

Alkemists Pharmaceuticals, Inc.

“The Plant Authentication Experts”



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Optimization of Extraction Methods of some of the pentacyclic triterpenes, sterols & linear alcohols & Quantification of β -Sitosterol in Pygeum aka Prunus africana (Hook. f.) Kalkman [Rosaceae] by High Performance Thin-Layer Chromatography (HPTLC) with comparative analysis by HPLC

Presented by:

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119th AOAC International Annual Meeting

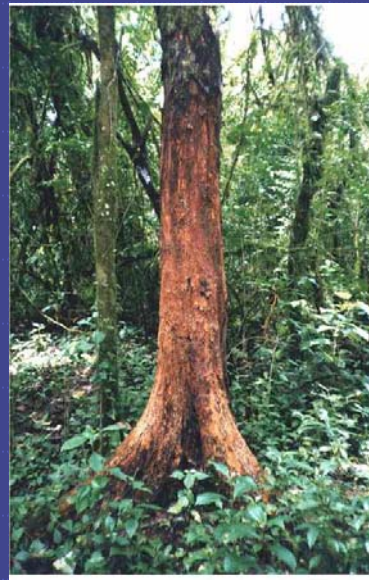
Wednesday September 14, 2005

Orlando, Florida USA



Pygeum I: Description, Relevance & Uses

- ◆ **Prunus africana (Hook. f.) Kalmk. [Rosaceae]**
 - Evergreen tree (40-60m tall) growing in the southern countries of Africa: Cameroon, Madagascar, Tanzania & Kenya in decreasing order of importation
 - Bark is dark brown to reddish brown



Pygeum I: Description, Relevance & Use

- ◆ Bark traditionally used for medicinal purposes in African countries as an infusion of powdered bark in milk or water as a remedy for treatment of bladder pains & micturition problems as well as for inflammation, kidney disease, malaria, stomach ache & fever
- ◆ Today, along with the root of stinging nettle & the fruits of saw palmetto, the bark of pygeum is valued in European phytotherapy for the treatment of benign prostatic hypertrophy

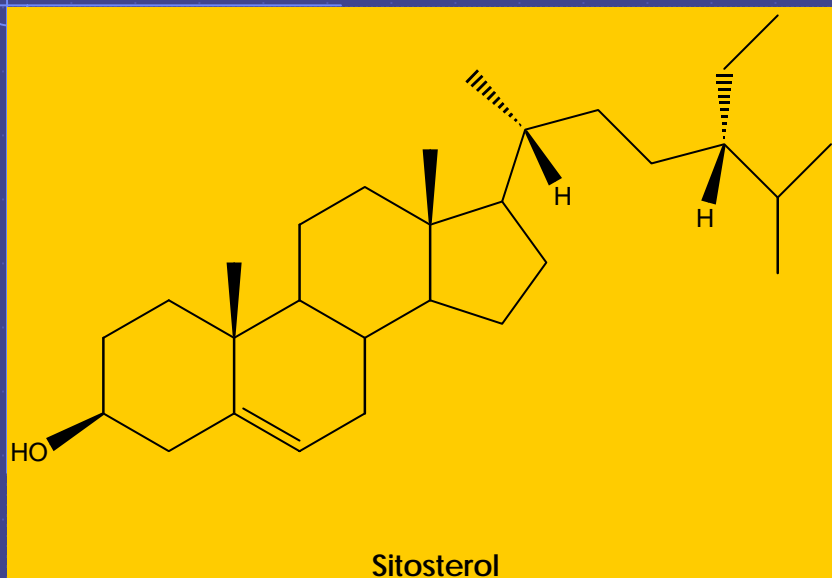
Pygeum I: Description, Relevance & Uses

- ◆ **Benign Prostatic Hypertrophy (BPH) affects most men over 50 & manifests as increased frequency of urination, inability to void, pain on passing urine & post urinary dribbling**
- ◆ **Causes seem to be related to a form of testosterone (dihydroxytestosterone, DHT)**
 - Produced from testosterone by the enzyme 5- α -reductase
 - Early stage defense consists of 5- α -reductase inhibition
 - Historic cure of castration, has long been abandoned!

