5-2007

ANALYSIS OF NUTRACEUTICALS FOR TOXIC METALS: DEVELOPMENT AND VALIDATION OF SAMPLE PREPARATION METHOD

Julia Cooper
Clemson University, jecoope@clemson.edu

ANALYSIS OF NUTRACEUTICALS FOR TOXIC METALS: DEVELOPMENT AND VALIDATION OF SAMPLE PREPARATION METHOD

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Chemistry

---

by
Julia Elizabeth Cooper
May 2007

Accepted by:
Dr. R. Kenneth Marcus, Committee Chair
Dr. George Chumanov
Dr. Steven Creager

ABSTRACT

With increasing numbers of health-conscious consumers purchasing nutraceutical supplements, total sales in the nutrition industry have soared to over $200 billion in 2006.[1] Approximately $50 billion of this was spent in the United States on functional foods and supplements, which are categorized as nutraceuticals and perceived to afford health benefits to the consumer. It is important to assess the safety of these nutraceutical products, with specific regards to toxic metals, including arsenic, cadmium, lead, and mercury. Because species of these metals may be more toxic than others, they can have hazardous health effects if ingested in excess quantities. Nutraceutical products can also vary by manufacturer, even if they contain the same active ingredient, and the plant ingredients used in manufacturing can be contaminated by pesticides or the soil in which they are cultivated.[2]

For many years, nutraceutical products fell under the category of "food" and, as such, they were not subject to strict restrictions, such as those established for "drugs." After the establishment of the Dietary Supplement Health and Education Act, which defined a dietary supplement, the U.S. Food and Drug Administration was able to set forth guidelines to establish good manufacturing practices (GMPs) for the production dietary or nutraceutical supplements. Though not focused directly on nutraceuticals, the State of California also enacted

Proposition 65, which establishes levels of toxic elements or compounds that must not be exceeded on a daily basis.

The work described in this thesis focuses on the development of a sample preparation method for nutraceutical products, specifically ethanolic tinctures and glycerin-based matrices, and analysis of these products by inductively coupled plasma atomic emission spectrometry (ICP-AES) for toxic metal content. This method was developed by utilizing microwave digestion, which allows high temperatures and pressures for complete digestion of difficult sample types. A method was then established using an ICP-AES instrument that allowed simple and fast analysis through the generation of calibration functions for each toxic metal. Botanical standard reference materials (SRMs) of Ephedra sinica stapf and Gingko biloba were analyzed for arsenic, cadmium, lead, and mercury to assess the validity of the developed sample preparation method.

DEDICATION

This work is dedicated to my parents, Richard and Jennifer Cooper, to my brother and sister, and to my husband, Bryan.

ACKNOWLEDGMENTS

I would like to thank Dr. R. Kenneth Marcus, my research advisor, for the opportunity to conduct this research and the experience of presenting research at national conferences. I would also like to thank my committee, Dr. George Chumanov and Dr. Stephen Creager.

I am extremely grateful to the Clemson University Institute for Nutraceutical Research for financial support for this research. I am appreciative of Gaia Herbs of Brevard, NC for supplying the nutraceutical samples for this study. I would also like to extend thanks to Lyondell Chemical Company of Newtown Square, PA for the use of their Spectroflame Module E ICP-AES instrument for completion of this study.

Special thanks go to Dr. John Kaup and Russell Hubbard of the Chemistry Department for their assistance with the JY 24 ICP.

I would also like to thank my colleagues in the Marcus group for their assistance, support, and friendship.

TABLE OF CONTENTS

Page

Table of Contents (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

Introduction to Nutraceuticals

The nutraceutical industry is a multibillion dollar per year industry, which encompasses herbal products, vitamins, dietary supplements, and tinctures.[1] It has undergone a steady rise in the number of manufacturers and products available to consumers, as well as the increased demand for supplements for the health-conscious. Some consumers use them as a part of a modified lifestyle, as a way of assisting a change to a healthier diet and more physically active life. Others use supplements as an alternative to prescription medicines and view them as a more natural remedy for ailments.[3] Because of the widespread use of, and growing demand for, nutraceutical products, the nutraceutical industry faces continued scrutiny in the areas of product supply, quality, and safety.

One of the major concerns in the nutraceutical industry is the potential presence of toxic metals, particularly arsenic, cadmium, lead, and mercury, in ingredients or final products. It is well known that these metals can cause adverse effects if they are ingested in large quantities or in their more toxic forms, insinuating the need for methods of detection and speciation of these metals. For example, inorganic forms of arsenic are more toxic than the organic form, the organic methyl mercury is more toxic than other mercury forms, while all forms of lead and cadmium are toxic.[4] These metals can be naturally occurring in soils

and can also be incorporated into the environment by contamination, via pesticide usage or chemical runoff. Therefore, it is easy to understand how they can be accumulated in the ingredients of nutraceutical supplements, especially those that primarily contain raw plant material.

In this thesis, a method for sample preparation of nutraceutical supplements by microwave digestion and determination of toxic metals by inductively coupled plasma atomic emission spectrometry (ICP-AES) is described. Chapter 1 details the challenges regarding the nutraceutical industry and the need for new methods for the assessment of product safety. Ideal characteristics of a newly developed method are described, along with basic principles of microwave digestion and ICP-AES. Chapter 2 presents research focused on development and validation of a method to analyze nutraceutical supplements for toxic metal content. It primarily focuses on the sample preparation for these supplements, a critical component of the analysis process, as well as the optimization of the ICP-AES system for analysis and quantification and the validation of the developed procedure. Finally, Chapter 3 discusses conclusions drawn from this study and proposes future studies for the continuation of this project.

Challenges Facing Nutraceutical Industry

One of the major challenges facing the nutraceutical industry is the implementation of the testing of products in the marketplace. Regulations by the government, with specific regards to these products, have not been heavily enforced. Until 1994, nutraceutical products fell under the heading of food

products and were subject to regulation under the Federal Food, Drug, and Cosmetic Act (FFDCA).[5] At that point, Congress passed the Dietary Supplement Health and Education Act (DSHEA), as an amendment to the FFDCA, to develop regulations on dietary supplements.[6] The DSHEA defined a dietary supplement as "a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients."[5] These ingredients include vitamins, minerals, herbs, botanicals, amino acids, and also combinations of these ingredients. In addition to defining the term "dietary supplement," the DSHEA also provided for the U.S. Food and Drug Administration (FDA) to establish good manufacturing practices (GMPs) for dietary supplements. These GMPs would be modeled after those that were already developed for foods; however, they would take several years to be developed.

In 2003, the FDA issued its Proposed Rule on Dietary Supplement GMPs.[5, 7] In the near-decade between the enactment of DSHEA and the FDA's proposal, there was communication between the FDA and the dietary supplement industry with regards to developing these GMPs. The industry sent the FDA information about its manufacturing practices in 1995, and the FDA published questions that it wanted the industry to answer in 1997, so the Proposed Rule was a long time in the making. However, these GMPs were only intended to address concerns in one area of safety of the dietary supplements. They were designed to ensure that manufacturers test their products for safety and effectiveness and that they indeed meet label claims as to what was included in the supplement. It only required that the final product be tested, however it did recommend testing of

individual ingredients and raw materials before manufacturing, as well as any material to be used for product packaging. The responsibility of the FDA is not to "approve" safety and effectiveness of a sample; however, the FDA can act to prove that a product is "unsafe" before actions are taken to remove it from the marketplace.

Even with DSHEA and the FDA's Proposed Rule, there still has not been any regulation on dietary supplements where safety, in the area of toxicity, is the major concern. Though not specifically aimed at dietary supplements and other nutraceuticals, the state of California's Proposition 65 established "levels of concern" for the exposure to hazardous chemicals and compounds. This Proposition 65 stems from California's Safe Drinking Water and Toxic Enforcement Act of 1986, which governs contamination of drinking water.[8-10] The law was updated in August 2003 to include Proposition 65, with the basic premise that a business has to warn a consumer of possible exposure to a substance that has been known to either cause cancer or reproductive problems.

Stipulations of the law include "Safe Harbor Levels", which indicate levels for each chemical or compound that must not be exceeded. Chemicals that are known or suspected to cause cancer are assigned "No Significant Risk Levels" (NSRLs). These represent "the daily intake level calculated to result in one excess case of cancer in an exposed population of 100,000, assuming lifetime (70 year) exposure at the level in question."[10] Reproductive toxicants are assigned Maximum Allowable Dose Levels (MADLs), which are the levels "at which the chemical would have no observable adverse effect assuming exposure at 1,000

times this level."[10] The safe harbor levels for some chemicals and compounds are still in development. The process for adopting an assigned safe harbor level is lengthy; therefore, priority levels have been established for those chemicals. Known safe harbor and priority levels for arsenic, cadmium, lead, and mercury are listed in Table 1.1. It is also important to note that the route of exposure is indicated for some of these levels. As indicated, the level that would cause adverse reproductive effects is higher than the level for carcinogenicity for cadmium, while the opposite is true for lead. All chemical forms of both of these metals are toxic, while there is a distinction made for the chemical forms of arsenic and mercury that are more toxic.

	As	**Cd**	**Hg**	**Pb**
NSRL (µg/day)	0.06 (inhalation) 10 (except inhalation)	0.05 (inhalation)	1st Priority for methyl mercury compounds	15 (oral)
MADL (µg/day)	1st Priority for inorganic oxides (2003 draft: 0.10 µg/day)	4.1 (oral)	1st Priority for methyl mercury compounds (1994 draft: 0.3 µg/day)	0.5

Table 1.1: Table of Proposition 65 Hazard Levels. NSRL indicates No Significant Risk Level. MADL indicates Maximum Allowable Dose Level.[10]

Lastly, for arsenic and mercury, some of the levels have not been fully established and priority levels, ranging from 1st to 3rd, have been assigned for their investigation. For these metals, draft levels have been given as a guideline until final levels are established.

Sample Preparation Techniques

Before sample analysis, sample preparation is typically a necessary step to modify the sample of interest for analysis. Different instruments require different types of sample modification, such as dissolution or digestion for wet chemistry techniques, or even the modification by chemical reaction after a digestion step. The type of modification that can be readily performed also depends heavily on the sample itself. Nutraceutical products exist in a variety of different forms and therefore have very complex matrices that must be digested or dissolved so that the sample is fully aqueous before analysis by ICP-AES.

Wet ashing and dry ashing are common methods of sample digestion procedures that utilize concentrated acids for the digestion of solid samples.[11] A traditional hot-plate can also be used for an open vessel digestion, whereby the sample is heated slowly in an open container. However, the most obvious drawbacks to these techniques are that they are very time-consuming, on the order of several hours to days, and increase the risk of sample contamination. Hot-plate digestions are also limited by the number of samples that can be digested at once, the uneven heating of each sample while on the hot-plate, and the loss of volatile analyte species.

Microwave digestion techniques can overcome the difficulties associated with more conventional digestion methods. First used in 1975, microwave digestion gained popularity because it can be performed in either open or closed vessels, which allow for higher pressures and temperatures to be achieved.[12] Early microwaves were modified home microwave appliances, but newer commercial systems have more safety features, including the ability to handle acid vapors and resist corrosion. The addition of pressure and temperature control in 1989 and 1992, respectively, allowed more adaptability and control of digestions.[13] Vessel technology has also progressed to allow pressures of up to 60-100 atm. Extensive investigation into microwave sample preparation has been studied and reviewed by Kingston and colleagues.[12-15]

Microwave digestion utilizes non-ionizing radiation, which causes the migration of ions and rotation of dipoles.[12] The frequency can range from 300-300,000 MHz, but 2450 MHz is most commonly used in commercial systems and corresponds to a wavelength of 12.2 cm, chosen so as not to interfere with typical telecommunication wavelengths.[13] The magnetron inside the microwave unit typically produces 600-700W of microwave energy. The construction of the microwave utilizes three classes of materials: reflective, transparent, and absorptive.[12] Reflective materials are used in the actual unit construction, are typically metals, and act to keep the microwave radiation inside the unit. Transparent materials are used in the components that are inside the microwave cavity and allow microwave radiation to pass through them. The sample

solutions, water, and acid are absorptive materials, and therefore absorb energy generated from the microwave radiation.

Although microwave unit construction has evolved since its early years, a generalized schematic of a microwave system is shown in Figure 1.1.[12, 13] The microwave generator is a magnetron, a cylindrical diode with an anode and a cathode upon which a magnetic field is imposed. A waveguide made of reflective material serves to propagate the microwave energy in the microwave cavity, where it is reflected from wall to wall. The mode stirrer, a fan-shaped blade, reflects and mixes energy and distributes it in various directions, while a turntable rotates the samples for even heating. The sample vessels used in microwave digestion vary and depend upon whether open vessel or closed vessel digestion is being performed.

