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Determination of Genistein and Protein Content in *Apios Carnea* and *Apios Fortunei* from China, and *Apios Americana*

Kenneth Brown[1], Jianhua Li[2], Katherine Wirth[1], Emily Wilkins[1], Jim Tufts[1] & Fernando Bahena[1]

[1] Department of Chemistry, Hope College, Holland, Michigan, USA

[2] Department of Biology, Hope College, Holland, Michigan, USA

Correspondence: Kenneth Brown, Hope College, Department of Chemistry, 35 East 12th Street, Holland, Michigan 49423, USA. Tel: 1-616-395-7173. E-mail: brownk@hope.edu

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Abstract

Several different groundnut *Apios* species obtained from various locations in China and North America were evaluated for genistein and total protein content. This preliminary research was conducted to determine the viability of these species as potential food sources. High performance liquid chromatography (HPLC) was the instrumentation used to determine the concentration genistein in *Apios (Fabaceae)* species including *A. carnea* and *A. fortunei* from China and *A. americana* from North America. The extracts of 80% ethanol were analyzed under HPLC isocratic conditions at a wavelength of 270 nm and the Lowry Protein Assay at 650 nm was utilized to determine the amount of protein in the samples. A linear calibration range of 0 ppm-340 ppm for genistein was obtained and the presence of genistein was also confirmed using LC-MS (M+, m/z 271). Genistein was present from 15-393 µg genistein/g plant, with the most genistein found within the groundnut tuber system. Of the samples analyzed, the *A. americana* plant contained the largest amount of genistein. The protein content ranged from 14-30 mg/g plant.

Keywords: genistein, groundnut tubers, apios, protein

1. Introduction

Isoflavones found in a number of different plant species have sparked wide-spread attentiveness and awareness in food chemistry and natural products chemistry. A primary food basis of isoflavnones comes from soybeans in the *Fabacceae* family. Research findings have reported that soybeans containing various isoflavones (diadzein, glycitein, and genistein) have potential health advantages such as inhibiting certain diseases, with antioxidant and antiosteoporosis properties [Saracino, Raggi, 2010; Wiseman et al., 2002]. Through phytochemical studies, genistein 5,7-Dihydroxy-3-(4-hydroxyphenyl) chromen-4-one (Figure 1), a polyphenol, has been identified in the tubers of *Apios americana* (groundnut) plant species [Nara, et al., 2011].

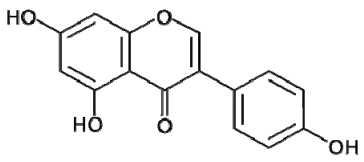

Figure 1. Chemical structure of 5,7-Dihydroxy-3-(4-hydroxyphenyl) chromen-4-one (genistein)

The same researchers also identified genistein-7-O-genitiobiside in *A. americana* Medik groundnut tubers, which was converted to genistein by ß-glucosidase [Nara et al., 2011]. Groundnut plants are vine-like or climbing recurrent plants ranging from 1-20 cm in diameter. In addition, groundnut tubers have been noted to have protein and carbohydrate content, and because of the presence of genistein, individuals consuming these groundnut

tubers may experience increased energy, reduction of postnatal illnesses, and lowered hypertension [Iwai, Matsue, 2007]. These biological properties make groundnut tubers an attractive alternative food source, especially for third-world developing countries. The projected global population will exceed 8 billion by the year 2030 and more than 9 billion by the year 2050 [International Programs (IP), http://www.census.gov/population/international/data/idb/worldpopgraph.php]. Most of this population growth will come from third-world developing countries in Latin American, Africa, and Asia [IP, http://www.census.gov/population/international/data/idb/worldpopgraph.php]. These groundnut tubers may play a role in meeting some of the food needs in these countries.

