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CHEMICAL CHARACTERIZATION OF BOTANICAL PRODUCTS: COMPREHENSIVE SPECIATION BY LIQUID CHROMATOGRAPHY PARTICLE BEAM MASS SPECTROMETRY

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CHEMICAL CHARACTERIZATION OF BOTANICAL PRODUCTS:

COMPREHENSIVE SPECIATION BY LIQUID CHROMATOGRAPHY

PARTICLE BEAM MASS SPECTROMETRY

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ABSTRACT

The dietary supplement industry has expanded and many of these supplements have become an important aspect of people's everyday lives. In 1994, the U.S. Dietary Supplement Health and Education Act (DSHEA) classified numerous nutraceutical/botanical products as dietary supplements because of their beneficial medicinal properties and provided the necessary regulation to the supplement producers. Since then, the interest of the scientific community towards dietary supplements has grown intensively and numerous studies have been carried out in order to understand the chemical behavior of the active molecules in the human body. The development towards analytical methods for the quantification of the active components and adulterants in the botanical products has acquired great interest.

Presented here is the chemical characterization of botanical products via a liquid chromatography particle beam mass spectrometry (LC-PB/MS) technique with dual ionization sources (electron ionization (EI) and glow discharge (GD)). More specifically, the catechin species in green tea and the caffeic acid derivatives in echinacea extracts have been characterized. As well, an arsenic speciation study was performed for the kelp and bladderwrack extract. Validation of the LC-PB/MS system was accomplished by the analysis of the ephedrine alkaloids using ephedra-containing dietary supplement standard reference materials (SRM's) 3241 Ephedra Sinica Stapf Native Extract and 3242 Ephedra Sinica Stapf Commercial Extract from NIST. Once validated, this analytical tool

was applied to the separation and characterization of green tea species in the NIST green tea SRM's which are under development. Finally, a selenium speciation method is applied to selenium enriched yeast and urine samples via LC-PB/EIMS.

Chromatographic methods (reversed-phase and ion-exchange) were developed and monitored by UV-absorbance and mass spectrometry. The GD source provides EI-like molecular fragmentation of each eluting compound. Therefore, a comparison between EI and GD sources can be carried out to contrast the mass spectra obtained. Quantification of the species is achieved by standard addition and internal standard approaches. Limits of detection in the nanogram level were obtained for the targeted species.

DEDICATION

I dedicate this work to my loved ones who believed in me and were always there when I needed them. It is through their support and motivation that I was able to complete my graduate degree at Clemson University.

To my friends from the University of Puerto Rico-Rio Piedras Campus, from Clemson University and to all of my family, thank you for all the love, support and the constant reminder that I can accomplish my goals. Especially to my parents, aunts and uncles, thanks for always lending your helpful word through a phone call, a card, or a visit. In loving memory of my grandmother, Mama Digna.

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CHAPTER ONE

INTRODUCTION

IMPORTANCE OF NUTRACEUTICALS

Beginning centuries ago, plants have been used for the prevention and treatment of disease due to the presence of naturally beneficial products. Even, Hippocrates (460-377 BC), the father of modern medicine, recognized such relationship and quoted “let food be thy medicine and medicine be thy food”.[1] Nowadays, reference to nutraceuticals and functional foods is very common in the nutrition industry due to the increase of consumer interest. Nutraceuticals, a word derived from “nutrition” and “pharmaceutical” in 1989 by DeFelice, can be defined as “a food or part of a food that provides medical or health benefits, including the prevention and/or treatment of a disease”.[2] Nutraceuticals also refers to biologically active components derived from functional foods.[3] On the other hand, functional foods are defined as food that is prepared with or without the knowledge of how or why it is being used.[2] Therefore, when the functional food is used for the prevention and/or treatment of diseases and/or disorders it is considered a nutraceutical, which can range from nutrients, dietary supplements, herbal products and processed foods.[2] These products have become part of the daily routine of many people worldwide, and so their safety is of great importance for the government and the scientific community. Currently, there is a relevant regulation in the United States that covers the sale and safety of foods including

botanical dietary supplements.[4, 5] A detailed description of this regulation is presented below.

Dietary Supplement Health and Education Act (DSHEA)

The dietary supplement health and education act was passed in 1994 by the US Congress with the purpose of delivering new regulations in the labeling and marketing of dietary supplements. DSHEA also defines that a dietary supplement:[4-6]

- is a product (other than tobacco) intended to supplement the diet that bears or contains one or more dietary ingredients such as vitamins, minerals, herbs and/or other botanicals, amino acids; a dietary substance for human consumption to supplement diet by increasing the total daily intake, or a concentrate, metabolite, constituent, extract, or combinations of these ingredients.
- is intended for ingestion in pill, capsule, tablet, or liquid form.
- is not represented for use as a conventional food or as the sole item of a meal or diet (i.e. a “meal replacement” is not a “dietary supplement”).
- is labeled as a “dietary supplement.”
- includes products such as a new drug, certified antibiotic, or licensed biologic that was marketed as a dietary supplement or food before approval, certification, or license (unless the Secretary of Health and Human Services waives this provision).

With DSHEA, the marketing of dietary supplements does not require approval from the Food and Drug Administration (FDA), but it is the producer and manufacturer responsibility to present the safety of the marketed products. As well, DSHEA kept the FDA's authority to issue regulations that require the manufacture of dietary supplements be in compliance with current good manufacturing practice (cGMP) standards, to ensure the quality of the products.[4] Since then, the interest of the scientific community towards dietary supplements has grown intensely and numerous studies have been carried out in order to understand the chemical behavior of active components in the human body. The development of analytical methods for the separation, detection and quantification of the active compounds, adulterants, and contaminants in the botanical products has acquired great interest. As well, the desire to obtain more than one type of chemical information from a single instrumentation device has always been a motivating force in analytical chemistry. Therefore, this research proposes the development of straightforward analytical methods that can provide qualitative and quantitative information for both organic and inorganic species present in dietary supplements. More specifically, a liquid chromatography system coupled to a mass spectrometer through a particle beam interface and that is capable of interchanging ionization sources (electron impact and glow discharge) is utilized for the comprehensive speciation of dietary supplements. This analytical tool will undergo optimization of the ion source parameters as well as, validation of the developed analytical approaches with NIST standard

reference materials for the chemical characterization of dietary supplements and botanical extracts.

SPECIATION ANALYSIS

In any particular system, it is important to determine the chemical form of the elemental constituents (e.g., oxidation state, molecular identity and ligand species) as these dictate their chemical, biological and toxicological properties. The different chemical states of a metal can range in their effects on the body from essential and necessary to toxic or carcinogenic. Chemical speciation is commonly defined as the analytical activity of identifying and/or measuring the quantities of one or more individual chemical species in a sample.[7] Speciation can be divided into three categories: 1) total elemental composition via the digestion of the material followed by element detection (e.g., atomic absorption, atomic emission or mass spectrometry), 2) basic speciation involving elemental quantification within separated fractions; more specifically, the use of a chromatographic separation with element-specific detection, and 3) comprehensive speciation which includes the identification and quantification of individual elemental and molecular species to obtain their chemical identity. The last of these has the greatest relevance as it provides a complete characterization of the species within a sample in a single run.

A variety of metal speciation techniques can be found in the literature. The most common speciation techniques involve some form of liquid-phase

separation (i.e., reversed phase or ion chromatography, or capillary electrophoresis) coupled to an inductively-coupled plasma mass spectrometry (ICP-MS).[8, 9] Even though ICP-MS provides great sensitivity, spectral simplicity, large dynamic range and high throughput analysis, it serves only as an elemental detector that is incapable of providing direct molecular species information. The ICP also has very little tolerance of high organic solvent compositions. Electrospray ionization mass spectrometry (ESI-MS) has generated interest of late in the speciation world because it is a soft ionization technique that can provide molecular weight information of the compounds without extensive fragmentation.[8, 9] The limitations associated with ESI-MS include the lack of molecular structure information, analyte signal suppression by complex matrices and poor elemental sensitivity in comparison to ICP-MS.[8] Researchers have used the complementary aspects of ICP-MS for elemental analysis and ESI-MS to obtain molecular species information.[8, 9] Nonetheless, the development of a single analytical method that could provide complete chemical speciation (elemental and molecular) information is something worth considering.

This dissertation describes the utilization of a liquid chromatography particle beam mass spectrometer (LC-PB/MS) with interchangeable ionization sources (glow discharge and electron impact) as an analytical tool for the *comprehensive speciation* analysis of solution-phase samples, providing elemental and molecular species information in a single separation. As well, application of the

LC-PB/MS towards the chemical characterization of nutraceuticals/botanical products is highlighted.

GLOW DISCHARGE

The application of glow discharge (GD) plasmas as ionization sources for mass spectrometry has a history dating back more than 80 years. In fact, in the 1920's and 1930's gas discharges were used by Aston, Thomson, Bainbridge and other scientists as ion sources for the first generation mass spectrographs. Even though gas discharges were well studied in the beginning of the last century, it was not until the 1970's that glow discharges were considered as analytical tools for mass spectrometry, optical emission spectroscopy and other analytical detection modes.[10]

Glow discharges are typically operated as low pressure plasmas (0.1 to 10 Torr),[11] although in recent years glow discharge plasmas have been also generated at atmospheric pressure.[12] In general, GD plasmas generate atoms, ions, electrons and photons based on the application of a voltage (500 to 2000 V) between two electrodes and subsequent break down of the discharge gas (most commonly argon).[13] Figure 1.1 shows a diagram of a simple diode dc glow discharge, showing the two regions of the plasma that are of concern; the cathode dark space and the negative glow. In analytical applications, abnormal glow discharges are the most common gas discharge and exhibit only these two regions, even though up to eight regions (depending on field distribution and

electron energy) can be present in a glow discharge.[14] In the abnormal GD, the surface of the cathode is fully covered by the discharge and is characterized by the current density and voltage increase as the current increase.[13, 14]

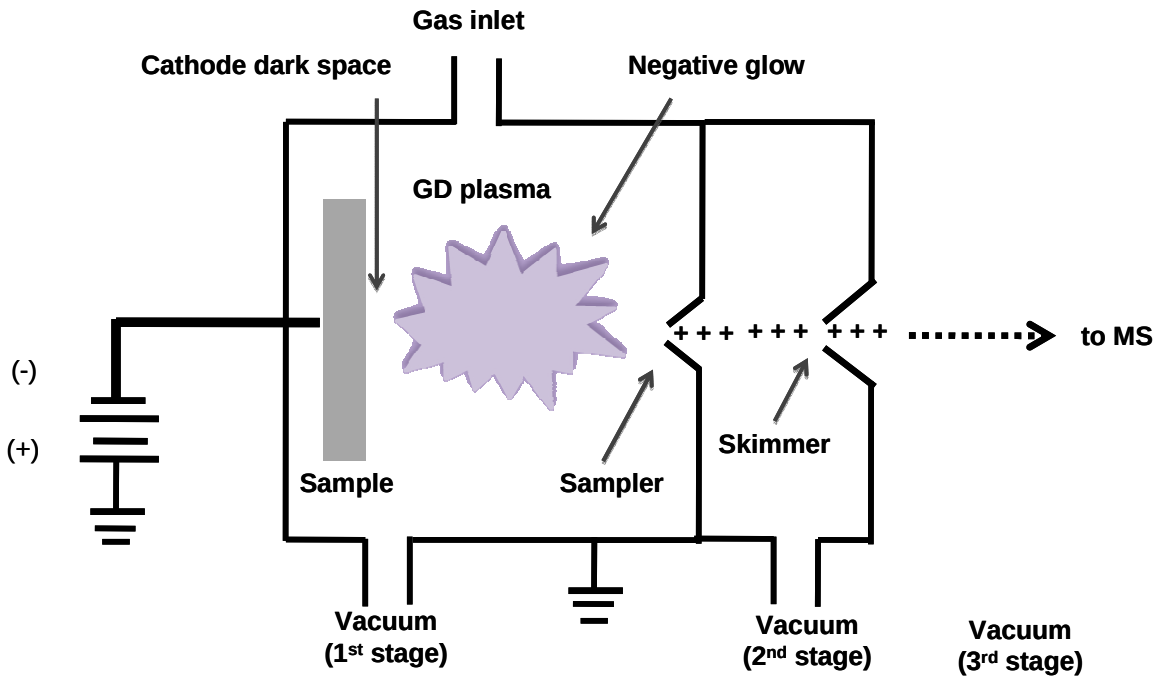

Figure 1.1. Schematic diagram of a simple glow discharge configuration coupled to a mass spectrometer.

Once the electron-ion pairs are formed in the GD, the positive ions accelerate to the cathode and hit the surface causing the emission of secondary electrons. These electrons are repelled by the negative potential of the cathode surface. As the secondary electrons accelerate they begin to gain kinetic energy and inelastic collisions occur with gas atoms, forming the cathode glow as the excited gaseous species relax. The electrons that pass the cathode glow without colliding, continue to accelerate and gain kinetic energy (~25 eV) resulting in little

excitation or ionization in the cathode dark space region.[15] Following the cathode dark space region is the negative glow, where most of the excitation and ionization collision processes take place. Due to the fact that this region is almost field-free, it is characterized for the presence of primary and secondary electrons. The collision processes occurring within this region provide the negative glow luminosity.

The ionization collisions within the GD generate the electron-ions pairs making the plasma self-sustaining. The ions in the GD plasma are of particular interest because besides contributing to self-sustain the plasma at the same time allow its use as an ionization source for mass spectrometry techniques. In fact, GDs are versatile sources that can serve for both sample atomization and ionization.[10, 11, 16-18] In addition the GD plasma sources can serve as speciation detector for gaseous and liquid samples due to their operation under reduce pressure, inert atmosphere, low power and low temperature environment.[19]

Kinetic Processes

Due to the operational pressures of GDs, collisional processes are responsible for creating the excited and ionized states required for analytical detection by OES and MS. In order to electronically excite or ionize the particles (atoms/molecules) that reach the negative glow region, potential or kinetic energy transfer must take place. This transfer of energy is accomplished through inelastic collisions with electrons, ions, and metastable atoms. The major

mechanisms of excitation and ionization for the analyte species are electron impact (Eq. 1), Penning collisions (Eq. 2) and charge transfer (Eq. 3) where Ar* represents a metastable argon atom:

$$M + e^- \rightarrow M^* + e^- / M^+ + 2e^- \quad (1)$$

$$M + Ar^* \rightarrow M^+ + Ar + e^- / M^* + Ar \quad (2)$$

$$Ar^+ + M \rightarrow M^+ + Ar \quad (3)$$

Figure 1.2 demonstrates the excitation and ionization processes that occur within the negative glow region. Electron impact involve inelastic collisions were transfer of kinetic energies between electrons and sputtered atoms occurs. On the other hand, Penning collisions involve the transfer of potential energy between the metastable Ar species due to their high-lying metastable states (11.5 and 11.7 eV for Ar) and the gas phase neutrals. Besides Ar, the rest of the noble gases can also be used as the GD gas. As mentioned earlier, Ar is the most common GD gas used due to its high metastable level energy and ionization potential (15.8 eV). The combination of these collisional processes involving metastable gas, ions and electrons occurring in the negative glow region allows for sample analysis by mass spectrometry as well as other spectroscopic detection modes (atomic absorption, atomic emission and atomic fluorescence).

Glow Discharge Mass Spectrometry

Over the last forty years or so, glow discharge mass spectrometry (GDMS) has been chiefly known for its use in the analysis of trace elements present in solid metal alloys and semiconductors, as well as the characterization of the ion population in the plasmas.[20] More recently, GDMS has been applied to solution and gas phase samples.[17, 21, 22] Figure 1.1 shows the basic arrangement for the coupling of the glow discharge ion source to a mass spectrometer. The discharge plasma environment is at a higher pressure than permissible to perform most MS analysis, therefore the ions must be transported from the plasma through a small orifice into an adjacent chamber at a much lower pressure, commensurate with the type of mass analyzer employed. Subsequently, the ions from the discharge gas and the sample are sorted in the mass spectrometer according to their mass-to-charge ratio (m/z), resulting in the collection of qualitative or quantitative data representative of sample composition.

As mentioned before, the glow discharge plasma typically operates between 0.1-10 Torr, while mass spectrometers typically require a vacuum pressure of less than 10^{-5} Torr to prevent the collision of ions with neutrals during their flight path, as well as electrical break down. Therefore, the ions formed in the plasma region are transported to the mass analyzer through a differential pumping system, meaning the GDMS instruments employ three vacuum regions (Fig. 1.1).[10] The first region is the location of the GD ionization source (~ 1 Torr), followed by an intermediate region ($\leq 10^{-4}$ Torr) and finally the mass analyzer

region (~ 10^{-6} Torr). Throughout the years, GD ion sources have been coupled to various mass analyzers, such as magnetic sectors,[10, 23] quadrupole,[24-26] ion traps,[27-29] time-of-flight[30-32] and Fourier transform ion cyclotron resonance.[33, 34]

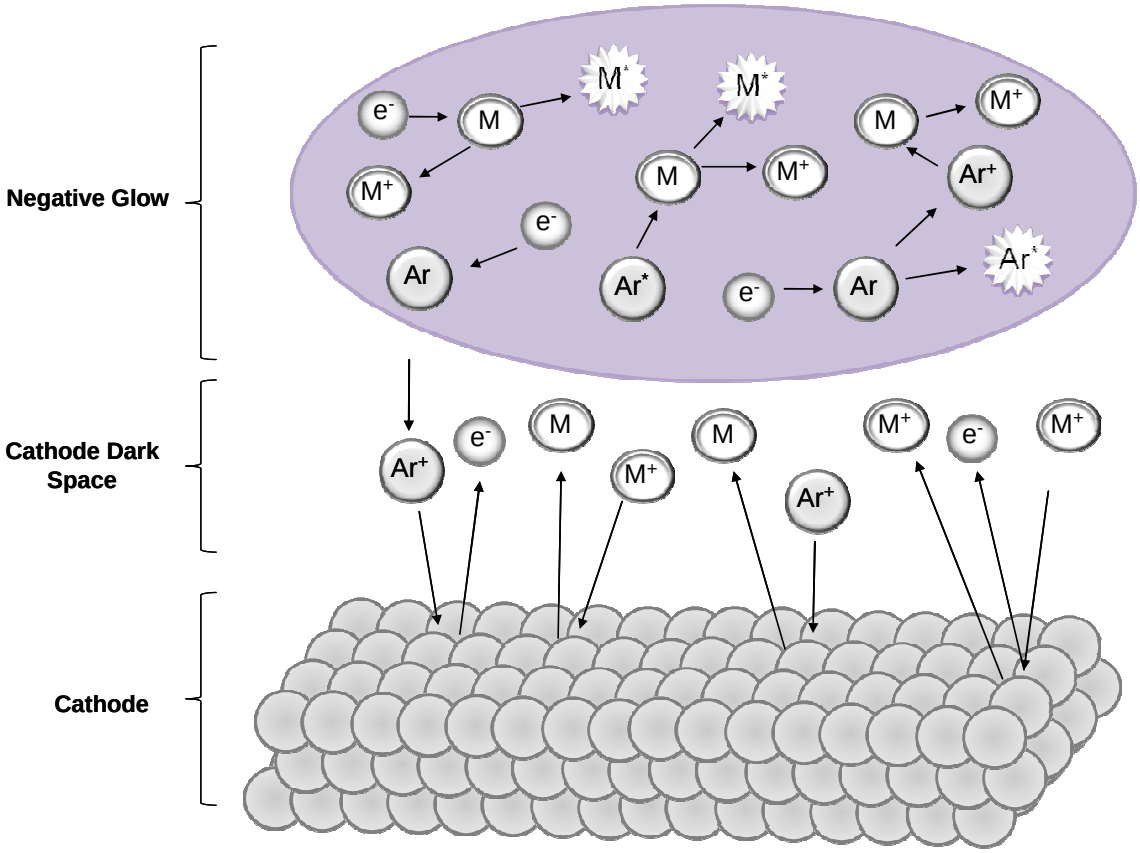

Figure 1.2. Collisional processes occurring in the glow discharge source. M = sputtered neutral, Ar* = argon metastable, Ar^+ = argon ion.

ELECTRON IMPACT IONIZATION

Electron impact (EI) ionization was first used by Dempster in 1918 and is one of the oldest and most common ionization modes for organic species with molecular weight less than 600 Da.[35] Electron impact is applicable for gas phase ionization and compounds with adequate volatility and thermal stability but causes extensive fragmentation therefore, in many cases the molecular ion of the species are not noticed. Figure 1.3 shows a diagram of an electron impact ionization source. In the electron impact source, a thin filament made of tungsten or rhenium wire can be resistively heated to generate a pool of electrons under high vacuum conditions (~10^{-6} Torr). The emitted electrons are repelled from the shield and attracted to the block, which is held at ground potential, therefore creating a potential difference that sets the kinetic energy of the electrons. When the vapor-phase analyte species (molecules) are subjected to a beam of electrons with sufficient energy (10-100 eV), an electron is abstracted from a molecular or atomic orbital, generally producing radical cations or molecular ion (Eq. 1). Residual vibrational energy in the ion that exceeds the individual bond energies can result in fragmentation. The resulting ionized analyte species exit the ion volume and enter the lens stack and then are mass filtered by the quadrupole mass analyzer. The standard EI acceleration voltage of 70 eV makes library comparison possible.

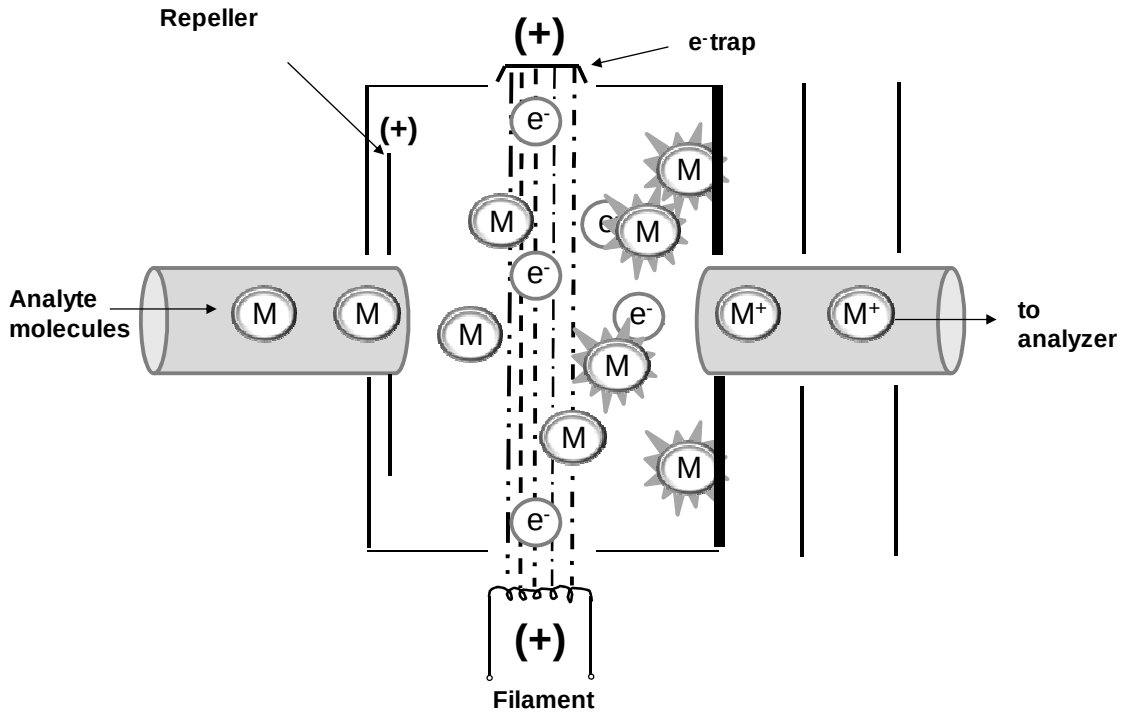

Figure 1.3. Schematic diagram of the electron impact source.

LC/MS "TRANSPORT-TYPE" INTERFACE

Over the years, gas chromatography mass spectrometry (GC/MS) has become a very common analytical technique that employs the electron impact ionization source because both methods handle volatile compounds. However, GC-MS is unpractical for nonvolatile and thermally labile compounds (unless chemically derivatized). Ideally, interfacing liquid chromatography mass spectrometry to EI is of great interest for the analysis of the less volatile or polar compounds not analyzable via GC/MS.

Liquid chromatography mass spectrometry (LC/MS) has become a topic of intense study since first introduced by Tal'roze *et al*. in 1969.[36] Originally, LC/MS

coupling seemed incompatible because liquid chromatography employs high pressures and mass spectrometry operates at high vacuum pressure. Other challenges encountered for LC/MS coupling are the flow-rate incompatibility as it needed to introduce 1 mL min^{-1} of liquid stream into the high vacuum MS and the fact that common ion sources (electron ionization and chemical ionization) cannot carry out desolvation and therefore, residual solvent vapor would cause analyte ion signal depression and spectral interference.[22, 37] The need for analytical techniques with the power of LC separations and the sensitivity and flexibility of mass spectrometric detection has made LC/MS coupling a subject of intense interest over the last two decades. The combination of liquid chromatography and mass spectrometry would provide the analytical community with an enhanced on-line system capable of handling samples that are not responsive to GC/MS. The use of GC/MS in environmental, agricultural and biological studies has been exhaustively applied, but many analytes, like some pesticides and other toxic substances cannot be easily analyzed due to their chemical properties and incompatibility with the GC environment.[38-41] The issues of coupling LC to MS were addressed with the introduction of a number of interfaces, which made possible on-line coupling of LC/MS. Of the interfaces, the moving belt [42] and the particle beam interface[43, 44] have been the most popular for liquid sample introduction. Although these two interfaces operate quite differently, both transport interfaces include aspects of on-line sampling, desolvation, solvent vapor removal and analyte delivery into the ion source low-

pressure (<10^{-4} Torr) environment at solution flow rates in the range 0.2-2.0 mL min^{-1} (as in the case of conventional LC separations).[22]

The moving belt interface is a transport device that physically carries the solute from the LC column outlet to the MS ion source via a stainless-steel moving conveyor chain.[22] The mobile phase is then removed during transportation by gentle heat and evaporation at reduced pressure.[37] Subsequently, the analyte is flash vaporized from the belt for ionization and detection and the belt undergoes a cleaning process with heat to remove residual solvent and nonvolatile materials. Although the moving belt was widely used for some years, it was superseded by the particle beam interface due to the fact that it suffered from memory effects and species-specific response characteristics in LC/MS applications.[22, 45]

Particle Beam Interface

This "transport-type" interface, first developed by Willoughby and Browner[44] and originally termed monodisperse aerosol generation interface for coupling (MAGIC) LC/MS, facilitated the continuous introduction of liquid samples into the electron ionization source while removing the residual solvent vapors and maintaining the chromatographic separation. Their main objective was the development of an interface compatible to a wide range of solvents and flow rates that efficiently allows liquid phase removal while maintaining the chromatographic integrity. The main advantage achieved during the LC particle

beam coupling for MS analysis is the fact that a clean, EI library searchable spectra can be produced.[46]

The particle beam (PB) interface (Fig. 1.4) is composed of a nebulizer, a heated desolvation chamber and a two stage momentum separator. First, the nebulizer transforms the LC effluent into a finely dispersed aerosol (spray mist) which is directed towards the desolvation chamber. Once in the desolvation chamber, the mist droplets begin to dry (volatile solvent evaporates) forming analyte particles which are drawn into the momentum separator. Besides the analyte particles, solvent vapor and nebulizer gas also find their way into the momentum separator, but are removed through the vacuum (i.e. two stage differential pumping system) yielding analyte particle enrichment. More specifically, the high mass/momentum particles maintain a linear path while the light weight species (solvent and nebulizer gas) move off trajectory and are pumped away.[22] Finally, dry, solvent free analyte particles enter the heated ion source region for vaporization and ionization, in this case electron impact and glow discharge. In this laboratory, the PB interface is used as a part of a LC quadrupole mass spectrometry system, which is described in the next section.

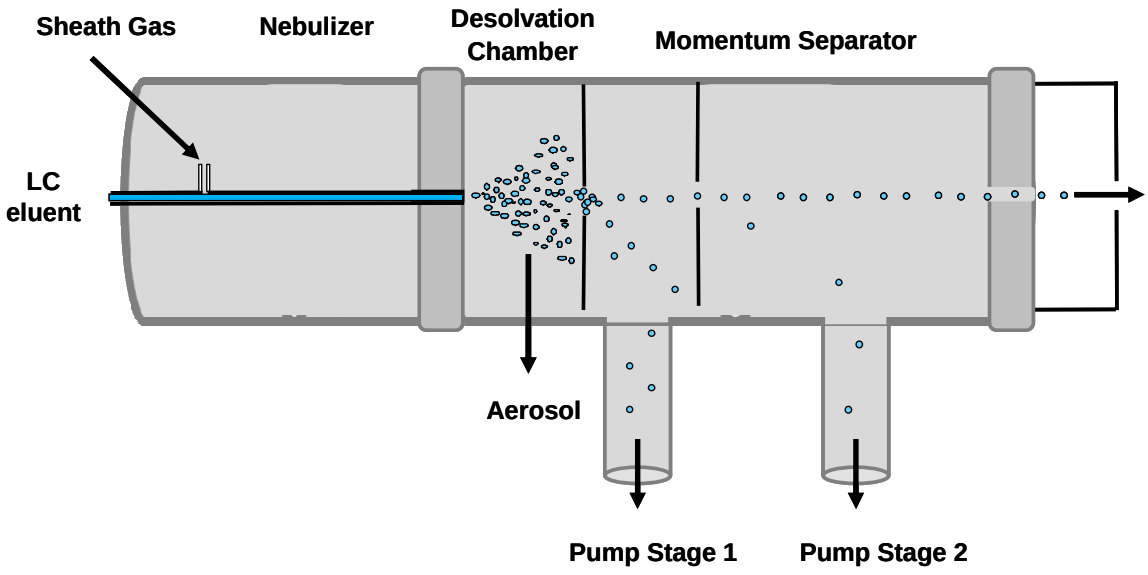

Figure 1.4. Schematic diagram of a particle beam interface.

LIQUID CHROMATOGRAPHY PARTICLE BEAM MASS SPECTROMETRY SYSTEM

The PB/MS system employed in this work is an Extrel (Pittsburgh, PA, USA) Benchmark Thermabeam LC/MS quadrupole mass spectrometer with two ionization sources (EI and GD) that are interchangeably mounted into the source block location shown in Fig. 1.5.

The PB interface (Thermabeam, Extrel Corp., Pittsburg, PA, USA) consists of a thermoconcentric nebulizer, a desolvation chamber and a two-stage momentum separator, which are employed to couple the liquid effluent from the chromatographic system to the ionization source. The thermoconcentric nebulizer, consisting of a 75 µm i.d. fused silica capillary within a 1.6 mm o.d.

stainless steel tube heated at a temperature of ~85℃ by applying a dc potential generates a finely dispersed aerosol. The temperature of the outer tube is regulated by the use of a temperature controller. Helium is employed as a sheath gas around the capillary in the steel tube to facilitate heat conduction and the introduction of a pneumatic nebulization effect. The aerosol is sprayed into a 35 mm i.d x 100 mm long steel spray chamber heated to ~110℃, undergoing desolvation. After exiting the spray chamber, the aerosol passes through the two-stage momentum separator across a pair of 1 mm diameter orifices (~10 mm apart) where residual solvent vapors are removed and the backing pressure is reduced. Finally, a beam of dry analyte particles (1-10 μm diameter)[45, 47, 48] reaches the heated (~ 275℃) source block of the EI or GD ion sources.

The electron impact ionization source consists of a tungsten filament that is resistively heated to generate a pool of electrons. The EI acceleration voltage is set to the standard EI voltage of 70 eV to make library comparison possible. The GD ionization source developed in this laboratory consists of a 12.5 mm diameter insertion probe (DIP) and a Cu cathode target inserted into the source block, perpendicular (45° surface angle) to the path of incoming particles through the mass spectrometer chamber via the solids probe inlet. The particles impinge on the cathode surface, are flash vaporized into the gas phase, and subsequently diffuse into the negative glow region to undergo ionization through various ionization processes such as, electron and Penning collisions. Ultra high purity argon (National Welders Supply Company, NC, USA) was use as the GD

plasma gas and a Spellman (Plainview, NY, USA) Model RHR5N50 high voltage power supply operating in the constant current mode was used to power the discharge. In both cases (EI and GD source), the resulting ionized analyte species exit through a 1 mm aperture into the quadrupole mass analyzer for subsequent detection by an electron multiplier.

The Extrel Merlin Automation (Pittsburgh, PA) Ionstation system software was used for the MS data acquisition. Specific details of each experiment are given in each pertinent chapter.

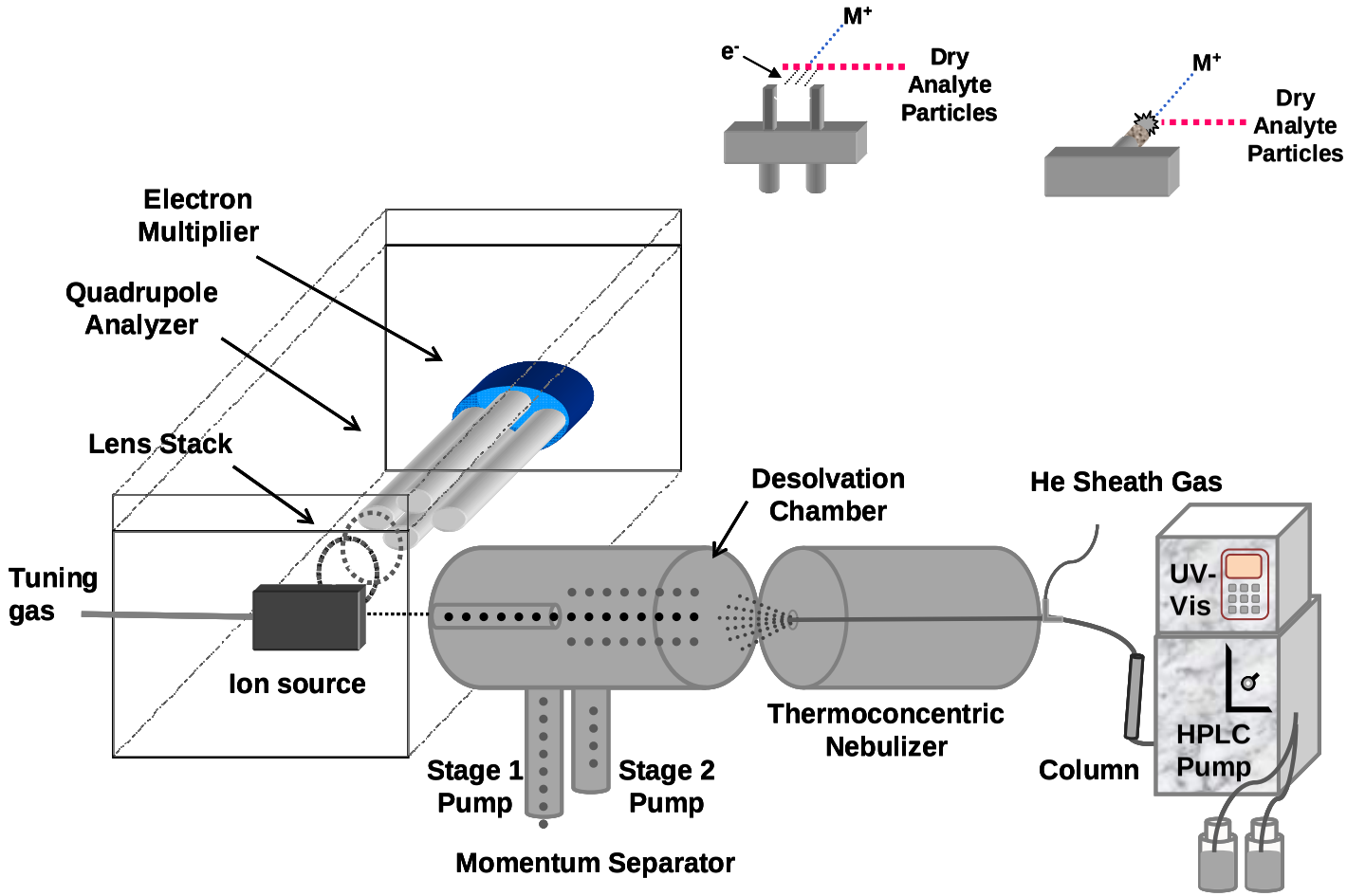

Figure 1.5. Schematic diagram of the Benchmark LC-PB/MS system with EI and GD ionization source.

SUMMARY

This chapter highlighted the interest and importance of dietary supplements in analytical applications. In addition, this chapter introduced the general concepts of the glow discharge, electron impact and particle beam interface as well as their roles involving liquid analysis by mass spectrometry. The research presented here points towards the development, growth and applicability of liquid sampling of real world samples by glow discharge and electron impact with the assistance of the particle beam as a liquid chromatography mass spectrometry interface. Chapter 2-6 describe the comprehensive speciation and chemical characterization of botanical products by LC-PB/MS. Chapter two has been accepted for publication in the *Analytical and Bioanalytical Chemistry* and covers the chemical characterization of the caffeic acid derivatives present in ethanolic *Echinacea* extract by using two ionization sources (EI and GD). Chapter three was published in the *Journal of Analytical Atomic Spectrometry* (M.V.B. Krishna, J. Castro, T.M. Brewer and R.K. Marcus, 2009, vol. 24, pp. 199-208) and discussed the speciation of arsenic species in ethanolic kelp and bladderwrack extracts by LC-PB/EIMS. The manuscripts for chapter four and five have been prepared and will be submitted for publication. Chapter four presents the validation of the LC-PB/MS system by the analysis of ephedrine alkaloids in *Ephedra* standard reference materials while chapter five deals with the separation of selenium species in two different matrices: selenium enriched yeast certified reference material and urine.

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CHAPTER TWO

ANALYSIS OF CAFFEIC ACID DERIVATIVES IN ECHINACEA EXTRACTS BY LIQUID CHROMATOGRAPHY PARTICLE BEAM MASS SPECTROMETRY (LC-PB/MS) EMPLOYING ELECTRON IONIZATION AND GLOW DISCHARGE IONIZATION SOURCES

INTRODUCTION

There is a great deal of consumer interest in herbs and botanicals as dietary supplements because of their purported beneficial health and medicinal properties. As a result, the sale of herbal products has grown by about 10-15% per year since 1994. The primary US government regulation of these products is through the Dietary Supplement Health and Education Act (DSHEA), passed in 1994.[1, 2] The growing use of dietary supplements of various forms brings up two primary sets of concerns of relevance to analytical chemistry.[3] The first involves the development of sound biochemical understandings of the metabolism and efficacy of supplement constituents. The second area of concern involves the consumer-oriented questions of product safety and authenticity. In both of these categories, the analytical challenges are greater than those encountered for pharmaceutical products because of the highly-complex natural product matrices and the variability across raw material sources and final product manufacturers.

Echinacea species have been used for centuries as herbal medicines because they provide favorable health effects, presumably by stimulating the

immune system.[3-7] *Echinacea* is now one of the most widely consumed herbal products in the United States and many other countries.[7] Currently, it is promoted for use in cold therapy and chronic infections of the respiratory system and the lower urinary tract.[4, 8-10] *Echinacea* can be found in the market as a dietary supplement in the form of capsules, tablets, powders, liquid tinctures, dried leaves and/or roots and in conventional foods (e.g., tea bags and drinks). Further, echinacea has also found application in a range of personal care items such as lip balms, toothpaste, and skin and hair care products.

Echinacea is a member of the *Compositae* (daisy) family, also known as the purple coneflower. Three species of *Echinacea* are in use medicinally: *E. purpurea, E. angustofilia* and *E. pallida*. The distribution of the key compounds varies between the three species of echinacea and also within the individual plant parts (roots, rhizomes, stems, leaves and flowers). Among the three species of *Echinacea*, *E. purpurea* has become the most cultivated species because the entire plant can be used (root, leaf, flower, and seed). The caffeic acid derivatives (i.e., polyphenolic compounds) present in echinacea include cichoric acid, caftaric acid, echinacoside, chlorogenic acid, and cynarine. Cichoric acid and caftaric acid are the major polyphenols in *E. purpurea,* with echinacoside being prominent in *E. angustofilia* and *E. pallida*. Chlorogenic acid and cynarine generally exists as the minor compounds in echinacea, but cynarine can only be found in the roots of *E. angustofilia*.[3] Of all the caffeic acid

derivatives, cichoric acid has been one of the most widely studied, and the only one to specifically show immunostimulatory properties.[7]

To acquire a high quality and authentic evaluation of the dietary supplements, analytical methods that can be standardized, detect adulterations and provide an effective and safer product to the consumer are necessary. [3] Currently, the most common method for the analysis of the active components in echinacea extracts is reversed-phase high performance liquid chromatography (RP-HPLC) because of its high resolution and fast analysis time. Normally, RP-HPLC is coupled to UV-Vis absorbance, electrochemical or mass spectrometry detection techniques.[3-6, 8, 11, 12] While being cost effective and analytically versatile, UV-Vis absorbance and electrochemical detection have major disadvantages, namely that they are not analyte-specific. As such, the retention times of the eluting analytes need to be compared with the retention times of their corresponding standards for identification, thus, the methods are only useful for QA/QC applications, and not for the determinations of unknowns. On the other hand, a considerable number of studies with mass spectrometry detection employing electrospray ionization (ESI) have been reported for the identification and quantification of the caffeic acid derivatives present in echinacea.[6, 8] While one of the strong points of ESI-MS is that it can provide molecular weight information of polar compounds without extensive fragmentation, ESI-MS-MS methods must be employed for the complete identification of specific compounds.[13] Another challenge to the use of ESI-MS in botanical product

characterization is the fact that conventional RP-HPLC methods developed for profiling (with UV-Vis detection) are not likely adaptable to the electrospray source as there can be large differences between solution flow rates and acceptable matrix/mobile phase compositions. Gas chromatography (GC),[14, 15] capillary electrophoresis (CE),[10] and micellar electrokinetic chromatography (MEKC)[16, 17] are less frequently used separation methods in the analysis of echinacea components. GC can only be employed for separation of the lipophilic species (alkylamides and polyacetylenes) present in echinacea,[14, 18] as the caffeic acid derivatives are too polar to efficiently separate.

There is increasing interest within the nutraceutical industry for analytical techniques that can perform a complete characterization of the chemical components in the herbal products in a single analysis. Over the last two decades, advances in metal speciation techniques have aided in the determination of metals and identification of organometallic species in biological and environmental systems.[19-21] Taken a step farther, c*omprehensive speciation,* defined as *the complete characterization of the metals, organometallic, and organic species in a single separation and detection experiment,* is the ultimate goal. Previous studies in this laboratory have shown that the use of a particle beam interface for the introduction of HPLC eluents into low-pressure ion sources (i.e., electron ionization and glow discharge plasmas) has great potential toward providing *comprehensive speciation*.[22-25]

The particle beam (PB) has been employed in this laboratory as a transport-type interface for liquid chromatography with glow discharge (GD) optical emission spectroscopy (OES) and mass spectrometry (MS) detection.[26-30] The GD mass spectra exhibit EI-like molecular fragmentation patterns for organic compounds as well as combined elemental/molecular information for organometallic compounds. For example, a comprehensive speciation study of organic and inorganic arsenic species through ion exchange chromatography PB/MS has been recently carried out.[23, 25] Additionally, the separation and identification of a series of catechins (polyphenols) in green tea tincture by electron ionization and glow discharge ionization LC/MS supports the present use of this analytical technique for the characterization of the caffeic acid derivatives present in echinacea extracts.[22]

Presented here is a RP-HPLC-PB/MS method for the separation, identification and quantification of the caffeic acid derivatives which are known to be constituents of echinacea extracts; caffeic acid (3,4-dihydroxy-cinnamic acid), caftaric acid, chlorogenic acid, and cichoric acid (Fig. 2.1). Two commercially available echinacea ethanolic extracts (i.e., tinctures) composed of a combination of *E. purpurea* and *E. angustofilia species* were used in this study. The optimal parameters for the EI source (electron energy and block temperature) and GD source conditions (discharge current and pressure) were determined by studying the response of the mass fragment intensities of the analytes over the tested range of conditions. As seen in Fig. 1, the common base structure of caffeic acid

in each provides a class specific signature ion that is present in all of the spectra. The mass spectra for the caffeic acid derivatives and the analytical response curves for each species were compared for the two sources. The separation of the caffeic acid derivatives in a standard solution as well as the commercial echinacea extract was accomplished by reversed-phase chromatography using a C_{18} column monitored by UV absorbance at 330 nm. Subsequently, the column effluent was coupled to the PB/MS apparatus equipped with the two ion sources. Quantification of the caffeic acid derivatives in the commercial product was achieved by standard addition. Taken as a whole, the LC-PB/MS approach with versatile, interchangeable EI and GD sources is believed to be a viable technique for the study of commercial botanical extracts and potential metabolites, and therefore should be well suited to other nutraceuticals/dietary supplements as well.