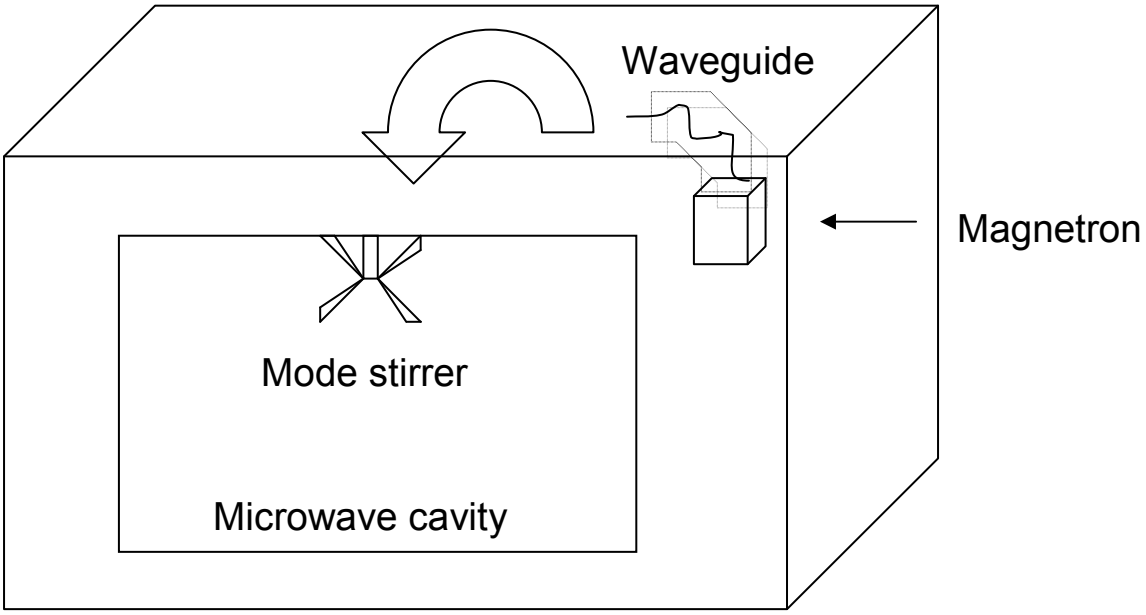


Figure 1.1. Generalized schematic of a commercially available microwave digestion system.

Containers used for open vessel microwave digestion can be the same used for hot-plate digestions, such as beakers and flasks.

Microwave digestion, like ashing techniques, employs acids for the breakdown and digestion of a sample, with the type of acid used depending heavily on the sample matrix. The most common acids used are nitric acid, hydrochloric acid, and perchloric acid, and also combinations of these acids.[12, 13] Nitric acid is the most suitable acid for digestion of biological and botanical samples because it is a very strong oxidizing agent. Hydrochloric acid is ideal for digestion of metal oxides, silicates, refractory oxides, and basic compounds. Hydrofluoric acid is usually added to a sample if silica is present, but it is not used in glass or quartz vessels due to etching. Care has to be taken when using sulfuric acid, as it can melt most plastics before it reaches its high boiling point, but it is advantageous because it can completely destroy organic compounds. A typical acid combination is aqua regia, a solution of 3:1 HCl to HNO_3, which is more powerful than either acid alone. Perchloric acid has a significant safety hazard in that it can react explosively with organic materials, but it can be used to attack metals when other acids are unsuccessful. Hydrogen peroxide 30% is sometimes used in combination with an acid to increase oxidation power, but because of its strong reactivity with organic matrices, like perchloric acid, it is added after a predigestion of the sample with acid. There are several examples in literature of different digestion protocol involving nitric acid only,[16, 17] nitric and perchloric acids,[18] as well as acid combinations with or without peroxide.[19, 20]

Basic Principles of ICP-AES

The theory for ICP-AES has been around since the 1960s, but the first instrument was not commercially available until the mid-1970s.[21] The modern ICP-AES systems on the market are capable of analysis of 70 different elements and have a large linear dynamic range of over 5 orders of magnitude. Traditionally, the multielemental capabilities were achieved through rapid sequential wavelength scanning, but newer technology now affords simultaneous wavelength measurements. Typical detection limits for ICP-AES range from 0.1 ppb, for magnesium and calcium, to 50 ppb, for arsenic and thorium, but this depends largely on instrument configuration and sample type.[22] Though ICP-AES operation costs are large due to the inert gas consumption, they are less than inductively coupled plasma mass spectrometry (ICP-MS), where more cost is incurred for the mass spectrometric detector and upkeep. The general schematic for an ICP-AES is shown in Figure 1.2. Flow of a sample through the ICP system begins with sample nebulization and uptake into the plasma, followed by excitation and emission of light from excited atoms. Separation of the emitted light into characteristic wavelengths occurs via an optics system, followed by detection and amplification of the light signal. The light signal is converted into the digital domain and a computer is used to store and analyze data.

Sample introduction into the ICP is typically achieved through the combination of a nebulizer and a spray chamber.[22] The most commonly used nebulizer and spray chambers are shown in Figure 1.3. The sample is most commonly in liquid form, which usually necessitates a dissolution step or an acid

digestion procedure beforehand. The nebulizer acts to convert the liquid sample into a fine aerosol, which is then carried into the spray chamber. The spray chamber filters out the larger sample droplets, which are collected as waste, from the smaller droplets, which are then carried by argon gas into the plasma. Efficiencies for nebulizer and spray chamber combinations are in the range of 1-3%. Standard pneumatic nebulizers are of the Meinhard style, in Figure 1.3a, which afford a stable signal because they can be self-aspirating, but they are not

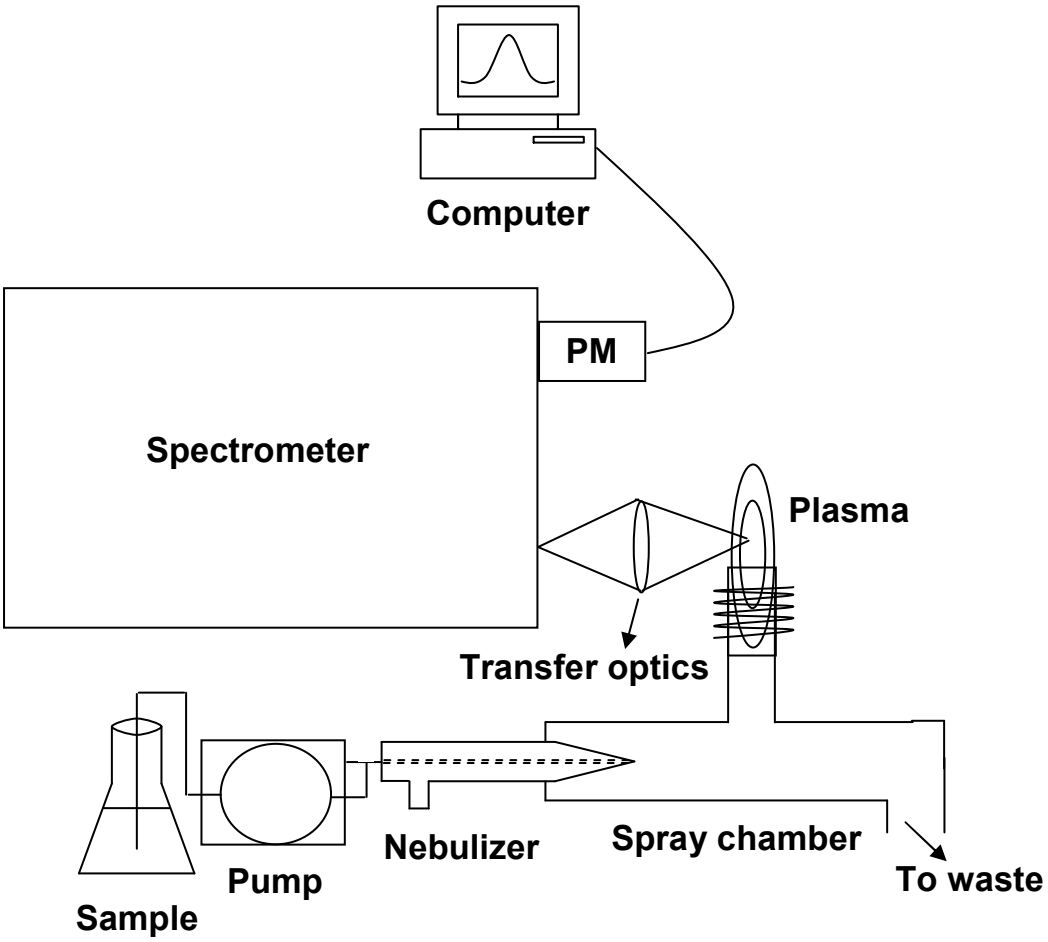


Figure 1.2. Schematic of an ICP-AES instrument.

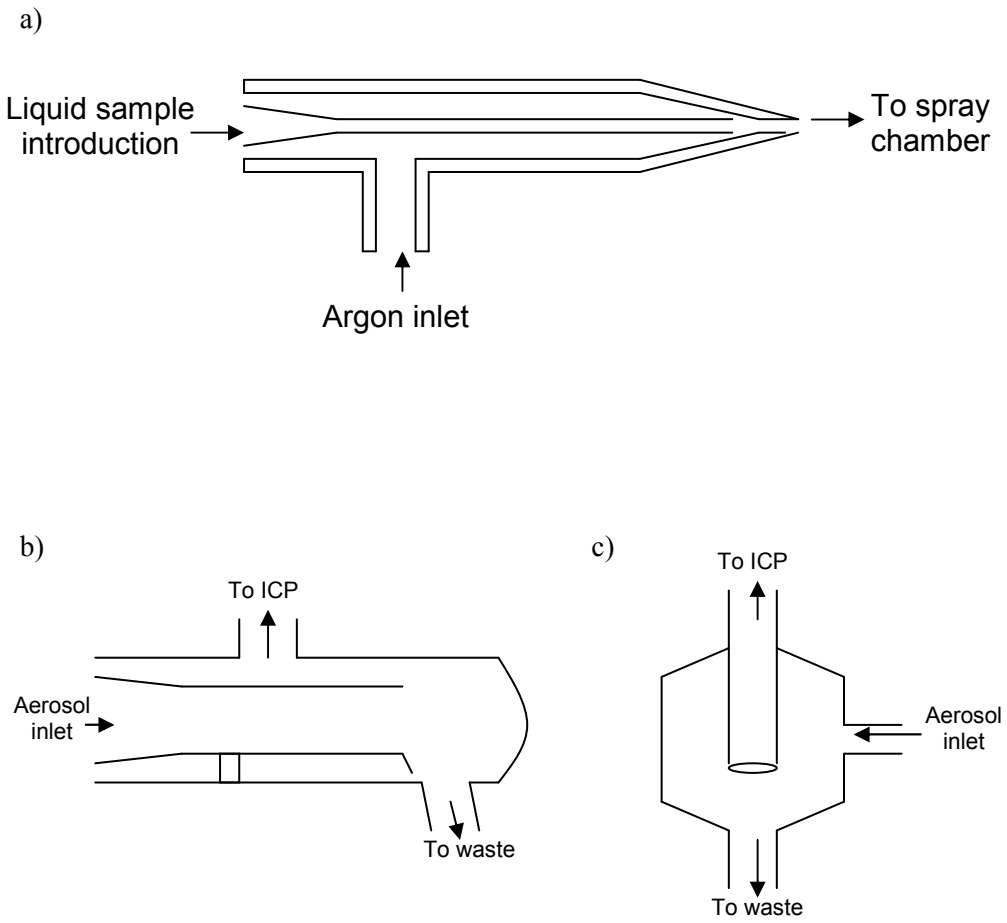


Figure 1.3: a) Schematic of a pneumatic nebulizer. b) Schematic of a Scott-type spray chamber and c) cyclonic spray chamber.

tolerant of particulates in the sample. Spray chambers are commonly one of two types: Scott type and cyclonic, Figures 1.3b and 1.3c, respectively. Scott type spray chambers are also known as "double-pass" and consist of two glass tubes. The aerosol passes into the inner tube and larger droplets are carried to waste while the smaller droplets are carried up into the plasma. An advantage of the Scott type is reduced aerosol turbulence, which increases signal stability, while the main disadvantages of this type are areas of dead volume inside the spray chamber, which lead to longer wash times and memory effects.[23] The cyclonic spray chamber is advantageous over the Scott type because of its smaller volume and significantly greater transport efficiency than other spray chamber types.[23, 24] Sample is introduced into the spray chamber, swirls downward, and is carried up into an internal spiral. This allows shorter wash times and therefore less sample is used and chances of memory effects are lower.[22]

The formation of the plasma is the critical step in ICP-AES.[21] Inert argon gas passes through the torch of the instrument, which has a copper coil connected to a radiofrequency (RF) generator. The frequency of the generator can range from 6-100 MHz, with most available instruments utilizing a 27.12 or 40.68 MHz frequency. Higher frequencies afford a toroidal shaped plasma. Utilizing the 40.68 MHz frequency allows greater coupling efficiency, greater stability, and improved plasma robustness.[24] However, shielding is required, to eliminate possible interferences with surrounding electrical equipment, and is more difficult with 40.68 vs. 27.12 MHz.[25] Typical operating powers are between 900 and 1500 W and depend upon the sample matrix. Plasma formation starts with an

alternating current that oscillates at a particular frequency, setting up a magnetic field at the top of the torch. A spark is applied from a Tesla coil, which acts to strip electrons from the argon gas atoms. These electrons then collide with other gas atoms, creating a plasma that is self-sustaining and toroidal in shape. In the tail of the plasma flame, the temperatures can be 5000-6000K, causing analytes to be excited and emit radiation at characteristic wavelengths.

While the high temperatures of the plasma prevent a lot of interferences, such as those arising from molecule formation from occurring, no spectrometric technique is truly interference-free.[25] Common interferences in ICP-AES include instrument drift, matrix effects, and spectral interferences.[21] Instrumental drift can be corrected by an internal wavelength calibration. Matrix effects, whereby the sample and standards have different transport efficiencies, i.e. they do not have the same matrix, can be remedied by matrix-matching of sample and standards as well as use of an internal standard. Spectral interferences can also occur, when the emission wavelength for the element of interest cannot be resolved from emission from another element, atom, or ions that are close by.[25] Due to the abundance of wavelengths for many elements, an alternate wavelength can usually be selected to eliminate this problem.

