7-2013

Analysis of Synephrine Content in Bitter Orange (Citrus aurantium L.) Dietary Supplements

Martha L. Newell

ANALYSIS OF SYNEPHRINE CONTENT IN BITTER ORANGE (CITRUS AURANTIUM L.) DIETARY SUPPLEMENTS

Martha L. Newell

Analysis of synephrine content in bitter orange (*Citrus aurantium* L.) dietary supplements

Martha L. Newell

A Thesis Submitted in Partial Fulfillment of Requirements of the CSU Honors Program for Honors in the Degree of

Bachelors of Science in

Biology

College of Letters and Sciences

Columbus State University

Thesis Advisor Jennifer Newbrey Date 7/8/13

Second Reader Julie Ballenger Date 8 July 2013

Director, Honors Program Cindy Tucker Date 7/8/13

Acknowledgements

I would first like to thank my thesis advisor and mentor, Dr. Jennifer Newbrey, for her tireless efforts in trying to overcome the difficulties we encountered during this project. Dr. Julie Ballenger served as my second reader, and gave excellent feedback and advice as the research progressed. I also thank Dr. Floyd Jackson for allowing me access to the HPLC, and Michael Anderson for his time training me and patiently answering my many, many questions. The faculty in the Biology department has been an amazing resource, and I believe every professor at one point or another had a hand in helping to get this research completed. Finally, I thank Dr. Cindy Ticknor and the Honors program for keeping me from going insane over the last four years, and for the chocolate.

This research was funded by the following organizations: The Beta Beta Beta Biological Honors Society Research Foundation, the Dr. Flora Clark Research Foundation, and the CSU Student Research and Creative Endeavors Grant.

Abstract

Many Americans are either currently taking, or have recently taken, non-prescription dietary supplements. These supplements claim to help prevent or relieve any number of ailments, but are not regulated by any governing body. One common use of dietary supplements is to aid in weight loss. Synephrine, the most abundant active component in bitter orange (*Citrus aurantium* L.) extracts, became a regular ingredient in weight loss supplements after the 2004 ephedra ban. The initial goal of this research was to use reverse phase high performance liquid chromatography (HPLC) to determine the synephrine content of five dietary supplements and compare to the reported content on the label. However, due to difficulties I encountered with the HPLC instrument, I instead used NanoDrop Spectrophotometry to analyze synephrine content. This technique was not as precise as HPLC, but data were obtained that indicated the supplements contained significantly less synephrine than the standard (1-Way ANOVA, $F_{4,21}$=60.042, P=<0.001). Additionally, my analysis indicated that the supplements had a lower concentration of synephrine than what was reported on their labels.

Introduction

The use of non-prescription dietary supplements is common among American consumers (Blank et al. 2007). The Food and Drug Administration (FDA) has rules and guidelines for accurate labeling of foods in relation to claims regarding health, nutrient content, and structure/function (Food and Drug Administration 2003). However, dietary supplement labels include a statement saying, "These claims have not been evaluated by the FDA. This product is not intended to diagnose, treat, cure, or prevent any disease." While the FDA has general warnings about dietary supplement use, the agency is not responsible for the regulation of dietary supplements due to the Dietary Supplement Health and Education Act of 1994 (US Public Law 103-417), in which supplements were defined as dietary ingredients, not as foods. The United States Pharmacopeial (USP) Convention has a dietary supplement verification program which offers voluntary testing to verify ingredients, to confirm a lack of contaminants, and to audit manufacturing practices (United States Pharmacopeial Convention 2012). However, few companies have taken advantage of this program and most products bearing the USP seal of verification are vitamins and minerals. Because they are not subject to mandatory governmental oversight, consumers are required to trust that the manufacturer's label contains an accurate description of the product, its uses and how it affects the human body.

While dietary supplements can be taken for a variety of purposes, one of the more common uses is to aid in weight loss. Approximately 30% of the United States is currently obese and three out of five adults are overweight, as determined by body mass index (Haaz et al. 2006). Weight loss supplements are a popular alternative to diet and exercise, bariatric surgery or other more invasive weight-loss techniques (Haaz et al. 2006). Ephedra-based supplements were widely used for weight loss and increased exercise performance until 2004 when ephedra was banned by

the FDA due to adverse cardiovascular events associated with its use (Gange et al. 2006). Following this ban, manufacturers started marketing "ephedra-free" supplements that could give consumers the same weight loss and performance enhancement as ephedra without the adverse side effects (Haller et al. 2008). Bitter orange (*Citrus aurantium* L.) is one of the "ephedra-free" supplements that gained popularity shortly after the ban. Bitter orange is native to eastern African and tropical Asia, and has been introduced to Florida, California, and the Mediterranean region. It has been used in traditional Chinese medicine for gastrointestinal problems and, in addition to weight loss, is currently used to treat heartburn, congestion, and paradoxically, to increase appetite (Fugh-Berman and Myers 2004).