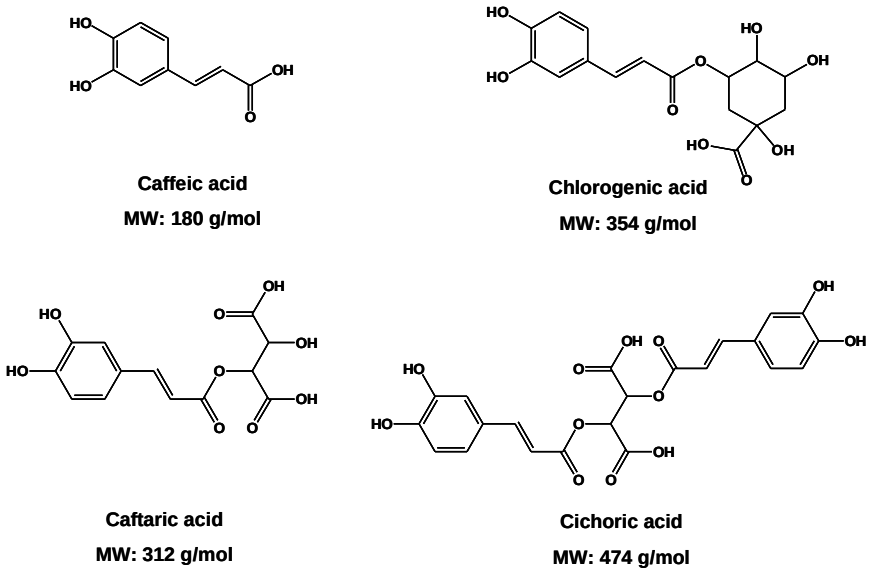


Figure 2.1. Chemical structures of the caffeic acid derivatives evaluated in this study.

EXPERIMENTAL

Particle Beam Mass Spectrometry System

The PB/MS system employed in this work has been described in detailed previously.[22, 23] The Extrel (Pittsburgh, PA, USA) Benchmark Thermabeam LC/MS quadrupole mass spectrometer with two ionization sources (EI and GD) that are interchangeably mounted into the source block location is shown in Fig. 1.5. ABB-Extrel Merlin (Pittsburgh, PA) Ionstation system software was used for the MS data acquisition. Total ion chromatograms (TIC) were acquired by scanning over a mass range of m/z 50-500 Da at a scan rate of 1.0 s per scan. Selected ion monitoring (SIM) chromatograms for specific masses could be extracted from the TIC data. Triplicate injections were carried out for each set of data points presented in the evaluation of experimental conditions and quantification characteristics. The data were then exported to Sigma Plot 8.02 (Systat Software, Inc., Richmond, CA) and Microsoft Excel (Redmond, WA) for final presentation.

The PB interface has been described in chapter one. Two ionization sources (electron ionization and glow discharge ionization source), were employed during this series of experiments (Fig. 1.5). The optimization of the operation parameters for the EI source (electron energy and source block temperature) and the GD source (discharge pressure and discharge current) has been described in previous work.[22]

Liquid Chromatography (LC) System

The separation of the echinacea extract components was performed via a Waters (Milford, MA) Model 600E HPLC system equipped with a Rheodyne (Cotati, CA, USA) Model 7125i injector with a 50 µL injection loop. The 250 mm x 4.6 mm Alltech Alltima C_{18} (5µm) reversed-phase column (Alltech Associates Inc., Deerfield, IL, USA) operating at room temperature and a mobile phase flow rate of 0.9 mL min^{-1} were used for the liquid chromatography separation. The HPLC solvents consisted of water (18.2 MΩ cm^{-1}, NANOpure Diamond, Barnstead International, Dubuque, IA) containing 0.1% v v^{-1} trifluoroacetic acid (TFA) and ACS-grade methanol (MeOH) (Fisher Scientific, Fair Lawn, NJ). A linear gradient method with a mobile phase composition varying from 75:25 (H_2O:MeOH) to 55:45 in 40 minutes was used for the separation of the target compounds. This gradient method provides comparable resolution to the many chromatographic methods reported in the literature.[3, 6, 8, 9, 12]

Sample Preparation

The 1000 µg mL^{-1} stock solutions of caffeic acid, chlorogenic acid (Sigma-Aldrich, St. Louis, MO, USA), cichoric acid, and caftaric acid (Chromadex, CA, USA) were prepared by weighing the appropriate amounts and dilution in a mixture of 75% water and 25% MeOH. The chemical structures of the respective compounds are shown in Fig. 2.1. The differences in these compounds are based on the pendant species affixed to caffeic acid through the

ester linkage. The echinacea test samples (50% ethanolic extracts) were supplied by Gaia Herbs (Brevard, NC, USA), and diluted 1:5 in the H_2O:MeOH solvent used to prepare the stock solutions. All solutions were stored in light-tight vessels at 4℃ and prepared fresh daily to ensure minimal degradation. Calibration curves were created by triplicate injections of the standard solutions into the LC system with spectral data acquired in TIC mode. The quantification of the caffeic acid derivatives in the commercial extract was achieved through a standard addition method to the extracts prior to the HPLC separation. The caffeic acid derivative stock solutions (1000 µg mL^{-1}) were prepared as mentioned previously and each one added in the amounts of 0.025 and 0.050 mL to 0.2 mL aliquots of the echinacea tinctures and diluted to 1 mL.

RESULTS AND DISCUSSION

Effect of EI and GD Source Operating Parameters on Analyte Responses

The ion volume (block) temperature and the kinetic energy of the electrons are the two primary controlling parameters for the EI source. Therefore, in order to determine the optimal conditions of these parameters, the analytical signal intensity and the MS fragmentation patterns of the caffeic acid derivatives require evaluation. A previous study of catechin species in green tea describes in detailed the evaluation of the source operating parameters,[22] but due to the difference in the structures of the compounds, the parameter optimization was

performed here as well. Caffeic acid and chlorogenic acid were taken as representative of the other test compounds.

The effect of ion source temperature (between 225-and-350℃) on the analyte intensities and fragmentation patterns for caffeic acid and chlorogenic acid were evaluated at a fixed electron energy of 70 eV. The TIC responses for both test compounds show a similar behavior, first increasing with block temperature, passing through a maximum, and then decreasing as the temperature is increased further. In the case of the caffeic acid, the strongest analyte response occurs at ~275 ℃, while the maximum for chlorogenic acid was at ~300 ℃. This general form of the response reflects a case where the initial increases in temperature affect greater vaporization, but beyond the maximum pyrolysis may be occurring. There were essentially no changes in the observed fragmentation characteristics for either compound as the source block temperature was changed. While the optimum temperatures for the two compounds are slightly different, a compromise block temperature of 275℃ value was chosen.

The effect of the electron energy on the analyte intensities and fragmentation patterns was evaluated over the range of 50 to 100 eV, at a block temperature of 275℃. As is typical of EI sources, an increase in the total signal intensity for both compounds is observed as the electron energy increases from 50 to 100 eV, though to a lesser degree at the upper end of the range. These results are very similar to the results obtained in our earlier PB/MS studies on

polyphenolic compounds,[22] hence, the data are not presented here. Also as is typical, there is an increase in the degree of fragmentation as the energy is increased. Ultimately, the standard electron energy of 70 eV was used for the completion of these studies to allow comparison with spectral libraries where such data exist.

In the case of the GD source, the discharge current and argon pressure are the two primary controlling parameters of analytical performance. As in the case of the EI source, the analytical signal intensity and fragmentation patterns were evaluated to determine the optimal source conditions, with caffeic acid and chlorogenic acid taken as representative of the target compounds. Figure 2.2a shows the effect of discharge current between 0.2 to 0.6 mA on the analyte intensities of total analyte signal for caffeic acid and chlorogenic acid at a fixed argon pressure of 0.5 Torr. The strongest analyte signal with respect to discharge current is at 0.2 mA followed by a gradual decrease in the intensity as the current is increased up to 0.6 mA. This trend contradicts the expectations based on the fact that electron density in the plasma should increase with current, but is consistent with previous PB/GDMS work.[31, 32] A better understanding of the role of the discharge current on analyte signal response was achieved when observing the trend of the 180/162 Da fragment ratios, shown in Fig. 2.2a. During the optimization studies with the EI and GD sources, it was observed that for any given compound, the obtained fragmentation pattern does not change significantly with variations in the respective operating

parameters. That is, while the total intensities change, the qualitative fragmentation patterns do not vary appreciably. The optimized discharge current obtained for this study at 0.2 mA is identical to the optimize discharge current obtained in the previous studies performed on green tea.[22] The fact that the fragment ion ratios do not change with the discharge current implies that the plasma energetics are consistent, and suggests that it is the sampling efficiency that is changed. Simply, as the current in the plasma is increased, the negative glow region will tend to withdraw back toward the cathode and away from the sampling orifice. Krishna and Marcus found this to be the case in detailed studies in PB/GDMS across a range of different cathodes and test compounds.[32]

Figure 2.2b shows the effect of argon discharge pressure between 0.3 to 0.7 Torr on the analyte intensities of the TIC signal for caffeic acid and chlorogenic acid at a fixed discharge current of 0.2 mA. Discharge pressure controls the discharge voltage and the frequency of gas phase collisions. The analyte signals increase with pressure for both test molecules until reaching a maximum at ~0.5 Torr and subsequently decreasing as the pressure goes to 0.7 Torr. The m/z 180/162 intensity ratio decreases slightly for chlorogenic acid as the collision frequency increases in the plasma, i.e. more fragmentation occurs. In this case, the optimum discharge pressure is slightly different that the one obtained previously for the green tea constituents (0.8 Torr).[22] Such variation in discharge pressure might be attributed to different thermodynamic properties of these compounds or slight differences in discharge geometry. A discharge

current of 0.2 mA and an argon source pressure of 0.5 Torr were used for the remainder of these studies.

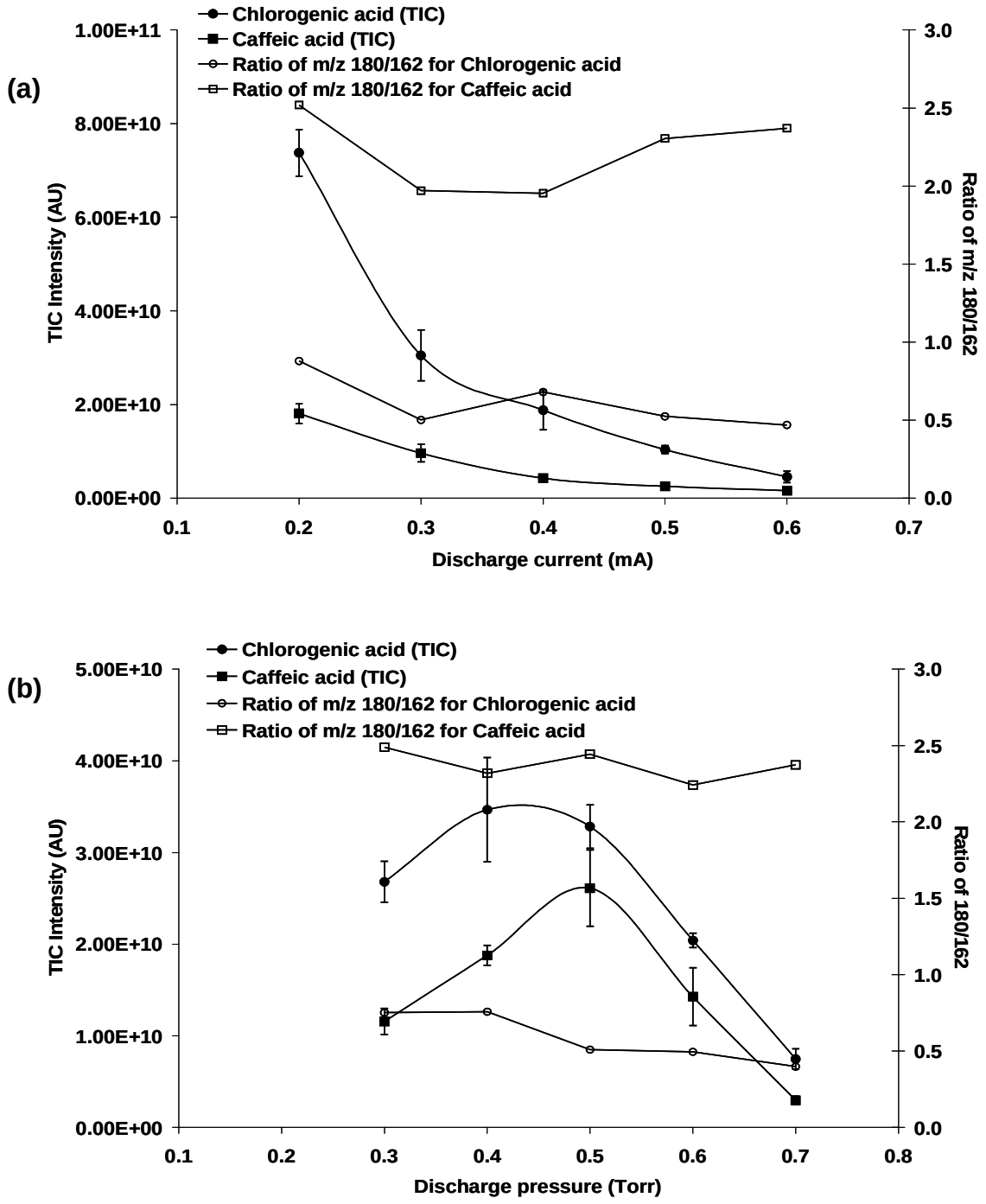


Figure 2.2. Effect of GD source operating conditions on TIC ion signal intensities for triplicate introduction of 200 µg mL^{-1} caffeic and chlorogenic acid a) effect of discharge current at a source pressure of 0.8 Torr argon and b) effect of discharge gas pressure at a discharge current of 0.2 mA. Injection loop = 50 µL; flow rate 0.9 mL min^{-1}.

Characteristic Mass Spectra for Caffeic Acid Derivatives

As mentioned previously, the PB interface provides efficient LC/MS coupling by removing solvent residues/vapors to affect a solvent-free environment within the respective ionization sources. This quality permits the acquisition of EI spectra that can be easily interpreted and compared to spectral libraries (where they exist). For these reasons, this laboratory has exploited the qualities of the PB interface for the use with GD plasma sources as well. Glow discharge sources are used specifically as they have been shown to provide both elemental and molecular information for trace metals analysis as well as the identification of organic compounds.[22, 23, 26, 29, 30, 33] This section presents a direct comparison of the spectral characteristics of the EI and GD sources for the caffeic acid derivatives anticipated to be present in the echinacea extract. The PB/EI and PB/GD mass spectra of caffeic acid, chlorogenic acid, caftaric acid, and cichoric acid obtained from 50 µL injections of 100 µg mL^{-1} solutions are presented in Figs. 2.3-2.6, respectively. The spectral acquisition conditions employed for both sources are the same, therefore direct comparisons can be made between the two. It is important to point out that only two of the caffeic acid compounds (caffeic and chlorogenic acid) studied here has sufficient volatility to allow analysis by standard GC/MS or direct probe methods. As such, it is only for those two compounds that NIST library spectra exist.

The EI and GD mass spectra of caffeic acid are presented in Fig. 2.3. The spectra are qualitatively very similar in terms of the identity of the fragment

species, though the extent of the fragmentation is less for the plasma source. In both cases, the base peak is reflective of the parent molecule, $(M-H)^+$ for the EI spectrum and $M^{\bullet+}$ for the GD spectrum. The m/z = 179 Da species in the EI spectrum is referred to as the caffeoate ion. In addition to the parent species, prominent fragment ions at m/z = 162 and 135 Da are seen in both spectra. (The m/z = 162 Da fragment is referred to as the caffeoyl group.) These fragments correspond to the loss of H_2O (18 Da) and the protonated carboxylate functionality (45 Da) from the parent molecule, both of which are typical of aromatic alcohols and carboxylic acids. Overall, the TIC response for the GD source is ~20% higher that the EI source, as is the base peak intensity in the extracted mass spectrum. Also shown in Fig. 2.3 is the NIST (EI) library spectrum created by plotting the tabulated peak intensities. (Note that peaks of <5% relative abundance are not plotted.) As can be seen, the prominent spectral peaks among the three spectra are quite similar, with the degree of fragmentation increasing from the standard library, PB/EI, and PB/GD sources. It is not surprising that the NIST spectrum exhibits more fragmentation because those molecules are exposed to continuous high temperatures and thus have greater internal vibrational energy prior to the ionization event. To be clear, the fact that the GD spectral are similar to those obtained via EI is not a requirement for successful use of the GD source, but it is most important that the same spectral interpretation rules can be employed to identify unknowns.

Figures 2.4 displays the mass spectra obtained for chlorogenic acid from the EI and GD ion sources, respectively, along with the NIST library spectrum. The mass spectra are very similar with simple and easily interpreted fragmentation patterns. In both cases, the deprotonated molecular ion $(M-H)^+$ for chlorogenic acid is seen at m/z = 353 Da. Prominent in both is the loss of the 273 Da six-member ring unit from the parent molecule, to yield the base caffeoate segment (180 Da). Below that mass are the signature ions for caffeic acid seen in Fig. 2.3, but with a higher level of fragmentation in both cases. Here again, the overall ion yield for the GD source is somewhat higher than of the EI source. In this case though, with diminished signal-to-noise characteristics to the EI sources.

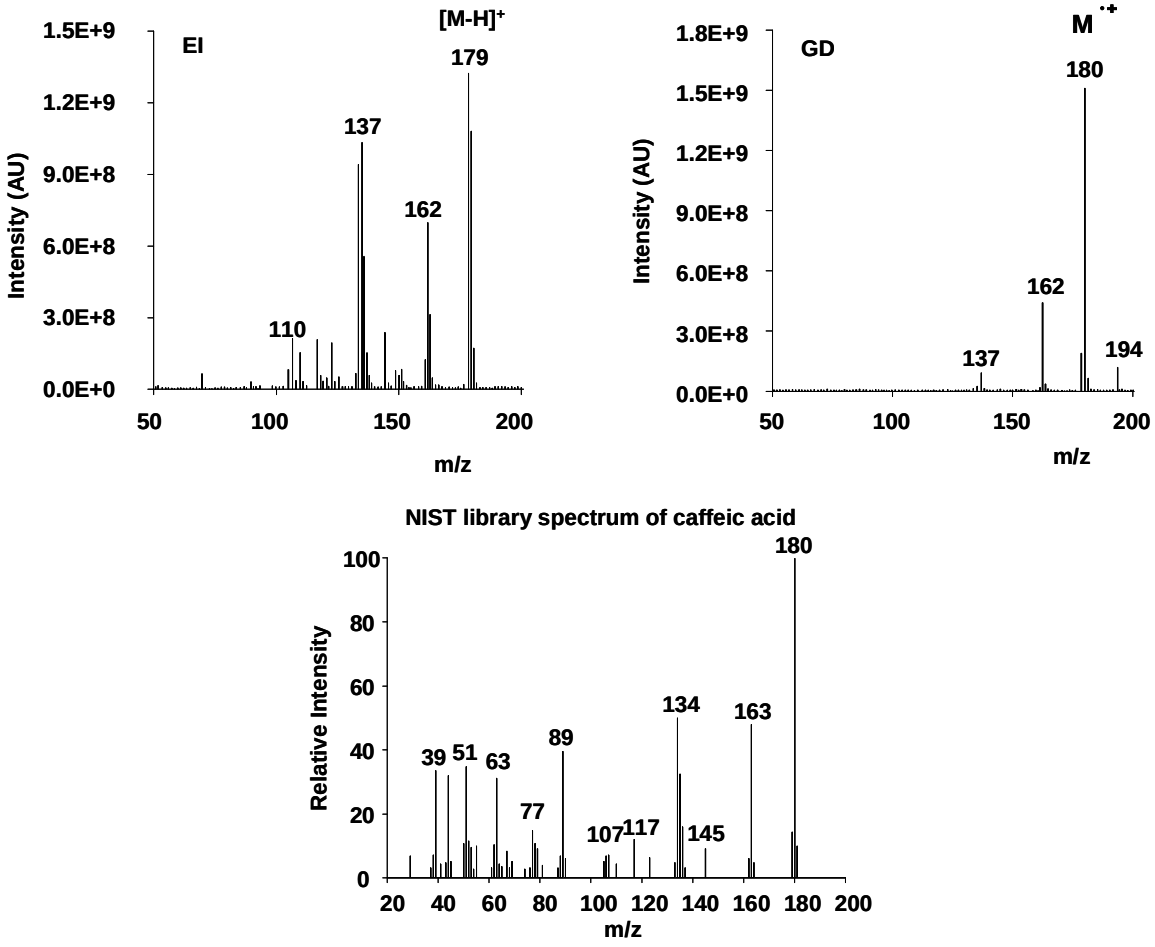
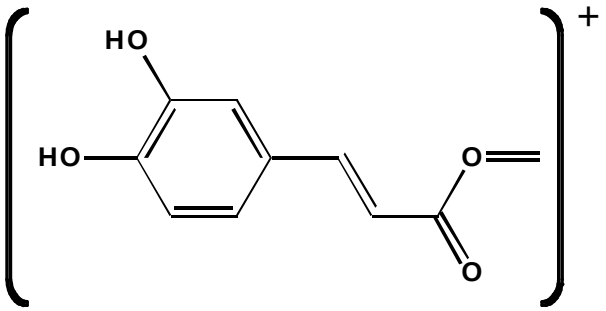


Figure 2.3. LC-PB mass spectra of 50 µL injections of 100 µg mL^{-1} solution of caffeic acid with the EI (electron energy = 70 eV, source block temperature = 275 ℃ and GD (discharge current = 0.2 mA, Ar pressure = 0.5 Torr) sources.

Figure 2.5 presents the mass spectra of caftaric acid obtained from the EI and GD ion sources, respectively. (No reference EI spectra are known to exist.) In this case, the mass spectra obtained from the two sources show greater differences in the fragmentation patterns. This is not surprising as the molecule has far greater complexity and degrees of freedom than caffeic and chlorogenic acid. Given this, it is surprising that both spectra do exhibit molecular ion signals.

In both instances, the fragmentation of the ester linkage yields the characteristic peaks of caffeic acid. Two additional fragment ions are seen here that are not present in the other caffeic acid derivatives, appearing at m/z = 114 and 137 Da. The first of these appears to be a fragment ion from the di-acid unit and the latter a rearrangement that is inclusive of the ester of the caffeic acid base unit. The structure of the m/z = 195 Da fragment present in the EI mass spectrum, and absent in the GD spectrum, is proposed below. The signal intensity of the base peak for the GD source shows an approximately 3x greater response than in the EI source spectrum, a far higher level of improvement than the other target compounds evaluated here.



The most complex of the caffeic acid derivatives is cichoric acid. Here again the molecule is likely to provide an EI standard spectrum with similar fragmentation patterns to the other caffeic acid derivatives. Inspection of the structure given in Fig. 2.1 shows that it is essentially a caffeic acid dimer, coupled through the di-basic unit seen in caftaric acid. Not surprisingly, as shown in Fig. 2.6, the EI spectrum for cichoric acid does not exhibit a molecular ion peak. On

the other hand, the GD spectrum for cichoric acid contains the $(M-H)^+$ at m/z 473 Da. For the most part, the EI spectrum is the same as that seen for caftaric; without the molecular ion. The same can be said for the GD spectrum, wherein the 115 Da fragment is the base peak, though there are additional peaks at 204 and 218 Da which may represent two methylene additions to the caffeic acid base unit (neither of which would be expected based on the structures of the other derivatives).

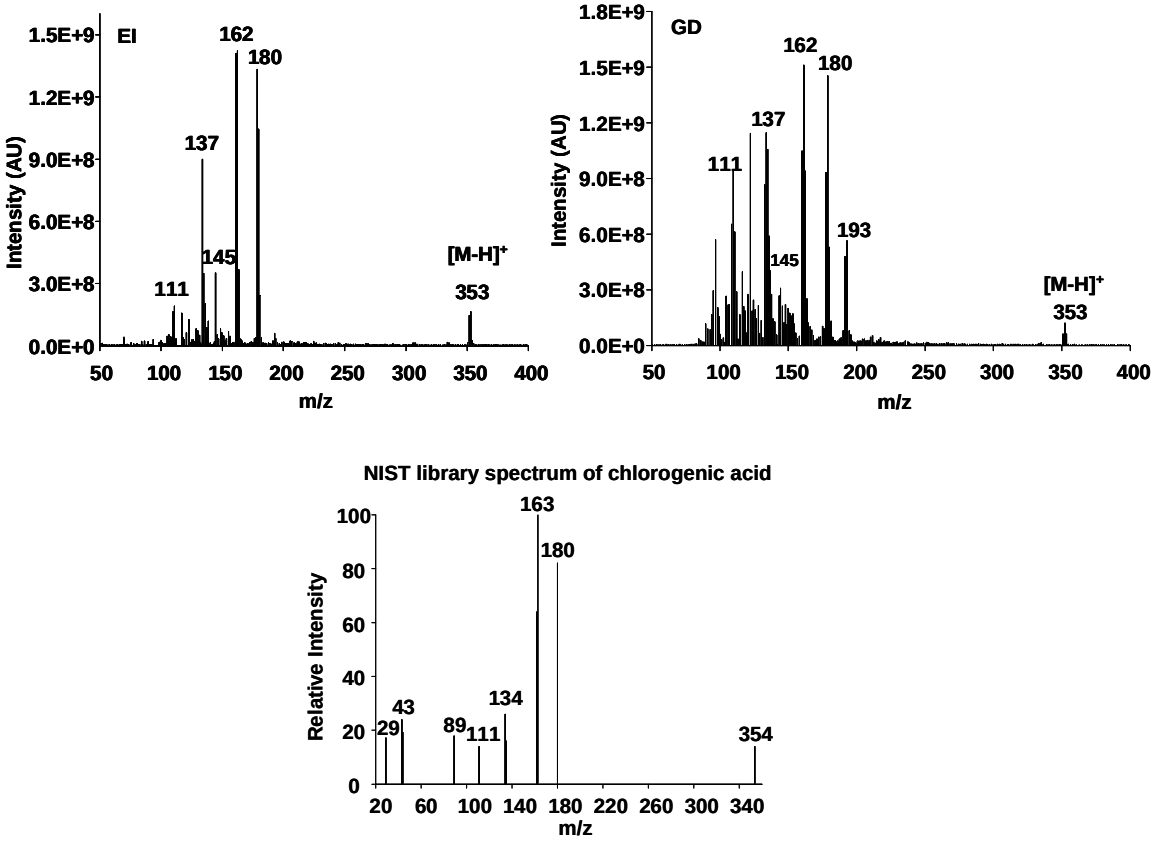


Figure 2.4. LC-PB mass spectra of 50 µL injections of 100 µg mL^{-1} solution of chlorogenic acid with the EI (electron energy = 70 eV, source block temperature = 275 ℃ and GD (discharge current = 0.2 mA, Ar pressure = 0.5 Torr) sources.

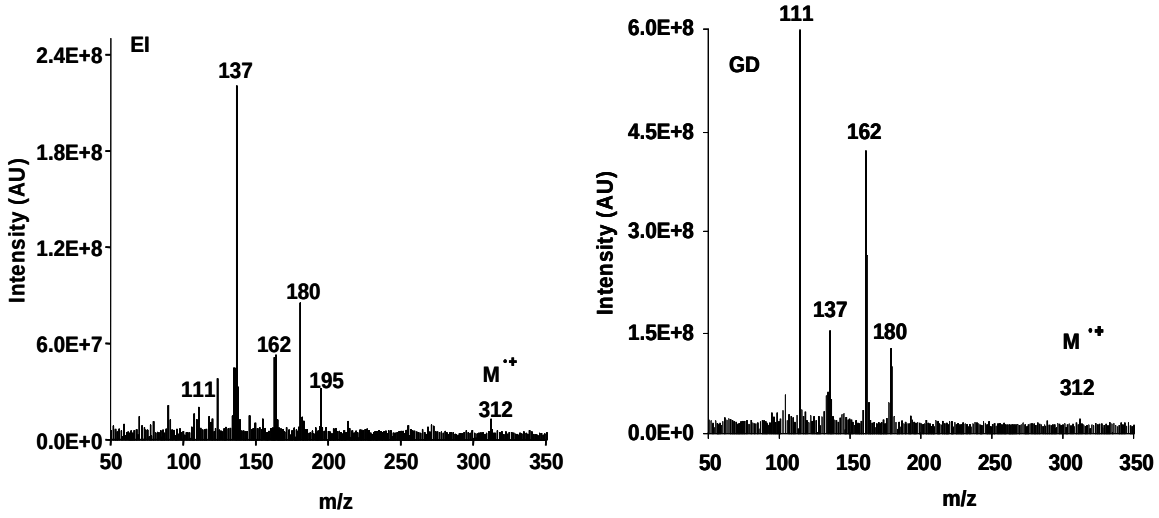


Figure 2.5. LC-PB mass spectra of 50 µL injections of 100 µg mL^{-1} solution of caftaric acid with the EI (electron energy = 70 eV, source block temperature = 275 ℃ and GD (discharge current = 0.2 mA, Ar pressure = 0.5 Torr) sources.

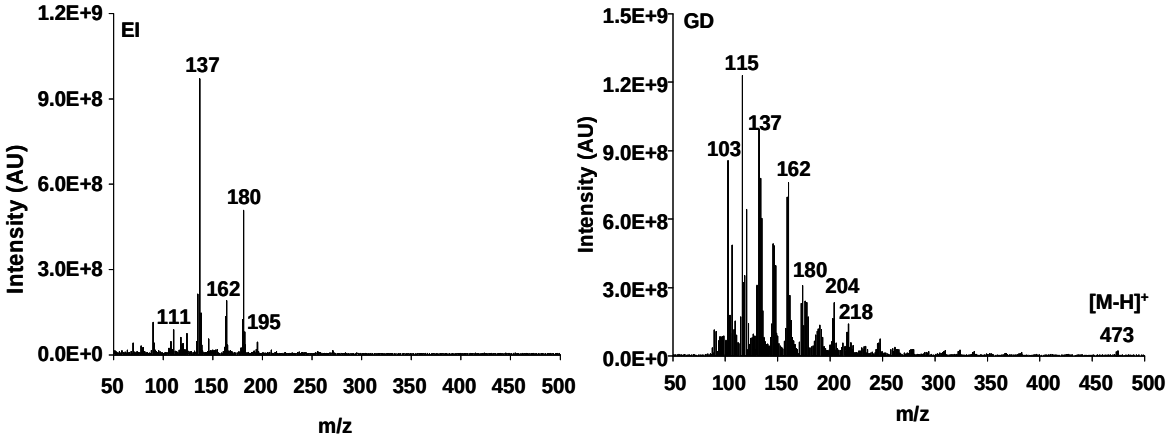


Figure 2.6. LC-PB mass spectra of 50 µL injections of 100 µg mL^{-1} solution of cichoric acid with the EI (electron energy = 70 eV, source block temperature = 275 ℃ and GD (discharge current = 0.2 mA, Ar pressure = 0.5 Torr) sources.

Overall, the EI and GD mass spectra acquired for the caffeic acid derivatives exhibit excellent correlation, with very similar fragment species, even though the relative intensities were not the same. Being able to make comparisons between the EI and GD ion sources spectra suggests that the

ionization energetics in the GD source are quite similar to the 70 eV EI. Most importantly, this allows the application of electron ionization spectral interpretation rules and the possibility of spectral library comparison. It is important to note that single-collision electron ionization is improbable in the GD source because its average electron energy is below 1 eV.[31] On the other hand, the metastable energy levels for Ar are 11.5 and 11.7 eV.[31] Hence, either multiple-electron or Penning-type ionization collisions would be the most probable ionization pathways occurring in the GD source. In general, the GD source provided high quality mass spectra with higher signal-to-noise ratios than the EI source.

Analytical Response Characteristics

Following the optimization of the PB/EIMS and PB/GDMS experimental conditions, the basic analytical response characteristics were obtained for the caffeic acid derivatives. Tables 2.1 and 2.2 show the analytical response data for caffeic, chlorogenic, cichoric and caftaric acids obtained for the EI and GD sources. Calibration functions using the TIC and the single ion monitoring (SIM) modes were generated for each of the caffeic acid derivatives through triplicate injections across the concentration range of 0 (i.e. analytical blank) to 100 µg mL^{-1} (involving 0.5, 1.0, 5.0, 10, 25, 50, 75 µg mL^{-1} concentrations). The SIM mode usually has a lower LOD than TIC as in the former mode the mass analyzer is set at a single m/z value for the duration of the experiment. In the

TIC mode, the mass analyzer is set to scan across a given mass range (m/z 50-500 Da) over the course of acquisition. Because the caffeic acid ion, $^{180}C_9H_8O_4^{\cdot+}$, appeared in the mass spectra of each of the caffeic acid derivatives, the quantitative data were acquired in SIM mode at m/z = 180 Da. The response functions for the species show good linearity with satisfactory correlation coefficients (R^2 values). It is almost universally true that the sensitivity of the EI source is superior to the GD source, with the resultant limits of detection being predominately set by the slopes, as opposed to variability in the blanks (i.e., precision). As seen in Table 2, monitoring of the analyte signals in the SIM mode generally yielded lower detection limits than TIC mode. The magnitude of the LODs obtained here are not relevant in terms of profiling of botanical extracts where concentrations are on the 100 µg mL^{-1} to percent levels, but are vital in metabolic studies.

Table 2.1. LC-PB/MS analytical response characteristics of the TIC signals for the caffeic acid derivatives with the EI and GD ion sources.

Analyte	Response Function	Accuracy (R^2)	Detection Limit (ng mL^{-1})	Absolute Mass (ng)
Caffeic acid				
EI	y = 2E+09x - 1E+08	0.9913	0.55	0.028
GD	y = 4E+08x - 5E+06	0.9971	3.70	0.19
Chlorogenic acid				
EI	y = 6E+08x - 2E+09	0.9749	5.50	0.03
GD	y = 4E+08x - 9E+07	0.9807	6.30	0.32
Cichoric acid				
EI	y = 1E+09x + 5E+09	0.9706	3.60	0.18
GD	y = 2E+08x + 6E+08	0.9812	7.20	0.36
Caftaric acid				
EI	y = 3E+09x + 3E+09	0.9925	3.96	0.13
GD	y = 2E+08x + 8E+08	0.9824	5.10	0.26

Table 2.2. LC-PB/MS analytical response characteristics at m/z = 180 Da fragment for the caffeic acid derivatives with the EI and GD ion sources.

Analyte	Response Function	Accuracy (R^2)	Detection Limit (ng mL^{-1})	Absolute Mass (ng)
Caffeic acid				
EI	y = 4E+08x - 3E+09	0.9995	0.97	0.05
GD	y = 2E+08x - 2E+08	0.9899	1.50	0.08
Chlorogenic acid				
EI	y = 8E+07x - 7E+08	0.9802	4.85	0.24
GD	y = 1E+08x - 2E+08	0.9816	3.10	0.16
Cichoric acid				
EI	y = 2E+08x - 9E+08	0.9663	1.94	0.10
GD	y = 1E+08x - 2E+07	0.9947	3.10	0.16
Caftaric acid				
EI	y = 6E+08x - 1E+09	0.9937	0.64	0.03
GD	y = 4E+07x - 6E+07	0.9844	7.70	0.38

While better LODs were obtained with the EI source than the GD source, the limits of detection for the GD source are still below 1 nanogram in every case. In the literature, the most commonly reported LODs are found for cichoric acid by UV absorbance detection and range from ~0.75 to 40 ng absolute.[4-6, 12] In the case of ESI detection, a reported absolute value of 0.15 ng for cichoric acid in SIM detection was also found.[4] Hence, the limits of detection obtained for the caffeic acid derivatives studied with the EI and GD sources are consistent with/or lower than the ones found in the literature. [4-6, 12] As mentioned previously, on most occasions ESI-MS only provides the spectral signature of the molecular ion, whereas the EI and GD sources provide fragmentation patterns which are useful in the identification of unknown compounds. Hence, the capabilities of these sources are better suited for applications in botanical product profiling and metabolic studies.

Chromatographic Separation and Quantification of Echinacea Constituents

The culmination of this study included the separation and quantification of two commercially available Echinacea Supreme extracts (lot number: 832011705B-OG and 832010308-OG). Figures 2.7 and 2.8 show overlays of the chromatographic separation of a 100 μg mL^{-1} mixture (2.5 μg, each) of the caffeic acid derivative standards and a 20% Echinacea Supreme extract (respectively) in the selected ion monitoring mode for both ion sources. Specifically, the signals of the m/z = 137, 162, and 180 Da characteristic fragment ions are extracted from the TICs, and co-added to yield simplified chromatograms. As seen in Fig. 2.7 for the synthetic mixture, the chromatographic separation with a linear gradient varying from 75:25 (H_2O:MeOH) to 55:45 results in a fully baseline-resolved separation in less than 30 minutes. The extracted mass spectra gathered at the respective elution times provide fragmentation patterns consistent with mass spectra of the standard compounds (Figs. 2.3-2.6). As such, the echinacea components can be unambiguously identified. The individual responses show quite good signal-to-noise characteristics, and the chromatographic integrity is very well maintained. There is a slight (<10 sec) delay in the appearance time of each of the peaks in the GD chromatogram which is due to the transit of analyte species from the cathode surface and through the plasma in the ~1 Torr plasma source. As can be seen, there are some species-specific differences in the responses for the two sources, which is not surprising given the different fragment ratios seen in Figs.

4-7. That said, the responses are fairly uniform, particularly given the disparity in the ionization methods.

The PB/EI and GD chromatograms of the commercial Echinacea Supreme extract are shown in Fig. 2.8. The caffeic acid derivatives of interest in this study are clearly identifiable in the traces (at this scale), with the exception of chlorogenic acid. Scale expansion followed by mass spectral examination (as well as spiking) confirmed that chlorogenic acid was indeed the compound with the retention time of ~14.0 min. Also labeled on the chromatogram is the simple caffeic acid derivative, cynarine, as well as echinacoside, a phenylpropanoid glycoside, not in our target list but readily identified with the instrument. Cynarine and echinacoside are prominent constituents of *Echinacea angustofilia*, but not in *Echinacea purpurea,* and are both known to be unstable in ethanolic extracts; thus their presence was something of a surprise.[34] The ability of the GD source to produce EI-like spectra allowed their ready identification from the expanded mass chromatogram as well as its relative position in the chromatogram. [3, 12, 35] Finally, echinacoside (whose structure is similar to cichoric acid, except the dimer is linked with a three-sugar unit) could not be identified unambiguously via its mass spectrum as there was no molecular ion (MW = 785 Da); this is not unexpected. Based on the extracted mass spectrum the compound was clearly a caffeic acid derivative, and so was identified based on the retention time of an echinacoside spike.

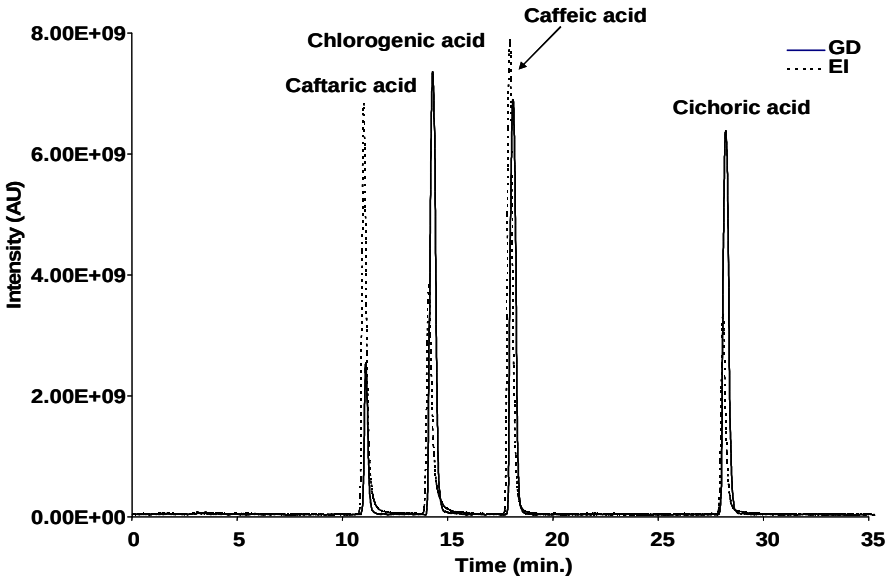


Figure 2.7. RP-HPLC separation of 100 µg mL^{-1} mixture of caffeic acid derivative standards in selected ion monitoring mode (m/z = 137, 162, and 180 Da) with EI and GD source. Gradient elution = 75:25 (H_2O:MeOH) to 55:45 in 40 minutes, flow rate = 0.9 mL min^{-1}., injection loop = 50 µL.

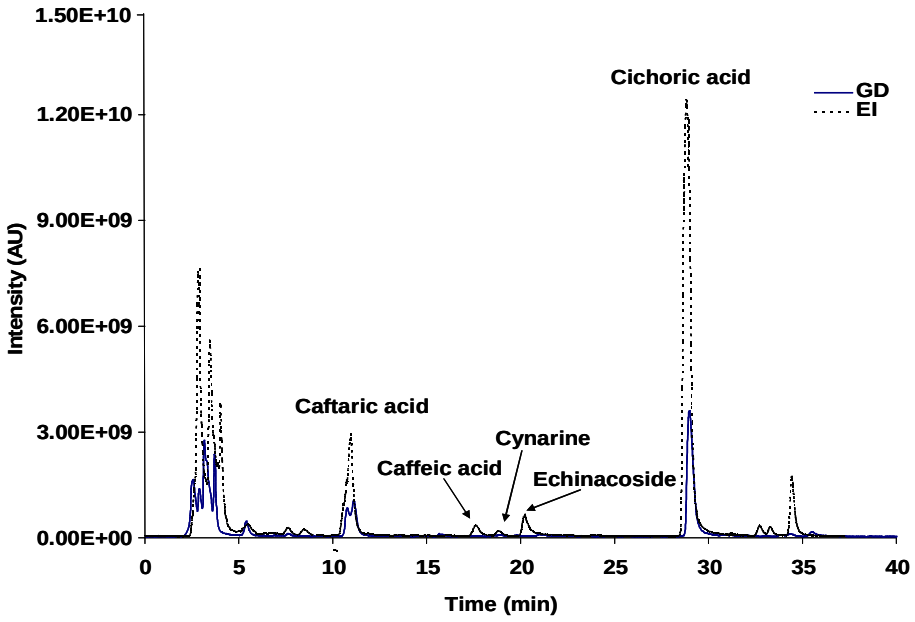


Figure 2.8. RP-HPLC separation of 20% Echinacea Supreme commercial ethanolic extract in selected ion monitoring mode (m/z = 137, 162, and 180 Da) with EI and GD sources. Gradient elution = 75:25 (H_2O:MeOH) to 55:45 in 40 minutes, flow rate = 0.9 mL min^{-1}., injection loop = 50 µL.