Pygeum I: Chemistry & Pharmacology

- ◆ **Synergistic effects of the known & unknown compounds responsible for its effects**
 - **Phytosterols**: (e.g. β -sitosterol) have anti-inflammatory effects by interfering with accumulation of pro-inflammatory prostaglandins in the prostate
 - **Pentacyclic triterpenes**: (ursolic & oleanolic acids) which have an anti-edema or decongesting action
 - **Trans-ferulic acid esters of long chain aliphatic alcohols**: (n-docosanol & tetracosanol) which reduce prolactin levels & block accumulation of cholesterol in the prostate (prolactin increases uptake of testosterone by the prostate & cholesterol increases binding sites for DHT)

Pygeum I: Chemistry & Pharmacology



◆ Pygeum chemistry:

- Extracts of Pygeum contain a number of lipid soluble substances
- Several fatty acids
- Sterols (β -sitosterol, β -sitosterol-3-O-glucoside, β -sitosterone)
- Pentacyclic triterpenoids (ursolic acid, oleanolic acid, and friedelin)
- Two linear alcohols (n-tetracosanol, n-docosanol)

Pygeum I Background: Optimization of Mobile Phase for HPTLC

- ◆ Various mobile phases evaluated for optimum separation of reference standards
- ◆ Initially literature reviewed for best separation
- ◆ Equi-elutotropic system of solvent mixture strength used to optimize separation & selectivity
- ◆ Calculations based on Touchstone, 1992 'Practice of Thin-Layer Chromatography'
 - Polarity index used to determine solvent strength P'
 - "Total P' " is $P' = F_a P'_a + F_b P'_b + F_c P'_c \dots$ where F is volume fraction of the pure solvents $a, b, c \dots$ while the P'_a , and so on, is the polarity index
 - Solvent strengths (total P') in the range 1.5 ~ 2.5 gave best separation
 - Selectivity/resolution can be varied by changing a solvent while maintaining ~ the same solvent strength

Pygeum I Background: Optimization of Sample Preparation

- ◆ 7 crude raw material samples & 3 powdered extracts prepared using multiple extraction solvents and methods
 - 0.5 grams of dried crude raw Pygeum bark, accurately weighed and extracted with 5 mL solvent
 - Extraction performed by sonicating 30 minutes and heating for 30 minutes at ~ 65° C in a dry block incubator
 - Extractions performed with simple, single solvents
 - ◆ ethanol, methanol, chloroform &
 - ◆ methanol:dichloromethane [1/1]
- ◆ Solid phase extraction using the EluChrome columns (Analytical Sales and Services, Pompton Plains, NJ) for multiple solvent extraction, from polar to non-polar solvents consisting of water/methanol, methanol, acetone, ethyl acetate, chloroform, cyclohexane & hexane

Pygeum I Background: Optimum Conditions, HPTLC

◆ Sample Preparation

- Accurately weighed amount of sample added to 100% grain EtOH, sonicated 30 minutes & heated 30 minutes in a dry block incubator @ 65° C ~ 1hr

◆ Mobile Phase

- Cyclohexane:chloroform:ethyl acetate:methanol [10/2.5/2/1]
- Toluene:ethyl acetate [8/2]