Synephrine is the most abundant active compound in bitter orange, and is frequently used in "performance stacks" that pair synephrine with other stimulants and non-steroidal anti-inflammatory drugs (Thomas et al. 2009). Manufacturers claim the compound promotes weight loss by reducing appetite, burning fat, increasing metabolism and energy, and/or having thermogenic properties. Synephrine is an α- and β-adrenergic antagonist and is structurally similar to ephedrine; however the cardiovascular effects have not been studied in great detail (Thomas et al. 2009). Synephrine, like ephedra, is a sympathomimetic, and its use has been linked to cases of variant angina (Gange et al. 2006), myocardial infarction, hypertension, (Thomas et al. 2009), and stroke (Bouchard et al. 2005) in otherwise active and healthy persons. The normal recommended dose is between 10 mg three times daily and 20 mg once daily, and it is commonly sold as a nearly pure powder or in capsules. Some capsules contain synephrine stacks for weight loss and others only contain bitter orange extract with a stated percentage of synephrine.

The purpose of this research is to determine the synephrine content of five dietary supplements reported to contain synephrine using high performance liquid chromatography (HPLC). The quantified amount of synephrine will be compared to the reported values on supplement labels.

Materials and Methods

HPLC

Materials

Five synephrine supplements were included in this study: Synephrine HCl 99% Bulk Powder (Build Your Own Supplements, York, PA), SyneBURN 100% Synephrine HCl (PrimaFORCE, Burlington, NC, Bitter Orange Herb 120 mg Capsule (unknown synephrine, Solaray, Park City, UT), Bitter Orange Standardized Extract (6% synephrine, Nature's Way, Lehi, UT). A pure synephrine standard (≥98% synephrine) was obtained from Sigma-Aldrich (St. Louis, MO). In addition, 4-(dimethylamino)pyridine, also from Sigma-Aldrich was to be used as an internal standard to improve quantification precision.

Sample Preparation and Synephrine Extraction

All extractions and preparations were conducted in glassware to avoid contaminating the organic solvents with plastic. Additionally, a bottle of working methanol was used for dilutions and extractions to avoid contaminating the methanol stock.

To prepare my standard, I measured 1mg of the ≥98% synephrine and dissolved it in 1mL HPLC-grade methanol. This was then used as the stock solution for a serial dilution, wherein the most dilute sample was 0.5μg/mL. The same serial dilution plan was followed for 4-(dimethylamino)pyridine, our internal standard.

Supplement capsules were opened, the mass was determined, and contents were combined to reach a total of 1g of material for each supplement. Tablets were prepared with a mortar and pestle and also combined to reach 1g of material. Synephrine was extracted following methods outlined in Arbo et al. (2008). In short, 4 mL of methanol was added and then samples were left at room temperature for 20 min. Afterwards, samples were centrifuged at 3,000 rpm for 15 min and then filtered through 2 Å syringe filters (National Scientific Company, Rockwood, TN). The extraction procedure was repeated for each sample and the first and second extracts were combined. A total of ten extracts were prepared for each supplement.

Chromatography

My plan was to identify the synephrine peak by comparing its retention time on the HPLC column with that obtained from the pure standard. I also planned to calculate the concentration of synephrine in samples by creating a calibration curve using known concentrations of the synephrine standard.

The details of HPLC method used were as follows: 20µL of each sample was injected into an Agilent 1100 HPLC system fitted with a C-18 column (150 x 4.6 mm i.d., 5 µm, Agilent Technologies, Santa Clara, CA) and a C-18 guard column (Phenomenex, Torrance, CA, method summarized in Table 1). The mobile phase A consisted of acetonitrile-water-trifluroacetic acid (5:95:0.01, v/v/v). Mobile phase B was pure acetonitrile. Gradient elution followed that outlined in Arbo et al. (2008): 0-8 min with 100 – 59% A, 8-10 min with 59 – 0% A, 10-12 min 0% A, 12-13 min 0-100% A, 13-18 min with 100% A, all at a constant flow rate of 0.6 mL/min. Synephrine was detected by UV absorption at 220 nm. The synephrine peak in the supplements was to be identified by comparing peak retention times with that obtained from the pure

standard. To calculate the concentration of synephrine in samples, I intended to create a calibration curve using known concentrations of the synephrine standard.

NanoDrop Spectrophotometry

Materials

All supplements and standards for this analysis were the same as have already been described above. The internal standard, 4-(dimethylamino)pyridine, was not required for this analysis.

Sample Preparation and Synephrine Extraction

The synephrine standard was prepared in a similar manner as described above, in that a 1mg sample was dissolved in 1mL HPLC-grade methanol. The dissolved sample was vortexed for 1 min, and 100µL was moved to a separate vial and left to dry overnight.

Supplement samples were measured out to 1g, and extracted with 500µL methanol by using the extraction protocol described above. A second extraction was completed for each supplement, and the extracts were combined for a total of 1mL methanol per sample. The total volume was vortexed for 1 min, and 100µL was moved to a separate vial and left to dry overnight.