The sensitivities observed upon injection of neat compounds can be different from that obtained under HPLC conditions. As such, a standard addition method was carried out to quantify the amounts of caffeic, chlorogenic, cichoric and caftaric acid in the two commercial extracts. The concentrations of the caffeic acid derivatives were evaluated based on triplicate HPLC separations for both ion sources. The results of those measurements are shown in Table 2.3. As was clearly seen in the chromatographic separation (Fig. 2.8) that cichoric acid is the major component in this ethanolic echinacea extract, followed by caftaric acid, caffeic acid, and chlorogenic acid. (The cynarine and echinacoside were not quantified due to limited quantities of the pure compounds.) The quantitative values obtained with the two sources are effectively the same for each of the compounds. As seen in Table 3, the quantification results obtained for the caffeic acid derivatives present in the two commercial Echinacea Supreme are different but the major components (cichoric and caftaric acid) are the same in both extracts. As well, the chlorogenic acid concentration is only determined in one of the extracts. The difference in concentration values and the absence of chlorogenic acid in one of the extract is due to the fact that two commercial extract have different lot numbers that were processed and manufactured in different years; perhaps under different extractions conditions and surely from different harvests. Thus, both PB/EI and GDMS approaches could be used to differentiate natural products.

Table 2.3. Quantification results for the caffeic acid derivatives by the standard addition method with LC-PB/MS using both ion sources.

Analyte	Quantity ($\mu g\ mL^{-1}$)			
	EI	% RSD	GD	% RSD
Echinacea Supreme (832011705B-OG)				
caffeic acid	43.5 ± 3.5	8.1	44.0 ± 6.0	14.0
chlorogenic acid	9.75 ± 0.35	3.6	10.7 ± 1.8	16.8
cichoric acid	770 ± 19	2.5	768 ± 14	1.8
caftaric acid	389 ± 16	4.0	370 ± 13	3.5
Echinacea Supreme (832010308-OG)				
caffeic acid	59.0 ± 4.9	8.3	56.3 ± 8.5	15.2
chlorogenic acid	not detected		not detected	
cichoric acid	687 ± 77	11.0	665 ± 56	8.4
caftaric acid	501 ± 42	8.3	563 ± 88	16

CONCLUSIONS

The data presented here support the use of LC-PB/MS with EI and GD ion sources as an analytical tool for the analysis and quantification of target compounds in botanical extracts, in this case *Echinacea*. The optimization of both of the ion sources was done by monitoring the response of the analyte molecular/fragments ion signal intensities. The mass spectra obtained for the caffeic acid derivatives via the EI and GD sources were similar to each other in terms of fragmentation patterns. This characteristic supports the use of standard EI spectral libraries in conjunction with GDMS as well as the use of EI spectral interpretation rules. The analytical response functions for the caffeic acid derivatives illustrate good linearity with satisfactory correlation coefficients (R^2 values) and LODs on the sub-nanogram level. A simple RP-HPLC method was

employed to separate the target compounds in the commercial products. Additional caffeic acid derivatives were readily identified based on their mass spectra and retention characteristics. Quantification of the caffeic acid derivatives in a pair of commercial extracts was performed by the standard addition method, with variabilities of less than 17% RSD for the two ion sources for triplicate mass chromatograms. The highest concentration values corresponded to cichoric acid followed by, caftaric acid, both known to be major components in *Echinacea purpurea* extracts. The ability to determine target compounds separated by HPLC based on easily interpreted mass spectra on concentrations ranging from the sub-ng ml^{-1} to high □g ml^{-1} levels is seen as having relevance for both commercial product developments as well as in fundamental metabolism studies.

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CHAPTER THREE

ON-LINE SEPARATION AND IDENTIFICATION OF INORGANIC AND ORGANIC ARSENIC SPECIES IN ETHANOLIC KELP AND BLADDERWRACK EXTRACTS THROUGH LIQUID CHROMATOGRAPHY PARTICLE BEAM ELECTRON IONIZATION MASS SPECTROMETRY (LC-PB/EIMS)

INTRODUCTION

The widespread acceptance and increased use of nutritional supplements can be demonstrated as the sale of natural supplements worldwide increased from $8 billion in 1995 to $19 billion in 2000.[1,2] It is widely recognized that the toxicological effects and biochemical functions of trace elements is strongly dependent on the chemical form (species) of the element.[3] Relative to other elements, arsenic has generated a great deal of interest because of the species-dependent toxicity of arsenic compounds and their existence in various environmental and biological specimens.[3, 4] The toxicity of arsenic species varies, ranging from relatively harmless organoarsenical compounds (e.g., arsenobetaine, arsenocholine and trimethylarsine oxide) to more potent organoarsenicals (i.e. monomethylarsonic acid and dimethylarsinic acid) as well as the inorganic arsenic species (i.e. arsenite and arsenate).[5, 6]

The two predominant pathways for human arsenic exposure are drinking water and dietary intake.[7] Although the arsenic levels in sea water are in the low nanogram levels, unusually large quantities of arsenic (1-100 µg) levels are

found in marine animals and plants (and consequently food that originates from marine sources) because of bioaccumulation and biotransformation of arsenic.[8-10] Therefore, regular consumption of marine-based food supplements, especially in combination with other kinds of seafood, can result in high daily intake of arsenic compounds; as high as several hundred micrograms per gram.[11, 12] The main arsenic compounds found in marine plants (e.g., seaweeds) are typically arsenoribosides (i.e., sugars), which are considered to be non-toxic.[13-15] However, some algae samples are known to contain high levels of the potentially toxic inorganic arsenic and organoarsenic compounds.[16, 17] Kelp *(Ascophyllum nodosum)* and bladderwrack *(Fucus vesiculosis)* are known to be some of the richest sources of micro-nutrients and minerals, and are often used as nutritional supplements.[18] Kelp is an especially good source of iodine and potassium, and has been useful in the treatment of under-active thyroid function as well as in treatments that alkalize blood chemistry. Bladderwrack (a type of brown sea weed also known as black tang, rockweed, and sea wrack) is part of the kelp family, and has been used to treat arteriosclerosis and iodine-deficiency ailments.[19] To be clear, the profile of the extracts from these (and any) botanical products will depend on the exact extraction conditions, including, solvent, temperature, time, and other issues.

Various speciation techniques have been developed to provide information that can be used to understand the distribution and fate of arsenic in biological and environmental systems.[5, 9, 20, 21] While gas chromatography-mass

spectrometry (GC-MS) methods are quite useful in the analysis of volatile arsenic compounds, these methods are not well suited for analyzing inorganic and ionic As species. High-performance liquid chromatography (HPLC) methods for arsenic speciation have been developed that can be interfaced with several types of detection systems including inductively coupled plasma mass spectrometry (ICP-MS),[9] electrospray ionization mass spectrometry (ESI-MS)[22-24] and tandem MS with selected reaction monitoring (SRM),[25-27] optical atomic spectroscopy,[28, 29] and to some extent voltametry.[30] While exhibiting outstanding elemental sensitivity, some of the limitations associated with ICP-MS detection are the need for complete chromatographic resolution of metal components present in the sample, poor compatibility with organic (e.g., reversed-phase) solvents, and identification that is purely based on matching chromatographic retention times rather than "molecular" characteristics. On the other hand, an advantage of using ESI-MS detection is the ability to produce pseudomolecular ions (M+H) of large molecules which has been exploited when analyzing arsenosugars.[24, 31] Unfortunately, when ESI-MS is compared to ICP for metal speciation, the limits of detection for ESI are three orders of magnitude higher than ICP-MS, and the analytical accuracy is generally much poorer.

An analytical technique that would provide accurate molecular weight, structural and elemental information (i.e. *comprehensive speciation)* about sample components using a single mass spectrometry ion source would be an asset in the study of metal species in biological specimens. To this end, a

particle beam (PB) LC/MS approach is being applied to the characterization of these materials.[32, 33] While HPLC-electron ionization (EI) MS methods have been successfully utilized to solve a variety of analytical problems,[34-36] these methods have not been sufficiently evaluated for elemental speciation purposes. This laboratory has been actively involved in the use of a PB-MS system equipped with interchangeable glow discharge (GD) and EI sources.[37] Recently PB-MS has been successfully utilized for online speciation of mercury through liquid chromatography and electron ionization.[38] The PB technique has also been used for the characterization of catechins and caffeine in green tea through EI and GD ionization LC/MS analysis.[39] These studies have demonstrated that the coupling of the PB interface to a GDMS ion source provides the ability to perform *comprehensive speciation* analysis of liquid mixtures that is not feasible with any other plasma MS source and that conventional EI analysis is also a viable approach for LC/MS analysis of botanical extracts.

This work focuses on the development of two liquid chromatography methods for the separation and identification of inorganic and organic arsenic species in commercial ethanolic extracts of kelp and bladderwrack using PB-EIMS detection. Inorganic arsenic (As (III) and (V)), dimethylarsinic acid (DMA), arsenobetaine (AB), and an arsenosugar (oxo-arsenosugar-glycerol, As 328) were used as the probe species. An isocratic reversed-phase (RP) HPLC method was developed using a C_{18} derivatized silica column which permits separation of the inorganic versus organoarsenicals and a complementary anion-

exchange chromatography separation was developed to allow separation of the constituents based on combined ionic/hydrophobic behavior. These two approaches demonstrate the versatility of the PB/EIMS approach. The instrument and chromatographic parameters were optimized to obtain the best sensitivity and resolution of the test compounds. Analytical response functions were obtained for each of the test compounds. The methods were applied to the separation, identification, and quantification of inorganic and organic arsenic species present in commercial ethanolic extracts of kelp and bladderwrack.

EXPERIMENTAL

Particle Beam Electron Ionization Mass Spectrometer System

The LC-PB/EIMS arsenic speciation was undertaken here on an Extrel (Pittsburgh, PA, USA) Benchmark mass spectrometer system (Fig. 1.5). A detailed description of the instrument has been provided in chapter one. The MS instrumentation and data acquisition were controlled using an Extrel Merlin data system. All common LC/MS detection modes such as single ion monitoring (SIM) and total ion chromatogram (TIC) modes were processed and the transient peak areas calculated, using the Merlin software. The data was then exported into Sigma Plot 8.02 (Systat Software, Inc., Richmond, CA) and MS Excel (Microsoft, Seattle, WA) for further processing. The mass spectrometer was repetitively scanned from 50-200 Da (50 – 350 Da for As 328) at 1.0 s per scan to obtain TIC responses as well as the temporally-resolved mass spectra and

SIM chromatograms. The operation parameters of the EI source (electron energy and block temperature) were optimized as described in previous work,[38, 39] with the eventual values of 70 eV and 300 ºC used throughout the analytical studies.

High Performance Liquid Chromatography

Chromatographic separations were carried out using a Waters (Milford, MA, USA) Model Series 600E liquid chromatography pump as the sample delivery system. Injections were carried out using a Rheodyne 9725 injection valve with a 5 µL injection loop (Rheodyne, CA, USA). The reversed-phase (RP) separation of the arsenic compounds was accomplished on a column made up of a C_{18} stationary phase on 5 µm silica diameter particles (Alltech Associated Inc. Deerfield, IL). The column geometry was 4.6 mm i.d. by 250 mm length. The separation was achieved under isocratic conditions with a mobile phase consisting of 0.1% TFA in a water:methanol (96:4) solvent. It was determined that rapid and efficient separations and identification could be achieved at a mobile-phase flow rate of 0.7 mL min^{-1}. In the case of the ion-exchange (IEC) separation, a Dionex Ionpac AS7 (Sunnyvale, CA, USA) anion-exchange column was employed using a gradient elution program with mobile phase compositions of (A) 0.5mM nitric acid (HNO_3) containing 2% methanol (MeOH) and (B) 50 mM HNO_3, as previously described by Guérin *et al*.[6] The separation was

accomplished in less than 8 minutes at a flow rate of 0.9 mL min^{-1}. The PB-EIMS and chromatographic operating conditions are given in Table 3.1.

Reagents and Solutions

Deionized water (NANOpure Diamond, Barnstead International, Dubuque, IA) of > 18 MΩ·cm, ACS-grade MeOH, trifluoroacetic acid (TFA) and HNO_3 (Trace Metal, Fisher Scientific, Fair Lawn, NJ) was used for the preparation of reagents and standards. The individual stock solutions (1000 µg mL^{-1} of arsenic (III) chloride (Aldrich, Milwaukee, WI), arsenic acid (sodium salt heptahydrate) (Sigma, St. Louis, MO), dimethylarsinic acid (Sigma, St. Louis, MO), arsenobetaine (Fluka, St. Louis, MO) were prepared in high purity (plasma grade) water. A standard solution of arsenosugar 328 (As 328) was kindly provided by the US-EPA, Cincinnati, OH, USA. All of the solutions were stored in sealed vials at a temperature of 4℃.

The test samples in this study were obtained from a commercial botanical products manufacturer in the form of tinctures. Specifically, 40% ethanolic extracts of kelp and bladderwrack were supplied as they would be delivered as over-the-counter nutraceutical products. The tinctures are clear liquids that are stored in amber bottles to minimize photodegradation of active compounds in the extracts. The sample preparation of extracts is described subsequently for the specific analytical procedures.

Table 3.1. Instrument operating parameters.

PB-EIMS	
Nebulizer tip temperature	85 ºC
Desolvation temperature	100 ºC
Source block temperature	300 ºC
Sheath gas (He) flow	500 mL min^{-1}
Mass range monitored	50-200 Da
Scan time	1 s
Number of scans averaged	5
HPLC	
Reversed-phase chromatography	
Flow rate	0.7 mL min^{-1}
Column	Alltech C_{18}
Mobile phase (isocratic)	96:4 H_2O:MeOH w/ 0.1% TFA
Ion-exchange chromatography	
Flow rate	0.9 mL min^{-1}
Column	Dionex AS7
Mobile phase (gradient)	(A) 0.5 mmol L^{-1} HNO_3, 2% MeOH (B) 50 mmol L^{-1} HNO_3

Determination of Total Arsenic Content in Kelp and Bladderwrack Extracts Using ICP-OES

An accurately weighed amount (~1 g) of ethanolic kelp extract was placed in a Teflon vessel containing 5 mL of concentrated HNO_3 and closed. The closed vessel was placed inside of a microwave digestion system (CEM Corporation, Mars 5 Express, NC, USA) where it was irradiated for total time of 15 min at 60 W power, equating to a constant temperature of 80 ℃. After this pre-digestion step, the sample was irradiated again at 300 W (100 %) to a temperature of 180 ℃ for 15 min using a 10 min ramp. Upon cooling the vessels were opened and the sample was diluted with high purity water to a final volume of 50 mL. The digests were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) for the determination of total arsenic content

using an external calibration method at the As (I) 193.66 nm transition. The digestion method was validated using NIST SRM 3241 *Ephedra sinica* Stapf Native Extract and SRM 3243 Ephedra-Containing Solid Oral Dosage Form which were weighed out and treated in the same manner as the ethanolic extracts.

RESULTS AND DISCUSSION

We have previously reported on the speciation of inorganic arsenic (As (III)), dimethylarsinic acid (DMA), and arsenobetaine (AB) from aqueous solutions using cation-exchange chromatography with PB/GDMS detection.[40] Although this was a simple and convenient method for arsenic speciation, the obtained detection limits were found to be very high and hence could not be applied for real biological specimens such as the commercial extracts studied here. In the case of the marine plants of interest here, it might be expected that arsenosugars would also be prominent arsenic species,[13, 14] and so a representative arsenoriboside (As 328) was added to the suite of target test species. The two different separation schemes were employed as a means of illustrating the versatility of the PB/EIMS detection method as well as serve as a further check that all species might be observed.

Characteristic Mass Spectra of Arsenic Compounds

The main limitation with most metal speciation techniques is the lack of accessible species-specific information for the detected compounds. As such, the qualitative means of identification in these approaches is solely based on the matching of chromatographic retention times. This shortcoming is the primary reason that the analytical methodologies using particle beam mass spectrometry in conjunction with GD and EI ionization sources are being developed. To illustrate the species-specific information of this approach, the PB/EI mass spectra of $AsCl_3$, DMA, AB and As 328 obtained in the flow injection mode (5 µL injection volumes) are presented in Figs. 3.1a-d, respectively. The spectra were acquired using the standard electron energy of 70 eV, allowing comparison with MS spectral libraries. As shown in Fig. 3.1a, the EI spectrum of $AsCl_3$ shows the protonated molecular ion $(M+H)^+$ at m/z = 181 Da, with prominent fragment ions seen at m/z = 145, 126 and 110 Da representing $AsCl_2^+$, $AsClO^+$, and $AsCl^+$, respectively. A significant peak appears at m/z = 91 Da, which is representative of AsO^+. $AsCl_3$ decomposes in water to form HCl gas and arsenous oxide,[41] which is introduced into EI source region in the form of dry particles. The qualitative power of EI is clearly demonstrated here as the parent pseudomolecular ion as well as the chemically relevant fragments are produced. The insert in Fig. 3.1a shows the mass spectrum of $AsCl_3$ from the National Institute of Standards and Technology (NIST) mass spectral database. The PB-EIMS spectrum of $AsCl_3$ shows a very good correlation with the NIST library

mass spectrum, having a qualitatively similar fragmentation pattern. An exact match to the NIST library is not required to affirm the qualitative potential of any ionization technique, but the similarities in the fragmentation patterns seen here provides greater confidence in the interpretation of unknown spectra.

Shown in Fig. 3.1b is the PB/EIMS spectrum of DMA ($(CH_3)_2AsOOH$). There is a prominent molecular ion at 138 Da along with various fragment peaks at 121, 106 and 91 Da. These peaks correspond to the loss of a hydroxyl group $^{121}(M\text{-}OH)^+$, the loss of a methyl group^{106} $(CH_3AsO)^+$ and the loss of second methyl group, respectively. These losses give rise to the $^{91}AsO^+$ ion, and ultimately result in the monoatomic $^{75}As^+$ ion. The mass spectrum obtained for DMA with the PB/EIMS system also shows excellent correlation with NIST library mass spectrum as seen in the inset.

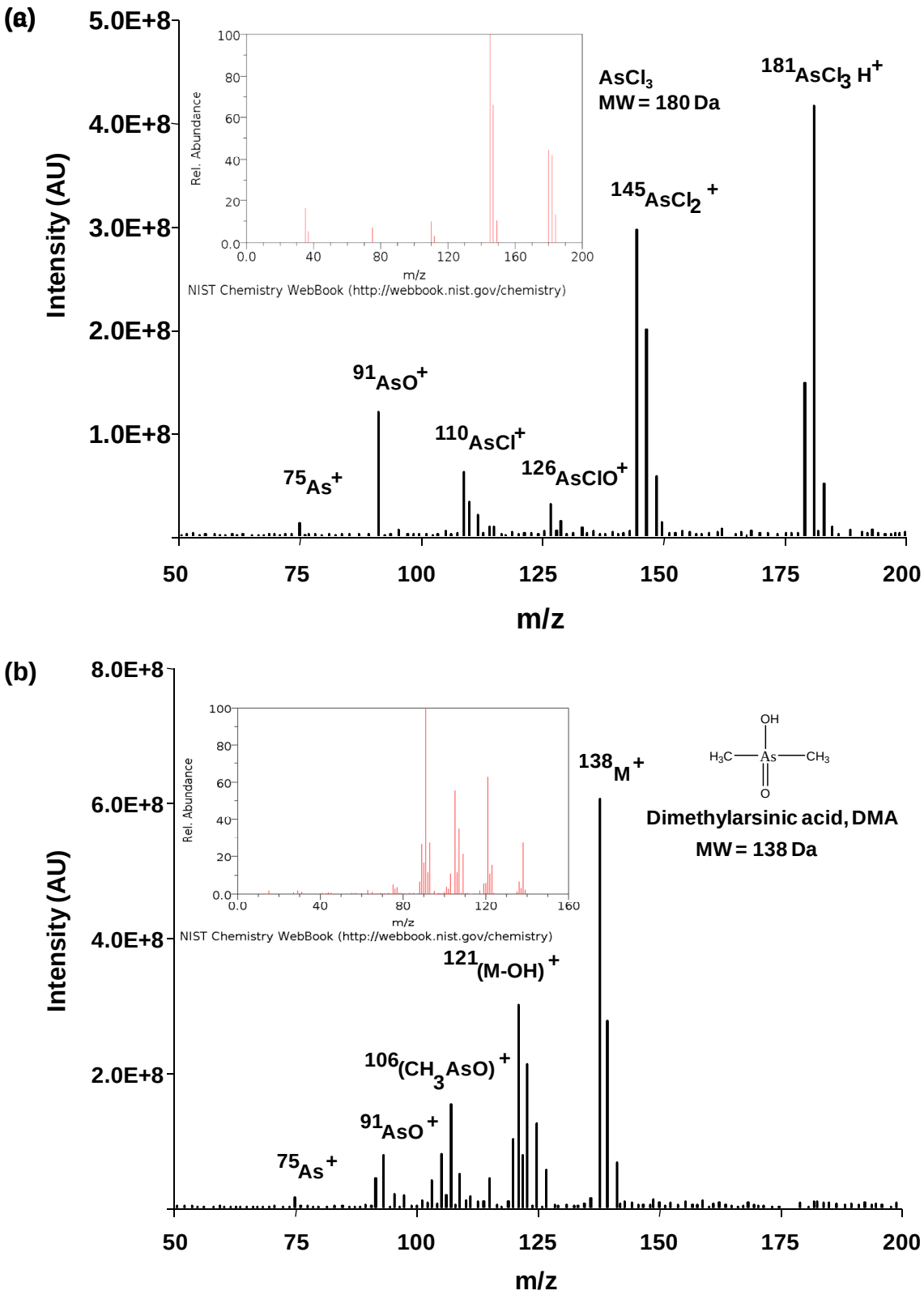
Figure 3.1c is the mass spectrum of arsenobetaine ($(CH_3)_3As\text{-}CH_2COOH$) obtained via PB-EIMS. A straight-forward fragmentation pattern containing the ion fragments of m/z = 160, 134, 121, and 105 Da along with molecular ion at m/z = 178 Da is seen in this spectrum. The respective clusters of peaks correspond to varying numbers of hydrogen atoms being present in the fragment ions. The most prominent fragment ions seen here correspond to the loss of CO_2 (m/z = 134 Da) from the molecular ion and trimethylarsonium ion $((CH_3)_3AsH)^+$ at mass 121 Da. The loss of the CO_2 neutral fragment is a class signature of carboxylic acids. There is a further loss of a methyl group, which gives rise to the cluster at 105 Da. Additionally, there is another peak in the spectrum

representing AsO^+ at m/z = 91 Da. Finally, the $^{75}As^+$ ion peak is observed, which represents the total dissociation of the ligand groups attached to the central arsenic atom. There is no equivalent EI library reference spectrum for this compound because of it limited volatility, illustrating the power of the PB/EI combination to produce straight forward fragmentation patterns that allow the identification of unknown compounds. On the other hand, the fragmentation behavior observed for DMA and arsenobetaine via PB/EIMS is similar to the ESI-SRM fragmentation transitions (parent ion → product ion) presented by Pergantis *et al*.[26, 27] In such examples, two SRM transitions for DMA (139→91 and 139→109) and arsenobetaine (179→120 and 179→105) are used for the identification of organoarsenic species.

While there are a number of potential arsenosugars that have been reported in marine plants, As 328 is the simplest and most commonly found.[13] It must be reiterated that this study is to determine the species present in the commercial ethanolic extracts, species present in the raw plant may not be present in a specific extract formulation. As 328 is included in this study to illustrate the ability to separate and detect this class of compounds if present in these tinctures. The PB/EI mass spectrum of As 328 is shown in Fig. 3.1d. There are various reports published on the ESI mass spectrometry of As 328 in the literature.[13, 14] To our knowledge, this is the first reported EI spectrum on an arsenosugar, demonstrating the utility of the PB interface for otherwise involatile species. The spectrum very clearly reveals the protonated molecular ion along

with a series structurally significant fragment ions. Among them, m/z = 97, 194, and 237 Da are the fragments of the base dimethylarsinylriboside, which is a common structural unit for arsenosugars. The most prominent fragment ion seen here at m/z = 104 Da corresponding to $(As(CH_3CH_2))^+$ ion. Additionally, a strong signal representing AsO^+ at 91 Da is observed. As mentioned in earlier sections, ESI-MS has ability to produce only molecular ions with very few fragmentation peaks, necessitating the use of MS-MS to obtain structural information and higher levels of validation. As a point of comparison, the ESI-MS-MS spectra also contain three fragment ion peaks (m/z = 97, 195 and 237 Da) in addition to the pseudomolecular parent ion.[15, 42]

It should be noted from Figs. 3.1a-d that molecular ion peaks of As(III) (m/z = 181 Da) and DMA (m/z = 138 Da) appeared as base peaks in their respective mass spectra. The molecular ion peaks for AB and As 328 are present only as a minor peak, implying that AB and As 328 are not as stable either in the vaporization or electron bombardment processes, preferentially yielding the trimethylarsonium ion $((CH_3)_3AsH)^+$ in the ion source. Extensive fragmentation is not a surprise in either case given the large number of degrees of vibrational freedom in each molecule. In addition, Devesa *et al.*[43] have made similar observations in their kinetic studies of arsenic species during heat treatment at temperatures >150 ℃. [43]


(a)
Intensity (AU)
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4.0E+8
3.0E+8
2.0E+8
1.0E+8
Rel. Abundance
m/z
NIST Chemistry WebBook (http://webbook.nist.gov/chemistry)
AsCl3
MW = 180 Da
181AsCl3 H+
145AsCl2 +
91AsO+
110AsCl+
126AsClO+
75As+
50
75
100
125
150
175
200
m/z
(b)
Intensity (AU)
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6.0E+8
4.0E+8
2.0E+8
Rel. Abundance
m/z
NIST Chemistry WebBook (http://webbook.nist.gov/chemistry)
138M+
121(M-OH) +
106(CH3AsO) +
91AsO +
75As+
OH
H3C
As
CH3
O
Dimethylarsinic acid, DMA
MW = 138 Da
50
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100
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150
175
200
m/z

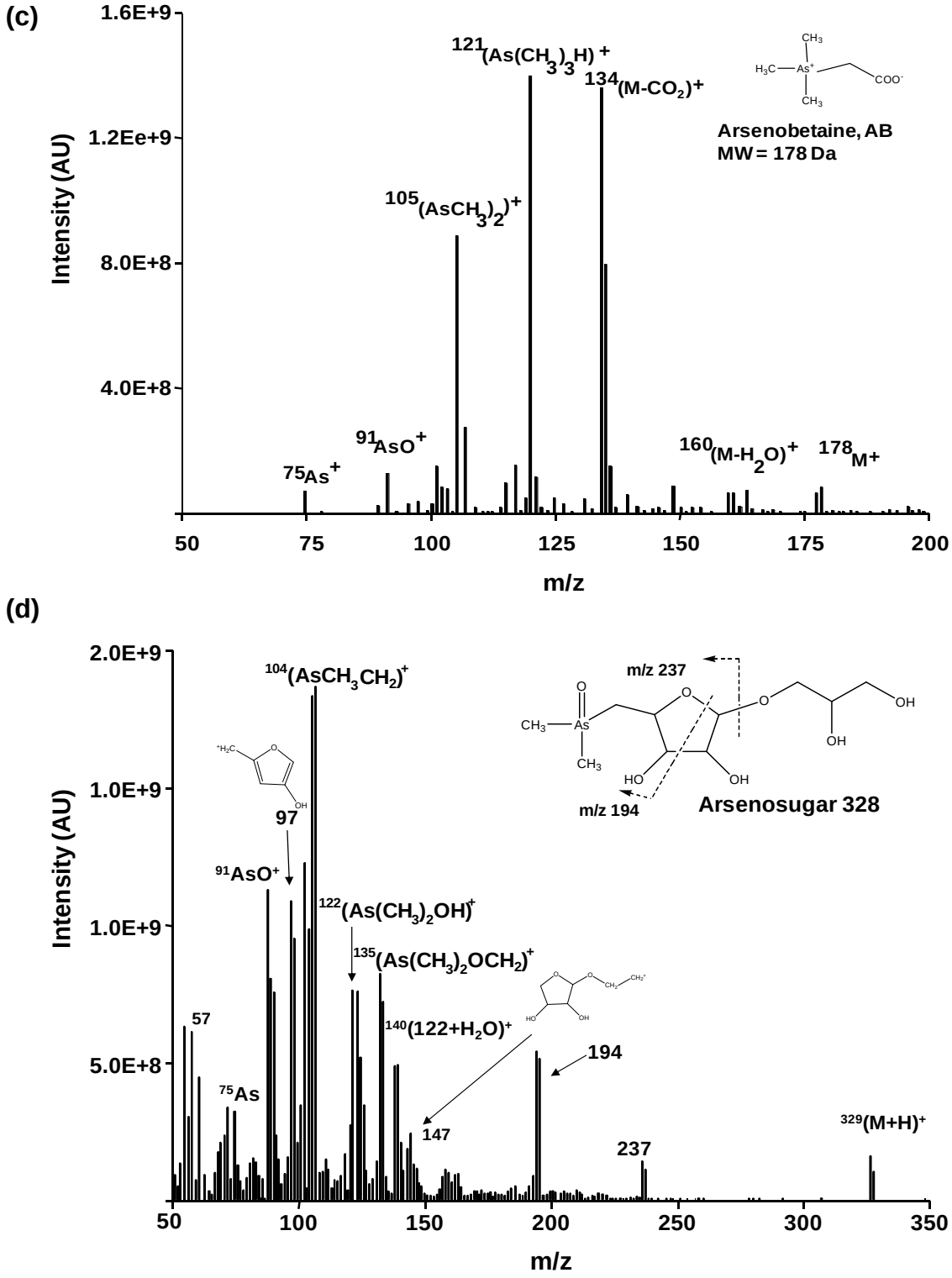

Figure 3.1. PB/EI mass spectra of a) $AsCl_3$, b) dimethylarsinic acid (DMA), c) arsenobetaine and d) arsenosugar 328. Concentration = 50 µg mL^{-1}, injection volume = 5 µL, ion volume temperature = 300 ^{o}C, and electron energy = 70 eV.

Analytical Performance

The performance of the LC-PB/EIMS method has been evaluated based on the linearity of standard calibration plots, the resultant limits of detection ($3\sigma_{blank}/m$), and the reproducibility of the chromatographic data. Initially, to evaluate the reproducibility of the PB/EIMS system, seven 5 µL replicate injections of the 10 µg mL^{-1} As 328 standard solution were acquired in SIM mode measuring the 91 Da signal. Very reproducible signal transients with an RSD of 3.9% (calculated using integrated peak areas) could be obtained with simple manual injections.

Calibration plots with standard solutions of each of the tested arsenic compounds were used to calculate limits of detection (LOD) for the PB/EIMS method. Two independent ways exist to determine the LODs in LC/MS: single ion monitoring (SIM) mode and full scan acquisition (TIC) mode. The main difference between SIM and TIC modes is that the former case has a much higher duty factor per unit of experiment time, while the latter accumulates data for all of the MS fragments (as well as background signals).

The calibration characteristics of each compound were determined from response functions derived over a concentration range of 0.1 to 100 µg mL^{-1} (as well as an analytical blank) in the SIM and the TIC modes. The arsenic oxide ion, $^{91}AsO^{+}$, commonly appeared in the mass spectra of each of the target arsenic compounds; therefore, quantitative data was acquired in the SIM mode at m/z = 91 Da. The respective instrument response functions, correlation

coefficients, and limits of detection of the four arsenic species are presented in Table 3.2. Based on the response functions, the absolute limits of detection of $AsCl_3$, DMA, AB and As 328 compounds in the SIM mode using the characteristic AsO^+ species were determined to be 0.03, 0.05, 0.008 and 0.005 ng respectively, while the LODs obtained in TIC mode scanning from 50-200 Da (m/z 50-350 Da was used for As 328), were calculated to be 0.10, 0.14, 0.04 and 0.01 ng, respectively. As would be expected, monitoring of analyte signal in the SIM mode yielded lower detection limits than TIC mode.

The third set of calibration response characteristics shown in Table 3.2 were generated using integrated peak areas (SIM) for the base peaks of the mass spectra for each of the compounds; the protonated molecular ion of $AsCl_3$ (m/z = 181 Da), the molecular ion of DMA (m/z = 138 Da), and the most prominent fragments of AB (m/z = 120 Da) and As 328 (m/z = 104 Da). As can be seen, the LOD values are comparable to the SIM monitoring of the AsO^+ ions. The sensitivity differences across the tested arsenic species are due to the combined effects of the relative volatility and/or ionization energies of the vaporized species.[40] The LOD values for DMA are somewhat higher (i.e. less sensitive) than the values obtained for the other test species. This variation in sensitivity for the organic arsenic species may be attributed to their differences in the physical and chemical properties. A complete assessment cannot be provided because the thermodynamic values corresponding to AB and As 328 could not be found. While the LODs obtained with TIC mode reported in Table

3.2 are comparable to many other reported methods used in arsenic speciation,[6, 8, 44-47] they are an order of magnitude higher than the values reported for liquid chromatographic separation methods coupled to ICP-MS[11] and ESI-SRM studies.[25-27] In this case, the use of SIM detection, the values become more closely in line. Although the LOD values are higher than ICP-MS, this system has the added advantage of providing species-specific information. (Studies in this laboratory have demonstrated the use of inorganic salts as carriers to improve the sensitivity in the PB/HC-OES and PB/EIMS determinations of proteins and mercury compounds, respectively, and so improvement might be expected.[38, 48]) ICP-MS generally provides unparalleled sensitivity, yet it does not provide structural identification of unknowns because analytes are dissociated to their elemental form in the high temperature plasma. Many arsenic speciation studies use the combination of ICP-MS for quantification and ESI-MS for qualitative arsenosugar identification.[13-15] In practice, these usually involve use of two very different separation processes for the two detection methods. On the other hand, Pergantis *et al*.[25-27] employs electrospray tandem triple quadrupole mass spectrometry in the selected reaction monitoring (MS-MS) for the quantification of arsenic species. The PB/EIMS is shown here to provide both high sensitivity and qualitative information not available by other single MS method, in a single LC separation (i.e. ESI-MS or ICP-MS).

Table 3.2. PB/EIMS response characteristics for $AsCl_3$, DMA, AB and As 328.

Species	Response Function	R^2	LOD (ng mL^{-1})	Absolute Mass (ng)
SIM mode (m/z = 91 Da)				
$AsCl_3$	$y = 1E^{+8} x + 2E^{+8}$	0.9954	6.0	0.03
DMA	$y = 3E^{+7} x + 2E^{+8}$	0.9891	10	0.05
AB	$y = 4E^{+8} x - 4E^{+8}$	0.9926	1.6	0.008
As 328	$y = 6E^{+8} x - 5E^{+8}$	0.9923	1.1	0.005
TIC mode (m/z = 50 - 200 Da)				
$AsCl_3$	$y = 8E^{+8} x + 3E^{+9}$	0.9938	20	0.10
DMA	$y = 6E^{+8} x + 4E^{+9}$	0.9797	27	0.14
AB	$y = 2E^{+9} x + 5E^{+9}$	0.9958	8	0.04
As 328	$y = 1E^{+10} x + 6E^{+9}$	0.9940	2	0.01
M^+ ion				
$AsCl_3$	$y = 2E^{+8} x + 8E^{+8}$	0.9912	9	0.045
DMA	$y = 1E^{+8} x + 2E^{+9}$	0.9730	12	0.06
AB	$y = 3E^{+8} x - 7E^{+8}$	0.9914	5	0.025
As 328	$y = 1E^{+9} x + 8E^{+9}$	0.9903	1	0.006

Reversed-phase, Ion-pairing Chromatography Separation of Arsenic Species

Two reversed-phase high-performance liquid chromatography (RP-HPLC) methods were evaluated to determine the optimal separation conditions for arsenic compounds in the target botanical mixtures.[49, 50] In the first set of experiments, five arsenic species (As (III), As (V), DMA, AB and As 328) in a synthetic mixture were separated on the C_{18} column using an isocratic RP method where a 95:5 H_2O:MeOH mixture was employed as the mobile phase. The two inorganic arsenic species were used here as both are known to be toxic and would certainly have different separation/detection characteristics than the organoarsenic compounds.[9, 51-54] In the resultant chromatogram, the arsenic species were not baseline-resolved and only three peaks were observed, including the co-elution of As (III) and As (V) as well as the AB and As 328

species. The former is not unexpected as both of the ionic, inorganic arsenicals would have a very low affinity for the hydrophobic stationary phase, and thus would not be retained. Subsequent studies were carried out with only four species (As (III), DMA, AB and As 328) as this study is mainly focused on separation and quantification of total inorganic and organic arsenic species. Differentiation between As (III) and As (V) is a separate issue, which is the forte of IEC methods as demonstrated in the next section.

The influence of various HPLC separation conditions were studied, to obtain the baseline resolution of the peaks, including methanol concentration, ion-pairing agent concentration and mobile phase flow rate for the three test compounds. Initially, the organic mobile phase composition was evaluated over the range of 3% to 8% (MeOH:H_2O) with the optimal composition being 4%. The compounds were not well-retained or resolved under most of these conditions, and the column selectivity was not sufficient for adequate separation. As expected, the inorganic compound, arsenic chloride ($AsCl_3$), was unretained and eluted with the injection volume. It is well known that ion-pairing agents can be used to alter the ionic or hydrophobic characteristics of the chromatographic support or the solutes themselves, and thus enhance the separation of compounds.[49] It has been found that TFA is well suited as an ion-pairing agent in particle beam mass spectrometry due to its high volatility (mp = -15℃ and bp = 72℃) when compared to other ion-pairing agents such as formic acid, hexane sulphonic acid and perchloric acid used by other research groups.[9, 28] The effect

of TFA concentration in the mobile phase on the chromatographic quality was evaluated over a range of 0.01% to 2% (v v^{-1}). The improvement in the resolution of the four arsenic species was pronounced as the concentration of TFA was increased to 0.05%, with the most efficient separation achieved using a mobile phase composition containing 0.1% TFA as ion-pairing agent, with the total analysis time being less than 8 min.

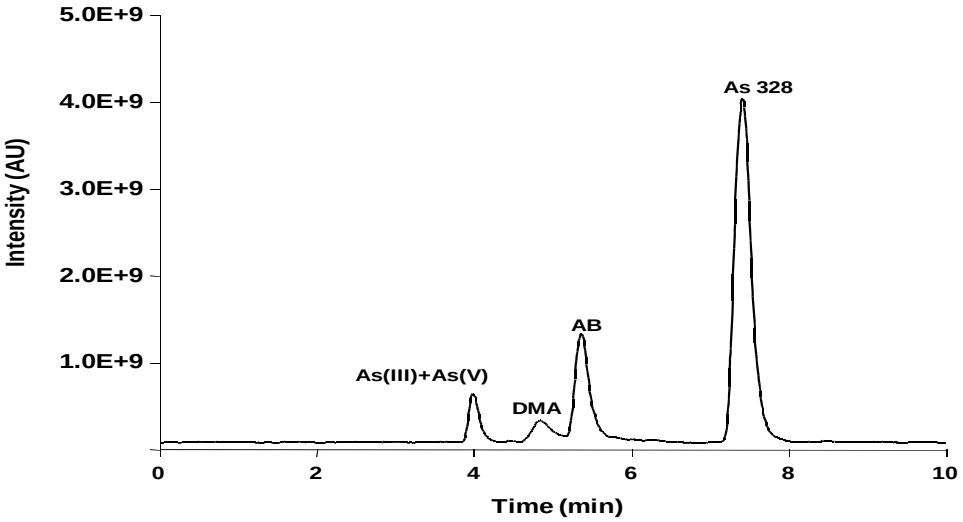

Figure 3.2. RP-PB/EIMS total ion chromatogram of a mixture of As(III)+As(V), DMA, AB and As 328 species. Mobile phase = 96:4 (H_2O:MeOH) containing 0.1% TFA, mass spectra acquisition = 50–350 Da at 1 s per scan, injection volume = 5 µL, flow rate = 0.7 mL min^{-1}, ion volume temperature = 300 ^oC, and electron energy = 70 eV.

The optimized chromatographic separation of the four anticipated arsenic species acquired in the TIC mode is shown in Fig. 3.2. The advantage of using EIMS in the TIC mode is the ability to extract the complete species' mass spectra to identify solutes based on the fragmentation patterns. Each elution peak in the TIC chromatogram could be expanded to yield mass spectra that were identical

to those of the individual arsenic compounds shown in Figs. 3.1a-d. This demonstrates the key benefit of using the PB/EIMS approach to arsenic speciation in comparison to ICP-MS analysis, as there is no direct molecular species information in the latter mass spectra.

Ion-exchange Chromatography Separation of Arsenic Species

While an RP method is an excellent way to differentiate between inorganic and organic As species, IEC is required to distinguish As III from As V. A more salient reason for changing separation modes (in general) is simply to isolate different species which may co-elute or are un-retained by another method. A variety of ion-exchange chromatography separation methods have been reported for the speciation of arsenic. The separation method published by Guérin *et al.*[6] was used for the separation of inorganic and organic arsenic species by ion-exchange with a minor modification. In this case, the methanol composition was changed from 1% to 2% and the flow rate was reduced from 1.35 to 0.9 mL min^{-1}. Figure 3.3 shows the SIM chromatogram collected at m/z = 91 Da for the separation of a synthetic mixture of the five arsenic species (As (III), As (V), DMA, AB and As 328), resulting in a baseline resolved separation of the As species in less than 8 minutes. It must be admitted here that the sensitivity in the IEC mode is compromised to some extent as a heavier solvent load is presented with the aqueous mobile phase. The elution order of the arsenic species is highly dependent on the pH of the mobile phase, as the arsenic species can be

in their neutral, anionic, cationic or zwitterionic form. In addition, the hydrophobicity of the stationary phase will play a role during the chromatographic separation, because the polymeric stationary phase provides capacity for hydrophobic interactions.[55-57] To better understand the chromatographic behavior of these species their acid dissociation constants (pK_a) need to be taken into consideration. The pK_a values for the arsenic species are as follow: As (III) (pK_a = 9.2), As (V) (pK_a = 2.2), DMA (pK_a = 6.2), AB (pK_a = 2.18) and As 328 (pKa not available).[57, 58] The HNO_3 concentration increases during the gradient elution therefore the elution order of the arsenic species should be: As (III), DMA, As (V) and AB. As seen in Fig. 3.3, the expected elution order is observed and such elution order is similar to that published by Mattusch *et al*.,[56] Pannier *et al*.[57] and Guérin *et al*.[6] More specifically, it is observed that the elution order of As (III), DMA and As (V) is governed by the anion-exchange mechanisms and that the later species (AB and As 328) are influenced by both anion-exchange and reversed-phase mechanisms. Again, the ability to obtain conclusive mass spectra allows ready assignment of these identities. This anion-exchange chromatographic separation, in conjunction with the ion-pair reversed-phase chromatography mode, demonstrates the capability of the PB interface to remove residual solvent vapors of different types, effect desolvation, and deliver analyte particles to the ionization source region.

