30.9	5.05	3.93	33.6			
Mean	5.65	4.07	30.8	5.06	3.97	33.7	5.35	4.02	32.2
SD	0.328	0.262	0.564	0.135	0.125	0.366	0.389	0.203	1.58
RSD_r, %	5.81	6.44	1.83	2.67	3.15	1.09	7.27	5.05	4.91
Target RSD_r low	4	5	3	4	5	3	4	5	3
Target RSD_r high	6	6	4	6	6	4	6	6	4
$PRSD_R$	8.67	9.11	6.73	8.82	9.15	6.64	8.75	9.13	6.68
HorRat	0.7	0.7	0.3	0.3	0.3	0.2	0.8	0.6	0.7
ProstActive Once Daily									
1	38.3	19.9	117	35.6	18.0	101			
2	35.3	18.5	109	35.0	18.1	100			
3	36.7	19.9	115	36.6	19.5	103			
4	34.8	18.6	108	36.3	19.2	102			
5	39.6	22.6	119	36.2	19.3	102			
6	35.3	18.2	109	36.0	19.3	100			
Mean	36.7	19.6	113	36.0	18.9	101	36.3	19.3	107
SD	1.92	1.63	4.75	0.572	0.666	1.21	1.40	1.25	6.86
RSD_r, %	5.23	8.32	4.20	1.59	3.52	1.20	3.86	6.48	6.41
Target RSD_r low	3	4	3	3	4	3	3	4	3
Target RSD_r high	4	5	4	4	5	4	4	5	4
$PRSD_R$	6.55	7.20	5.53	6.57	7.24	5.63	6.56	7.21	5.58
HorRat	0.8	1.0	0.8	0.2	0.5	0.2	0.6	0.9	1.0
ProstActive Plus saw palmetto combined with nettle root extract									
1	31.8	10.4	129	34.4	9.42	117			
2	31.5	9.25	128	36.0	10.2	119			
3	32.0	10.1	130	35.6	10.1	119			
4	31.3	9.17	121	34.5	9.90	116			
5	30.3	9.37	119	36.5	10.5	120			
6	32.3	10.5	126	34.1	10.0	115			
Mean	31.5	9.80	126	35.2	10.0	118	33.4	9.91	1.22
SD	0.700	0.604	4.51	0.983	0.359	1.97	2.07	0.488	5.26
RSD_r, %	2.22	6.16	3.58	2.79	3.59	1.67	6.20	4.92	4.31
Target RSD_r low	3	4	3	3	4	3	3	4	3
Target RSD_r high	4	5	4	4	5	4	4	5	4
$PRSD_R$	6.70	7.99	5.44	6.59	7.96	5.50	6.64	7.97	5.47
HorRat	0.3	0.8	0.7	0.4	0.5	0.3	0.9	0.6	0.8
Prostasan Prostrate Capsules									
1	39.8	20.2	116	40.4	20.7	108			
2	39.6	19.3	117	40.2	20.8	108			
3	37.8	19.6	113	40.1	20.4	110			