Multiple methods have been developed for the determination of genistein in different substances. Genistein in blood plasma of individuals was determined using HPLC with electrochemical detection [Saracino, Raggi, 2010]. Yang and researchers utilized HPLC equipped with UV-Vis detection to determine genistein in a JiangYaBiFeng tablet from China [Yang et al., 2011]. Another research group used capillary electrophoresis to determine several isoflavones including genistein in Japanese plants from Estonia [Vaher, Koel, 2003].

In this paper, we report on the isolation of genistein in several plant species, namely, *A. carnea*, *A. fortunei*, and *A. americana*. Sections of the roots, stems, leaves, tuber skin, and tuber-cortexes were examined for the presence of genistein and this information was correlated to the origin of the plant. The results presented herein are representative of samples from 1) Tianmu Mountain in Zhejlang, China, 2) Changle, a province of Guangxi, China, 3) Jordan Creek, Missouri, USA, and 4) Sugar Creek, Indiana, USA.

2. Method

2.1 Reagents and Extraction Method

Genistein was purchased from Sigma-Aldrich (St. Louis, MO). Standard concentrations of genistein were prepared in HPLC-grade methanol and ranged from 0-340 ppm. The groundnut samples, *Apios* species (*Apios carnea, Apois fortunei, and Apois americana*), were collected from various locations in China and in North America. Two different sampling sites were used for Changle, Guangxi, China, and Jordan Creek, Missouri, whereas one sampling site was used for Sugar Creek, Indiana. Prior to extracting genistein, the skin of the groundnut tubers was removed and the groundnut tubers were then sliced into 5 mm sections. The skin, cortex of the groundnut tubers, roots, stems, and leaves were freeze-dried separately at the same time for 48 hours. After freeze drying, the samples were pulverized into a powder using a mortar and pestle in the presence of liquid nitrogen. Following the procedure of Nara et al [2011], the samples (100 mg) were then processed using 80% ethanol in the amount of 10 mL in centrifuge tubes, and placed on a Daigger shaker for 60 minutes. The centrifuge tubes were vortexed for 3 minutes and placed in a centrifuge for 5 minutes at 2000 RPM. Finally, the extractants were filtered using a Sterlitech 0.45 micron filter (17 mm) for analysis.

2.2 HPLC Analysis with UV-Vis Detection and Protein Assay

The amount of genistein in the tubers was determined under isocratic HPLC conditions. The HPLC mobile phase consisted of 57% HPLC grade water with 2% acetic acid and 43% HPLC grade methanol and a wavelength of 270 nm (Perkin Elmer, UV Detector, Series 200) was used in all determinations of genistein in the samples. For the HPLC system, a dual-piston pump (Perkin Elmer, LC Series 200) delivered the mobile phase at 0.75 $mL \cdot min^{-1}$ and a pulse damper was used in-line to minimize background noise arising from pump pulsations. Twenty microliter injections were made using a Perkin Elmer Series 200 autosampler with a delivery precision <0.5% (RSD). Between all injections, the sampling needle was flushed with purified water and the mobile phase. The injection system utilized a Rheodyne 7725-stainless steel valve, and a Phenomenex, 5-micron, C-18 column (150 mm x 4.6 mm-I.D.) was used for all separations. The determination of protein in the samples was based on the Lowry Protein Assay [Lowry, Rosebrough, Farr, Randall, 1951], using Bovine Serum Albumin (BSA) as the standard to prepare the calibration curve. All reagents for the Lowry Protein Assay were purchased from Sigma-Aldrich. The BSA standards were prepared with 2% sodium carbonate in 0.1 M NaOH, 1% potassium tartrate, and 0.5% copper(II) sulphate pentahydrate in purified water. The standards and samples were allowed to incubate for 10 minutes at room temperature before diluting with the Folin-Phenol reagent. Afterwards, the samples and standards were allowed to react for 30 minutes at room temperature before measuring the Beer's Law absorbance at the wavelength of 650 nm.

3. Results and Discussion

A UV-Vis spectrum of a genistein standard solution (1.00×10^{-5} M) in the mobile phase was used to determine the appropriate wavelength of 270 nm for HPLC analysis of samples. A 100 ppm genistein standard in Figure 2 gave a retention time of c.a. 22.7 minutes and representative chromatogram of a real

sample taken from the groundnut tuber cortex is shown in Figure 3.