After drying, all samples were dissolved in 100µL of water and vortexed for 3 min. The final dilution for each supplement and the standard varied slightly due to differences in absorptivity (Table 2). Samples were vortexed after each subsequent dilution.

Statistical Analysis

Upon completion of the spectrophotometry analysis, collected data were analyzed in SPSS using a One-Way ANOVA. Additionally, the coefficient of variation was calculated in Excel to determine the amount of variation that occurred across subsamples of each supplement.

Results

HPLC

Data were unable to be obtained using HPLC. Due to problems with the available HPLC, both synephrine and the internal standard had nearly identical chromatograms (Fig 1). Based on a paper by Takei et al. (1999), synephrine should have come off the column at approximately 9 min, and pyridine should have come off at least 1 min before. To correct for any introduced error or contamination, the samples were remixed with new solvent and only glassware was used for transfer and storage of solvents and dilutions. Additionally, a new, factory-clean column was put on the instrument and the guard column was changed. Before injecting synephrine, the column was rinsed with 100% methanol (Fig. 2a). A trial run with 4μg synephrine in methanol was then conducted (Fig. 2b). Due to the difficulties I encountered, it was decided to use NanoDrop Spectrophotometry to complete the analysis in place of HPLC.

NanoDrop Spectrophotometry

The NanoDrop spectrophotometer measures absorbance along a continuous wavelength gradient from 220-750nm (Fig. 3). An ANOVA was conducted to determine if there were significant differences between supplements and the standard. Post-hoc tests revealed significant differences across the supplements (1-Way ANOVA, $F_{4,21}$=60.042, P=<0.001, Figure 4). The

coefficient of variation was high for all samples, including the standard (Table 3). However, the coefficient of variation within the subsamples was much lower than the overall sample variation (Table 4). A composite graph for 10 samples is shown in Fig. 5.

Discussion

The initial objective of this research was to use HPLC to quantify the synephrine concentration in a variety of commercial supplements and to compare the concentrations that I obtained with those reported by the manufacturers on the supplement labels. However, during HPLC method development, I encountered several problems. Without running a sample through the column, the instrument consistently showed a peak at 2.5-3 min. It was decided to move ahead with obtaining calibration curves for the standards, as the unidentified peak did not overlap with the time that synephrine and pyridine were expected to show on the chromatogram. The chromatograms for these trials are shown in Figures 1 and 2. After changing the column, guard column and attempting to correct for potential introduced error, the chromatograms shown in Figures 3 and 4 were obtained from an injection of pure methanol and a synephrine trial, respectively.

The problems encountered with the HPLC may be attributed to a number of factors. The major contributors seem to be the unknown instrument history and unknown column history. The instrument is housed in a different department, and has been used primarily for basic classroom work for a number of years. It had recently been certified clean by the manufacturer, but there may have been some residual compound in the injection loop or elsewhere within the instrument that reacted with the solvents used in this method. Similarly, the first column used was donated by an outside pharmaceutical lab. It is not known what the column was used for in the past or

what solvents had been run through it. However, if the history of the column results in the problems I encountered, then they should have been resolved when I switched to using a brand new column, which was not the case. Due to time constraints, it was decided to instead quantify the synephrine content using a NanoDrop Spectrophotometer. With this different method there was no need for an internal standard, so the data reported only include the four supplements and synephrine standard. As noted, a very high coefficient of variation was calculated for all samples using this method, including the standard. With the standard, this may be explained by the tiny amount used for the analysis. A 1mg sample was measured out on a balance that read to 0.1mg. Because of the small amount needed and relatively imprecise balance used, any fluctuations in mass across the samples would have caused high variation in absorbance.

This cannot explain the high variation in the supplements, however, as those were measured to 1g on a balance that read to 0.001g. One potential source of added variation is contaminants in the capsules and tablets. If there were other fillers or compounds in the supplements that dissolved in methanol, those would have been extracted with the synephrine and may have interfered with synephrine absorption. If more extraneous compounds were extracted in some sub-samples than others, those would have higher absorption and could contribute to the high variation. It is possible there was variable synephrine content across the capsules which contributed to the calculated variation, although I would not have expected the variation to be as high as was present. Variation within each sub-sample was low in most cases; it only became high when all sub-samples were averaged together. Another source of variation may be from how well water redissolved the synephrine following extraction. If synephrine was unable to fully dissolve in some of the sub-samples, it would have decreased overall absorbance and led to an increase in variation.

Variation may also have been introduced when taking absorption measurements. The method developed for HPLC analysis had synephrine detection set at 220nm. The spectrophotometer used for this research was able to detect at that wavelength, but it was at the low extreme for the instrument. Absorption was instead detected at 230nm and 240nm, with the decision that the measurements at 230nm would be used as the synephrine absorbance. In my absorbance graphs it was apparent that the maximum absorbance for my samples occurred at 220 – 225nm, with the line for 230nm falling on the down slope of the absorbance curve (Fig. 3). Variance may have been introduced if the slope of the curve changed slightly across samples or sub-samples (Fig. 5). This figure shows the composite graphs for Nature's Way, though some other samples are included in the composite and can be ignored for the purposes of this discussion. Many of the graphs are very similar, as I would expect if they are from the same supplement. However, small changes to the height of the peak would alter the rate of change on the slope, thereby affecting the measured absorbance at that point.