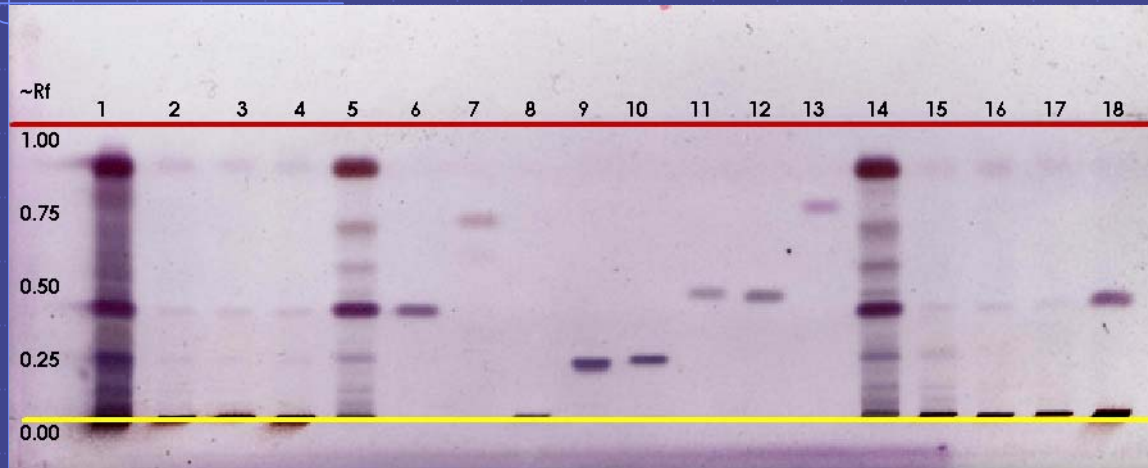
◆ Stationary Phase

- 10 x 10 & 20 x 10 cm EM Science glass backed silica gel F₂₅₄ HPTLC plates
- Prewashed with methanol, activated @ 105° C, 20 minutes

◆ Derivatization/Detection

- 10% EtOH/H₂SO₄ spray reagent → 110° C 5 min → vis light → UV 365 nm
- Vanillin/H₂SO₄ spray reagent → 110° C 5 min → vis light → UV 365 nm

Pygeum I: HPTLC-System 1



◆ Mobile Phase

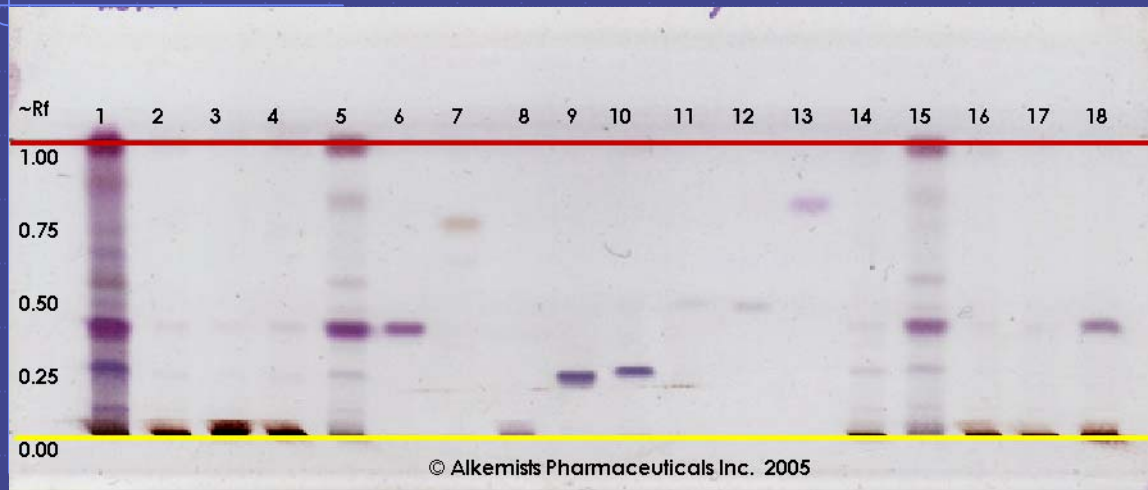
- Cyclohexane: 10
- chloroform: 2.5
- ethyl acetate: 2
- Methanol: 1

- Lanes 1, 5, 14: powdered extracts
- Lanes 2, 3, 4, 15, 16, 17, 18: crude raw materials
- Lane 6: β-sitosterol
- Lane 7: β-sitosterone
- Lane 8: β-sitosterol-D-glucoside
- Lane 9: ursolic acid
- Lane 10: oleanolic acid
- Lane 11: 1-tetracosanol
- Lane 12: n-docosanol
- Lane 13: friedelin

◆ Derivatization/Detection

- Vanillin/H₂SO₄ spray reagent →
- 110° C 5 min → vis light

Pygeum I: HPTLC-System 2



◆ Mobile Phase

- toluene: 8
- ethyl acetate: 2

Lanes 1, 5, 14:	powdered extracts
Lanes 2, 3, 4, 15, 16, 17, 18:	crude raw materials
Lane 6:	β -sitosterol
Lane 7:	β -sitosterone
Lane 8:	β -sitosterol-D-glucoside
Lane 9:	ursolic acid
Lane 10:	oleanolic acid
Lane 11:	1-tetracosanol
Lane 12:	n-docosanol
Lane 13:	friedelin