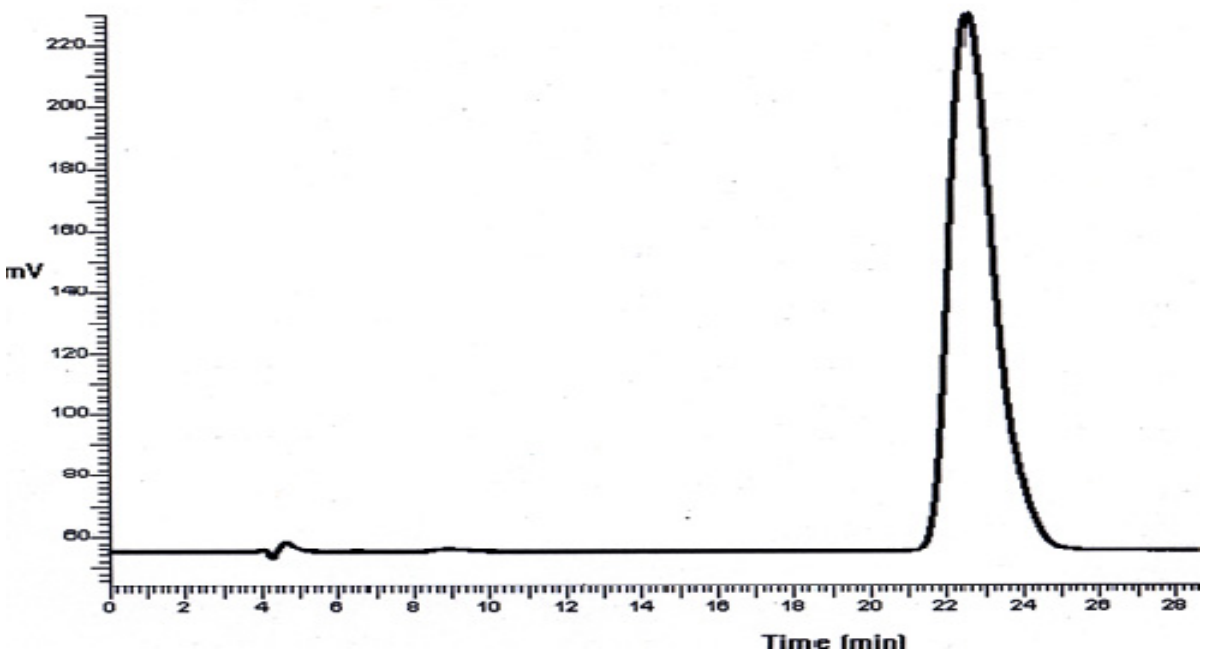

Figure 2. HPLC of genistein standard (100 ppm); retention time is 22.7 minutes

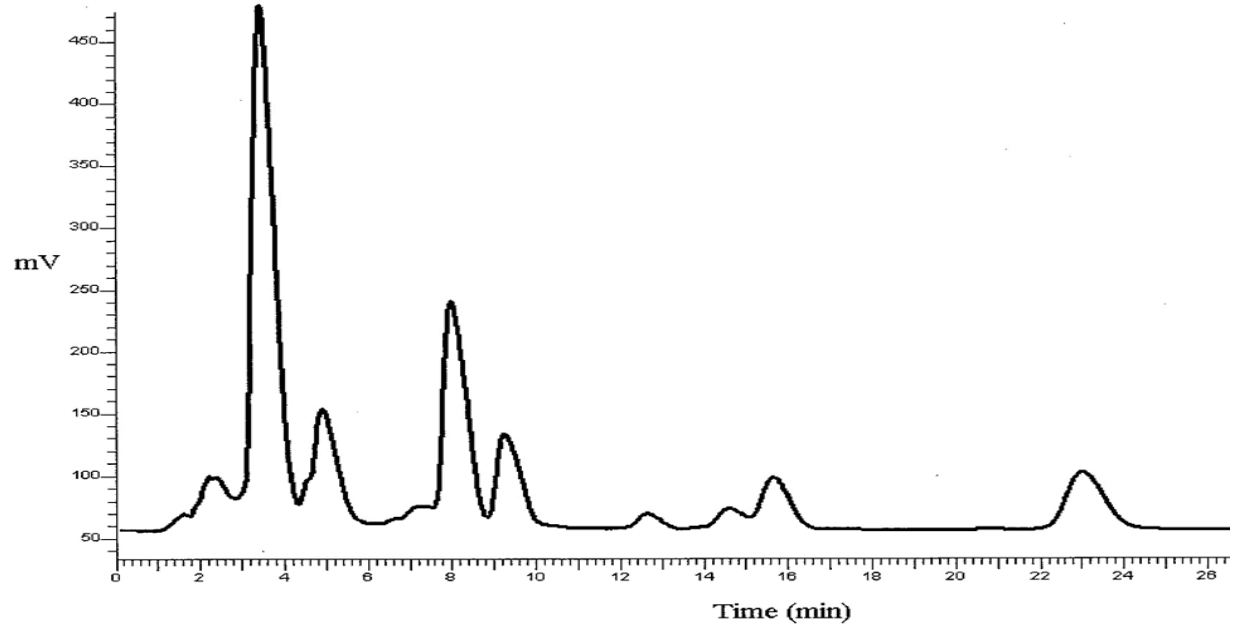

Figure 3. HPLC of a representative sample of a groundnut tuber cortex; retention time is 22.8 minutes

Genistein was detected with a retention time of c.a. 22.8 minutes within the samples. Concentrations of standard solutions from 0-340 ppm produced a linear calibration curve (Figure 4, correlation coefficient, R^2= 0.9998), which was used to determine genistein concentrations. In addition, LC/MS analysis of the 75 ppm standard and sample showed a m/z (M+) peak at 271 mass units, which confirmed the presence of genistein. The results shown in Table 1 provide a summary of the samples tested for the presence of genistein and protein content. Although genistein is present in the root system of the plant species, most of the genistein bioaccumulates within the tuber cortex. *Apios americana* contains the greatest amount of genistein, followed by the two Asian species, *A. fortunei*, and *A. carnea,* which contains the least amount of genistein. *Apios carnea* and *A. fortunei* have

amounts of genistein ranging from 15-101 µg genistein/g plant and 20-163 µg genistein/g plant, respectively. *Apios americana* contains 88-393 µg genistein/g plant and this is consistent with observations that *A. carnea* has an immature tuber. The *Apios* plants contain comparable amounts of genistein found in other food sources such as nuts, but Liggins and researchers