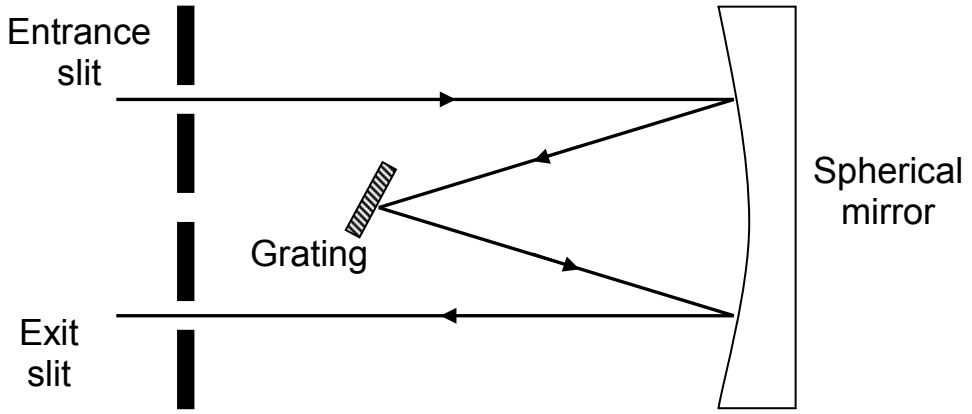
The orientation of the plasma can be one of two different geometries. In the radial geometry, the plasma is vertically oriented and optically thin. This geometry affords the probability of fewer interferences because of the short distance that the emitted light has to travel to the optics system.[21] The other geometry possibility is axial, where the plasma is horizontal, pointed towards the

optics system. Usually this type of geometry involves a shear gas that cuts off the tail end of the plasma to dissipate the hot gases. Axial plasmas have better sensitivity, as the emitted light is collected with greater efficiency.[21]

There are two main classes of dispersive optics systems for an ICP-AES instrument: monochromators, which isolate a single wavelength band at a time, and polychromators, which are capable of isolating many wavelength bands simultaneously.[21] Typical monochromators include the Ebert style, with one large mirror, and Czerny-Turner, which uses two mirrors. These monochromators are shown in Figure 1.4. In the Czerny-Turner configuration, light enters the monochromator through an entrance slit and is then reflected off a collimating mirror and onto a diffraction grating. Diffraction gratings have groove densities ranging from 600-4200 grooves/mm, with higher resolving powers achieved at the larger densities. In a sequential scanning monochromator, the diffraction grating is moveable and rotates to different angles corresponding to different wavelengths. From the diffraction grating, the light passes onto another collimating mirror and is focused onto the exit slit placed in front of the detector.

Polychromator optics systems, shown in Figure 1.5, allow more rapid analyses due to their ability to simultaneously differentiate and detect many wavelengths. In the Rowland circle polychromator with Paschen-Runge mounting, the grating and slits are aligned and fixed permanently along the circumference of a circle. The detectors, typically photomultiplier tubes (PMTs) are fixed behind the exit slits. The main advantage of this type of mounting is its very large wavelength coverage.

a)



b)

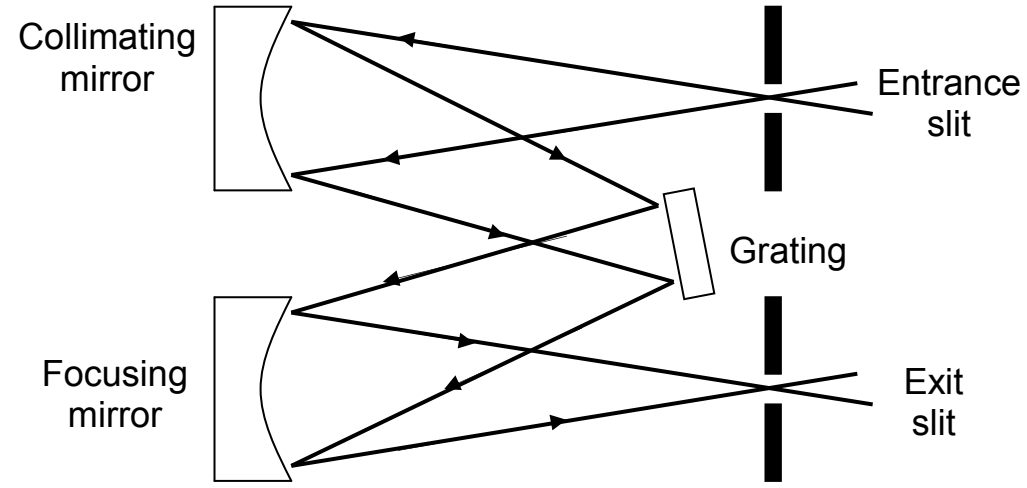


Figure 1.4: a) Schematic of the Ebert monochromator. b) Schematic of the Czerny-Turner monochromator.

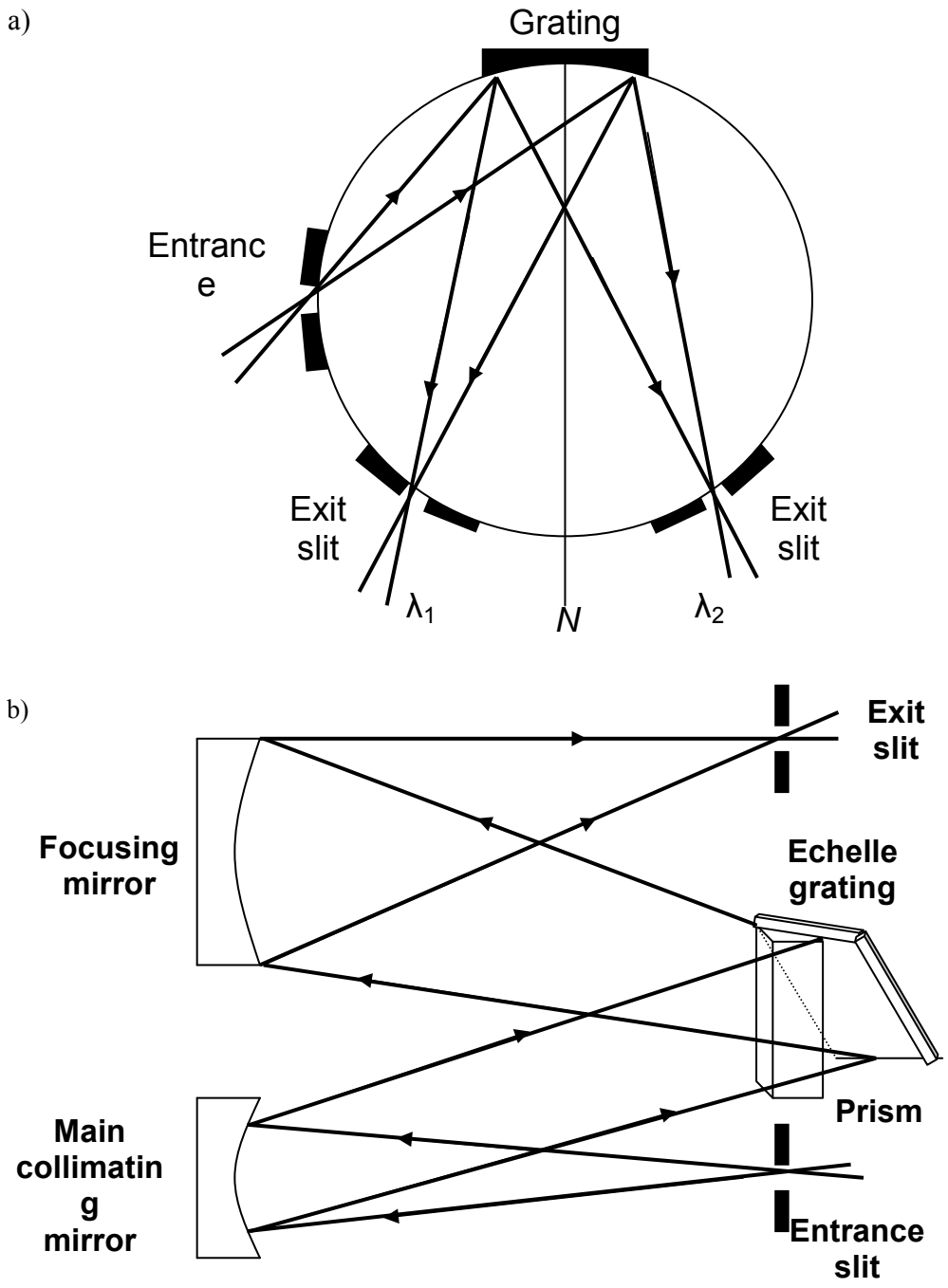


Figure 1.5: a) Schematic of the Rowland circle polychromator with Paschen-Runge mounting. b) Schematic of the Echelle polychromator.

Also available on newer, commercial instruments is the Echelle polychromator. In this configuration, a diffraction grating and a prism are set up perpendicular to each other. The grating has a low groove density, which allows the production of multiple overlapping orders of light. The orders of light are then separated by the prism into a 2-dimensional pattern that is focused onto the detector.

The most common detector, until recently, is the PMT.[21] PMTs are advantageous due to large wavelength coverage, from 160-900 nm, and their operating premise is fairly simple. Emitted light enters the PMT, strikes the photocathode, and ejects electrons. These electrons cascade down a dynode chain, emitting more electrons, which are collected by the anode. Typical PMTs have 9-16 dynodes, with signal amplification of $10^{6\text{-}10}$x. The electrons measured correspond to the current measured at the anode, which is proportional to the number of photons striking the cathode, which is, in turn, proportional to the concentration of analyte in the sample.

Solid state charge-coupled (CCD) and charge-induced devices (CID) are detectors used for instruments with simultaneous measurement abilities.[22] These detectors are composed of pixels and have 3 parts: a photosensitive area, a storage area, and readout register. Photons from incident radiation strike the photosensitive area, where they are then pulled away from the surface and amassed in the storage area. Upon accumulation, the charges are then transferred to the readout register. CCDs and CIDs are well-suited for use with an Echelle spectrometer, where the ability to produce 2-D information is advantageous. However, the main disadvantage of this type of detector is the potential for

blooming, where charges spill over onto adjacent pixels, but newer instruments have implemented anti-blooming technology to prevent this.[22]

Other Techniques for Analysis of Aqueous Samples

There are several analytical techniques available for analyzing the same sample types that are discussed in this text. It is useful to include a brief overview of some of these techniques.

Inductively Coupled Plasma Mass Spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS) was developed in the early 1980s and utilizes the same atomization principles as ICP-AES. Simplistically, it consists of an inductively coupled plasma that is interfaced with a mass analyzer. The technique has advantages of high sensitivity and low backgrounds, which allow low limits of detection on the order of sub-ng/L.[21]

Sample introduction into an ICP-MS is capable of being changed to analyze samples that are solid, liquid, or gas. A liquid sample can be nebulized in the same manner as in ICP-AES, but uptake can also be facilitated by flow injection, aerosol desolvation, or direct injection.[21] Solid samples can be atomized by laser ablation, spark ablation, or slurry nebulization, while gaseous samples are adapted for analysis by cold vapor or hydride generation. Flow rates are lower than ICP-AES and range from 0.1-0.5 mL/min, while typical powers used are 900-1500W, similar to ICP-AES .[21, 22, 25]

Formation of the plasma occurs under the same principles as in ICP-AES. A schematic of an ICP-MS instrument is shown in Figure 1.6. Between the plasma and the mass analyzer is an interface region, consisting of a step-down

vacuum stage and two cones, a sample and a skimmer cone, that each has a small orifice.[21] This stage allows a representative sample of the ion population in the plasma to be extracted and then transferred to the high vacuum region. Once in the high vacuum region, the ions are focused through a series of electrostatic lenses before reaching the analyzer.

The next components of the ICP-MS system are the mass analyzer and the detector. The quadrupole mass analyzer is the most common for inorganic MS. It separates ions based on mass/charge (m/z) ratios over a mass range of 4-260 amu, with a resolution of 1 Da, and is relatively small in size.[21, 24] Other mass analyzers include the magnetic sector analyzer, which has a curved ion flight path, and the time-of-flight (TOF). The magnetic sector analyzer can obtain mass resolution higher than unit resolution and has limits of detection 5-10X lower than the quadrupole.[21] Time-of-flight analyzers are simultaneous, making them advantageous when coupled to chromatographic and laser ablation sample introduction.[24] The most common detector used in ICP-MS is the electron multiplier (EM), which operates much like a photomultiplier tube (PMT).

One of the major applications of ICP-MS is in the area of agriculture and the environment.[21] Trace element analysis of food and geological specimens allow for a "fingerprint" for each sample type for comparison to other samples of the same types.

