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Chromatographic methods development for the characterization of phytopharaceutical materials

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CHROMATOGRAPHIC METHODS
DEVELOPMENT FOR THE CHARACTERIZATION
OF PHYTOPHARMACEUTICAL MATERIALS

Melanie A. Levesque
December, 1998
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THESIS
SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE
OF
MASTERS OF SCIENCE

APPROVED:

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December, 1998
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DEDICATION

To my family

Far away there in the sunshine
are my highest aspirations. I may not
reach them but I can look up and see
their beauty, believe in them, and
try to follow them.

- Louisa May Alcott
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ABSTRACT

The increased popularity of alternative medicine treatments has prompted resurgence in the use of traditional herbal remedies. Among this vast number of herbs, ginseng is purchased by a considerable number of consumers; especially three species: *Panax ginseng, Panax quinquefolium*, and *Eleutherococcus senticosus*. Many of the physiological effects claimed by ginseng manufacturers tend to mimic those of central nervous system stimulants. This led to the purpose of this research, which was to develop an HPLC chromatographic method in order to determine if any specific CNS stimulants are within ginseng supplements. Specifically we found a series of CNS stimulants, methylxanthines (caffeine, theophylline, and theobromine), do exist in varying concentrations. The identity and concentration of the specific methylxanthines varied with the type of ginseng and the manufacturer.

The final conditions for the efficient chromatographic separation are through the use of a Hewlett-Packard Model 1100 HPLC with a Zorbax C18-SB column, a mobile phase composition of 21% methanol: 79% (2%) acetic acid, a column temperature of 35°C, a flow rate of 1.0 ml/min, and a UV wavelength detection at 280 nm. The levels of methylxanthines ranged from 0-1 mg/dose to 15-50 mg/dose. Identification of the methylxanthines was confirmed via standard addition, GC-MS and/or LC-MS.
I. INTRODUCTION

Holistic medicines, homeopathic remedies and dietary supplements are the current catch phrases important to the burgeoning area of alternative medicine therapy. Many people disillusioned with the complexities of modern medicine are looking for alternatives to their HMO and primary care physician. Many of these individuals are utilizing a whole series of different “medications” that can best be described as phytopharmaceuticals. Phytopharmaceuticals are supplements derived from medicinal plants and herbs that are meant to be ingested. Hundreds of these herbal compositions are available to the consumer and promise to maintain good health as well as improve performance in literally all aspects of life from libido lifts to stress relief to carcinoma cures. Many of these claims are based on anecdotal or cultural histories where the herbs have been used for centuries as medicines and remedies. While many individuals claim that the phytopharmaceuticals are the wonder drugs of the 90’s, many of the physical effects are scientifically unsubstantiated. This is not to say that herbs cannot be potent medications, many modern day medicines are derived from herbal extracts. In 1994, the Dietary Supplement Health and Education Act (DSHEA) removed dietary supplements from the FDA’s food additive category shifting the burden of proof to the FDA to prove lack of safety rather than falling on the supplement manufacturer to prove safety. Since the FDA does not regulate phytopharmaceuticals for their quality or composition, animal studies are not required for the determination of safety and there are no set requirements for dosage or composition of the supplements. In most cases, the complete chemical composition of these compounds is not even known. Finally, the few clinical and chemical studies associated with many of
these products are typically contradictory and/or inconclusive.³ For instance, studies on the effects of ginseng on stress with rats has shown that a daily dose does indeed reduce stress levels in rats, however studies with human subjects are inconclusive with regards to any substantial physiological effect.⁴,⁵ It is interesting that long term exposure to ginseng can cause several side effects including: blood pressure fluctuations, nervousness, insomnia, heart palpitations, anxiety, and dermatitis.³ Therefore, documentation of the physiological benefits or dangers of phytopharmaceuticals is a definite need for the consumer. Consequently, a chemical characterization and quantification of the basic components within many of these products is a real and critical need. We have chosen to exclusively focus this research on the characterization of a single supplement of widespread use, ginseng.

Ginseng has been used for nearly 5000 years as a mainstay of Chinese medicine. The earliest mention of the use of ginseng is in the 2000 year old herbal of Seng Nong.⁶ Ginseng is claimed to be a cure-all panacea, an aphrodisiac and an adaptogen (producer of a state of increased resistance to body stress). There are three common species of ginseng: 1) Asian Ginseng (Panax pseudoginseng Wallich); 2) American Ginseng (Panax quinquefolius L.); 3) Siberian Ginseng (Eleutherococcus senticosus) (Figure 1). Ginseng preparations are derived from the dried root of two species of the Araliaceae family, *Panax ginseng* C.A. Mey (Chinese ginseng) which is native to the northeastern region of China and *Panax quinquefolium* L. (American ginseng) which is native to northeastern North America.⁶,⁷ The Latin name *Panax* means "panacea" or all-healing. These plants are now rare in nature after years of over-collection and are now considered endangered
Figure 1. Ginseng and Ginseng Root
species. Currently, most preparations of ginseng are derived from cultivated plants, however tradition indicates that wild plants will be more effective. Another ginseng product, Siberian ginseng, is derived from *Eleutherococcus senticosus* a plant also from the ivy family (Araliaceae) found in Russia. This closely related plant seems to produce the same physiological effects as *Panax*.\(^6\)

Ginseng samples typically contain numerous compounds. These include ginseng saponins, ginseng oil, phytosterol, sugars, carbohydrates, acids, nitrogenous substances, amino acids, peptides, vitamins, minerals, ferments, and enzymes. Among these, it is assumed that ginseng saponins contribute the most, if not all, of the claimed pharmacological activities of ginseng extract preparations.\(^5,8-21\) Therefore, analysis of ginseng products has centered on the isolation and determination of the concentrations of the various saponin (sapogenin) glycosides within the herb.

Saponins are adducts of a sugar (glucose, fructose, galactose, etc.) and a steroid or terpene (Figure 2). The saponins are mainly of plant origin, but also occur in a number of marine animals. Saponins are chemically diverse compounds that have a particular set of chemical properties, including formation of colloidal solutions in water, bitter taste, sternutatory and irritant properties to the mucous membrane and hemolytic action against red blood cells. Although most saponins are non-toxic and appear to have no deleterious physiological effects, very high doses of saponins can cause intestinal irritations and lesions. While further information about saponins is of interest, they do not explain many of the traditional effects claimed by ginseng users. The amount of saponins
Figure 2. Representative structure of ginseng saponins.\textsuperscript{11}

\begin{table}[h]
\centering
\begin{tabular}{llll}
\hline
 & $R_1$ & $R_2$ & $R_3$ \\
\hline
Ginsenoside $Rg_1$ & H & Glc-O- & Glc- \\
Ginsenoside $Rg_2$ & H & Rha-Glc-O- & H \\
Ginsenoside $Re$ & H & Rha-Glc-O- & Glc- \\
Ginsenoside $Rf$ & H & Glc-Glc-O- & H \\
Ginsenoside $Rh_1$ & H & Glc-O- & H \\
Ginsenoside $Rb_1$ & Glc-Glc- & H & Glc-Glc- \\
Ginsenoside $Rb_2$ & Glc-Glc- & H & Ara(p)-Glc- \\
Ginsenoside $Re$ & Glc-Glc- & H & Xyl-Glc- \\
Ginsenoside $Rd$ & Glc-Glc- & H & Ara(f)-Glc- \\
Ginsenoside $Rg_3$ & Glc-Glc- & H & H \\
\hline
\end{tabular}
\end{table}

* Glc- Glucose, Rha- Rhamnose, Ara- Arabinose, Xyl- Xylose
within ginseng have been analyzed using several techniques although the most traditional method is reverse phase high performance liquid chromatography, HPLC.\textsuperscript{8-13} Components are identified by comparison to retention times of ginsenoside standards. The goal of this study is to chromatographically determine the identity of chemical complexes, other than the saponins, within the various types of ginseng materials. Specifically, analysis will focus on the determination of the concentration levels of methylxanthines (stimulants) within ginseng plants.\textsuperscript{22-24}