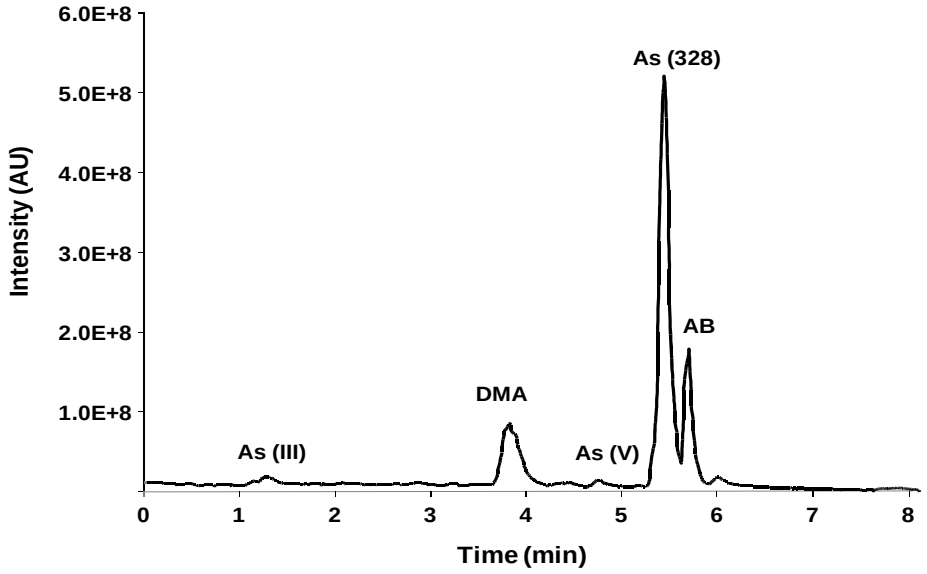

Figure 3.3. IEC-PB/EIMS total ion chromatogram of a mixture of As(III), DMA, As(V), As 328 and AB species. Mobile phase = A) 0.5mM HNO_3 containing 2% MeOH and B) 50 mM HNO_3, mass spectra acquisition = 50–350 Da at 1 s per scan, injection volume = 20 µL, flow rate = 0.9 mL min^{-1}, ion volume temperature = 300 ^{o}C, and electron energy = 70 eV. Step gradient (A:B): 100:0 hold for 2.5 min., 90:10 hold for 3 min., 80:20 hold for 5 min.

Arsenic Speciation in Ethanolic Kelp and Bladderwrack Extracts

Commercial ethanolic kelp and bladderwrack extracts were used as test samples to demonstrate the applicability of this LC/PB-EIMS approach to identify the chemical forms of arsenic. Initially, an EI mass spectrum (shown in Fig. 3.4) was obtained for the raw ethanolic kelp extract after diluting it with the mobile phase to a final concentration of 1%. The EI mass spectrum shows a number of prominent ion fragments, which without some form of chemical separation cannot be interpreted. Based on the complexity seen here, it is not surprising the signals were present at masses identified in Figs. 3.1a-d, but of course cannot be

assigned as such. A similarly complex mass spectrum was obtained for the ethanolic extract of bladderwrack; hence it is not shown here.

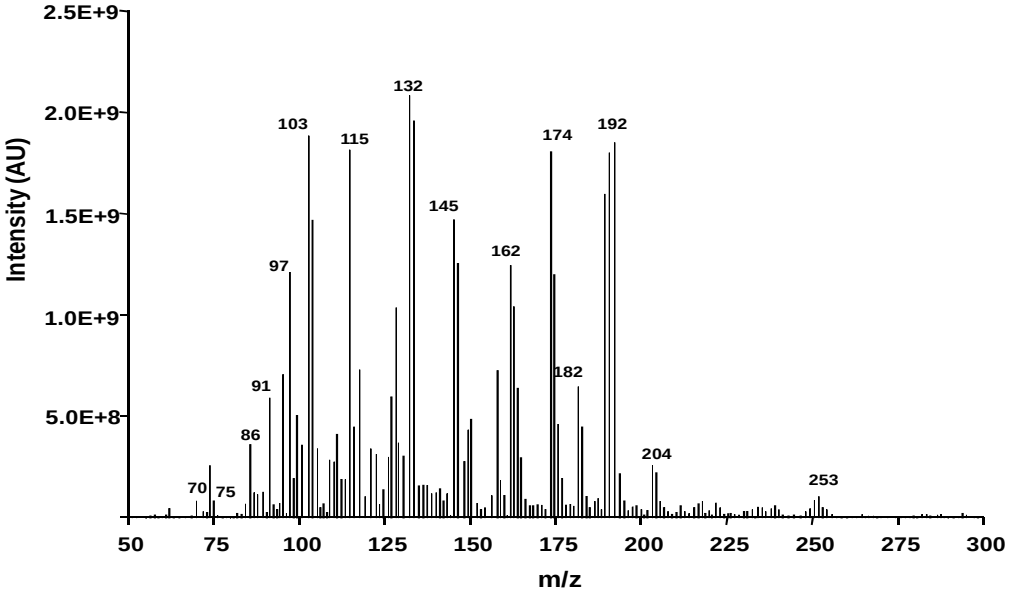

Figure 3.4. PB/EI mass spectrum of 1% ethanolic kelp extract. Injection volume = 5 µL, flow rate = 0.7 mL min^{-1}, ion volume temperature = 300 ^{o}C, and electron energy = 70 eV.

Initially, the crude ethanolic extracts (diluted to a 10% composition in the mobile phase) were injected onto the C_{18} column for arsenic speciation. The resulting ion chromatograms included irregularly-shaped and split peaks, which could be due to overloading of the column or clogging of frits. In addition, the strength of the sample solvent (5% ethanol at this point) would likely affect the chromatographic characteristics. Both of these phenomena could be corrected by further dilution of the sample, of course at the expense of diluting the arsenic species in the test sample. In order to overcome these problems, the matrix was modified by evaporating 10 mL samples of both the ethanolic kelp and

bladderwrack extracts in a water bath at a temperature of ~60 ℃ to near dryness. The residual sample was re-solubilized by dilution to 2 mL with the mobile phase and centrifuged. As such, a pre-concentration factor of 5 was attained and the resulting chromatographic quality much improved but the distribution of the species is not perturbed. In the same manner, good recoveries were obtained when the ethanolic extracts were spiked with the arsenic analytical standards before evaporation and re-solubilization, ensuring the efficiency of the matrix modification process employed during these experiments. As suggested in studies by Montoro *et al*.,[43] and shown here, the EIMS mass spectral characteristics and relative retention times indicated that exposure to elevated temperatures did not change the chemical form of the arsenic species or their distribution.

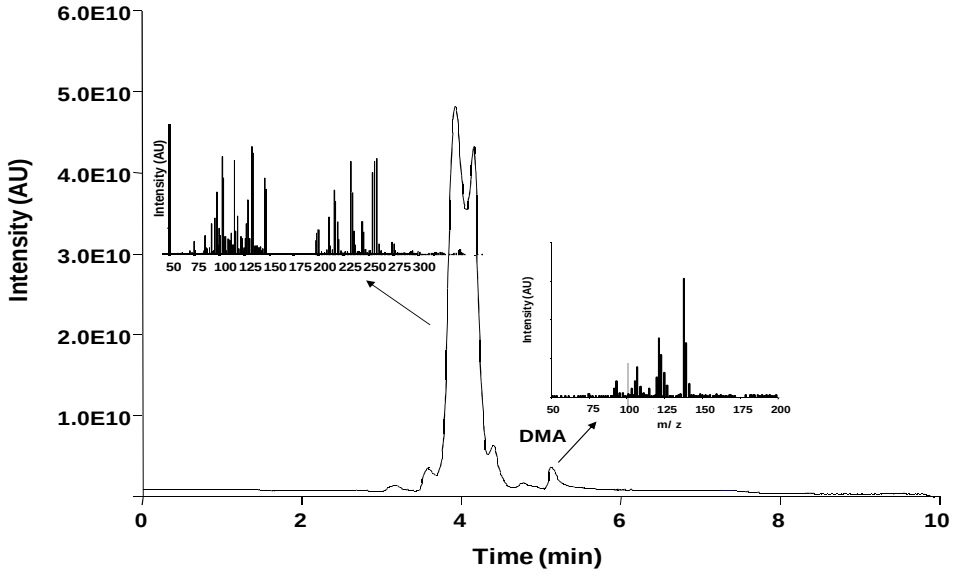

Figure 3.5. RP-PB/EIMS total ion chromatogram (TIC) obtained for ethanolic kelp extract after sample pretreatment step. Mobile phase = 96:4 (H_2O:MeOH) containing 0.1% TFA, mass spectra acquisition = 50–350 Da at 1 s per scan, injection volume = 5 µL, flow rate = 0.7 mL min^{-1}, ion volume temperature = 300 ^{o}C, and electron energy = 70 eV.

The LC-PB/EIMS total ion chromatogram obtained for the kelp extract by reversed-phase chromatography is shown in Fig. 3.5. As can be seen a high intensity split peak appears in the region of the injection peak. Expansion of the TIC into a single mass spectrum at t=4.0 min yields a mass spectrum qualitatively similar to that shown in Fig. 3.4, reflecting (not surprisingly) the high levels of polar species in the extract. On the other hand, spectral expansion of the peak eluting at a retention time of ~5.2 min reveals the presence of DMA as the spectrum is identical to that of Fig. 3.1b. Monitoring the target analyte signals in the SIM mode can eliminate many of these signals and leads to greater chromatographic simplicity. There is a compromise since the SIM mode can only provide limited molecular information, though with higher sensitivity. The mass spectra obtained for the four arsenic compounds (Figs 3.1a-d) contain the common peak at m/z = 91 Da corresponding to the AsO^+ ion, making it a logical target to identify which regions of the chromatogram may contain arsenic species. This is a very common approach to target analysis in organic mass spectrometry.

The SIM chromatograms collected at m/z = 91 Da are shown in Figs. 3.6a and 3.6b for 5 µL injections of the kelp and bladderwrack extracts, respectively, using the optimized RP method. Also shown are the chromatograms that result from the injection consisting of 4 µL of the extract and 1 µL of a spike containing 25 µg mL^{-1} of each of the arsenic species. In both cases, the 91 Da signature ion is seen at the retention times corresponding to As (III) and DMA species. The

identity of the DMA was confirmed mass spectrometrically in both cases. In actuality, it can only be said that the first peak is inorganic arsenic (either As (III) or (V)). On a semi-quantitative basis, the majority of the arsenic species (90-95%) is present in the tested samples in the form of inorganic arsenic, with very minor amounts present in the form of DMA (5-10% of the total As). There was no detectable amount of the AsO^+ species (i.e., above the LOD) corresponding to the AB and As 328 compound elution times.

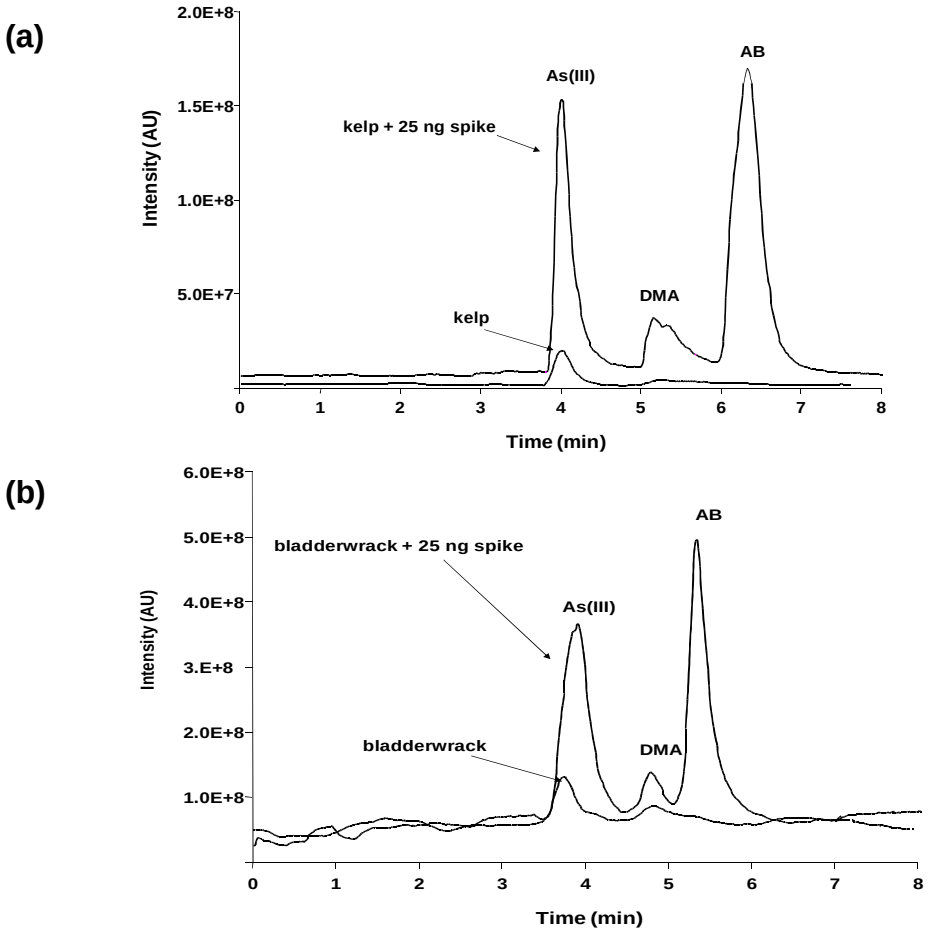

Figure 3.6. RP-PB/EIMS single ion chromatograms (m/z = 91 Da) of arsenic species present in a) kelp and b) bladderwrack. The overlapped chromatograms were obtained with samples spiked with mixture of 25 ng (absolute) of each arsenic species. Mobile phase = 96:4 (H_2O:MeOH) containing 0.1% TFA as an ion-pairing agent, injection volume = 5 µL, flow rate = 0.7 mL min^{-1}, ion volume temperature = 300 ^oC, and electron energy = 70 eV.

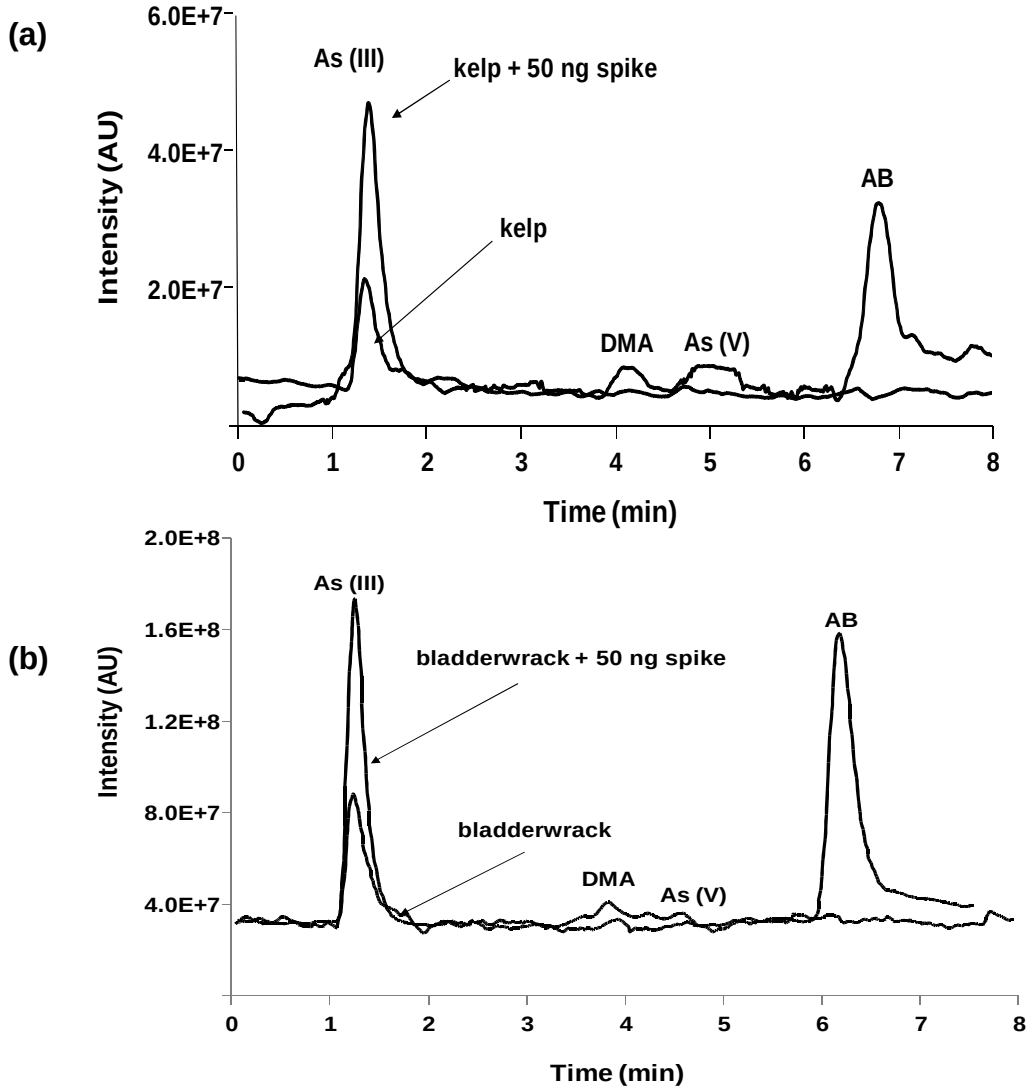

Figure 3.7. IEC-PB/EIMS single ion chromatograms (m/z = 91 Da) of arsenic species present in a) kelp and b) bladderwrack extracts. The overlapped chromatograms were obtained with samples spiked with mixture of 50 ng (absolute) of each arsenic species. Mobile phase = A) 0.5 mM HNO_3 containing 2% MeOH and B) 50 mM HNO_3, injection volume = 20 µL, flow rate = 0.9 mL min^{-1}, ion volume temperature = 300 ^{o}C, and electron energy = 70 eV. Step gradient (A:B): 100:0 hold for 2.5 min, 90:10 hold for 3 min., 80:20 hold for 5 min.

In the same manner, the SIM chromatograms (m/z = 91 Da) for the kelp and bladderwrack extract were collected for the separations performed by ion-exchange chromatography (Figs. 3.7a and b). Although in the case of the kelp extract the inorganic arsenic species (As (III) and (V)) were separated by ion-

exchange, the final speciation result is the same for both chromatographic modes (reversed-phase and ion-exchange). Meaning that for both chromatographic separations the same species were observed, with no arsenosugars or arsenobetaine detected. While the previously cited works identified arsenosugars to be prominent components of algae and kelp,[13-15] they are not present in these ethanolic extracts. The LC-PB/EIMS data presented previously clearly demonstrated that if present at measurable levels (Table 3.2), these compounds would be seen. The discrepancies in the identified species arises from the differences in the primary extraction methodologies (water, methanol or water:methanol mixture) found in the literature,[5, 8] whereas in this particular kelp and bladderwrack extracts, pure grain alcohol (i.e. ethanol) was used as the extraction solvent in the product formulation.

Quantitative Analysis

The quantification of inorganic arsenic and DMA in the ethanolic extracts was accomplished using a standard addition method, as this allows for better matrix and chromatographic matching than the use of response functions as done in the method characterization depicted in Table 3.2. Based on the standard addition analysis, the concentrations of inorganic arsenic and DMA in the kelp and bladderwrack extracts are shown in Table 3.3 for both of the separation strategies. In the case of the RP separation, the values reflect the respective inorganic and organic fractions, while for the IEC separation the As

(III) and As (V) values are displayed explicitly. Also presented in the table are the total As values as obtained by ICP-OES, allowing assessment of the recoveries of the respective analyses.

In the kelp extract, the two inorganic species which are clearly identified and quantified constitute 87% of the total arsenic. These results are similar to the findings reported by Salgado *et al.*[17] in their arsenic speciation studies in kelp powder extracts. The inorganic fraction in the bladderwrack extract represents 91% of the total arsenic content. The total arsenic species concentration determined by RP-PB/EIMS for the kelp and bladderwrack extracts were found to be 7.1 ± 0.6 μg mL^{-1} and 6.8 ± 0.4 μg mL^{-1}, respectively. In the case of the IEC-PB/EIMS, 6.5 ± 1.5 μg mL^{-1} and 7.1 ± 0.2 μg mL^{-1} of total arsenic were obtained for the kelp and bladderwrack extracts. The recoveries of the arsenic species were validated as the total arsenic concentrations in the ethanolic kelp and bladderwrack extracts were found to be 7.0 ± 0.4 μg mL^{-1} and 6.5 ± 0.3 μg mL^{-1}, respectively via ICP-OES. As such, there is a great deal of confidence in both the qualitative and quantitative aspects of the LC-PB/EIMS method. Based on the assumption that a typical dosage of these sorts of tinctures might be of the order of 1-3 mL per day, these materials fall well below the maximum permissible levels of arsenic ingestion recommended by the Food and Agricultural Organization/World Health Organization (FAO/WHO) proposed a tolerable weekly intake of 15 μg inorganic arsenic/kg body weight.

Table 3.3. Quantification results for inorganic and organic arsenic by standard addition with LC-PB/EIMS and ICP-OES.

Species	Kelp ($\mu g\ mL^{-1}$)	Bladderwrack ($\mu g\ mL^{-1}$)
Reversed-phase Chromatography		
Inorganic As	6.1 ± 0.6	6.2 ± 0.4
DMA	0.96 ± 0.12	0.62 ± 0.08
Total As	**7.1 ± 0.6**	**6.8 ± 0.4**
Ion-exchange Chromatography		
As (III)	4.3 ± 1.0	6.4 ± 0.2
As (V)	1.9 ± 1.0	not detected
DMA	0.66 ± 0.30	0.73 ± 0.10
Total As	**6.9 ± 1.5**	**7.1 ± 0.2**
ICP-OES		
Total As	**7.0 ± 0.4**	**6.5 ± 0.3**

CONCLUSIONS

The applicability of LC-PB/EIMS system to the separation, identification, and quantification of inorganic and organic arsenic species in commercial kelp and bladderwrack extracts has been demonstrated. The use of the particle beam interface allows for efficient solvent removal with the ultimate introduction of dry particulates into the EI volume. As such, the different arsenic species effectively yield mass spectra that allow ready identification, i.e. the method allows unambiguous, *comprehensive speciation*. The limits of detection for the different arsenic species approach those afforded by ICP-MS, with the added advantage that they can be determined with species specificity. Another advantage with LC/PB system is that it can accept a wide range of mobile phases operating at

normal HPLC flow rates (~1 mL min^{-1}) making it well suited for various chromatographic modes.

The complementary aspects of using both reversed-phase and ion exchange chromatography were employed to provide different types of separation characteristics. In this way, chances of missing a given constituent are minimized as well. The mass chromatograms obtained show the presence of inorganic arsenic, with a minor amount (about 5-10% of total arsenic content) of DMA detected in both the extracts. By choosing either the SIM or TIC data acquisition mode, the user can get either limited molecular information (if target species are known) or full mass spectrum at every chromatographic data point. The capability of detecting and identifying other known seaweed constituents, namely arsenobetaine and As 328, illustrated the potential of the method for profiling extractions performed under different conditions. The results obtained in this study, and those presented previously, clearly demonstrate that PB/EIMS is a viable on-line detection method for comprehensive arsenic speciation analysis and suggest its application to other natural matrices to safeguard human health and allow for metabolism and nutritional studies of greater information content.

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CHAPTER FOUR

VALIDATION OF A LIQUID CHROMATOGRAPHY-PARTICLE BEAM ELECTRON IONIZATION MASS SPECTROMETRY SYSTEM FOR THE ANALYSIS OF BOTANICAL EXTRACTS: EVALUATION OF EPHEDRINE ALKALOIDS IN STANDARD REFERENCE MATERIALS

INTRODUCTION

Consumer interest in botanical products as dietary supplements has grown intensely because of their suggested medicinal properties and health benefits.[1, 2] The nutritional supplement industry sales are governed in the United States by the Dietary Supplement Health and Education Act (DSHEA) passed by Congress in 1994. In summary, DSHEA's objective is to ensure that the identity, purity, quality and strength of the products are reflected in the labels.[1-3] DSHEA also states that the proof of safety regarding the dietary supplements falls in the hands of the Food and Drug Administration (FDA). For example, ephedra containing dietary supplements gained popularity in the US due to the use in weight loss and management, as well as athletic performance and/or energy enhancement.[4, 5] However, different adverse side effects such as heart attacks, stroke, seizure and death were linked to the consumption of ephedra.[4] The frequency of these incidents provided the FDA with enough reason to prohibit the sale of any ephedra containing products and the supplements were banned from the market in 2004.[6]

The clearance of contaminants such as pesticides, heavy metals and adulterants from dietary supplements has become of great concern in the nutritional industry, government agencies and the public.[7] For that reason, government agencies and laboratories are working together in the development of standard reference materials to target the evaluation and validation of new and existing analytical methods used in the analysis of the dietary supplements. The first suite of standard reference materials containing ephedra were introduced in 2005 and developed by the National Institute of Standards and Technology (NIST) in collaboration with the National Institute of Health Office of Dietary Supplements (NIH-ODS) and FDA.[5] The suite of SRMs is composed of SRM 3240 *Ephedra sinica* Stapf Aerial Parts, SRM 3241 *Ephedra sinica* Stapf Native Extract, SRM 3242 *Ephedra sinica* Stapf Commercial Extract and SRM 3244 Ephedra-Containing Protein Powder, representing the variety of matrices extracted and processed in different manners.[5, 8]

Ephedra herba (*Ma-Huang*) plants have been used in traditional Chinese medicine for over 5000 years to reduce fever, treat cough and asthma.[4] These plants are widely known for being a source of ephedrine alkaloids, which are naturally occurring ingredients used as stimulant and diet aids (as mentioned above). The ephedrine alkaloids are composed of three pairs of diastereomers with primary (norephedrine and norpseudoephedrine), secondary (ephedrine and pseudoephedrine) and tertiary (methylephedrine and methylpseudoephedrine) amine functionality. The chemical structures of the ephedrine alkaloids with their

respective molecular weight are shown in Fig. 4.1. From the six alkaloids, ephedrine and pseudoephedrine are the most abundant species (over 80% of total alkaloids) found in most ephedra plant materials.[9, 10]

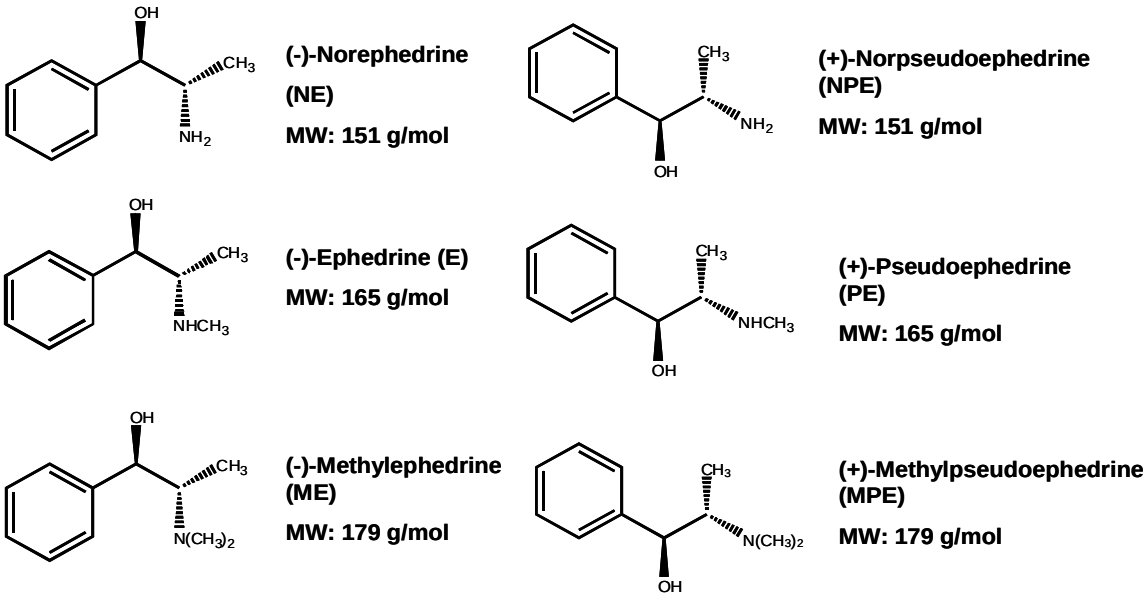


Figure 4.1. Chemical structures of the ephedrine alkaloids.

The concern for the safety in the use of ephedra-containing supplements as well as other dietary products has led to the development of numerous analytical methods for the analysis of the active components, such as the ephedrine alkaloids in this case. High performance liquid chromatography (HPLC) using phenyl[5, 11, 12] or C_{18} [5, 13, 14] columns with UV absorbance[5, 13-16] and/or mass spectrometry[5, 11, 12, 16, 17] detection are the most common methods reported for the separation and identification of ephedrine alkaloids in plant material, urine matrix and commercial products. However, when coupling the LC

eluent to UV-Vis absorbance detectors the species identification is not analyte specific and analytical standards are necessary to perform retention time matching. On the other hand, mass spectrometry is a very powerful detection method due to the fact that it provides molecular weight and structural information of the analyte species in a given sample. Both electrospray ionization (ESI)[11, 17] and atmospheric pressure chemical ionization (APCI) mass spectrometry[11, 12] have been used for the identification of ephedra alkaloids. These two mass spectrometry approaches are very sensitive and provide great ionization stability but when coupled to common LC mobile phases the ionization processes are quenched.[18] For that reason, modifications to the chromatographic conditions (i.e. mobile phase, ion pairing agent) are necessary. As well, the difference in flow rates is troublesome and changes are needed due to the fact that ESI and APCI operate under µL min^{-1} flow rates and the standard LC flow rates are mostly in the mL min^{-1} range.[18, 19] Other methods used in the analysis of ephedrine alkaloids include gas chromatography mass spectrometry (GC-MS)[10, 20, 21] with and without derivatization of the ephedrine alkaloids and capillary electrophoresis.[5, 9, 22]

Although numerous analytical methods can be found in the literature, the need for simple and easy to operate instrumentation that can also provide a full analysis of the sample of interest (in this case botanical supplements) drives research towards development of new analytical tools. In this laboratory, the particle beam has been employed successfully as a liquid chromatography mass

spectrometry interface for the analysis of many organic, organometallics, inorganic and biological species by employing a glow discharge ionization source.[23-26] In recent years, this unique analytical tool, which has the capability of interchanging ionization sources (electron ionization and glow discharge), has been focused on comprehensive speciation studies.[19, 27, 28] More specifically, the chemical characterization of botanical extracts such as kelp,[28] bladderwrack,[28] green tea[19] and echinacea has been performed. Table 4.1 provides a list of the various herbal products with their respective chemical components targeted during analysis by LC-PB/MS in this laboratory. This coupling takes advantage of the ease of operation, solvent compatibility (wide range of polarities and flow rates) and efficient solvent removal of the PB interface.[23, 29, 30]

Table 4.1. Botanical products with their respective chemical components characterized by LC-PB/MS system.

Nutraceutical/Botanical Products		
Green Tea **(Catechins & xanthines)**	***Echinacea*** **(Caffeic acid derivatives)**	***Kelp/Bladderwrack*** **(Arsenic species)**
catechin	**cichoric acid**	**As (III)**
epicatechin	**caftaric acid**	**As (V)**
epigallocatechin	**chlorogenic acid**	**dimethylarsinic acid**
epigallocatechin gallate	**cynarin**	**arsenobetaine**
epicatechin gallate	**echinacoside**	**arsenosugar 328**
caffeine		

This work presents the validation of the LC-PB/MS system with the electron ionization source by analyzing the ephedrine alkaloids present in the ephedra-containing standard reference materials. Figure 4.2 depicts a flow chart of the analytical method development process carried out in this laboratory for all the botanical studies done to date. In this case, special emphasis is given to ephedrine, pseudoephedrine, norephedrine and methylephedrine present in SRM 3241 *Ephedra sinica* Stapf Native Extract and SRM 3242 *Ephedra sinica* Stapf Commercial Extract. Mass spectra for each of the ephedrine alkaloids were obtained using analytical standards, their molecular ion and specific signature ions identified and then compared to the NIST EI library spectra (when available). Calibration curves for all the species of interest were generated and their respective detection limits determined. The development of the chromatographic separation for the alkaloids was accomplished by RP-LC using a phenyl column and monitored by UV absorbance at 210 nm. Once the optimal separation was achieved, the separation column was coupled to the PB/EIMS system for the quantification and validation by a standard addition method. This validation demonstrates that the PB/EIMS detection method is a viable approach for the chemical characterization of botanical extracts.

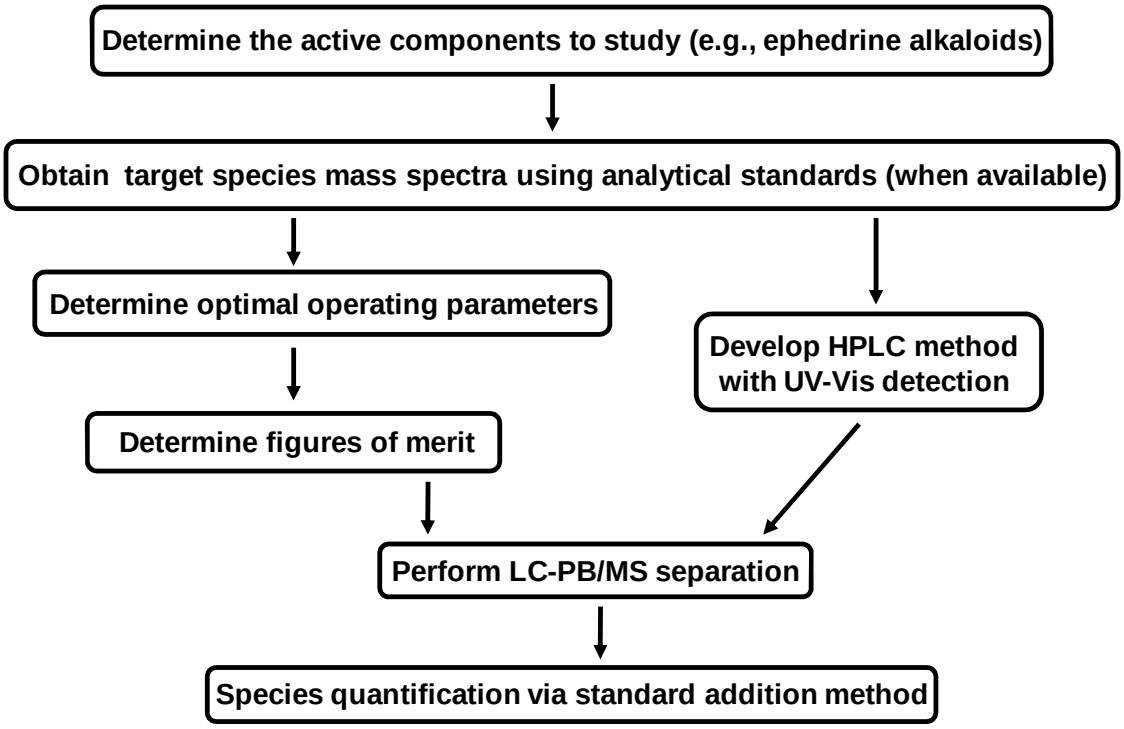


Figure 4.2. Flow chart demonstrating the LC-PB/MS analytical method development.

EXPERIMENTAL

Instrumentation and Chromatographic Conditions

The chromatographic separation of the ephedrine alkaloids was performed via a Waters (Milford, MA) Model 600E HPLC system and a Waters Model 2487 dual wavelength absorbance detector (Milford, MA) equipped with a Rheodyne (Cotati, CA, USA) Model 7125*i* injector with a 50 µL injection loop. The 250 mm x 4.6 mm Alltech Alltima Phenyl (5µm) reversed-phase column (Alltech Associates Inc., Deerfield, IL, USA) operating at room temperature and a mobile phase flow rate of 1.0 mL min^{-1} were used for the LC separation. The HPLC solvents consisted of water (18.2 MΩ cm^{-1}, NANOpure Diamond, Barnstead

International, Dubuque, IA) containing 0.1% v v^{-1} trifluoroacetic acid (TFA) with HPLC-grade methanol (Fisher Scientific, Fair Lawn, NJ) or HPLC-grade acetonitrile (Fisher Scientific, Fair Lawn, NJ). A linear gradient method with a mobile phase composition varying from 5 to 20 percent MeOH over 15 minutes was used for the separation of the alkaloids.

The PB/MS system used in this study for the alkaloid detection, identification and quantification was an Extrel (Pittsburgh, PA, USA) Benchmark Thermabeam LC/MS quadrupole mass spectrometer with an electron ionization source, depicted in Fig. 1.5, and has been previously described in detail in Chapter 1 and literature[19, 23, 25, 31]. Detailed explanation of the Thermabeam particle beam interface (Extrel Corp., Pittsburg, PA, USA) has also been described previously in Chapter 1 and literature.[23, 26, 31] The nebulizer is heated to a temperature of ~85℃, the desolvation chamber at ~110℃ and the source block is held at a temperature of 200℃. The optimization of the operating parameters for the EI source (electron energy and source block temperature) has been described in previous work.[19]

Total ion chromatograms (TIC) were acquired using the Extrel Merlin (Pittsburgh, PA) Ionstation system software by scanning over a mass range of m/z = 50-200 Da at a scan rate of 1.0 s per scan. Selected ion monitoring (SIM) chromatograms for specific masses could be extracted from the TIC data for background correction and peak integration. Triplicate injections were carried out for each set of data points presented in the evaluation of experimental

conditions and quantification characteristics. The LC and MS data was exported to Sigma Plot 8.02 (Systat Software, Inc., Richmond, CA) and presented using Microsoft Excel and PowerPoint (Redmond, WA).

Reagents and Solutions

The 1000 µL mL^{-1} stock solutions of (-)-ephedrine, (+)-pseudoephedrine, (-)-norephedrine and (-)-N-methylephedrine (Sigma-Aldrich, St. Louis, MO, USA) were prepared by weighing the appropriate amounts of the analytes and diluting in a mixture of 0.1% water containing TFA. Calibration curves were created by triplicate injections of the standard solutions into the LC system (without column present) with spectral data acquired in total ion chromatogram (TIC) mode. The ephedra containing dietary supplement standard reference materials were supplied by NIST (Gaithersburg, MD, USA).

Quantification of the ephedrine alkaloids present in the NIST SRMs was achieved through a standard addition method. Stock standard solutions (1.0 mg mL^{-1}) of ephedrine, pseudoephedrine, norephedrine and N-methylephedrine were added in the amounts of 0.050 and 0.10 mL to aliquots of the ephedra reference materials and diluted to 1.0 mL. The ephedra aliquots were 0.10 and 0.20 mL and diluted up to 1.0 mL making 10% and 20% solutions. All solutions were stored in light-tight vessels at $4^{o}C$ and fresh dilutions were prepared as necessary.

Extraction Procedure

Approximately 0.5 grams of SRM 3241 and 3242 materials were accurately weighted, added to 50 mL polypropylene tubes and extracted in 19 mL of methanol by sonication for one hour and thirty minutes. After extraction, the samples were centrifuged at 4000 rpm for 30 minutes and filtered using a 0.45 µm PTFE filter (Alltech Associates Inc Deerfield, IL, USA) for final analysis.

Moisture Assessment

Moisture content of SRM 3241 and 3242 was determined by drying in an oven at ~ 100˚C for 24 hours. Conversion factors were determined based on dry-mass/original mass and used to report the quantification values on a dry-mass basis.

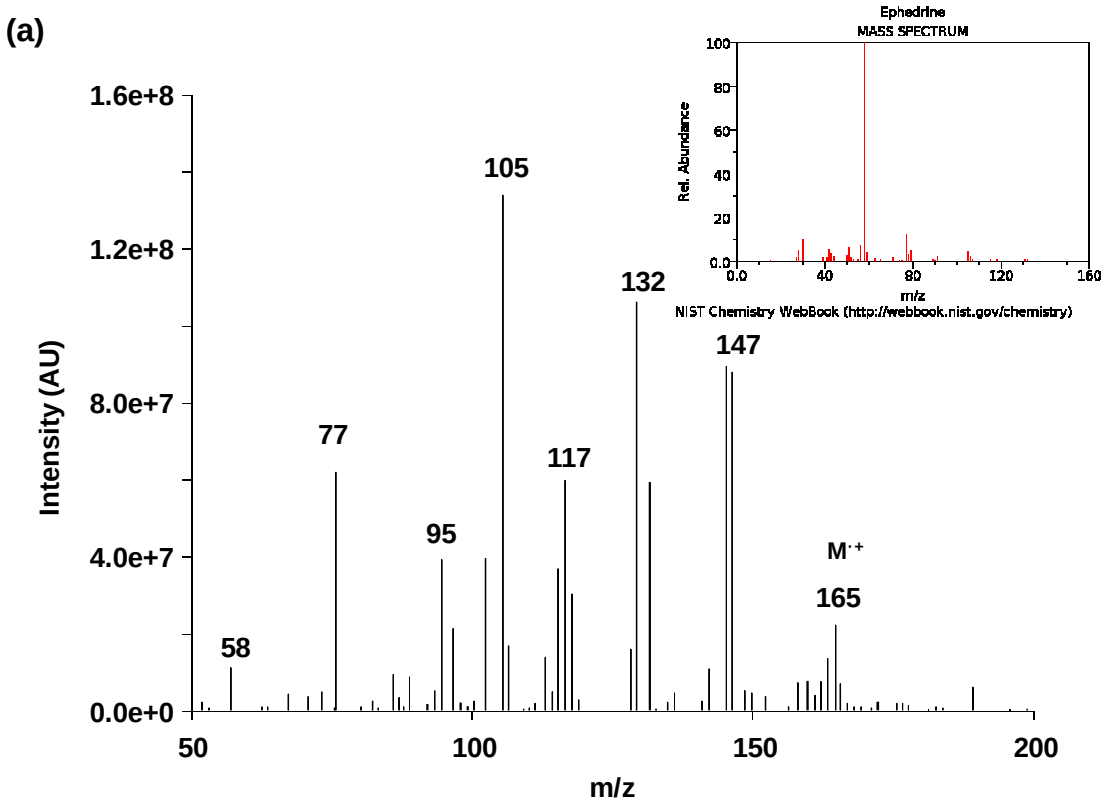
RESULTS AND DISCUSSION

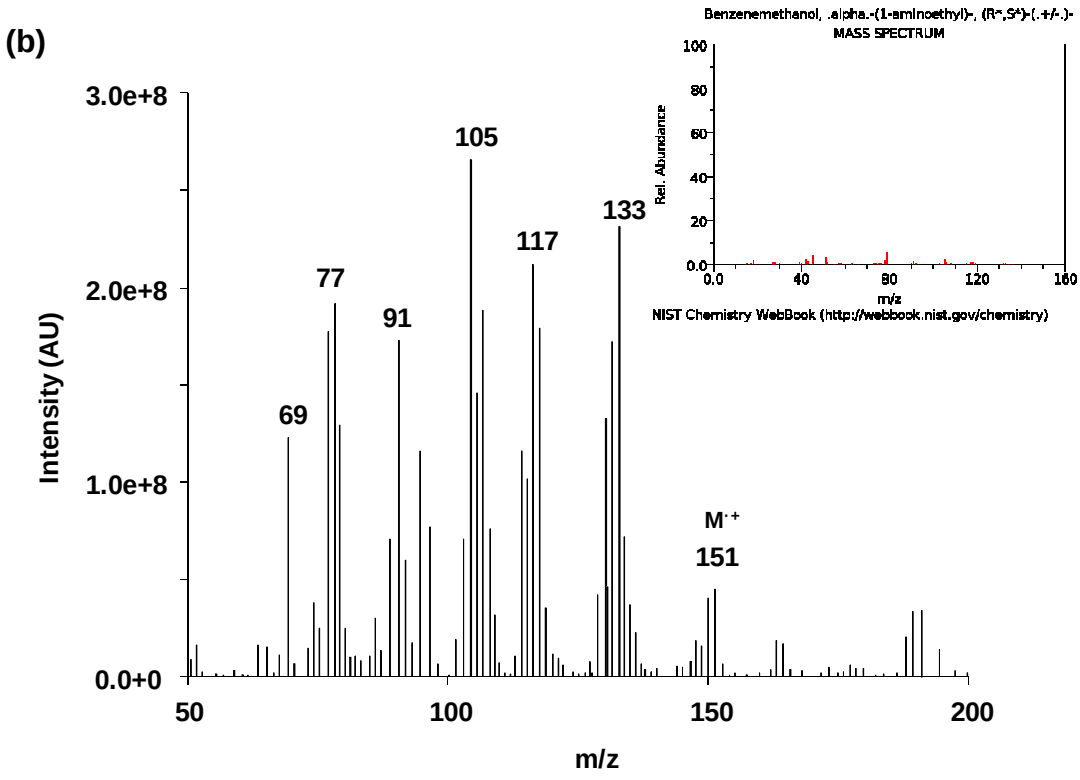
Ephedrine Alkaloids Mass Spectra

The ability to acquire and easily interpret mass spectra for species of interest is a powerful advantage supplied by using the PB interface. As mentioned previously, the PB interface can efficiently couple to LC/MS and deliver dry analyte particles to the source housing by removing solvent residues/vapors. To illustrate this important characteristic of the PB interface, the PB/EI mass spectra of ephedrine, norephedrine and methylephedrine obtained in the flow injection mode (50 µL injection loop) are shown in Figs. 4.3a-c,

respectively. The spectra were acquired using the standard electron energy of 70 eV, allowing comparison with MS spectral libraries. The insert in each figure shows the equivalent mass spectrum from the NIST mass spectral database acquired by GC-MS.