◆ Derivatization/Detection

- Vanillin/H₂SO₄ spray reagent →
- 110° C 5 min → vis light

Pygeum II: HPLC & HPTLC

- ◆ Initially testing was intended to quantitate by HPTLC the various markers in Pygeum
 - HPTLC was not achieving the necessary resolution for ursolic/oleanolic acids & docosanol/tetracosanol
 - Since β -sitosterol, being a significant sterol, was easily identified & separated by HPTLC, & in appreciable quantity, it could be easily quantified & potentially validated for use as an important marker for the quality control of Pygeum bark in extracts & raw material
 - To the best of our knowledge the available literature contains no data on quantification of β -sitosterol in Pygeum by HPTLC
 - The reliability of the methods was estimated by comparison of results obtained by use of the two approaches

Pygeum II: HPLC & HPTLC

- ◆ Several experiments performed to illustrate extraction efficiency
- ◆ 1 gm. sample (XA16005AHP-8) accurately weighed:
 - Method 1: Sonicated in 6 ml 100% grain undenatured EtOH 30 min, made up to 10 ml in vol. flask & centrifuged
 - ◆ Samples A, B, C
 - Method 2: Sonicated in 4 ml 100% grain undenatured EtOH for 15 min. x 2, made up to 10 ml in vol. flask & centrifuged
 - ◆ Samples D, E, F
 - Method 3: Sonicated in 3 ml 100% grain undenatured EtOH for 10 min. x 3, made up to 10 ml in vol. flask & centrifuged
 - ◆ Samples G, H, I

Pygeum II: Comparison of extraction efficiency using HPTLC

Method 1:

Sample	Concentration µg/mL	% β-sitosterol
A	32.4278	0.03182
B	30.1475	0.03010
C	29.0962	0.02839
	Average %	0.03010
	Std. Dev.	0.00171
	% RSD	5.68951

Compare methods 1 to 2:

t-Test: two-sample assuming equal variances

df: 4

t₄ Critical: 2.78 (P=0.05)

|t| = 2.70

Since experimental value of |t| is < the critical value, the null hypothesis is retained.

There is no statistical difference between the means of the two methods of extraction.

Compare methods 2 to 3:

t-Test: two-sample assuming equal variances

df: 4

t₄ Critical: 2.78 (P=0.05)

|t| = 7.282

Null hypothesis is rejected, the means of these two methods of extraction are statistically different.

Method 2:

Sample	Concentration µg/mL	% β-sitosterol
D	29.4866	0.02879
E	26.8199	0.02680
F	28.0251	0.02777
	Average %	0.02779
	Std. Dev.	0.00099
	% RSD	3.58014

Pygeum II: comparison of extraction efficiency using t-Test for two means

Method 3:

Sample	Concentration µg/mL	% β-sitosterol
G	38.2357	0.03849
H	34.0375	0.03404
I	39.3471	0.03934
	Average %	0.03729
	Std. Dev.	0.00285
	% RSD	7.62964

Compare methods 1 to 3:

t-Test: two-sample assuming equal variances

df: 4

t_4 Critical: 2.78 (P=0.05)

$|t| = 4.99$

Since experimental value of $|t|$ is > the critical value.

Null hypothesis is rejected, concluding these two methods of extraction are statistically different.

Conclusion:

Methods for A-C (1) & D-F (2) are not as efficient as for G-I & therefore Method 3 was used for the experiments to follow for comparison of the two methods of analysis.