Methylxanthines are well known for their stimulatory effects on the central nervous system and cardiovascular system. This is probably the reason that plant extracts such as coffee, tea, and chocolate have been used for centuries. Unlike other secondary products produced uniquely by only one plant species, methylxanthines appears to be a widespread secondary product found in a variety of unrelated plant species.\textsuperscript{25} There are numerous methylxanthines, however, we will focus on the most abundant: theobromine, theophylline, and caffeine\textsuperscript{26-28} (Figure 3). The threshold of stimulation is quite different for each methylxanthine as seen in Table 1.\textsuperscript{27}

Theobromine (3,7-dimethylxanthine) is an alkaloid generally found in cocoa beans and hence chocolate. It is the least active as a central nervous stimulant of the three naturally occurring methylxanthines. It was used for the treatment of asthma, as a cardiac stimulant, as a diuretic, and in veterinary medicine as a vasodilator.\textsuperscript{27,28}

Theophylline (1,3-dimethylxanthine) is an alkaloid found in plants such as the leaves of the tea bush. It is primarily used as a drug for the treatment of asthma. The major pharmacological actions of theophylline include stimulation of the cardiac muscle
Figure 3. Structures, nomenclature, and abbreviations of the natural methylxanthines.

![Structures of methylxanthines](image)

Table 1. Comparison of thresholds of stimulation by the most abundant methylxanthines.

<table>
<thead>
<tr>
<th>METHYLXANTHINES</th>
<th>Threshold of Stimulation (μmol/kg)</th>
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<tbody>
<tr>
<td>Theophylline (1,3-Dimethylxanthine)</td>
<td>10</td>
</tr>
<tr>
<td>Caffeine (1,3,7-Trimethylxanthine)</td>
<td>25</td>
</tr>
<tr>
<td>Theobromine (3,7-Dimethylxanthine)</td>
<td>250</td>
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causing more complete emptying of the heart; relaxation of the bronchial muscle; acting as a diuretic increasing urine output and stimulation of the central nervous system.

The metabolism of theophylline has been extensively studied. Approximately 10% is excreted unchanged in the urine while the remaining 90% of the drug is converted to other compounds before it is eliminated from the body. The biologic half-life of theophylline varies from 3.5 hours in children to 8-9 hours in most adults. It is substantially prolonged in the presence of liver disease and/or cardiac decompensation. The amount of theophylline required to produce the desired benefits is 10-20 µg/ml with toxic side effects common at levels above 20 µg/ml. The toxic effects on the central nervous system are evident as wakefulness, restlessness, and agitation. These symptoms may progress to seizures, coma and death. The toxic effects on the myocardium consist of increased heart rate and cardiac arrhythmia.\textsuperscript{27,28}

Caffeine (1,3,7-trimethylxanthine) is a naturally occurring substance found in the leaves, seeds or fruits of more than 60 plants including coffee beans, cocoa beans, kola nuts and tea leaves. These products are commonly used to make many favorite beverages such as coffee, tea and cola drinks, and foods such as chocolate. People differ greatly in their innate sensitivity to caffeine. Many individuals can drink several cups of coffee within an hour and notice no effects, others may feel some effect after one serving.\textsuperscript{29-32} Caffeine does not accumulate in the bloodstream or body and is normally excreted within several hours following consumption.

The determination of methylxanthines in various ginseng supplements will be analyzed via reverse phase HPLC. While the initial conditions for HPLC stem from AOAC Official Method, Theobromine and Caffeine in Cacao Products\textsuperscript{22}, further methods
development to improve the procedure will be studied. Other analyses will include GC-MS, UV-Vis, and LC-MS of the supplements for further assurance of the presence of one or more methylxanthine.

A. CHROMATOGRAPHIC THEORY

High Performance Liquid Chromatography, HPLC, is one of the prevalent analytical separation tools used for medical, industrial and academic applications. By definition, HPLC comprises all liquid chromatographic techniques that require the use of elevated pressures to force the liquid through a packed bed of the stationary phase. Through a series of chemical and physical interactions, materials can be separated into less complex mixtures and matrices. After purification either interfaced or external processes can be used to complete the characterization.

During the 1960's, gas, column and thin layer chromatography were the main instruments for separation science. The need for a chromatographic technique suitable for the routine and fast analysis of non-volatile compounds was essential for many different types of industrial applications. The application of chromatographic theory to the development of column liquid chromatography led to the use of very small particles for the solid adsorbent stationary phase. The advent of efficient packing technology in the 1970's made it feasible to reproducibly prepare high-efficiency HPLC columns from 5 to 10-μm particles. Prior to this development, packing techniques had failed to give good results for particles smaller than about 30 μm. The term HPLC was first defined as High Pressure Liquid Chromatography in relevance to the need of sufficient pressure to produce a more rapid flow rate in correlation to the low permeability of the packing material at that time.
Soon this name was replaced by High Performance Liquid Chromatography corresponding to the new instrumental technique having better “performance” in terms of resolving power, detection, quantification and speed.

There exist several types of chromatography with the most traditional form of liquid chromatography referred to as normal-phase chromatography. It involves a polar adsorbent such as silica or alumina and a nonpolar mobile phase. The mechanism for separation consists of the interaction of the polar functional groups of the analytes with polar sites of the packing.

Reversed-phase chromatography developed in the late 70’s, involves a nonpolar stationary phase, typically silica-based, and a polar, usually aqueous, mobile phase. Although the separation mechanisms in reverse phase chromatography are still under discussion, basic retention is a function of the hydrophobicity of the analyte. In turn, the hydrophobicity of the analyte is a direct function of the size of the hydrophobic area of a molecule. Therefore, reversed-phase chromatography is ideal for the separation of compounds of a homologous series. For instance, not only can similar analytes be separated by reversed-phase chromatography but at times, it is also possible to assign the order of elution based on their structures. Another advantage of reversed phase HPLC is that equilibration of the columns with the aqueous mobile phases is rapid; in most cases only a few column volumes are needed. For the majority of analytes, good peak shapes and reproducible retention times are obtained without difficulty. Reverse phase chromatography is by far the most popular HPLC technique.33-35

An HPLC system consists of 8 major components: (1) the mobile phase reservoir; (2) the pump, (3) the injector/autosampler, (4) the column, (5) the detector; (6) the data
system, (7) the connective tubing, and (8) the waste reservoir (Figure 4). Each component has a specific function. The mobile phase transports the analyte to the column and must be degassed/filtered to keep the system free of insoluble particulates that could clog tubing. The pump is capable of delivering pulse-free flow at pressures up to 6000 psi. The type and length of column vary according to the separation procedure of interest. The detector must give a linear response to the solute of interest over the range of concentrations to be analyzed. The data system simply handles the signal that is generated from the detector. Finally, the connective tubing must ensure zero volume, which is essential to minimize dead volumes, band broadening and enhance resolution.

**Figure 4.** Representative HPLC System.\(^{33}\)

In order to understand the applications and terminology of HPLC, the basic fundamentals must be thoroughly understood. The following is a list of essential definitions to the chromatographer in understanding the applications of HPLC.
Elution Volume and Peak Width:

Elution volume and peak widths are the two basic measurements required to develop the theory and definitions of HPLC.

Figure 5. Representative chromatogram.

![Diagram of chromatogram with annotations: V0, V1, V2, V3, W2, W3.]

where \( V_0 \) is the elution volume of unretained compound which equals one column volume, \( V_1 \) is the elution volume of compound 1, \( V_2 \) is the elution volume of compound 2, and \( W_2 \) is the peak width of compound 2 in terms of volume.

Isocratic Elution:

Isocratic elution indicates a constant solvent composition at a constant temperature.

Gradient Elution:

Gradient elution refers to varying one or more parameters continuously. Typically, only one parameter is varied. The type of gradient that is used most often is mobile phase composition from low elution strength to high elution strength. Elution strength is a measure of the ability of the mobile phase to elute more strongly adsorbed solutes from the column.