The PB/EIMS spectrum for ephedrine, presented in Fig. 4.3a, shows the molecular ion ($M^{\cdot+}$) at m/z = 165 Da followed by the loss of water $(M\text{-}H_2O)^+$ at m/z = 147 Da. Other prominent fragment ions (following the loss of water) seen at m/z = 132, 117, and 105 Da represent the loss of a methyl group, followed by the loss of the primary amine and the loss of a second methyl group, leading to the phenylium ion at m/z = 77 Da. The mass spectra for pseudoephedrine is identical to the one obtained for ephedrine due to the fact that the only structural difference between the species is the stereocenter configuration (hence spectrum not shown). Figure 4.3b corresponds to the PB-EIMS spectrum of norephedrine with the molecular ion present at m/z = 151 Da along with various fragment peaks at m/z = 133, 117, 104 and 77 Da. As in the case of ephedrine, the fragment ion transition from 151→133 corresponds to the loss of water from the molecular ion. Finally, Fig. 4.5c shows the PB-EIMS mass spectrum of methylephedrine. As seen in the previous two spectra, a very similar and straight-forward fragmentation pattern containing the ion fragments of m/z = 161, 133, 117, 105 and 77 Da along with the $M^{\cdot+}$ at m/z = 179 Da is observed. The ion transition (179→161 Da) corresponding to $(M\text{-}H_2O)^+$ is also present.


(a)
Ephedrine
MASS SPECTRUM
Rel. Abundance
m/z
NIST Chemistry WebBook (http://webbook.nist.gov/chemistry)
Intensity (AU)
1.6e+8
1.2e+8
8.0e+7
4.0e+7
0.0e+0
105
132
147
77
117
95
M·+
165
58
50
100
150
200
m/z


(b)
Benzenemethanol, .alpha.-(1-aminoethyl)-, (R*,S*)-(.+/-.)-
MASS SPECTRUM
Rel. Abundance
m/z
NIST Chemistry WebBook (http://webbook.nist.gov/chemistry)
Intensity (AU)
3.0e+8
2.0e+8
1.0e+8
0.0+0
105
133
117
77
91
69
M·+
151
50
100
150
200
m/z

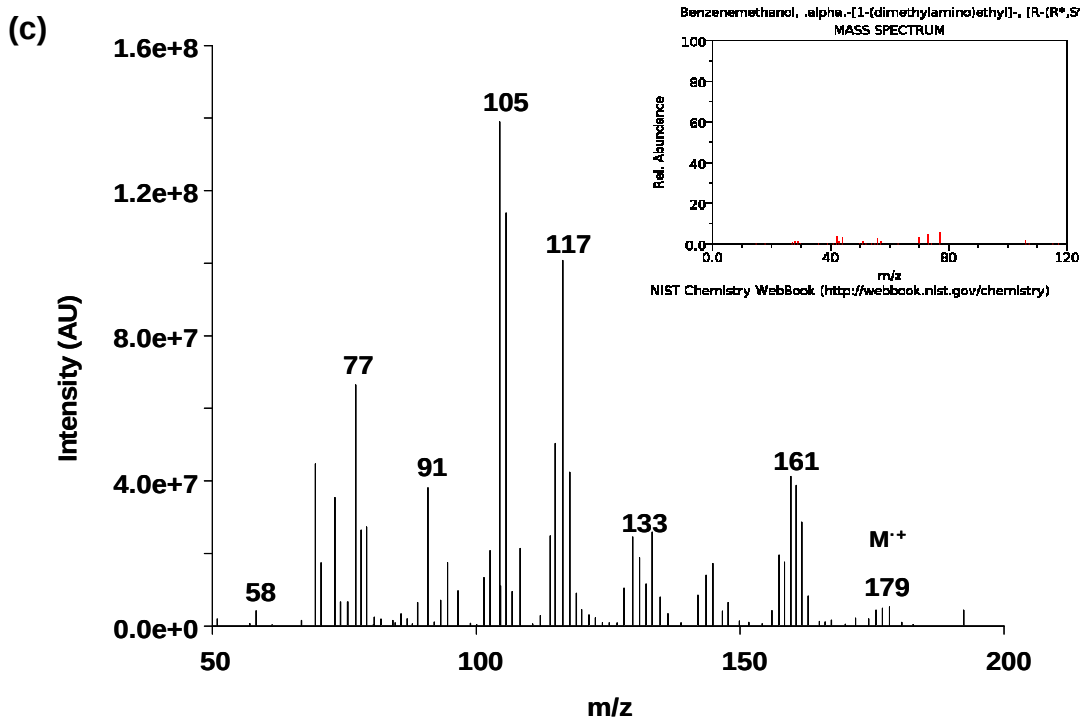


Figure 4.3. LC-PB/EI mass spectra of a) ephedrine, b) norephedrine, and c) methylephedrine. Electron energy = 70 eV, block temperature = 200 ℃, concentration = 100 µg mL^{-1}, 50 µL injection loop.

The NIST mass spectra, which were acquired by GC-MS with electron ionization at 70 eV, lack the molecular ion corresponding to the ephedrine alkaloids and only a few fragment ions can be compared due to the limited volatility and thermal stability. As well, the PB interface allows the introduction and subsequent in-source vaporization for ionization. Hence, the acquisition of real EI spectra for the ephedrine alkaloids via LC-PB/MS clearly provides an advantage over GC-MS.

Analytical Performance

Table 4.2 shows the figures of merit obtained for the ephedrine alkaloids by the LC-PB/EIMS system. Response curves using the total ion chromatogram (TIC) were generated through triplicate injections across the concentration range of 0 (i.e. the analytical blank) to 100 µg mL^{-1}. Each of the corresponding response functions shows acceptable linearity with satisfactory correlation coefficients (R^2 values). The limits of detection ($3\sigma_{blank}/m$) determined for the ephedrine alkaloids are all below 1 nanogram, absolute. These LODs obtained for the ephedrine alkaloids via LC-PB/EIMS are consistent with/or lower than the values reported in the literature by using GC-MS (0.01-0.7 ng absolute)[10, 21] and ESI-MS (0.03-0.8 ng absolute)[17] detection.

Table 4.2. Analytical response characteristics of ephedrine alkaloids by LC-PB/EIMS.

Analyte	Response Function	Accuracy (R^2)	Detection Limit (ng mL^{-1})	Absolute Mass (ng)
(-)-Ephedrine	y = 2E+09x + 1E+10	0.9997	2.04	0.10
(-)-Norephedrine	y = 2E+09x + 1E+08	0.9930	2.70	0.13
(+)-Pseudoephedrine	y = 1E+09x + 1E+10	0.9816	3.20	0.16
(-)-Methylephedrine	y = 5E+08x + 5E+09	0.9909	4.40	0.22

Chromatographic Separation of Ephedrine Alkaloids

As mentioned earlier, HPLC (reversed phase, ion-pairing or strong cation exchange) with UV absorbance and/or MS detection are the most common

method used for the analysis of ephedrine alkaloids.[5, 11, 13, 14, 17] Two ion-pairing reversed-phase liquid chromatography methods were evaluated to determine the best separation conditions for the ephedrine alkaloids present in the ephedra SRMs. From work reported in the literature as well as consideration of the functional groups of the ephedrine alkaloids it has been determined that the best stationary phase for the separation of the ephedrine alkaloids would likely be a phenyl column. Once, the chromatographic column was chosen, two different organic modifiers were evaluated for the separation of the ephedrine alkaloids. During the first chromatographic separation of an ephedrine alkaloid synthetic mixture containing 100 µg mL^{-1} of each of the species a full linear gradient, varying from 5 to 95% ACN (1% min^{-1} rate change) and 0.1% TFA in water, was performed. The chromatographic evolution of the separation was monitored by UV-Vis absorbance at 210 nm. The four ephedrine alkaloids eluted in the first 15 minutes with good baseline resolution although ephedrine and pseudoephedrine elute very close to each other. Figure 4.4a shows the LC-PB/EIMS chromatographic separation of the ephedrine alkaloid synthetic mixtures in the selected ion monitoring (SIM) mode at m/z = 105 and 132 Da. The signal of the two fragment ions used for the SIM mode are extracted from the TIC mode and co-added to yield simplified chromatographic separation.

Even though using acetonitrile as the elution solvent delivered a good separation of the ephedrine alkaloids, methanol was attempted to determine if better resolution of the ephedrine/pseudoephedrine pair could be achieved.

Besides the elution solvent all of the other chromatographic parameters were kept constant. Figure 4.4b shows the LC-PB/EIMS chromatographic separation of the ephedrine alkaloid synthetic mixtures in the SIM mode (m/z = 105 and 132 Da) using methanol as the organic modifier. From the resultant chromatograms, it can be observed that by using methanol a better separation is obtained. A linear gradient method varying from 95:5 (0.1% TFA in water: MeOH) to 80:20 over 15 minutes at 1 mL min^{-1} is used during the remainder of the study.

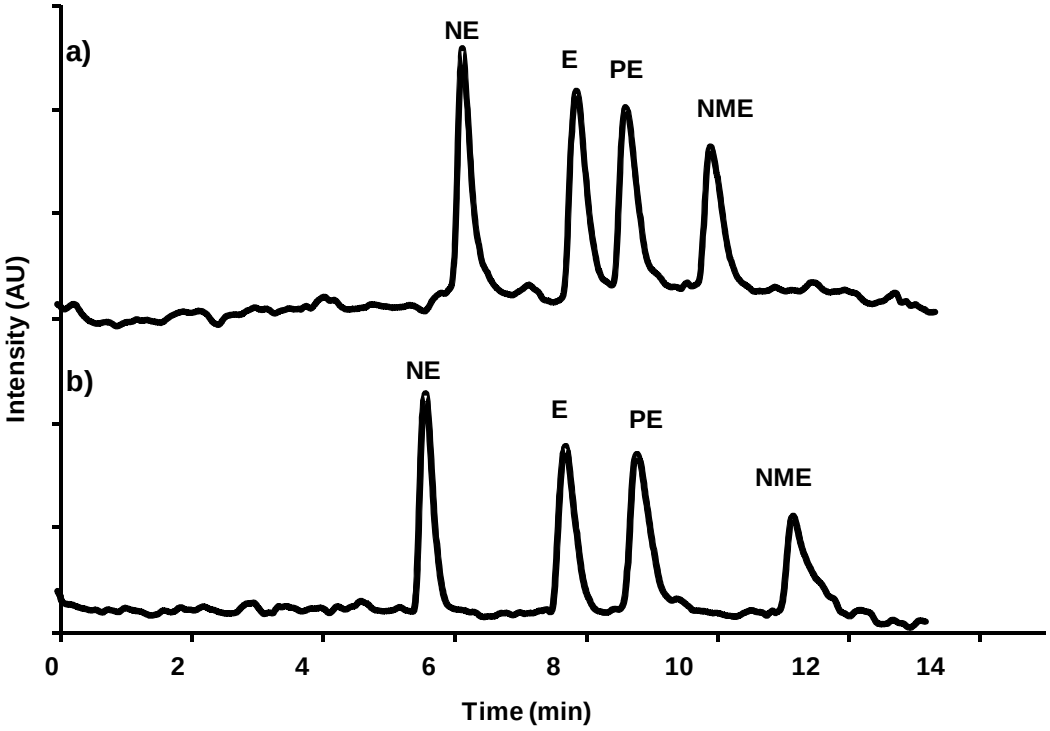


Figure 4.4. LC-PB chromatographic separation of 100 µg mL^{-1} mixture of ephedrine alkaloids in SIM mode (m/z = 105 and 132 Da) using a) methanol and b) acetonitrile as part of the gradient elution mode. Electron energy = 70 eV, block temperature = 200 ℃, 50 µL injection loop.

Quantification of the Ephedrine Alkaloids in NIST Standard Reference Materials

Once acceptable chromatographic conditions were achieved, the ephedra containing dietary supplement reference materials were analyzed and the ephedrine alkaloids quantified by standard addition method. Figure 4.5a and b show overlays of the chromatographic separation of a 20% SRM 3241 and 10% 3242 solutions along with their 50 µg mL^{-1} spiked solutions in SIM mode (m/z = 105 and 132 Da), respectively. The mass spectra extracted from the chromatogram for each eluted species provided fragmentation patterns similar to the spectra shown in Figs. 4.3a-c. A standard addition method was performed for the quantification of the ephedrine alkaloids, ephedrine, pseudoephedrine and methylephedrine in the two ephedra reference materials. The calculated values obtained for the ephedrine alkaloids were based on triplicate chromatographic separations and are shown in Table 4.3. For the ephedrine alkaloids, the experimental values obtained by the standard addition method were comparable to the certified values provided by NIST, with recoveries of ≥ 86% and relative standard deviations (RSDs) of ≤14% (*n = 3*). The experimental values for norephedrine could not be determine (ND) because after extraction and dilution for the quantification analysis the SIM signals fall below the detection limits. The high recoveries achieved during the quantification analysis of the ephedrine alkaloids clearly demonstrate that the developed chromatographic method coupled to the PB/EIMS system is a viable approach for the assessment of botanical extracts.

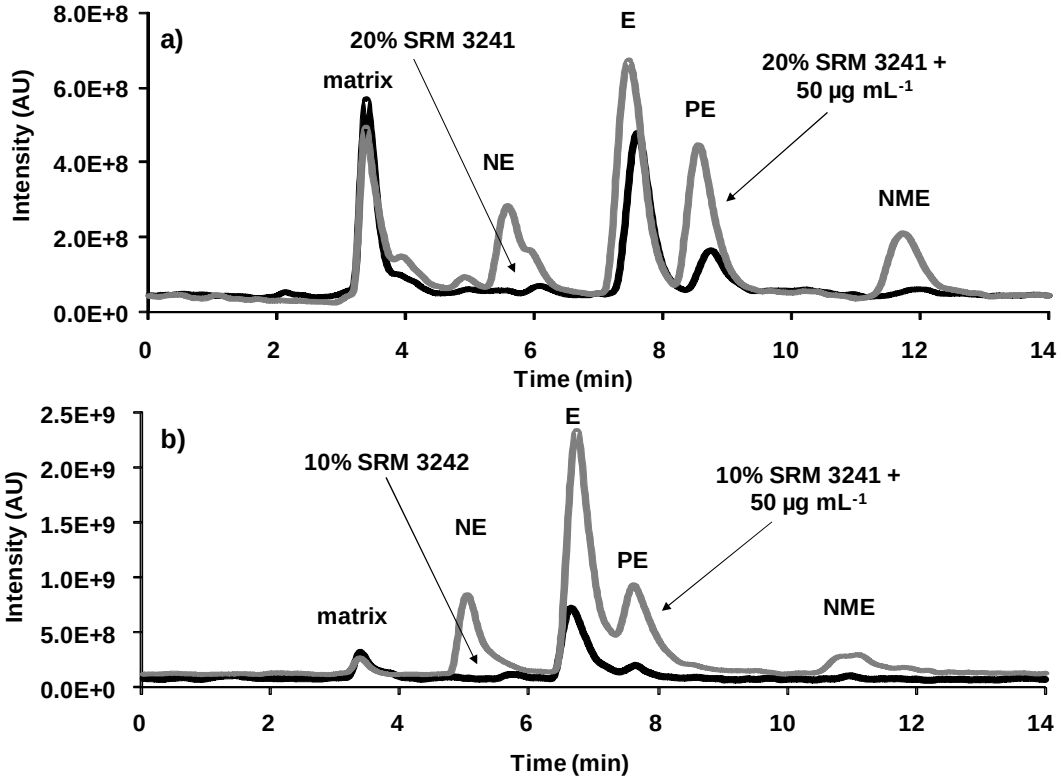


Figure 4.5. LC-PB chromatographic separation of a) 20% SRM 3241 and b) 10% SRM 3242 in SIM mode (m/z = 105 and 132 Da) . The overlapped chromatograms correspond to samples spiked with 50 µg mL^{-1} of each ephedrine alkaloid. Electron energy = 70 eV, block temperature = 200 ℃, 50 µL injection loop.

Table 4.3. Validation results for ephedrine alkaloids in NIST SRMs 3241 and 3242 using the standard addition method.

Ephedrine Alkaloids	Certified Values (mg g^{-1})	Calculated Values (mg g^{-1}) (n=3)	%RSD	%Recovery
SRM 3241 *Ephedra Sinica* Stapf Native Extract				
(-)-Ephedrine	28.86 ± 1.17	24.92 ± 2.60	10.4	86
(+)-Pseudoephedrine	10.74 ± 1.11	9.80 ± 0.35	3.6	91
(-)-Methylephedrine	2.61 ± 0.51	2.33 ± 0.05	2.1	90
(-)-Norephedrine	0.48 ± 0.20	ND		
SRM 3242 *Ephedra Sinica* Stapf Commercial Extract				
(-)-Ephedrine	78.80 ± 2.30	73.50 ± 10.20	13.9	94
(+)-Pseudoephedrine	9.27 ± 0.94	9.31 ± 0.66	10.3	91
(-)-Methylephedrine	2.77 ± 0.57	2.52 ± 0.01	0.4	100
(-)-Norephedrine	0.57 ± 0.18	ND		

CONCLUSIONS

The validation of the LC-PB/EIMS system as an analytical tool for the chemical characterization of botanical extracts was achieved by the analysis of a NIST Ephedra-containing dietary supplement SRM. Mass spectra for the ephedrine alkaloids were obtained, including the molecular ion and significant fragmentation patterns. Response functions with satisfactory linearity were generated and LODs in single nanogram level were determined. A straightforward and simple chromatographic separation was developed for the separation of the ephedrine alkaloids in the Ephedra NIST SRM 3241 and 3242. Quantification and validation of ephedrine, pseudoephedrine and methylephedrine was performed by standard addition with recoveries of ≥ 86% and RSDs of ≤14%.

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CHAPTER FIVE

SELENIUM SPECIATION BY LIQUID CHROMATOGRAPHY PARTICLE BEAM MASS SPECTROMETRY (LC-PB/MS): APPLICATION TO BOTANICAL AND URINE MATRICES

INTRODUCTION

Selenium (Se) plays an important role in the human body as an essential trace element that is also shown to provide numerous health benefits such as anti-carcinogenic and anti-oxidative properties.[1-4] Selenium, as selenocysteine, is required for the activity of antioxidant enzymes such as glutathione peroxidase and thioredoxin reductase.[5] The intake amount of Se has a narrow range between deficiency and toxicity, as well as the chemical form in which it is present.[6] A daily consumption of less than 0.1 mg kg^{-1} of body weight results in Se deficiency and levels above 1 mg kg^{-1} are deemed toxic.[3, 7] The most common chemical forms of selenium available in the environment in order of increasing toxicity are selenate (Se^{VI}), selenomethionine (SeMet), selenocystine ($SeCys_2$) and selenite (Se^{IV}).[8, 9] Selenium is introduced into the food chain through plants which uptake Se via compounds present in the soil.[10] However, due to the fact that the Se concentration in soil varies widely for regions all over the world, Se-enriched food supplements have gained interest and popularity.[10] For example, selenite, selenate, hydrogen selenite, selenomethionine and Se-(methyl)selenocysteine (Se-MeSeCys) can be found in commercially available

Se supplements.[2] Selenium-enriched yeast is the most common plant matrix found in these supplements, in which SeMet is usually the primary form of Se absorbed and stored within the human body.[2, 11]

Over the years, the nutritional bioavailability and toxicity of Se supplements has become a topics of interest in the scientific community.[3, 12] Therefore many analytical approaches have been developed, as well as reviewed in the literature, for the separation and determination of inorganic and organic Se species.[2, 10, 13] These encompass coupling gas chromatography[9, 12, 14, 15] or liquid chromatography (e.g., ion-pairing reversed phase and ion-exchange chromatography)[3, 6, 10, 11, 15-18] to various detection modes, with the most common being inductively coupled plasma mass spectrometry (ICP-MS)[4, 15, 19-22] for elemental analysis and/or electrospray ionization mass spectrometry (ESI-MS)[1, 4, 6, 10, 13, 17] for molecular information. While ICP-MS sensitivity for chromatographic separations is excellent, it can only provide elemental information because of the complete dissociation of the species in the high temperature plasma.[10, 15, 23, 24] Therefore, detection methods such as ESI-MS are necessary to obtain a complete chemical characterization of the species, particularly when retention times comparison to analytical standards is not possible. Other limitations surrounding ICP-MS are the need for complete chromatographic resolution of the metal components in the sample and high percentage organic solvent incompatibility.[15, 23, 25] On the other hand, ESI-MS is a soft ionization technique that can provide molecular weight information without

extensive fragmentation.[23, 26] The limitations associated with ESI-MS include lack of molecular structure information, analyte signal suppression by complex matrices, and lower sensitivity than ICP-MS.[23, 26, 27] For that reason, ESI-MS and ICP-MS are used as complementary techniques.[18, 28] Nonetheless, the development of a single analytical tool that could provide elemental and molecular information in one analysis needs to be considered.

Previous work in this laboratory implemented the liquid chromatography particle beam mass spectrometry (LC-PB/MS) system for the analysis of a large number of organic, inorganic, organometallic and biological compounds in neat solutions and real world samples such as botanical products (e.g., green tea, echinacea, kelp) using interchangeable ionization sources (electron ionization and glow discharge).[23, 24, 26, 29-31] These studies have demonstrated that the coupling of the PB interface to electron ionization mass spectrometry (EIMS) or glow discharge mass spectrometry (GDMS) ion sources provide the capabilities to accomplish *comprehensive speciation* analysis (i.e. the identification and quantification of individual elemental and molecular species) that is necessary for metabolic studies, regulatory compliance and quality control.[23, 24, 26, 29] Consequently, as part of the ongoing studies in this laboratory, the present work focuses on the separation and identification of organic and inorganic Se species in Se-enriched yeast certified reference material (SELM-1) and urine. An ion-pairing reversed phase LC method using a C_{18} column coupled to UV-Vis absorbance detector (λ = 210 and 254 nm) was initially evaluated for the

chromatographic separation. After determining the best chromatographic conditions, the liquid stream was interfaced to the PB/MS system for analysis of SELM-1 and urine. Mass spectra were acquired for each of the species of interest using analytical standards, and characteristic fragmentation patterns and signature ions were identified for each of the species. Instrumentation parameters were optimized and calibration curves generated for the species to determine their respective analytical figures of merit. Quantification of methionine (Met) and SeMet content in SELM-1 was accomplished by standard addition. On the other hand, the total selenium content was determined by using a microwave digestion and ICP-OES method recently published by this laboratory.[32]

EXPERIMENTAL

Reagents and Solutions

Stock solutions (1000 µg mL^{-1}) of sodium selenate, sodium selenite, selenomethionine, selenocystine, Se-(methyl)selenocysteine, methionine and creatinine (Sigma-Aldrich, St. Louis, MO, USA) were prepared in water containing 0.1% trifluoroacetic acid (TFA) (Fisher Scientific, Fair Lawn, NJ). The synthetic urine solution was prepared as reported by Gammelgaard and Jons[33] containing 55 mM of sodium chloride, 67 mM of potassium chloride, 2.6 mM of calcium sulfate, 3.2 mM of magnesium sulfate, 19.8 mM of sodium dihydrogen sulfate, 29.6 mM of sodium sulfate, 310 mM of urea and 9.8 mM of creatine in

water. These reagents were all purchased from Sigma-Aldrich, except for potassium chloride and magnesium sulfate (Fisher Scientific, Fair Lawn, NJ). All solutions were stored at 4^{o}C and fresh dilutions prepared as necessary.

Instrumentation

The PB/MS system used in this study was an Extrel (Pittsburgh, PA, USA) Benchmark Thermabeam LC/MS quadrupole mass spectrometer with an electron ionization source, depicted in Fig. 1.5, and has been previously described in chapter one and literature.[24, 26, 34] Total ion chromatograms (TIC) were acquired using the Extrel Merlin (Pittsburgh, PA) Ionstation system software by scanning over a mass range of m/z = 50-350 Da at a scan rate of 1.0 s scan^{-1}. Selected ion monitoring (SIM) chromatograms for specific masses were extracted from the TIC data for background correction and peak integration. The data was exported to Sigma Plot 8.02 (Systat Software, Inc., Richmond, CA) and presented using Microsoft Excel and PowerPoint (Redmond, WA). Details on the particle beam interface have also been described greatly in the literature[24, 26, 34, 35] and in chapter one. In the current study, the nebulizer is set at a temperature of ~85℃, the desolvation chamber at ~110℃ and the source block at ~275℃.

A Waters (Milford, MA) Model 1525 HPLC binary system equipped with a Waters Model 2487 dual wavelength absorbance detector and a Rheodyne (Cotati, CA, USA) Model 7125*i* injector and a 50 µL injection loop were used for the chromatographic separation. An Alltech Alltima C_{18} (250 mm x 4.6 mm ,

5µm) reversed-phase column (Alltech Associates Inc., Deerfield, IL, USA) and guard column (All-Guard Holder with Alltima C_{18} Cartridge, Alltech Associates Inc., Deerfield, IL, 7.5 mm x 4.6 mm, 5 µm) operated at room temperature and a flow rate of 0.9 mL min^{-1} was utilized. The LC solvents consisted of water (18.2 MΩ cm^{-1}, NANOpure Diamond, Barnstead International, Dubuque, IA) containing 0.1% v v^{-1} trifluoroacetic acid (TFA) and HPLC-grade methanol (Fisher Scientific, Fair Lawn, NJ). A linear gradient method with a mobile phase composition varying from 5-15% MeOH over the first 10 minutes followed by a 15-40% MeOH over the next 10 minutes was used for the separation.

Determination of Met and SeMet in SELM-1 via PB/EIMS

Approximately 0.25 grams of SELM-1 (Institute for National Measurement Standards, National Research Council Canada) in powdered form and 24 mL of 4M methanesulfonic acid (99.5%, Aldrich, St. Louis, MO, USA) were added to a 50 mL round bottom flask and extracted by reflux for 16 hours.[12, 20] After extraction, the samples were centrifuged at 4000 rpm for 60 minutes and filtered using a 0.22 µm PVDF filter (Alltech Associates Inc Deerfield, IL, USA) for final analysis.

Quantification of the SELM-1 was achieved through a standard addition method. Stock standard solutions (1.0 mg mL^{-1}) of SeMet and Met were added in 0.025 and 0.050 mL aliquots to SELM-1 and diluted to 1.0 mL. The SELM-1 aliquots were 25% and 50% solutions. The moisture content of SELM-1 was

determined by drying in an oven at ~100˚C for 24 hours. Conversion factors were determined based on dry-mass/original mass and used to report the quantification values on a dry-mass basis.

Determination of Total Selenium in SELM-1 using ICP-OES

For the total Se content, approximately 0.9 grams of SELM-1 and 5 mL of trace metal nitric acid (Fisher Scientific, Fair Lawn, NJ) were placed in a 75 mL microwave Teflon vessel. The vessels were positioned inside a MARS Xpress microwave digestion system (CEM Corporation, Matthews, NC, USA) for the pre-digestion step consisting of irradiation for 15 minutes at a temperature of 80℃ (power at 300 W). Subsequently, the digestion step irradiated the sample to a temperature of 180℃ for 15 minutes using a ramp time of 10 minutes. Once the vessels were cooled to room temperature the sample was transferred to a 50 mL volumetric flask and diluted to the mark with Milli-Q water. The elemental analysis of the digested sample was performed by ICP-OES (Jobin-Yvon Ultima 2, Longjumeau, France) using an external calibration method with detection at the 196.026 nm Se (I) transition.

RESULTS AND DISCUSSION

Characteristic Mass Spectra

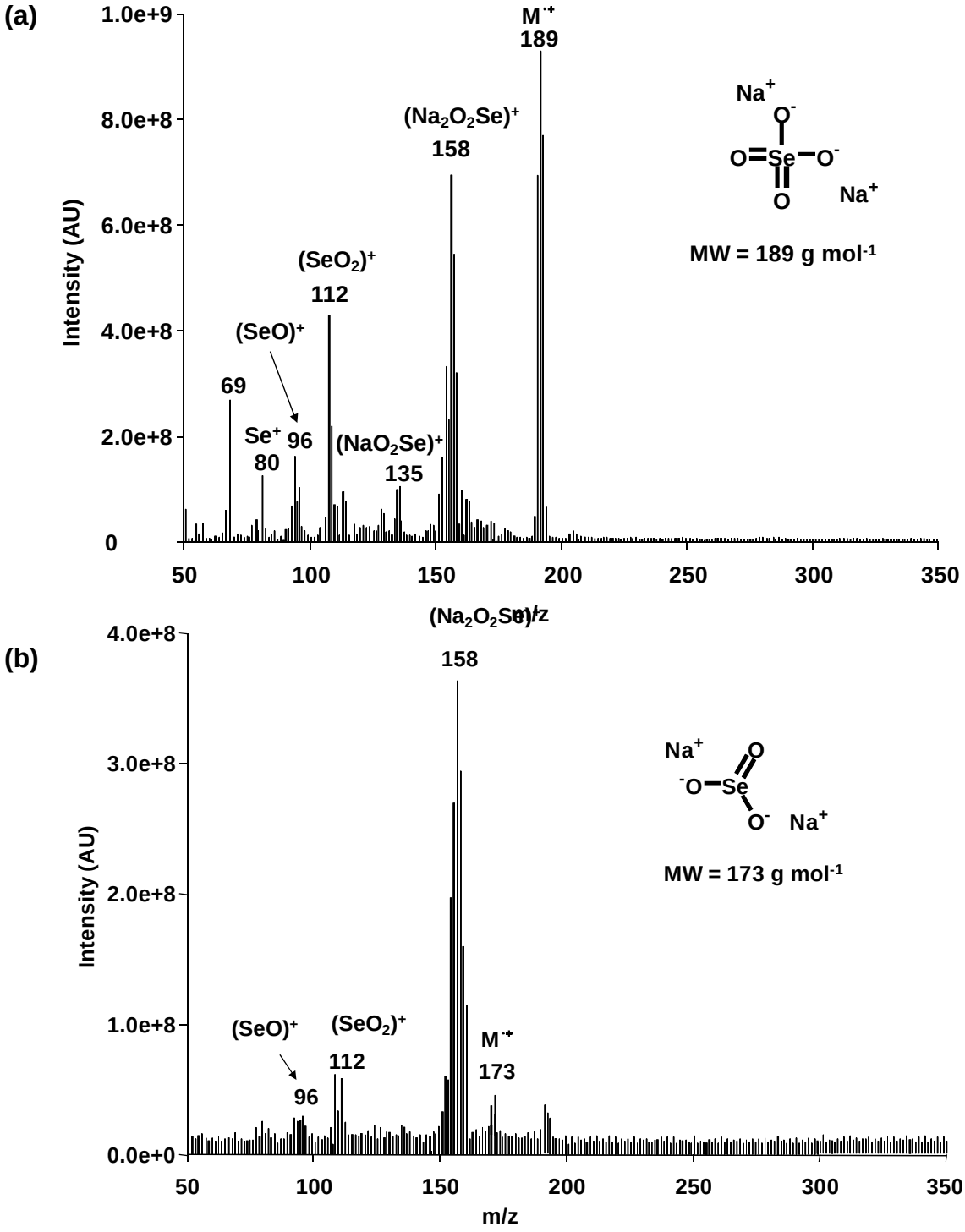
As mentioned previously, most metal speciation techniques lack accessible species-specific information for detection. During qualitative

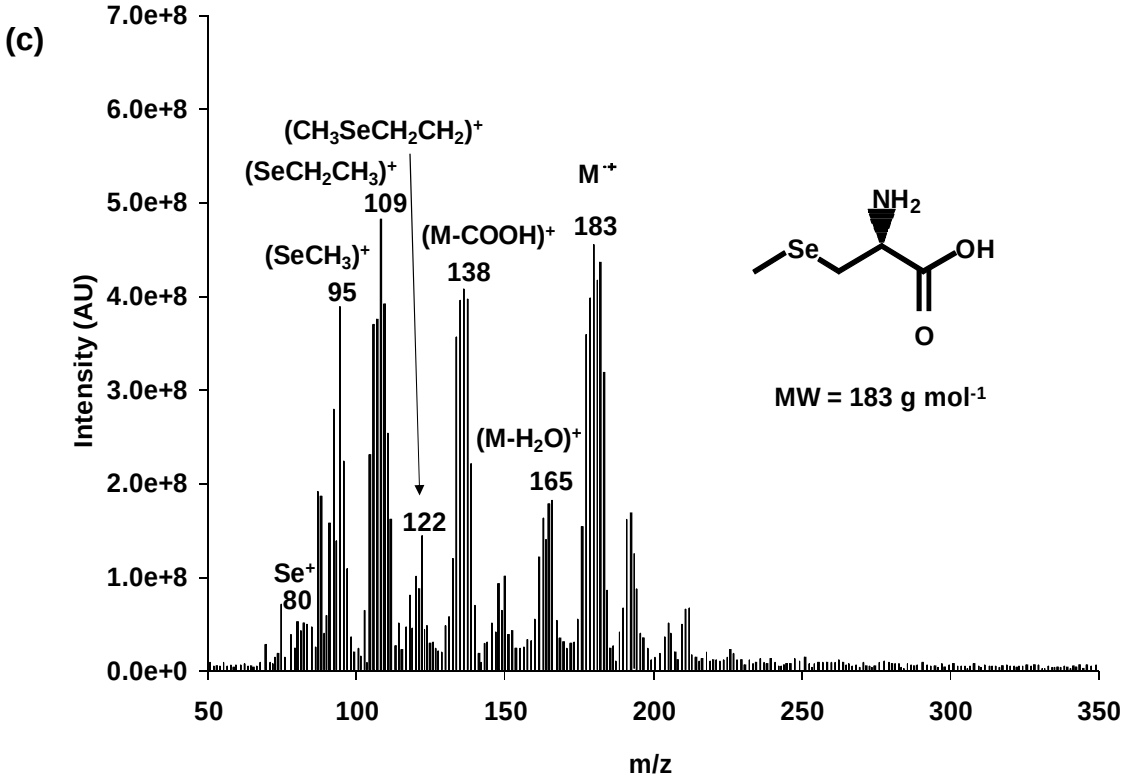
analyses, the identification of the compounds by these approaches is based on matching chromatographic retention times of analytical standards. For this reason analytical methods using the PB/EIMS and/or PB/GDMS are being developed. The PB interface allows the acquisition of simple and easily interpreted EI and GD spectra making spectral library comparison possible when available and at the same time maintaining chromatographic integrity by efficiently removing solvent residues/vapors. Figures 1a-f show the PB/EI mass spectra (along with their respective chemical structures) for sodium selenate, sodium selenite, Se-(methyl)selenocysteine, selenocystine, selenomethionine and methionine obtained in the flow injection mode from 50 µL injections of 100 µg mL^{-1} stock solutions. Each spectrum shows the molecular ion with clear and simple fragmentation patterns with the exception of $SeCys_2$, suggesting that $SeCys_2$ is not stable under the operating conditions. Figures 1a-b correspond to the EI spectra of the inorganic Se species, sodium selenate and sodium selenite. Each spectrum shows their respective molecular ion at m/z = 189 Da (sodium selenate) and m/z = 173 Da (sodium selenite) as well as very similar fragment ions at m/z = 158, 112, 95 and 80 Da representing $(Na_2O_2Se)^+$, $(SeO_2)^+$, $(SeO)^+$ and Se^+, respectively.

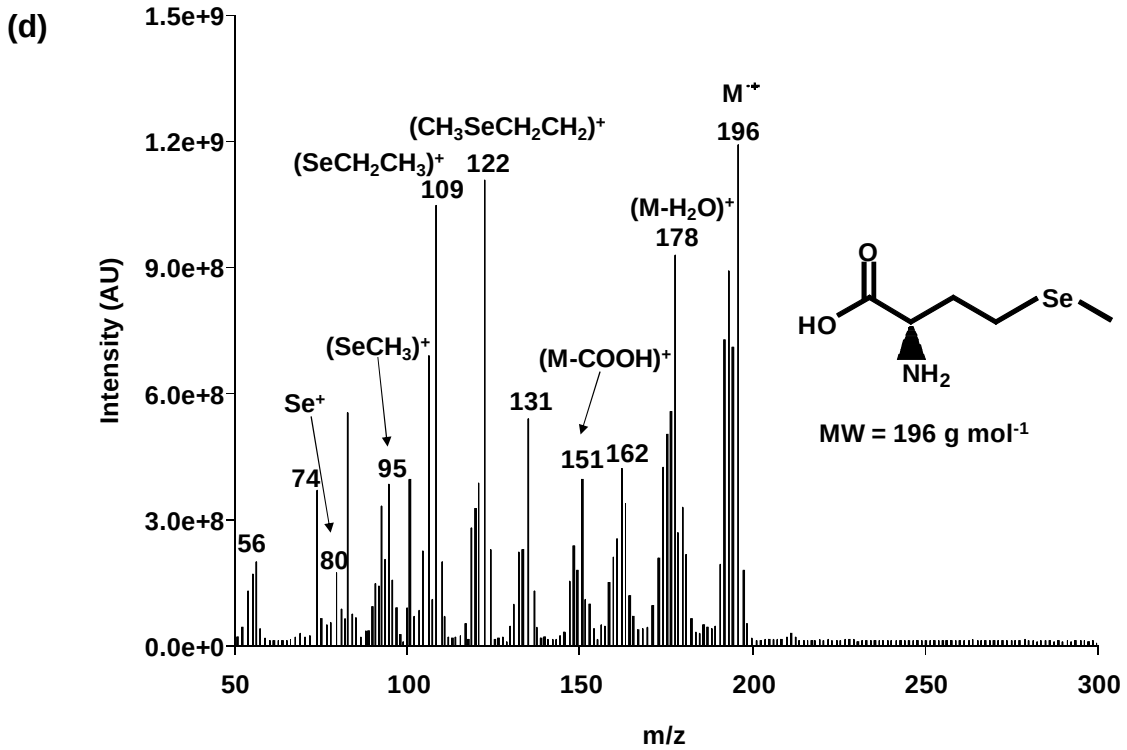
Shown in Fig. 1c is the PB/EIMS spectrum of Se-MeSeCys. This mass spectrum shows the molecular ion at m/z = 183 Da along with prominent fragment peaks at 165, 138, 109, 95 and 80 Da. These peaks correspond to the loss of water $(M-H_2O)^+$, the loss of a carboxylic acid group $(M-COOH)^+$, followed

by the loss of the NH_2 group to yield $CH_3SeCH_2CH_2^+$, the loss of CH_2 group to yield $(CH_3SeCH_2)^+$**,** the loss of a second CH_2 group $(SeCH_3)^+$ and the loss of CH_3 group forming the Se^+. Figure 1d depicts the mass spectrum for SeMet acquired via PB/EIMS with the molecular ion at m/z = 196 Da including a very clear and similar fragmentation pattern to Se-MeSeCys. The fragment peaks at m/z = 178, 151, 122, 109, 95 and 80 Da represent $(M\text{-}H_2O)^+$, $(M\text{-}COOH)^+$, $(CH_3SeCH_2CH_2)^+$**,** $(CH_3SeCH_2)^+$ and Se^+, respectively. In addition, Fig. 1e shows the PB/EIMS spectrum corresponding to $SeCys_2$. As mentioned earlier, the mass spectrum obtained for $SeCys_2$ lacks the molecular ion peak at m/z = 334 Da, although it depicts the ion fragment where the molecule is cleaved (in half) at the Se-Se bond. The other fragment ions (m/z = 183, 138, 109, 95 and 80 Da) observed are comparable to the fragment peaks described in the previous spectra (Se-MeSeCys and SeMet). Finally, Fig. 1f introduces the PB/EIMS spectrum of Met which presents a straight forward fragmentation pattern containing fragment ions at m/z = 132 and 104 Da along with the molecular ion at m/z = 149 Da. These two fragment ions correspond to the loss of a hydroxyl group $(M\text{-}OH)^+$ and the loss of a carboxylic group $(M\text{-}COOH)^+$ from the molecular ion. The insert in Fig. 1f shows the mass spectrum of Met from the NIST mass spectral library and it can be clearly seen that the PB/EIMS spectrum of Met presents a similar fragmentation pattern. In the case of the selenium species no equivalent NIST EI library spectra are available due to their limited volatility. Therefore, it is clearly

demonstrated that the PB/EIMS generates clear fragmentation patterns allowing the identification of unknown species.