Test material identification/replicates	Day 1 precision mg/100 g			Day 2 precision mg/100 g			Days 1 and 2 precision combined, mg/100 g		
	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol
4	38.6	19.6	117	39.6	20.3	107			
5	37.7	19.4	115	41.5	20.4	108			
6	39.2	19.9	118	39.8	20.8	108			
Mean	38.8	19.7	116	40.3	20.6	108	39.5	20.1	112
SD	0.900	0.333	1.79	0.668	0.225	0.983	1.08	0.542	4.32
RSD_r, %	2.32	1.69	1.54	1.66	1.09	0.910	2.73	2.7	3.86
Target RSD_r low	3	4	3	3	4	3	3	4	3
Target RSD_r high	4	5	4	4	5	4	4	5	4
$PRSD_R$	6.50	7.19	5.51	6.46	7.14	5.57	6.48	7.17	5.54
HorRat	0.4	0.2	0.3	0.3	0.2	0.2	0.4	0.4	0.7
Pygeum and saw palmetto standardized herbal extracts									
1	21.5	8.07	264	22.3	8.26	278			
2	21.8	8.91	266	22.9	8.68	277			
3	23.3	9.76	281	21.8	8.47	274			
4	22.5	9.13	269	22.9	8.53	278			
5	21.9	8.91	270	22.1	8.35	273			
6	22.2	8.32	267	22.0	8.39	276			
Mean	22.2	8.85	270	22.3	8.45	276	22.3	8.65	273
SD	0.639	0.600	6.02	0.468	0.148	2.10	0.538	0.467	5.48
RSD_r, %	2.88	6.78	2.23	2.10	1.75	0.761	2.41	5.40	2.01
Target RSD_r low	4	4	2	4	4	2	4	4	2
Target RSD_r high	5	5	3	5	5	3	5	5	3
$PRSD_R$	7.06	8.11	4.86	7.06	8.17	4.84	7.06	8.14	4.85
HorRat	0.4	0.8	0.5	0.3	0.2	0.2	0.3	0.7	0.4
Saw palmetto pygeum lycopene complex tablets									
1	112	100	268	114	102	269			
2	113	101	268	129	115	300			
3	114	101	268	115	102	271			
4	116	103	274	117	103	274			
5	109	98.1	263	121	107	280			
6	118	104	279	125	112	293			
Mean	114	101	270	120	107	281	117	104	276
SD	3.14	2.11	5.62	5.95	5.56	12.6	5.66	4.98	11.0
RSD_r, %	2.75	2.09	2.08	4.96	5.20	4.48	4.84	4.79	3.99
Target RSD_r low	3	3	2	3	3	2	3	3	2
Target RSD_r high	4	4	3	4	4	3	4	4	3
$PRSD_R$	5.53	5.63	4.86	5.48	5.58	4.83	5.51	5.60	4.84
HorRat	0.5	0.4	0.4	0.9	0.9	0.9	0.9	0.9	0.8
Saw palmetto (*Serenoa repens*) dried fruit CO_2 extracts									
1	55.5	28.7	176	56.6	28.9	174			
2	55.1	29.1	178	55.5	28.7	172			
3	55.6	28.7	178	55.9	28.3	175			
4	55.4	29.1	176	55.9	28.6	173			
5	53.8	27.8	171	56.3	28.7	174			
6	53.9	27.6	171	55.1	28.5	173			
Mean	54.9	28.5	175	55.9	28.6	174	55.4	28.6	1.74

Test material identification/replicates	Day 1 precision mg/100 g			Day 2 precision mg/100 g			Days 1 and 2 precision combined, mg/100 g		
	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol
SD	0.818	0.648	3.22	0.538	0.204	1.05	0.842	0.462	2.42
RSD_r, %	1.49	2.27	1.84	0.962	0.713	0.603	1.52	1.62	1.39
Target RSD_r low	3	3	3	3	3	3	3	3	3
Target RSD_r high	4	5	3	4	5	3	4	5	3
$PRSD_R$	6.17	6.80	5.18	6.15	6.80	5.19	6.16	6.80	5.19
HorRat	0.2	0.3	0.4	0.2	0.1	0.1	0.2	0.2	0.3

[a] SD = Standard deviation.

[b] RSD_r = Intralaboratory relative standard deviation.

[c] $PRSD_R = 2\ C^{-0.15}$, where C is the mean concentration expressed as a mass fraction.

[d] HorRat = $RSD/PRSD_R$.