Temperature gradient:

Temperature gradient refers to varying the temperature in order to aid separation. Usually the gradient goes from low temperature to high temperature to improve separation. Typically used in gas chromatography.
Band Shapes

Peak fronting:

Peak fronting occurs when too much sample has been applied to the column. The excess solute affects the physical properties of the stationary phase and alters the partitioning from the mobile to stationary phase. This "overloading" of the column results in a gradual rise and an abrupt end of the chromatographic peak.

Figure 6. Overloading band shape.$^{34}$

Peak tailing:

Peak tailing occurs when small quantities of solute are retained more strongly than most of the solute. It leads to an abrupt rise and a long tail of the chromatographic peak. A cause of tailing may be due to the presence of sites at which the solute is held strongly by the stationary phase.

Figure 7. Tailing band shape.$^{34}$

Band broadening:

The more time the solute spends on the column the longer it will take to elute. This results in a broad peak. To minimize band broadening from occurring outside the chromatography column, all dead space and tubing lengths should be minimized.
Peak Shape

Asymmetry factor ($A_s$):

Asymmetry factors are used to measure the tailing of the peaks. The factors may be calculated in several different ways.

Figure 8. Chromatogram showing an asymmetric peak.

\[ A_s = \frac{B}{A} \]  \hspace{1cm} (1)

where $B$ is the width of the tail of the peak and $A$ is the width of the front of the same peak.

Void volume ($V_0$):

Void volume represents the total volume of the liquid retained within the column.

Linear flow rate:

The linear flow rate ($u$) refers to the distance, usually in cm, of column length traveled in one minute by the solvent.

Retention Relationships

Retention time ($t_r$):

Retention time is the time it takes the solute from injection onto the column until that component reaches the detector.
**Time spent in mobile phase** \((t_m)\):

\[ t_m \text{ is the time needed for the mobile phase to travel the length of the column. An unretained solute would be eluted in time } t_m. \]

**Volume Flow Rate** \((F)\):

Volume flow rate is the number of milliliters of solvent per minute that travel through the column.

**Retention Volume** \((V_r)\):

Retention time is the volume of mobile phase required to elute a particular solute from the column. This can be related to retention time and volume flow rate by equation 2.

\[ V_r = t_r * F \quad (2) \]

**Capacity Factor** \((k')\):
(a measure of retention)

The capacity factor is a direct measure of how strongly a sample adsorbs to the packing material of the column. The longer a component is retained by the column, the greater the capacity factor. Large capacity factors favor good separation but also increase elution time and bandwidth.

\[ k' = \frac{t_r - t_m}{t_m} \quad (3) \]

**Plate Theory**

**Theoretical Plates** \((N)\):
(a measure of efficiency)

Plate theory is described as the idea that the column is divided into \(N\) Segments. Within each “segment” an equilibration occurs. In order to measure the number of theoretical plates; the retention and the bandwidth of a component must first be determined.

\[ N = \frac{16t_r^2}{w^2} = \frac{5.54t_r^2}{w^{1/2}} \quad (4) \]

where \(t_r\) is the retention time of the peak and \(w\) is the width of the band.
Particle size:

Generally the smaller the particle size the more efficient the column. Smaller particles give more theoretical plates per length of column.

Height Equivalent to a Theoretical Plate (H.E.T.P.):

Injection of a small volume of analytes onto a column results in a narrow band on the top of the column. The band becomes broader as the analyte migrates through the column. In a uniform bed, the peak width increases with the square root of the length that the band has traveled.

If a mixture of two components is injected, they will both form the same narrow band on top of the column. If they have slightly different affinities to the column, they will migrate down the column at slightly different speeds. As they migrate down the column, the distance between the centers of both bands increases in direct proportion to length that the bands have traveled. In addition, the width of each band increases only with the square root of this length. Thus, the bands will at some point be completely separated. The farther they migrate, the more resolved they would be.\(^{35}\)

\[
\text{H.E.T.P.} = \frac{L}{N} \tag{5}
\]

where \(L\) is the total length of the column and \(N\) is the theoretical plates of the column.

Reduced Plate Height (h):

It is the comparison of different length columns that are also packed with different sized particles.

\[
h = \frac{H}{d_p} \tag{6}
\]

where \(H\) is an abbreviation for HETP and \(d_p\) is the diameter of the particles.

- Theoretical plates can be controlled by many factors however; the most important one is flowrate.

Figure 9. Plates vs. Flowrate
Rate Theory of Chromatography

The van Deemter equation describes the dependence of the Height Equivalence of a Theoretical Plate, HETP, on linear velocity. It assumes that the HETP is composed of three different, independent contributions. These contributions are independent to one another and thus form the observed curvilinear equation.

\[ H = A + \frac{B}{\mu} + C\mu \quad (\mu = \text{linear velocity}) \]  

(7)

where \( A, B, \) and \( C \) are constants for a given column and mobile phase. The \( A \) term represents the multiple paths of different lengths possibly traveled by the solute. The \( B \) term is inversely proportional to the linear velocity and it signifies longitudinal diffusion of a solute as it travels through the column. The \( C \) term is directly proportional to the linear velocity and it arises from the finite time required for the solute to equilibrate between the mobile and stationary phases.\(^{34}\)

**Linear velocity (μ):**

This is the rate at which the mobile phase passes through the column. It can sometimes be defined as linear flow rate or as volume flow rate because each is proportional to the other.\(^ {35}\)

**Separation**

**Selectivity (\( α \))**:  
(a measure of peak separation)

Selectivity is a measure of peak separation and describes how differently two compounds react in the same chromatographic system.

\[ α = \frac{V_2 - V_0}{V_1 - V_0} \]  

(8)

where \( V_1 \) and \( V_2 \) are the elution volumes of compounds 1 and 2 respectively and \( V_0 \) is the volume of an unretained solute, which equals one column volume.

**Resolution (\( R_s \))**:  
(Degree of Magnitude of Separation)

Resolution is defined as the separation between two peaks. Two peaks are assumed to be completely resolved when the resolution is equal to 1.5.
Table 2. % Overlap between two peaks at various resolution values.

<table>
<thead>
<tr>
<th>RESOLUTION</th>
<th>% OVERLAP BETWEEN 2 PEAKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>16%</td>
</tr>
<tr>
<td>1.0</td>
<td>2.3%</td>
</tr>
<tr>
<td>1.5</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

\[ R_s = \frac{\Delta t_c}{w_{ave}} \]  
(9)

However, it can be shown that the resolution is given by:

\[ R_s = N \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k'}{1 + k'} \right) \]  
(10)

where \( N \) is the number of theoretical plates, \( \alpha \) is the relative retention, \( k' \) is the capacity factor of the component of interest.

- Resolution depends on the capacity factor \( k' \)

Table 3.

<table>
<thead>
<tr>
<th>( k' ) VALUE</th>
<th>( k' ) TERM</th>
<th>( k' ) RESOLUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1/2</td>
<td>.50</td>
</tr>
<tr>
<td>2</td>
<td>2/3</td>
<td>.67</td>
</tr>
<tr>
<td>3</td>
<td>3/4</td>
<td>.75</td>
</tr>
<tr>
<td>10</td>
<td>10/11</td>
<td>.91</td>
</tr>
<tr>
<td>20</td>
<td>20/21</td>
<td>.95</td>
</tr>
</tbody>
</table>

Figure 10. Capacity Factor \( k' \) Ideal Range graph

As \( k' \) increases, resolution increases until \( k'/k'+1 \) approaches a value of one.
Resolution depends on selectivity

Example:
\[
\alpha = 1.1 \quad \frac{1.1 - 1}{1.1} = 0.09
\]
\[
\alpha = 1.4 \quad \frac{1.4 - 1}{1.4} = 0.29
\]

When the selectivity value is small, small changes in selectivity produce large changes in resolution. The selectivity term approaches one when alpha is large.