(c)
7.0e+8
6.0e+8
5.0e+8
4.0e+8
3.0e+8
2.0e+8
1.0e+8
0.0e+0
Intensity (AU)
50
100
150
200
250
300
350
m/z
(CH3SeCH2CH2)+
(SeCH2CH3)+
109
M·+
183
(SeCH3)+
95
(M-COOH)+
138
(M-H2O)+
165
122
Se+
80
NH2
Se
OH
O
MW = 183 g mol-1


(d)
1.5e+9
1.2e+9
9.0e+8
6.0e+8
3.0e+8
0.0e+0
Intensity (AU)
50
100
150
200
250
300
m/z
M·+
196
(CH3SeCH2CH2)+
(SeCH2CH3)+
122
109
(M-H2O)+
178
(SeCH3)+
(M-COOH)+
Se+
131
74
95
151
162
56
80
O
HO
Se
NH2
MW = 196 g mol-1

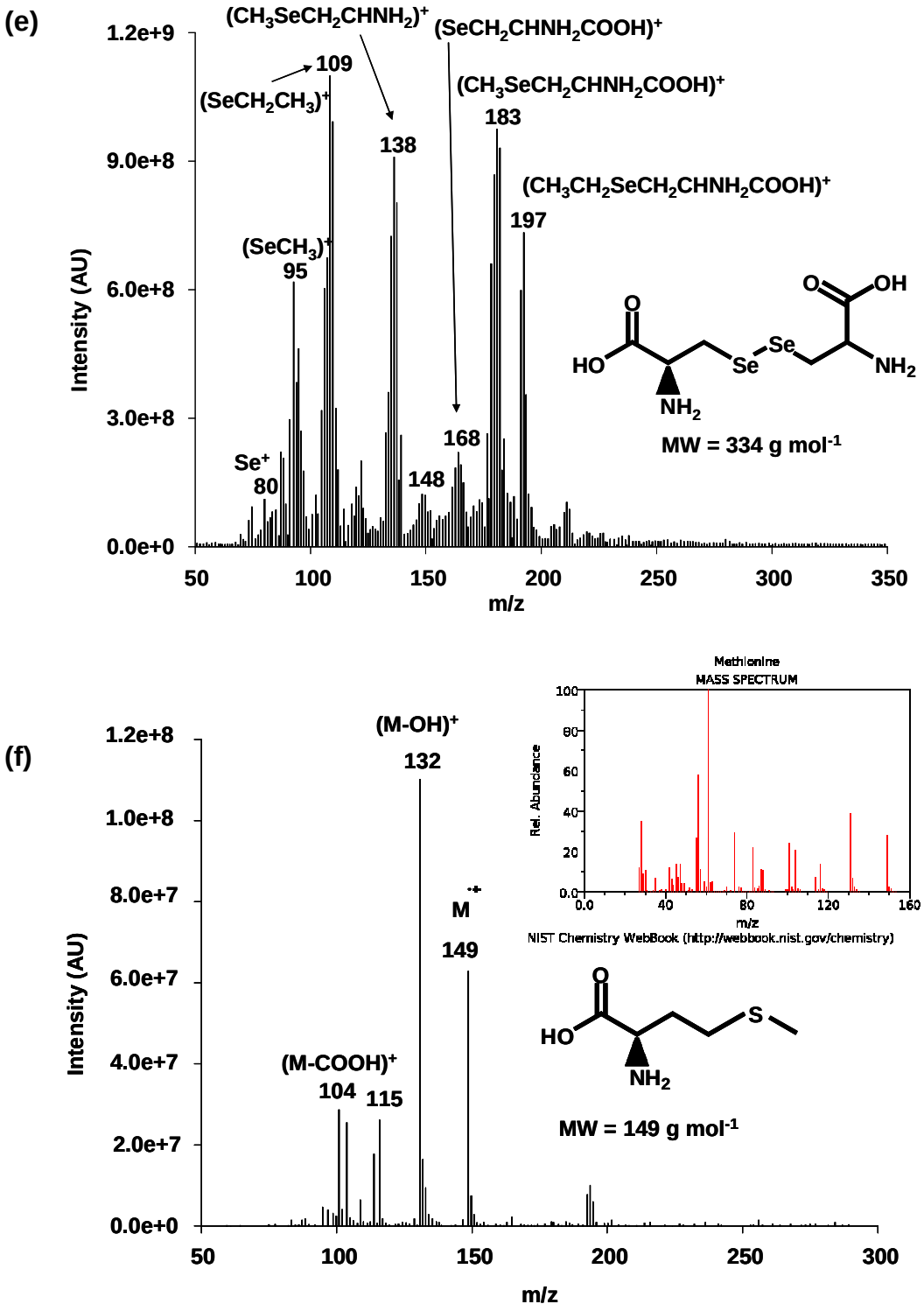


Figure 5.1. LC-PB/EI mass spectra of a) sodium selenate, b) sodium selenite, c) Se-(methyl)selenocysteine, d) selenomethionine, e) selenocystine and f) methionine. Electron energy = 70 eV, block temperature = 275 ℃, concentration = 100 µg mL^{-1}, 50 µL injection loop.

Analytical Response Characteristics

Calibration curves were generated for the various selenium species and methionine using the total ion chromatogram (TIC) with triplicate injections across the concentration range of 0 (i.e. analytical blank) to 100 µg mL^{-1} (involving 1.0, 5.0, 10, 25, 50, 75 µg mL^{-1} concentrations). Each of the corresponding response functions shows acceptable linearity with satisfactory correlation coefficients (R^2 values). Table 1 shows the instrument response functions, correlation coefficients and the limits of detection for the selenium species and methionine. The limits of detection ($3\sigma_{blank}/m$) determined are on the sub-nanogram level. The LODs obtained for the selenium species and methionine are slightly higher than the values reported in the literature obtained by ICP-MS (e.g., 0.08-0.80 ng mL^{-1})[36, 37] but the PB/EIMS system has the advantage of providing structural identification of the compounds as well. On the other hand, the PB/EIMS LODs are appreciably lower than the ESI values reported in the literature (3-28 ng mL^{-1}),[8, 17, 38] for the organic and inorganic Se species.

Table 5.1. LC-PB/EIMS analytical response characteristics for methionine and the selenium species.

Analyte	Response Function	Accuracy (R^2)	LOD (ppb)	Absolute Mass (ng)
Selenocystine	$y = 3E^{+09}x + 1E^{+10}$	**0.9972**	**1.7**	**0.09**
Selenomethionine	$y = 2E^{+09}x - 2E^{+10}$	**0.9938**	**2.2**	**0.11**
Se-methyl-selenocysteine	$y = 2E^{+09}x + 3E^{+09}$	**0.9917**	**3.2**	**0.16**
Sodium selenate	$y = 7E^{+08}x + 8E^{+09}$	**0.9880**	**6.7**	**0.34**
Sodium selenite	$y = 3E^{+08}x + 9E^{+08}$	**0.9875**	**8.6**	**0.43**
Methionine	$y = 3E^{+07}x + 2E^{+08}$	**0.9828**	**6.4**	**0.32**

Ion-pairing Reversed Phase Chromatographic Separation

As mentioned earlier, a number of researchers have reported methods for the separation of inorganic and organic selenium using a combination of ion-exchange or ion-pairing reversed phase chromatography coupled to ICP-MS and/or ESI-MS. Previous work in this laboratory demonstrated the separation of three organic selenium species using a C_{18} column with an isocratic mode composed of H_2O-TFA-MeOH.[30] In addition, TFA has served successfully as an ion pairing agent in this laboratory for the analysis of many botanical samples and its high volatility (mp = -15 C° and bp = 72 C°) is suitable in PB/MS analysis.[23] At the same time, ion-pair chromatography facilitates the separation of the ionic species and uncharged molecular species. Therefore, this mobile phase composition (H_2O-TFA-MeOH) was evaluated for the separation of the inorganic and organic Se species as well as Met by varying the 5 MeOH . Figure

2 shows the PB/EIMS chromatographic separation a synthetic mixture composed of the five Se species and Met in the selected ion monitoring mode (SIM) at m/z = 149, 158 and 196 Da). The signal of the three fragment ions used for the SIM mode are extracted from the TIC mode and co-added to yield the simplified chromatogram. The separation of the species was achieved using a linear gradient method varying form 95:5 (0.1% TFA in H_2O: MeOH) to 85:15 for 10 minutes followed a gradient change of 85:15 (0.1% TFA in H_2O: MeOH) to 60:40 another 10 minutes at 0.9 mL min^{-1}. In the resultant chromatogram, Se species are fully baseline resolved with the exception of Se (IV), and $SeCys_2$. However, the resolution of the unresolved species was adequate for qualitative purpose. In this study special emphasis was given for the quantification of SeMet and Met. Again, the ability to acquire a mass spectrum for each of the eluting compound allows for easy identification.

The elution order of the inorganic selenium species is dependent on the pH of the mobile phase because Se (IV) (pK_1 = 2.5, pK_2 = 7.3) and Se (VI) (pK_2 = 1.7) are present in solution as anions with one or two negative charges. In the case of Met and the organic Se species, the pH of the mobile phase and the hydrophobicity of the stationary phase play a role on the elution order, with the latter being more pronounce. The elution order of the species observed in the resultant chromatogram were as expected and similar to that published by Zheng *et al.*[16, 39]

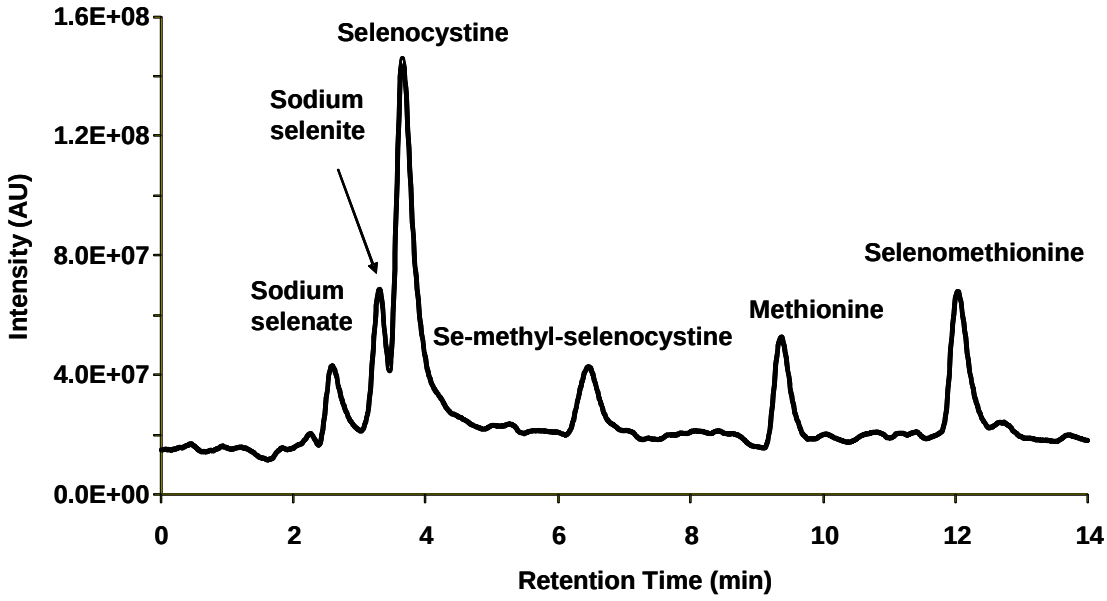


Figure 5.2. LC-PB chromatographic separation of 100 µg mL^{-1} mixture of methionine and selenium species in selected ion monitoring mode at m/z = 149, 158 and 196 Da. Electron energy = 70 eV, block temperature = 275 ℃, 50 µL injection loop.

LC-PB/MS Analysis of Se-enriched Yeast Certified Reference Material

As mentioned earlier, Se-enriched yeast is most commonly used for the production of Se dietary supplement. At the same time, SeMet is the dominant Se species found the foods and one of the most bioavailable.[11] The inorganic Se added for enrichment to the yeast growth medium intrudes on the sulfur assimilation plant pathway forming SeMet, which is believed to nonspecifically incorporate into the plant proteins in the place of Met.[11] Subsequently, leaching of the Se species from the proteins was necessary to obtain a complete characterization of the Se content and species in the supplements.[7] Many pretreatment procedures have been reported in the literature for the evaluation of Se distribution and speciation; such as hot water, enzymatic or acid reflux

extractions.[7, 9, 20, 22] Sample preparation in this work was performed by methanesulfonic acid reflux because of the reagent accessibility as well as the reported satisfactory results.[9, 11, 12, 14, 40]

After sample preparation by acid reflux extraction and achieving acceptable chromatographic conditions, the Se-enriched yeast certified reference material was analyzed and Met and SeMet quantified by standard addition method. Figure 3 shows the chromatographic separation of a 25% SELM-1 solution and a 50 µg mL^{-1} spike solution in the SIM mode (m/z = 149 and 196 Da) corresponding to the molecular ion of Met and SeMet, respectively. The quantification results obtained for Met and SeMet in SELM-1 based on triplicate chromatographic separations are depicted in Table 2. A comparison between the experimental values obtained by standard addition and the certified values provided by NRC show recoveries of 93% (RSD = 9%, n=4) and 97% (RSD=11%, n=4) for SeMet and Met, respectively. Clearly, the high recoveries achieved during the analysis of SELM-1 demonstrate that the LC-PB/EIMS system is a viable on-line detection method for the comprehensive speciation of Se species and therefore suggesting its application to other matrices such as urine.

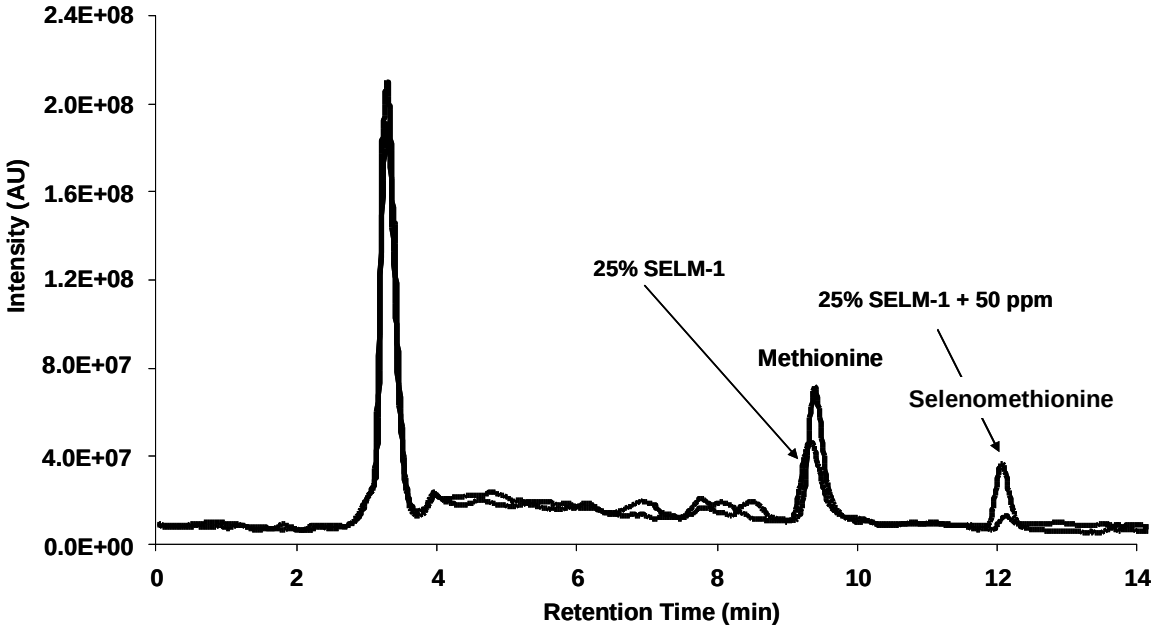


Figure 5.3. LC-PB chromatographic separation of a 25% SELM-1 in SIM mode (m/z = 149 and 196 Da). The overlapped chromatogram corresponds to a sample spiked with 50 µg mL^{-1} of methionine and selenomethionine. Electron energy = 70 eV, block temperature = 275 ℃, 50 µL injection loop.

Table 5.2. Validation results for selenium-enriched yeast CRM (SELM-1).

Analyte	Certified values (mg/kg)	RSD (%)	Calculated values (mg/kg) n=4	RSD (%)	Recovery (%)
Methionine (*149 Da*)	5758 ± 277	5.1	5344 ± 468	8.8	93
Selenomethionine (*196 Da*)	3389 ± 173	4.8	3293 ± 375	11	97
Total Se (ICP-OES)	2059 ± 64	3.1	2084 ± 40	1.9	101

LC-PB/MS Evaluation of Urine

Selenium content as it is excreted from the body in urine reflects the Se absorption from food as well as the metabolic changes characterizing the boundary between essential and toxic concentrations.[41, 42] For that reason, investigations into the Se content and Se metabolites in urine have been a major area of research.[8, 19, 21, 41] Selenium compounds such as SeMet, $SeCys_2$, selenocystamine, trimethylselenonium, selenosugars and many other species have been determined in urine by liquid chromatography coupled to ICP-MS or ESI-MS.[21, 37] However, the spectral interferences for Se and chloride are troublesome by conventional ICP-MS.[15] Therefore, the LC-PB/MS technique is evaluated here for the analysis of urine, which is a complex matrix containing high concentrations of urea, proteins, chloride, sodium and potassium.[37] In this particular case only preliminary studies have been carried out. Figure 4 shows the PB/EIMS chromatographic separation of a 10% synthetic urine solution containing 50 µg mL^{-1} of SeMet. The overlapped chromatograms correspond to the fragment ion traces of urea (m/z = 60 Da), creatininine (m/z = 113 Da) and SeMet (m/z = 196 Da). It is important to mention that no other procedure besides sample filtration and dilution was performed to minimize matrix interferences. Although further work involving the analysis of human urine before and after Se supplementation needs to be performed, the resultant chromatogram present here clearly demonstrates that the PB/MS system is applicable to such complex matrices.

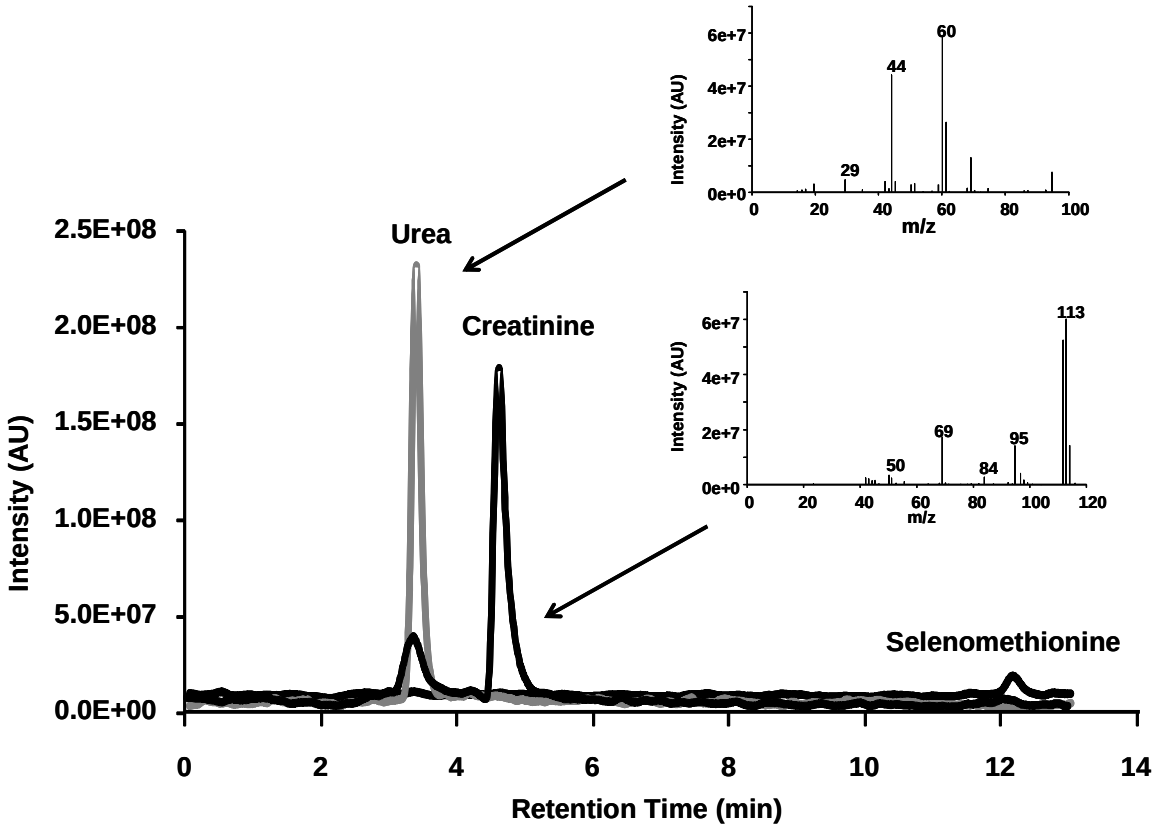


Figure 5.4. LC-PB chromatographic separation of a 10% synthetic urine solution containing 50 µg mL^{-1} of selenomethionine. The overlapped chromatograms correspond to fragment ion traces at m/z = 60, 113 and 196 Da. Electron energy = 70 eV, block temperature = 275 ℃, 50 µL injection loop.

CONCLUSIONS

The LC-PB/EIMS system has been shown to serve as an analytical tool for the comprehensive speciation of a selenium-enriched botanical sample as well as a urine matrix. Mass spectra for the inorganic and organic Se species as well as Met, creatinine and urea were acquired and such included their corresponding molecular ion with simple fragmentation patterns. Calibration curves were generated with satisfactory linearity and LODs in nanogram level. An ion-pairing reversed-phase chromatographic method was developed for the separation and characterization of the species of interest in SELM-1 and urine. Quantification by a standard addition method was carried out on the SELM-1 for SeMet and Met with recoveries of 93% and 97%, respectively. Total selenium content was evaluated by ICP-OES with a recovery of 100%.

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SUMMARY

The basis of the research presented here demonstrates the advantages and advances that have been accomplished for an LC/MS coupling technique; the particle beam (PB) interface. The ability to interchange between two different ionization sources; electron impact ionization and glow discharge ionization allows comprehensive chemical information of botanical products that are employed as dietary supplements. Chapter 1 outlined the importance of dietary supplements in people's daily lives as well as in the research communities. Subsequently, the fundamental aspects of glow discharge plasma, electron impact ionization and transport-type LC/MS interfaces are covered in addition to their application for the analysis of liquid analytes in flowing streams. Chapter 1 also introduced the analytical instrumentation used in this work that made possible the operation of glow discharge or electron ionization sources. This analytical technique was evaluated under several conditions, all of which were able to maintain chromatographic integrity and exhibit efficient analyte ionization. This dual mode LC-PB/MS technique is not currently commercially available but would expand the options available to researchers for qualitative and/or quantitative analysis where both elemental and molecular information is required.

Chapter 2 discussed the use of both EI and GD ionization sources coupled to the LC-PB/MS technique by the chemical characterization of the caffeic acid derivatives present in ethanolic Echinacea extract. The generated PB/EI and PB/GD mass spectra, followed common fragmentation rules in mass

spectrometry allowing the identification of known and unknown species as well as spectral library comparison when available. The work presented in Chapter 2 also demonstrated that using either an EI or GD source enables both the identification and quantification of the caffeic acid derivatives in Echinacea extract.

Chapter 3 demonstrated the ability of the LC-PB/EIMS system to simultaneously ionize inorganic and organic arsenic species for their identification and quantification in commercially available kelp and bladderwrack extracts. The work presented in this chapter clearly shows the advantages of using the particle beam interface which can accept a wide variety of separation modes (i.e. reversed phase and ion-exchange chromatography) by performing efficient solvent removal while maintaining the chromatographic integrity. The ability to generate EI spectra for compounds (in this case arsenobetaine and arsenosugar) that are not found in spectral libraries due to their poor volatility/thermal stability is another benefit of using the LC-PB/MS techniques.

It is of great importance to demonstrate that the PB/MS methodology is a reliable approach for the study of botanical products. Therefore, the validation of this technique through the use of NIST Ephedra-containing dietary supplement SRMs and a standard addition method was presented in Chapter 4. EI mass spectra for the ephedrine alkaloids, including the molecular ion and discernible fragmentation patterns, were obtained. Quantification by means of standard addition also allows for the confirmation of the expected retention times for

species not seen in the ion chromatograms. The validation results obtained during this study certainly showed the capacity of the PB/MS technique. Chapter 5 continued to demonstrate the power and flexibility of the PB/MS system for the comprehensive chemical characterization of botanical products/dietary supplements and introduced the preliminary evaluation of another natural matrix (i.e. urine).

Currently, the complete characterization of the organic and inorganic components of botanical products requires the use of two different chemical separations methods (ICP-MS and ESI-MS), with each optimized to their respective ionization source. The research presented here addressed the development of a practical analytical tool that can identify elemental and molecular solutes and provide quantitative information of the botanical product components in a single analysis. This approach allows the analysis of small molecules (molecular weights ≤ 700 Da) that do not warrant the expense or complexity of ESI-MS, which would require MS-MS analysis to obtain fragmentation pattern data and does not provide elemental information. On the other hand, ICP-MS provides the necessary elemental information but identification of the species is based on matching chromatographic retention times rather than “molecular” spectral characteristics. In addition, the LC-PB/MS allows the analysis of highly polar molecules that are not feasible by GC-MS. The ease of operation of the PB interface and the fact that a wide variety of separation modes can be employed without affecting the product ionization

characteristics allows for the optimization of the chromatographic separation independent from detection. Hence, LC-PB/MS can be employed as another alternative or complementary technique for the already established chromatographic separations of botanical products.

Both ionization sources (EI and GD) can provide spectra for organic, organometallic, and inorganic species and therefore perform comprehensive profiling of the species of interest. The sources are also able to generate mass spectra that are simple and easy to interpret, allowing the use of spectral interpretation rules and electronic spectral libraries. This dual ionization mode capability is not currently available in any commercial instrumentation and could find application in nutritional, environmental, and toxicological areas where both elemental and molecular species information is required. The unique combination of liquid chromatography sample introduction and two versatile ion sources provides for the *comprehensive speciation* that is necessary for fundamental metabolic studies as well as regulatory compliance and quality control.

Future work in this laboratory will continue through the collaboration with NIST on the chemical characterization and certification of other dietary supplements. Additionally, fundamental metabolic studies will be continued, as well as the development and optimization of the GD source geometries (direct insertion probe and hollow cathode) for the analysis of botanical products.

APPENDICES

APPENDIX A

DETERMINATION OF CATECHINS AND CAFFEINE IN GREEN TEA STANDARD REFERENCE MATERIALS BY LIQUID CHROMATOGRAPHY PARTICLE BEAM ELECTRON IONIZATION MASS SPECTROMETRY (LC-PB/EIMS)

INTRODUCTION

Green tea (*Camellia sinesis*) is one of the most consumed drinks worldwide, becoming part of the daily routine of many people and a significant source of antioxidants, which can provide diverse health benefits.[1-3] The major class of active compounds in green tea is the polyphenols, more specifically the catechins (also known as flavan-3-ols) which make up 30% (mass fraction) of green tea leaves.[4] The most abundant catechin species in green tea include (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-gallocatechin, (-)-gallocatechin gallate and (-)-epigallocatechin gallate. Other compounds present in green tea are phenolic acids (gallic acid, chlorogenic acid and caffeic acid), flavanols (quercetin, kaempferol and myricetin) and xanthines (caffeine and theophylline).[5] The consumption of polyphenols has acquired a great deal of attention because of their strong antioxidant properties, which have been shown to be beneficial in the prevention of cancer and cardiovascular diseases. Other reported medicinal benefits of the polyphenols include anti-inflammatory, anti-arthritic and anti-angiogenic properties.[2, 6, 7]

Botanical supplements such as green tea, echinacea and goldenseal have become an important part of people's nutrition due to their numerous health benefits. For that reason, it is of most importance that the producers and manufactures of such products provide accurate information of safety. In 1994, the Dietary Supplement Health and Education Act (DSHEA) assigned the United State Food and Drug Administration to regulate the production of these supplements. DSHEA ensures the safety of the supplements by providing a legal definition of dietary supplements, establishing guidelines for displaying the ingredients on the labels and allowing the FDA to present good manufacturing practice (GMP) regulations.[8, 9] After DSHEA, the Office of Dietary Supplements (ODS) was established within the National Institutes of Health (NIH) to promote scientific research as well as the development of Standard Reference Materials (SRM) for botanical supplements in order to achieve product consistency throughout the raw material characterization as well as the identification of potential adulterants and contaminants.[10, 11] The production of these SRMs also allows the validation of new analytical methods for the characterization and quantification of the main components present in botanical supplements.

Among the various analytical methods that can be found in the literature, reversed-phase liquid chromatography (RP-LC) is the method of choice for the separation and identification of the green tea species (polyphenols).[1, 12-15] The chromatographic separations are most commonly followed by UV-visible absorbance[1, 13, 16] or mass spectrometry (MS)[1, 3, 6, 17] detection, although

electrochemical[18-20] and fluorescence[21, 22] detection have also been used. However, UV-absorbance, electrochemical and fluorescence detection methods mentioned above are not very analyte-specific. Therefore, the identification of the analyte peaks requires matching their chromatographic retention times with analytical standards. On the other hand, MS has been demonstrated to be very powerful by allowing the identification, confirmation and quantification of multiple species present in a complex biological matrix. More specifically, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been reported for the identification and quantification of the catechin species present in green tea.[3, 23, 24] While, ESI-MS can provide molecular weight information of the polar compounds without extensive fragmentation, and in many cases the addition of MS-MS methods are necessary for the complete species-specific identification. Another important challenging aspect that needs to be considered during ESI-MS experiments is the fact that conventional RP-LC methods are not easily interfaced to the electrospray source because of the differences between solution flow rates and matrix/mobile phase compositions.[16]

In this laboratory, the particle beam mass spectrometry technique has been employed successfully for the detection and determination of an assortment of organic, organometallics, inorganic and biological compounds by the application of a glow discharge ionization source.[25-29] The ease of operation and efficient solvent removal of the PB interface allows the EIMS or GDMS ion sources the ability to perform *comprehensive speciation*, meaning the separation

of elemental and molecular species in a single run. More recently, this unique analytical tool has been complemented with the capability of interchanging ionization sources (electron ionization and glow discharge) to affect the comprehensive speciation of organic and inorganic arsenic species for the analysis of ethanolic bladderwrack and kelp extracts as well as the chemical characterization of green tea extracts.[30, 31] As well, the LC-PB/MS detection method has been validated for the ephedrine alkaloids present in the ephedra-containing NIST dietary supplement standard reference materials by a standard addition method.[32]

Presented here is a RP-LC-PB/EIMS method for the chemical characterization of green tea's main constituents. More specifically, this approach is employed for the quantification of caffeine and catechin species present in three NIST standard reference materials (SRM 3254 *Camellia sinesis* Leaves, SRM 3255 *Camellia sinesis* Extract and SRM 3256 Green Tea-containing Oral Dosage Form) currently under development. Mass spectra for each of the target species were obtained using analytical standards (when available) and their class-specific signature ions identified. Calibration curves for all the species of interest were generated and their respective detection limits determined. The chromatographic separation for green tea extracts was accomplished by RP-LC using a C_{18} column and monitored by UV absorbance at 210 and 254 nm. Once the optimal separation was achieved, the column effluent

was coupled to the PB/EIMS system for the quantification of caffeine and catechins by standard addition and the internal standard approach.

EXPERIMENTAL

Particle Beam Electron Impact Mass Spectrometer System

The PB-MS system used in this study was an Extrel (Pittsburgh, PA, USA) Benchmark Thermabeam LC/MS quadrupole mass spectrometer with an electron ionization source, depicted in Fig. 1.5. The particle beam serves as a "transport-type" interface for LC/MS. This allows for continuous sample introduction into the ionization source (in this case EI) in the form of dry particles by removal of the residual solvent vapors and at the same time maintaining the chromatographic integrity of the separation. The PB-MS system is equipped with a tungsten filament set at an acceleration voltage of 70 eV, the standard voltage for EI, making spectral library comparisons possible.

Data acquisition for the MS was performed under the control of the Extrel Merlin (Pittsburgh, PA) Ionstation system. Total ion chromatograms (TIC) were typically acquired over the mass range of 50-500 Da in a scan time of 1.0 s. The chromatographic (temporal) trace of a particular mass can be isolated from the TIC for background correction and peak integration. The data was then exported to Sigma Plot 8.02 (Systat Software, Richmond, CA), Microsoft (Redmond, WA) Excel, and Power Point for further processing.

The Thermabeam interface (Extrel Corp., Pittsburg, PA, USA), employed the introduction of the liquid flow through a thermoconcentric nebulizer, a desolvation chamber, and a two-stage momentum separator. The aerosol generated by the nebulizer (~86°C tip temperature) passes through the heated desolvation chamber (~130 °C), were the wet droplets begin to dry and the solutes form particles. As the particle/gas mixture passes through a pair of 1 mm differential pumping orifices (one per stage), the low-mass solvent molecules are dispersed and pumped away because they have low momentum, while the heavier analyte-containing particles are able to pass through to the next orifice. Once the particles leave the interface there is little or no solvent vapor remaining. The resulting beam of dry analyte particles then moves into the heated (~275 °C) source block region. The optimization of the operating parameters for the EI source (electron energy and source block temperature) had been performed and described in previous work.[30, 31, 33]

Sample Preparation and Delivery

A 1000 µg mL^{-1} stock solutions of catechin, epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), gallic acid (GA), proxyphylline (Sigma-Aldrich, St. Louis, MO, USA), caffeine (Aldrich Chemical Co. Inc, Milwaukee, WI, USA) and trimethyl-$^{13}C_3$ caffeine (Cambridge Isotope Laboratories Inc., Andover, MA, USA) were prepared by weighing appropriate amounts and diluting in a mixture of 95% water

and 5% 2:1 methanol (MeOH) :acetonitrile (ACN). Working standard solutions were prepared fresh daily to ensure minimal degradation. The green tea SRM's analyzed were supplied by NIST, which are part of the family of SRM's under development. All solutions were stored in light-tight vessels at 4^{o}C and fresh dilutions were prepared as necessary.

The samples were introduced into the PB interface via a Waters (Milford, MA) Model 1525 HPLC binary system equipped with a Rheodyne (Cotati, CA, USA) Model 7125i injector and a 50 µL injection loop. A fixed flow rate of 0.9 mL min^{-1} was used throughout this work. The liquid output passed directly through a Waters Model 2487 dual wavelength absorbance detector (Milford, MA) monitoring at 210 and 254 nm during the development of the chromatographic separation. Liquid chromatography separation of caffeine and the catechin compounds was accomplished using an Alltima C_{18} reversed-phase chromatography column (Alltech Associates Inc., Deerfield, IL USA, 250 mm x 4.6 mm, 5 µm) and guard column (All-Guard Holder with Alltima C_{18} Cartridge, Alltech Associates Inc., Deerfield, IL, 7.5 mm x 4.6 mm, 5 µm) operated at room temperature. The initial composition of LC mobile phase consisted of 95 % water (18.2 MΩ cm^{-1}, NANOpure Diamond, Barnstead International, Dubuque, IA) containing 0.1% TFA (A) and 5% 2:1 MeOH:ACN (B). A linear gradient of 5 to 10% B from 0 to 5 min, followed by a linear gradient of 10 to 35% B from 5 to 50 min was used for separation of the species.

Quantification of caffeine and the catechin species were performed using a standard addition method and the internal standard approach. For the standard addition method stock standard solutions (1.0 mg mL^{-1}) of caffeine, catechin, EC, EGC, EGCG, ECG, and GA were added in the amounts of 0.025 and 0.050 mL to aliquots of the green tea tincture and diluted to 1.0 mL. The green tea aliquots were of 50, 100 and 200 µL and diluted up to 1.0 mL making 5, 10 and 20% solutions. In the case of the internal standard approach, stock standard solutions (1.0 mg mL^{-1}) of caffeine, catechin, EC, EGC, EGCG, ECG, and GA were utilized to prepare a calibration solution with final concentrations of 100 and 150 µg mL^{-1}. The internal standards proxyphylline and trimethyl-$^{13}C_3$ caffeine utilized for the quantification of the catechins and caffeine were added to the calibration solutions to achieve a concentration of 100 and 50 µg mL^{-1}, respectively. NIST SRM 3260 Bitter Orange-Containing Solid Oral Dosage Form was analyzed as a quality control sample for caffeine.

Extraction Procedure

The extraction procedures performed for the preparation of the green tea SRMs were provided by NIST. Approximately 0.2 grams of SRM 3255 (*Camellia sinesis* Extract) material were accurately weighted, added to 15 mL polypropylene tubes, combined with the internal standard solutions containing proxyphylline and trimethyl-$^{13}C_3$ caffeine and dissolved in 2 mL of 30% MeOH solution by shaking for one minute. After extraction, the sample was filtered

using a 0.45 µm PTFE filter (Alltech Associates Inc Deerfield, IL, USA) for final analysis.

In the case of SRMs 3254 (*Camellia sinesis* Leaves) and 3256 (Green Tea-containing Oral Dosage Form), approximately 0.3 grams of material and 0.1 grams of diatomaceous earth (Fisher Science Education, Rochester, NY) for sample dispersal were accurately weighted, combined with the internal standard solutions and added to 50 mL polypropylene tubes. SRM 3256 was extracted in 6 mL of 30% MeOH using a rotary inversion extraction system, a laboratory built apparatus, at ~60 rpm over a period of 3 hours. After extraction, the sample was centrifuged at 4000 rpm for 30 minutes. The supernatant was decanted and stored at 4℃. Subsequently, 3 mL of 30% MeOH were added and the material was re-extracted in the same manner. The supernatant volumes were added together and filtered (0.45 µm PTFE filter) for final analysis. In a similar manner, SRM 3254 was extracted in 4 mL of 30% MeOH and 3mL of 0.1% EDTA by the rotary inversion extraction system, at ~60 rpm over a period of 3 hours. After extraction, the sample was centrifuged at 4000 rpm for 30 minutes. The supernatant was decanted and stored at 4℃. Subsequently, 1 mL of 30% MeOH and 1 mL of 0.1% EDTA are added and the material was re-extracted in the same manner. The supernatant volumes were added together and filtered (0.45 µm PTFE filter) for final analysis.

Determination of Moisture

The moisture content of SRM 3254, 3255 and 3256 was determined by drying in an oven at ~ 95 ˚C for 24 hours. Conversion factors were determined based on dry-mass/received mass and used to report the quantification values on a dry-mass basis.

RESULTS AND DISCUSSION

Electron Ionization Mass Spectra

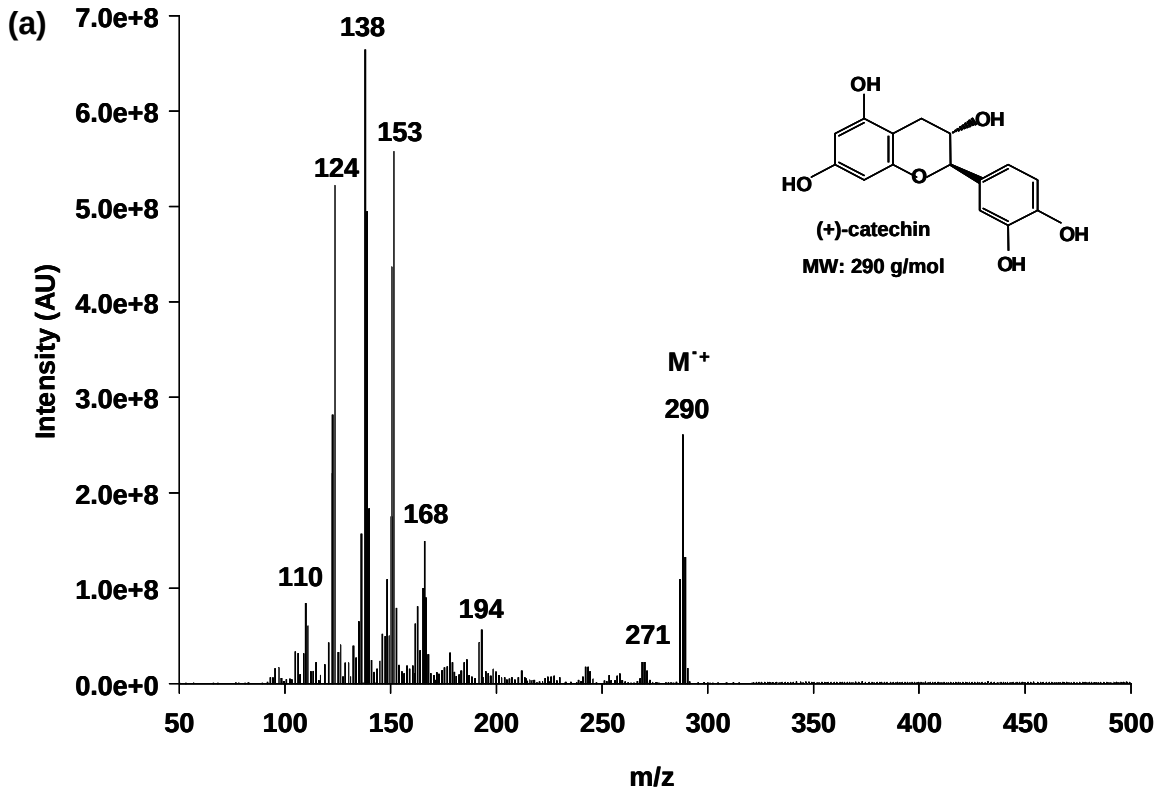
The acquisition of simple and easily interpreted EI spectra via the PB interface allows spectral library comparison (when available) and demonstrates the efficiency of the interface to remove solvent residues/vapors while maintaining chromatographic integrity. Figures A.1a-h depict the individual mass spectra obtained from 50 μL injections of 100 μg mL^{-1} solutions of catechin, EGC, gallic acid, caffeine, ECG, EGCG, proxyphylline and trimethyl-$^{13}C_3$ caffeine with their respective chemical structures. The spectra show the molecular ion ($M^{\cdot +}$) for each of the species with the exception of ECG and EGCG. The catechin compound spectra (catechin, EGC, ECG, and EGCG) are very similar with easy to interpret fragmentation patterns, as would be expected, because the family of catechin species have specific signature fragment ions. The absence of the molecular ion for ECG and EGCG suggests that these compounds are not stable under the operating parameters.

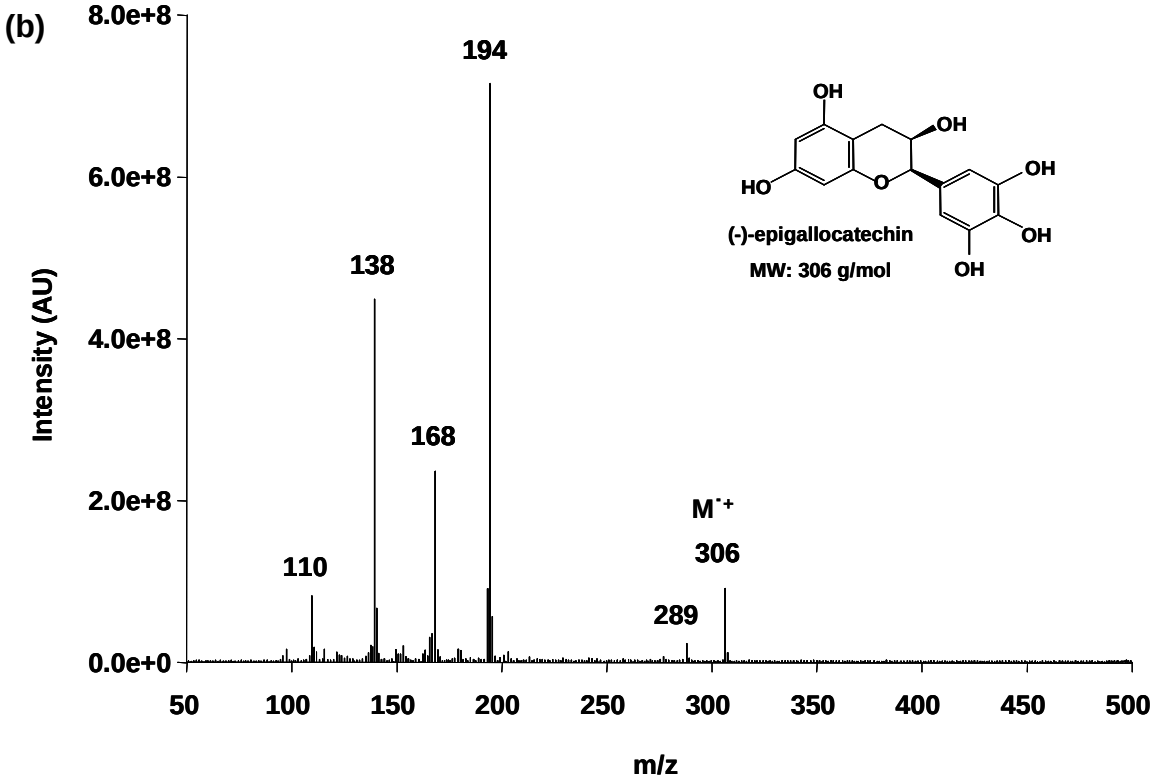
The EI spectrum of catechin (Fig. A.1a) shows the molecular ion at m/z = 290 Da, with a base peak at m/z = 139 Da and other prominent fragments seen at m/z = 168, 153 and 124 Da. The fragment ion at m/z = 124 Da represents the cleavage of the bi-phenol ring from the catechin molecular ion. The mass spectra obtained for EC and catechin are indistinguishable, because their only structural difference is the chirality of the stereocenter (hence the spectrum for EC is not shown here).

