DATA MINING USING DIRECT INJECTION TRIPLE QUADRUPOLE MASS SPECTROMETRY, INFRARED SPECTROSCOPY, INDUCTIVELY COUPLED PLASMA OPTICAL EMISSION SPECTROSCOPY, AND POLYMERASE CHAIN REACTION FOR THE RAPID IDENTIFICATION OF NUTRACEUTICALS AND CONTAMINANTS

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There has been a rapid surge toward “organic” products devoid of GMOs, MSGs, and other common compounds found in processed foods that continue to indicate an association with an increased risk for disease. These consumers seek nutrients and vitamins that are lacking in their diet and lifestyle in the form of nutraceuticals for disease prevention and treatment as well as overall lifestyle enhancement. However, these products generally lack clinical evidence as well as legal definition. Due to this ambiguity, nutraceuticals are neither considered a food product nor a pharmaceutical product. Furthermore, due to their alleged natural properties allowing for safe, therapeutic effects, nutraceuticals are being eagerly sought after by consumers in the place of pharmaceuticals. Additionally, since nutraceutical substances are “naturally” derived, there is a general lack of regulation regarding the manufacturing and distribution process. This mismanagement leads to lack of quality assurance (QA) and quality control (QC) protocols strictly implemented to define appropriate production and storage parameters. Without these critical measures, consumers are subjected to contamination of their products resulting from improper storage conditions and unmanaged production. These contaminants often include heavy metal impurities, pesticides, bacterial activity, and may also be adulterated with illicit drugs, all leading to detrimental health and environmental effects.

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I dedicate this research and this honor to my dad, Mike Morgan. You will always be my Superman whose faith is unwavering and truly inspiring. I love you endlessly.

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CHAPTER 1

INTRODUCTION

1.1 Nutraceuticals and Nootropics

As epidemiological experimentation continues to indicate the rise of adverse health effects within the human population, the need for versatile health intervention becomes critical. Chronic age-related diseases persist in the form of cardiovascular diseases, neurodegenerative diseases, diabetes, and various types of cancer [1]. Furthermore, most of these conditions and increased risk for compromised health have been directly related to food products and dietary habits from epidemiological studies. Modern agriculture maintains sustainable food production for the world's continued increase in population by using Genetically Modified Organisms (GMOs). Utilizing these transgenic plants can quickly produce higher yield, enhance nutrition through higher quality, and create tolerance to biotic and abiotic stressors. However, these advancements consequently create unintentional gene alteration to wild plants, environmental pollution, impede crop genetic diversity, and introduce new viruses and toxins, along with a multitude of other unknown ramifications [2]. For these reasons, there has been a rapid surge toward "organic" products devoid of GMOs, MSGs, and other common compounds found in processed foods that continue to indicate an association with an increased risk for disease. These same individuals seek nutrients and vitamins that are lacking in their diet and lifestyle in the form of nutraceuticals. The term "nutraceutical" indicates that it is made from parts of food that provide health benefits [3]. Another similarly used term is "nootropics" which typically refers to supplements that are used with the intent of enhancing cognitive functions specifically [4]. There's a wide range of uses for these compounds including physical and mental performance

enhancement, vital vitamins for metabolic functions, and aiding in disease treatment or prevention.

Nutraceuticals and nootropics are commonly used interchangeably, however there are some technical differentiations between the two terms. Nutraceuticals typically involve plant-derived foods in the form of concentrated bioactive components in a non-food matrix. The active component that exerts a wide range of potentially beneficial biological activities are secondary metabolites referred to as phytochemicals. These metabolites are present in significant parts of diet at low potency. Phytochemicals associated with health benefits include glucosinolates, the compounds of the Alliaceae family that contain sulfur, terpenoids, and various polyphenol groups. Their bioactive benefits relate to their antioxidant properties which are involved in various chronic degenerative diseases including inflammation, LDL oxidation, aging, and plaque development. Furthermore, carotenoid lycopene, onion, and garlic extracts, more commonly known as Alliaceae extracts, Sulphur derivative extracts such as alliin and allicin, phytosterols, flavanols, ellagic acid (EA), and ellagitannins (ETs) are commonly manufactured nutraceuticals containing these phytochemicals, among others. On the other hand, nootropics are used with the intent of enhancing cognitive function by utilizing drugs, supplements, nutraceuticals, or functional foods that support and increase memory, cognition, attention, and concentration. Multiple mechanisms are employed such as increasing brain circulation, providing precursor neurotransmitters, improving overall neuronal function, and providing constant and sustainable energy for brain usage. These mechanisms are initiated from various dietary sources and supplements that increase glucose levels in the brain. Common sources are from vitamins, Omega-3 fatty acids, antioxidants, amino acids, caffeine, hormones, and various herbs and flowers. While these compounds may produce desirable effects, their wide array of products

include drugs that could pose detrimental health risks. Certain racetams act as positive allosteric modulators of AMPA receptors as well as acetyl-cholinergic system modulators. However, despite piracetam, aniracetam, and nefiracetam being accepted as “pharmacologically safe,” serious health risks have surfaced. Similarly, productivity and performance enhancing nootropics act only while the drugs are present in the blood. To achieve “maximum focus,” stimulants are used including amphetamines, eugeroics, cholinergic, and xanthine compounds. These groups utilize a range of drugs from nicotine (cholinergic) and caffeine (xanthine) to Modafinil (eugeroic) and methamphetamine. Other compounds used as nootropics are reuptake inhibitors, such as methylphenidate, metabolic function enhancers, such as vinpocetine, racetams, such as piracetam, and secondary enhancers, such as dehydroepiandrosterone (DHEA). Other explicitly illegal, but still popularly sought after, nootropic stimulants in circulation include phenibut, a GABA-mimetic, and N-Methyltyramine HCL, a weak alpha-2 adrenoreceptor antagonist. Each of these drugs are associated with high risk for disease as well as other complications that vary depending on an individual’s pre-existing health conditions [5].

Regardless of whether a drug is being used as a nutraceutical to supplement their dietary needs and prevent risk for a myriad of potential diseases, or it is used as a nootropic to enhance cognitive performance, these products generally lack clinical evidence as well as legal definition. Due to this ambiguity, nutraceuticals/nootropics are neither considered a food product nor a pharmaceutical product. This gray area allows for these compounds to be presented as a pharmaceutical in the form of a capsule or tablet, or in food form as a functional food component of a recipe [6]. Furthermore, due to their alleged natural properties allowing for safe, therapeutic effects, nutraceuticals are being eagerly sought after by consumers in the place of pharmaceuticals. Additionally, since nutraceutical substances are “naturally” derived, there is a

general lack of regulation regarding the manufacturing and distribution process. This mismanagement leads to lack of quality assurance (QA) and quality control (QC) protocols strictly implemented to define appropriate production and storage parameters. Without these critical measures, consumers are subjected to contamination of their products resulting from improper storage conditions and unmanaged production. These contaminants often include heavy metal impurities, pesticides, bacterial activity, and may also be adulterated with illicit drugs, all leading to detrimental health and environmental effects [7, 8].

1.2 Heavy Metals

During the synthesis of pharmaceuticals and supplementary compounds, inorganic impurities are frequently deposited in the produced samples through unregulated industrial activities. Furthermore, due to the growing demand and use of nutraceuticals, herbal alternatives, and organic dietary supplements, emerging contamination arise from the original sources themselves. These natural, plant-derived, and herbal compounds are attractive to health-conscious consumers assuming that there are no affiliated side effects with phytotherapy. Since pharmaceuticals and other mass-produced medicines are synthesized, the Food and Drug Administration (FDA) clearly defines the parameters to ensure the health and safety of their affiliated users. Additionally, these compounds require well-established, intensive research to establish associated risks and benefits for the public's knowledge. However, products derived from plants and other naturally occurring sources are not well-defined by the FDA regarding the requirements for quality and regulation of these products. Furthermore, any compound that is not directly considered a drug or food for human consumption is not required by the FDA to be researched or tested for contamination. Producers are only required to perform general analyses of apparent and hazardous metals such as lead, arsenic, and mercury, as well as levels of

microbiological purity in raw plant extracts. Unfortunately, a lot of the more severe and impactful metals, including arsenic, cadmium, lead, and mercury naturally occur in the environment at toxic and even lethal levels, and are not accurately analyzed using generic panels. These heavy metals naturally occur in the soil and migrate into groundwater, air, and land, where they eventually deposit and accumulate. Artificial sources of heavy metals are common as well through industrial production, in addition to everyday automobile emissions and combustion by-products [9, 10].

Regardless of if the metal is naturally or artificially occurring, both biologically essential and non-essential metals are produced within the environment. Essential metal elements can either include metals that are required to perform vital biochemical functions or can be metals that are largely beneficial to these processes. For an element to be considered essential, it must be present in human tissue, able to be supplemented to normalize altered levels and physiological function in the body, and lead to irreversible damage to vital organs when totally absent. On the other hand, elements are recognized as a beneficial element and not essential if they only improve functionality but are not directly required for functionality. Some examples of essential heavy metals include iron, zinc, copper, and nickel. These metals are important constituents of several main enzymes within the body and play essential roles in oxidation-reduction reactions [9].

1.2.1 Iron

Elemental iron (Fe) is naturally found in abundance throughout various layers of the earth’s crust and concentrates in iron ores and minerals. It is distributed throughout the soil in trace amounts which further dissolves into the groundwater. Additionally, humans consume ideally seven milligrams of dietary iron daily mainly through iron-rich foods such as beef, red

wine, and eggs. However, to prevent excess levels of exposure, uptake of iron is relatively low. Approximately only 25% of consumed iron is absorbed into the body to avoid generating destructive reactive oxygen species. Despite its low absorption rate, iron is an essential trace element involved in over one hundred enzymatic reactions including electron transport, DNA synthesis, and the transportation of oxygen through hemoglobin. Due to the vital roles of iron, the human body has established an effective way of storing between one to three grams of iron in the body while eliminating approximately one milligram daily to maintain appropriate levels. Approximately 80% of iron content circulates via hemoglobin and myoglobin [9]. Iron is mainly absorbed by entering the stomach from the esophagus. It is then oxidized into Fe^{3+}. This form is normally insoluble; however, gastric acidity and solubilizing agents prevent precipitation from occurring. The iron is then absorbed by intestinal mucosal cells in the duodenum and the upper jejunum. Iron is then coupled to transferrin, a blood-plasma glycoprotein, which delivers the iron to various cells throughout the body. Certain factors, such as other heavy metal competitors, can negatively influence the absorption of iron [11]. When the absorption of iron is diminished, iron homeostasis is disrupted which produces iron deficiency (ID). Since iron is an essential element, diminished activity results in total-body ID, specifically depletion of macrophage and hepatocyte iron stores. When these stores are exhausted and an excessive reduction in erythropoiesis occurs, ID frequently results in iron deficiency anemia (IDA) [12].

1.2.2 Zinc

Elemental zinc (Zn) is naturally found in abundance in the Earth's crust. From there, zinc is distributed into the soil which is further dispersed into the air and groundwater. An ideal dietary amount of 15 milligrams of daily zinc is consumed mainly in the form of red meat, poultry, shellfish, nuts, whole grains, and dairy products; however, zinc is most plentiful in

oysters [9, 13, 14]. The body retains trace amounts of zinc, approximately two grams, that concentrates in the brain, bones, and muscles. Additionally, zinc homeostasis is mainly regulated by the small intestine, liver, and pancreas [15]. The bioavailability of zinc is largely dependent on the ratio of refined and unrefined grains consumed. Unrefined diets decrease bioavailability and range from 18-26% whereas refined diets have a higher bioavailability of 26-34% [16]. Zinc is an essential trace element that is required to carry out over one-hundred vital enzymatic chemical reactions. Some of its major roles include DNA synthesis, tissue repair, immune system support, and cell growth. Furthermore, zinc plays a catalytic role in acid-base reactions in association with RNA polymerases. Zinc fingers function by interacting with nucleic acids and are observed to organize tertiary structure of proteins. Additionally, Zn^{2+} ions perform regulatory functions through inert metal ions that have signaling capacity, as well as act as messengers to facilitate communication among cells [9]. Following ingestion, zinc ions are released, and are absorbed by the small intestine. Once zinc uptake occurs, it is transported from the lumen into enterocytes. From there, approximately 70% of the zinc ions are released into the portal blood where they bind to albumin, a blood protein produced by the liver, which ultimately distributes the metal throughout the body [16]. Reduced amounts of zinc results in deficiency affecting vital organ systems including the gastrointestinal, integumentary, immune, skeletal, and reproductive systems. Furthermore, when dysfunction occurs in the reproductive and skeletal system, hypogonadism and dwarfism may occur. Additionally, dysfunction in humoral and cell-mediated immunity results in an increase of susceptibility to infection and apoptosis in major cells [9, 17].

1.2.3 Copper

Copper (Cu) is an elemental metal released from decaying vegetation, fossil fuel burning, and windblown dust. This metal is further distributed throughout the environment by attaching to

particulate matter in the air, dissolving in water, and attaching to soil where it is then taken up by plants. Aquatic life, such as fish and mollusks, bioaccumulate copper in their biological systems. Humans consume dietary copper in the form of copper-rich foods including meats, oysters, mushrooms, and nuts. Humans require only a few milligrams of daily elemental copper since the body only retains approximately one-hundred milligrams of copper. Due to its minimal dietary requirement, homeostatic mechanisms are highly essential and effective at regulating both uptake and export. These mechanisms ensure essential copper supplies are being received as well as minimizing toxicity from excess uptake. Copper is a vital trace element for enzymes involved in aerobic metabolism, neovascularization, neuroendocrine functionality, and iron metabolism. For example, mitochondrial cytochrome c oxidase is a metalloprotein that contains three copper centers per monomeric unit and is the final electron acceptor in the electron transport chain that produces ATP. Additionally, copper contributes toward the formation of connective tissue in the form of collagen and keratin [9, 18]. Once ingested, copper is partially absorbed in the stomach where bound copper ions are freed due to the highly acidic environment. The largest part of absorption occurs in the duodenum and ileum due to the high solubility of copper in the intestine through chelators and organic acids. From there, copper ions enter the intestine's mucosal cells via simple diffusion. Severe copper deficiency can result from low dietary intake or genetic defects, and is associated with impaired immune function, anemia, cardiac hypertrophy, and weak connective tissue. Additionally, copper deficiency can result in insufficient oxygen production in the brain which has the potential for permanent brain damage. On the other hand, an excess amount of copper can accumulate in the liver and brain as in the case of Wilson disease. This progressive genetic disease is incapable of regulating copper homeostasis

appropriately, and leads to hepatoxicity, central nervous system damage, and ultimately death if left untreated [9, 19].

Just as iron, zinc, and copper are essential trace metals naturally present in the environment, other non-essential heavy metals are also present in the environment that produce toxic and sometimes lethal effects. Some common toxic metals, such as cadmium, lead, and mercury, readily interact with essential metals within biological systems. Other non-essential metals, such as arsenic, produce lethal effects by disrupting and ultimately inhibiting biochemical reactions that are vital for life.

1.2.4 Cadmium

Cadmium (Cd) is a toxic, nutritionally non-essential element found in most soil and rocks as well as coal and mineral fertilizers. However, cadmium is versatile and mainly used in the manufacturing of batteries, plastics, and tobacco. Tobacco leaves contain a substantial amount of cadmium leading to doubling the cadmium intake for frequent cigarette smokers. In addition to acute exposure in the environment, humans typically consume quantities of cadmium from foods such as grains and leafy vegetables. Once ingested, cadmium accumulates in the liver and the kidneys, and targets the renal and skeletal system by interacting with essential metals, especially calcium. Cadmium produces nephrotoxicity in the kidneys by interfering with calcium and vitamin D metabolism. Additionally, cadmium deposits in osteoid tissue and interferes with calcification, decalcification, and bone remodeling. Cadmium also interacts with zinc through the binding of both elements to metallothionein. Metallothionein is a low molecular weight protein that binds zinc and copper for transport and storage. When cadmium is in plasma and binds to metallothionein, renal tubule toxicity occurs during excretion. Finally, iron deficiency increases the absorption of cadmium in the gastrointestinal tract [20, 21]. Cadmium ultimately interferes

with DNA repair mechanisms and affects cell proliferation by generating reactive oxygen species (ROS) and inducing cell apoptosis and necrosis [22].