Resolution depends on theoretical plates (N)

Table 4.

<table>
<thead>
<tr>
<th>N, PLATE COUNT</th>
<th>(N) RESULTS</th>
<th>% CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 1000</td>
<td>31.6</td>
<td></td>
</tr>
<tr>
<td>N = 2000</td>
<td>44.7</td>
<td>↑ 41%</td>
</tr>
<tr>
<td>N = 3000</td>
<td>54.7</td>
<td>↑ 73%</td>
</tr>
<tr>
<td>N = 5000</td>
<td>70.7</td>
<td></td>
</tr>
<tr>
<td>N = 10,000</td>
<td>100</td>
<td>↑ 41%</td>
</tr>
<tr>
<td>N = 15,000</td>
<td>122.4</td>
<td>↑ 73%</td>
</tr>
</tbody>
</table>

Resolution increases proportionally with the square root of the theoretical plates (refer to equation 7).

HPLC Methods Development

In carrying out quantitative analysis in a set of samples, it is necessary to develop a protocol suitable for a specific separation. First, the separation problem must be defined and all information essential to the problem is collected. Typically this would include:

- details of the physiochemical properties of the analyte;
- the physiochemical properties of the other components in the matrix;
- features (desirable and undesirable) of the available methods for separation.

Major aspects of method development include chromatographic expertise in which the researcher needs to have as many potential solutions to the problem as possible at his/her disposal. Optimization involves manipulation of experimental variables relating to the mobile phase until the desired separation has been achieved. Method validation consists of\(^{33,35}\).
Specificity: An assay is specific if the analytical response arises from the analyte of interest only and no other compound that may be in the sample can have the same response.

Linearity: A method is linear if there is a linear relationship between the analytical response and concentrations of analyte in the sample solutions over a specified range of concentration of the analyte.

Robustness: A robust or rugged method is one, which is not greatly affected by minor changes that might reasonably be expected during the course of an assay.

Precision: the precision of a method is the degree of scatter of the results and is usually reported as a relative standard deviation. It is often subdivided into repeatability and reproducibility.

Accuracy: An assay is accurate if the mean result from the assay is the same as the true value.

Limit of detection: The limit of detection is the amount of analyte, which can be reliably detected under the stated experimental conditions.

Limit of quantitation: The limit of quantitation is the amount of analyte, which can be reliably quantified under the stated experimental conditions.

Stability in solution: It is important for the analyte to be sufficiently stable in solution in the solvent used in order to carry out reliable analysis.

System suitability: It is important to know if the chromatographic technique of choice is efficient for the methods development of the compound of interest.
In this research project, methods development was accomplished by varying many parameters of the HPLC instrument. Initial parameter changing will begin with mobile phase composition. Once a good separation is established other parameters can be varied such as column type, flow rate, column temperature, UV wavelength detection, volume of sample injected, and concentration of the sample. A brief example of methods development is described in the following text.

Compound X is only known structurally. The assignment is to obtain peak purity of compound X via HPLC. This can be done in the following way: first a literature search is established on the compound itself as well as on a similar compound. With this an initial
protocol may be determined and can be applied to compound X. It is from this point that parameters can be varied in order to obtain the best separation. Mobile phase composition is usually the starting point; generally the organic solvents are increased rather than the aqueous solvents. The next factor considered could be column type. Simply changing column length can drastically expand the separation of the compound from other constituents that may be in the sample. Next flow rate can be increased or decreased which allows you to see the effects this can cause on the separation. Usually a flow rate of 1.0 ml/min is sufficient. Column temperature can sometimes decrease retention time, decrease resolution, and sharpen peaks. Finally, the maximum absorbance of compound X can be determined via UV-vis, which in turn can then be transferred over to the HPLC and then analysis of compound X is obtained with all the appropriate conditions. All these variations and optimizations of the different parameters constitute methods development.
II. EXPERIMENTAL SECTION

The experimental procedures can be broken down into three main elements; obtaining samples, extraction, and HPLC analysis (includes methods development).

Various samples of ginseng herbal supplements were purchased from several retail stores in the Rochester, NY area. Several trips were conducted to obtain samples manufactured from different manufacturer lots. Another set of samples was purchased from Chinese apothecaries in New York City and Hong Kong. These were nonprocessed root samples. All materials remained sealed in their appropriate containers until analysis. A total of 40 unique ginseng supplements were obtained and analyzed.

Prior to HPLC analysis the methylxanthines were extracted from the bulk material with a slight variation of the accepted procedure described by AOAC for the extraction of methylxanthines from Cocoa beans.\textsuperscript{22} In the case of solid samples, the contents of one capsule of the ginseng supplement were removed from the capsule and weighed; the mass was typically 500 mg. The sample was then ground in a mortar and pestle and the resultant fine powder was placed in 10-25 ml of HPLC-grade water (Baker Chemical Co.), heated to boil in an 800-watt microwave oven (Sharp) and then sonicated for 5 minutes in a VWR Scientific Sonicator. The resultant heterogeneous solution was filtered first through a 0.45 \textmu m and then a 0.20 \textmu m filter disk (Gelman Acrodisks). The samples were then ready for HPLC analysis.

In the case of liquid samples, the neat samples were heated as described above to facilitate dissolution of the methylxanthines. The resultant solution was then filtered and prepared as above.
Both internal and external standards were used in the HPLC analysis. Each standard was weighed and dissolved in hot HPLC grade water. The concentrations ranged from 0.01-0.1 mg/ml (see Table 5). The solutions were then sonicated for 5 minutes and heated in a microwave oven for an additional 45 seconds to ensure complete dissolution of the solid standard compound. The solutions were filtered first through a 0.45 μm and subsequently through a 0.20 μm filter disk. The external standard solutions were then ready for HPLC analysis. Note that the external standard solutions were analyzed at the beginning and at the end of each analysis run. For internal standards the following protocol was followed: 0.10 μl of the standard solution and 0.90 μl of water were added to 1.0 ml total volume of the ginseng solution. The internal standard solution was then analyzed as indicated above.

**Table 5.** Absorbance response in correlation with concentration of the standard solutions.

<table>
<thead>
<tr>
<th>METHYLXANTHINE</th>
<th>CONCENTRATION</th>
<th>ABSORBANCE RESPONSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theobromine</td>
<td>0.01 mg/ml</td>
<td>160.97</td>
</tr>
<tr>
<td></td>
<td>0.05 mg/ml</td>
<td>847.46</td>
</tr>
<tr>
<td></td>
<td>0.1 mg/ml</td>
<td>1680.65</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0.01 mg/ml</td>
<td>148.90</td>
</tr>
<tr>
<td></td>
<td>0.05 mg/ml</td>
<td>785.98</td>
</tr>
<tr>
<td></td>
<td>0.1 mg/ml</td>
<td>1559.10</td>
</tr>
<tr>
<td>Caffeine</td>
<td>0.01 mg/ml</td>
<td>130.73</td>
</tr>
<tr>
<td></td>
<td>0.05 mg/ml</td>
<td>730.60</td>
</tr>
<tr>
<td></td>
<td>0.1 mg/ml</td>
<td>1472.49</td>
</tr>
</tbody>
</table>

HPLC columns were purchased from Burdick and Jackson, Phenomenex, and Hewlett Packard. The B&J column was an Octadecyl; C18 bonded type OD5 column. The silica substrate had a particle size of 5 μm and a 4.6 mm x 25 cm configuration. The Phenomenex column was a C8 Luna column with a particle size of 5 μm and a 4.6 mm x
15 cm configuration. The HP Zorbax C18-SB column contained StableBond silica packed under high-pressure slurry loading technique to give optimum column efficiency. The silica substrate had a particle size of 5 μm and a porosity of 80 A. This column is a C18 stable bond with 4.6 mm x 25 cm configurations.