Table 4

Accuracy data (percent)

Test material identification/replicates	Day 1 accuracy			Day 2 accuracy			Days 1 and 2 accuracy combined		
	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol
Spiking level		1X			1X			1X	
Saw palmetto 45% powdered extract									
1	82.9	89.7	85.2	103	98.4	101			
2	100	101	107	103	101	102			
3	100	95.7	101	103	99.9	102			
4	112	101	113	104	103	101			
5	98.0	104	94.2	102	102	99.1			
6	105	103	109	101	102	98.6			
Mean	99.7	99.1	102	103	101	101	101	100	101
SD[a]	9.64	5.41	10.4	1.03	1.67	1.45	6.72	3.96	7.08
RSD_r, %[b]	9.67	5.46	10.2	1.00	1.65	1.44	6.65	3.96	7.01
Alcohol-free saw palmetto									
1	101	107	113	99.8	101	100			
2	98.0	102	109	101	96.2	103			
3	99.4	111	108	101	93.8	102			
4	96.9	101	101	99.9	7.34[c]	101			
5	94.9	106	95.0	100	101	101			
6	97.3	102	101	102	102	100			
Mean	97.9	105	105	101	98.8	101	99.3	102	103
SD	2.11	3.87	6.63	0.868	3.59	1.17	2.09	4.75	4.86
RSD_r, %	2.16	3.69	6.31	0.859	3.63	1.16	2.10	4.66	4.72
Liquid Herbal Extract: saw palmetto									
1	99.9	101	96.1	101	100	100			
2	98.8	100	93.9	101	101	101			
3	99.8	101	98.3	102	104	101			
4	99.5	95.5	97.5	101	102	102			
5	101	113	103	101	101	97.5			
6	98.4	114	102	101	100	97.5			
Mean	99.6	104	98.5	101	101	99.8	100	103	99.2
SD	0.914	7.58	3.48	0.408	1.51	1.91	1.07	5.40	2.77
RSD_r, %	0.918	7.29	3.53	0.404	1.50	1.91	1.07	5.24	2.79
ProstActive Once Daily									
1	129	143	120	100	95.6	93.3			
2	105	86.7	97.4	103	98.2	99.7			
3	110	116	110	102	95.7	98.2			
4	103	98.8	96.8	100	96.2	96.5			
5	107	94.5	104	99.4	95.7	95.6			
6	109	113	104	98.3	94.8	94.6			
Mean	111	109	105	100	96.0	96.3	105	102	101
SD	9.42	20.2	8.67	1.73	1.15	2.35	8.32	15.1	7.68
RSD_r, %	8.49	18.5	8.26	1.73	1.20	2.44	7.92	14.8	7.60