reported no genistein present in 43 of 80 different foods assayed [Liggins, Mulligan, Runswich, Bingham, 2002]. Genistein has been previously reported in *A. Americana*, but it has not been reported in other *Apios* plants. In addition, few studies have been done to determine the distribution of genistein in the plants. Recent phylogenetic studies suggest that *A. carnea* detached first from the residual populations, indicating that the genistein increased during the evolutionary history of *Apios* [Li et al., 2014]. In all samples, regardless of species, the stems and leaves contains the lowest amount of genistein and in some cases, no genistein was detected. It was not possible to completely remove the skin from the cortex and therefore remnants of the cortex remained on the skin. The skin was subjected to the same extraction protocol used for the cortex and produced 0.1-0.7 µg genistein/g plant.

Table 1. Summary of genistein and total protein content

Species	Location	µg Genistein/g	mg protein/g*
A. Carnea	Changle, Guanxi, China	101±21	19.5±0.8
A. Carnea	Changle, Guanxi, China	15±5	14.4±0.6
A. fortunei	Zhejiang, China	20±3	17.6±0.8
A. fortunei	Zhejiang, China	163±26	20.3±0.7
A. americana	Jordan Creek, Missouri	321±51	30±1
A. americana	Jordan Creek, Missouri	88±14	15.4±0.6
A. americana	Sugar Creek, Indiana	393±62	20.6±0.9

Table 1 shows a summary of the genistein and protein content in the various *Apois* plants. Each reported value is based on n=4. *Values are reported as average±standard deviation.