Analysis was performed on a Hewlett Packard Model 1100 liquid chromatograph equipped with HP ChemStations software and a variable-wavelength detector. The final set of conditions used for complete analysis of all the ginseng supplements consisted of a mobile phase composition of 21% methanol: 79% (2%) acetic acid, a column temperature of 35°C, a flow rate of 1.0 ml/min, a UV wavelength of 280 nm and an average pressure of 150 atm. The column used for all analyses was the HP Zorbax C18-SB. Injections of all samples were performed manually or with the use of an autosampler with the injection volumes set at 5 μl or 10 μl. Each sample was analyzed at least twice.
III. RESULTS/DISCUSSION

Methods development involves a strategic approach to an analytical problem through the use of numerous steps. First and foremost, before any analysis can be done, any and all pertinent information about the compound(s) of interest must be gathered. After the knowledge base is established an initial experimental protocol can be mapped out. In this research project, information on the methylxanthines as well as ginseng was obtained from the literature. Our initial protocol was obtained from a standard method for the determination of caffeine and theobromine in cocoa beans. This gave us the starting conditions for the separation of the methylxanthine standards. Using the Burdick and Jackson OD5 Octadecyl HPLC column, the conditions used for this initial attempt consisted of a mobile phase composition of 65% methanol: 35%(1%) acetic acid, column temperature of 25°C, flow rate of 1.0 ml/min, and UV detection wavelength of 254 nm. Figure 12 is a chromatogram of numerous methylxanthines at a concentration of 0.1 mg/ml with the above parameters. As observed, some methylxanthines are not well separated and some peaks are ill-defined; specifically the peaks at 7.337 and 7.609 minutes show extensive overlap and the peaks at 2.894 and 7.609 minutes exhibit prominent peak tailing. Therefore, an experimental approach to resolve the peaks by achieving better separation while simultaneously improving peak shape is the first goal. After, several different mobile phase compositions with the B&J column were tried with limited success, Figures 13 and 14, it was decided that success could not be achieved with the B&J column. There are several factors that need to be considered when choosing the right column. Typically this involves column design features such as packing material, length,
Figure 12. Chromatogram of various methylxanthine standards.

HPLC analysis of numerous methylxanthines at a concentration of 0.1 mg/ml with a Burdick and Jackson column and HPLC conditions of 65% methanol: 35% (1%) acetic acid, flow rate of 1.0 ml/min, column temperature of 25°C and a UV wavelength of 254 nm. Compounds are as labeled.
**Figure 13.** Chromatogram of various methylxanthine standards.

HPLC analysis of numerous methylxanthines at a concentration of 0.1 mg/ml with a Burdick and Jackson column and HPLC conditions of 90% acetonitrile: 10% (1%) acetic acid, flow rate of 1.0 ml/min, column temperature of 25°C and a UV wavelength of 254 nm. Compounds are as labeled.

**Figure 14.** Chromatogram of various methylxanthine standards.

HPLC analysis of numerous methylxanthines at a concentration of 0.1 mg/ml with a Burdick and Jackson column and HPLC conditions of 10% acetonitrile: 90% (1%) acetic acid, flow rate of 1.0 ml/min, column temperature of 25°C and a UV wavelength of 254 nm. Compounds are as labeled.
and diameter, as well as particle size, shape, and distribution. The initial column used, was the Burdick and Jackson OD5 C18 bonded phase with a column length of 25 cm, an inner diameter of 0.46 cm, and a packing of 5 μm. The inability to improve separation and especially peak shapes on the B&J column is assumed to be caused by the irregular-shaped particles of the packing material within the column. Although the B&J column does have particles of a size range 4.8-5.2 microns, there is no effort made to regulate particle shapes. This varies the linear velocity and pathway components within the van Deemter equation. The net result of the multiple pathways is broad irreproducible peaks. Also the compounds we are examining tended to adsorb to the surface of the column because of the irregular particle shapes, which caused the peaks to tail and be ill-defined. This lack of quality control of solid-support particle shapes also led to variability when columns were changed. Therefore, a column packed with a more uniform particle shape was purchased, a Phenomenex Luna column. The Luna column consists of a C8 bonded phase with a column length of 15 cm, an inner diameter of 0.46 cm, and packing of 5 μm. This type of column is packed with spherical shaped particles, which provide more uniformity in the reproducibility from one packing to another. The change from a C18 to a C8 column was also varied in hopes of reducing the retention times of the methylxanthines thus providing a shorter analysis time. In addition to its higher quality, the Luna column provided better resolution, enhanced peak definition as well as shorter retention times which is as expected with the shorter length and lower MW stationary phase (Figure 15). Through calculations you can see the comparison of the value of N for the Luna column (N) vs. the B&J column (N): 2274.23 to 788.81, respectively. Although much improvement was obtained
Figure 15. Chromatogram of theobromine, theophylline, and caffeine standard solution.

HPLC analysis of a theobromine, theophylline, and caffeine solution at a concentration of 0.1 mg/ml with a Phenomenex Luna column and HPLC conditions of 65% methanol: 35 (1%) acetic acid, flow rate of 1.0 ml/min, column temperature of 25°C and a UV wavelength of 254 nm. Compounds are as labeled.
using the Luna column, after multiple injections and over time a loss in reproducibility was observed. Specifically, the retention times of the methylxanthines began to vary with loss of resolution and peak shape. The data suggest that with our experimental conditions, the bonded phase began to break down, leaving silica sites to interact with the solute. In analytical chemistry consistent reproducible results are extremely important thus a more chemically resistant column was needed. Through the literature another bonded phase column was described that apparently could withstand more acidic conditions than the Luna, an HP Zorbax column. This column consisted of a C18 stable bonded phase with a column length of 25 cm, an inner diameter of 0.46 cm, and packing of 5 μm. Although the Zorbax column was very similar to the Luna column in particle shape, it is the binding of the stationary phase to the solid support that allowed the use of more rigorous conditions. The coating, Rx-SIL, sterically protects the bonded phase allowing it to remain stable even at extremely low pH. Upon use, the Zorbax column was very efficient with remarkable reproducibility (Figure 16). Since it provided stability, well-resolved methylxanthine peaks, and enhanced peak definition; the Zorbax column was determined as the column of choice.

The next phase of the methods development was to “fine tune” the separation of the methylxanthines by varying other experimental parameters: mobile phase composition, temperature, and flow rate. The goal is to have maximum resolution in the minimum timeframe possible. While one usually only changes one variable at a time, in some instances it was necessary to vary two or more of the above. In reversed phase chromatography, varying mobile phase goes from a low elution strength to a high elution strength. In this manner even the weakly bound materials will initially bind to the column
Figure 16. Chromatogram of theobromine, theophylline, and caffeine standard solution.

HPLC analysis of a theobromine, theophylline, and caffeine solution at a concentration of 0.1 mg/ml with a Hewlett Packard Zorbax column and HPLC conditions of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C and a UV wavelength of 280 nm. Compounds are as labeled.
for short periods of time. It is after the evaluation of the results from the low elution strength that it is slowly varied to higher values either through step-function or gradient conditions. The higher elution strength is accomplished by increasing the hydrophobicity of the solvent mixture. In other words, the polarity of the solvent system is decreased over time via a gradient. In this study since we were only concerned with water-soluble materials the elution strength was lowered by slowly increasing the percentage of the aqueous acetic acid component until desired resolution and plate count was achieved. As the amount of aqueous component (1% acetic acid) increased, the more resolved and the more defined the methylxanthine peaks appear, Figures 17-18. In Figure 18 the peaks are completely resolved, however it was found that when the ginseng samples were analyzed under these conditions other materials in the sample interfered with the methylxanthine peaks. It was through this pattern of slowly increasing the aqueous component that the final mobile phase composition was determined to be 21% methanol: 79% (2%) acetic acid (Refer to Figure 16).