I had planned to quantify the synephrine content in the tested supplements. The spectrophotometer is not as precise an instrument as HPLC, but absorption for supplements as compared to the standard could be used as a rough quantification. However, the variation was so high that I determined quantification would not produce meaningful data.

Tables and Figures

Table 1: Chromatography method used for synephrine detection in both a known standard and in commercial supplements.

Instrument	Agilent 1100 HPLC		
Column	C-18 column (150 x 4.6 mm i.d., 5 µm, Agilent Technologies, Santa Clara, CA) and a C-18 guard column (Phenomenex, Torrance, CA).		
Mobile Phase A	Acetonitrile:water:trifluroacetic acid (5:95:0.01, v/v/v)		
Mobile Phase B	HPLC Grade Acetonitrile		
Gradient Arbo et al. (2008)	Minutes	%A	%B
	0	100	0
	8	59	41
	10	0	100
	12	0	100
	13	100	0
	18	100	0
Injection Volume	20uL		
Flow Rate	0.6mL/min		
Detection	220nm UV		

Table 2: For spectrophotometry, a stock solution was mixed with a known synephrine concentration. These solutions were diluted with water until I found a concentration that was able to be measured by the instrument. The dilutions in the third column are the micrograms/milliliter, and the final column shows the total concentration for the 3µg used in analysis.

Sample ID	**Stock**	**Dilution for NanoDrop**	**Amount in final sample**
Standard	1µg/µL	0.25µg/µL	0.75µg
Solaray	100µg/µL	6.7µg/µL	20µg
Nature's Way	100µg/µL	10µg/µL	30µg
SyneBURN	100µg/µL	20µg/µL	60µg
Bulk Powder	100µg/µL	1µg/µL	3µg

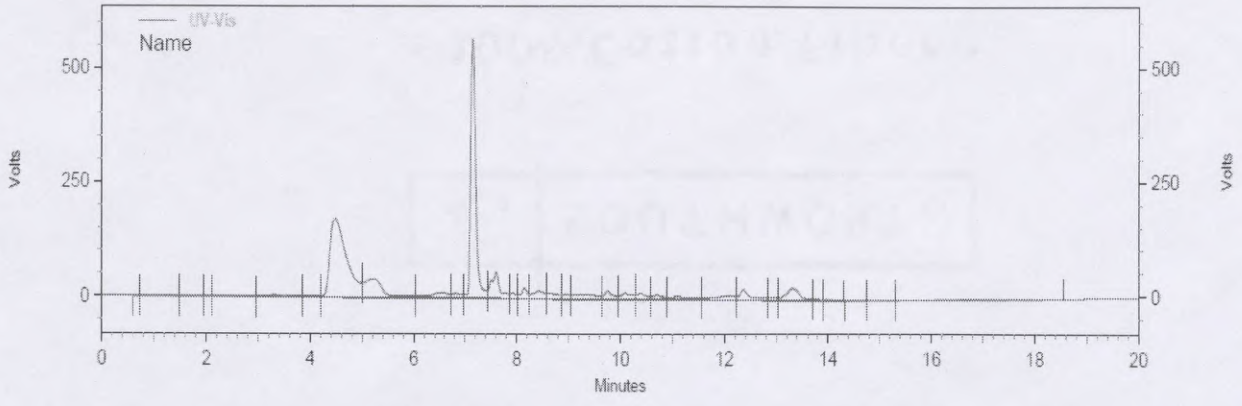
Table 3: Overall mean absorbance per gram, standard deviation, and coefficient of variation for each supplement.

Supplement	Mean (abs/g)	Standard Deviation	Coefficient of Variation
Standard	0.8343	0.1838	22%
Solaray	0.0713	0.0171	24%
Nature's Way	0.0362	0.0151	41%
SyneBURN	0.0202	0.0035	17%
Bulk Powder	0.1811	0.0566	31%

Table 4: Mean absorbance per gram, standard deviation, and coefficient of variation for each sub-sample. Solaray, Nature's Way and SyneBURN had 6 sub-samples analyzed each, while the standard and Bulk Powder each had 4 sub-samples.