1.2.5 Lead

Lead (Pb) is one of the earliest discovered metals, and possesses unique properties such as ductility, low melting point, resistivity to corrosion, and high malleability. These characteristics all contribute to its ubiquitous nature [25]. Furthermore, lead is one of the most hazardous non-essential metals that deposits in soil and water and distributes through the air in the form of dust particles. A significant source of soil contamination is from deteriorated outdoor paint from buildings, furniture, and fences that crack under environmental stress, and deposit into the soil. Another source is from past use of lead in gasoline depositing in the soil near major roads and highways which then accumulates. Water contamination arises from the accumulation of lead as it passes through plumbing systems. Additionally, plumbing installed before 1930 may contain lead pipes, plumbing installed before 1986 may contain lead-based solder in the copper joints of the plumbing system, and any brass faucets may contain lead. In addition to the previously listed sources, lead particle dust is the leading source of lead exposure among humans. Lead dust is commonly created during home renovations, as well as when lead-based paints crack, chip, or peel. Lead-based paints is no longer used but can still be found in homes built before 1980 if they have not been properly removed or renovated. Furthermore, children can be exposed through antiques or imported toys that have been manufactured with lead-based paint. Finally, imported goods including spices, foods, antiques, and ceramics as well as some traditional or cultural medicines and cosmetics potentially contain lead. This is due to the more lenient environmental and manufacturing regulations of developing countries outside of the United States [23, 24]. Once the particles enter the body, they interfere with multiple biological

functions, primarily the central nervous, hematopoietic, hepatic, and renal systems. In the nervous system, lead reduces motor activity in the form of peripheral neuropathy by degrading the myelin sheath surrounding the nerves. This ultimately impairs signal transduction of nerve impulses. Lead also interferes with the synthesis of hemoglobin which directly affects the hematopoietic system. This interference occurs by inhibiting key enzymes involved in the heme synthesis pathway leading to lipid peroxidation in the form of hemolytic anemia. Nephropathy can occur at acute or chronic levels. Acute nephropathy expresses itself in the form impaired tubular transport mechanisms. Lead causes the tubular epithelium to degenerate as well as produce nuclear inclusion bodies which contain lead protein complexes. This leads to Fanconi's syndrome where abnormal extraction of glucose, phosphates, and amino acids in the urine occur. On the other hand, chronic nephropathy causes glomerular and tubulointerstitial changes, leading to irreversible damage through renal breakdown as well as hyperuricemia. Regarding reproductive impairment, lead reduces motility and number of sperm in men, as well as creates abnormalities in prostate function and testosterone serum. In women, lead increases the susceptibility of infertility, miscarriages, pre-eclampsia, and premature delivery. Additionally, lead directly influences the development of the fetus. All toxicological effects from lead exposure are expressed via cellular, intracellular, and molecular mechanisms. The leading cause of lead toxicity is by producing oxidative stress. This biological imbalance of free radicals is caused by generating reactive oxygen species (ROS) while simultaneously depleting antioxidant reserves. Specifically, glutathione (GSH) is a vital antioxidant that quenches free radicals. Lead interacts with GSH by binding with its sulfhydryl groups, and ultimately inactivates its antioxidant properties. Additionally, lead directly causes red blood cell (RBC) hemolysis by inhibiting δ-aminolevulinic acid dehydratase (ALAD) which increases δ-aminolevulinic acid

(ALA) concentrations in the blood and in the urine. These elevated levels generate more ROS ultimately leading to cell death [24, 25, 26].

1.2.6 Arsenic

Arsenic (As) is a ubiquitous metalloid that is a naturally occurring component of the earth's crust as well as from anthropogenic sources. This compound exists in an inorganic and an organic form. Inorganic arsenic compounds are found in groundwater, soil, and sediment resulting from industrial and agricultural activity, weathering rock, ore smelting, and mining. The organic form is mainly found in dietary sources such as fish and shellfish at significantly lower concentration levels. The organic form found in fish poses a less toxic risk than its toxic inorganic form. Exposure to elevated levels of inorganic arsenic results from ingesting contaminated drinking water, consuming food prepared with contaminated water, as well as inhaling air emissions from pesticide and other manufacturing facilities [33, 34, 35]. Arsenic enters the body mainly in its trivalent inorganic form, referred to as arsenite, via simple diffusion, and is either further methylated to form monomethylarsonic acid [MM(V)] or is demethylated to form dimethylarsinic acid [DMA(V)]. Trivalent inorganic arsenic interacts with and binds to the sulfhydryl groups of the subunits within the pyruvate dehydrogenase (PDH) complex. This interference impairs cellular respiration and induces cell death through apoptosis and necrosis. Furthermore, trivalent arsenic inhibits GSH production which leads to oxidative stress and cellular damage through the formation of reactive oxygen species. Acute arsenic poisoning results in nausea, vomiting, and severe abdominal pain. However, chronic exposure can cause cardiovascular abnormalities, nephrotoxicity, neurotoxicity, and induce hepatotoxicity [33, 35, 36, 37].

1.2.7 Mercury

Mercury (Hg) predominantly exists either in an inorganic or organic form. Its inorganic form includes metallic mercury and mercury vapor. Elemental mercury (Hg) takes on a metallic state and is also referred to as ‘quicksilver’ due to its silvery-white color. Metallic Hg is a heavy, liquid metal that readily vaporizes in the air to become mercury vapor. Mercury naturally resides within the earth’s crust and is released into the environment from the weathering of rocks as well as volcanic activity. However, the main release of mercury is anthropogenic through industrial activity, coal combustion activity, and mining [27, 28]. The main route of exposure of inorganic mercury is via inhalation of elemental mercury vapor particles in the air from industrial and manufacturing production. Approximately 70-85% of mercury ions are absorbed via the lungs; however, 3% of mercury will also be absorbed dermally, and 0.1% will be absorbed by the gastrointestinal (GI) tract. These inorganic Hg salts are corrosive to both the skin and GI tract and could potentially induce nephrotoxicity. Mercury that enters oceans, lakes, and rivers settles into the water sediment and is transformed by aquatic biota into the organic form of mercury. This occurs when inorganic mercury is methylated to produce methylmercury. Methylmercury (MeHg) is a persistent substance that is highly toxic due to its ability to bioaccumulate within organisms. As concentration levels elevate within organisms, other wildlife and humans are exposed to MeHg through biomagnification within the aquatic food chain. The fish with the greatest ability to bioaccumulate are larger predatory fish at the top of food chains [29]. The main route of MeHg exposure is through ingestion of contaminated fish from both salt and fresh water. In addition to GI tract absorption, MeHg is also absorbed through the skin and lungs. Once these ions are absorbed and distributed into circulation, MeHg enters the erythrocytes where they mainly bind to hemoglobin (Hb) in addition to a small amount that binds to plasma

proteins. The MeHg oxidizes the Hb iron to its ferric state which inhibits oxygen binding. Instead, methemoglobin forms which inhibits oxygen transportation. Furthermore, this complex reduces blood oxygenation ultimately leading to impaired aerobic respiration and neurotoxicity in the central nervous system. Additionally, MeHg directly interacts and interferes with the thiol group of the glutathione (GSH) antioxidant system responsible for mediating and preventing neurotoxicity. MeHg decreases GSH levels, leading to the formation of reactive oxygen species, and oxidative damage within vital biomolecules. This ultimately results in neurotoxicity and brain tissue damage [30, 31, 32].

Despite the toxicity that metals pose to biological systems, specific metals have been deemed essential for a variety of biochemical and physiological functions. Furthermore, deficient amounts of essential heavy metals can result in a variety of diseases and complications. However, the properties of these metals that exert beneficial functions are the same characteristics potentially leading to toxicity. On the other hand, trace amounts of non-essential heavy metals continuously circulate in the environment, and directly interfere with cellular functions, enzymatic metabolism, detoxification, and general biochemical reactions. However, their bioavailability is dependent on multiple physical factors including temperature, sequestration, and adsorption. These factors influence the degree of toxicity that can occur within the body. The distinguishing factor that differentiates between essential, toxic, and even lethal heavy metals is the dosage. Furthermore, any metal becomes toxic at a high enough dose, even essential metals. For example, copper is an essential co-factor for various vital enzymes related to monitoring oxidative stress as well as involved in carbohydrate metabolism and forming hemoglobin. However, excessive exposure of any essential elements leads to cellular and tissue damage. In the case of copper, excessive exposure leads to Wilson disease where copper

accumulates in the liver, brain, and other vital organs, and can cause hepatotoxicity. The concentration range is narrow when differentiating between beneficial and toxic doses of these trace elements [37, 38].

Regardless of if heavy metals are being consumed at beneficial doses, they accumulate in the environment and resist breakdown due to their persistent nature. Not only do they accumulate in the environment, but they also contaminant food chains in the biota. As these compounds build-up from both naturally occurring and anthropogenic sources, bioaccumulation occurs within the organisms of the ecosystem, ultimately leading to biomagnification within the food web. At this point, the apex predators have accumulated toxic and even lethal doses of the heavy metals consumed by the lower trophic levels. These toxic concentrations interfere with the chemical stimuli in organisms that are crucial for communication in their environment. Furthermore, the toxic interactions produced by the heavy metals within the organism can lead to deformities which negatively affect natural predator-prey dynamics in the population [33].

1.3 Microorganisms

In addition to the inorganic impurities produced during the synthesis of pharmaceuticals and supplements, as in the case of heavy metals, organic impurities can be produced in the form of microorganism contaminants. This form of contamination, like its inorganic relatives, emerges from lack of proper regulatory protocol. The presence of microorganisms typically results from two related sources: lack of quality control and quality assurance, and failure to establish effective identification and preventive measures. Despite the concern for the presence of unintentional microorganisms in supplementary compounds, some microorganisms are naturally occurring and are vital for every day biological processes. Like heavy metals, essential and non-essential microorganisms exist in the environment. Essential microbes contribute beneficial

properties by producing primary metabolites including amino acids, vitamins, and nucleotides. These metabolites have the ability of producing secondary metabolites as well which provide essential compounds required for agriculture. Furthermore, one of the essential roles of microbes today is in the production of antibiotics and other drug therapies by utilizing microbial secondary metabolites, which account for approximately half of the pharmaceuticals circulating in the market [40].

Microbial properties are not only applicable to the external environment, but also to the internal environment. Gut microbiota are essential constituents, along with nutrients and host cells, in the complex ecosystem that exists within the human gastrointestinal tract. The human immune system recognizes commensal microbes to be able to achieve gut homeostasis while maintaining trigger responses against entero-pathogens. Gut microbes assist in maintaining the integrity of the mucosal barrier in addition to providing nutrients and protect against pathogens. Homeostasis is maintained between the mucosal immune system and the commensal microbiota via pattern recognition receptors within epithelial cells. These receptors recognize molecular factors produced by the intestinal microbes responsible for mediating and resolving inflammation, discriminating between beneficial and pathogenic bacteria, and regulating the number of immune cells and pattern recognition receptors [41].

In addition to normal flora, infectious bacteria capable of causing host damage attempt to reside within the human ecosystem. This type of microbial activity is indicative of pathogens [42]. These organisms, like any other organism, strive to live and procreate, typically at the expense of its host, via a parasitic relationship. Humans become ideal hosts due to desirable properties including a nutrient-rich, uniform, warm, and moist environment. However, these pathogens must be able to successfully infiltrate their host, establish a nutritionally compatible

niche within the environment, avoid innate immune response and normal flora regulatory mechanisms, replicate within the invaded environment using natural resources, and exit their current host in order to target a new host. Pathogens accomplish this either by gaining access through weakened immune systems, or by developing highly specialized mechanisms that initiate a response from the host.

Despite the vast phylogenetic diversity of bacteria, only a small portion are dedicated pathogens. Most bacteria are free-living and are reliant on their host for nutrition; however, they perform most of their metabolic functions themselves. Furthermore, distinct diversity exists even within each class of pathogen [43]. Within the scope of pathogenic bacteria, familiar types of genera have been associated with various clinical diseases observed in humans.

1.3.1 *Staphylococcus*

The genus *Staphylococcus* contains clump-forming, gram-positive cocci bacteria that are pathogenic to humans and other mammals. This genus is divided into two groups based on if the bacteria is capable of performing the coagulase reaction. The inability to clot blood plasma, coagulase-negative staphylococci (CNS), constitutes over thirty other species, while staphylococci able to clot blood plasma, coagulase-positive staphylococci (CPS), comprises the most pathogenic species known as *Staphylococcus aureus*. This species' pathogenesis is multifactorial and is the main bacterial cause of nosocomial and community-acquired infections. Additionally, this species commonly produces styes, furuncles, and boils, among other superficial skin infections. Furthermore, more severe infections caused by *S. aureus* include pneumonia, endocarditis, mastitis, and meningitis [42]. Another species that is characterized as CPS is *Staphylococcus warneri*. These bacteria are gram-positive spheres that appear in clusters. Unlike *S. aureus*, *S. warneri* is an infrequent pathogen. Instead, it is a common commensal

constituent of mammalian skin flora. When infectious, *S. warneri* is a rare microbial factor in urinary tract infections (UTI), and typically only occurs in immunocompromised host states as well as prosthetic devices and catheters situations [44, 45]. In the other group of coagulase-negative staphylococci (CNS), *Staphylococcus epidermidis* consists of non-motile, gram-positive cocci that cluster in grape-like, raised, white colonies. This species is a commensal epithelial microflora in humans but can be associated with nosocomial infections relating to prosthetic devices and catheters [46, 47]. Additionally, a potential identifying characteristic of *S. epidermidis* is its production of "slime" when isolated. However, controversy surrounds why this slime is produced. Many perspectives attribute it to the type of growth medium used, whereas others identify it as a virulence marker based on in vitro manifestation and its ability to form a biofilm in vivo [44]. Another species in the CNS group is *Staphylococcus hominis*. These bacteria are commensal epidermis bacteria that are non-motile, gram-positive clusters of spherical cells and are considered infrequent pathogens [48]. *S. hominis* is found in niches containing apocrine glands and can lead to nosocomial infections when immunocompromised states occur within the host [44, 49].

1.3.2 *Bacillus*

The genus of *Bacillus* consists of rod-shaped bacterial species that are either aerobic and form endospores or are facultative anaerobes. Furthermore, one endospore is formed per cell and is resistant to extreme environmental conditions including temperature, radiation, moisture, and disinfectants. Despite most of these species being gram-positive, some species cultures may become gram-negative over time. A diverse physiology exists within Bacillus allowing for life in every natural environment [50]. *Bacillus anthracis* is a non-hemolytic, non-motile bacterial species acquired through direct or indirect contact with infected herbivores. Infection occurs

indirectly by handling infected material, ingestion of infected meat, or inhalation of dust containing spores, leading to cutaneous anthrax in the form of eschar with edema, intestinal anthrax, and pulmonary anthrax, respectively. *B anthracis* is the causative agent of anthrax and is the only permanent pathogen within the genus *Bacillus* capable of causing an epidemic disease in mammals. Its principal virulence factors are its capsule as well as a three-component toxin which are both encoded on plasmids [50, 51]. Within the same genus, *B cereus* is a motile, hemolytic, facultative anaerobe commonly found in soil, food, and vegetation, that is toxin-producing [52]. Two food poisoning syndromes result from infection including an emetic syndrome with rapid onset, and a diarrheal syndrome with a slower onset [50]. The emetic syndrome typically results from the consumption of rice products as well as other starchy foods, whereas the diarrheal syndrome generally results from consuming contaminated meats, fish, milk, and vegetables. *B cereus* produces various aggressions, hemolytic and non-hemolytic components, and enterotoxins that lead to necrosis by activating inflammatory caspases within the infected tissue [52]. Another bacterial species within this genus is *Bacillus subtilis*, also referred to as hay or grass *bacillus*. This species is a soil-dwelling, gram-positive, non-pathogenic, commensal gut microbe that is found in a range of environments. Common uses for *B. subtilis* include probiotics for intestinal and immune health as well as pharmaceutical antibiotics against *Heliobacter pylori*. The only potential pathogenic activity associated with this species is septicemia, endocarditis, meningitis, and a myriad of infections occurring in the GI, urinary, or respiratory tract that occur only in rare circumstances [50, 53].

1.3.3 *Pseudomonas*

The genus of *Pseudomonas* consists of 191 species of gram-negative, aerobic, Gamma-proteobacteria. Additionally, they are motile via a single polar flagellum that commonly reside in

soil, fresh water, and general marine environments, and rapidly colonize in hospital-related materials. *Pseudomonas aeruginosa* is an emphasized species within this genus due to its opportunist pathogenic nature. This species accounts for nearly 80 percent of opportunistic nosocomial infections by *Pseudomonads*. Of those, approximately 50 percent fatality occurs. The most common infections produced by *P. aeruginosa* include endocarditis, UTIs, pneumonia, central nervous system, and musculoskeletal system. Additionally, this bacterial species targets hospitalized patients with cystic fibrosis and cancer as well as burn patients. *P. aeruginosa* causes neutropenia in cancer patients along with patients receiving immunosuppressive drugs by producing an exotoxin similar to diphtheria known as toxin A [54, 55].

1.3.4 *Salmonella*

The genus *Salmonella* consists of gram-negative, rod-shaped bacteria, and is divided into two species: *enterica* and *bongori*. *S. enterica* is the type species within this genus which is further divided into six subspecies. Furthermore, *S. enterica* is a zoonotic, facultative intracellular pathogen capable of colonizing within animals, plants, and humans. The species causes salmonellosis and can reside within the environment. Transmission of this bacterial species is typically foodborne by ingesting contaminated food or water; however, infection can occur through direct contact with infectious animals and other individuals. Ingested pathogenic salmonellae can withstand passing through gastric acid to ultimately invade intestinal mucosa and produce toxins. The foodborne form of salmonellosis is referred to as enteric salmonellosis, or non-typhoidal, and is typically self-limiting. Associated characteristics include vomiting, diarrhea, and abdominal pain. The typhoidal form is referred to as Typhoid fever and is spread interpersonally via the fecal-oral route. Associated characteristics with Typhoid fever include lethargy, anorexia, and headache with limited to no intestinal symptoms [56, 57].