In chromatographic separations raising column temperature decreases retention time, decreases resolution, and most importantly sharpens peaks. First the column temperature was raised from 25°C to 30°C, Figure 19 shows the results of well-resolved standards but the caffeine peak is not as sharp as it should be. Again the column temperature was raised but this time from 30°C to 40°C. Increasing the temperature to 40°C caused the methylxanthines to elute faster from the column and the caffeine peak is slightly sharper as seen in Figure 20. As expected raising the column temperature resulted in a shorter analysis time, reduced selectivity of the peaks, and sharper peaks. Although shorter retention times were obtained from these conditions, desirable separation of the
Figure 17. Chromatogram of theobromine, theophylline, and caffeine standard solution. HPLC analysis of a theobromine, theophylline, and caffeine solution at a concentration of 0.1 mg/ml with a Hewlett Packard Zorbax column and HPLC conditions of 40% methanol: 60% (1%) acetic acid, flow rate of 1.0 ml/min, column temperature of 25°C and a UV wavelength of 254 nm. Compounds are as labeled.

Figure 18. Chromatogram of theobromine, theophylline, and caffeine standard solution. HPLC analysis of a theobromine, theophylline, and caffeine solution at a concentration of 0.1 mg/ml with a Hewlett Packard Zorbax column and HPLC conditions of 30% methanol: 70% (1%) acetic acid, flow rate of 1.0 ml/min, column temperature of 25°C and a UV wavelength of 254 nm. Compounds are as labeled.
Figure 19. Chromatogram of theobromine, theophylline, and caffeine standard solution VWD1A.

- **Wavelength**: 254 nm (MAYW0^2000-D)
- **mAU**: 100-900

HPLC analysis of theobromine, theophylline, and caffeine at a concentration of 0.1 mg/ml with a Hewlett Packard Zorbax column and HPLC conditions of 30% methanol:70% (1%) acetic acid, flow rate of 1.0 ml/min, column temperature of 30°C and a UV wavelength of 254 nm. Compounds are as labeled.

Figure 20. Chromatogram of theobromine, theophylline, and caffeine standard solution VWD1A.

- **Wavelength**: 254 nm (MAYW0^2000-D)
- **mAU**: 100-900

HPLC analysis of theobromine, theophylline, and caffeine at a concentration of 0.1 mg/ml with a Hewlett Packard Zorbax column and HPLC conditions of 30% methanol:70% (1%) acetic acid, flow rate of 1.0 ml/min, column temperature of 40°C and a UV wavelength of 254 nm. Compounds are as labeled.
methylxanthine peaks from the many components in the ginseng supplements could not be attained when applying these conditions. Taking into consideration the results determined from the column temperature of both 30°C and 40°C, the final column temperature setting was determined at 35°C (Figure 16). Note the column temperature was determined when the mobile phase composition was at 30% methanol: 70% (2%) acetic acid.

Flow rate is an important factor in the separation of compounds. When doing a “quick and dirty” analysis, resolution is not usually top priority. Therefore, a faster flow rate can be applied to the analysis. This generally results in a quicker analysis time since it will elute at a faster pace the compound(s) that bind more strongly to the stationary phase. Other times, resolution of compounds is an important part of the analysis. Sometimes the peaks are difficult to separate thus; the flow rate can be reduced. This often results in band broadening since the compound(s) will be retained longer on the column. Taking these facts into consideration, perhaps the peaks could be resolved to a greater extent, which in turn would result in a shorter analysis time. In order to resolve the peaks to a greater extent the flow rate was reduced from 1.0 ml/min to 0.5 ml/min as shown in Figure 21. As a result, the methylxanthines were well resolved however most exhibit band broadening as well as extensive peak tailing. In Figure 22, the flow rate was set to 2.0 ml/min, although this improved peak definition, the methylxanthines could not be resolved from other components within the ginseng supplements. Consequently a flow rate of 1.0 ml/min was determined to be most efficient. Note the flow rate was determined using the Phenomenex Luna column. The detection system for the analysis has to exhibit a linear response over a wide range of possible concentrations. The choice for materials similar to
Figure 21. Chromatogram of various methylxanthine standards.

HPLC analysis of various methylxanthines at a concentration of 0.01 mg/ml with a Phenomenex Luna column and HPLC conditions of 65% methanol: 35% (1%) acetic acid, flow rate of 0.5 ml/min, column temperature of 25°C and a UV wavelength of 254 nm. Compounds are as labeled.

Figure 22. Chromatogram of theobromine, theophylline, and caffeine standard solution.

HPLC analysis of theobromine, theophylline, and caffeine at a concentration of 0.1 mg/ml with a Phenomenex Luna column and HPLC conditions of 50% methanol: 50% (1%) acetic acid, flow rate of 2.0 ml/min, column temperature of 25°C and a UV wavelength of 254 nm. Compounds are as labeled.
methylxanthines is an Ultraviolet-visible absorption detection system. To choose a wavelength for detection the first step is to make samples and use a UV-Vis spectrophotometer. In this case, a Hewlett Packard model 8453 UV-Vis photodiode array spectrometer was used and the $\lambda_{\text{max}}$ for caffeine, theophylline and theobromine are 280 nm, 274 nm, and 274 nm, respectively.

Gathering all the information on the different parameters together and applying them to the HPLC instrument, the final conditions for the best analysis of the ginseng supplements were as follows: Zorbax C18 SB column, mobile phase composition of 79% (2%) acetic acid: 21% methanol, column temperature of 35°C, flow rate of 1.0 ml/min, and UV wavelength of 280 nm. Figure 16 shows the chromatogram of the three methylxanthines with the final conditions.

It is important in HPLC analysis to verify the accuracy of the column. Through time the column packing can deteriorate which would reduce the number of theoretical plates. This in turn will affect the resolution of the peaks and reproducibility is diminished. In order to determine the physical condition of the column a calibration check must be established and performed daily. It was in this manner that the loss of plate number was found for the Luna column. Calibration and check curves can be constructed by analyzing a standard sample at different concentration levels. A response is considered acceptable if the plot of the analytical response versus the concentration of the analyte in the sample is linear. Calibration curves were obtained for the Zorbax column for injection volumes of both 20 μL and 5 μL (Figures 23 and 24). As shown linearity was achieved for each methylxanthine. After least squares analysis, the equation of the line obtained from the graphs was used to determine the amount of methylxanthine each supplement contained.
Figure 23. Calibration curve of the methylxanthines.

<table>
<thead>
<tr>
<th></th>
<th>Caffeine</th>
<th>Theophylline</th>
<th>Theobromine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equation</td>
<td>$y = 50844x$</td>
<td>$y = 53962x$</td>
<td>$y = 55119x$</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.9992</td>
<td>0.9988</td>
<td>0.9987</td>
</tr>
</tbody>
</table>

Calibration curves for theobromine, theophylline, and caffeine at 20 µl injection volumes. HPLC analysis of the methylxanthines was done with a Hewlett Packard Zorbax column and HPLC conditions of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C and a UV wavelength of 280 nm.
Figure 24. Calibration curve of the methylxanthines.

Calibration curves for theobromine, theophylline, and caffeine at 5 µl injection volumes. HPLC analysis of the methylxanthines was done with a Hewlett Packard Zorbax column and HPLC conditions of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C and a UV wavelength of 280 nm.
For complete results of the methylxanthines in the ginseng supplements refer to Table 6.

In order to determine the linear detection limits of the methylxanthines in the ginseng supplements, the analysis of samples after serial dilutions was performed (see Figures 25 and 26). As shown the lowest possible level of linear detection was found to be at 0.001 mg/ml for theobromine, theophylline, and caffeine.

After the conditions for analysis are established for a series of standards, real samples must be analyzed so that any/all matrix effects can be evaluated. In order to determine if there were any significant matrix effects within the ginseng extracts on the methylxanthines chromatographic response, internal standards were added to the ginseng supplement. Then the response from the internal standards was evaluated via standard addition analysis and the initial concentration of the component was determined (Figure 27). From the internal vs. external standard analyses it was found that the results correlated within ± 2%. The following chromatograms are examples of an internal standard addition to a ginseng supplement. Figure 28 represents the NGE ginseng supplement alone followed by Figure 29, which is the NGE sample with theophylline as an internal standard. Figures 30-34 represents the ZKG ginseng supplement as caffeine is slowly being added as an internal standard addition. Refer to Figure 27 for the standard addition curve for the ZKG sample.