Pygeum II: HPTLC Repeatability Data

Crude Raw Material: (XA16005AHP-8)

Assay	Conc/10 µl	Actual Conc
1	359.18	
2	355.84	
3	355.41	
4	344.95	
5	341.57	
6	341.69	
Average	349.773	34.977
SD	7.912	
%RSD	2.262	

Powdered Extract: (XA33505CHR-11)

Assay	Conc/2 µl	Actual Conc
1	657.43	
2	661.66	
3	663.5	
4	660.68	
5	653.9	
6	639.1	
Average	656.045	328.022
SD	8.969	
%RSD	1.367	

Pygeum II: HPTLC 'Intermediate' Precision

Crude Raw Material: (XA16005AHP-8)

Assay	Conc/10 µl	Actual Conc
1	335.398	
2	362.44	
3	344.631	
4	349.77	
5	340.375	
6	350.934	
7	350.287	
Average	347.6911	34.77
SD	8.690	
%RSD	2.50	

Powdered Extract: (XA33505CHR-11)

Assay	Conc/2 µl	Actual Conc
1	601.517	
2	612.107	
3	656.05	
4	676.712	
Average	636.595	318.30
SD	35.674	
%RSD	5.60	

Pygeum II: HPLC Repeatability

Crude Raw Material: (XA16005AHP-8)

Assay	Concentration
1	35.3
2	35.07
Average	35.185
SD	0.16
%RSD	0.462

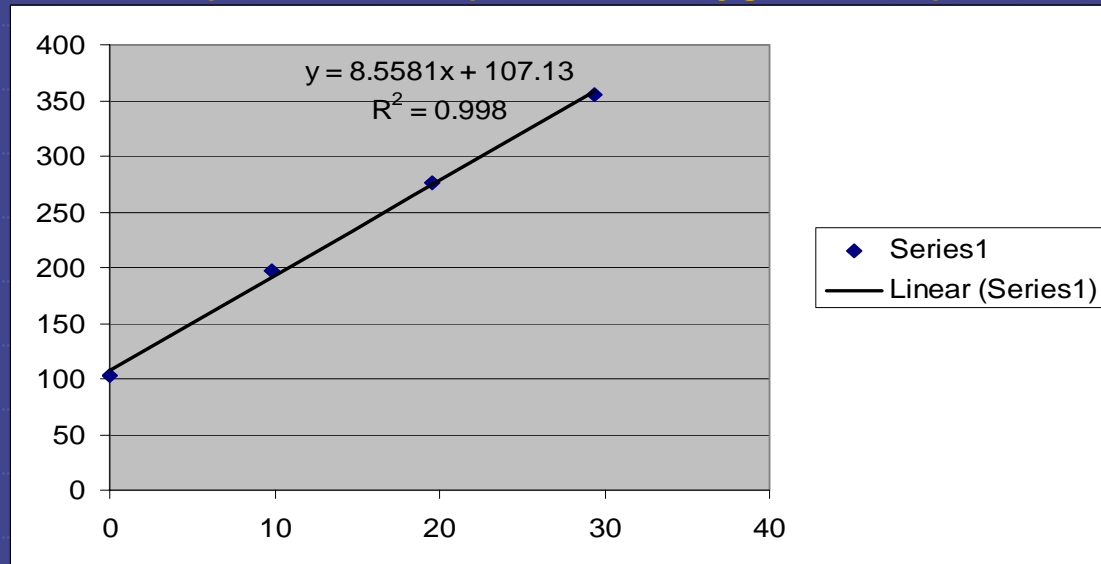
Powdered Extract: (XA33505CHR-11)

Assay	Concentration/30
1	9.8818
2	9.7527
3	9.7708
Average (actual)	294.053
SD	0.07
%RSD	0.02377