Sub-Sample	Mean (abs/g)	Standard Deviation	Coefficient of Variation
Standard			
A	1.033	0.0038	0.4%
B	0.9683	0.0208	2.1%
C	0.5607	0.004	0.7%
D	0.7763	0.0101	1.3%
Solaray			
A	0.0564	0.0012	2.1%
B	0.065	0.004	6.2%
C	0.046	0.0009	1.9%
D	0.0857	0.0064	7.5%
E	0.0799	0.0057	7.1%
F	0.0813	0.0026	3.2%
Nature's Way			
A	0.04	0.0024	6.0%
B	0.0277	0.0009	3.2%
C	0.0196	0.0005	2.6%
D	0.0193	0.0004	2.1%
E	0.0554	0.0026	4.7%
F	0.0515	0.0006	1.2%
SyneBURN			
A	0.0279	0.0003	1.1%
B	0.0189	0.0012	6.3%
C	0.1932	0.0003	0.2%
D	0.0182	0.0013	7.1%
E	0.0187	0.0009	4.8%
F	0.0181	0.0006	3.3%
Bulk Powder			
A	0.1698	0.0012	0.7%
B	0.155	0.0032	2.1%
C	0.1246	0.0019	1.5%
D	0.2749	0.0049	1.8%

1a



1b

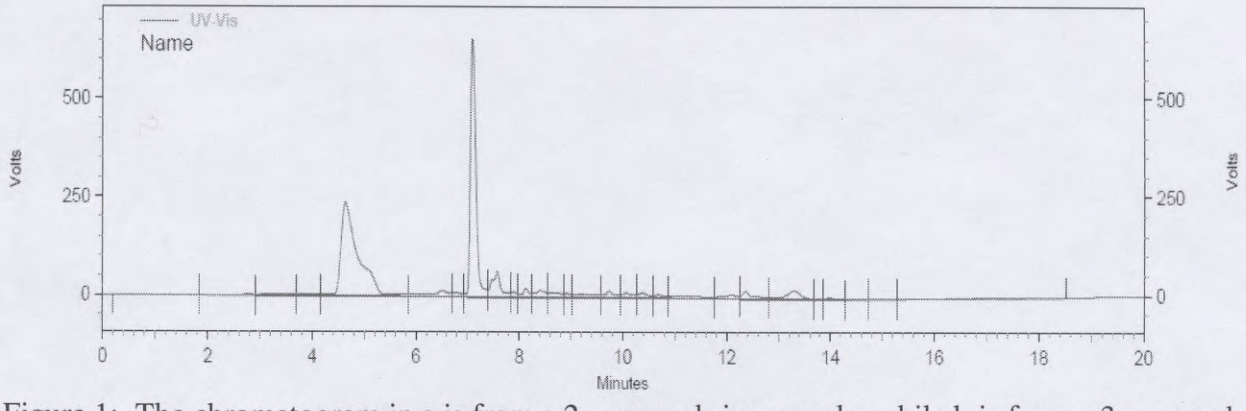
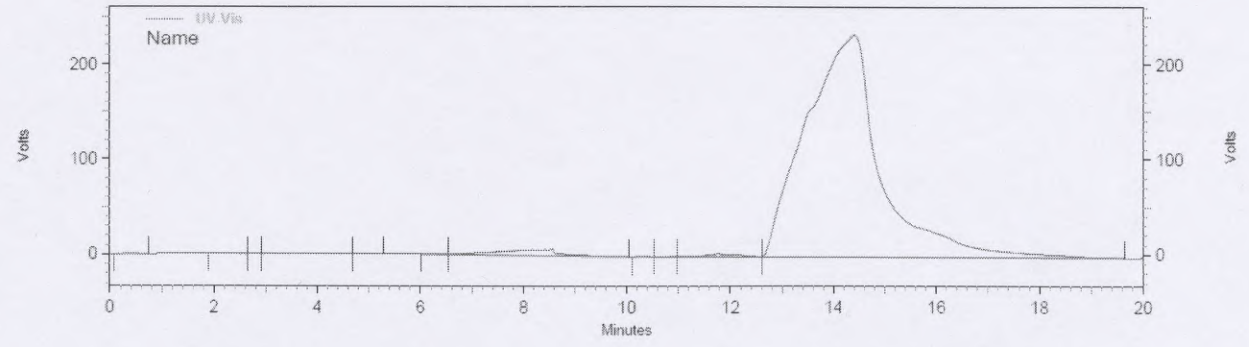


Figure 1: The chromatogram in a is from a 2µg synephrine sample, while b is from a 3µg sample of 4-(dimethylamino)pyridine. The chromatograms are almost identical for both standards.