1.3.5 *Escherichia coli*

Escherichia consists of five motile, gram-negative, rod-shaped, coliform species, the type species being *E. coli*. *E. coli* is the most common facultative anaerobe found in the lower intestine, and typically resides as a nonpathogenic, commensal microbe in the human GI tract. Infectious *E. coli* strains are opportunistic pathogens that take advantage of immunocompromised host states resulting from ingesting contaminated food or water. These strains can cause urinary tract infections (UTIs), blood and central nervous system infections in the form of sepsis and meningitis, and enteric disease. Diarrheagenic *E coli* (DEC) strains are associated with characteristic virulence factors and include five pathotypes: enterotoxigenic *E coli* (ETEC), enteropathogenic *E coli* (EPEC), Shiga toxin-producing *E coli* (STEC), enteroaggregative *E coli* (EAEC), and enteroinvasive *E coli* (EIEC). Symptoms often include diarrhea with a consistency ranging from watery to bloody, abdominal pain, and cramps [58, 59].

Commensal bacterial relationships are essential between certain microorganisms and humans for maintaining gut homeostasis as well as the mucosal immune system. Furthermore, when intentionally and appropriately utilized, microbes assist the human environment through the development of probiotics and antibiotics. However, when unregulated, these same beneficial microbes can become opportunistic pathogens, and take advantage of their immunocompromised host. Regulatory measures are vital when utilizing the same sources where bacterial species naturally reside to avoid unintended consumption. Without effective identification and deliberate application, the same beneficial microbes that contribute toward human health are the same bacteria that can exert toxicity and result in community-acquired infections.

1.4 Regulation

The current era of consumerism exists with a high level of accessibility to information

attainable through a mere web search. Reviews, comments, and photos for nearly any product can be found within seconds. Furthermore, within the scientific community, research, data, and publications have become equally as accessible to the public. As the concern for human health continues to grow, this accessibility becomes vital to not only the scientific community, but to all fields of research, as well as the average consumer. Fast-paced individuals require concentrated time put toward supplementing any lacking areas of health including exercise, dietary, and physiology in general that result in illness. Common chronic diseased states such as cardiovascular, obesity, diabetes, blood pressure, anxiety, and hyperactivity, all interfere with daily life. As a result, consumers strive for enhanced health and wellness to prevent the onset of mental and physiological stressors. Some individuals find solutions by using medications and pharmaceuticals; however, numerous consumers experience negatively associated side effects. These undesirable effects can either result from interactions with other medications being taken, or from individual contraindications that prevent using the drug. Additionally, chronic use of these drugs can become costly and unaffordable. An alternative approach to avoid the use of pharmaceuticals is through plant and nutrition-based nutraceuticals. These compounds are naturally derived and use bioactive compounds to achieve the same results as synthetic medications [60]. Due to the natural derivation of nutraceuticals, their bioactive constituents and mechanisms are not fully researched and understood. Additionally, presence of microbial and metal contamination could persist from their originating environment. As the demand for more natural solutions increase, information and safety levels become essential in establishing therapeutic and toxic ranges of these compounds. Within this defining process, these alternative compounds create difficulty surrounding the category they most appropriately reside in. The FDA defines a nutraceutical as a food-derived supplementary compound that benefits health

through the prevention and treatment of disease. Since the term “nutraceutical” originates from both classifications of nutrition and pharmaceuticals, it is technically not regulated as a pharmaceutical drug, and tends to fall into the largely inclusive class of food despite its large constituents of cognitive enhancers, physical enhancers, vitamins, supplements, pre- and post-workouts, and hormone enhancers [3]. This gray area of classification quickly becomes complicated when determining appropriate and safe levels of consumption for consumers. Due to the lack of explicit definition, inadequate research is conducted regarding nutraceuticals which inherently results in an overall absence of information. Consequently, general ranges and vague safety measures have become implemented as a substitute for scientific data and values. Additionally, any established data concerning contamination levels of microbes or metals as well as potentially toxic ingredients found in nutraceuticals is buried within dense federal documents. This inaccessibility to credible data does not allow for informed decisions to be made regarding health on both an individual and group level.

Microbial safety levels and regulation are difficult due to their diverse properties allowing them to proliferate in a wide variety of biological environments that can be used as bioactive sources. To establish a general level of microbial contamination, random sampling is implemented which does not accurately express existing contamination levels [59]. Furthermore, microbiological criteria pertaining to canned, chilled, cooked, baked, dairy products, and raw products are well established and readily indicate satisfactory, unsatisfactory, and borderline values associated with health and safety [62, 63, 64]. Unfortunately, these same resources fail to address any microbial contamination levels within compounds that fall within the classification of naturally derived nutraceuticals.

Similarly, heavy metal contamination has been extensively researched and analyzed as it

relates to environmental impacts as well as overall health and safety. Effective regulation has been established by defining permissible exposure limits (PEL) in addition to recommended levels. These values are maintained and updated by the Occupational Safety and Health Administration (OSHA), the National Institute for Occupational Safety and Health (NIOSH), and the American Conference of Governmental Industrial Hygienists (ACGIH) [83]. However, due to the ubiquitous nature of metals, their presence in food and drug-related supplements is inevitable. Despite the FDA mentioning general provisions for lead (as Pb), arsenic (as As), and mercury (as Hg) in the Code of Federal Regulations as it pertains to food and drugs, this document fails to mention any other impactful metals such as nickel, copper, and cadmium, among others. Furthermore, it fails to discuss compounds that do not classify as food or drugs which is essential is properly regulating the broad class of nutraceuticals [66, 67, 68].

Current regulatory measures for “dietary supplements,” which includes any supplementary compound regardless of its function, verbally prohibits manufacturing and distribution of supplements that either contain adulterants or are misbranded in some way. To ensure that manufacturers comply with the terms of the FDA and the Dietary Supplement and Education Act (DSHEA), the firms are responsible for evaluating the safety of their own products and labelling them appropriately as pertaining to the Federal Food, Drug, and Cosmetic Act. At no point during this process is there quality control or assurance required to be analyzed or approved by outside regulation. Intervention and action occur only when adulterated or misbranded products have been distributed and reached the market. Additionally, these disciplinary actions include issuing a warning letter to the offending company to inform them that they are in violation. From there, further ramifications are not defined. Furthermore, the FDA declares that no actions should impose regulatory barriers that would limit the distribution

of safe products to consumers. However, they do not define any preventative parameters that ensures the safety and accuracy of the products before reaching consumers. Intervention occurs only when violation and injury has occurred [69, 70].

Ultimately, by not distinguishing between nutraceuticals, food, and pharmaceuticals, this gray area cannot be appropriately researched and analyzed. Until then, general understanding and lack of proper regulation will continue to result in lack of information and injury. Preventative measures require testing the original, raw product source, followed by rigorous testing before processing and manufacturing, and confirmation following processing and manufacturing to ensure maximum quality and safety of product. At that point, reliable and scientifically based data can be generated and accessed to a variety of dependent consumers, particularly sensitive population groups such as infants, children, and the elderly [71].

1.5 References

[1] Dept. of Health and Human Services, Centers for Disease Control and Prevention, National Center for Health Statistics. (2014). *Health, United States, 2013: With special feature on prescription drugs*.

[2] Salvador DM (2021) GMOs and Human Health: A Review. Health Sci J. 15 No. 10: 894.

[3] Manikandaselvi, S., V. Vadivel, and P. Brindha. "Nutraceuticals–An overview." *Int J Pharm Bio Sci* 7.1 (2016): 284-296.

[4] Iyen, Sandra, I., Ogofotha, Oghenekeno, G., & Odiba, J. (2019). Advances in nutraceuticals. *International Journal of Medicinal Plants and Natural Products*, *5*(2). https://doi.org/10.20431/2454-7999.0502002

[5] Espín, J. C., García-Conesa, M. T., & Tomás-Barberán, F. A. (2007). Nutraceuticals: Facts and fiction. *Phytochemistry*, *68*(22-24), 2986–3008. https://doi.org/10.1016/j.phytochem.2007.09.014

[6] Nasri, H., Baradaran, A., Shirzad, H., & Rafieian-Kopaei, M. (2014, November 6). *New Concepts in nutraceuticals as alternative for pharmaceuticals*. International journal of preventive medicine. Retrieved September 28, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4336979/

[7] Williamson, E. M., Liu, X., & Izzo, A. A. (2019, December 4). *Trends in use, pharmacology, and clinical ... - wiley online library*. Trends in use, pharmacology, and clinical applications of emerging herbal nutraceuticals. Retrieved September 30, 2022, from https://bpspubs.onlinelibrary.wiley.com/doi/abs/10.1111/bph.14943

[8] Gil, F., Hernández, A. F., & Martín-Domingo, M. C. (2021, January 29). *Toxic contamination of Nutraceuticals and Food Ingredients*. Nutraceuticals (Second Edition). Retrieved September 30, 2022, from https://www.sciencedirect.com/science/article/pii/B9780128210383000689

[9] Filipiak-Szok, A., Kurzawa, M., & Szłyk, E. (2014, November 4). *Determination of toxic metals by ICP-MS in Asiatic and European medicinal plants and dietary supplements*. Journal of Trace Elements in Medicine and Biology. Retrieved September 30, 2022, from https://www.sciencedirect.com/science/article/pii/S0946672X14001886

[10] Wu, W., Wu, P., Yang, F., Sun, D.-ling, Zhang, D.-X., & Zhou, Y.-K. (2018, February 20). *Assessment of heavy metal pollution and human health risks in urban soils around an electronics manufacturing facility*. Assessment of heavy metal pollution and human health risks in urban soils around an electronics manufacturing facility Author links open overlay panel. Retrieved September 30, 2022, from https://www.sciencedirect.com/science/article/pii/S0048969718305825

[11] Bridges, K. R. (2015, February 16). *Iron Deficiency* . Iron absorption. Retrieved September 30, 2022, from https://onlinelibrary.wiley.com/doi/10.1002/9780470015902.a0002277.pub2

[12] Bermejo, F., & García-López, S. (2009, October 7). *A guide to diagnosis of iron deficiency and iron deficiency anemia in digestive diseases*. World journal of gastroenterology. Retrieved September 30, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2754511/

[13] Schroeder, H. A., Nason, A. P., Tipton, I. H., & Balassa, J. J. (1966, December 29). *Essential trace metals in man: Zinc. relation to environmental cadmium*. Journal of chronic diseases. Retrieved September 30, 2022, from https://pubmed.ncbi.nlm.nih.gov/6023228/

[14] Salgueiro, M. J., Zubillaga, M., Lysionek, A., Sarabia, M., Caro, R., Paoli, T. D., Hager, A., Weill, R., & Boccio, J. (2000, July 6). *Zinc as an essential micronutrient: A Review*. Nutrition Research. Retrieved September 30, 2022, from https://www.sciencedirect.com/science/article/pii/S0271531700001639

[15] Krebs, N. F. (2000, May 1). *Overview of zinc absorption and excretion in the human gastrointestinal tract*. The Journal of Nutrition. Retrieved September 30, 2022, from https://academic.oup.com/jn/article/130/5/1374S/4686378

[16] Maares, M., & Haase, H. (2020, March 13). *A guide to human zinc absorption: General overview and recent advances of in vitro intestinal models*. Nutrients. Retrieved September 30, 2022, from

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7146416/#:~:text=Intestinal%20zinc%20absorption%20is%20mainly,the%20portal%20blood%20%5B83%5D%20

[17] Tuerk, M., & Fazel, N. (2009, March). *Zinc deficiency*. Current Opinion in Gastroenterology. Retrieved September 30, 2022, from https://journals.lww.com/co-gastroenterology/Fulltext/2009/03000/Zinc_deficiency.9.aspx

[18] Brunori, M. (2001, June 22). *Iron and copper in cytochrome oxidase: Spectral and kinetic studies*. Inorganica Chimica Acta. Retrieved September 30, 2022, from https://www.sciencedirect.com/science/article/pii/S002016930092108X

[19] National Academy Press. (2000). *Copper in drinking water*.

[20] Goyer, R. A. (1997). Toxic and essential metal interactions. *Annual Review of Nutrition*, *17*(1), 37–50. https://doi.org/10.1146/annurev.nutr.17.1.37

[21] Pinot, F., Kreps, S. E., Bachelet, M., Hainaut, P., Bakonyi, M., & Polla, B. S. (2000, July 1). *Cadmium in the environment: Sources, mechanisms of biotoxicity, and biomarkers*. Reviews on Environmental Health. Retrieved September 30, 2022, from https://www.degruyter.com/document/doi/10.1515/REVEH.2000.15.3.299/html

[22] Rafati Rahimzadeh, M., Rafati Rahimzadeh, M., Kazemi, S., & Moghadamnia, A.-A. (2017, April 8). *Cadmium toxicity and treatment: An update*. Caspian journal of internal medicine. Retrieved September 30, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5596182/#:~:text=Mechanism%20of%20Toxicity%3A%20Cadmium%20affects,induction%20of%20apoptosis%20(15).

[23] Body, P. E., Inglis, G., Dolan, P. R., & Mulcahy, D. E. (2009, January 9). *Environmental lead*. Taylor & FrancisCritical Reviews in Environmental Control. Retrieved September 30, 2022, from https://www.tandfonline.com/doi/abs/10.1080/10643389109388403

[24] *Common sources of lead.* in the Home- EH: Minnesota Department of Health. (n.d.). Retrieved September 30, 2022, from https://www.health.state.mn.us/communities/environment/lead/fs/common.html

[25] Flora, G., Gupta, D., & Tiwari, A. (2012, June 5). *Toxicity of lead: A review with recent updates*. Interdisciplinary toxicology. Retrieved September 30, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3485653/

[26] Manna, K., Debnath, B., & Singh, W. S. (2019). Sources and toxicological effects of lead on human health. *Indian Journal of Medical Specialities*, *10*(2), 66. https://doi.org/10.4103/injms.injms_30_18

[27] Philbert, M. A. (2010). Introduction to the nervous system and behavioral toxicology. *Comprehensive Toxicology*, 1. https://doi.org/10.1016/b978-0-08-046884-6.01301-4

[28] Bernhoft, R. A. (2012). Mercury toxicity and treatment: A review of the literature. *Journal of Environmental and Public Health*, *2012*, 1–10. https://doi.org/10.1155/2012/460508

[29] National Research Council, Commission on Life Sciences, Board on Environmental Studies and Toxicology, Committee on the Toxicological Effects of Methylmercury. (2000). *Toxicological effects of Methylmercury*. National Academy Press.

[30] Kutayli, W. M., & Silberstein, P. (2014). Methemoglobinemia. *Reference Module in Biomedical Sciences*. https://doi.org/10.1007/springerreference_109708

[31] The Toxicology of Mercury Larry A. Broussard, PhD, DABCC, FACB,1Catherine A. Hammett-Stabler, PhD, DABCC, FACB,2Ruth E. Winecker, PhD,2,3 Jeri D. Ropero-Miller, PhD

[32] Farina, M., Rocha, J. B. T., & Aschner, M. (2011). Mechanisms of methylmercury-induced neurotoxicity: Evidence from experimental studies. *Life Sciences*, *89*(15-16), 555–563. https://doi.org/10.1016/j.lfs.2011.05.019

[33] Hughes, M. F. (2002). Arsenic toxicity and potential mechanisms of action. *Toxicology Letters*, *133*(1), 1–16. https://doi.org/10.1016/s0378-4274(02)00084-x

[34] Ratnaike, R. N. (2003). Acute and chronic arsenic toxicity. *Postgraduate Medical Journal*, *79*(933), 391–396. https://doi.org/10.1136/pmj.79.933.391

[35] Kaur, T., Singh, A. P., & Goel, R. K. (2011). Mechanisms pertaining to arsenic toxicity. *Toxicology International*, *18*(2), 87. https://doi.org/10.4103/0971-6580.84258

[36] Jomova, K., Jenisova, Z., Feszterova, M., Baros, S., Liska, J., Hudecova, D., Rhodes, C. J., & Valko, M. (2011). Arsenic: Toxicity, oxidative stress and human disease. *Journal of Applied Toxicology*. https://doi.org/10.1002/jat.1649

[37] Luch, A. (2012). Molecular, clinical and environmental toxicology. *Experientia Supplementum*. https://doi.org/10.1007/978-3-7643-8340-4

[38] Borzelleca, J. F. (2000). Paracelsus: Herald of modern toxicology. *Toxicological Sciences*, *53*(1), 2–4. https://doi.org/10.1093/toxsci/53.1.2

[39] Ali, H., Khan, E., & Ilahi, I. (2019). Environmental chemistry and ecotoxicology of hazardous heavy metals: Environmental persistence, toxicity, and bioaccumulation. *Journal of Chemistry*, *2019*, 1–14. https://doi.org/10.1155/2019/6730305

[40] Demain, A. L., & Sanchez, S. (2009). Microbial Drug Discovery: 80 years of progress. *The Journal of Antibiotics*, *40*(19), 5–16. https://doi.org/10.1002/chin.200919215

[41] Hevia, A., Delgado, S., Sánchez, B., & Margolles, A. (2015). Molecular players involved in the interaction between beneficial bacteria and the immune system. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.01285

[42] Casadevall, A., & Pirofski, L.-A. (2009). What is a pathogen? *Annals of Medicine*, *34*(1), 2–4. https://doi.org/10.1080/07853890231733858O

[43] Wilson, J. H., & Hunt, T. (2002). *Molecular biology of the cell, 4th edition: A problems approach*. Garland Science.