Because of the ability to differentiate between m/z ratios of species present in a sample, ICP-MS is a valuable technique for analyzing samples where a particular species may be a concern, i.e., it is more toxic than another species. This is especially important in regards to contamination in the environment, which can include pesticide usage and runoff.[17, 21, 26]

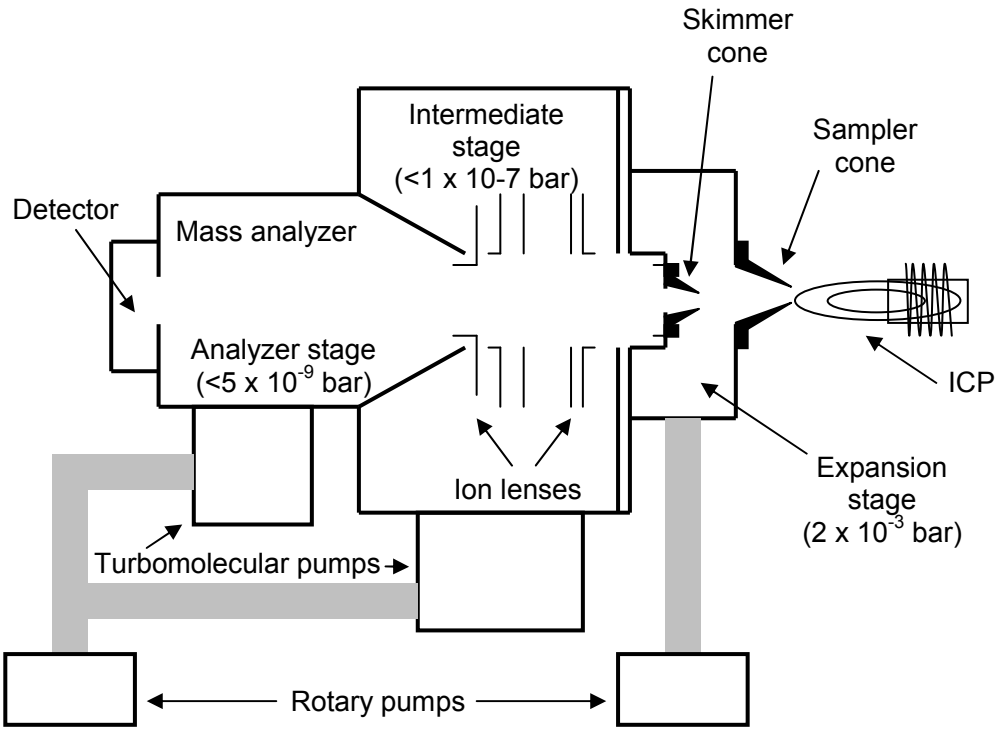


Figure 1.6: Generalized schematic of an ICP-MS instrument.

Other applications for use of ICP-MS are in the field of semiconductors, for detection of both dopants and contaminants, as well as in clinical and pharmaceutical settings, where primary sample types are body fluids and tissues.[21]

Atomic Absorption Spectrometry

Atomic absorption instruments employ a line source, most commonly a hollow cathode lamp (HCL).[21, 22] A HCL contains atoms of the element of interest, an inert gas, and a cathode and anode. Cathodic sputtering produces the atomic vapor, causing radiation emission and absorption by the atoms in the flame. Detection in AA techniques is accomplished by photomultiplier tubes (PMTs), like those used in ICP-AES, as well as monolithic solid-state devices, which are more recent advancements that afford simultaneous measurements. A variation in instrumental design utilizes a double-beam spectrometer as the optics system, which reduces baseline noise and improves detection limits.

Atomic absorption spectrophotometry (AAS) is generally classified by the type of atomizer used: a high temperature flame or an electrothermal atomizer. Graphite tubes or cup furnaces are commonly used in electrothermal AAS.[22] In graphite furnace AAS, a liquid sample of known volume, 5-50 μL, is placed in the furnace and undergoes a multistep temperature program. This temperature program acts to separate out the analyte of interest from other matrix components by vaporization. The temperature is increased to the point of atomization and it is at this point that an atomic absorption measurement is made. The temperature program consists of a drying step, followed by pyrolysis, which vaporizes the matrix of the sample. A cool-down step after pyrolysis allows greater sensitivity and a reduction in peak tailing because a flat temperature profile is achieved. Also, cooling allows an increase in temperature range, thereby increasing the heating rate. Atomization then completely dissociates the sample and converts

the sample into a vapor of free atoms. The final step is a cleanout procedure, which burns off any remaining sample residue.

A second technique for atomic absorption utilizes long, thin flames. Flame AA is a very rugged technique for liquid samples, is relatively inexpensive, and affords detection limits at the mg/L level and lower.[21] The flames in this technique are most commonly composed of air-acetylene or nitrous oxide-acetylene. Sample introduction into the instrument occurs via nebulization into a spray chamber, where oxidant and fuel gases mix.[21, 22] As with ICP-AES, the spray chamber also filters out larger sample droplets. Once in the flame, the solvent evaporates and leaves a salt behind, which later produces atoms as a byproduct of flame reactions.

Hydride generation can be coupled with spectrometric techniques to afford better detection of volatile elements, such as arsenic, selenium, bismuth, and others.[27] The basic premise of hydride generation (HG) involves the conversion of the volatile element into a stable hydride, either in a reaction vessel or in a continuous flow or flow injection mode.[21, 22] A flow of reducing agent, typically sodium borohydride, $NaBH_4$, is reacted with an acidified sample, producing the covalent metal hydride and excess hydrogen. This method of sample introduction affords greater transport efficiencies, up to 80% versus 1-3% in typical ICP instrumentation, because it eliminates the nebulizer/spray chamber set-up, i.e., the vapor is carried directly to the plasma.[23] Other advantages include lower detection limits for these elements and essentially matrix-free detection; however, the experimental procedure is more complicated and the excess hydrogen requires

removal, by nitrogen trap.[21, 22] Some recent applications of this technique include determination of arsenic and selenium in biological and herbal samples.[18, 28]

Cold vapor generation (CV) is used to aid in the analysis of mercury by conversion of inorganic mercury(II) to elemental mercury.[22] It involves a similar setup to that of HG. More commonly, however, stannous chloride, $SnCl_2$, is used as the reducing agent rather than sodium borohydride. A disadvantage of this technique is water mist entering the observation cell, when coupled to AAS, which causes scattering. To eliminate this, a trap is used to collect the mist or the carrier gas and observation cell is heated.[29] Major advantages to this technique are a lower detection limit for mercury, on the order of single-digit ng/L, and, by using $SnCl_2$, excess hydrogen generation is avoided.[22]

As with any spectrometric technique, AA measurements can suffer from interferences. Spectral interferences tend to dominate because of significant background absorption, which can sometimes overlap the absorption of the analyte.[21, 22] Matrix modification can be performed to control the matrix background. Flame AAS suffers from flicker noise, due to fluctuation of the flame. A common nonspectral interference in graphite furnace AAS occurs in the condensed-phase, when the analyte of interest forms a volatile compound, which is then lost during the pyrolysis step. Spike recovery experiments can reveal gas-phase interferences, where the matrix affects the atomization efficiency of the analyte. Advantages of graphite furnace AAS include low detection limits and increased sensitivity versus flame AAS. The sample size and gas consumption is also decreased in graphite furnace AAS. However, graphite furnace AAS is met

with several disadvantages, a major one of which is analysis time. Analysis is very slow and the working range of the instrument is very narrow, around 3 orders of magnitude. Applications of AAS include the determination of major constituents, such as calcium, iron, and magnesium, in food, biological, and herbal samples, where this technique is used in conjunction with ICP-AES or ICP-MS to determine concentrations of minor or trace constituents.[11, 16]

CHAPTER TWO

ANALYSIS OF NUTRACEUTICALS FOR TOXIC METALS: DEVELOPMENT AND VALIDATION OF SAMPLE PREPARATION METHOD

Introduction

In 2006, the nutrition industry was responsible for $210 billion in total global sales.[1] This was increased by approximately $20 billion from the previous year. Approximately one-third of the total global sales were attributed to supplements, with the remaining two-thirds attributed to functional foods, natural and organic foods, natural and organic personal care, and household products. Sales of these products in the United States alone comprised $75 billion of the total global sales, with $21 billion of it spent on supplements. It is apparent from this data that the nutrition industry is experiencing a continual rise in product manufacturing and usage by consumers.

With the financial boom in the nutrition industry, a lot of attention has been focused on the safety of nutritional products. Nutraceutical products, a branch of nutritional products comprised of functional foods and dietary supplements, are perceived to afford some health benefit to the user and these benefits may be promoted on product labels. However, the validity of these health benefit claims, as well as the overall safety of these products, has generally not been tested and confirmed. There have been strides toward establishing rules

and regulations on the manufacturing and testing of nutraceutical products in recent years, but due to the diversity of products available, as well as the large number of manufacturers, creation of these regulations and testing protocol to be applicable to all products is very difficult and time-consuming.

The most recent developments in supplement product regulation include the Dietary Supplement Health and Education Act (DSHEA), which defined a dietary supplement with specific criteria, and the Food and Drug Administration's Proposed Rule on Dietary Supplement Good Manufacturing Practices (GMPs), established to address safety concerns with regards to label claims of dietary supplements.[5-7] In addition to these federal regulations, the state of California has enacted Proposition 65, an amendment to the Safe Drinking Water and Toxic Enforcement Act of 1986.[8-10] This amendment establishes "Safe Harbor Levels" for many substances and compounds that are known or suspected to cause cancer or adverse reproductive effects. While Proposition 65 is not specifically aimed at nutraceutical products, it does set forth specific guidelines for daily maximum exposure to toxic species, some of which can potentially be found in nutraceutical products.

Toxic metals that can be present in nutraceutical products include arsenic, cadmium, lead, and mercury. However, for some of these elements, there are species that pose more risk of toxicity than others. For example, organic methyl mercury is more toxic than other mercury species, because elemental mercury and other mercury compounds are converted to methyl mercury upon ingestion. The converse is true for arsenic, however, in that the inorganic arsenic oxides are more

toxic than organic arsenic species. There is no distinction made between species of lead and cadmium, as all forms of both metals have toxic qualities. Because toxicity varies by species for arsenic and mercury, but not for cadmium and lead, it is important to determine total metal concentration in nutraceutical supplements, as well as concentrations for specific species.

Nutraceutical products are available in a wide variety of matrices, including ethanolic tinctures, glycerin-based supplements, powders, and tablets. With such a spectrum of sample types, it is a big challenge to develop methods for the analysis of toxic metals that are applicable to most, if not all, of these different matrices. The first and foremost focus of the method development is on the preparation required before sample analysis. The various nutraceutical product types provide obstacles in this challenge, due to variability in matrix type, as well as variation within each matrix.

Recently, several research groups have reported digestion and analysis procedures for dietary and botanical supplements, as well as for food and other biological samples.[2, 11, 16, 17] Sample preparation of these matrices generally consist of either wet and dry ashing or microwave digestion. Atomic absorption spectrometry (AAS) , inductively coupled plasma mass spectrometry (ICP-MS), and inductively coupled plasma atomic emission spectrometry (ICP-AES) were utilized for the determination of major and minor constituents, because atomic spectrometric methods are most suitable for the elemental analysis of these types of samples.

In this laboratory, recent attention has focused on the development and validation of a method of sample preparation for nutraceutical samples analysis by ICP-AES. First, a microwave digestion procedure was investigated for preparation of ethanolic tinctures and glycerin-based nutraceutical samples. It was found that microwave digestion lessened the sample decomposition time and allowed more samples to be prepared at the same time. Secondly, an analysis procedure was developed for quantification of toxic elements, namely arsenic, cadmium, lead, and mercury, by ICP-AES. Here, it was found that a fast and simple analysis could be performed via generation of calibration functions. Lastly, the sample preparation and analysis procedure was validated using nutraceutical standard reference materials (SRMs).

Experimental Procedure

Inductively Coupled Plasma AES Systems

A JY 24 (Horiba Jobin Yvon, Longjumeau, France) sequential ICP spectrometer was used for preliminary method development experiments and routine sample analysis. A peristaltic pump and high-solids concentric nebulizer were used for sample introduction into a Scott-type double pass spray chamber. This instrument utilizes a fully demountable radial observation torch. The ICP-AES employs a 1 m Czerny-Turner sequential monochromator, a holographic diffraction grating with 2400 grooves/mm, and is coupled to a single PMT detector. Data acquisition is controlled by JY Analyst v5.2 and WinImage software.

A Spectroflame Modula E (Spectro Analytical Instruments, GmbH) ICP spectrometer was utilized in this study for method validation experiments. Sample introduction into the ICP system was accomplished through use of a pneumatic nebulizer coupled to a cyclonic spray chamber. Sample was pumped into, and waste pumped out of, the spray chamber by a computer controlled peristaltic pump. The ICP utilizes a one-piece axially oriented torch, with measurements taken "end-on". A Neslab CFT-75 refrigerated recirculator operating at 16°C was used to cool the torch. Instead of mirrors and lenses, fiber optics guide the light into the spectrometer through four entrance slits. Also, a gas-filled (nitrogen) spectrometer enables detection below 190 nm, without the need for vacuum or flushing. The monochromator optics system is a Paschen-Runge mount with direct wavelength drive (DλD), which lessens the distance the detectors move. On the Rowland circle, six exit slits, each with a PMT detector, are set up on a 7° arc. The diffraction grating utilized in this instrument has 2400 grooves/mm and the wavelength range of the spectrometer is 120-800 nm, with a focal length of 750 mm. Data acquisition was controlled through Spectro System Software Smart Analyzer v2.20. Instrument operation parameters for each ICP system are shown in Table 2.1.

Microwave Digestion System

The system used for microwave sample digestion was a MARS XPress (CEM Corporation, Matthews, NC) with MARS XPress sample digestion vessels. The microwave system is internally coated with a fluoropolymer and includes an impact resistant door with safety interlocks. The microwave digestion system

utilizes a 40-place sample rotor for use with PFA Teflon sample digestion vessels and is capable of temperature control via an infrared sensor that the vessels pass over during rotation. The PFA Teflon sample digestion vessels used in this study are 75-mL capacity, operable at temperatures up to 260°C, and are capable of venting and resealing.

Sample Preparation Procedures

Nutraceutical supplements, in the form of ethanolic tinctures and glycerin-based samples, were provided for this study by Gaia Herbs (Brevard, NC). The ethanolic tinctures consisted of 25-75% ethanol and either single herbs or herbal blends. Ethanolic tinctures are designed to deliver effectiveness very quickly, as they do not need to be digested by the body first.