Test material identification/replicates	Day 1 accuracy			Day 2 accuracy			Days 1 and 2 accuracy combined		
	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol
Spiking level		1X			1X			1X	
ProstActive plus saw palmetto combined with nettle root extract									
1	105	103	104	104	103	94.5			
2	106	99.1	105	105	99.5	89.3			
3	99.9	99.7	105	107	104	94.7			
4	101	96.0	97.2	94.9	99.7	77.5			
5	103	98.6	106	102	103	85.7			
6	101	104	99.8	104	104	97.2			
Mean	103	100	103	103	102	89.8	103	101	96.3
SD	2.44	2.96	3.51	4.20	2.06	7.34	3.28	2.68	8.73
RSD_r, %	2.37	2.96	3.41	4.08	2.02	8.17	3.18	2.65	9.07
Prostasan Prostrate Capsules									
1	108	99.5	108	100	99.3	97.3			
2	98.6	95.2	105	98.5	95.4	92.8			
3	95.2	92.3	101	97.0	93.9	92.6			
4	106	95.8	108	97.7	94.8	93.5			
5	88.5	87.3	93.4	99.2	95.7	94.3			
6	103	94.8	107	99.3	95.9	96.1			
Mean	99.9	94.2	104	98.6	95.8	94.4	99.3	95.0	99.1
SD	7.30	4.08	5.71	1.11	1.85	1.89	5.02	3.14	6.33
RSD_r, %	7.31	4.33	5.49	1.13	1.93	2.00	5.06	3.31	6.39
Pygeum and saw palmetto standardized herbal extracts									
1	91.2	89.6	78.7	101	103	77.9			
2	115	84.7	104	106	103	81.7			
3	118	83.6	95.5	104	102	84.2			
4	112	100	84.8	90.4	102	73.9			
5	96.5	111	85.4	103	108	86.5			
6	119	85.5	97.1	104	103	73.1			
Mean	109	92.4	90.9	101	104	79.6	105	98.0	85.2
SD	11.8	10.9	9.46	5.63	2.26	5.49	9.60	9.49	9.47
RSD_r, %	10.8	11.8	10.4	5.57	2.17	6.90	9.14	9.68	11.1
Saw palmetto pygeum lycopene complex tablets									
1	92.1	94.3	91.5	100	98.4	94.7			
2	94.9	95.8	93.4	106	105	107			
3	105	106	104	104	102	102			
4	75.6	80.5	72.7	107	103	106			
5	111	107	106	98.4	96.8	89.9			
6	99.8	103	101	88.1	87.5	77.7			
Mean	96.4	97.8	94.8	101	98.8	96.2	98.5	98.3	95.5
SD	12.3	9.94	12.2	6.97	6.30	11.2	9.76	7.95	11.2
RSD_r, %	12.8	10.2	12.9	6.90	6.38	11.6	9.91	8.09	11.7
Saw palmetto (*Serenoa repens*) dried fruit CO_2 extracts									
1	110	106	103	99.6	99.7	106			

Test material identification/replicates	Day 1 accuracy			Day 2 accuracy			Days 1 and 2 accuracy combined		
	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol
Spiking level		**1X**			**1X**			**1X**	
2	110	102	97.7	97.7	99.7	100			
3	104	93.4	93.5	96.2	97.0	94.4			
4	113	104	102	98.9	97.4	99.4			
5	108	103	101	99.7	97.6	101			
6	109	105	99.5	91.7	93.9	91.2			
Mean	109	102	99.5	97.3	97.6	98.7	103	99.9	99.1
SD	2.97	4.55	3.46	3.04	2.14	5.21	6.75	4.18	4.24
RSD_r, %	2.72	4.46	3.48	3.12	2.19	5.28	6.55	4.18	4.28
Spiking level		1X			1/2X			2X	
Powdered saw palmetto berry (Day 1 accuracy)									
1	104	96.1	89.6	108	89.3	77.8	108	97.8	102
2	97.5	113	79.5	90.6	68.1	43.7	102	96.8	98.3
3	108	110	91.7	116	110	82.4	105	100	100
4	103	81.0	86.6	100	89.9	66.7	104	103	102
5	103	125	84.7	100	100	69.1	104	93.6	103
6	105	101	90.9	104	68.9	68.7	106	95.1	98.3
7	96.1	75.4	78.9	116	110	60.4	108	98.7	101
Mean	102	100	86.0	105	90.9	67.0	105	97.9	101
SD	4.19	17.7	5.23	9.21	17.4	12.6	2.21	3.13	1.86
RSD_r, %	4.11	17.7	6.08	8.77	19.1	18.8	2.10	3.20	1.84
Powdered saw palmetto berry (Day 2 accuracy)									
1	102	106	88.9	114	99.2	96.1	107	104	105
2	102	116	85.8	118	97.6	97.7	96.1	97.7	101
3	103	101	85.6	113	99.1	98.0	101	98.7	101
4	99.5	96.5	78.8	115	80.3	88.9	98.3	98.0	104
5	103	106	89.5	103	91.3	91.9	104	97.7	103
6	89.8	171	81.6	118	95.5	92.7	99.3	98.9	103
7	95.3	117	78.2	120	92.3	99.6	104	102	108
Mean	99.2	116	84.1	114	93.6	95.0	101	99.6	104
SD	4.97	25.3	4.59	5.62	6.65	3.89	3.81	2.46	2.44
RSD_r, %	5.01	21.8	5.46	4.93	7.10	4.09	3.77	2.47	2.35
Powdered saw palmetto berry (Days 1 and 2 accuracy combined)									
Mean	101	108	85.0	110	92.3	81.0	103	98.7	102
SD	4.71	22.5	4.83	8.83	12.7	17.1	3.61	2.84	2.57
RSD_r, %	4.66	20.8	5.68	8.03	13.8	21.1	3.50	2.88	2.52

[a] SD = Standard deviation.