2a



2b

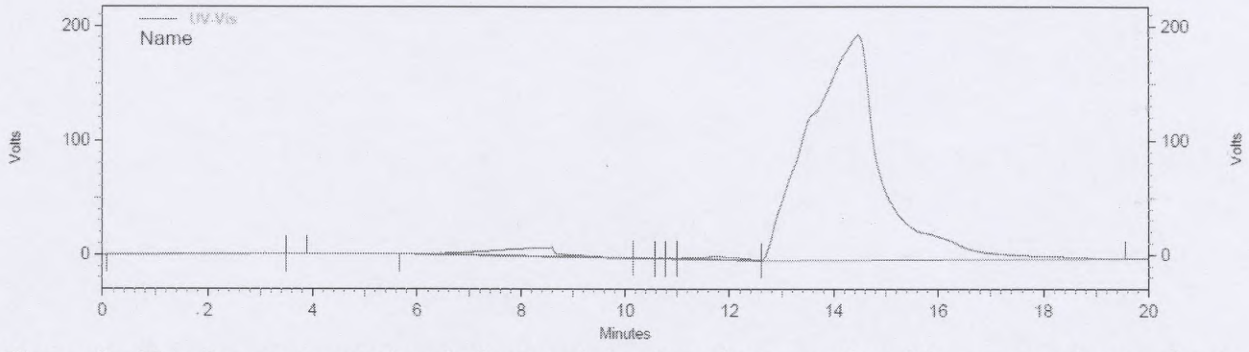


Figure 2: The chromatogram in a is from an injection of pure methanol in a new column. A run with 4µg synephrine was then conducted, resulting in the chromatogram in b. As before, the chromatograms from both trials were the same.

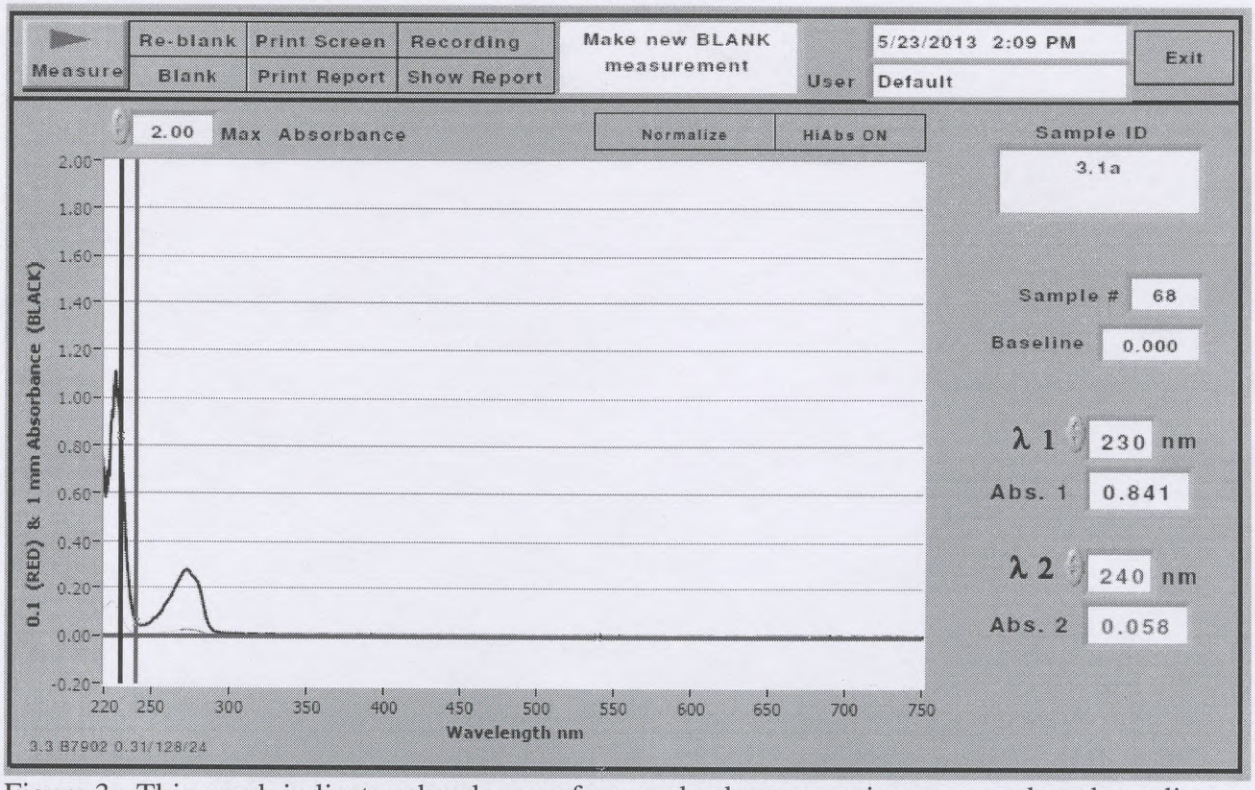


Figure 3: This graph indicates absorbance of a sample along a continuous wavelength gradient using the NanoDrop spectrophotometer. Absorbance was specifically recorded at 230nm and 240nm for all samples.