In Figure 3, multiple peaks are present, and some of the peaks after 8 minutes are well-resolved. However, during the earlier stages of the separation between 2-6 minutes, the peaks are not clearly resolved. The use of a gradient method would help provide better resolution for these earlier peaks. For quantitative purposes, the peak due to genistein at approximately 23 minutes is clearly separated and resolved from other peaks. The resolution for the genistein peak and the adjacent peak at 15.8 minutes is much greater than 1.5, which is the criterion for using a chromatographic peak for quantitative purposes. Although the peak due to genistein is fairly symmetrical, there is a very small degree of peak tailing, which indicates that some sites on the column adsorb genistein slightly stronger than others. In general, this isocratic method seems suitable for the separation of genistein within these samples.

In the Lowry method, the nitrogens on the peptide linkages of the protein react with Cu^{2+} ions under basic pH conditions to form a complex. The Cu^{2+} ions are then reduced to Cu^{+} and reacted with Folin-Ciocalteay reagent to produce heteropolymolybdenum blue. The absorbance of the Beer's Law plot for the heteropolymolybdenum blue complex was monitored at 650 nm after full color development. The Beer's Law plot shown in Figure 5 for the Lowry protein method is linear over a range of 0-0.014 mg/mL with R^2=0.9916. The equation of the line used for the determination of the protein content is: 8.77x + 0.0028, and the method provides the total protein content within the *Apios* plants. All samples tested had protein levels between 14-21 mg protein/g sample. Potatoes have protein content ranging from 17-26 mg/g [Washington State Potato Commission, http://www.potatoes.com/nutrtion], which is comparable to the amount found in these groundnut tubers. Regardless of the species, the groundnut tubers contain similar quantities of protein that are present in potatoes, although the amount of genistein varies between the Asian and North American species.

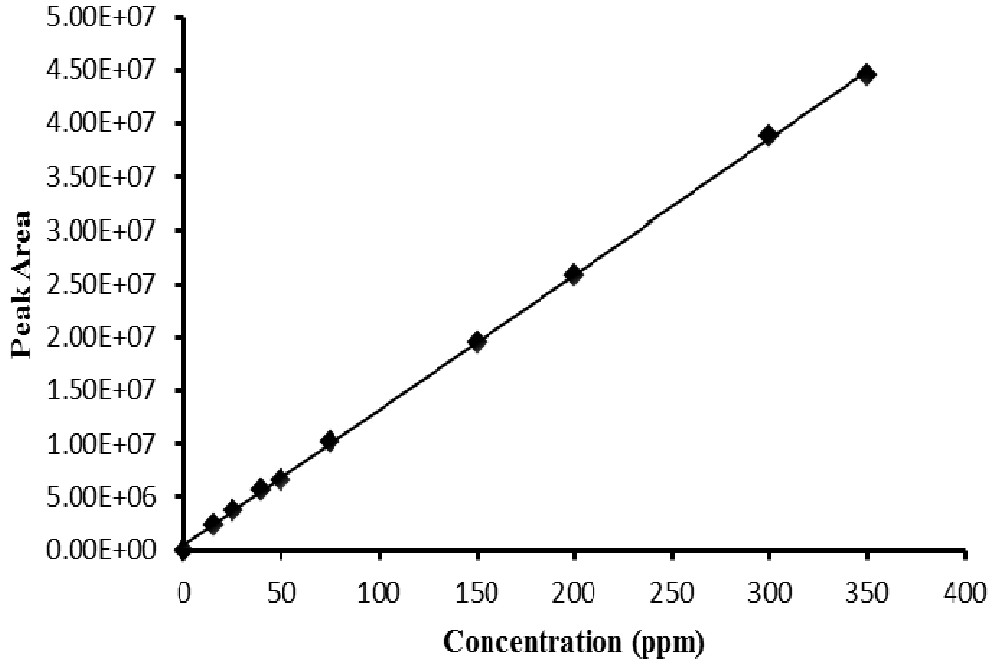

Figure 4. Calibration curve for genistein at 270 nm based on HPLC, UV-Vis detection. concentration of standards range from 0-340 ppm; $R^2 = 0.9998$