In depth identification of a compound within a sample can be determined through several techniques. At this point it was important to verify the identity of the methylxanthines found in the various ginseng supplements. This was done using two different techniques. The first technique was through GC-MS. Figure 35 shows the
Table 6. Amount of methylxanthines per tablet in each Herbal Supplement

<table>
<thead>
<tr>
<th>Herbal Supplement Code/ Figure #</th>
<th>Mass of theobromine in mg/tablet</th>
<th>Mass of theophylline in mg/tablet</th>
<th>Mass of caffeine in mg/tablet</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME/45</td>
<td>0.040</td>
<td>0.050</td>
<td>53</td>
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<tr>
<td>CSE/58</td>
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<td>0.030</td>
<td>0.040</td>
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<td>BF/43</td>
<td>16</td>
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<td>0.030</td>
</tr>
<tr>
<td>PMU/67</td>
<td>0.030</td>
<td>0.040</td>
<td>39</td>
</tr>
<tr>
<td>EF/56</td>
<td>0.020</td>
<td>**</td>
<td>0.080</td>
</tr>
<tr>
<td>LF/57</td>
<td>0.030</td>
<td>0.10</td>
<td>0.040</td>
</tr>
<tr>
<td>ZGK/30</td>
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<td>0.040</td>
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<tr>
<td>CRG/61</td>
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<td>0.050</td>
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<td>KyG/66</td>
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<td>0.010</td>
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<td>GPM/40</td>
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<td>HP/64</td>
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<td>0.10</td>
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<tr>
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<td>0.010</td>
<td>0.10</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>GK/65</td>
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<td>0.050</td>
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<tr>
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<td>**</td>
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<td>0.020</td>
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<td>HASG*76</td>
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<tr>
<td>NMG/71</td>
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<td>**</td>
</tr>
<tr>
<td>SGES*73</td>
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</tr>
<tr>
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<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>KG/60</td>
<td>0.030</td>
<td>**</td>
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</tr>
<tr>
<td>Ga/70</td>
<td>0.20</td>
<td>0.10</td>
<td>**</td>
</tr>
</tbody>
</table>

a. in mls
** below linear detection range
Figure 25. Detection Limit graph

Linear detection graph for theobromine, theophylline, and caffeine.
HPLC analysis of the methylxanthines was done with a Hewlett Packard Zorbax column and HPLC conditions of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C and a UV wavelength of 280 nm.
Expanded detection limit graph of theobromine, theophylline, and caffeine.
HPLC analysis of the methyloxanthines was done with a Hewlett Packard Zorbax column and HPLC conditions of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C and a UV wavelength of 280 nm.
Figure 27. Internal Standard addition curve of caffeine to the ZKG ginseng supplement.

HPLC analysis of the methylxanthines was done with a Hewlett Packard Zorbax column and HPLC conditions of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C and a UV wavelength of 280 nm.
Figure 28. Chromatogram of the NGE Ginseng Supplement.

HPLC analysis of NGE ginseng supplement at a concentration of 53.64 mg/ml with the Zorbax column and the following conditions: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm. Compounds are as labeled.

Figure 29. Chromatogram of the NGE Sample “spiked” with Theophylline.

HPLC analysis of NGE ginseng supplement at a concentration of 53.64 mg/ml with theophylline as an internal standard at a concentration of 0.1 mg/ml. The Zorbax column and the following conditions were used: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm. Compounds are as labeled.
Figure 30. Chromatogram of the ZKG Ginseng Supplement.

HPLC analysis of ZKG ginseng supplement at a concentration of 49.34 mg/ml with the Zorbax column and the following conditions: mobile phase composition of 21% methanol: 79% acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm. Compounds are as labeled.

Figures 31-34. Chromatograms of the ZKG Sample at various Standard Addition Levels of Caffeine.
HPLC analysis of ZKG ginseng supplement at a concentration of 49.34 mg/ml with an internal standard addition of caffeine at a concentration of 0.5 mg/ml. The Zorbax column and the following conditions were used: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm. Compounds are as labeled.
Figure 35. GC-MS analysis of the ZKG Ginseng Supplement.
overall scan of the sample and the identification of the peak as caffeine. The second method used for identification of the methylxanthines was LC-MS. Figure 36 shows the HPLC chromatogram and the MS spectrum of the ZKG ginseng supplement. The methylxanthines of interest were identified as theobromine and caffeine.

Reproducibility is an extremely important factor in the analysis of compounds. Therefore each ginseng supplement was analyzed at least twice. It was also of interest to see if there was a difference between tablets of the same brand of ginseng as well as tablets of the same brand but which had a different lot number. In order to determine if there was a difference between tablets of the same brand, 3 tablets were uniformly prepared and analyzed twice under the same conditions in a continuous fashion. As shown in the following figures, there were no significant differences (Figures 37-39). For example the area of caffeine/mg/ml sample is very similar for each tablet, 2.53e4/50.09, 2.54e4/49.59, 2.40e4/47.24, respectively. In order to determine if there was a difference between tablets of the same brand but which had different lot numbers, one tablet from each bottle was uniformly prepared and analyzed under the same conditions. Again no significant differences were observed (Figures 40 and 41).

The following figures are a few examples of ginseng supplements in order to view the differences between the different samples (Figures 42-45). As shown each ginseng supplement varies in concentration and intensity in its components of saponins and methylxanthines. All chromatograms of each ginseng supplement can be seen in Appendix A.
HPLC analysis of ZKG ginseng supplement at a concentration of 49.04 mg/ml with the Zorbax column and the following conditions: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm.
Figure 37. Chromatogram of Tablet 1 of the ZKG Ginseng Supplement at a concentration of 50.09 mg/ml.

HPLC analysis was obtained with the Zorbax column and the following conditions: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm.

Figure 38. Chromatogram of Tablet 2 of the ZKG Ginseng Supplement at a concentration of 49.59 mg/ml.

HPLC analysis was obtained with the Zorbax column and the following conditions: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm.
Figure 39. Chromatogram of Tablet 3 of the ZKG Ginseng Supplement at a concentration of 47.24 mg/ml.

HPLC analysis was obtained with the Zorbax column and the following conditions: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm.
Figure 40. Chromatogram of the GPM Ginseng Supplement lot # 1433 at a concentration of 48.35 mg/ml.

Figure 41. Chromatogram of the GPM Ginseng Supplement lot # 2816 at a concentration of 49.42 mg/ml.

HPLC analysis was obtained with the Zorbax column and the following conditions: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm.
Figure 42. Chromatogram of Pure Ginseng Root Hairs at a concentration of 30.10 mg/ml

![Chromatogram of Pure Ginseng Root Hairs](image)

Figure 43. Chromatogram of BF Ginseng Supplement at a concentration of 50.20 mg/ml

![Chromatogram of BF Ginseng Supplement](image)

HPLC analysis was obtained with the Zorbax column and the following conditions: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm.
Figure 44. Chromatogram of GGK Ginseng Supplement at a concentration of 48.38 mg/ml

Figure 45. Chromatogram of ME Ginseng Supplement at a concentration of 29.18 mg/ml

HPLC analysis was obtained with the Zorbax column and the following conditions: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm.
IV. CONCLUSION

The main objective of this study was to develop a method that would provide a means of determining the amount of methylxanthines, (theobromine, theophylline, and caffeine), within various ginseng supplements. This goal was successfully met using reversed-phase High Performance Liquid Chromatography with the following conditions: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and a UV detection wavelength of 280 nm. The column used was a Hewlett Packard Zorbax SB-C18. The samples were injected in 5-20 µl aliquots depending on concentration. These conditions, when applied to the ginseng supplements, produced well-resolved methylxanthine peaks, which were identified by spiking sample aliquots with standards, with GC-MS and with LC-MS. Using calibration curves, the amount of each methylxanthine contained in all the ginseng supplements was determined. The reproducibility of each supplement was very consistent and fell within ± 2%.