[44] Baron, S. (1996). *Medical microbiology* (4th ed.). University of Texas Medical Branch at Galveston.

[45] Kanuparthy, A., Challa, T., Meegada, S., Siddamreddy, S., & Muppidi, V. (2020). Staphylococcus warneri: Skin commensal and a rare cause of urinary tract infection. *Cureus*, *12*. https://doi.org/10.7759/cureus.8337

[46] Otto, M. (2009). Staphylococcus epidermidis — the 'accidental' pathogen. *Nature Reviews Microbiology*, *7*(8), 555–567. https://doi.org/10.1038/nrmicro2182

[47] Vuong, C., & Otto, M. (2002). Staphylococcus epidermidis infections. *Microbes and Infection*, *4*(4), 481–489. https://doi.org/10.1016/s1286-4579(02)01563-0

[48] Kloos, W. E., & Schleifer, K. H. (1975). Isolation and characterization of Staphylococci from human skin II. descriptions of four new species: Staphylococcus warneri, Staphylococcus capitis, Staphylococcus hominis, and Staphylococcus simulans. *International Journal of Systematic Bacteriology*, *25*(1), 62–79. https://doi.org/10.1099/00207713-25-1-62

[49] Elsevier, Academic Press. (2016). Staphylococcus Hominis. In *Encyclopedia of Food and Health*. essay.

[50] Turnbull, P. (1996). Bacillus. In S. Baron (Ed.), *Medical Microbiology*. (4th ed.). University of Texas Medical Branch at Galveston.

[51] Thorne, C. B. (1993). Bacillus anthracis. *Bacillus Subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics*, 113–124. https://doi.org/10.1128/9781555818388.ch8

[52] McDowell, R. H., Sands, E. M., & Friedman, H. (2022). Bacillus Cereus. In *Statpearls*. essay, National Library of Medicine.

[53] Kovacs, A. T. (2019). Bacillus subtilis. *Trends in Microbiology*, *27*(8), 724–725. https://doi.org/10.1016/0966-842x(93)90082-3

[54] Michel-Briand, Y., & Baysse, C. (2002). The pyocins of pseudomonas aeruginosa. *Biochimie*, *84*(5-6), 499–510. https://doi.org/10.1016/s0300-9084(02)01422-0

[55] Iglewski, B. H. (n.d.). Pseudomonas. In *Medical Microbiology* (4th ed.). essay, University of Texas Medical Branch at Galveston.

[56] Knodler, L. A., & Elfenbein, J. R. (2020). Salmonella enterica. *Trends in Microbiology*, *27*(11), 964–965. https://doi.org/10.1016/j.tim.2019.05.002

[57] Giannella, R. A. (1996). Salmonella. In *Medical microbiology* (4th ed.). essay, University of Texas Medical Branch at Galveston.

[58] Liu, D. (2019). Escherichia coli. In *Encyclopedia of microbiology* (4th ed.). essay, Elsevier Science Direct.

[59] Nataro, J. P., & Kaper, J. B. (1998). Diarrheagenic Escherichia coli. *Clinical Microbiology Reviews*, *11*(1), 142–201. https://doi.org/10.1128/cmr.11.1.142

[60] Shahgholian, N. (2022). Introduction to nutraceuticals and natural products. *Handbook of Nutraceuticals and Natural Products*, 1–14. https://doi.org/10.1002/9781119746843.ch1

[61] Pérez-Lavalle, L., Carrasco, E., & Valero Diaz, A. (2020). Microbiological criteria: Principles for their establishment and application in food quality and safety. *Italian Journal of Food Safety*, *9*(1), 8543. https://doi.org/10.4081/ijfs.2020.8543

[62] Food and Agriculture Organization of the United Nations, & World Health Organization. (2021). Microbiological risk assessment – guidelines for food. *Food and Agriculture Organization of the United Nations*, 288. https://doi.org/10.4060/cb5006en

[63] Food and Agriculture Organization of the United Nations, & World Health Organization. (2005). *Codex alimentarius General Requirements for Food Hygiene* (2nd ed.). Food and Agriculture Organization of the United Nations.

[64] *CFR - Code of Federal Regulations Title 21*. U.S. Food and Drug Administration. (n.d.). Retrieved October 3, 2022, from https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=112.55

[65] United States Department of Labor. (n.d.). *Permissible Exposure Limits*. Permissible Exposure Limits – OSHA Annotated Table Z-1 | Occupational Safety and Health Administration. Retrieved October 3, 2022, from https://www.osha.gov/annotated-pels/table-z-1

[66] Ciociola, A. A., Cohen, L. B., Kulkarni, P., Kefalas, C., Buchman, A., Burke, C., Cain, T., Connor, J., Ehrenpreis, E. D., Fang, J., Fass, R., Karlstadt, R., Pambianco, D., Phillips, J., Pochapin, M., Pockros, P., Schoenfeld, P., & Vuppalanchi, R. (2014). How drugs are developed and approved by the FDA: Current process and future directions. *American Journal of Gastroenterology*, *109*(5), 620–623. https://doi.org/10.1038/ajg.2013.407

[67] Agency for Toxic Substances and Disease Registry. (2019, July 2). *Lead (PB) toxicity: What are U.S. standards for lead levels?* Environmental Health and Medicine Education. Retrieved October 3, 2022, from https://www.atsdr.cdc.gov/csem/leadtoxicity/safety_standards.html

[68] Mensah, E., Kyei-Baffour, N., Ofori, E., & Obeng, G. (n.d.). Influence of human activities and land use on heavy metal concentrations in irrigated vegetables in Ghana and their health implications. *Appropriate Technologies for Environmental Protection in the Developing World*, 9–14. https://doi.org/10.1007/978-1-4020-9139-1_2

[69] U.S. Department of Health and Human Services. (n.d.). *Office of dietary supplements - dietary supplement health and education act of 1994*. NIH Office of Dietary Supplements. Retrieved September 30, 2022, from https://ods.od.nih.gov/about/dshea_wording.aspx

[70] Center for Food Safety and Applied Nutrition. (n.d.). *Dietary supplements*. U.S. Food and Drug Administration. Retrieved September 30, 2022, from https://www.fda.gov/food/dietary-supplements

[71] Neuwirth, L. S., Cabañas, E., Cadet, P., Zhu, W., & Markowitz, M. E. (2022, May 10). *Cereal and juice, lead and arsenic, our children at risk: A call for the FDA to re-evaluate the allowable limits of lead and arsenic that children may ingest*. Environmental Research and Public Health. Retrieved September 30, 2022, from https://www.mdpi.com/1660-4601/19/10/5788

CHAPTER 2

INSTRUMENTATION

2.1 Materials

All the nutraceutical samples analyzed (Table 2.1) were provided by the Food and Drug Administration (FDA) (Dallas, TX), and were compared against deer antler velvet powder capsules (Tobin's Farm, Springboro, PA), deer antler velvet extract (Antler Farms, Canterbury, NZ), and methandienone (Cerilliant, Round Rock, TX) for ingredient evaluation. The samples in powder-form were analyzed directly on a Nicolet iS 10 Fourier-transform infrared spectrometer (FTIR) coupled with a Smart iTR Attenuated Total Reflectance (ATR) accessory (ThermoFisher Scientific, Waltham, MA) using disposable, double-ended spatulas (USA-Scientific Inc, Ocala, FL). The liquid samples were analyzed using the ATR-FTIR as well by directly pipetting 10 μL (Eppendorf, Hamburg, Germany) of sample that had been filtered using 0.45 μm polyether sulfone (PES) syringe filters (ThermoFisher Scientific, Waltham, MA).

Sample Name	Designated #	Sample Name	Designated #	Sample Name	Designated #
Bucked Up Pre-Workout	1	Fast Acting Extenze Maximum Release Male Enhancement	8	Bucked Up Pre-Workout	15
Woke AF Nootropic Pre-Workout	2	Bucked Up Post-Workout Deer Antler Extract Spray	9	Prejym High Performance Pre-Workout (Rainbow Sherbet Flavor)	16
Beyond Raw Nootropic	3	Performix SST v2X Extreme Thermogenic Fat Burner Weight Loss Supplement	10	Beyond Raw Nootropic	17
C4 Ultimate Pre-Workout Performance (Cotton Candy Flavor)	4	C4 Ultimate Pre-Workout Performance (Cotton Candy Flavor)	11	Bucked Up Pre-Workout Deer Antler Extract Spray	18
Prejym High Performance Pre-Workout (Rainbow Sherbet Flavor)	5	Woke AF Nootropic Pre-Workout	12	Fast Acting Extenze Maximum Release Male Enhancement	19
Total War Pre-Workout	6	Total War Pre-Workout	13	Performix SST v2X Extreme Thermogenic Fat Burner Weight Loss Supplement	20
MuscleTech Shatter Pre-Workout (Rainbow Fruit Candy Flavor)	7	MuscleTech Shatter Pre-Workout (Rainbow Fruit Candy Flavor)	14		

Table 2.1: **The analyzed nutraceutical sample brand names and designated sample number**

The solvent mixtures used for direct-injection mass spectrometry (DIMS) were 1 ppm solutions using 1:1(v/v) of chloroform and acetonitrile with 1% ammonium acetate (ThermoFisher Scientific, Waltham, MA), 1:1(v/v) of acetonitrile and methanol with 1% glacial acetic acid (ThermoFisher Scientific, Waltham, MA), and 1:1(v/v) of methanol and water (Millipore, Billerica, MA) and 1% glacial acetic acid (ThermoFisher Scientific, Waltham, MA), respectively. The mass spectrometric analysis of each sample was performed on an API 4000 Q Trap system (AB Sciex, Framingham, MA) equipped with an electrospray ionization source as well as direct injection according to the parameters specified in Table 2.2.

Carrier Gas	Nitrogen (N_2)
Mass Value Range (amu)	20-1,000
Polarity	Positive
Scan type	Q1
Scan Duration	1 minute
Scan Cycle Time (seconds)	0.6
Solvent Delivery Rate (mL/min)	0.05

Table 2.2: **API 4000 Q Trap Instrumentation Parameters for direct-injection mass spectrometry of nutraceutical samples**

Heavy metal analysis of the samples was conducted using an iCAP 7000 Series ICP Spectrometer (ThermoFisher Scientific, Waltham, MA). Sample solutions were prepared by dissolving 0.5 grams of sample into 10 mL of 2% nitric acid stock solution (ThermoFisher Scientific, Waltham, MA). Standard metal solution concentrations of 1 ppm, 5 ppm, and 10 ppm were prepared using zinc, beryllium, iron, chromium (SCP Science, Quebec, CA), arsenic (Inorganic Ventures, Christiansburg, VA), Lead (ThermoFisher Scientific, Waltham, MA), cadmium, mercury (SPEX CertiPrep, Metuchen, NJ), nickel (Sigma-Aldrich, St. Louis, MO), zinc, and copper (PerkinElmer, Austin, TX).

Microorganism detection was performed by culturing each sample in tryptic soy broth (Soybean-Casein Digest Medium) (Fisher Scientific, Waltham, MA) in a shaking incubator at 230 rpm at 37℃ for 24 hours. These cultures were then streaked onto Difco nutrient agar plates (Fisher Scientific, Waltham, MA), and incubated at 37℃ for 24 hours. Eleven bacterial primer pair reaction solutions were prepared using nuclease-free water, GoTaq Master Mix (Promega, Madison, WI), eleven specific primer pairs (Integrated DNA Technologies, Newark, NJ), and a small amount of sample bacteria. Colony-PCR was conducted on these reactions using an Eppendorf Mastercycler Nexus Thermal Cycler (Fisher Scientific, Waltham, MA). The PCR products were visualized on a 1% gel using an Owl EasyCast B1 Gel Electrophoresis System (ThermoFisher Scientific, Waltham, MA), and 1x TAE buffer.

2.2 Mass Spectrometry

Mass spectrometry (MS) is an analytical technique originating in the field of physics with the intent of discovering electrons by J.J. Thomson during his cathode array experimentation in 1897. Thomson's apparatus was initially designed to measure the mass-to-charge (*m/z*) ratios of electrons. Two years later, an updated version of the apparatus was built that could indirectly measure the mass of the electron. Thomson's cathode ray experimentation established the foundation of mass spectrometry, and lead to the creation of the first mass spectrometer that measured the masses of charged atoms. This prototype generated ions from gas discharge tubes which then travelled through parallel electric and magnetic fields. These ions were deflected into parabolic trajectories and were detected using a photographic plate [72].

Mass spectrometers consist of an ion source, a mass analyzer, and a detector. The ion source produces gaseous ions from the sample. Mass analyzers characterize and separate ions into their characterized mass components based on their *m/z*. Finally, the detector is responsible

for detecting the produces ions and recording their relative abundance. Through this process, the structure, and chemical properties of unknown components within samples can be identified through the conversion of the samples into gaseous ions. This is done by generating multiple ions from the sample which are then separated according to their specific *m/z*. The relative abundance of each type of ion is then recorded and plotted. Mass spectrometers produce ions from the sample injected into the ionization source which are then separated according to their *m/z*. The fragments are then fragmented and analyzed in a second mass analyzers which are then detected by the detector as they emerge, and their abundance is converted into an electrical signal. This signal is finally transmitted to a computer where the data can be analyzed [73].

2.3 Triple Quadrupole Mass Spectrometry

The mass analyzer is the component of the mass spectrometer that is responsible for taking ionized masses and separating the ions based on their specific *m/z* which are then output to the detector [80]. These signals are further converted into a digital output for spectral analysis. Multiple mass analyzer systems have the ability of characterizing ions based on their *m/z* values including quadrupole (Q), quadrupole ion trap (QIT), time of flight (ToF), and Fourier transform ion cyclotron resonance (FT-ICR). These analyzers differ in size, resolution, price, mass range, and the ability to perform tandem mass spectrometry (MS/MS) [74]. These variances in instrumentation ultimately determine the most appropriate analyzer based on the properties of the analyte of interest.

Triple quadrupole mass spectrometers (QQQ) utilize three quadrupole mass analyzers (Q1-Q3) each consisting of four rods where DC and RF voltages are applied. Only ions of specific *m/z* are stable and permitted to pass through the quadrupoles when a specific DC/RF voltage is applied [75]. QQQ is considered a tandem MS (MS/MS) technique where Q1 and Q3

are used as working mass filters while Q2 acts as a collision cell [76]. This method generates several ionic species from a sample where only ions of a specific mass are further fragmented and mass analyzed, allowing for the separation and identification of various compounds in a single instrument. The entire instrument, as shown in Figure 2.1, is in series, consisting of an electrospray ionization source, the first quadrupole mass filter (Q1), the quadrupole collision chamber (Q2), the third quadrupole mass filter (Q3), and a particle multiplier. Q1 is responsible for selecting the ions that are further fragmented in Q2 via collision-induced dissociation (CID). Furthermore, Q3 selects the product ions fragmented from Q2, which are then sent to the particle multiplier where the ions are detected [77].

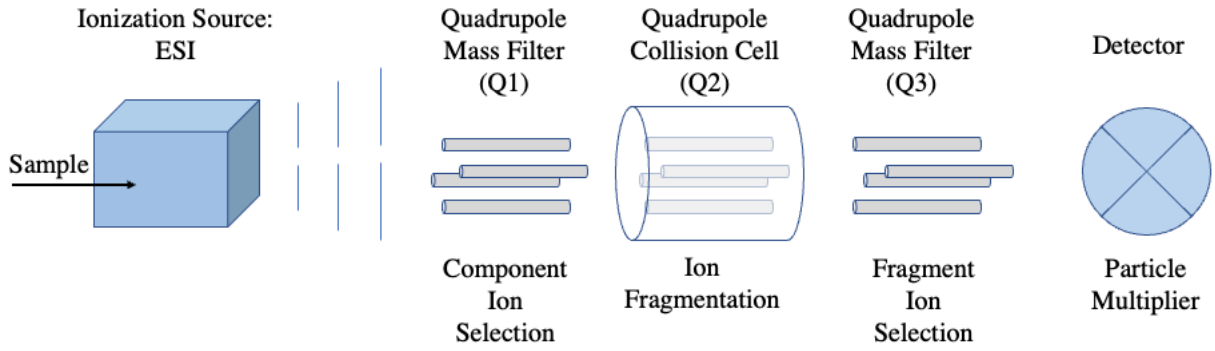

Figure 2.1: **Triple Quadrupole Mass Spectrometer Schematic**

Two modes can be used in quadrupoles: scanning and filtering. During scanning mode, DC an RF voltages are ramped resulting in a full scan of the mass spectra. This mode is more commonly used for qualitative data analysis due to its low sensitivity and slow scan speed, whereas filtering mode is used for quantitative analysis. Single quadrupoles typically utilize selected ion monitoring (SIM) where a fixed set of DC and RF voltages is applied to the quadrupole. However, this method excludes ions with different *m/z* and only allows for a single *m/z* to pass through. On the other hand, triple quadrupole MS/MS utilizes multiple reaction monitoring (MRM) for quantitative analysis which allows for enhanced sensitivity and

selectivity by using multiple quadrupoles. Furthermore, Q1 filters specific precursor ions of interest. The collision cell (Q2) uses CID to generate a product ion by colliding the filtered precursor ion into a neutral collision gas, such as nitrogen. These characterized product ions continue into Q3 where only ions with specific *m/z* can pass through. This double mass filtration system significantly reduces noise as well as increase selectivity [76]. Furthermore, QQQ enhances discrimination of ion fragmentation when compared to a single quadrupole MS system, and obtains molecular structural information, allowing for quantitation [75].