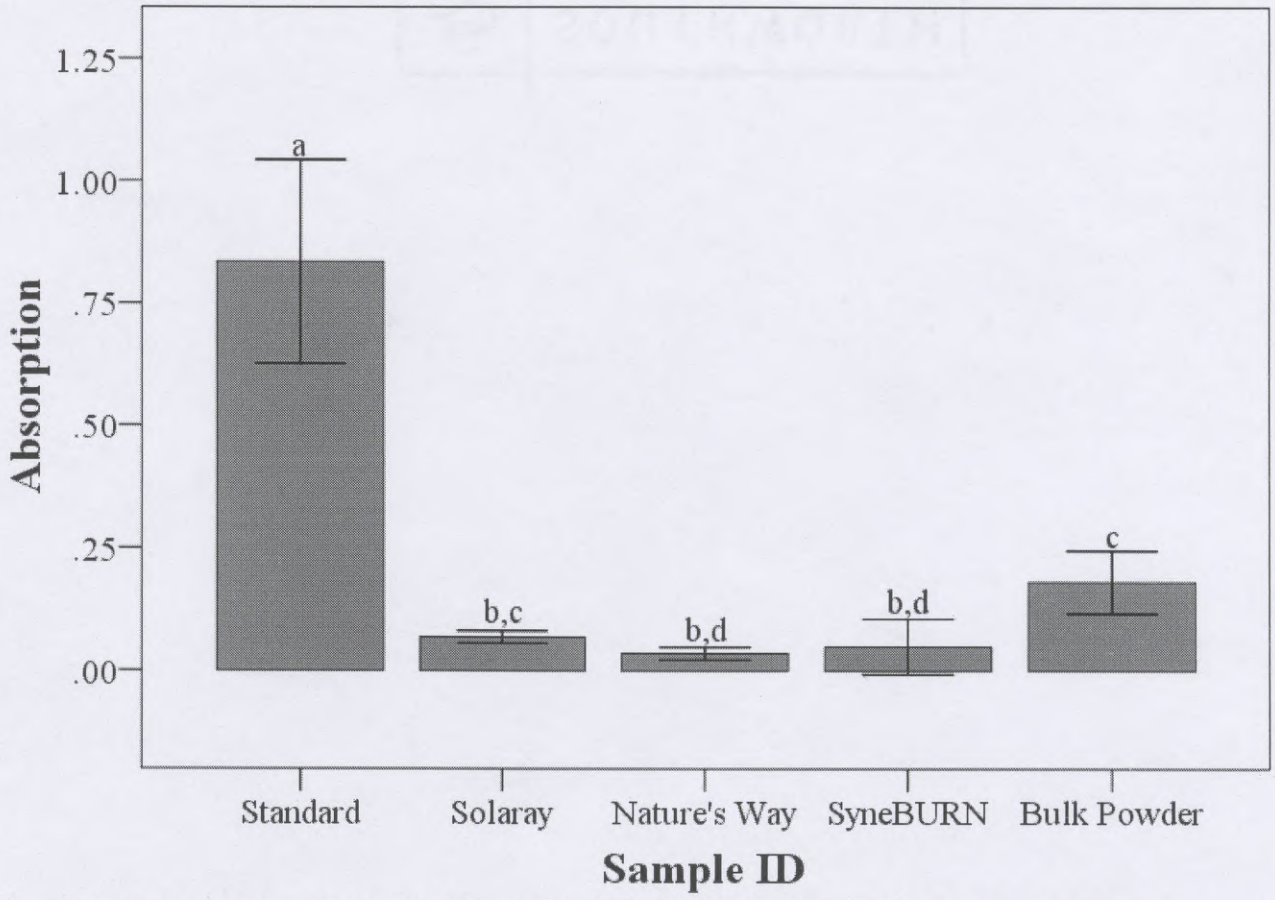


Figure 4: Spectrophotometer absorbance (+/- 1S.E.) across the supplements and standard. Letters above the bars denote significance, in that bars with a common letter are not significantly different from each other.