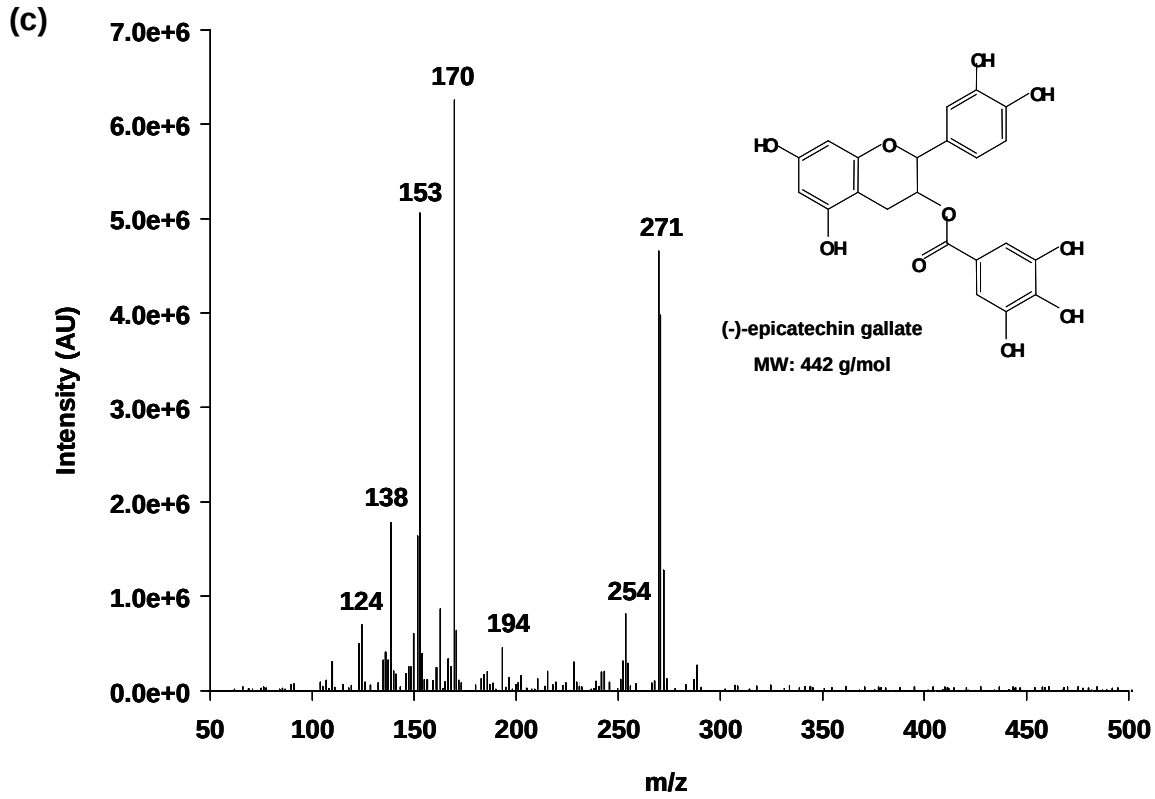
The mass spectra of EGC (Fig. A.1b) presents the molecular ion at m/z = 306 Da with a base peak at m/z = 194 Da. The difference between catechin and EGC is simply an additional hydroxyl group on the polyphenol ring. The transition observed from the molecular ion to the fragment peak at 289 Da represents the loss of a water molecule (M – 18 Da), followed by the fragmentation of the fused ring system as the major fragments appear at m/z = 168 and 139 Da. The mass spectra of ECG and EGCG (Figures A.1c and d) have consistent fragmentation patterns between each other, with base peak at m/z = 170 and 194 Da, respectively.

Besides the catechins, caffeine is a xanthine alkaloid and an important component in green tea extracts because of its stimulant properties. As seen in Fig. A.1e, the mass spectrum of caffeine shows a base peak corresponding to the molecular ion at m/z = 194 Da with characteristic fragment peaks at m/z = 165, 138, and 109 Da. Figure A.1f shows the spectra for gallic acid with a molecular ion at m/z = 170 Da and fragment peaks at m/z = 153 and 124 Da

corresponding to the loss of water and the carbonyl group, respectively. Lastly, Fig. A.1g and A.1h show the spectra for the two internal standards (proxyphylline and trimethyl-$^{13}C_3$ caffeine) with molecular ion at m/z = 238 and 197 Da, respectively. The spectra obtained for caffeine, gallic acid, catechin and epicatechin are similar to the NIST library spectra. In the case of the other catechin species, the NIST library spectra are not available due to their limited volatility and thermal stability. There is also the difference between the spectra presented here with those of ESI-MS and APCI-MS techniques, where the molecular ion is obtained almost exclusively and collisional dissociation (MS-MS) is required for the acquisition of structural information.[23, 24, 31, 34]




(b)
8.0e+8
6.0e+8
4.0e+8
2.0e+8
0.0e+0
Intensity (AU)
194
138
168
110
289
M·+
306
50
100
150
200
250
300
350
400
450
500
m/z
OH
OH
HO
O
OH
OH
OH
(-)-epigallocatechin
MW: 306 g/mol


(c)
7.0e+6
6.0e+6
5.0e+6
4.0e+6
3.0e+6
2.0e+6
1.0e+6
0.0e+0
Intensity (AU)
170
153
271
138
124
194
254
50
100
150
200
250
300
350
400
450
500
m/z
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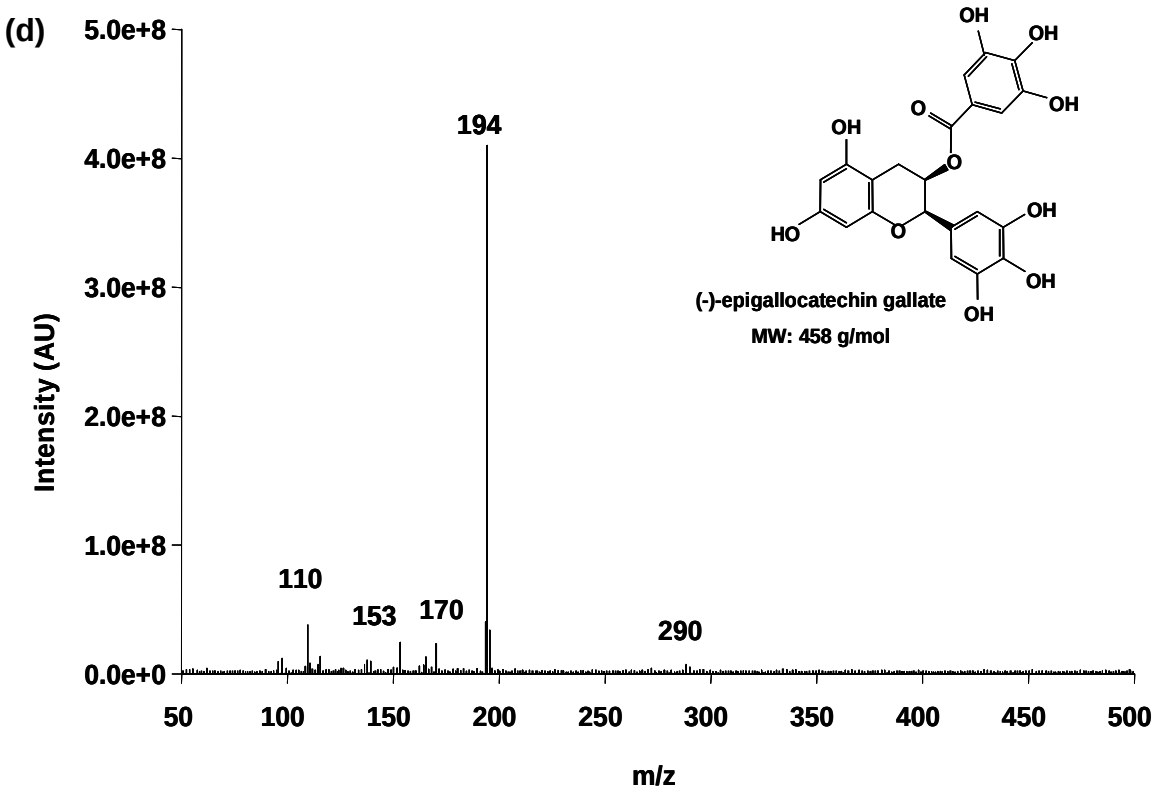
MW: 442 g/mol


(d)
5.0e+8
4.0e+8
3.0e+8
2.0e+8
1.0e+8
0.0e+0
Intensity (AU)
194
110
153
170
290
50
100
150
200
250
300
350
400
450
500
m/z
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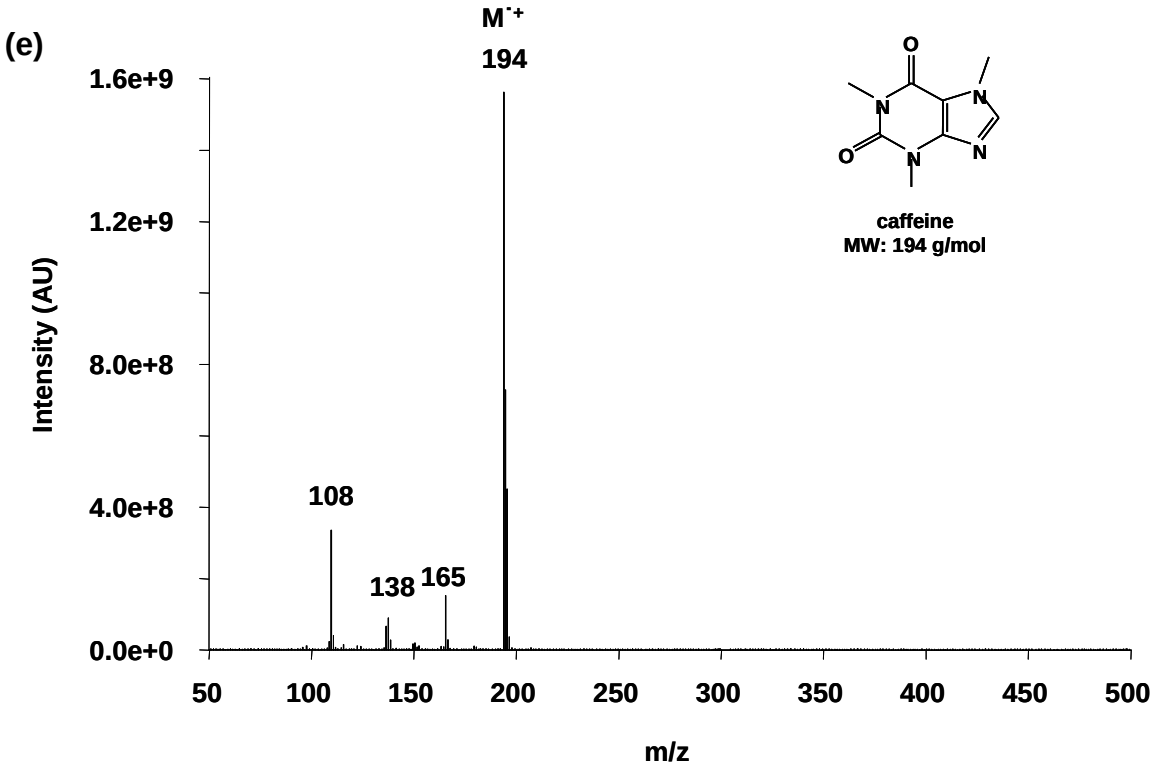
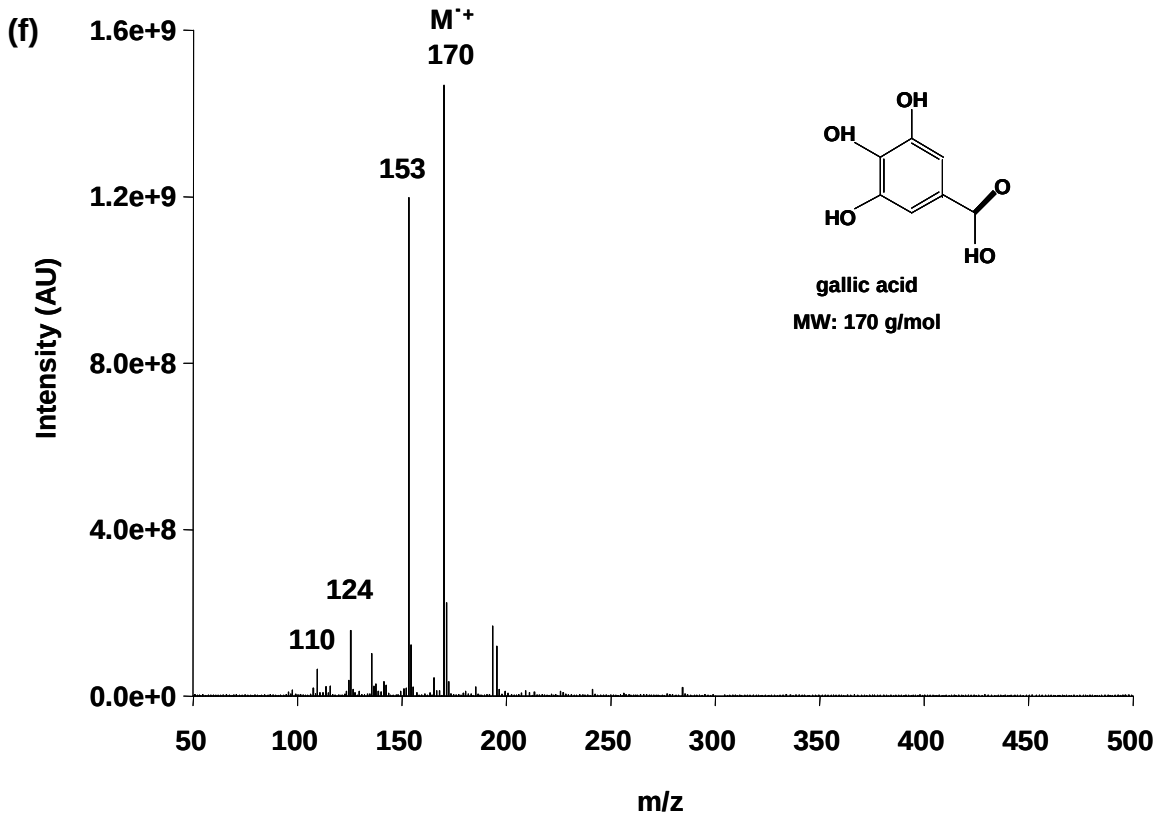
OH
OH
O
OH
O
HO
O
OH
OH
OH
(-)-epigallocatechin gallate
MW: 458 g/mol


(e)
M·+
194
1.6e+9
1.2e+9
8.0e+8
4.0e+8
0.0e+0
Intensity (AU)
108
138
165
50
100
150
200
250
300
350
400
450
500
m/z
O
N
N
O
N
N
caffeine
MW: 194 g/mol


(f)
1.6e+9
1.2e+9
8.0e+8
4.0e+8
0.0e+0
Intensity (AU)
M·+
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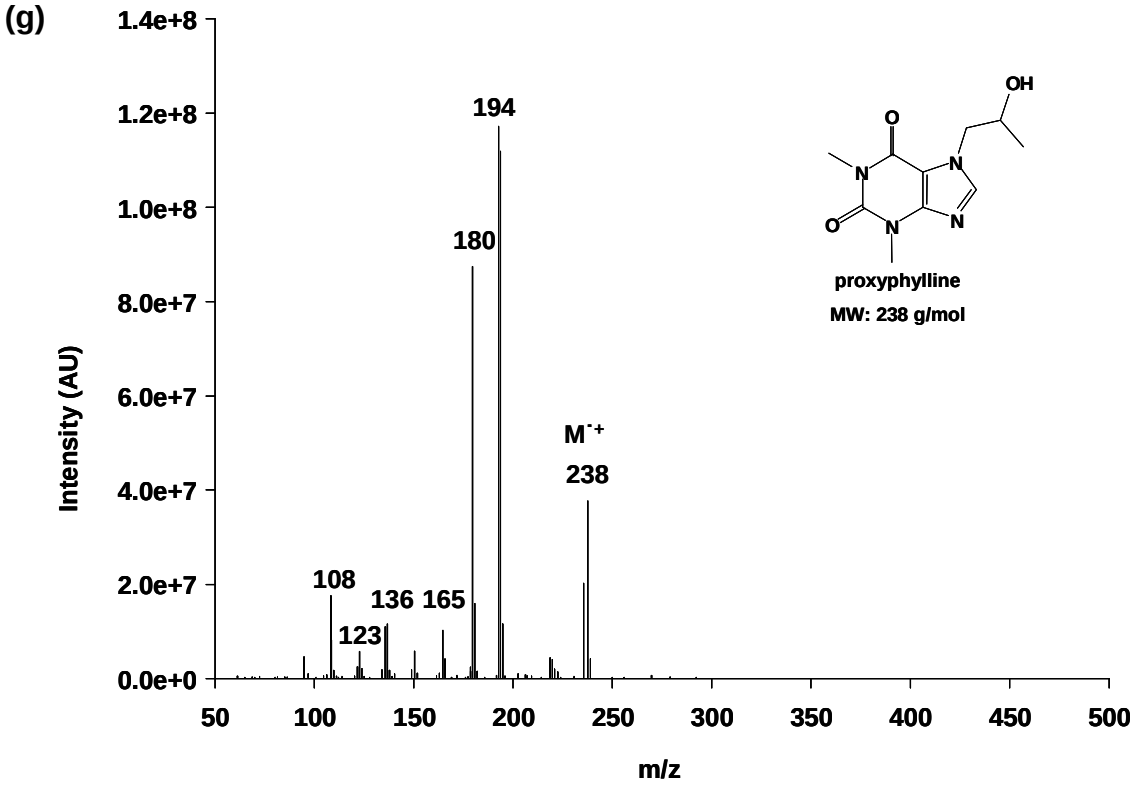
153
124
110
OH
OH
HO
O
HO
gallic acid
MW: 170 g/mol
50
100
150
200
250
300
350
400
450
500
m/z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1.2e+8
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6.0e+7
4.0e+7
2.0e+7
0.0e+0
Intensity (AU)
194
180
M·+
238
108
136
165
123
OH
O
N
N
O
N
N
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100
150
200
250
300
350
400
450
500
m/z

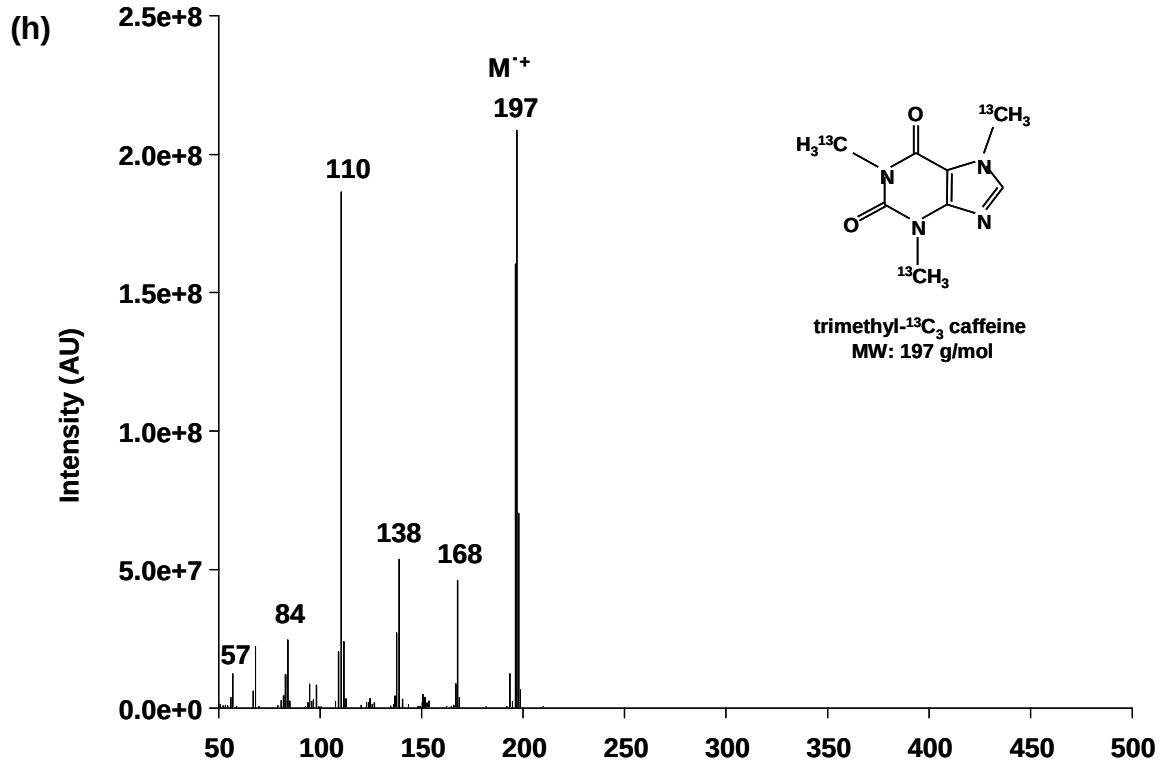

Figure A.1. LC-PB/EI mass spectra of a) catechin, b) EGC, c) ECG, d) EGCG, e) caffeine and f) gallic acid, g) proxyphylline and f) trimethyl-$^{13}C_3$ caffeine. Electron energy = 70 eV, block temperature = 275 ℃, concentration = 100 µg mL $^{-1}$, 50 µL injection loop.

Figures of Merit

Table A.1 shows the analytical response characteristics for caffeine and the catechin species obtained by the LC-PB/EIMS system. Response curves using the TIC and selected ion monitoring modes were generated through triplicate injections across the concentration range of 0.1 to 100 µg mL^{-1} (including the analytical blank). More specifically, for the generation of the selected ion monitoring mode calibration curves the molecular ion and base peak responses of each target species were considered. Each of the species'

response functions show good linearity with acceptable correlation coefficients (R^2 values). Overall results show that the LC-PB/EIMS limits of detection ($3\sigma_{blank}/m$) fall in the nanogram level for all the species. Such values are consistent with and/or higher in comparison to the LODs reported by researchers for UV-absorbance (0.2 to 4 ng absolute) and ESI-MS (0.4 to 0.7 ng absolute) detection. Nevertheless, the magnitude of the LODs obtained here are not relevant in terms of profiling botanical extracts where concentrations of the species are in the µg mL^{-1} to percent levels, but are relevant in metabolic studies.

Table A.1. Analytical response characteristics for green tea species with LC-PB/EIMS.

Analyte	Response Function	Accuracy (R^2)	Detection Limit (ng mL^{-1})	Absolute Mass (ng)
TIC mode (m/z = 50-500 Da)				
Catechin	y = 4E+08x – 1E+09	0.9925	1.9	0.094
Epicatechin	y = 4E+09x – 2E+10	0.9794	0.85	0.043
EGC	y = 8E+06x – 5E+07	0.9623	8.7	0.87
Caffeine	y = 1E+09x – 5E+09	0.9901	4.3	0.23
EGCG	y = 1E+08x – 3E+08	0.9821	20	1.0
Gallic Acid	y = 3E+09x – 1E+10	0.9860	9.5	0.47
ECG	y = 5E+06x – 6E+07	0.9788	53	5.3
M^+ ion				
Catechin (290 Da)	y = 2E+07x – 5E+07	0.9823	31	15
Epicatechin (290 Da)	y = 8E+07x – 6E+08	0.9470	43	2.1
EGC (306 Da)	y = 5E+05x – 1E+06	0.9530	74	7.4
Caffeine (194 Da)	y = 5E+08x – 2E+09	0.9940	3.4	0.17
Gallic Acid (170 Da)	y = 8E+08x – 2E+09	0.9913	5.8	0.29
Base peak				
Catechin (138 Da)	y = 1E+08x – 4E+08	0.9940	7.5	0.38
Epicatechin (138 Da)	y = 8E+08x – 5E+09	0.9559	4.3	0.21
EGC (194 Da)	y = 1E+07x – 3E+07	0.9727	138	14
EGCG (194 Da)	y = 1E+07x – 2E+07	0.9911	218	11
ECG (170 Da)	y = 1E+06x – 1E+07	0.9856	263	26

Reversed-Phase Chromatographic Separation of Green Tea Species

Three reversed-phase liquid chromatography methods were evaluated to determine the best separation conditions for the target species in the green tea materials. During the first set of chromatographic separations, a green tea synthetic mixture containing 50 µg mL^{-1} of each of the green tea species was separated on the C_{18} column using the method previously published by this laboratory.[31] More specifically, a linear gradient method varying from 75:25 (0.1% TFA in water) to 55:45 over 12 minutes was performed and the progress of the separation monitored by UV-Vis absorbance at 210 and 254 nm. The resultant chromatographic separation (Fig. A.2a) demonstrates that the previously published gradient method was not able to fully-baseline resolve all of the targeted species. Gallic acid and EGC, as well as caffeine and epicatechin, co-elute at t_r = 3.75 min and ~ 5.0 min, respectively. The second set of chromatographic conditions attempted were provided by NIST, consisting of a linear gradient varying from 97:3 (0.1% phosphoric acid in water: 2:1 MeOH:ACN containing 0.1% phosphoric acid) to 68:32 over 75 minutes at 1.0 mL min^{-1}. Figure A.2b shows the chromatographic separation of a 50 µg mL^{-1} synthetic green tea mixture using the method provided by NIST. As in the previous method, the green tea species do not completely separate, with EGC and catechin (t_r = 46.0 min), as well as EGCG and epicatechin (t_r = 60.0 min) co-eluting during the analysis. Another drawback of this chromatographic method is the long run time of the gradient.

The third and optimal set of chromatographic conditions was based on modifications made to the previous method (provided by NIST). More specifically, the ion pairing agent was changed to TFA and the flow rate to 0.9 mL min^{-1}. Previous work done in this laboratory had demonstrated the use of TFA as a viable ion pairing agent for chromatographic separation. Hence, the optimized chromatographic separation conditions for the analysis of green tea SRMs include a linear gradient varying from 95:5 (0.1% TFA in water: 2:1 MeOH:ACN containing 0.1% TFA) to 90:10 over 5 min, followed by a linear gradient of 90:10 (0.1% TFA in water: 2:1 MeOH:ACN containing 0.1% TFA) to 65:35 from 5 to 50 min. Figure A.3 shows an overlay of the UV-Vis absorbance (254 nm) and MS (m/z = 170 and 194 Da) chromatographic responses of a 100 µg mL^{-1} synthetic mixture of the green tea species. The MS trace is shown at m/z = 194 and 170 Da due to the fact that it is a characteristic fragment ion of the catechin species (consistent with all of the spectra) and the molecular ion for gallic acid. A proposed structure corresponding to fragment ion m/z = 194 Da has been published previously by this laboratory.[31] In comparison to the two chromatographic method previously attempted, the green tea species are baseline resolved and the analysis run time is reduced in comparison to the NIST method.

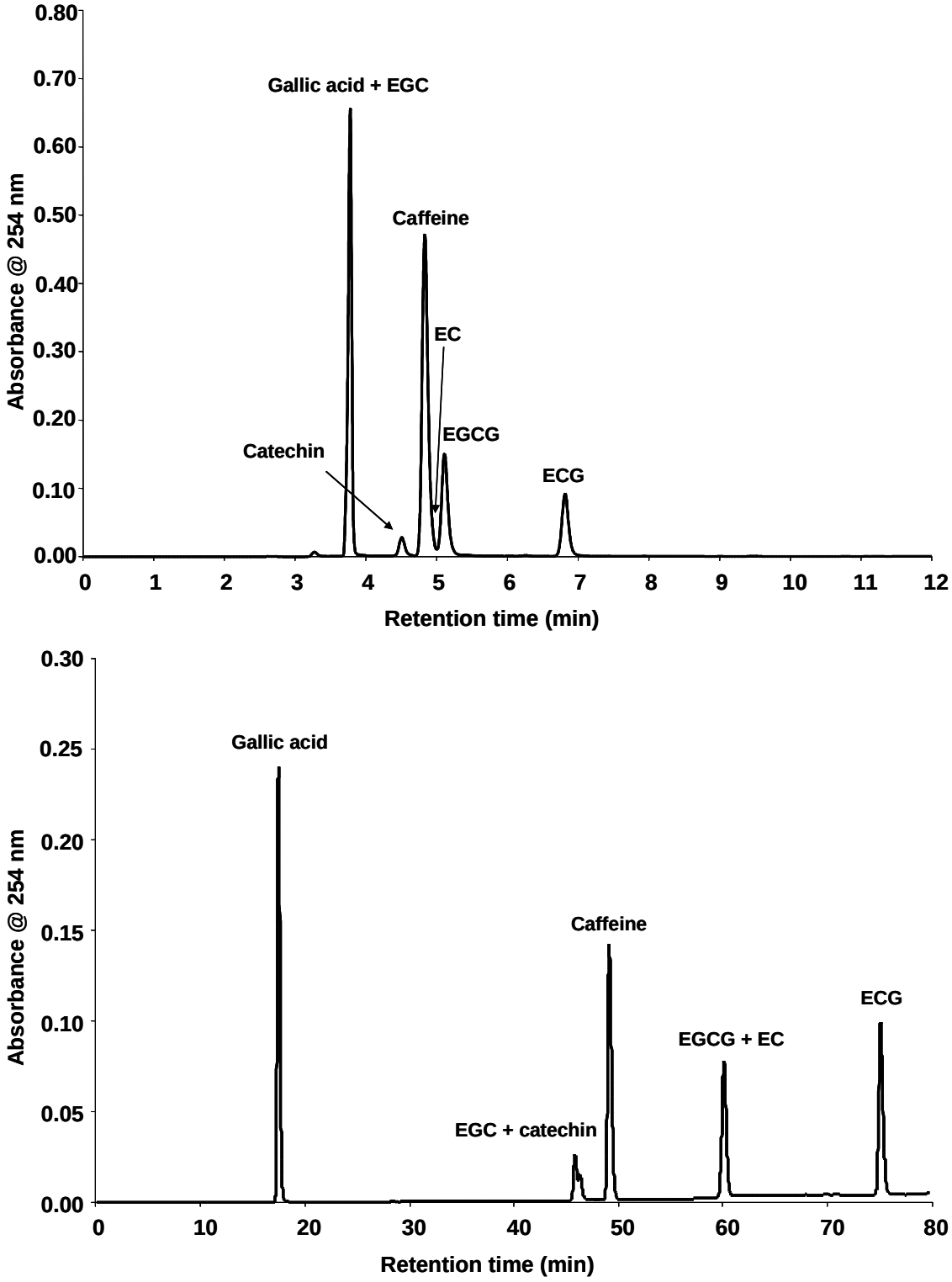

Figure A.2. Reversed- phase chromatographic separation of 50 µg mL^{-1} mixture of green tea standards using a) published method[31] and b) NIST method. Injection loop = 50 µL.

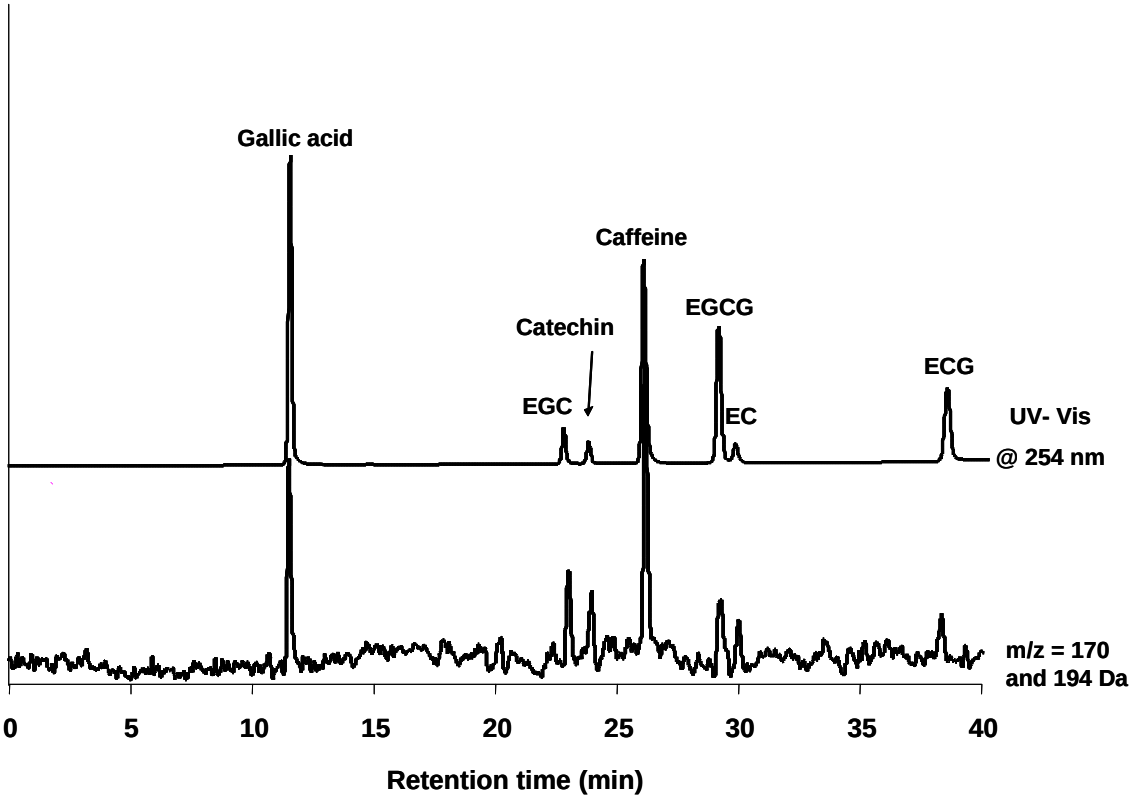

Figure A.3. Reversed- phase chromatographic separation of 100 µg mL^{-1} mixture of green tea standards with UV-absorbance at 254 nm *(top)* and selected ion mode at 170 and 194 Da *(bottom).* Electron energy = 70 eV, block temperature = 275 ℃, 50 µL injection loop.

Quantification Analysis

Once suitable chromatographic conditions have been achieved, the green tea reference materials (six different boxes of the three SRMs) will be analyzed and the targeted species quantified by standard addition and the internal standard approach. Figure A.4 shows an overlay of the LC-PB/EIMS chromatogram of a 5% SRM 3255 solution in TIC mode and extracted traces of selected fragment ions m/z = 194, 290 and 306 Da. As shown in Fig. A.1a-f, the m/z = 138 and 194 Da are common fragment peaks in all the species tested. As

well, m/z = 194 Da also corresponds to the molecular ion of caffeine. All the target species are labeled on the chromatogram as well as, gallocatechin (t_r = 19.0 min) which is also part of the catechin family. The ability to extracted mass spectral information for each of the eluting peaks allows the identification of gallocatechin, which has a molecular ion at m/z = 306 Da and similar fragment ions to the catechin species.

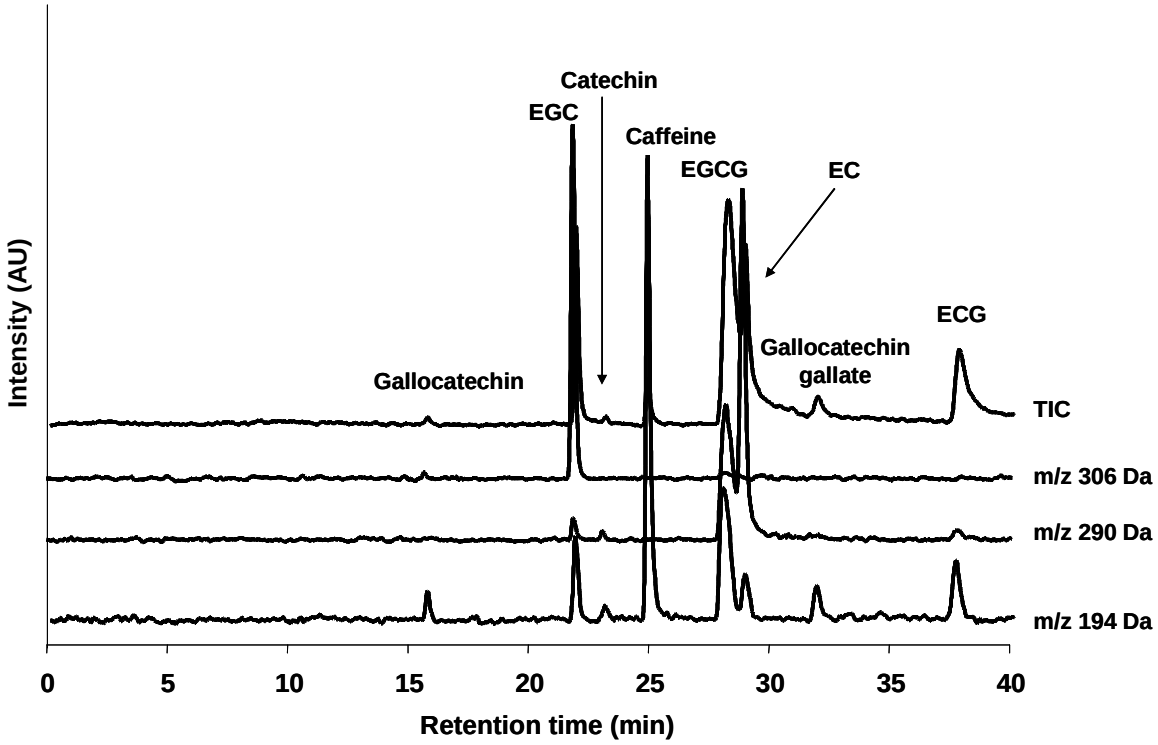

Figure A.4. LC-PB chromatographic separation of 5% SRM 3255 in TIC mode and three traces of fragment ions. Electron energy = 70 eV, block temperature = 275 ℃, 50 µL injection loop.

Figure A.5 shows an overlay of the chromatographic separation of a 5% (SRM 3255) and 20% (SRM 3254 and 3256) solutions of green tea reference

material at m/z = 194 Da. The mass spectra extracted from the eluted species provided consistent fragmentation patterns to the spectra acquired from the analytical standards, therefore allowing the identification of the species of interest. As in the case of SRM 3255, gallocatechin can also be observed during the analysis of SRM 3256. A standard addition method and an internal standard approach will be perform for the quantification of gallic acid, EGC, EC, caffeine, EGCG, catechin and ECG in the three green tea reference materials. The SRM 3260 Bitter Orange-Containing Solid Oral Dosage Form is use as a quality control sample. The control sample was only tested for caffeine due to the fact that is one of the few available dietary supplement reference materials already validated by NIST. Recovery values of 22% and 86% were obtained for caffeine in SRM 3260 by the standard addition method and the internal standard approach, respectively. For the internal standard approach trimethyl-$^{13}C_3$ caffeine will be used as the internal standard. The reproducibility response between the different boxes can be seen in Fig. A.6 with the overlay of three LC-PB/EIMS chromatograms corresponding to 5% solutions of SRM 3255.

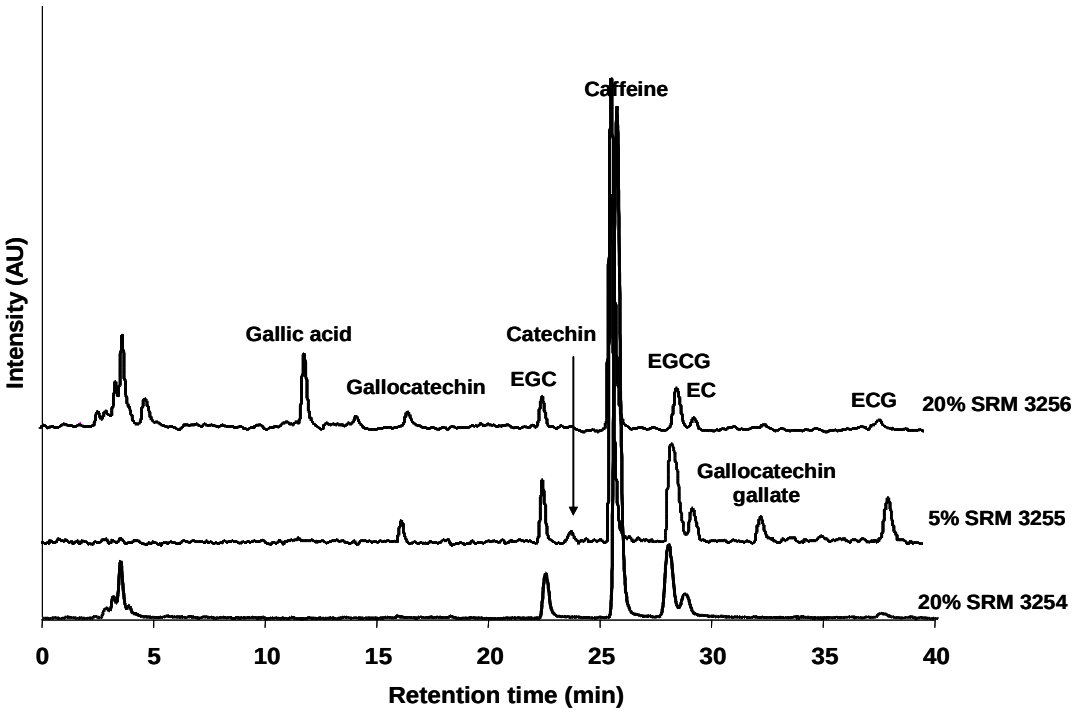

Figure A.5. Overlay of LC-PB chromatographic separation of three green tea standard reference materials at m/z = 194 Da. Electron energy = 70 eV, block temperature = 275 ℃, 50 µL injection loop.

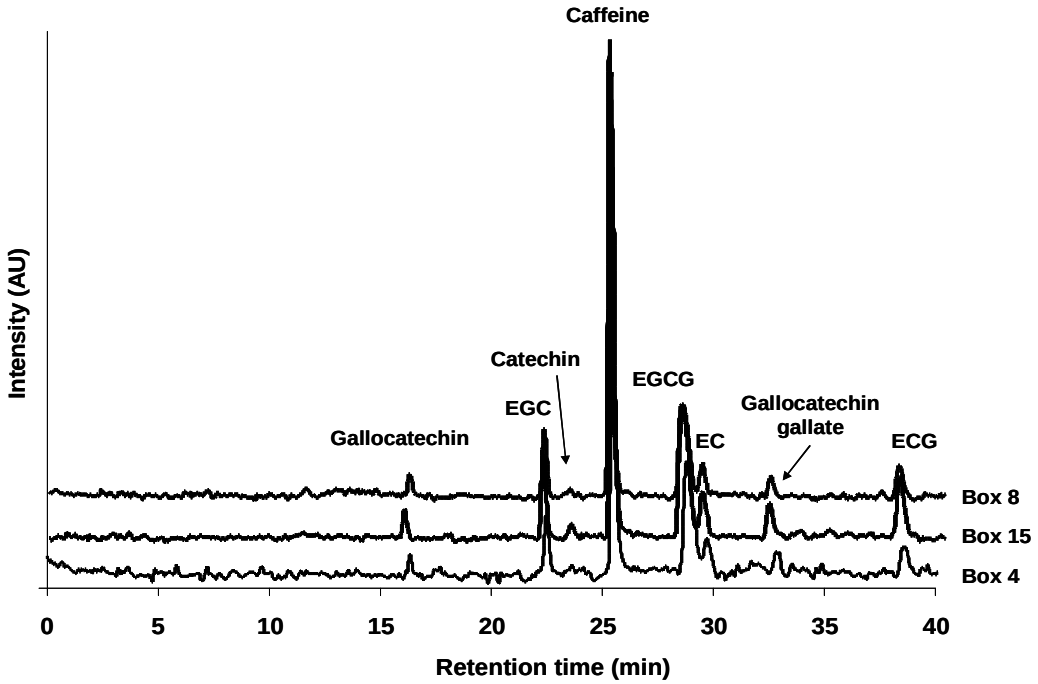

Figure A.6. LC-PB chromatographic separation overlay of three different boxes of 5% SRM 3255 at m/z = 194 Da. Electron energy = 70 eV, block temperature = 275 ℃, 50 µL injection loop.