2.4 Electrospray Ionization

The role of the ion source in a mass spectrometer is to produce gaseous ions which can be accomplished through differing methods. The analyte is transferred into the gas phase through ionization either concurrently or through separate processes [78]. Common ionization methods include electron ionization (EI), chemical ionization (CI), matrix-assisted laser desorption/ionization (MALDI), and electrospray ionization (ESI). Each method is specific to certain molecular masses, ranges, and states as well as the desired application of the ionized analyte. Hard ionization techniques, such as EI, bombard high quantities of residual energy onto the analyte to produce a high degree of fragmentation. This technique is appropriate for smaller, organic molecules with relatively lower molecular weights below 600. Although EI is effective for many gaseous molecules, it leads to extensive fragmentation and obscures the molecular ion. On the other hand, soft ion sources, such as ESI, impart less residual energy on the analyte which produces less fragmentation. This technique ionizes the macromolecule into small droplets instead of fragmenting into smaller charged particles. Because of this, soft ionization techniques are more appropriate for larger biological molecules with higher molecular weights [80].

Before soft ionization methods such as ESI were discovered, no ionization method could

overcome extensive analyte fragmentation. Furthermore, as research of large, biological molecules, such as proteins, emerged, the need for molecular mass precision in experimentation became imperative. Hard ionization techniques were not viable options when handling nonvolatile, thermally labile molecules that often lead to destruction of the molecular structures. However, in 1989, John Fenn introduced the revolutionary soft method of ESI which maintained the integrity of proteins and other large molecules. This method produces little residual energy onto the analyte which produces little fragmentation and preserves very weak noncovalent interactions in the gas phase. ESI allows for mass spectrometric analysis to rapidly identify molecular mass as well as structural characteristics of compounds. Ultimately, coupling ESI with mass spectrometry led to the development of proteomics [81].

As previously mentioned, ESI is a soft ionization technique that involves nonvolatile molecular ions that produce gaseous ions, and is typically applied to larger, thermally fragile molecules. Since this technique is a soft method, very little fragmentation of the formed molecular ions is produced, and weak bonds are preserved. This allows for the analysis of post-translationally modified proteins and nucleic acids [79]. Three major processes occur when ESI is used to transfer the analyte from its aqueous, solution state, to its gaseous state. The first process produces charged droplets from the high-voltage capillary tip where the analyte solution is injected. The second process repeats the evaporation of the solvent from the charged droplet where it is disintegrated. This results in a small, charged droplet. The last process employs the mechanism of forming the gas-phase ion [81]. The production of ESI ions requires the dispersal of highly charged droplets at atmospheric pressure followed by a set of conditions that evaporate the droplets. By applying a high electric field to a small flow of liquid away from the capillary tube, an accumulation of charge is produced on the surface of the liquid at the terminus of the

capillary [82]. The liquid surface is pulled into an elliptic shape which becomes a pointed cone at a certain voltage due to electrostatic Coulomb attraction. This tapered cone is referred to as a Taylor Cone, named after Sir Geoffrey Taylor, who demonstrated that an equilibrium of forces that derive surface tension and electrostatic forces can be reached when a liquid cone has an opening angel of 49.3º [83]. The high electric field disrupts the liquid surface leading to the formation of highly charged liquid droplet which is emitted from the tip of the Taylor Cone as shown in Figure 2.2 [82].

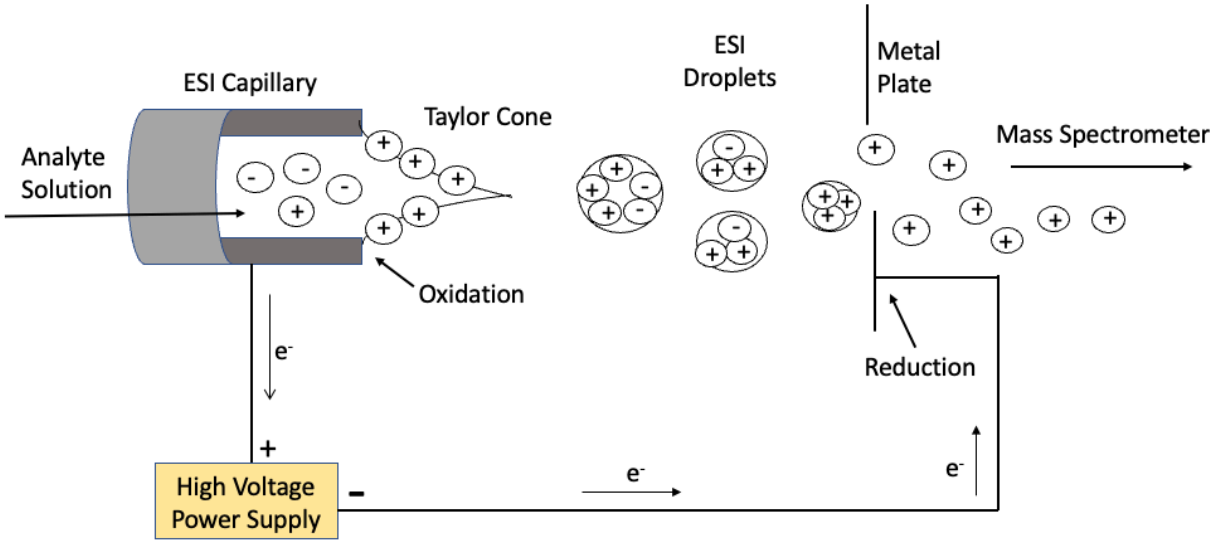

Figure 2.2: **Electrospray Ionization (ESI) Schematic**

The exact ESI mechanism that describes the formation of gas-phase ions from evaporating droplets remains undetermined after nearly four decades of application. The beginning steps of ESI are understood to include the formation of charged droplets that are approximately micro-meter sized that then undergo solvent evaporation and jet fission. This ultimately produces smaller charged droplets approximating nanometer radii. From there, several models are debated regarding the mechanism that forms and releases gaseous ions. Presently, there are three proposed models: the ion evaporation model (IEM), the charge residue model

(CRM), and the chain ejection model (CEM). Most controversy focusses on the two competing ideas of the IEM and the CRM since the CEM focusses on unfolded proteins. The current distinguishing factor is the application of the IEM for small, preformed ions while the CRM is applied to the behavior of larger globular species. However, controversy still surrounds what the defining parameters are for what constitutes “small” and “large” analytes. The cutoff size widely varies ranging between 100 Da to 5 MDa. The mechanism of the IEM suggests that the electric field that emanates from the droplet surface subject the ions to desorption. However, this model requires the ejected ion to overcome certain obstacles including surface deformation, solvent polarization, and ion-droplet coulombic repulsion [83, 84, 85]. On the other hand, the CRM is characterized by the release of the analyte ions via droplet evaporation to dryness [84]. Finally, the CEM, as stated previously, applies to unfolded proteins. This model proposes that protein chains are driven to the droplet surface by exposed nonpolar residues and proceed to gradually migrate out from the droplets [85].

2.5 Collision-Induced Dissociation

Collision-induced dissociation (CID) is a common technique used to fragment gaseous ions produced from soft-ionization methods, such as ESI. This technique is particularly effective when fragmenting ions from larger, non-volatile molecules such as nucleic acids and proteins. CID implements the use of a strong electric field to accelerate a kinetically excited precursor molecule into a neutral buffer gas such as helium, nitrogen, or argon. Each ion will collide with these gaseous molecules several times which will gradually convert into enough internal energy to break the weakest covalent chemical bonds. The resulting fragmented ions are then extracted from the collision chamber and injected into a mass analyzer which further produces a specific fragmentation spectrum. This spectrum reflects the relative energies of the intact bonds as well

as the structure of the precursor and product ions as shown in Figure 2.3.

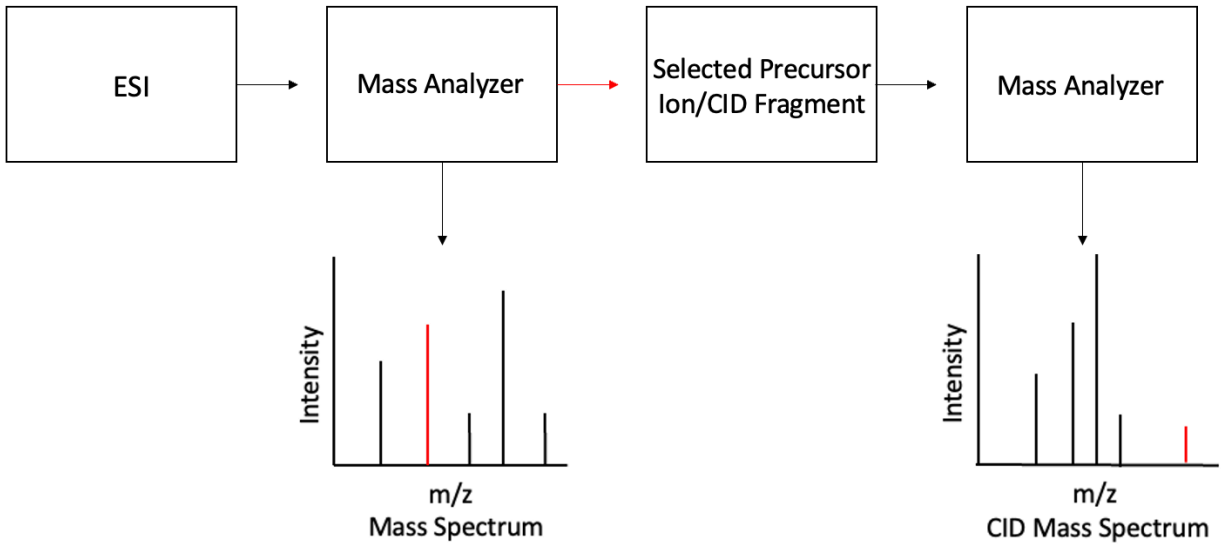

Figure 2.3: **Illustration of isolating precursor ions based on their m/z by the mass analyzer which are then fragmented. The fragmented ions result in product ions that are analyzed again to produce the final fragmentation spectrum for the isolated ion.**

Furthermore, functional groups and linkages can be determined based on the masses of the fragment ions, allowing for the identification of molecules of interest and their chemical structure based on their associated fragmentation pattern [78]. The process of fragmenting precursor ions of interest into product ions is referred to as tandem mass spectrometry (MS/MS) and is commonly coupled with ESI and CID to identify and analyze the chemical composition of large molecules. This method is also used for drug and metabolite identification by scanning and comparing various drug's fragmentation patterns and structures.

2.6 Inductively Coupled Optical Emission Spectroscopy

Due to the risk that heavy metal impurities pose to human health, instrumentation methods such as inductively coupled mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectroscopy (ICP-OES) have been implemented to routinely monitor and quantify levels of heavy metals present in pharmaceuticals and supplements. Atomic

spectrometric techniques are commonly used for trace element analysis and utilizes various methods depending on analyte concentration availability. ICP-OES is among the most sensitive analytical techniques that has become a popular method for elemental speciation due to its ability of determining trace and ultra-trace levels of multiple elements within different sample matrices [87]. Additionally, since ICP-OES quantifies by measuring excited atoms and ions at their characteristic wavelengths within a high-temperature plasma, this method is the most specific multi-element analysis technique. Since this technique is compatible with diverse sample matrices, ICP-OES is highly effective for drug and pharmaceutical trace analysis.

ICP-OES instrumentation consists of inductively coupled plasma and an optical emission spectrometer. The components of these parts include a sampler, pump, nebulizer, spray chamber, ICP torch, mono or polychromator, and detector, as shown in Figure 2.4. Argon gas is used as the supply gas for the torch tube which generates a high frequency magnetic field around the coil.

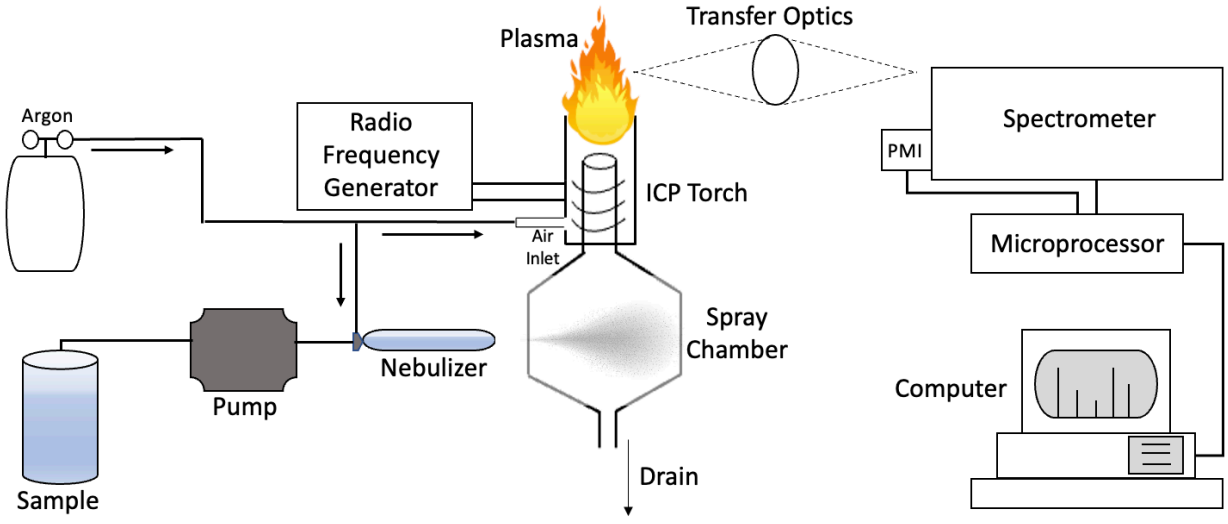

Figure 2.4: **Illustration of Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) Instrumentation**

This strong magnetic field initiates the ionization of the argon in the torch tube. The ionized

argon gas produces plasma with high temperature, energy, and electron density, and is used to excite and emit the molecules in the sample. The sample is typically liquid and is introduced into the nebulizer using a peristaltic pump. The nebulizer transforms the liquid sample into droplets of various sizes. The smaller droplets are directed into the plasma whereas the larger sample droplets are discarded into the drain. The small droplets then pass through the narrow tube in the torch tube where it is atomized by the hot plasma which excites the atoms to a higher energy state. When these atoms return to their low energy state, emission rays corresponding to each metal's wavelength are released. The light waves are then transferred to the optical emission system or spectrometer (OES). Then, an oscillating current is produced inside an induction coil which resides in the plasma tube by a radiofrequency generator (RF). When exposed to the plasma gas, this current develops into an oscillating magnetic field in the sample ions, and the prisms within the OES separate the light into specific wavelengths associated with the elements being analyzed. The detector converts the emitted light from the metal atoms in the plasma into an electrical signal for quantification. Finally, the light is resolved into its constituents by a diffraction grating for further light intensity measurement by a photomultiplier tube (PMT) [88, 89].

Due to the low detection limits for multi-element analysis, ICP-OES is highly advantageous when compared to other elemental analysis techniques. Additionally, ICP-OES has good stability which produces limited spectral interference and low matrix effects. This method can be applied to quantitate nearly all the periodic elements ranging from trace to high concentrations [90].

2.7 Infrared Spectroscopy

The development of infrared (IR) spectrophotometers in the 1940s was initially restricted

as an analytical instrument used for research and developmental applications on organic polymers and compounds, specifically in the field of petrochemistry. These original dispersive spectrophotometers transmitted light through the sample which was then dispersed by a diffraction grating. The intensity of each wavelength was then sequentially detected using a thermocouple [91]. Thermocouples measure temperature by using a sensor consisting of two different types of metals, typically bismuth and antimony, that are joined together at one end. The Peltier Effect occurs when a voltage is generated as the junction is heated by IR radiation, which is proportional to the applied temperature at the junction [92]. Dispersive IR spectrophotometers utilize a double-beam optical configuration to perform an automatic background correction in real time to account for the loss of light intensity [91]. IR spectrophotometers record the relative amount of energy of the IR radiation as it passes through the sample as a function of wavelength/frequency. Furthermore, due to the different chemical structural fragments of various samples, these structural differences in the IR absorption spectrum allow for the identification of different samples [93]. Since functional groups consistently absorb the same frequency range of IR radiation regardless of the other molecule's components, by measuring the amount of IR radiation each compound's chemical bonds absorb, these unique properties can be used to identify different compounds. This correlation between the molecular functional groups and their absorbed IR radiation absorption allows for the identification of molecules, as well as any structural or chemical changes of the molecule [94].