	JY 24 Sequential ICP Spectrometer	**Spectroflame Modula E ICP Spectrometer**
Power (W)	1000	1350
Gases: Nebulizer Coolant Auxiliary	 0.35 mL/min 12 L/min 0.2 L/min	 22* 26* 35*
Peristaltic Pump Speed (rpm)	20.5	120
Wavelengths (nm): As Cd Pb Hg	 193.695 214.438 220.353 194.227	 193.695 214.438 220.353 184.950

Table 2.1.: Instrument settings for the two ICP-AES instruments utilized in the nutraceutical study and validation.
* = Gas settings for Spectroflame Modula E ICP Spectrometer are particular to that instrument brand. Units and conversion factors are unknown.

The glycerin-based samples consisted of pure alcohol-free liquid extracts and comprise the contents of vegetable gel capsules upon final product manufacturing. The glycerin content in the samples varies from 50-60% and these samples consist of more raw plant material than the ethanolic tinctures. The glycerin-based samples are packaged in gel capsules, which require digestion by the body upon consumption, and are therefore designed for timed-release.

Ethanolic tinctures were prepared for analysis by one of two methods. In the preliminary method, 1 mL of tincture was heated by hotplate in a 50 mL volumetric flask. To the tincture, 5 mL of concentrated *trace metal grade* nitric acid (Fisher Scientific) was added and the sample was heated until a vigorous reaction, including bubbling of nitric acid and sample mixture and the generation of red nitrogen dioxide fumes, had taken place. The sample was then cooled and diluted to volume with *plasma grade* water (Fisher Scientific; VWR Scientific Products). The second, ultimate, method utilized a microwave digestion procedure. Approximately 1 g of tincture was accurately weighed on an analytical balance to 4 decimal places and placed into the bottom of the 75 mL Teflon microwave digestion vessels. To eliminate the ethanol in the sample, concentrated *trace metal grade* nitric acid was slowly added to the uncapped vessel 0.5 mL at a time, up to a total volume of 5 mL. After the reaction between the nitric acid and ethanol was complete, producing reddish-brown nitrogen dioxide gas, the vessels were placed in the microwave system with the caps not fully torqued down. The microwave program consisted of a predigestion step, where the samples were heated at 80°C for 10 minutes. After cooling, the vessel

caps were tightened and the samples were placed in the microwave system again and ramped to a temperature of 180°C in 10 minutes, followed by a temperature hold for 15 minutes. Sample digestion was deemed complete when no raw plant material was visible in the vessel and the digestate was yellow in color. The samples were then cooled and vented. They were quantitatively transferred to 50 mL volumetric flasks and diluted to volume with *plasma grade* water. Prior to use, all volumetric flasks were soaked overnight in a 20% nitric acid bath and rinsed with *plasma grade* water. For storage, digested samples were transferred to 2 ounce (60 mL) amber Nalgene bottles (Fisher Scientific) that had been rinsed with *plasma grade* water and dried.

Glycerin-based nutraceuticals were also prepared by microwave digestion. A sample size of approximately 1 g was accurately weighed on an analytical balance to 4 decimal places and placed into the bottom of a 75 mL Teflon microwave digestion vessel. Because the digestion primarily takes place in the very bottom of the digestion vessel and the digestion volume is very small, care was taken to be sure that the sample was in the bottom of the vessel and little sample was on the sides of the vessel. Concentrated *trace metal grade* nitric acid, 5 mL total volume, was added to each sample. The samples were allowed to remain in a fume hood to predigest. Afterward, the samples were placed into the microwave system and digested using the aforementioned microwave digestion program. The samples were then cooled and vented. They were quantitatively transferred to 50 mL volumetric flasks and diluted to volume with *plasma grade*

water. For storage, digested samples were transferred to 2 ounce (60 mL) amber Nalgene bottles.

Calibration solutions were routinely prepared from aqueous multielement standards. Standards of 20 ppm arsenic, cadmium, lead, and mercury (High Purity Standards, Charleston, SC) were used to make a 1 ppm stock solution. This stock solution was used to prepare calibration standards on a daily basis. Calibration standards, including a blank solution of 10% nitric acid and increasing elemental concentrations up to 0.300 ppm, were prepared with the same acidity (10% nitric acid) as the digested samples. Calibration standards were run in 5 replicates and plotted as a linear function for each element.

Samples used for validation of the method by the Spectro ICP were NIST SRM 3241 ephedra sinica stapf native extract and SRM 3247 gingko biloba extract. A preliminary validation experiment was performed on the JY 24 ICP, utilizing 0.9871 g SRM 3243 ephedra sinica stapf solid oral dosage form that was digested in the same manner as the glycerin-based nutraceutical samples. Masses of 0.9957 g ephedra sinica stapf native extract and 0.7592 g gingko biloba were accurately weighed on an analytical balance and each placed into the bottom of separate 75 mL Teflon microwave digestion vessels. Because the level of mercury in NIST SRMs 3241 and 3247 was very low, each SRM was spiked with 20 ppb (final concentration) aqueous mercury standard before digestion. To each sample, 5 mL of Ultrex ultrapure concentrated nitric acid (J.T. Baker) was added. Analytical blanks, consisting of 5 mL Ultrex concentrated nitric acid, were also prepared and digested along with the nutraceutical samples. All samples were

digested in the same manner as the glycerin-based nutraceutical samples and prepared to 50 mL total volume in volumetric flasks. Aqueous single element standards of 1000 ppm each arsenic, cadmium, lead, and mercury (Inorganic Ventures) were used to prepare a stock solution containing 10 ppm of each element. This 10 ppm solution was used for standard addition spikes of 50, 100, and 200 ppb of each element in 10 mL total volume of digested sample. The original sample and the spiked samples were run in succession and the optical responses were plotted as a linear function. The original sample concentration of each element was determined by calculation of concentration in the sample analyzed, with a correction for sample dilution.

Results and Discussion

Development of Sample Preparation Procedure

The crucial objective of sample preparation is to ensure that the prepared sample is in a matrix or format compatible with the chosen method of analysis with the highest possible yield. For the ethanolic extracts and glycerin-based supplements, it was necessary to manipulate the sample in such a way to both acidify and make totally aqueous before introduction into the ICP-AES torch. The ethanol and glycerin in the starting materials are not compatible with the current ICP-AES instrumentation available for use. It was also important to develop a procedure that could be adapted to other nutraceutical product matrices in addition to the ethanolic tinctures and glycerin-based samples, such as raw plant materials, tablets, and powders. The desired procedure would be both efficient and easy to perform on a daily basis.

Since samples that are analyzed by ICP-AES are typically acidic, the first step in developing the sample preparation procedure was to determine the acid that would best digest the sample. The most common acid used for organic samples is nitric acid. Concentrated nitric acid was added to the ethanolic samples and they were heated in open volumetric flasks on a hotplate. While the reaction of the nitric acid with ethanol succeeded in digesting all plant material present, it should be noted here that the reaction is very violent, producing nitrogen dioxide gases (reddish-brown in color). For some samples, it did not require a total of 5 mL nitric acid, but the volume of acid used for each sample was kept consistent. Though this procedure was successful at digesting the plant material present in the ethanolic tincture, there were several disadvantages, including possible sample loss, because of the open vessels, and the time-consuming nature of the reaction. The procedure was limited to the number of flasks that would fit onto a hotplate, which was six, plus the time needed to thoroughly heat the sample until the digestion was complete, which was several hours.

Since the hotplate procedure was successful at digesting the ethanolic tinctures, it was then attempted with the glycerin-based supplements. The same sample preparation procedure was not as successful with the glycerin-based samples as it was with the ethanolic tinctures. Because of the viscosity differences between samples, i.e. different percentages of glycerin in each sample, it was rather difficult to measure out the glycerin-based samples quantitatively. It was nearly impossible to measure out 1 mL of sample volume, even after heating

the sample thoroughly, so an approximately 1 g sample, accurate to 4 decimal places, was weighed out instead. Upon addition of the nitric acid and heating of the sample in volumetric flasks, the reaction was still incomplete, with undigested sample and an oily residue remaining. Since 6 M hydrochloric acid is found in the human stomach and these glycerin-based samples are typically ingested orally, both 6 M HCl and concentrated HCl were tried and were both unsuccessful at fully digesting the glycerin-based samples. Concentrated sulfuric acid was also tried and resulted in the same problems. It was apparent that a different sample preparation procedure would be needed that would be more efficient in digesting the glycerin-based samples.

The procedure developed here was modified from digestions performed by other research groups, namely nitric acid digestions of other nutraceutical products and standard reference materials[17], as well as mixed-acid digestions of plant reference materials.[19] However, these digestions by other research groups did not specifically include ethanolic tinctures and glycerin-based samples. Microwave digestion has seen a steady rise in the number of sample types and applications it can be used for, including food and nutraceutical samples.[16, 17, 30] Because microwave digestion systems allow the samples to be heated much quicker and under pressure, they are more efficient at total sample digestion than wet and dry ashing and open-vessel digestions. A Mars XPress (CEM Corporation) microwave digestion system was purchased for digestion of the glycerin-based samples, as well as other nutraceutical samples. The microwave digestion system enabled the design of procedures that could be tailored to

specific matrix types and adapted when the need arose, such as one based on the digestion of the ethanolic tinctures could be modified for the glycerin-based nutraceuticals. The same volume of concentrated nitric acid, 5 mL, was used for each matrix type. If less than 5 mL of acid was used, the samples were not completely digested. Some samples would react immediately with the acid and some would react with the acid after a short time sitting in the fume hood. However, there were samples that did not exhibit this behavior, so a predigestion step was programmed into the digestion procedure. This predigestion step heated all samples to 80°C for 10 minutes to jump-start the initial reaction. Because pressure could build up very quickly in the vessels, the caps on the samples were not torqued down during this step. By not tightening the caps on the vessels, there is a chance that volatiles may escape, and the probability of this happening could be determined by spiking experiments. However, tightening the vessels would be dangerous, as an increase in pressure beyond vessel maximum could result in vessel explosion. The total digestion protocol was much more successful after the predigestion step was implemented. The typical digestion protocol included a temperature ramp to 180°C in 10 minutes, followed by a temperature hold for 15 minutes. The digestion was complete when no undigested sample remained in the vessel and the digestate was yellow in color. The final digestate was diluted to 50 mL total volume to achieve 10% acidity. By using microwave digestion, more samples could be digested at once and the digestion proceeded at a much faster pace.

As mentioned, the microwave digestion procedure could be adapted depending on sample type. Even within the glycerin-based samples, there was variation in the ingredients in each sample. For example, some samples also contained soy lecithin as an ingredient in addition to the glycerin, which made them more difficult to digest. The digestate remaining after the microwave digestion for glycerin-based samples contained oily sample residue and solid sample material, indicating an incomplete digestion. The oily residue that remained was most likely remnants of the soy lecithin, whose structure is comprised of phospholipids. The microwave digestion procedure was modified to include the addition of more acid, 10 mL total, as well as an increase in the temperature to 210°C, to fully digest the entire sample.

With the digestion procedure now different for the glycerin-based samples and the ethanolic tinctures, it was desirable to modify the procedure for the ethanolic tinctures so that one method could ultimately be used for all nutraceutical digestions. As previously mentioned, the reaction between nitric and ethanol is very violent. This was evident during a reaction of nitric acid with some ethanolic tinctures, as microwave digestion vessels exploded when the vessels were capped and pressure accumulated too quickly for the vessels to vent properly. For subsequent digestions involving ethanolic tinctures, the ethanol was reacted with the nitric acid, producing nitrogen dioxide gas, by adding the acid very slowly in 0.5 mL increments up to 5 mL acid total, while the vessel was left uncapped. This prevented runaway accumulation of pressure in the vessels from the evolution of nitrogen dioxide and the reaction was less violent than when the

full volume of acid was added at once. This acted as the predigestion step for these samples and after reacting, they were digested by the same microwave digestion protocol as the glycerin-based samples.

In summary, the ultimate digestion procedure for the ethanolic tinctures and glycerin-based nutraceutical samples utilized microwave digestion of an approximately 1 g sample, accurately weighed to 4 decimal places, and 5 mL *trace metal grade* HNO_3 for each sample. The acid was added to the glycerin-based nutraceuticals at once, but was slowly added to the ethanolic tinctures, while the vessels were not capped. Samples were capped and allowed to predigest in a fume hood before being predigested in the microwave system for 10 minutes at 80°C. A final digestion program, consisting of a temperature ramp to 180°C in 10 minutes, followed by a hold for 15 minutes, digested samples and resulted in a yellow colored digestate. Samples were then diluted to 50 mL before analysis.

Selection of Instrument Parameters for ICP-AES Instruments

Before the nutraceutical samples could be analyzed, development of an experimental procedure for the ICP-AES instrument was required. Since ICP-AES is an emission technique, it was vital to select the best wavelengths for emission of the chosen analytes. The JY 24 ICP has a wavelength database that contains multiple wavelengths for selected elements. However, this database contains wavelengths that are not sensitive, in addition to the most sensitive ones. The JY 24 ICP is equipped with WinImage software, which allowed scanning of the wavelengths from 165-765 nm in approximately 4 minutes. The advantage of

this software is the ability to overlay spectra of differing concentrations to evaluate signal-to-noise characteristics at each wavelength. To determine the best wavelengths for arsenic, cadmium, lead, and mercury, analytical blanks and standards with known elemental concentrations were scanned in WinImage and the five most abundant wavelengths for each element were viewed. Elemental interferences at each wavelength were checked via both WinImage scanning and wavelength databases. Wavelengths that were examined on the JY 24 ICP for arsenic, cadmium, lead, and mercury are shown in Table 2.2, including the wavelengths that were ultimately chosen.