It was determined that the identity and concentration of the methylxanthines of interest vary with the type of ginseng (Asian, American, or Siberian) as well as the manufacturer. The levels of methylxanthines in the various supplements range from 0-1 mg/dose to 15-50 mg/dose. With the recommended daily dosages listed on the manufacturer’s bottles the higher end dose would be equivalent to drinking a “pot” (8-10 cups) of caffeinated coffee per day. With this amount of caffeine within the supplements, it is apparent that some of the “energy” effects attributed to ginseng may be caused by the presence of methylxanthines. It is also important to note that some
individuals have a low tolerance to caffeine. Therefore the effect would depend upon the dosage, supplements with small doses would in fact increase energy, while the higher concentrated supplements could cause the consumer ingesting these some physiological discomfort such as nausea, intestinal pain, and maybe even vomiting. This presence of methylxanthines may be due to natural production by the plant and/or by the actual addition of methylxanthines to the ginseng preparations by the manufacturer. In either case, the presence of methylxanthines in these supplements has been brought to the consumer’s attention through an article in the Democrat and Chronicle last fall.

An area of continuing research in our laboratory is to determine if the levels of methylxanthines in the supplements are natural or additive. It will be necessary to grow our own ginseng plants and submit them to different types of stress. In the case of other plants, part of the plant’s defense mechanism to external predators/stress is to increase production of methylxanthines. Initially we plan to start by stressing the plant with arid and moist conditions while simultaneously varying the amount of direct sunlight. After these preliminary investigations various plants will be subject to various diseases and parasites. An HPLC analysis would then be performed on the plant to determine if the stresses would cause an increase or a decrease in methylxanthine levels.

A final area of great interest would be the evaluation of herbal supplements for various salicylates and ephedrine-like compounds. We would emphasize our analysis to those supplements, which promise clarity in thought, and over all long-term well being. These two claims could be the result of low doses of ephedrine and aspirin, respectively. Preliminary studies on the gingko supplements, which claim to increase brain function, have been completed. The initial conditions determined thus far consist of a gradient
elution method. For the first six minutes, the mobile phase composition is set at 50% (2%) acetic acid and 50% methanol. At six minutes, the gradient begins and, at ten minutes, a final mobile phase composition of 100% methanol is reached. The flow rate is 1.0 ml/min, column temperature of 25°C, and a wavelength of detection at 254 nm. With these conditions we found what appears to be ephedrine within the Gingko supplements. Further analysis such as LC-MS, and internal standard addition need be completely in order to accurately identify the peak as being ephedrine and to determine the amount the supplement contains chromatograms and data relating to the initial studies can be found in Appendix B.
APPENDIX A

The following ginseng supplements have all been analyzed with the Hewlett Packard Zorbax column and the following conditions: mobile phase composition of 21% methanol: 79% (2%) acetic acid, flow rate of 1.0 ml/min, column temperature of 35°C, and UV wavelength of 280 nm.

Figure 46. Chromatogram of the GGKC Ginseng Supplement at a concentration of 46.81 mg/ml.

Figure 47. Chromatogram of the SVSGR Ginseng Supplement at a concentration of 40.85 mg/ml.
Figure 48. Chromatogram of the WSGR Ginseng Supplement at a concentration of 41.93 mg/ml.

Figure 49. Chromatogram of the KWGR Ginseng Supplement at a concentration of 56.71 mg/ml.
Figure 50. Chromatogram of the WHSG Ginseng Supplement at a concentration of 50.40 mg/ml.

Figure 51. Chromatogram of the CC Ginseng Supplement at a concentration of 51.83 mg/ml.
Figure 52. Chromatogram of the HEP Ginseng Supplement at a concentration of 57.46 mg/ml.

Figure 53. Chromatogram of the SKG Ginseng Supplement at a concentration of 24.22 mg/ml.
Figure 54. Chromatogram of the SGR Ginseng Supplement at a concentration of 41.98 mg/ml.

Figure 55. Chromatogram of the KGR Ginseng Supplement at a concentration of 47.63 mg/ml.
Figure 56. Chromatogram of the EF Ginseng Supplement at a concentration of 48.38 mg/ml.

Figure 57. Chromatogram of the LF Ginseng Supplement at a concentration of 62.21 mg/ml.
**Figure 58.** Chromatogram of the CSE Ginseng Supplement at a concentration of 42.90 mg/ml.

**Figure 59.** Chromatogram of the AG Ginseng Supplement at a concentration of 51.34 mg/ml.
Figure 60. Chromatogram of the KG Ginseng Supplement at a concentration of 53.04 mg/ml.

Figure 61. Chromatogram of the CRG Ginseng Supplement at a concentration of 50.50 mg/ml.
Figure 62. Chromatogram of the SJWE Ginseng Supplement at a concentration of 57.18 mg/ml.

Figure 63. Chromatogram of the SJP Ginseng Supplement at a concentration of 45.84 mg/ml.
**Figure 64.** Chromatogram of the HP Ginseng Supplement at a concentration of 49.01 mg/ml.

**Figure 65.** Chromatogram of the GK Ginseng Supplement at a concentration of 49.23 mg/ml.
**Figure 66.** Chromatogram of the KyG Ginseng Supplement at a concentration of 45.16 mg/ml.

**Figure 67.** Chromatogram of the PMU Ginseng Supplement at a concentration of 32.55 mg/ml.
Figure 68. Chromatogram of the NGE Ginseng Supplement at a concentration of 54.61 mg/ml.

Figure 69. Chromatogram of the DQ Ginseng Supplement at a concentration of 56.19 mg/ml.
Figure 70. Chromatogram of the Ga Ginseng Supplement at a concentration of 38.53 mg/ml.

Figure 71. Chromatogram of the NMG Ginseng Supplement at a concentration of 37.50 mg/ml.
**Figure 72.** Chromatogram of the NASJW Ginseng Supplement at a concentration of 50.68 mg/ml.

**Figure 73.** Chromatogram of the SGES Ginseng Supplement at a concentration of 50.36 mg/ml.
Figure 74. Chromatogram of the ZAG Ginseng Supplement at a concentration of 51.34 mg/ml.

Figure 75. Chromatogram of the ZSG Ginseng Supplement at a concentration of 50.55 mg/ml.
Figure 76. Chromatogram of the HASG Ginseng Supplement at a concentration of 50.04 mg/ml.

Figure 77. Chromatogram of the GGTGR Ginseng Supplement at a concentration of 62.08 mg/ml.
Figure 78. Chromatogram of the HPMG Ginseng Supplement at a concentration of 46.91 mg/ml.

Figure 79. Chromatogram of the SHG Ginseng Supplement at a concentration of 52.98 mg/ml.
Figure 80. Chromatogram of the Ex Ginseng Supplement at a concentration of 27.58 mg/ml.
APPENDIX B

The following gingko samples have been analyzed with the conditions determined thus far, which consist of a gradient elution method. For the first six minutes, the mobile phase composition is set at 50% (2%) acetic acid and 50% methanol. At six minutes, the gradient begins and, at ten minutes, a final mobile phase composition of 100% methanol is reached. The flow rate is 1.0 ml/min, column temperature of 25°C, and a wavelength of detection at 254 nm.

Figure 81. Chromatogram of the Pure Ephedrine standard at a concentration of 0.01 mg/ml.

Figure 82. Chromatogram of the NGB Gingko Supplement at a concentration of 56.96 mg/ml.
Figure 83. Chromatogram of the GP Gingko Supplement at a concentration of 59.32 mg/ml.

Figure 84. Chromatogram of the GBL Gingko Supplement at a concentration of 29.14 mg/ml.
REFERENCES

Ginseng References


7. S. Foster, Herbs for Your Health., Interweave Press, Loveland, CO. 48


Gingko References