In the 1980s, IR spectroscopy instrumentation was revolutionized by the introduction of Fourier transform infrared (FTIR) spectrometers [95]. The invention of the FTIR was made possible by the Michelson interferometer as shown in Figure 2.5. Albert Abraham Michelson created this optical equipment which is capable of accurately measuring the wavelengths of light.

This instrument works on the principle that the division of light amplitude from an extended source is divided into equal parts of intensity by partial reflection and refraction [96]. This is accomplished by using a beam splitter composed of specific material responsible for transmitting half of the radiation and reflecting the other half. One of the beams is transmitted to the fixed mirror while the second beam is reflected off the beam splitter onto the moving mirror. Both the fixed and the moving mirror reflect the radiation back to beam splitter resulting in one beam passing to the detector, while the other passes back to the source [95]. Unlike a dispersive monochromator, as in IR spectrometry, FTIR uses an interferometer which allows for all the sample's wavelengths to be simultaneously detected. This spectral data is obtained by using the interferometer to collect the resulting interference waveform which is referred to as an interferogram. The interferogram can then be translated into the IR spectrum by using Fourier transform to calculate the intensity at each wavelength [93]. Fourier transform separates the different frequencies of overlapping waveforms [91].

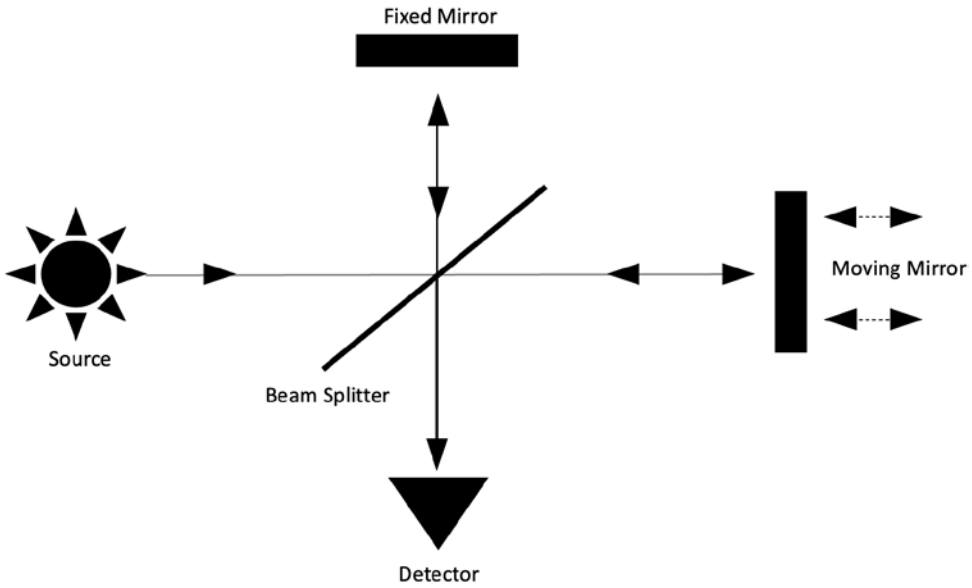

Figure 2.5: **Schematic of Michelson interferometer Instrumentation**

FTIR is an analytical technique allowing for greater signal-to-noise ratio, high resolution,

and higher throughput [93]. Furthermore, it provides qualitative and quantitative information from solid, liquid, and gaseous samples. Initially, FTIR was restricted to spectroscopic techniques to identify functional groups of chemical compounds; however, it is now frequently applied for rapid and direct measurement, identification, quality control, and manufacturing procedures of pharmaceuticals, supplements, and illicit drugs [93, 98].

Additionally, attenuated total reflection (ATR) is an FTIR direct contact sampling method that can be used in conjunction with IR spectroscopy to allow for direct examination of solid or liquid samples without requiring preparation. ATR operates by measuring the changes occurring within an internally reflected infrared beam interacts with a sample. This technique also refines the bulk methodology of IR spectroscopy by enhancing surface sensitivity [95].

2.8 Polymerase Chain Reaction

Before the development of polymerase chain reaction (PCR), attempting to amplify DNA for identification purposes was unfeasible. The basic principle of using two primers for DNA replication was described by Gobind Khorana in 1971, however establishing this technique was impeded at that point due to difficulty with primer synthesis and purifying polymerase. It was not until 1984 when American biochemist, Kary Mullis, was able to overcome these obstacles and establish the now common and indispensable technique of PCR. PCR revolutionized the field of molecular biology for DNA analysis, and Mullis was awarded the Nobel Prize in 1993. This technique used in vitro enzymatic synthesis to amplify specific DNA fragments from miniscule quantities of source DNA material. PCR is a quick, straightforward, inexpensive method that allows for even relatively poor-quality samples to be amplified for analysis.

The principle of PCR is based on the chain reaction that occurs by taking advantage of specific enzymatic proteins, referred to as polymerases, that create a DNA template to assemble

a complementary strand. These strands are formed by the polymerases stringing together individual DNA nucleic acids in addition to using primers. Primers are small fragments of the DNA that attach the nucleic acids together to create a single-stranded sequence of nucleotides referred to as an oligonucleotide. Each primer is complementary to either end of the original DNA strand. The cycling process of PCR occurs in three steps: denaturation of double-stranded DNA, primer annealing, and primer extension, as shown in Figure 2.6. Each cycle approximates 3-5 minutes and is typically repeated between 30-40 times. The first step is when DNA is denatured by using high temperatures ranging between 90-100°C. This process is responsible for separating the complementary strands of DNA that are held together by hydrogen bonds. The second step is when the primers are annealed to the dissociated strands of the DNA template to prime the extension that occurs in step three. Primer extension is when the ends of the annealed primers extend to create the complimentary DNA strand copy. This result doubles the DNA quantity which can continue to be doubled up to an unlimited number of cycles.

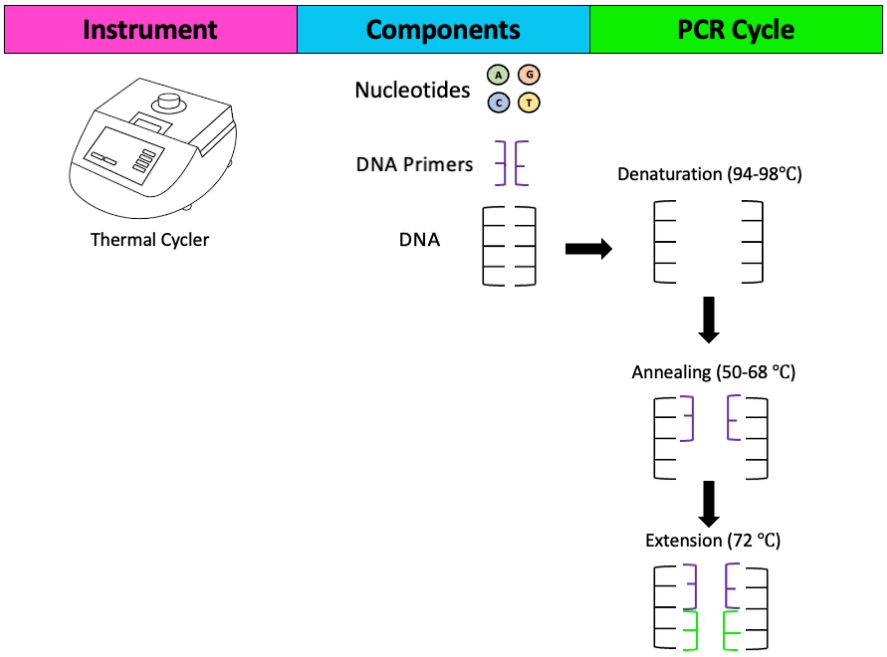

Figure 2.6: Illustration of Polymerase Chain Reaction Instrumentation, Components, and Mechanism for One Cycle

Originally, *Escherichia coli* was the standard polymerase used in PCR. However, this bacterium's thermostability was deemed unfit for the temperatures necessary for DNA denaturation. As a substitute, Taq polymerase has become the common substitute polymerase due to its thermostable properties isolated from *Thermus aquaticus*, a thermophilic bacterium tolerant of high temperatures. This polymerase contributes to the high yield, throughput, automation, and specificity achieved by PCR today. However, cycle number is limited based on depletion of reagents leading to plateau, typically occurring after approximately thirty-five cycles. The produced amplified target DNA sequences can be visualized using a variety of techniques based on how much amplified DNA is present. Gel electrophoresis and ethidium bromide staining can be used if a significant amount of DNA was able to be amplified; however, if not enough DNA was amplified, assays that utilize hybridization can be used. In this case, the DNA that was able to be amplified is hybridized to a synthetic probe in a solution. The probe is labeled at its 5' end using a radioactive phosphate group. Additionally, oligomer restriction (OR) is a common detection method that can detect amplified DNA from human immunodeficiency virus (HIV) [99, 100, 101, 102].

Within molecular biology, various types of PCR can be utilized depending on sample type and desired product. One of the most common types of PCR is real-time PCR (RT-PCR), also referred to as quantitative real-time PCR (Q-RT PCR). This specific method can be used to simultaneously amplify and quantify targeted DNA, and is useful for gene expression analysis [99, 103]. Other types of PCR techniques include Multiplex PCR, Qualitative PCR, and Colony PCR. This versatile technique has extensive diagnostic applications for not only research in biological sciences and medicine, but also in forensics and criminology. Furthermore, PCR has future implications for infectious disease identification in agricultural science, genetic profiling,

and anti-viral therapy, among others [99, 101, 103].

Colony-PCR is a high-throughput, rapid PCR method for screening bacterial colonies that have been cultured on selective media. This method is more time and cost-effective compared to conventional PCR which requires confirmation of DNA insertion during cloning, followed by bacterial transformation, DNA purification, and restriction digest. Additionally, colony-PCR is compatible with methodology that is sequence and restriction independent. Comparable to conventional PCR, the setup is simple; however, colony-PCR is more rapid since it does not require extraction and further purification of the DNA before analysis. Colony-PCR uses the bacterial colonies of interest as the template DNA. As shown in Figure 2.7, these colonies are directly scooped up and added to the reaction tubes containing a master mix of deoxyribonucleotide triphosphates (dNTPS), reaction buffer, Taq polymerase, specific complimentary forward and reverse primers, and nuclease-free water. From there, the two sets of species-specific primers amplify the bacterial DNA, which is then visualized using agarose gel electrophoresis. After evaluation, the PCR products are sequenced to analyze the sequence of the bacteria DNA present in the sample [105, 106].

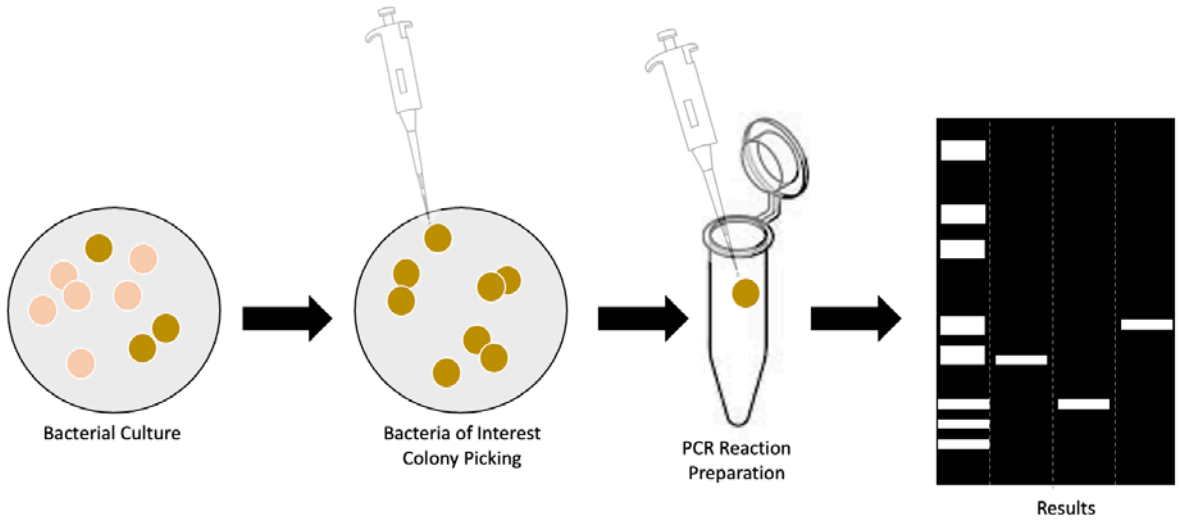

Figure 2.7: **Schematic of Colony PCR Methodology**

2.9 References

[72] Griffiths, J. (2008). A brief history of mass spectrometry. *Analytical Chemistry, 80*(15), 5678–5683. https://doi.org/10.1021/ac8013065

[73] Premier Biosoft . (n.d.). *Mass Spectrometry*. Mass spectrometry: Introduction, principle of mass spectrometry, components of mass spectrometer, applications. Retrieved October 3, 2022, from http://www.premierbiosoft.com/tech_notes/mass-spectrometry.html

[74] El-Aneed, A., Cohen, A., & Banoub, J. (2009). Mass spectrometry, review of the basics: Electrospray, MALDI, and commonly used mass analyzers. *Applied Spectroscopy Reviews, 44*(3), 210–230. https://doi.org/10.1080/05704920902717872

[75] *Triple Quadrupole Mass Spectrometer (QqQ MS)*. Labcompare. (n.d.). Retrieved October 3, 2022, from https://www.labcompare.com/Mass-Spectrometry/131-Triple-Quadrupole-Mass-Spectrometer-QqQ-MS/#:~:text=A%20triple%2Dquadrupole%20mass%20spectrometer,either%20SIM%20or%20scan%20mode.

[76] Schreiber, A. (n.d.). *Advantages of using triple quadrupole over single quadrupole mass spectrometry to quantify and identify the presence of pesticides in water and soil samples*. Retrieved October 3, 2022, from https://www.selectscience.net/application-articles/advantages-of-using-triple-quadrupole-over-single-quadrupole-mass-spectrometry-to-quantify-and-identify-the-presence-of-pesticides-in-water-and-soil-samples/?artid=28421

[77] Yost, R. A., & Enke, C. G. (1979). Triple quadrupole mass spectrometry for direct mixture analysis and structure elucidation. *Analytical Chemistry, 51*(12), 1251–1264. https://doi.org/10.1021/ac50048a002

[78] Johnson, A. R., & Carlson, E. E. (2015). Collision-induced dissociation mass spectrometry: A powerful tool for natural product structure elucidation. *Analytical Chemistry, 87*(21), 10668–10678. https://doi.org/10.1021/acs.analchem.5b01543

[79] Desiderio, D. M., Kraj, A., & Nibbering, N. M. (2009). *Mass spectrometry: Instrumentation, interpretation, and applications*. Wiley.

[80] Munson, B. (2006). Chemical ionization mass spectrometry: Theory and applications. *Encyclopedia of Analytical Chemistry*. https://doi.org/10.1002/9780470027318.a6004

[81] Mazumdar, S. (2012). Electrospray ionization mass spectrometry: A technique to access the information beyond the molecular weight of the analyte. *International Journal of Analytical Chemistry, 2012*, 1–40. https://doi.org/10.1155/2012/282574

[82] Smith, R. D., Loo, J. A., Edmonds, C. G., Barinaga, C. J., & Udseth, H. R. (1990). New developments in biochemical mass spectrometry: Electrospray ionization. *Analytical Chemistry, 62*(9), 882–899. https://doi.org/10.1021/ac00208a002

[83] Wilm, M. (2011). Principles of electrospray ionization. *Molecular & Cellular Proteomics*, *10*(7). https://doi.org/10.1074/mcp.m111.009407

[84] Daub, C. D., & Cann, N. M. (2011). How are completely desolvated ions produced in electrospray ionization: Insights from molecular dynamics simulations. *Analytical Chemistry*, *83*(22), 8372–8376. https://doi.org/10.1021/ac202103p

[85] Lübbert, C., & Peukert, W. (2021). Characterization of electrospray drop size distributions by mobility-classified mass spectrometry: Implications for ion clustering in solution and Ion Formation Pathways. *Analytical Chemistry*, *93*(38), 12862–12871. https://doi.org/10.1021/acs.analchem.1c00727

[86] Konermann, L., Metwally, H., Duez, Q., & Peters, I. (2019). Charging and supercharging of proteins for mass spectrometry: Recent insights into the mechanisms of electrospray ionization. *The Analyst*, *144*(21), 6157–6171. https://doi.org/10.1039/c9an01201j

[87] Ammann, A. A. (2007). Inductively coupled plasma mass spectrometry (ICP MS): A versatile tool. *Journal of Mass Spectrometry*, *42*(4), 419–427. https://doi.org/10.1002/jms.1206

[88] Sneddon, J., & Vincent, M. D. (2008). ICP-oes and ICP-MS for the determination of metals: Application to oysters. *Analytical Letters*, *41*(8), 1291–1303. https://doi.org/10.1080/00032710802013991

[89] Iti, S. (2020). ICP-OES: An advance tool in Biological Research. *Open Journal of Environmental Biology*, 027–033. https://doi.org/10.17352/ojeb.000018

[90] National Academies Press. (2004). Compositional Analysis. In *Forensic analysis weighing bullet lead evidence* (pp. 12–15). essay.