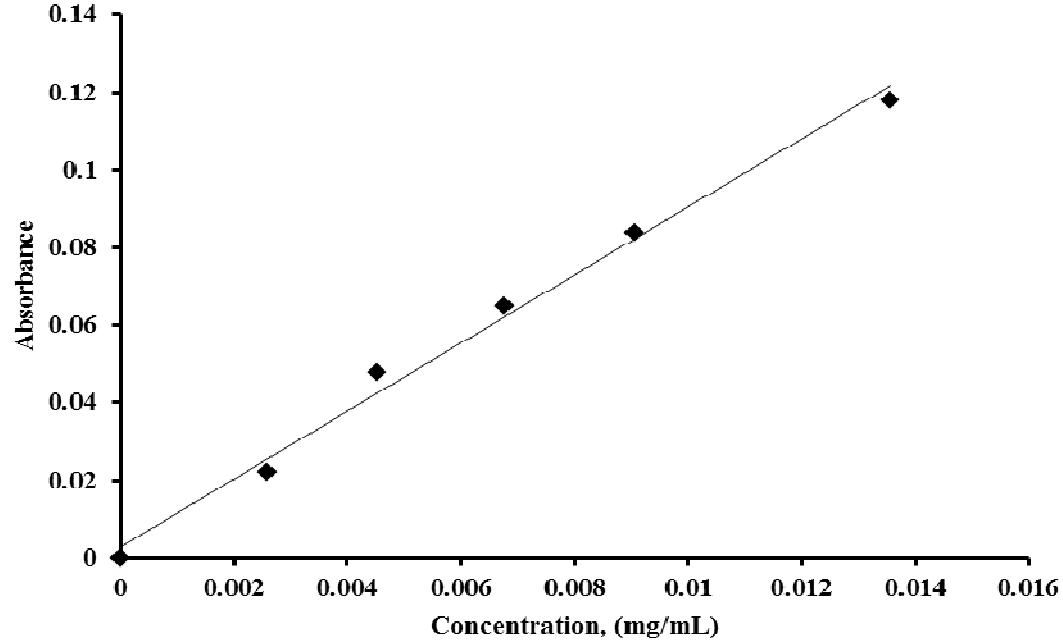

Figure 5. Calibration curve for protein determination using the Lowry Protein Assay at 650 nm; concentration of standards ranged from 0-0.014 mg/mL; $R^2 = 0.9916$

4. Conclusion

Differences in genistein concentrations may be related to factors including geographical, ecological, morphological, and biological differences, wherein the latter pertains to phylogenetic divergent sequences. *Apios carnea* is dispersed in Vietnam, Thailand, northern India, and central and southern China, whereas *A. fortunei*

appears in southwestern and central China. The results indicate that *Apios* plants have good potential as a food source in various habitats. More research is needed to determine the impact of geographical, ecological, and morphological differences on the presence of genistein, protein, and other vital nutrients in these *Apios* species. Genistein occured in all species of *Apios* tested, but the concentrations vary with the least amount in *A. carnea* and the most in *A. Americana*. The amount of genistein varies within different parts of the *Apios* plants, with the most genistein being found in the tuber cortex and the least in the leaves. Although the groundut tubers that were tested are much smaller than potatoes, they do contain similar amounts of protein that are in potatoes. Therefore, *Apios* is considered a promising alternative food source in areas of various habitats. Current efforts are underway to determine the other vital compounds and nutrients within the *Apios* species.

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