Since the method validation experiments were performed using a different ICP-AES system, a Spectroflame Modula E, it was necessary to reconfirm the selected wavelengths. The Spectro ICP software allowed scanning and overlaying of spectra at the wavelengths of interest to determine whether the wavelength was acceptable for use. The wavelengths for arsenic, cadmium, and lead were acceptable for use on both instruments, but the mercury wavelength used for the JY 24 ICP was not acceptable for use on the Spectro ICP because a 0.300 ppm aqueous solution of mercury could not be distinguished from the background. A sensitive mercury wavelength for the Spectro ICP was found at 184.950 nm and this was used during the validation experiments. The JY 24 ICP has two power settings: P1, set to 1000 W for aqueous samples, and P2, set to 1250 W for organic samples. The P1 setting was utilized on the JY 24 ICP for this study. The power setting for the Spectro ICP is computer-controlled and was set at 1350 W for aqueous samples. The gas settings for both ICP instruments

were not changed from those used for routine, everyday analyses and are indicated in Table 2.1, shown in the Experimental section.

Element	Wavelength (nm)	Observation
As	189.042	Below 190 nm, requires purging
	193.695*	Peak for 1 ppm distinguishable from background (Best S/N)
	193.759	No difference between signal and background
	228.812	Interference with Cd 228.802 nm
	236.967	No difference between signal and background
Cd	214.438*	Peak for 1 ppm distinguishable from background (Best S/N)
	226.502	No difference between signal and background
	228.802	Interference with As 228.812 nm
	326.106	No difference between signal and background
	361.051	No difference between signal and background
Pb	168.155	Below 190 nm, requires purging
	197.179	Interference with As
	205.088	Interferences with Ni, Fe
	220.353*	Peak for 1 ppm distinguishable from background (Best S/N)
	283.307	Doublet peak
Hg	184.887	Below 190 nm, requires purging
	194.227*	Peak for 1 ppm distinguishable from background (Best S/N)
	253.652	No difference between signal and background
	313.155	Doublet peak
	365.015	Peak present for both blank and 1 ppm Hg

Table 2.2: Signal-to-noise characteristics for wavelengths on the JY 24 ICP in the wavelength selection process. (Wavelengths chosen for use are denoted with *.)

Sample Quantification Parameters for ICP-AES Instruments

Emission spectrometry techniques operate on the premise that the intensity of light emitted at a particular wavelength for an element is proportional to the concentration of that element present in a sample. The simplest way to determine this relationship is through the generation of calibration functions for each element of interest. By generating calibration functions, routine sample analysis becomes relatively fast and efficient. While calibration functions that are linear

are ideal, non-linear functions can occur and require careful establishment of the relationship between intensity and concentration. In developing a method for analyzing nutraceutical samples, a challenge presents itself in that there are very few standards that exist in the representative sample matrices and none exist that can be universally used for all matrix types. Due to this obstacle, a calibration function generated through analysis of aqueous elemental standards would be the most universally applicable technique; however, they would not be matrix-matched. Before generating the calibration functions, the wavelengths for arsenic, cadmium, lead, and mercury were scanned with the highest concentration standard, as well as a calibration blank, to produce a line profile for each element at the respective wavelengths. From this profile, spectral background positions were set and could be checked and adjusted on a daily basis. In preliminary experiments, the concentration range for the calibration functions was from a calibration blank to 2 ppm, but this was re-evaluated upon realizing that the levels of each element of interest that would be expected in the nutraceutical samples lied in the lower end of this concentration range. Subsequent calibration functions utilized calibration standards that were prepared from calibration blank to 0.300 ppm, including low concentrations of 0.025 and 0.050 ppm. The intensity vs. concentration was plotted for each element to generate linear calibration functions.

Calibration functions prepared on the Spectro ICP and JY 24 ICP are shown in Figures 2.1 and 2.2. All of the calibration functions have correlation coefficients that are greater than 0.99, with the exception of cadmium on the JY

24 ICP, and the intercept of the each function crosses the y-axis at very close to zero. Spectral background complexity at the cadmium and mercury wavelengths on the JY 24 ICP warranted weighting the cadmium function by $1/(\text{concentration})^2$ to reduce variance, while the mercury function was forced through zero. Statistically, the weighting factor of $1/(\text{concentration})^2$ is appropriate because of the approximate proportionality of replicate measurement variance to the concentration at each measurement point, if measurements are made within the calibration range of the instrument.[31] The correlation coefficient of the unweighted cadmium function on the Spectro ICP was 0.9997. The correlation coefficient of zero for the JY 24 data means that the weighted function has no correlation with the linear function. The mercury function was forced through zero by setting the y-intercept to zero and allowing the function to pass closely to the higher concentration points. Y-axis error bars, for 5 replicate analyses, are included for each concentration analyzed and the errors are generally small, ≤ 10% RSD, for most points with only a few exceptions. The limits of detection for each element were determined using the calibration functions generated for each element and are listed in Table 2.3. Limits of detection reflect the concentration of an element that must be present in a sample such that it can be detected with reasonable certainty.[23] The limits of detection were calculated Equation 1, with n = 5.

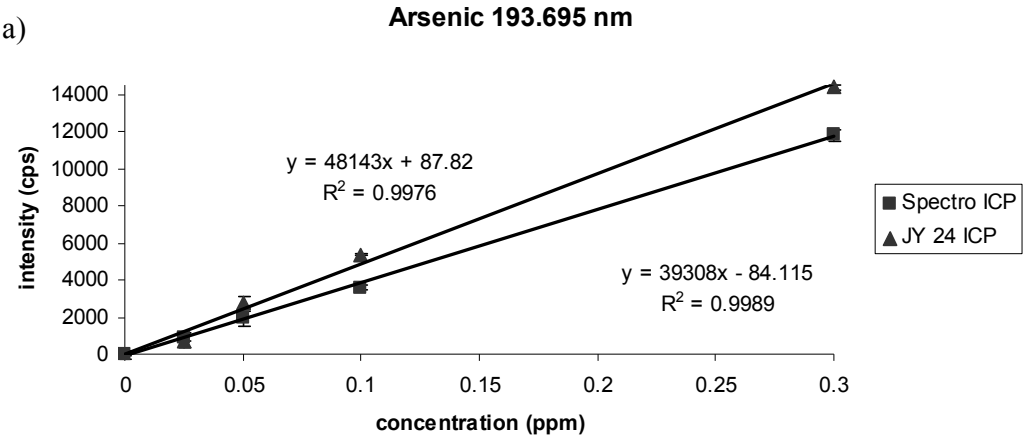
$$\text{LOD} = 3s_x \qquad (1)$$

where x represents the 0.050 ppm calibration standard and s is the standard deviation. The 0.050 ppm measurement was used instead of a blank measurement

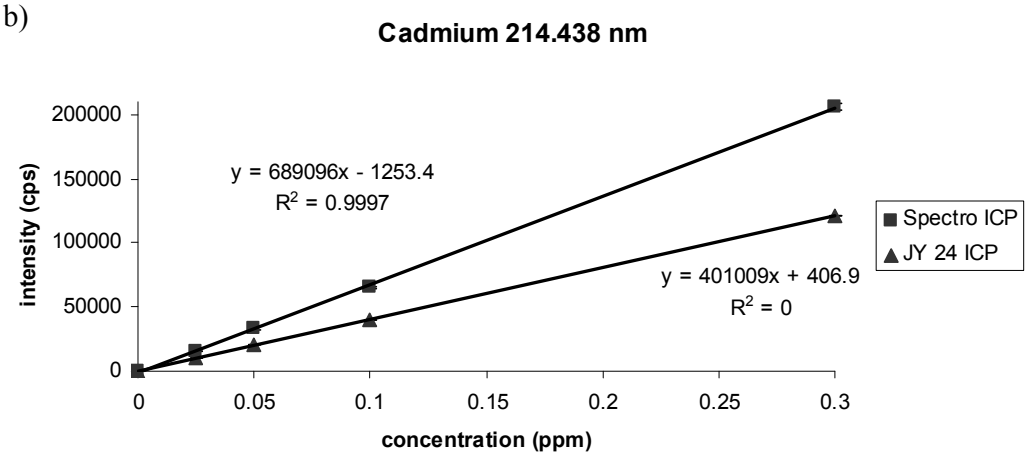
because 0.050 ppm represented a reasonable concentration expected to produce a reproducible intensity, thus variation in this intensity is more representative of variation in a measured sample. The detection limits calculated using the JY 24 ICP are reasonably low for cadmium, lead, and mercury; however, it is very high for arsenic, indicating that a large concentration of arsenic has to be present in the sample to be detected with certainty. The Spectro ICP yields very low detection limits for cadmium and mercury and only moderately low detection limits for arsenic and lead. Detection limits calculated for each element using 5 replicate measurements of the blank sample were comparable for cadmium, lead, and mercury for the Spectro ICP, but higher for arsenic. For the JY 24 ICP, detection limits calculated with blank measurements are comparable to those calculated with the 0.050 ppm measurement for all elements. The standard deviations experienced at each concentration measurement are similar, to yield such results, except in the case of arsenic on the Spectro ICP, where standard deviation is less at the 0.050 ppm measurement.

The differences in slopes of the calibration functions on the two instruments are indicative of differing sensitivities. Calibration sensitivity refers to the slope, m, in the function $y = mx + b$. A larger slope is indicative of a large change in intensity for a small change in concentration.[29] For arsenic, the calibration sensitivities for the JY 24 ICP and the Spectro ICP are very similar, indicating that similarly large changes in intensity represent similarly small changes in concentration.

a)



b)



c)

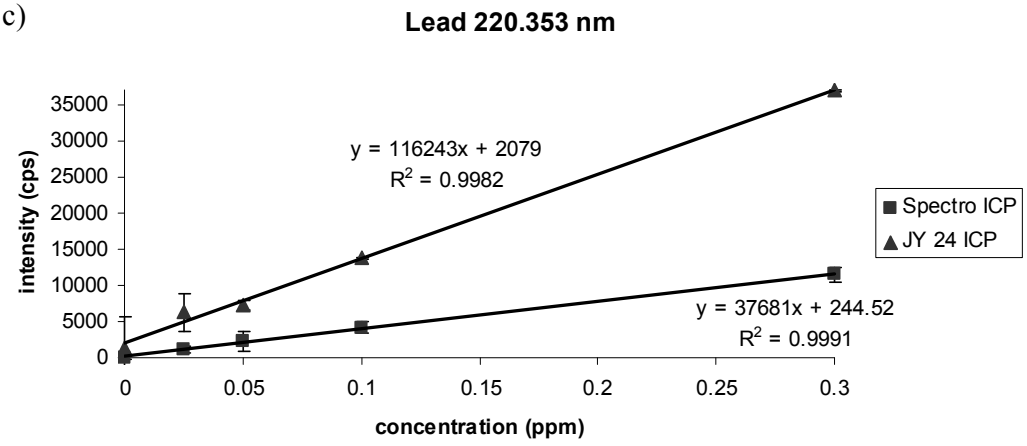
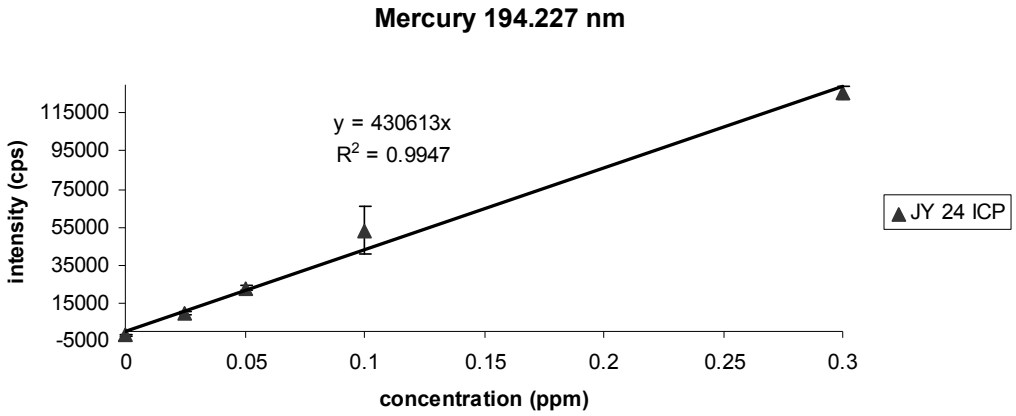


Figure 2.1: Calibration functions of a) arsenic, b) cadmium, and c) lead on the JY 24 ICP-AES and the Spectro ICP-AES instruments.

a)



b)

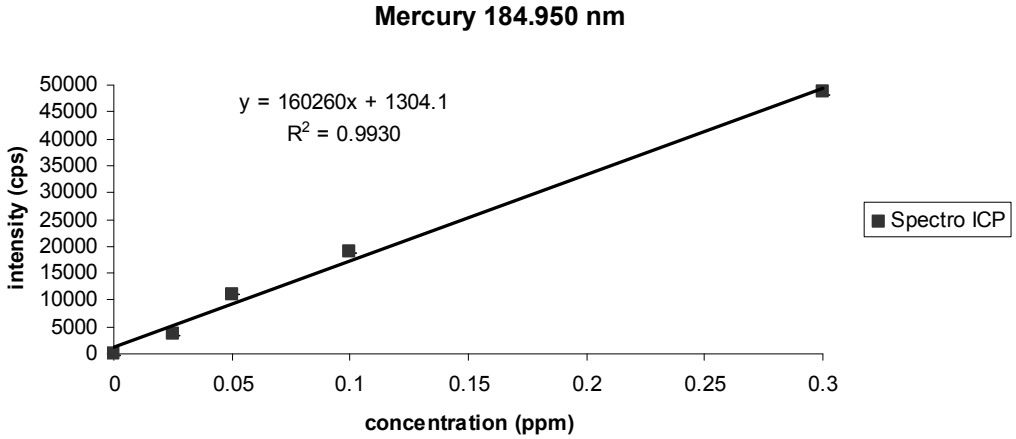


Figure 2.2: Calibration functions for mercury on the a) JY 24 ICP-AES, with the function forced through zero, and on the b) Spectro ICP-AES instruments.