[91] *Principles of infrared spectroscopy (2) history of IR spectrometers: Jasco Global*. JASCO Inc. (2022, September 16). Retrieved October 3, 2022, from https://www.jasco-global.com/principle/principles-of-infrared-spectroscopy-2-history-of-ir-spectrophotometers/#:~:text=Infrared%20spectrophotometers%20were%20developed%20in,Institute%2C%20the%20predecessor%20of%20JASCO.

[92] Tiffany, W. B. (1976). Introduction And Review of Pyroelectric Detectors. *Modern Utilization of Infrared Technology I*, *62*. https://doi.org/10.1117/12.954445

[93] Willey, R. R. (1976). Fourier transform infrared spectrophotometer for transmittance and diffuse reflectance measurements. *Applied Spectroscopy*, *30*(6), 593–601. https://doi.org/10.1366/000370276774456642

[94] Ng, L. M., & Simmons, R. (1999). Infrared Spectroscopy. *Analytical Chemistry*, *71*(12), 343–350. https://doi.org/10.1021/a1999908r

[95] Blum, M.-M., & John, H. (2011). Historical perspective and modern applications of attenuated total reflectance - fourier transform infrared spectroscopy (ATR-FTIR). *Drug Testing and Analysis*, *4*(3-4), 298–302. https://doi.org/10.1002/dta.374

[96] Ling, S. J. (2016). *University physics. volume 3* (Vol. 3). OpenStax College Rice University.

[97] Rossini, J. (2020, August 15). *FTIR: Hardware*. Chemistry LibreTexts. Retrieved October 3, 2022, from https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_(Analytical_Chemistry)/Instrumental_Analysis/Spectrometer/How_an_FTIR_instrument_works/FTIR%3A_Hardware#:~:text=Michaleson%20Interferometer-,History,of%20the%20wavelengths%20of%20light.

[98] Bunaciu, A. A., Aboul-Enein, H. Y., & Fleschin, S. (2010). Application of fourier transform infrared spectrophotometry in pharmaceutical drugs analysis. *Applied Spectroscopy Reviews*, *45*(3), 206–219. https://doi.org/10.1080/00387011003601044

[99] Joshi, M., & Deshpande, J. D. (2011). POLYMERASE CHAIN REACTION: METHODS, PRINCIPLES AND APPLICATION. International Journal of Biomedical Research, 2(1). https://doi.org/10.7439/IJBR.V2I1.83

[100] Templeton, N. S. (1992). The polymerase chain reaction history methods, and applications. *Diagnostic Molecular Pathology*, *1*(1), 58–72. https://doi.org/10.1097/00019606-199203000-00008

[101] Schochetman, G., Ou, C.-Y., & Jones, W. K. (1988). Polymerase Chain Reaction. *The Journal of Infectious Diseases*, *158*(6), 1154–1157. http://www.jstor.org/stable/30137034

[102] Schochetman, G., Ou, C.-Y., & Jones, W. K. (1988). Polymerase chain reaction. *Journal of Infectious Diseases*, *158*(6), 1154–1157. https://doi.org/10.1093/infdis/158.6.1154

[103] Marmiroli, N., & Maestri, E. (2007). Polymerase chain reaction (PCR). *Food Toxicants Analysis*, 147–187. https://doi.org/10.1016/b978-044452843-8/50007-9

[104] Rajalakshmi, S. (2017). *Different types of PCR techniques and its applications*. International Journal of Pharmaceutical, Chemical and Biological Sciences. Retrieved October 3, 2022, from https://www.scribd.com/document/553020306/Different-Types-of-Pcr-Techniques-and-Its-Applications

[105] Bergkessel, M., & Guthrie, C. (2013). Colony PCR. *Methods in enzymology*, *529*, 299–309. https://doi.org/10.1016/B978-0-12-418687-3.00025-2

[106] Woodman, M. E. (2008). Direct PCR of intact bacteria (colony PCR). *Current Protocols in Microbiology*, *9*(1). https://doi.org/10.1002/9780471729259.mca03ds9

[107] Cole, C. (2010). *Cut: A guide to adulterants, bulking agents and other contaminants found in illicit drugs*. Centre for Public Health, Faculty of Health and Applied Social Sciences, Liverpool John Moores University.

[108] Lichtenstein, A. H., & Russell, R. M. (2005). Essential nutrients: Food or supplements? *JAMA*, *294*(3), 351. https://doi.org/10.1001/jama.294.3.351

CHAPTER 3

RAPID IDENTIFICATION OF NUTRACEUTICAL AND SUPPLEMENTARY COMPOUNDS USING FOURIER TRANSFORM INFRARED SPECTROSCOPY AND DIRECT INJECTION MASS SPECTROMETRY

3.1 Introduction

The public need for alternative medical solutions regarding disease prevention and overall enhanced health has continued to gain popularity due to lack of consumer fulfillment in commonly prescribed medications [1, 7]. As unique lifestyles change and continue to develop, there is a need for individualized treatment specific to genetic pre-dispositions that ultimately influence dietary, physical, and mental needs. Generalized medications typically do not account for these variations between consumers. Interest in more natural medical derivations has directed focus toward nutraceuticals [60, 108]. This generalized class includes compounds made from parts of food that have been observed to supplement human health. This vague definition encompasses products that not only prevent or treat diseased states, but also enhance all biological scopes including both physical and cognitive function [3, 4]. These compounds are administered in a myriad of forms such as capsules, tablets, sprays, and powders, tailored to the preference of the consumer. Furthermore, this health-conscious demographic desires to fill their needs with specific supplements in a convenient, affordable way that nutraceuticals can provide compared to its traditional, prescription counterparts. As these custom products become more attainable, production demand surges. Furthermore, to be able to manage large production, an increase in manufacturing efficiency becomes vital. Due to the generality of nutraceuticals and its dual properties of both food derivation and pharmaceutical-like actions, ambiguity surrounds associated legality and regulatory parameters [3]. Additionally, since market competition has

emerged between nutraceuticals and well-established, thoroughly tested pharmaceuticals, no priority has been directed toward the scientific testing and analyses of nutraceuticals. This information is vital in understanding appropriate dosing, related toxicity, and establishing any contraindications or adverse effects associated with nutraceuticals [6]. These actions ultimately allow for effective comparison between nutraceuticals and pharmaceuticals as well as influence determining appropriately prescribed actions for public health. Since no clinical evidence has been established for nutraceuticals, requisite quality control and quality assurance has become fluid, and has led to lenient regulation. Despite lacking scientific credentials and associated legal definition, the demand for nutraceuticals continues to grow. Furthermore, since no standards have been established in this emerging classification of products, no effective preventative actions are defined and enforced to protect eager consumers from health injuries [61]. Vulnerable individuals look to these products hoping to enhance their lives, and instead become victim to unmanaged manufacturing and distribution of products that are claimed to be safe and effective. Consumers are ultimately exposed to products with no associated regulation that ensure proper quality, and purity of products confirmed to be devoid of contamination resulting from improper storage and handling, as well as potential tampering resulting in adulterated products [8, 107]. Information regarding the process and condition of post-market products needs to not only be defined, but also accessible to understand the ingredients responsible for resulting health. To properly assess the state of this broad class of supplements, sample ingredients need to be analyzed to identify any questionable variance of ingredient amounts, as well as ensure that the listed ingredients are congruent with the ingredients that are physically included. Specific instrumental analytical techniques can accomplish these objectives by accurately identifying sample constituents to confirm their presence or absence in respect to listed ingredients.

Additionally, these techniques can further quantitate the levels of the included ingredients confirmed to ensure that the amounts specified are accurate. One of these methods is mass spectrometry (MS) which identifies the constituents and their respective structures within a given sample based on each compound's mass to charge ratio. This process is accomplished by converting an injected, liquid sample into gaseous ions from the ion source. From there, the converted ions are then characterized and separated based on their mass to charge ratio. Finally, each ion's abundance is recorded and plotted in the form of a mass spectrum [73, 74]. Another analytical technique used for sample identification is Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR). This instrumentation provides qualitative information for both solid and liquid samples. Furthermore, ATR-FTIR can be utilized to rapidly identify sample's functional groups. This ultimately establishes quality assurance measures to indicate if product tampering and adulteration has occurred [94, 95, 98].

3.2 Methods

Prior to analysis, all samples listed in Table 2.1 were mixed thoroughly to ensure proper homogenization. Each sample was then directly analyzed using ATR-FTIR spectroscopy, using appropriate background respective to each sample's medium, as well as thorough methanol sterilization of the instrument's stage before and after each run to avoid contamination. After the spectral molecular fingerprint of each sample was obtained, three different solutions were prepared to rapidly analyze the ingredients present in each sample using direct inject mass spectrometry (DIMS). The generated mass spectra were then annotated and compared to their product labels to analyze the presence, absence, or variation of ingredients shown on each mass spectrum relative to the label's listed ingredients and amounts. Based on the annotated spectra, any discrepancies between what the label claimed to be present and what was observed to be

present was analyzed through quantitation. This was accomplished by creating a concentration curve with a standard sample of the suspected adulterant, and plotting the concentration observed in the tampered samples along the curve to quantify the amount of adulterant present in each sample. To ensure that the suspected adulterated peaks were not the result of experimental error, variations of the sample amount were analyzed to observe any variation in adulterant concentration.

3.2.1 Homogenization

To ensure proper sampling, all powder samples were homogenized using multiple methods. Each powder sample was manually inverted and shook vigorously for one minute, followed by three minutes of mixing using a mechanical mixing lab stirrer. A 1-gram aliquot was then taken, and the previous two mixing methods were repeated to ensure no variance within the sample. For the liquid samples, each sample was inverted and shaken vigorously for 10 seconds. Each liquid sample was then filtered using 0.45 μm PES syringe filters.

3.2.2 ATR-FTIR Analysis

After the samples were properly homogenized and filtered to ensure no variation within the samples, they were analyzed using an FTIR direct contact sampling method via Attenuated Total Reflectance (ATR). The stage was sterilized using methanol before and in-between sample runs. Each sample was analyzed by directly placing a small amount of sample onto the ATR crystal diamond using either a disposable spatula, if powder, or a pipet, if liquid. The resulting ATR-FTIR spectra for each sample was then analyzed to gather information regarding the functional groups of the compound’s molecular constituents.

3.2.3 Triple Quadrupole Analysis

To ultimately assess the accuracy and legitimacy of the sample compositions, the presence, absence, and variance between the ingredients claimed on the product's labels and the actual sample's constituents were compared against standard ingredients. Since the nutraceutical samples of interest attribute their health effects to the use of deer antler velvet products, a sample of deer antler velvet powder (Tobin Farms) and deer antler velvet extract (Antler Farms) were obtained. Additionally, a lab-grade reference methandienone standard (Cerilliant) was obtained for comparison against samples with suspected steroid adulterants. Each nutraceutical sample was prepared using three different solution methods to establish the most appropriate solvent choice for spectral analysis. Each sample, including the deer velvet antler powder and extract, was initially prepared in a 1 mg/mL solution of a 50:50 (v/v) chloroform/acetonitrile mixture with 1% ammonium acetate, a 50:50 (v/v) acetonitrile/methanol mixture with 1% glacial acetic acid, and a 50:50 (v/v) methanol/ water mixture with 1% glacial acetic acid, respectively. These three solutions of each sample were then filtered using a 0.45 μm PES syringe filter and were further diluted to achieve a final concentration of 1 ppm. The methandienone standard was directly prepared as a 1 ppm solution. All the prepared samples were directly injected onto an API 4000 Q Trap system using the specified instrument parameters listed in Table 2.2. The deer antler velvet powder sample, deer antler velvet extract, and methandienone mass spectra were compared and annotated to establish working "standard" spectra for analysis and identification of the prepared nutraceutical samples. Each of the three spectra associated with each sample's solvent preparation were observed to identify the optimal solvent conditions. Once the ideal sample solvent mixture was established, each sample's spectrum fragments were compared relative to its respective product label, any duplicate samples, as well as the deer antler velvet

spectra, and methandienone spectrum. All produced spectra were annotated with respect to the ingredients that were confirmed, as well as additional peaks that were not associated with the product label, for further analysis. After the presence, absence, and any variation of ingredients was assessed for each spectrum, suspected adulterated peaks identified within specific samples were further analyzed by varying sample amount. Each sample of interest was prepared in incremental quantities while maintaining a 1 ppm concentration to assess any variation in the adulterated peak of interest. After this peak persisted with no indication of variance, this adulterated peak was quantified by creating a calibration curve using the methandienone standard. Solutions of 0.1 ppm, 1 ppm, 10 ppm, and 50 ppm of methandienone were prepared in a 50:50 (v/v) chloroform/acetonitrile solvent mixture with 1% ammonium acetate. Each sample was run using the API 4000 Q Trap system following the same parameters as before, and the resulting absolute intensity values of the peak of interest were constructed into a calibration curve. Each sample containing the adulterant peak of interest was re-run using the same run conditions and solution preparation. The absolute intensities of each sample's peak of interest were plotted along the methandienone calibration curve to quantify the adulterant concentration in each sample.

3.3 Results

Prior to identifying the ingredients of samples 1-20, ATR-FTIR was performed on each sample to provide preliminary spectral information. Each spectrum expresses a unique molecular fingerprint respective to the sample's constituents based on the functional groups within the compound. The resulting spectra for the three working standards are compared in Figure 3.1. Each sample was directly analyzed and repeated, shown in Figure 3.2, using spectral comparison, listed in Table 3.1, to observe any noticeable variance potentially indicative of

product tampering, and ensuring reproducibility.

Run #	Relative Intensity (%) of Peak 1091 (cm^{-1})	Relative Intensity (%) of Peak 1410[a] (cm^{-1})	Relative Intensity (%) of Peak 1550[a] (cm^{-1})	Relative Intensity (%) of Peak 1410 and 1550[b] (cm^{-1})
1	95.5	97	97	99.5
2	89	95	97	98
3	96	97	97	99.7
4	92	95	95	99.5
5	93	96	97	99.5
6	93	96	96	99.5
7	94	96	98	99.5
8	92	95	96	99.5
9	87	93	94	99
10	89	94	95	99

Table 3.1: **The relative intensities of peaks 1091, 1410, and 1550 associated with each repeated Bucked Up pre-workout run shown in Figure 3.2 using ATR-FTIR spectral analysis where [a] are the relative intensities of peaks 1,410 and 1,550 relative to the main peak (1,091), and [b] are the relative intensities of peaks 1,410 and 1,550 relative to each other**

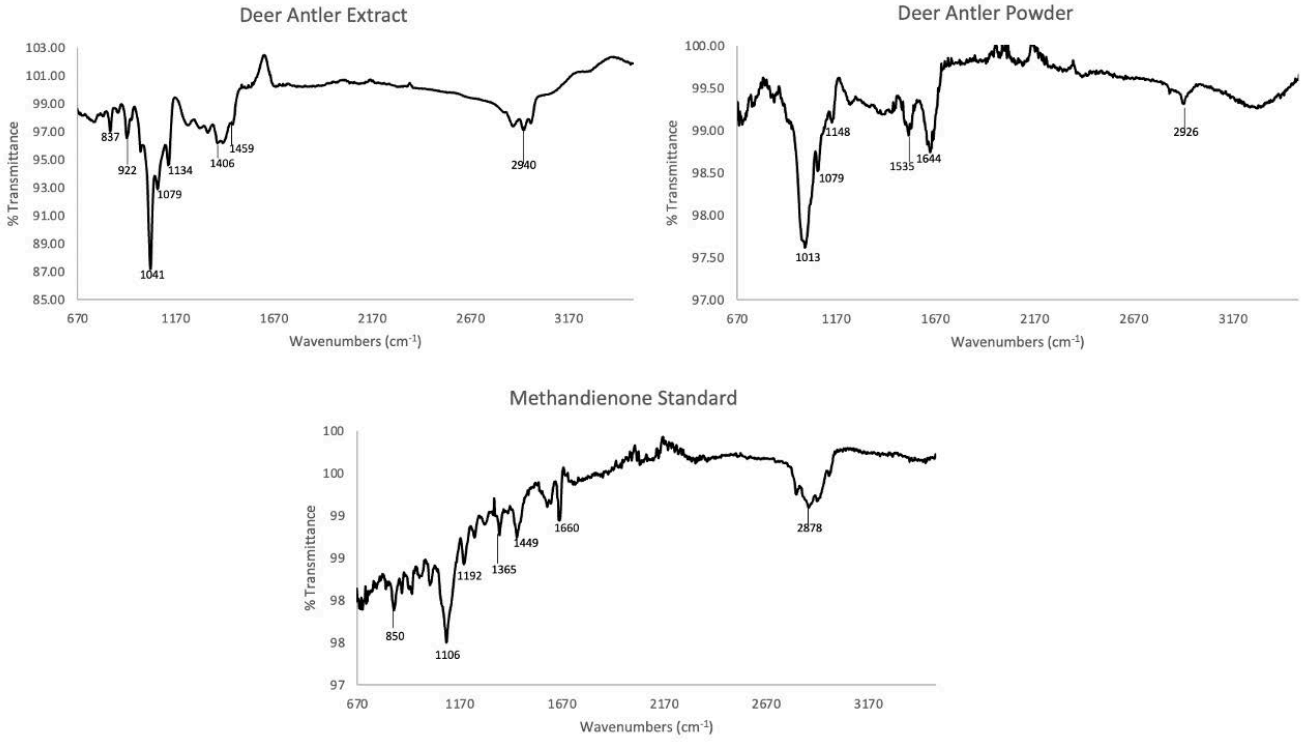

Figure 3.1: **ATR-FTIR spectral analysis comparing the functional groups of deer antler velvet extract (top left), deer antler velvet powder (top right), and methandienone (center).**