CONCLUSIONS

The data presented here demonstrate the capabilities of the LC-PB/EIMS as an analytical tool for the certification of green tea reference materials. The mass spectra obtained for caffeine and the catechin species demonstrates clear and easy to interpret fragmentation patterns. Calibration curves were generated and the analytical figures of merit acquired, illustrating good linear responses and LODs in the nanogram level. A HPLC chromatographic method was developed for the separation of the target species in the green tea reference materials. Additional catechin species (gallocatechin and gallocatechin gallate) present in the green tea materials were identified based on their mass spectra and retention characteristics. Finally, the quantification of the target species is currently underway by a standard addition method and an internal standard approach, for six boxes of the three different green tea SRMs.

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APPENDIX B

METAL ANALYSIS OF BOTANICAL PRODUCTS IN VARIOUS MATRICES USING A SINGLE MICROWAVE DIGESTION AND INDUCTIVELY COUPLED PLASMA OPTICAL EMISSION SPECTROMETRY (ICP-OES) METHOD

INTRODUCTION

The marketing, sale, and consumption of botanical products (aka, dietary supplements or nutraceuticals) has been on the upsurge over the last 20 years because of their perceived health benefits towards heart disease, cancer and other conditions. In 2007, the US nutritional product market was responsible for $94 billion in consumer sales, an approximately 11% increase from 2006.[1] In the past, the overall assurance of product safety and the subsequent health effects claimed on the labels required no substantiation. With the increase in sales, the safety and efficacy of these products has become a very important issue. For decades there have been efforts toward the establishment of rules and regulations on the manufacturing and testing of the botanical products. Because of the variety of products, compositions, and manufacturer processes available, the creation of these regulations is very arduous and time-consuming process. In addition, the wide diversity of product sources and analytical capacities makes the development of unified standards quite difficult.

There are two distinct aspects to the regulation of botanical product commerce: truth in labeling and quality/safety assurance. In 1994, the Dietary

Supplement Health and Education Act (DSHEA) introduced new regulations for dietary supplements.[2] This act defined the specific criteria that dietary supplements should meet and began to address several quality/safety concerns of supplements in the market place. In 2003 the Food and Drug Administration (FDA) proposed regulations that would make dietary supplement manufacturing, packaging, and storage be in compliance with current good manufacturing practices (cGMPs). Overall, the cGMPs address the safety concerns with regards to the claims made on the products label.[2-4] 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 which establishes "Safe Harbor Levels" for many substances and compounds that are known or suspected to cause cancer or adverse reproductive effects.[5, 6] Although this California law does not target botanical products, it provides specific guidelines for the daily maximum exposure to toxic species (e.g. heavy metals), some of which can potentially be found in botanical extracts. Specifically, the maximum allowable dose levels in Proposition 65 for arsenic, cadmium, lead and mercury are 0.1, 4.1, 0.5 and 0.3 μg day^{-1}, respectively.

Botanical products can be found in a wide variety of forms/matrices; including ethanolic tinctures, soft gels, tea bags, powders, capsules and tablets. Ideally, the monitoring of the elemental components in different types of sample matrices could be carried out by a single sample preparation and analytical determination method. However, due to the nature of the various matrices, the

development of such a methodology is very challenging. Several laboratories have reported digestion and analysis procedures for dietary and botanical supplements, as well as for food and other biological samples.[4, 7-9] The sample preparation and detection techniques used for these matrices generally consist of either wet and dry ashing or microwave digestion with atomic absorption spectrometry (AAS), inductively coupled plasma optical emission spectrometry (ICP-OES), or inductively coupled plasma mass spectrometry (ICP-MS). To this point, the concept of broad-ranging matrix capabilities has not been demonstrated.

The present work describes the development and validation of a single botanical product preparation and analysis method using a microwave digestion procedure that is applied to three diverse matrices (powdered dried raw material, liquid-phyto caps, and ethanol-based tinctures) analyzed by ICP-OES for As, Cd, Hg, Pb, Fe, Na, Ca, P and Zn. Once the optimization of the digestion parameters was achieved, NIST standard reference material (SRM) 3241 *Ephedra sinica* Stapf Native Extract, SRM 3243 Ephedra-Containing Solid Oral Dosage Form and SRM 3246 *Ginkgo biloba* (leaves) were employed for the validation of this method by generating calibration curves with aqueous standard solutions and by the standard addition method. Special emphasis during the course of this study is given to the heavy metal content in the commercial botanical products. It is believed that this straightforward, unified approach provides a cost-effective

alternative to the use of multiple, matrix-specific approaches to dietary supplement analysis.

EXPERIMENTAL

Instrumentation

Digestion of the samples was performed with a MARS Xpress microwave digestion system (CEM Corporation, Matthews, NC, USA). The system was equipped with a 40-place sample rotor (turret) capable of holding 75 mL PFA-Teflon sample digestion vessels operable at temperatures of up to 260˚C and 500 psi. Temperature control was achieved through feedback via an infrared sensor. Temperatures ranging from 50˚C to 80˚C in combination with hold times of 10, 15 and 20 minutes were evaluated for the pre-digestion step with the power set at 300 W. In the case of the digestion step (power at 1200 W), temperatures ranging from 150˚C to 210˚C with ramp and hold time variations of 10, 15, and 20 minutes were evaluated. Caution must be taken to allow pressurized vessels to come to room temperature before opening to atmosphere. Table B.1 presents the optimal microwave digestion system operating conditions employed in the quantitative evaluation of the method.

The quantitative elemental analysis of the botanical extracts was performed by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Jobin-Yvon Ultima 2 (Longjumeau, France) equipped with a radial-view plasma, a Meinhard concentric glass nebulizer and a cyclonic spray

chamber. The Ultima 2 spectrometer consists of a 1.0 m Czerny-Turner monochromator equipped with 2400 grooves mm^{-1} holographic grating, controlled by JY Analyst v5.2 data acquisition software. In order to obtain the optimal ICP-OES performance, the experimental conditions (i.e. power, sample introduction rate, nebulizer gas flow rates and the emission wavelengths) need to be considered. For the sake of simplicity, each of the parameters, with the exception of the emission wavelength, was set to the manufacturer's default values and held constant throughout the course of the entire study. For the selection of the best emission wavelength, all or some of the transitions were selected from the software database and evaluated with a 1.0 μg mL^{-1} multi-element standard solution containing all of the target and elements present in the botanical extracts. The wavelength responses were evaluated based on their sensitivity, absence of spectral interferences, and detection limits. Table B.2 shows the ICP-OES operation parameters and wavelengths used here.

Table B.1. Optimized microwave digestion system.

Stage	Power (W)	Ramp Time (min.)	Temperature (°C)	Hold Time (min.)	Cool Down Time (min.)
Pre-digestion	300	0	80	15	15
Digestion	1200	10	180	15	15

Table B.2. ICP-OES operation conditions.

Parameters	Condition
Power (W)	1000
Ar gas flow rate (L/min)	12.0
Nebulizer (L/min)	0.02 at 1.0 bar
Sheat gas flow rate (L/min)	0.20
Peristaltic pump speed (rpm)	20.0
Replicates	5
Element	**Wavelength (nm)**
As	193.695
Cd	214.438
Pb	220.353
Hg	194.950
Ca	211.276
Zn	213.856
Na	588.995
P	213.618
Fe	259.940

Materials

All samples and standards were digested in *trace metal* grade nitric acid (HNO_3) (Fisher Scientific, Fair Lawn, NJ, USA) and diluted in MilliQ-water (18.2 MΩ cm^{-1}, NANOpure Diamond, Barnstead International, Dubuque, IA). The samples were stored in 60 mL amber Nalgene bottles (Fisher Scientific, Fair Lawn, NJ, USA) prior to analysis. Single and multielement solutions (certified reference materials) used in the preparation of standards were obtained from High Purity Standards, Charleston, SC, USA.

NIST standard reference material (SRM) 3241 *Ephedra sinica* Stapf Native (hot water) Extract, SRM 3243 Ephedra-Containing Solid Oral Dosage Form and SRM 3246 *Ginkgo biloba* (Leaves) (all in powdered form) were used for the validation of the method. Botanical extracts in the form of ethanolic

tinctures (single herbs or blends) consisting of 25 to 75 percent ethanol, liquid phyto-cap samples consisting of 50 to 60 percent glycerin, and powdered raw material used for this study were provided by Gaia Herbs (Brevard, NC).

Sample Preparation

Approximately one gram of each botanical extract (ethanolic tinctures and liquid phyto-cap samples) was accurately weighted and placed in a 75 mL Teflon microwave digestion vessels. One mL of concentrated HNO_3 was carefully added to the vessel to prevent an explosive reaction. Once the initial reaction had come to completion, an additional 4 mL of HNO_3 was added to the vessel. (In the case of the glycerin-based samples, the entire 5 mL of HNO_3 was added in one step.) After the reaction between the HNO_3 and the ethanolic extract was completed, the vessels were placed in the microwave system with the caps un-torqued (not fully sealed) for the pre-digestion step. Once cool, the vessel caps were tightened and the samples were placed back in the microwave system for the final digestion step. After the conclusion of the digestion step, the vessels were allowed to cool to room temperature, vented and the samples transferred to 50 mL volumetric flasks and diluted to volume with MilliQ-H_2O. In the case of the powdered raw material, use of 1 gram of sample resulted in an undigested residue (i.e. particulate present in solution). Therefore, various amounts of the powdered raw material were investigated, with a mass of ~0.85 g resulting in complete digestion of the various raw materials.

A 1.0 µg mL^{-1} stock solution of the heavy metals (As, Cd, Pb, and Hg) was routinely prepared in MilliQ-H_2O from aqueous multielement standards of 20 µg mL^{-1} and further used to prepare the aqueous calibration standards on a daily basis. For the other elements (Fe, Na, Ca, P and Zn) a 1000 µg mL^{-1} multielement standard was used for the preparation of the standard solutions. The calibration standards were prepared to contain the same acidity (10% nitric acid) as the digested samples. For the standard addition method, a 10 µg mL^{-1} stock solution including As, Cd, Pb and Hg was prepared and amounts of 0.050, 0.100 and 0.200 mL were added to 10 mL of the digested sample. In the case of Fe, Na, Ca, P and Zn amounts of 0.200, 0.400 and 0.600 mL from the 1000 µg mL^{-1} multielement standard were added to 10 mL of the digested sample.

RESULTS AND DISCUSSION

Development of Digestion Procedure

In order to obtain correct elemental quantification, it is crucial to ensure that the prepared samples are in a suitable matrix that can be subsequently analyzed by the instrument of choice (ICP-OES in this case). To be the most practical in implementation, it was desired to develop a procedure that can be applied to multiple matrices (i.e. ethanolic tinctures, raw material, tablets and/or powder forms). The ultimate developed procedure should be simple, efficient, and easy to perform on a regular basis while providing high yields and reproducibility. Initially, open vessel hotplate methods where evaluated, wherein

HNO_3 was added to the ethanolic samples for digestion and heated in open volumetric flasks.[10, 11] The reaction of HNO_3 with ethanol fully digested the samples, but it should be noted that the reaction is very violent, producing nitrogen dioxide gases. While this procedure was successful for the digestion of the ethanolic tinctures, there are several disadvantages, including possible analyte (vapor) loss from the open vessels and the time-consuming (3-4 hours) nature of the reaction if done under mild conditions. Because the hotplate procedure was moderately effective for the ethanolic tinctures, the liquid phyto-cap samples were digested in the same manner, but with no success. The glycerin-based sample digestions were incomplete with undigested and oily residue material remaining. One limitation may be due to the fact that the glycerin-based samples are more concentrated with respect to botanical material than the ethanolic tinctures. In addition, each sample has different degrees of viscosity because each extract contains a different percentage of glycerin. Various nitric acid digestion procedures found in the literature for nutraceutical products[9] and mixed-acid digestion procedures of plant materials[12] were applied for the hot plate digestion of the glycerin-based samples. It was hoped the procedures from the literature would be applicable to the different sample-types (i.e. ethanolic tinctures and liquid phyto-cap samples), but they were attempted with no success. Hydrochloric acid and sulfuric acid were also unsuccessfully explored for the use in sample digestion,[4, 13-15] therefore the application of microwave digestion was considered.

Microwave digestion has is widely applied to the analysis of numerous types of samples, including the botanical product and dietary supplement fields.[8, 9, 16] The application of microwave enhanced chemistry for sample preparation allows for shorter reaction times (i.e. digestion), reduction in the number of discrete sample preparation steps, greater sample homogeneity after digestion, increased sample throughput and better precision.[10, 17, 18] The processes are also very well suited for standardization and automation during method development.[10]

During the development of the digestion procedure, the microwave operation parameters (e.g., run time and temperature) were evaluated for the different botanical matrices. Given the diversity of materials, a particular digestion was deemed successful when an optically homogeneous, and temporally stable, solution was produced. The same amount of concentrated nitric acid (5 mL) used while evaluating the hotplate digestion was used for each sample-type during the microwave digestion. After the addition of the nitric acid to the samples, the initial reaction time was varied from matrix-to-matrix (0 to 30 min). Therefore, a pre-digestion step (first stage) was added to the microwave program to initiate the reaction between the matrix and acid, followed by the second stage of digestion (Table B.1) were the temperature is ramped from 80℃ to 180℃ over the course of ten minutes at a power of 1200 W, following a hold time at 180˚C for 15 minutes and cool time for another 15 minutes.

Analytical Response Characteristics

Once the optimization of the operation parameters for the primary dissolution was achieved, the analytical response characteristics were determined for each of the elements of interest using aqueous multielement standard solutions. The calibration curves were generated for each of the elements through the acquisition of five intensity measurements across a concentration range from 0 (i.e. analytical blank) to 300 ng mL^{-1} for the heavy metals and 0 to 50 μg mL^{-1} for Fe, Na, Ca, P and Zn. Good linearity and satisfactory coefficients of correlation (R^2 values) were observed for each of the elemental response functions. The limits of detection (LOD = $3\sigma_{blank}/m$) were also calculated from each calibration response. Table B.3 shows the analytical response characteristics obtained by ICP-OES for each of the elements of interest based on the use of aqueous calibration standards.

Table B.3. ICP-OES analytical response characteristics.

	Pneumatic nebulization			***CMA spray chamber***		
Element	**Response Function**	**R^2**	**LODs ($ng\ mL^{-1}$)**	**Response Function**	**R^2**	**LODs ($ng\ mL^{-1}$)**
Fe	$y = 9E^{+4}x + 3E^{+5}$	0.9995	5.0			
Na	$y = 7E^{+3}x + 2E^{+4}$	0.9998	15.0			
P	$y = 7E^{+2}x + 2E^{+2}$	0.9999	100.0			
Zn	$y = 5E^{+4}x + 5E^{+5}$	0.9935	19.0			
Ca	$y = 5E^{+2}x + 2E^{+3}$	0.9994	15.0			
As	$y = 6E^{+4}x - 6E^{+2}$	0.9996	6.0	$y = 1E^{+5}x - 8E^{+2}$	0.9989	3.0
Cd	$y = 1E^{+5}x - 4E^{+2}$	0.9999	4.0	$y = 2E^{+5}x - 2E^{+2}$	0.9999	4.0
Pb	$y = 1E^{+5}x - 1E^{+3}$	0.9989	6.0	$y = 9E^{+4}x - 9E^{+1}$	0.9994	15.0
Hg	$y = 1E^{+5}x - 4E^{+2}$	0.9982	5.0	$y = 1E^{+6}x - 6E^{+3}$	0.9995	5.0

The determination of some metals/metalloids can be achieved with better sensitivity through the use of hydride generation sample introduction. In addition to the conventional solution nebulization described above, determination of the heavy metal concentrations was also performed following hydride generation using the concomitant metal analyzer (CMA) spray chamber (Jobin-Yvon, Longjumeau, France). When using the CMA spray chamber, the reaction of sodium borohydride and an acidic solution (i.e. hydride formation) takes place in the chamber after being delivered by a peristaltic pump. One gram of sodium borohydride was dissolved in 100 mL of water, with three different hydrochloric acid (HCl) concentrations (0.5, 1.0 and 3.0 M) evaluated to determine the best acid composition. Calibration curves were obtained for As, Cd, Hg, and Pb over the concentration range of 0 to 300 ng mL^{-1}, at each HCl concentration. The best elemental responses during the hydride generation experiments were observed with a 1M HCl concentration. As in the case of using conventional nebulization, good linearity and satisfactory correlation coefficients were observed for each response functions, as shown in Table B.3. Overall, the limits of detection for As were improved by a factor of 2, but at the expense of a ~3x increase in Pb LOD. In the case of Hg and Cd, no changes in the LODs were observed. Due to the fact that the LODs obtained without the CMA chamber are in the low ng mL^{-1} levels, and fall below the Prop 65 guidelines, the quantitative elemental analysis of the various botanical extracts was carried out without hydride generation.

Method Validation

Upon development of the singular digestion procedure, it is necessary to ensure the procedure's efficiency to digest the samples in such a way that an accurate representation of elemental concentrations is obtained. The ultimate goal of this study was to validate the developed digestion procedure by analysis of standard reference materials (SRMs). The selected SRMs for the validation experiments need to be in a suitable matrix that is representative of the botanical extracts. However, because commercial botanical products have only recently come under scrutiny, very few SRMs targeting botanical products exist. The National Institute of Standards and Technology (NIST) had initiated the development of dietary supplement SRM suites, encompassing materials from the different preparation/production steps (e.g., harvest to final manufactured product).[19] The first two suites of NIST botanical SRMs available in the market were *Ephedra sinica* and *Gingko biloba.* Three reference materials; SRM 3241 *Ephedra sinica* Stapf Native (hot water) Extract, SRM 3243 Ephedra-Containing Solid Oral Dosage Form and SRM 3246 *Ginkgo biloba* (ground leaves) were employed during the validation experiments. The primary material for SRM 3241 was prepared by hot water extraction of the plant material under pressure, followed by filtration and concentration to produce the native product.[20] The materials making up SRM 3243 and SRM 3246 were prepared from various commercially available sources, ground and sieved for production of the packaged SRM.[20, 21] The certified values for these SRMs include reports of the

active organic components, as well as the trace levels of toxic metals and nutrients. Due to the very low concentrations of the heavy metals in these SRMs (where certified at all) it was necessary to validate the method by inclusion of nutrient elements (Fe, Na, P, Zn, Ca) to the analyte list. Thus, the method can be validated over a very wide range of elemental concentrations, as well as physical and chemical characteristics.

Table B.4. Elemental recoveries for aqueous standard solutions and the three commercial botanical product matrix types taken through microwave digestion process and ICP-OES analysis.

Element	Recovery (%)				
	Aqueous Standards (n=5)	**Ethanolics (n=16)**	**Phyto-caps (n=14)**	**Raw (n=10)**	**Cumulative (n=40)**
Fe	103	102	105	96	102
Na	100	106	107	100	105
P	109	110	104	100	105
Zn	103	108	106	103	106
Ca	103	110	106	105	107
As	96	97	97	96	97
Cd	97	99	97	101	99
Pb	94	92	91	92	92
Hg	95	60	62	61	61
Total	**100 ± 5**	**98 ± 16**	**97 ± 14**	**95 ± 13**	**97 ± 14**

Prior to validating the entire digestion and ICP-OES analysis method for botanical products, it was first necessary to do so for the test elements present in neat aqueous (standard) solutions. To do so removes the chemical digestion efficiency aspect of the process, but includes aspects of solution preparation, transfer among the various vessels, and performing the ICP-OES quantification procedure. The first column of Table B.4 shows the recovery values analysis (were n represents the number of intensity measurements taken for each element) obtained for a mixture of the aqueous standards (100 ng mL^{-1} each) taken through the complete sample preparation (microwave digestion) and ICP-OES analysis. Recoveries of 94% and higher were obtained for each of the elements, with sample-to-sample variabilities of ≤ 4% RSD, demonstrating that there was minimal elemental loss during the sample preparation procedures. There is a question as to why the recoveries of the nutrient elements are all above 100%, albeit not by much. These elements are the most likely to be present in the de-ionized water used in the solution preparations, thus leading to somewhat elevated blank levels.

The validation of the microwave digestion procedure developed for the three different matrices was accomplished using both the external calibration and standard addition methods, which are the most common approaches for ICP-OES measurements. Table B.5 shows the validation results obtained for the nutrient elements in SRMs 3241 and 3243, using the external calibration and standard addition procedures. (Values are not certified for these elements in

SRM 3246.) Overall, good recoveries were obtained for these elements, with values of 86% and higher, as well as having variabilities of ≤ 15% RSD. The precision here is in fact better than provided on the SRM certificates of analysis (overall variability of ≤ 21% RSD). Table B.6 presents the validation results obtained for As, Cd, Hg, and Pb using external calibration and standard addition. For the detectable elements, As and Pb (in most cases), the determined values were comparable to the certified values provided by NIST, with recoveries of ≥ 95% obtained by external calibration and standard addition. The precision is not as good with the heavy metals here in comparison to the NIST values, presumably due to the use of less sensitive ICP-OES than ICP-MS used for NIST quantification. The goal behind using both calibration techniques was to determine the potential effects of the different botanical matrices on the ICP analysis; i.e, are there potential matrix effects that make calibrations curves unsuitable, and only standard addition is a viable means of quantification? Because both validation procedures provided good results and the fact that the number of botanical samples to be analyzed is high, the analysis of the botanical extracts was performed by using the external calibration method.

Table B.5. Validation results for nutrient elements in NIST SRMs 3241 and 3243.

Element	Reference Values (mg kg^{-1})	Calculated Values (mg kg^{-1})	% Recovery	Calculated Values (mg kg^{-1})	% Recovery
		Aqueous calibration standards		*Standard addition method*	
		SRM 3241			
Fe	900 ± 100 (11%)	803 ± 58 (7%)	90	955 ± 89 (9%)	106
Na	2480 ± 280 (11%)	2446 ± 103 (4%)	99	2849 ± 351 (12%)	115
P	not certified	3169 ± 173 (5%)	-	3173 ± 177 (6%)	-
Zn	not certified	ND	-	ND	-
Ca	8450 ± 500 (6%)	9103 ± 241 (3%)	108	9063 ± 1098 (12%)	107
		SRM 3243			
Fe	760 ± 160 (21%)	787 ± 27 (3%)	104	650 ± 20 (3%)	86
Na	1960 ± 140 (7%)	1915 ± 98 (5%)	98	2283 ± 48 (2%)	116
P	6800 ± 1000 (15%)	6863 ± 120 (2%)	101	7070 ± 464 (7%)	104
Zn	3250 ± 310 (10%)	3851 ± 102 (3%)	118	3993 ± 350 (9%)	123
Ca	1.03 ± 0.05[a] (5%)	1.06 ± 0.14 (13%)	103	1.44 ± 0.21 (15%)	140

[a] Reference value in percentage
(X%) = relative standard deviation

Table B.6. Validation results for heavy metal elements in NIST SRMs 3241, 3243, and 3246.

Element	Certified Values (mg kg^{-1})	Calculated Values (mg kg^{-1})	% Recovery	Calculated Values (mg kg^{-1})	% Recovery
		Aqueous calibration standards		*Standard addition method*	
		SRM 3241			
As	1.29 ± 0.08 (6%)	1.27 ± 0.01 (1%)	99	1.332 ± 0.081 (6%)	104
Cd	0.0587 ± 0.0036 (6%)	ND	-	ND	-
Pb	0.241 ± 0.012 (5%)	ND	-	ND	-
Hg	0.00383 ± 0.00029 (8%)	ND	-	ND	-
		SRM 3243			
As	0.554 ± 0.018 (3%)	0.612 ± 0.035 (6%)	110	0.599 ± 0.013 (2%)	108
Cd	0.122 ± 0.003 (2%)	ND	-	ND	-
Pb	0.692 ± 0.056 (8%)	0.764 ± 0.086 (11%)	114	0.671 ± 0.112 (17%)	97
Hg	0.00900 ± 0.00044 (5%)	ND	-	ND	-
		SRM 3246			
Cd	20.8 ± 1.0 (5%)	ND	-	ND	-
Pb	995 ± 30 (3%)	1121.0 ± 0.2 (0.01%)	112	1063 ± 154 (14%)	107
Hg	23.1 ± 0.2 (1%)	ND	-	ND	-

(X%) = relative standard deviation

Quantification of Botanical Extracts

After completion of the method validation, the three different matrices of botanical samples underwent microwave digestion and were analyzed for As, Cd, Hg, Pb, Fe, Na, Ca, P and Zn by ICP-OES. Tables B.7-9 show the concentration values obtained for the elements of interest from the powdered raw material, glycerine-based and ethanolic tinctures samples, respectively. The toxic metals (As, Cd, Hg, Pb) were not detected (ND) in the glycerin-based samples and the ethanolic tinctures, indicating their safety. In the case of powdered raw materials, a few of the samples (for example; Bilberry P.E. and Burdock Root) provided detectable levels of As and Pb. Because, in many situations the powdered-raw materials are employed for the production/preparation of other consumable matrices (e.g, capsules, tablets, tinctures) the amount of the heavy metals would have to be accounted for in the final preparation.

In order to corroborate the fact that the ND assignments for many of the heavy metals were not the result of systematic errors, each of the botanical samples was spiked with a standard aqueous solution containing each of the test elements prior to the addition of nitric acid and the microwave digestion. Table B.4 also shows the recovery values obtained for each of the elements for the three sample matrix types were n represents the number of botanical samples analyzed. Recoveries of 90% and higher were observed for each of the elements with the exception of mercury, which resulted in a 61% recovery for the different sample matrices. The uniformity of the elemental recoveries across the

different matrix forms is firm validation of the efficacy and utility of the developed digestion procedure. The loss of mercury during the experiments could be due to the volatility of the element or adsorption to the digestion vessel walls or the components of the ICP sample introduction system. Based on the fact that the recovery for Hg was the same as the other elements in the case of the aqueous standard solutions (Table B.4), it seems quite clear that volatile Hg species are formed in the initial nitric acid decomposition of organomercury compounds prior to the sealing of the microwave vessels. Unfortunately, processing in this manner is required as the mixture of HNO_3, with ethanol in particular, is quite rapid and exothermic. There may be some improvement in Hg recoveries by using lower concentrations of the acid, but this would occur at the expense of longer digestion times.

Table B.7. Elemental composition of powdered raw botanical product materials.

Sample	Fe ($\mu g\ g^{-1}$)	Na ($\mu g\ g^{-1}$)	P ($\mu g\ g^{-1}$)	Zn ($\mu g\ g^{-1}$)	Ca ($\mu g\ g^{-1}$)	As ($\mu g\ g^{-1}$)	Cd ($\mu g\ g^{-1}$)	Pb ($\mu g\ g^{-1}$)	Hg ($\mu g\ g^{-1}$)
Rhodiola Rosea	8.56 ± 0.26	99.5 ± 3.0	93.2 ± 3.0	ND	727 ± 5	ND	ND	ND	ND
Milk Thistle Dry Extract	3.71 ± 0.43	4.12 ± 0.48	1354 ± 36	ND	326 ± 88	ND	ND	ND	ND
Bayberry P.E.	414 ± 13	137 ± 3	230 ± 6	ND	184 ± 3	ND	ND	ND	ND
Ashwagandha Powder	28.2 ± 1.7	285 ± 6	833 ± 20	7.48 ± 0.54	85.6 ± 3.5	ND	ND	ND	ND
Hawthorn Berry	47.5 ± 1.6	ND	618 ± 15	ND	1587 ± 15	ND	ND	0.750 ± 0.170	ND
Licorice Root	242 ± 4	522 ± 12	347 ± 27	ND	22295 ± 357	ND	ND	ND	ND
Green Tea	140 ± 2	ND	3622 ± 38	ND	2805 ± 61	ND	ND	1.10 ± 0.23	ND
Schizandra Berry	55.1 ± 2.1	12.7 ± 0.3	2036 ± 42	ND	961 ± 10	ND	ND	ND	ND
Chinese Skullcap	51.4 ± 1.0	1893 ± 29	1432 ± 18	ND	2844 ± 65	ND	ND	ND	ND
Bilberry P.E.	131 ± 2	33.5 ± 0.7	502 ± 6	ND	81.7 ± 2.5	ND	ND	24.5 ± 0.9	ND
Ttribulus	43.4 ± 3.4	16.3 ± 0.5	4177 ± 94	ND	46537 ± 1616	ND	ND	ND	ND
Bitter Orange Peel	137 ± 4	47.2 ± 0.7	726 ± 26	ND	14269 ± 120	ND	ND	ND	ND
Holy Basil	433 ± 7	6548 ± 60	3632 ± 119	15.6 ± 0.6	24278 ± 280	0.966 ± 0.353	ND	ND	ND
Burdock Root	189 ± 5	3.37 ± 0.70	949 ± 18	ND	3684 ± 74	0.877 ± 0.190	ND	ND	ND

Table B.8. Elemental composition of glycerine-based botanical product extracts.

Sample	Fe (μg g^{-1})	Na (μg g^{-1})	P (μg g^{-1})	Zn (μg g^{-1})	Ca (μg g^{-1})	As (μg g^{-1})	Cd (μg g^{-1})	Pb (μg g^{-1})	Hg (μg g^{-1})
Whole Body Defense	51.6 ± 1.3	628 ± 10	535 ± 16	ND	1878 ± 20	ND	ND	ND	ND
Echinacea Goldenseal	ND	153 ± 2	434 ± 21	ND	501 ± 5	ND	ND	ND	ND
Holy Basil	ND	2187 ± 28	474 ± 16	ND	42.6 ± 3.4	ND	ND	ND	ND
Saw Palmetto	ND	3.19 ± 0.26	14710 ± 223	ND	511 ± 17	ND	ND	ND	ND
Milk Thistle Seed	ND	34.8 ± 0.5	4885 ± 47	ND	170 ± 6	ND	ND	ND	ND
Antioxidant Supreme	1.68 ± 0.22	93.8 ± 1.9	1089 ± 43	ND	346 ± 11	ND	ND	ND	ND
Kava Kava Root	ND	95.8 ± 1.5	5728 ± 126	ND	239 ± 3	ND	ND	ND	ND
Cinnamon	ND	ND	712 ± 16	ND	143 ± 3	ND	ND	ND	ND
Ginkgo	ND	ND	258 ± 13	ND	42.2 ± 1.9	ND	ND	ND	ND
Thyroid Support	15.9 ± 0.9	6742 ± 68	1411 ± 45	ND	451 ± 9	ND	ND	ND	ND
Green Tea	ND	ND	660 ± 20	ND	ND	ND	ND	ND	ND
Valerian	1.23 ± 0.74	269 ± 4	719 ± 21	ND	74.3 ± 2.0	ND	ND	ND	ND
Olive Leaf	ND	30.3 ± 0.6	133 ± 5	ND	131 ± 4	ND	ND	ND	ND
Motor Oil for Men w/ Zn	2.52 ± 0.24	345 ± 3	4955 ± 41	7247 ± 105	1462 ± 15	ND	ND	ND	ND
Male Llbido	3.24 ± 0.33	101 ± 4	515 ± 25	ND	405 ± 14	ND	ND	ND	ND

Table B.9. Elemental composition of ethanolic tinctures of botanical products.

Sample	Fe ($\mu g\ g^{-1}$)	Na ($\mu g\ g^{-1}$)	P ($\mu g\ g^{-1}$)	Zn ($\mu g\ g^{-1}$)	Ca ($\mu g\ g^{-1}$)	As ($\mu g\ g^{-1}$)	Cd ($\mu g\ g^{-1}$)	Pb ($\mu g\ g^{-1}$)	Hg ($\mu g\ g^{-1}$)
Maitake Gold	2.37 ± 0.19	ND	390 ± 11	ND	4.63 ± 0.72	ND	ND	ND	ND
Milk Thistle	ND	15.6 ± 0.6	365 ± 11	ND	16.7 ± 1.1	ND	ND	ND	ND
Lobelia Herb & Seed	4.70 ± 0.34	30.7 ± 0.6	96.1 ± 6.9	ND	447 ± 11	ND	ND	ND	ND
Ginkgo Leaf Extract	7.21 ± 0.29	ND	949 ± 19	ND	465 ± 8	ND	ND	ND	ND
Sangre de Drago	ND	ND	24.4 ± 1.8	ND	192 ± 4	ND	ND	ND	ND
Sanito	53.5 ± 0.9	13.3 ± 0.4	168 ± 12	ND	267 ± 5	ND	ND	ND	ND
Valerian Root	4.38 ± 0.20	10.9 ± 0.2	93.0 ± 5.6	ND	23.1 ± 1.4	ND	ND	ND	ND
Echinacea Supreme	ND	86.9 ± 0.4	172 ± 2	ND	65.8 ± 1.7	ND	ND	ND	ND
Licorice Root Extract	ND	629 ± 10	95.5 ± 7.9	ND	355 ± 8	ND	ND	ND	ND

CONCLUSIONS

A single microwave digestion method has been successfully applied for the elemental analysis of three different botanical matrices (powder raw material, glycerin-based samples and ethanolic tinctures) by ICP-OES. In addition, method validation was carried out by external calibration and standard addition using three NIST standard reference materials. Both calibration techniques provided good results, but due to the high number of samples, the external calibration was the technique of choice. Recovery results obtained by the addition of element standard solutions to the botanical matrices prior to addition of nitric acid and microwave digestion and carried through every step demonstrate that the presented methodology is uniform and can be applied for the elemental analysis of different botanical product matrices.

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APPENDIX C

PARTICLE BEAM GLOW DISCHARGE MASS SPECTROMETRY: SPECTRAL CHARACTERISTICS AND FIGURES OF MERIT FOR THE EPHEDRINE ALKALOIDS

EXPERIMENTAL

The PB/MS system used for the analysis of the ephedrine alkaloids was an Extrel (Pittsburgh, PA, USA) Benchmark Thermabeam LC/MS quadrupole mass spectrometer with a GD ionization source, depicted in Fig. 1.5, and described in Chapter 1. Total ion chromatograms (TIC) were acquired using the Extrel Merlin Ionstation software by scanning over a mass range of m/z = 50-200 Da at a scan rate of 1.0 s per scan. The MS data was exported to Sigma Plot 8.02 (Systat Software, Inc., Richmond, CA) and presented using Microsoft Excel and PowerPoint (Redmond, WA). Detailed explanation of the PB interface and the GD ionization source have been described in Chapter 1. The nebulizer is heated to ~85℃, the desolvation chamber at ~110℃ a nd the source block is held at a temperature of 200℃. The GD operating p arameters were ~0.3 Torr and 0.2 mA for the discharge pressure and current, respectively.

The 1000 µL mL^{-1} stock solutions of (-)-ephedrine, (+)-pseudoephedrine, (-)-norephedrine and (-)-N-methylephedrine (Sigma-Aldrich, St. Louis, MO, USA) were prepared by weighing the appropriate amounts of the analytes and diluting in a mixture of 0.1% water containing TFA. Calibration curves were created by

triplicate injections of the standard solutions into the LC system (without column present) with spectral data acquired in total ion chromatogram (TIC) mode.

EPHEDRINE ALKALOIDS FIGURES OF MERIT

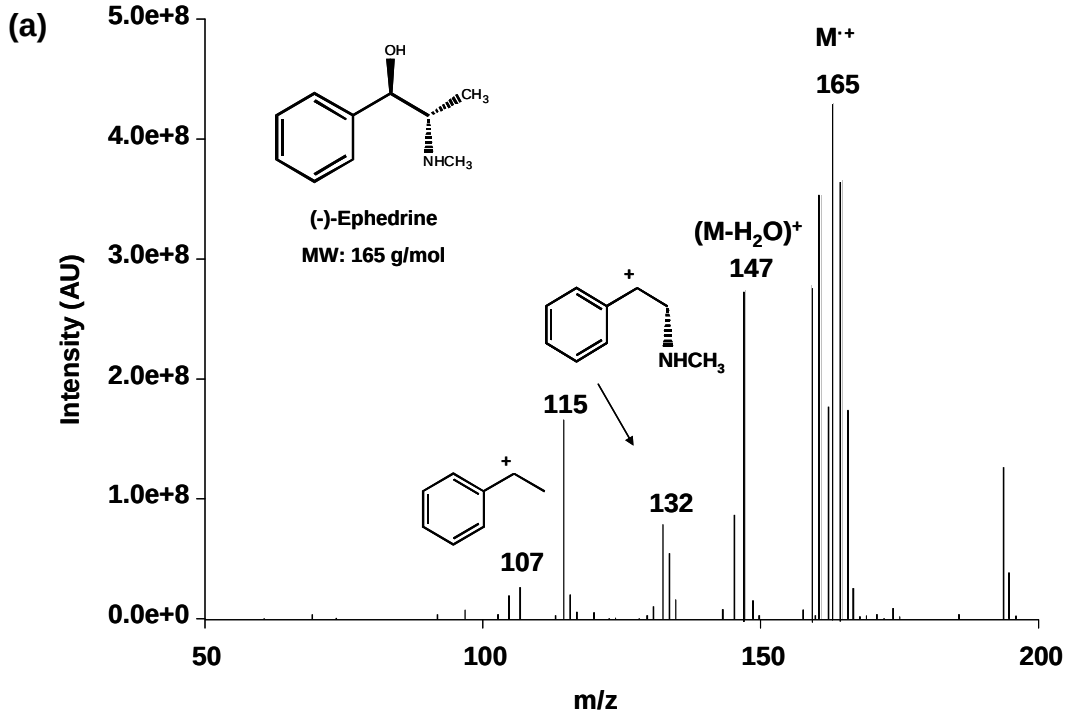
Table B.1 shows the figures of merit obtained for the ephedrine alkaloids by the LC-PB/GDMS system. Response curves were generated in a similar manner as mentioned in Chapter 4. Each of the corresponding response functions shows acceptable linearity with satisfactory correlation coefficients (R^2 values). The limits of detection ($3\sigma_{blank}/m$) determined for the ephedrine alkaloids are all below 1 nanogram, absolute. The LODs obtained for the ephedrine alkaloids using the PB/GDMS are consistent with the LODs corresponding to PB/EIMS as well as to the values reported in the literature for GC-MS and ESI-MS (shown in Chapter 4).

Table C.1. Analytical response characteristics of ephedrine alkaloids by LC-PB/GDMS.

Species	Response Function	Accuracy R^2	Detection Limits (ng mL^{-1})	Absolute Mass (ng)
Ephedrine	y = 4E+08x - 8E+09	0.9994	6.6	0.66
Pseudoephedrine	y = 5E+08x + 4E+09	0.9942	4.3	0.43
Norephedrine	y = 2E+08x + 2E+09	0.9874	9.6	0.96
N-methylephedrine	y = 2E+08x - 6E+08	0.9853	8.6	0.86

GLOW DISCHARGE SPECTRA FOR EPHEDRINE ALKALOIDS

As demonstrated in Chapter 2, comparison between the EI and GD sources' spectral fragmentations can be accomplished due to the fact that the GD source yields spectra that obey EI fragmentation rules. Figure B.1a-c show the PB/GDMS spectra obtained from a 50 µL injection of a 100 µg mL^{-1} solutions of ephedrine, norephedrine and methylephedrine. The spectra obtained by both EI (Chapter 4) and GD sources for the ephedrine alkaloids tested show similar and simple fragmentation patterns including their molecular ion. Chapter 4 presented a detail explanation of the fragmentation pattern observed for the ephedrine alkaloids which corresponds with the fragmentation behavior seen in the GD spectra.



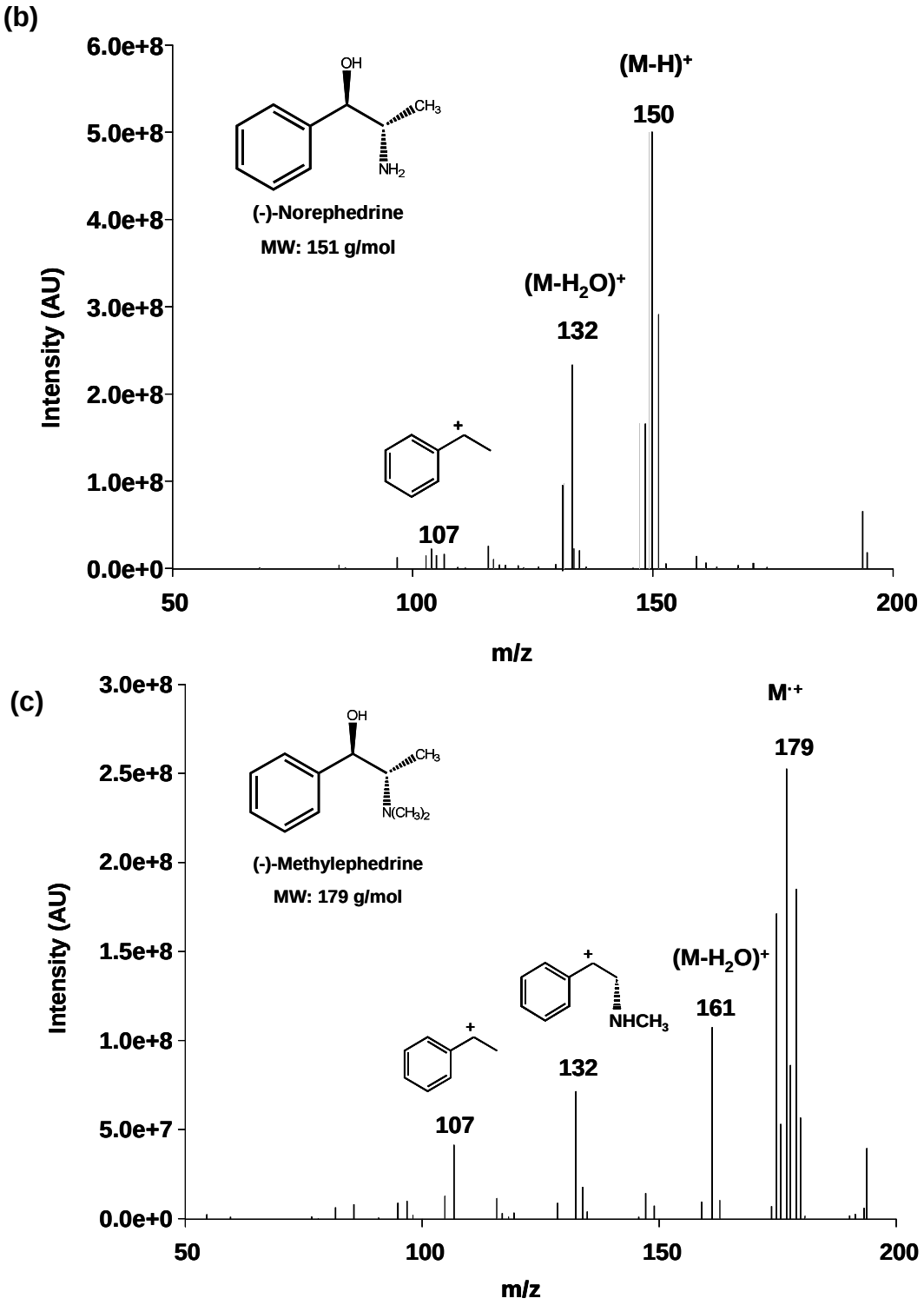


Figure C.1. LC-PB/GD mass spectra of a) ephedrine, b) norephedrine, and c) methylephedrine. Discharge current = 0.2 mA, discharge pressure = 0.3 Torr, block temperature = 200 ℃, concentration = 100 µg mL^{-1}, 50 µL injection loop.

APPENDIX D

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