Pygeum II: HPTLC Standard Addition of raw material

Standard addition plot of 'least squares fit' for Pygeum sample XA16005AHP-8

Area of β -sitosterol
chromatographic
peak



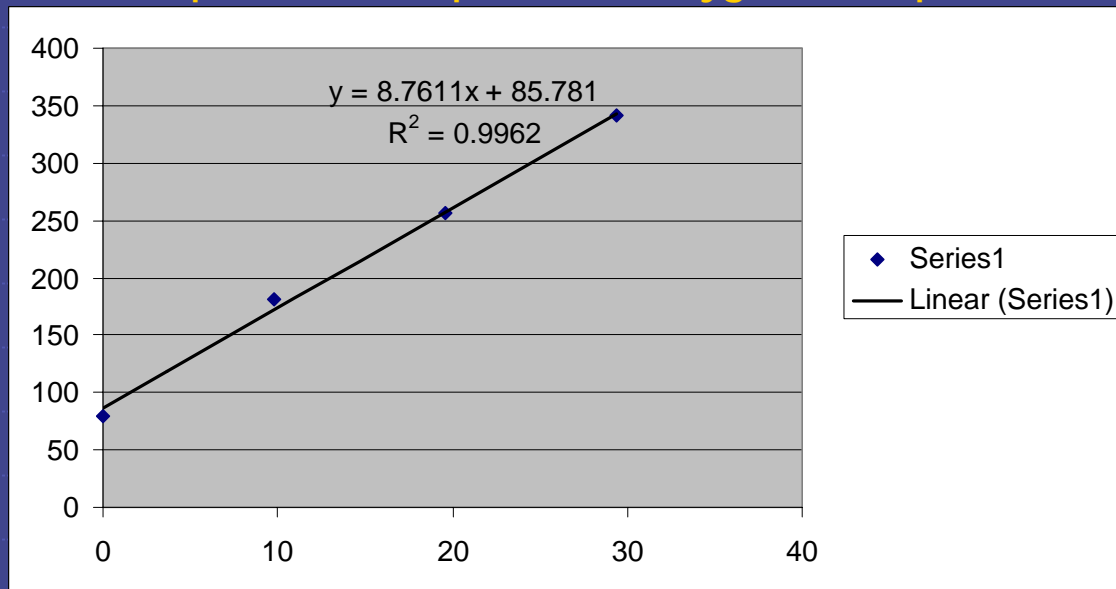
concentration of β -sitosterol ($\mu\text{g/ml}$)

Value of β -sitosterol from standard addition curve: 12.518 $\mu\text{g/ml}$
Concentration of β -sitosterol in sample 8: 37.554 $\mu\text{g/ml}$

Pygeum II: HPTLC Standard Addition of extract

Standard addition plot of 'least squares fit' for Pygeum sample XA33504CHR-11

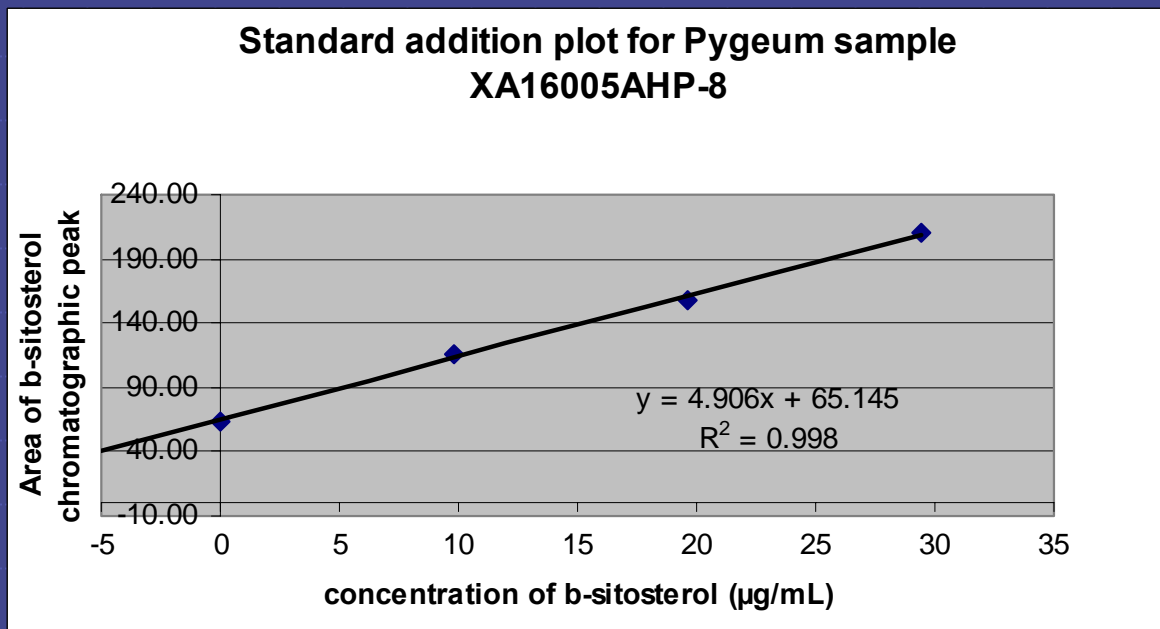
Area of β -sitosterol
chromatographic
peak



concentration of β -sitosterol ($\mu\text{g}/\text{mL}$)