Element	JY 24 Sequential ICP Spectrometer (ppb)	Spectroflame Modula E ICP Spectrometer (ppb)
As	47.1	20.8
Cd	4.7	3.3
Pb	2.5	16.6
Hg	2.8	2.7

Table 2.3: Limits of detection for each ICP-AES system used in this nutraceutical study and validation. LOD = $3s_x$, where x represents the 0.050 ppm calibration standard and s is the standard deviation. (n = 5)

The calibration sensitivity is greater for cadmium on the Spectro ICP versus the JY 24 ICP, while the reverse is true for lead and mercury. Differences in detection limits between the JY 24 ICP and the Spectro ICP could be due to instrumental configuration differences. Axial ICPs are known to produce better, i.e. lower, detection limits when compared to radially viewed ICPs.[32] The detection limits are slightly lower for cadmium and mercury, and much lower for arsenic, on the Spectro ICP. However, the detection limit for lead is lower on the JY 24 ICP, due to the much greater sensitivity for lead on the JY 24 ICP. Also, even though the detection limits for arsenic on the Spectro ICP are lower than the JY 24 ICP, both detection limits are still higher than what would be considered "trace".

The accuracy of concentration determination by use of calibration functions can be evaluated by analysis of a spiked sample. The concentration of the spike, as determined by the calibration function, is compared to the known

spike concentration. To determine spike recoveries for each element, an acid blank that was digested with the standard reference materials was spiked with 0.100 ppm of each element and analyzed by the generated calibration functions for the Spectro ICP-AES system. An acid blank was used as the sample matrix due to the unavailability of an appropriate standard reference material, i.e. an unfortified sample matrix. Spike recoveries for each element on the Spectro ICP are shown in Table 2.4. Average spike recoveries are near 100% for arsenic, cadmium, and lead, while they are less for mercury. For a spiked acid blank, the recoveries should be at or near 100%, since there should be no interfering species in the sample as well as no sample loss. The low recovery for mercury means that the calibration function is not capable of accurately determining concentrations near the spike value. Since the recoveries of the other three elements analyzed are near 100%, there may have been an error in the concentration of the mercury spike. The closer to 100% recovery indicates the calibration function is accurate in determining the concentration of element present in aqueous solutions.

Method Validation by Standard Addition

The ultimate goal of this research project was to develop a method of preparing nutraceutical products for analysis by ICP-AES. Upon development of a digestion procedure, it is necessary to be sure that this procedure effectively digests the nutraceutical products in such a way as to convey an accurate representation of elemental concentrations. Therefore, it was important to validate the developed digestion procedure by analysis of a certified standard reference material and assess the efficiency of the developed procedure.

Standard addition experiments are very accurate at determining trace levels of elements present in a sample, provided the instrument response to increasing concentration levels is linear. Standard addition is performed by adding increasing, known concentrations, called spikes, of the elements of interest to aliquots of a sample solution before sample analysis. For example, spikes of 50, 100, and 200 μL of a 10 ppm lead solution were added to 10 mL aliquots of a digested nutraceutical sample, to yield spike concentrations of 50, 100, and 200 ppb lead, respectively.

Element	**Average Spike Recoveries (%)**
As	94.2
Cd	97.6
Pb	107.5
Hg	71.3

Table 2.4.: Average spike recoveries (%) for arsenic, cadmium, lead, and mercury as determined by the Spectro ICP-AES instrument. Spike recoveries were determined by analysis of 0.100 ppm spike, where n = 5.

The samples, including an unspiked sample, were analyzed by ICP-AES and the response function was plotted for each element. The unspiked sample should yield intensity, I_x, representative of the concentration, $[X]_i$, of lead, for example,

present in the digested sample. The spike concentration, *S*, should yield intensity $I_{s+x.}$ The form of the standard addition equation is then as follows[33],

$$\frac{[X]_i}{[S]_f + [X]_f} = \frac{I_x}{I_{s+x}} \qquad (2)$$

where $[S]_f$ and $[X]_f$ represent final values. The initial concentration present in the sample, $[X]_i$, can be determined from the equation.

Graphically, the concentration of lead in a sample can be determined by extrapolation of the line back to the x-axis or by solving the equation for the line of regression. Using data from SRM 3247 gingko biloba extract, found later in this section in Figure 2.5b, the concentration of lead in the digested sample was determined by setting y = 0, giving

x = -1226.9 / 24206 = -0.0507 ppm

This value is the concentration of lead present in the digested sample. The value is negative because it resides on the negative side of the x-axis, but the sign is dropped to give the concentration. To determine the total lead concentration in the SRM, the dilution volume and mass of SRM digested are taken into account, as follows

0.0507 ppm x 50.00 mL = 2.535 μg

and 2.535 μg / 0.7592 g = 3.339 μg/g (ppm)

The value of 3.339 ppm represents the concentration of lead in the original SRM as determined by microwave digestion and analysis by ICP-AES. The standard deviation of the measurement was determined by using the following equation[33]

$$s = \sqrt{\frac{\sum_i \left(x_i - \bar{x}\right)^2}{n - 1}} \qquad (3)$$

where n = 5. All calculations for concentration determination by standard addition were performed in this manner and calculated values are given in Table 2.5.

Before validating the sample digestion procedure, a certified standard reference material was chosen for standard addition experiments. It is important that the sample upon which to perform the standard addition experiment is a suitable matrix for the application, in this case a nutraceutical sample. It is also desirable that this sample contain known certified levels of the element of interest in the range that could be expected to be detected by the method of analysis. However, because nutraceutical samples have only recently come under scrutiny for their safety, both in active ingredients and toxic metals, very few certified reference materials exist that are applicable to this type of procedure. The National Institute of Standards and Technology (NIST) has recently developed two suites of nutraceutical standard reference materials (SRMs). These SRMs encompass several examples of extracts and final manufactured supplements of ephedra sinica stapf and gingko biloba and are certified for active organic components and trace levels of toxic metals.

Given the detection limits for the toxic metals in this study, the samples analyzed for validation would need to have or exceed levels of 50 ppb arsenic, 20 ppb lead, and 5 ppb each of cadmium and mercury to be able to accurately be detected. Due to varying levels of toxic metals in each standard, depending on the

particular extract, there was not one single SRM that could be used for validation that had levels for arsenic, cadmium, lead, and mercury that were all above the detection limits, upon sample digestion and dilution, capable of the ICP-AES instrument. In order to evaluate the validity of the sample preparation procedure, three SRMs were selected for use in standard addition experiments. A preliminary validation experiment was performed on the JY 24 ICP utilizing SRM 3243 ephedra sinica stapf solid oral dosage form. Validation experiments were also performed on the Spectro ICP for SRM 3241 ephedra sinica stapf native extract and SRM 3247 gingko biloba extract. The certified concentrations of each toxic metal studied in each SRM are given in Tables 2.4a-c. Mercury concentrations were very low, below instrument detection limits, in all SRMs in both sample suites. Because of this, each SRM analyzed by the Spectro ICP was spiked with mercury before digestion, to yield a final concentration of 20 ppb after dilution, as a carrier study. The SRM was then subjected to the digestion procedure and analysis, to determine the possibility and extent of elemental loss during the sample preparation procedure.

The standard addition functions generated for each detectable toxic metal in the SRMs are shown in Figures 2.3a-e. The standard addition functions display correlation coefficients greater than 0.99 for lead and mercury in SRM 3247, as well as cadmium in SRM 3243, indicating very linear agreement between intensity and concentration. Lead in SRM 3243 and mercury in SRM 3241 have lower correlation coefficients of 0.9886 and 0.9723, respectively, because of the larger standard deviations in some of the measurements.

a)

	SRM 3243 Ephedra sinica stapf Solid Oral Dosage form Certified values (mg/kg)	Calculated values from standard addition experiments (mg/kg)
Arsenic	0.554 ± 0.018	ND
Cadmium	0.122 ± 0.0033	0.096 ± 0.005
Lead	0.692 ± 0.056	0.586 ± 0.102
Mercury	0.009 ± 0.00044	ND

b)

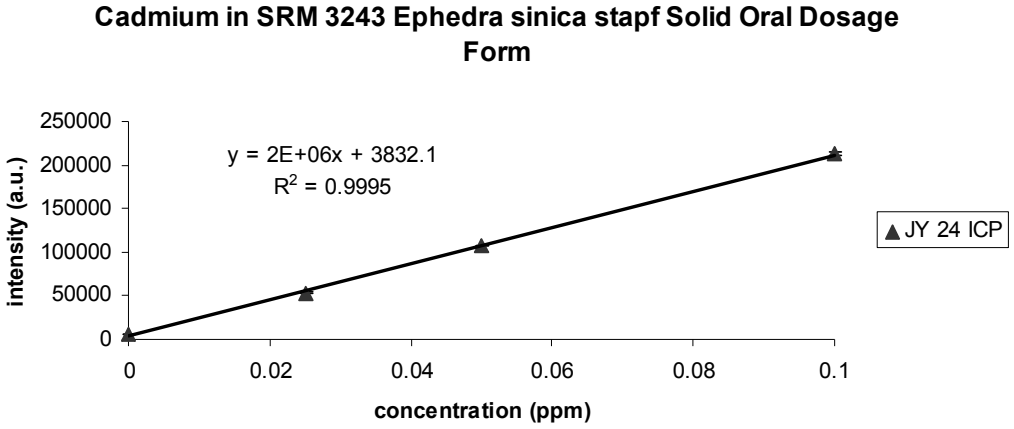
	SRM 3241 Ephedra sinica stapf Native extract Certified values (mg/kg)	Calculated values from standard addition experiments (mg/kg)
Arsenic	1.285 ± 0.081	ND
Cadmium	0.0587 ± 0.0036	ND
Lead	0.241 ± 0.012	ND
Mercury*	0.00383 ± 0.00029	0.020 ± 0.006

c)

	SRM 3247 Gingko biloba extract Certified values (mg/kg)	Calculated values from standard addition experiments (mg/kg)
Arsenic	0.31421 ± 0.0124843	ND
Cadmium	0.007532 ± 0.000139	ND
Lead	4.2728 ± 0.0313	3.339 ± 0.495
Mercury*	0.000980 (information value)	0.021 ± 0.005

Table 2.5: a) Certified and calculated values for NIST SRM 3243 by JY 24 ICP. b) and c) Certified and calculated values for NIST SRMs 3241 and 3247 by Spectro ICP.
(* denotes samples were spiked before digestion to yield 20 ppb Hg, final concentration)

a)



b)

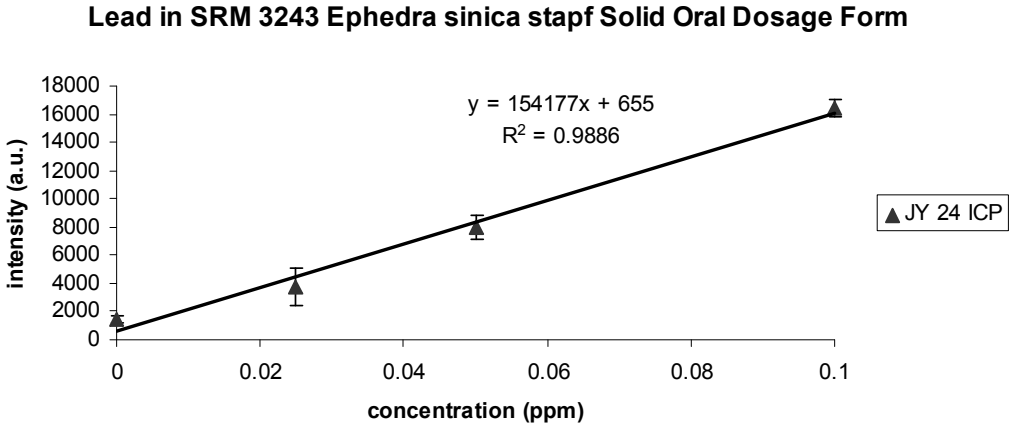
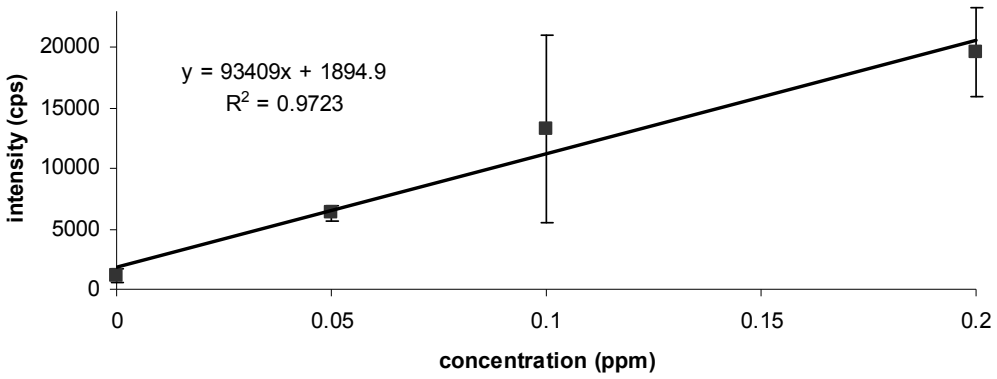
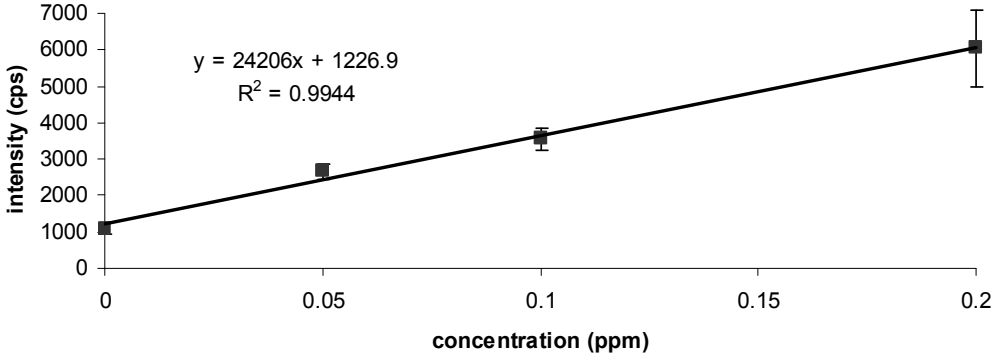


Figure 2.3: Standard addition functions for a) cadmium and b) lead in SRM 3243 in preliminary validation experiments on the JY 24 ICP.