[b] RSD_r = Intralaboratory relative standard deviation.

[c] Rejected based on the Dixon Test.

Table 5

Second analyst precision (ruggedness test)

Test material identification/replicates	Day 1 precision, mg/100 g			Day 2 precision, mg/100 g			Days 1 and 2 precision combined, mg/100 g		
	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol	Campesterol	Stigmasterol	beta-Sitosterol
Saw palmetto pygeum lycopene complex tablets									
1	117	108	316	122	119	328			
2	122	112	329	131	121	348			
3	132	121	351	122	117	329			
4	116	108	315	125	117	335			
5	124	116	324	119	109	320			
6	122	115	328	125	118	337			
Mean	122	113	327	124	117	333	123	115	330
SD[a]	5.74	5.05	13.1	4.10	4.12	9.54	4.85	4.76	11.3
RSD_r, %[b]	4.70	4.47	4.01	3.31	3.52	2.86	3.94	4.14	3.42
Target RSD_r low[b]	3	3	2	3	3	2	3	3	2
Target RSD_r high[b]	4	4	3	4	4	3	4	4	3
$PRSD_R$[c]	5.47	5.53	4.72	5.46	5.51	4.71	5.46	5.52	4.71
HorRat[d]	0.9	0.8	0.8	0.6	0.6	0.6	0.7	0.8	0.7
Saw palmetto (*Serenoa repens*) dried fruit CO_2 extracts									
1	40.4	22.7	145	43.2	25.6	157			
2	42.0	26.6	151	42.6	24.0	152			
3	42.7	25.5	153	43.1	24.7	155			
4	42.8	25.8	154	43.9	25.2	159			
5	42.6	24.9	153	44.3	25.1	159			
6	44.0	25.9	157	40.3	25.5	145			
Mean	42.4	25.2	152	42.9	25.0	155	42.7	25.1	153
SD	1.18	1.36	4.02	1.41	0.591	5.36	1.27	1.01	4.68
RSD_r, %	2.78	5.40	2.64	3.29	2.36	3.46	2.97	4.02	3.06
Target RSD_r low	3	3	3	3	3	3	3	3	3
Target RSD_r high	4	5	4	4	5	4	4	5	4
$PRSD_R$	6.41	6.93	5.29	6.40	6.94	5.28	6.40	6.94	5.29
HorRat	0.4	0.8	0.5	0.5	0.3	0.7	0.5	0.6	0.6

[a] SD = Standard deviation.

[b] RSD_r = Intralaboratory relative standard deviation.

[c] $PRSD_R = 2\ C^{-0.15}$, where C is the mean concentration expressed as a mass fraction.

[d] $HorRat = RSD_r/PRSD_R$.

Table 6

Saponified solution stability (ruggedness test)

	Timepoints	Campesterol	Stigmasterol	beta-Sitosterol
		mg/100g		
Saw palmetto (*Serenoa repens*) dried fruit CO_2 extracts	Day 1[a]	42.4	25.2	152
	Day 3	40.4	24.1	146
	Day 7	42.4	23.6	151
	Mean	41.7	24.3	150
	SD[b]	1.15	0.819	3.21
	RSD, %[c]	2.76	3.37	2.14

[a] Mean from 2nd analyst Day 1 precision.

[b] SD = Standard deviation.

[c] RSD = Relative standard deviation.