Value of β -sitosterol from standard addition curve: 9.791 $\mu\text{g}/\text{mL}$
Concentration of β -sitosterol in sample 11 (dil 1:10): 29.373 $\mu\text{g}/\text{mL}$
Concentration of β -sitosterol in sample 11: 293.73 $\mu\text{g}/\text{mL}$

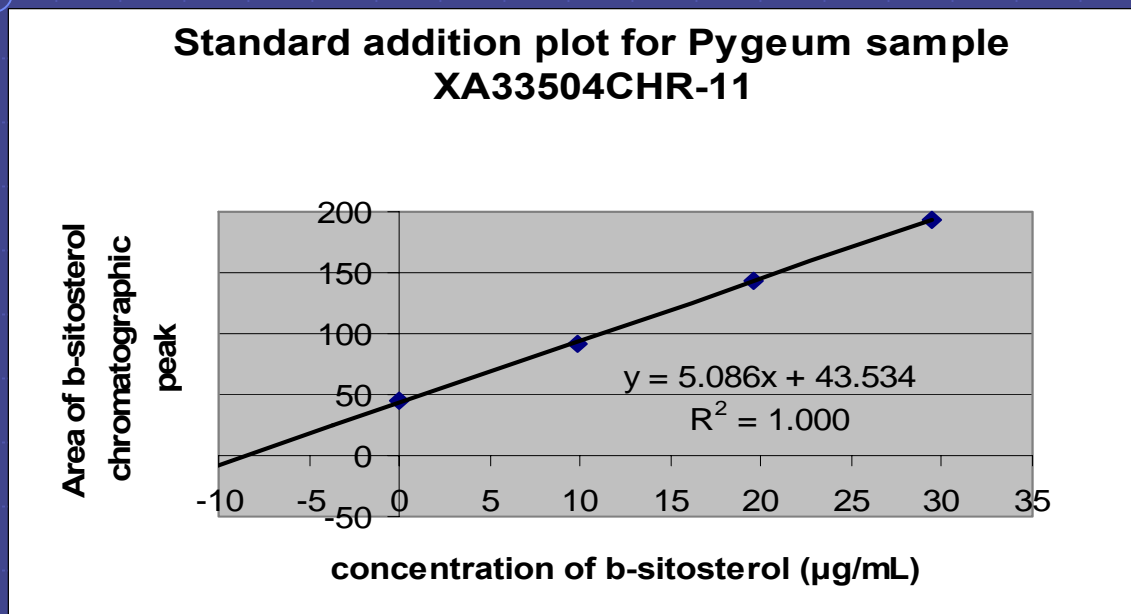
Pygeum II: HPLC Standard Addition of raw material



Value of β -sitosterol from standard addition curve: 12.639 $\mu\text{g/ml}$

Concentration of β -sitosterol in sample 8: 37.918 $\mu\text{g/ml}$

Pygeum II: HPLC Standard Addition of extract



Value of β -sitosterol from standard addition curve: 8.560 $\mu\text{g/ml}$

Concentration of β -sitosterol in sample 11 (dil 1:10): 25.679 $\mu\text{g/ml}$

Concentration of β -sitosterol in sample 11: 256.79 $\mu\text{g/ml}$

Pygeum II: HPTLC % Recovery of raw material

Sample	Sample Total µg/ml β-sitosterol	Spike µg/ml β-sitosterol	Total µg/ml β-sitosterol, Actual	% Recovery
8	35.0934	0	35.0934	na
8m	35.0934	29.4	35.4492	101.01
8n	35.0934	58.8	35.5173	92.66
8o	35.0934	88.2	28.5561	81.37
		Average	32.1742	91.68
		SD	3.1793	
		%RSD	10.75	

Pygeum II: HPTLC % Recovery of extract

Sample	Sample Total µg/ml β-sitosterol	Spike µg/ml β-sitosterol	Total µg/ml β-sitosterol, Actual	% Recovery
11	276.276	0	276.276	na
11p	276.276	29.4	265.182	95.98
11q	276.276	58.8	220.356	79.76
11r	276.276	88.2	201.246	72.84
		Average	228.928	82.86
		SD	35.756	
		%RSD	14.34	