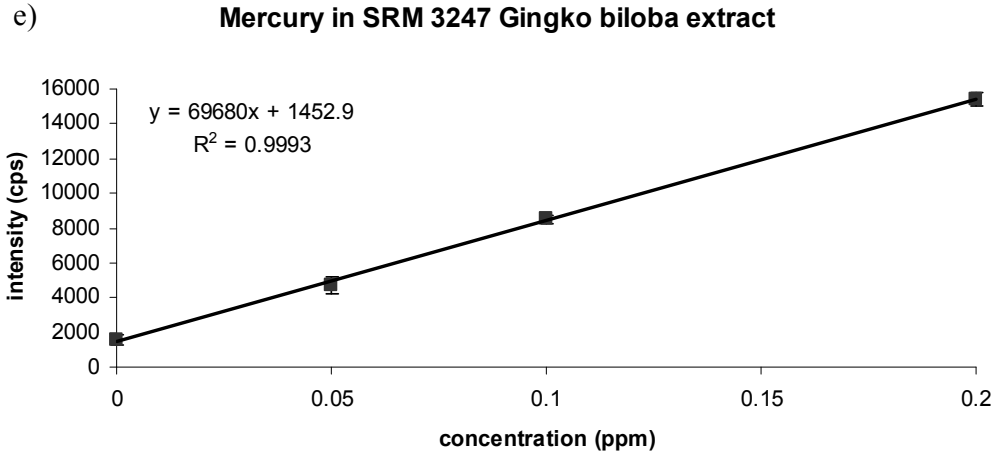
c)



d)



e)



Figures 2.3 (continued): Standard addition functions for c) mercury in SRM 3241 and d) lead and e) mercury in SRM 3247 as analyzed on the Spectro ICP.

The calculated concentrations for each element in the two SRMs evaluated with the Spectro ICP, as well as the preliminary validation results from the JY 24 ICP, are given in addition to the certified concentrations in Table 2.5a-c. The preliminary validation results, determined using the JY 24 ICP, indicated that the method was applicable for determination of cadmium and lead; however, the calculated values were lower than the certified values. In standard addition experiments using the Spectro ICP, the only elements that could be validated were lead and mercury in SRMs 3241 and 3247. Again, these values were less than certified values. Standard deviations in calculated concentrations were low for cadmium and mercury, while they were higher for lead. In SRM 3243, the error in lead may be larger due to the decreased correlation coefficient of the standard addition function. For SRM 3247, the correlation coefficient for lead is very high, and so the lower calculated concentration and error may be due to sample inhomogeneity. In cases where the element was not detected, such as arsenic, it was due to the certified concentrations being lower than the detection limits. To determine how efficient the method was at determining known concentrations, recoveries for the NIST SRMs were calculated by ratioing the calculated concentration to certified concentration. The recoveries were in the range of ~80-85%, except for mercury in SRM 3247, and are shown in Table 2.6. This indicates that the digestion method is not capable of 100% recovery, i.e. the calculated concentration is only representative of ~80% of the actual concentration in the sample, for these solid NIST SRMs.

Element	SRM	Recovery (%)
Cadmium	3243	79
Lead	3243	85
	3247	78
Mercury	3241	85
	3247	99

Table 2.6: Recoveries (%) for detected elements in standard reference materials.

The recoveries of the mercury spike in the carrier study were 85% for SRM 3241 and 99% for SRM 3247, as shown in Table 2.6.

Since the sample was spiked before digestion and recovery after analysis is reasonable, the digestion method may still be applicable and not responsible for the lower calculated values. Also, the SRMs that were used in the validation experiments are solid (powder) nutraceutical samples, and as such, the method may still be valid for the glycerin-based and ethanolic nutraceutcal samples. The standard addition experiments performed to assess the validity of the sample preparation procedure give a reasonable indication that the procedure is applicable to cadmium, lead, and mercury detection. However, it also indicates that the procedure needs modification because of the low recoveries. Calculated concentrations that are lower than certified concentrations can mean that the procedure is not 100% effective at complete digestion or that there is loss of important elements during the digestion procedure.

It is also important to note that the validation experiments utilized solid samples, not glycerin-based or ethanolic samples, and as such, may only be valid assumptions for solid matrix nutraceuticals, necessitating the need for standard reference materials in other matrices.

In addition, it would be useful to locate a standard reference material that contained arsenic at a higher concentration to assess the validity of the sample digestion procedure for arsenic and to make recommendations on digestion procedure modification.

CHAPTER THREE

CONCLUSIONS

Microwave digestion has been utilized as a sample preparation technique for nutraceutical products, specifically ethanolic tinctures and glycerin-based samples, prior to analysis for toxic metals by inductively coupled plasma atomic emission spectrometry (ICP-AES). The use of ICP-AES as an analysis technique necessitates a sample preparation method that can digest all solid material, such that the sample is totally aqueous before introduction into the instrument. Because of the variation in sample matrices of nutracutical products, i.e. powders, tablets, tinctures, and glycerin-based samples, a sample preparation method utilizing microwave digestion was developed that can be adjusted depending on sample type and, thus, can be applicable to the many nutraceutical sample types. The ultimate sample preparation method microwave digests a 1 g sample with a small volume of nitric acid (5 mL). It is capable of digesting up to 40 samples in less than 2 hours.

Calibration functions were generated by ICP-AES for arsenic, cadmium, lead, and mercury in aqueous solutions and reasonably low detection limits for cadmium, mercury, and lead were determined for a Spectro ICP with an axially oriented torch. Certified standard reference materials (SRMs) of nutraceutical products were prepared using the developed sample preparation method and analyzed by ICP-AES for toxic metals. Preliminary and final validation results

suggest that the sample preparation method is may be successful for glycerin-based and ethanolic nutraceuticals but is only ~80% efficient for cadmium, lead, and mercury in solid standard reference materials (SRMs). The concentrations of arsenic in the SRMs were too low to be detected accurately by the ICP-AES instruments.

The sample preparation method could not be fully validated because the efficiency is lower than 100%, suggesting possible incomplete digestion, loss of elements of interest during digestion, or loss of elements during dilution. However, mercury spikes before digestion exhibit reasonable high recovery, indicating that the digestion method may not involve loss of elements. To assess the problems with the efficiency of the method for solid nutraceuticals, experiments should be performed that adjust the digestion protocol by the addition of more nitric acid, addition of other different acids, increased temperature, and increased temperature hold time. Careful preparatory and transfer procedures should also be established and carried out to lessen any chance for sample loss and contamination. It would also be useful to locate a certified nutraceutical SRM that has a significantly high concentration of arsenic present in it so that the sample preparation method can be evaluated with respect to the arsenic concentration. If such an SRM cannot be located, exploration into hydride generation as a sample introduction technique for these nutraceutical samples should be investigated, as it affords greater efficiency and lower detection limits for arsenic.

REFERENCES

(1) In *Nutrition Business Journal*; Penton Media, Inc.

(2) Raman, P.; Patino, L. C.; Nair, M. G. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 7822-7827.

(3) Cardellina II., J. H. *Journal of Natural Products* **2002**, *65*, 1073-1084.

(4) Berman, E. *Toxic metals and their analysis*; Heyden: London ; Philadelphia, 1980.

(5) 21 U.S.C. §321.

(6) Public Law 103-417. §4,108 Stat. 4325. Oct. 25, 1994.

(7) Jaksch, F.; Wang, M.; Roman, M.; American Pharmacists Association. *Handbook of analytical methods for dietary supplements*; American Pharmacists Association: Washington, D.C., 2005.

(8) California. Legislature. Senate. Committee on Local Government.; California. Legislature. Assembly. Committee on Local Government. *Proposition 65 : Local Taxpayers and Public Safety Protection Act : the summary report from the informational hearing*; Senate Publications: Sacramento, CA, 2004.

(9) California. Legislature. Senate. Toxics and Public Safety Management Committee.; Mizak, E.; California. Legislature. Senate. Committee on Budget and Fiscal Review. Subcommittee No. 3 - Health Human Services and ALRB *Joint hearing, Senate Toxics and Public Safety Management Committee and Senate Budget and Fiscal Review Committee [i.e. Subcommittee] No. 3, State of California : Proposition 65 implementation : State Capitol, Room 2040, Sacramento, California, Monday, March 16, 1987, 10:00 A.M*; Joint Publications: Sacramento, CA (Box 90, State Capitol, Sacramento 95814), 1987.

(10) *Safe Drinking Water and Toxic Enforcement Act of 1986: Proposition 65.*; Office of Environmental Health Hazard Assessment: State of California, 2003.

(11) Miller-Ihli, N. J. *Journal of Agricultural and Food Chemistry* **1996**, *44*, 2675-2679.

(12) Kingston, H. M.; Jassie, L. B. *Introduction to microwave sample preparation : theory and practice*; American Chemical Society: Washington, DC, 1988.

(13) H.M. (Skip) Kingston and Stephen J. Haswell, E. *Microwave-Enhanced Chemistry: Fundamentals, Sample Preparation, and Applications*; American Chemical Society: Washington, DC, 1997.

(14) R.C. Richter, D. L., H.M. Kingston *Analytical Chemistry* **2001**, 30A-37A.

(15) Kingston, H. M.; Jassie, L. B. *Analytical Chemistry* **1986**, *58*, 2534-2541.

(16) Lozak, A.; Soltyk, K.; Ostapczuk, P.; Fijalek, Z. *The Science of the Total Environment* **2002**, *289*, 33-40.

(17) Dolan, S. P.; Nortrup, D. A.; Bolger, P. M.; Capar, S. G. *Journal of Agricultural and Food Chemistry* **2003**, *51*, 1307-1312.

(18) Liu, Z. F.; Sun, H. W.; Shen, S. G.; Li, L. Q.; Shi, H. M. *Analytica Chimica Acta* **2005**, *550*, 151-155.

(19) Sucharova, J.; Suchara, I. *Analytica Chimica Acta* **2006**, *576*, 163-176.

(20) Melaku, S.; Gelaude, I.; Vanhaecke, F.; Moens, L.; Dams, R. *Microchimica Acta* **2003**, *142*, 7-12.

(21) Cullen, M. *Atomic spectroscopy in elemental analysis*; Blackwell Pub. ; CRC Press: Oxford, UK; Boca Raton, FL, 2004.

(22) Broekaert, J. A. C. *Analytical atomic spectrometry with flames and plasmas*; Wiley-VCH: Weinheim, 2001.

(23) Dean, J. R. *Practical Inductively Coupled Plasma Spectroscopy*, 1st ed.; John Wiley and Sons, Ltd.: West Sussex, England, 2005.

(24) Hill, S. J. *Inductively coupled plasma spectrometry and its applications*; Sheffield Academic Press ;CRC Press: London. Boca Raton, FL, 1999.

(25) Montaser, A.; Golightly, D. W. *Inductively coupled plasmas in analytical atomic spectrometry*, 2nd ed.; VCH Publishers: New York, N.Y., 1992.

(26) Melaku, S.; Dams, R.; Moens, L. *Analytica Chimica Acta* **2005**, *543*, 117-123.

(27) Pohl, P. *Trends in Analytical Chemistry* **2004**, *23*, 87-101.

(28) Sun, H. W.; Liu, Z. F.; Wu, W. J.; Li, L. Q.; Shi, H. M. *Analytical and Bioanalytical Chemistry* **2005**, *382*, 1060-1065.

(29) Ingle, J. D.; Crouch, S. R. *Spectrochemical analysis*; Prentice Hall: Englewood Cliffs, N.J., 1988.

(30) Allen, L. B.; Siitonen, P. H.; Thompson, H. C. *Journal of Agricultural and Food Chemistry* **1997**, *45*, 162-165.

(31) U.S. Environmental Protection Agency, Office of Solid Waste, 1998.

(32) Brenner, I. B.; Zander, A. T. *Spectrochimica Acta Part B* **2000**, *55*, 1195-1240.

(33) Harris, D. C. *Quantitative chemical analysis*, 5th ed.; W.H. Freeman: New York, 1999.