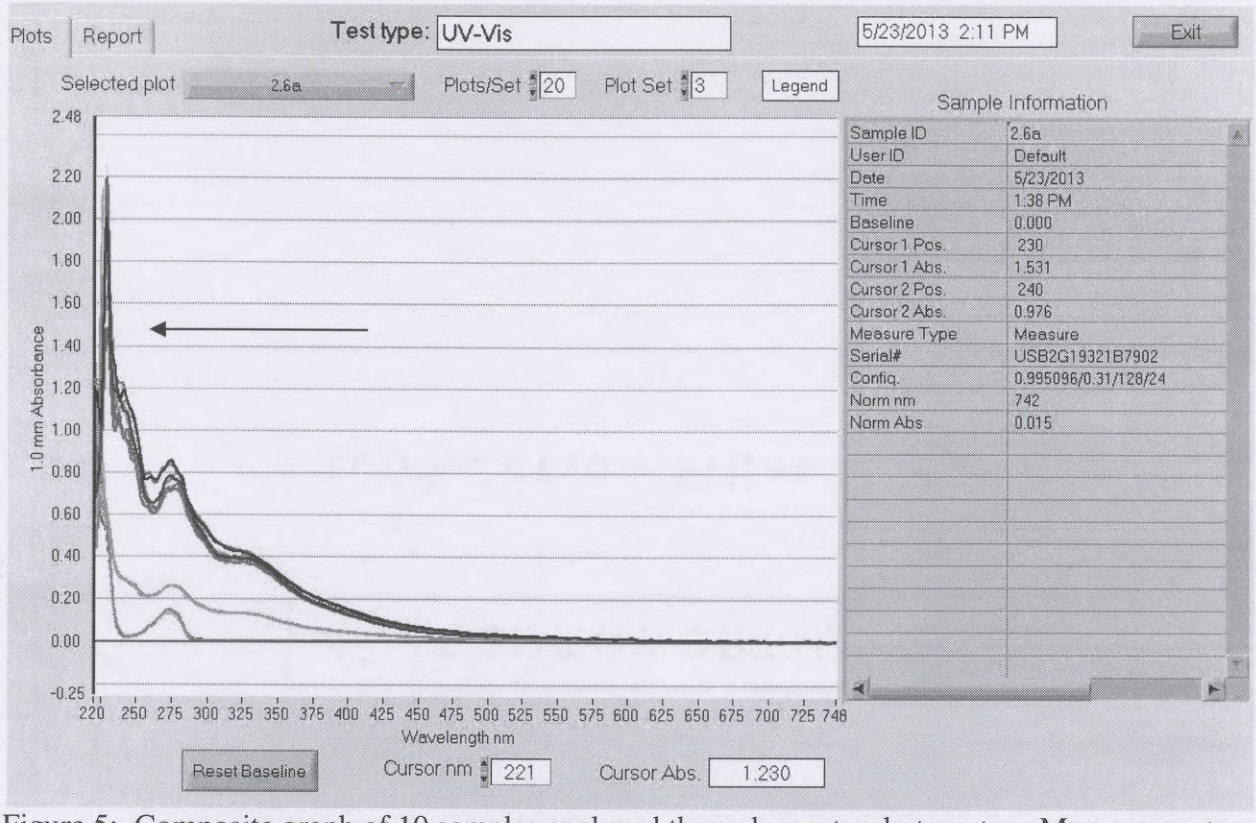


Figure 5: Composite graph of 10 samples analyzed through spectrophotometry. Measurements were taken at 230nm. The curves marked by the arrow are from the same supplement (Nature's Way).

Works Cited

Arbo, M.D., E.R. Larentis, V.M. Linck, A.L. Aboy, A.L. Pimentel, A.T. Henriques, E. Dallegrave, S.C. Garcia, M.B. Leal, R.P. Limbeger. 2008. Concentrations of *p*-synephrine in fruits and leaves of *Citrus* species (Rutaceae) and the acute toxicity testing of *Citrus aurantium* extract and *p*-synephrine. Food and Chemical Toxicology 46: 2770-2775

Blank, H.M., M. Serdula, C. Gillespie, D.A. Galuska, P.A. Sharpe, J.M. Conway, L.K. Khan, B.E. Ainsworth. 2007. Use of non-prescription dietary supplements for weight loss is common among Americans. Journal of the American Dietetic Association 3: 441-447

Bouchard, N.C., M. Howland, H. Greller, R. Hoffman, L. Nelson. 2005. Ischemic stroke associated with use of an ephedra-free dietary supplement containing synephrine. Mayo Clinic Proceedings 4: 541–545.

U.S. Food and Drug Administration. 2003. Claims that can be made for conventional foods and dietary supplements. Washington, DC, http://www.fda.gov/food/labelingnutrition/labelclaims/ucm111447.htm

Fugh-Berman, A., A. Myers. 2004. *Citrus aurantium,* an ingredient of dietary supplements marketed for weight loss: current status of clinical and basic research. Experimental Biology and Medicine 229: 698-704

Gange, C.A., C. Madias, E. Felix-Getzik, A.R. Weintraub, N.A. Mark Estes. 2006. Variant angina associated with bitter orange in a dietary supplement. Mayo Clinic Proceedings, 4: 545-548

Haaz, S., K. Fontaine, G. Cutter, N. Limdi, S. Perumean-Chaney, D. Allison. 2006. *Citrus aurantium* and synephrine alkaloids in the treatment of overweight and obesity: an update. Obesity Reviews 7: 79-88

Haller, C., A., M. Duan, P. Jacob III, N. Benowitz. 2008. Human pharmacology of a performance enhancing dietary supplement under resting and exercise conditions. British Journal of Clinical Pharmacology 6: 833-840

Takei, H., M. Hirabuki, F. Yoshizaki. 1999. Analysis of synephrine in the peel of citrus fruit, immature citrus fruit and decoctions of Chinese medicinal prescriptions containing these crude drugs by capillary electrophoresis. Analytical Sciences 15: 1017-1020

Thomas J.E., J. Munir, P. McIntyre, M. Ferguson. 2009. STEMI in a 24-Year-Old Man after Use of a Synephrine-Containing Die/tary Supplement: A Case Report and Review of the Literature. Texas Heart Institute Journal 6: 586–590

United States Pharmacopial Convention. 2012. USP verified dietary supplements. United States Public Law 103-417, 103rd Congress. 1994. Dietary Supplement and Health Education Act of 1994. http://ods.od.nih.gov/About/DSHEA_Wording.aspx