Pygeum II: HPLC % Recovery of raw material

Sample	Sample Total µg/ml β-sitosterol	Spike µg/ml β-sitosterol	Total µg/ml β-sitosterol, Actual	% Recovery
8	35.185	0	35.185	na
8m	35.185	29.4	39.26	111.57
8n	35.185	58.8	33.39	94.90
8o	35.185	88.2	31.49	89.48
		Average	34.71	98.65
		SD	4.05	
		%RSD	11.67	

Pygeum II: HPLC % Recovery of extract

Sample	Sample Total µg/ml β-sitosterol	Spike µg/ml β-sitosterol	Total µg/ml β-sitosterol, Actual	% Recovery
11	294.053	0	294.053	na
11p	294.053	29.4	262.1	95.98
11q	294.053	58.8	249.8	79.76
11r	294.053	88.2	232.8	72.84
		Average	248.2	82.86
		SD	2.59	
		%RSD	10.42	

Pygeum II: Comparing Statistical Data

Repeatability & Accuracy for the crude raw material (XA16005AHP-8)

Sample 8	Calibration Plot Data	Standard Add'n Data
HPTLC	34.77 (n=7) (SD=8.69)	37.55 (n=9) (SD=2.24)
HPLC	35.18 (n=3) (SD=0.16)	37.92 (n=9) (SD=1.95)
$F_{\text{critical}(P=0.05)}$	39.33 ($F_{6,2}$)	4.102 ($F_{8,8}$)
F_{calc}	2,949.85	1.318

This data shows that there is a difference (Null Hypothesis rejected) between the variances of the 'Calibration Plot Data', reflecting the two methods differ in their precision but the 'Std. Add'n Data' reveals no statistical difference (Null Hypothesis retained) between the two variances.

Pygeum II: Comparing Statistical Data

Repeatability & Accuracy for the extract (XA33504CHR-11)

Sample 11	Calibration Plot Data	Standard Add'n Data
HPTLC	318.30 (n=4) (SD=35.67)	293.73 (n=9) (SD=2.59)
HPLC	294.05 (n=4) (SD=0.07)	256.79 (n=9) (SD=6.32)
$F_{\text{critical}(P=0.05)}$	15.44 ($F_{3,3}$)	4.102 ($F_{8,8}$)
F_{calc}	259,721.3	5.971

The data for the powdered extract reflects that there is a statistical difference between both 'Calibration Plot Data' & 'Standard Add'n Data' in the case of the extract, indicating potential matrix interference due to greater amounts of matrix constituents in the PE.

Conclusions

1. While HPTLC is more environmentally friendly, has higher throughput & is cheaper to run, it still is not a widely accepted quantitative method, due to comparable costs of instrumentation & difficulty in getting the system to work, with a lot more potential for variability or 'systematic' error.
2. Although HPTLC has poorer capacity for separation it may not be an issue in the botanical field. It may actually be a better 'fit for purpose'.
3. One potential solution, in my humble opinion, would be to work on a system of determining the 'measurement uncertainty' of our measurements, with any quantitative analysis of botanicals, in particular. This would at least give the end-user a level of confidence in the measurements that should match the needs of an industry that seems determined to get quantitative results, at any cost.

Conclusions & Discussion

- ◆ "Statistics is never having to say you're certain"
 - American Statistical Association bumper sticker
- ◆ More than 30 years ago John K. Taylor wrote:
 - "The objective of quality assurance programs for analytical measurements is to reduce measurement errors to tolerable limits and to provide a means of ensuring that the measurements generated have a high probability of acceptable quality."
 - "Quality is a subjective term. What is high quality in one situation may be a low or unacceptable quality in another case. Clearly the tolerable limits of error must be established for each case. Along with this there must be a clear understanding of the measurement process and its capability to provide the results desired."
- ◆ Therefore it is necessary to determine the quality of results with the 'end-user' & decide on – 'the fitness for purpose' – of the measurements!

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"It's tough to predict, especially the future."

Casey Stengel

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