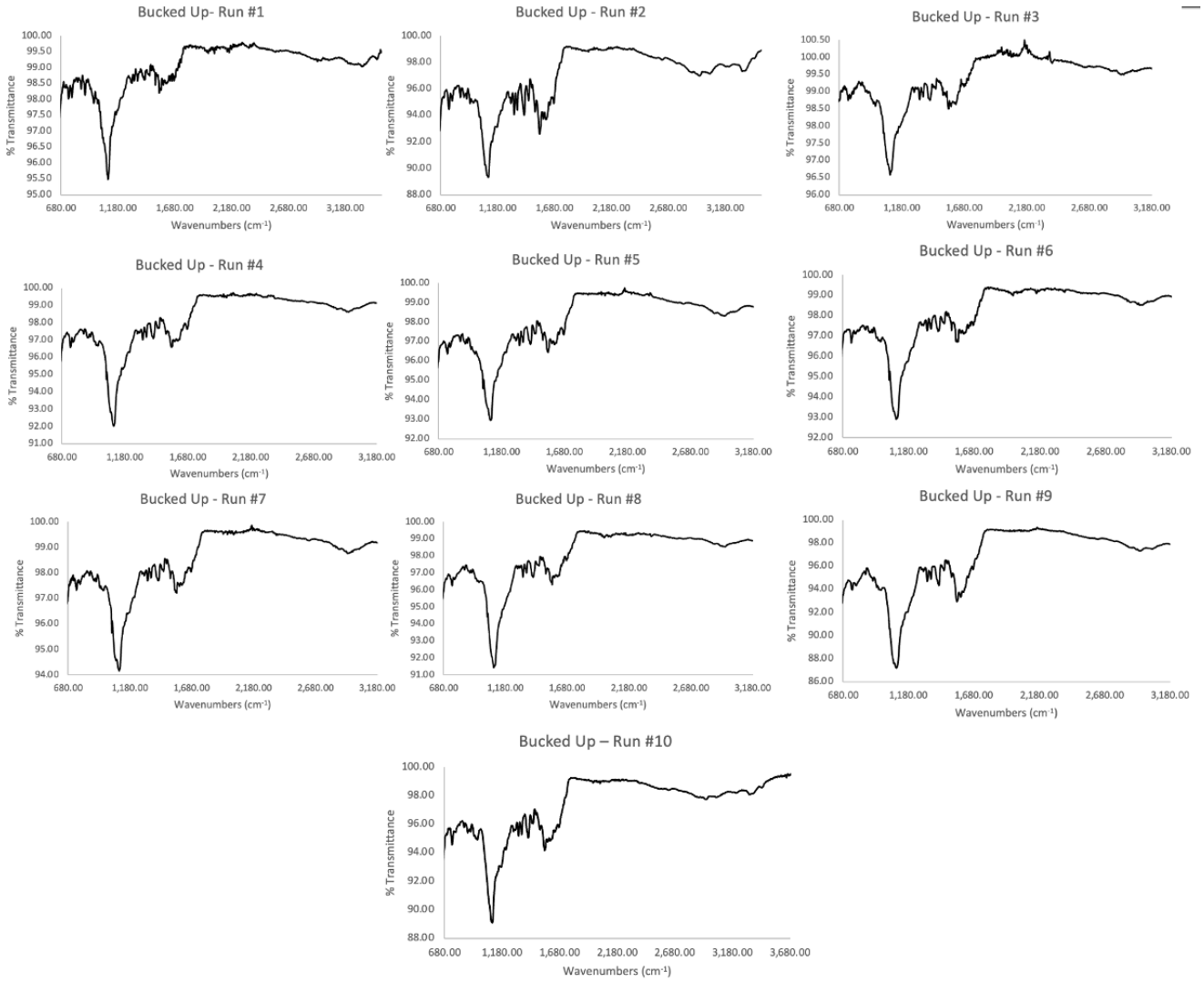

Figure 3.2: **ATR-FTIR reproducibility analysis of Bucked Up pre-workout using spectral comparison**

Preliminary assessment of the three potential solvent mixtures indicated more organic solubility affiliation of the sample's ingredients of interest. This observation initiated further analysis to focus mainly on the data resulting from sample preparation using the chloroform/acetonitrile solvent mixture. Accurate sample analysis first required a baseline standard pertaining to the active ingredient(s) responsible for each sample's effects. Specifically, the brand Bucked Up attributes their results to their staple ingredient, deer antler velvet extract. Furthermore, Bucked Up claims that they specifically use the extract from deer antler velvet versus a powder form due

to the heightened levels of Insulin Growth Factor-1 (IGF-1) present in the extract. To evaluate this claim, deer velvet antler powder and deer antler velvet extract were obtained to establish working standards. Both samples were filtered and prepared as 1 ppm solutions and analyzed using direct-injection mass spectrometry (DIMS) following the parameters listed in Table 2.1. The resulting mass spectra comparison is shown in Figure 3.3 and confirms that the deer antler velvet powder and the deer antler velvet extract are the same compound indicated by the identical fragmentation patterns. Furthermore, this comparison confirms that the extract form of deer antler velvet does contain higher constituent concentration levels comparative to the deer antler velvet powder as observed by the absolute intensities shown in the mass spectra. Additionally, this comparison suggests a parent peak mass reference of 305 m/z for deer antler velvet products. This information, once properly evaluated and confirmed, can be used to either validate or invalidate sample labels claiming the use of deer velvet antler in their products.

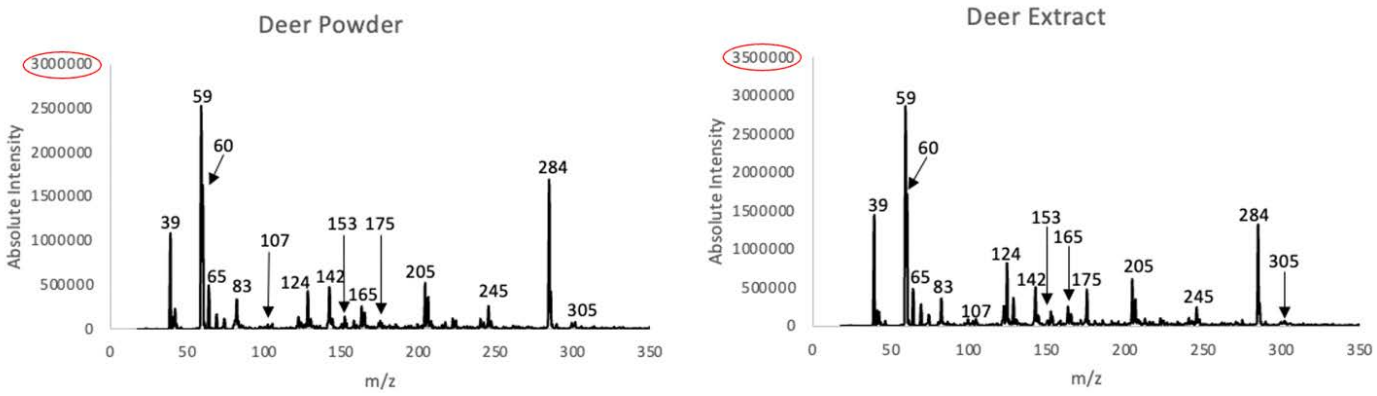

Figure 3.3: The mass spectra of deer antler velvet powder (left) and deer antler velvet extract (right) comparing parent and fragment peaks as well as the difference in ingredient concentration levels using direct-injection mass spectrometry (DIMS)

Manufacturers have discovered similarity between naturally synthesized IGF-1 found in deer antler velvet, and common steroids used for rapid muscle mass growth. This information established “deer antler velvet extract” as a novel steroid-alternative used to market toward consumer’s desiring physical enhancement.

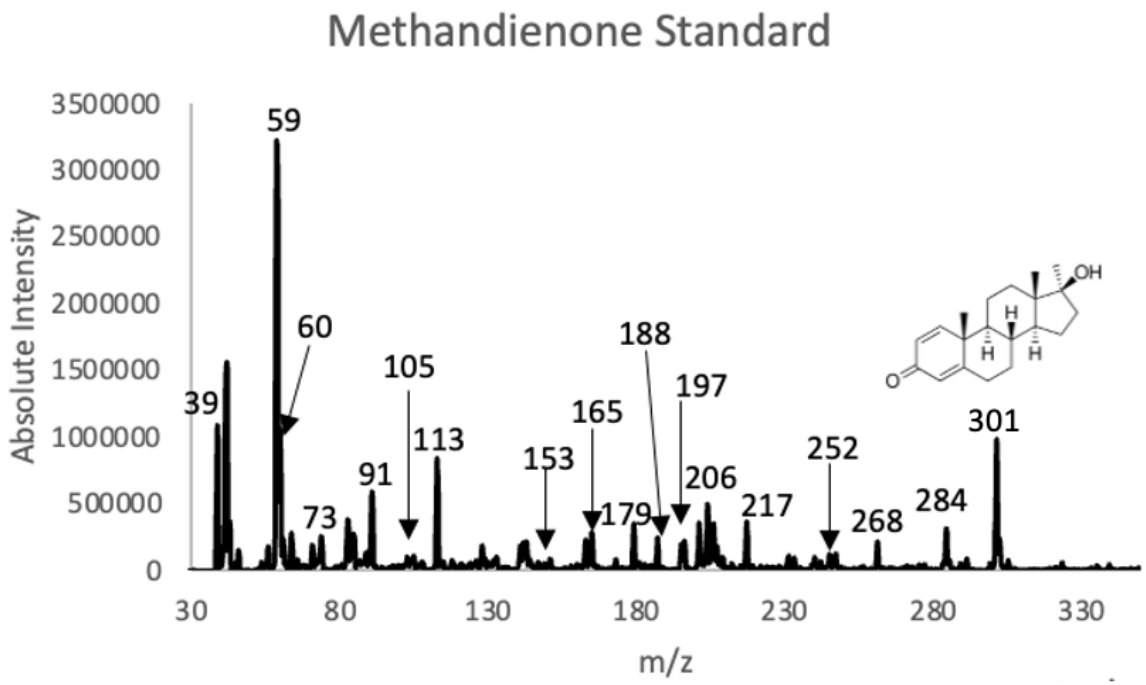

Figure 3.4: **The produced mass spectrum of the suspected steroid adulterant, methandienone, present in Bucked Up dietary supplements.**

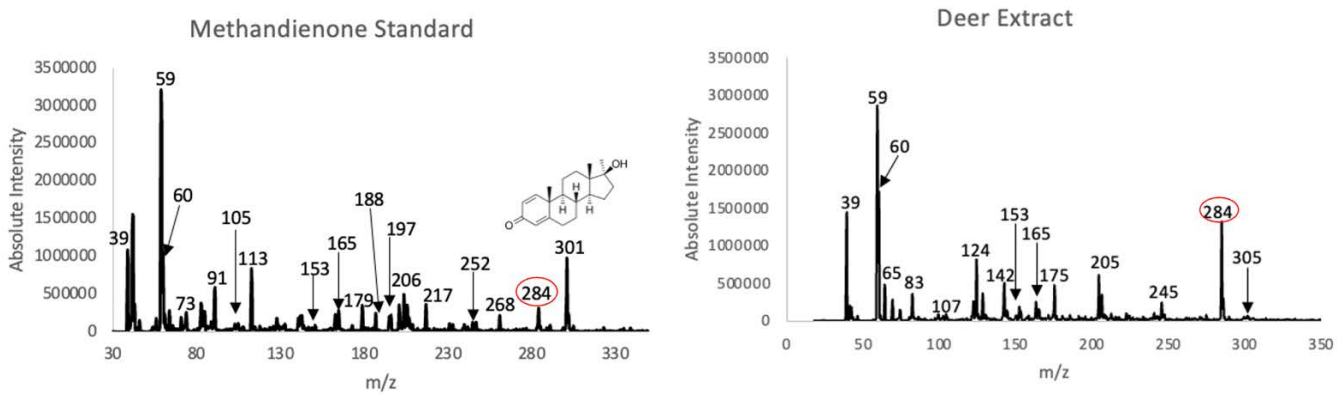

Figure 3.5: **Comparing the mass spectra of the deer antler velvet extract (right) and the suspected steroid adulterant, methandienone (left).**

Due to the striking similarity between the product mass, properties, fragmentation, and effects between IGF-1 and anabolic steroids, dubious manufacturers, such as Bucked Up, have been accused of substituting IGF-1 ingredients with steroids to be able to provide a highly effective product. To gauge potential levels of steroid adulteration, specifically in products originating from Bucked Up, a 1 ppm methandienone standard was prepared and run using DIMS, as shown

in Figure 3.4. By comparing Figures 3.3 and 3.4, shown in Figure 3.5, differentiating fragments between the deer antler velvet and the potential steroid adulterant, methandienone, could be established. Additionally, the observation of peak 284 in the deer antler velvet extract suggests that methandienone is potentially incorporated into manufactured and distributed deer antler velvet products themselves.

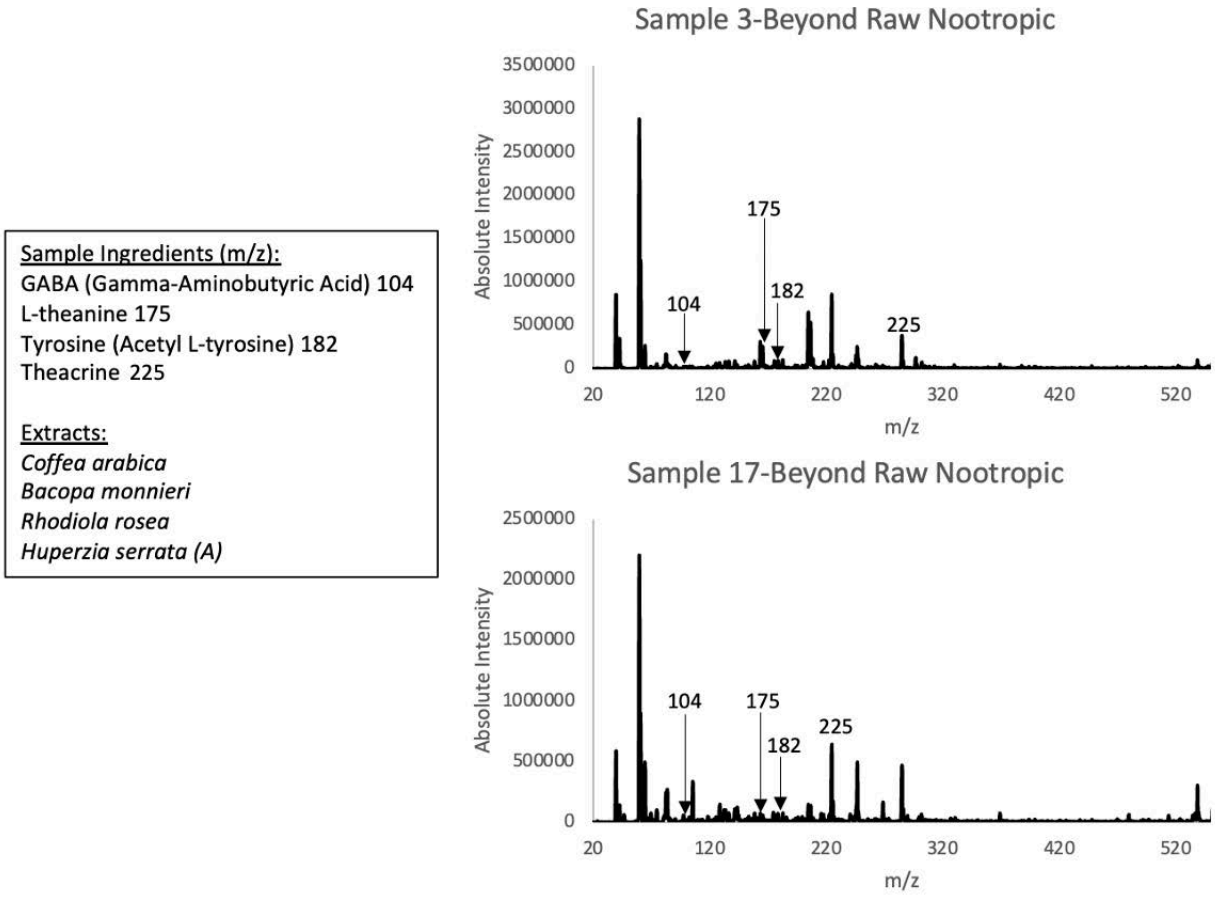

Figure 3.6: Schematic showing the workflow of analyzing each sample's listed ingredients correlating to specific mass spectrum peaks as well as comparing pairs of duplicate samples to observe any variance

Following deer antler velvet and steroid sample analysis, samples 1-20 were prepared and analyzed using DIMS following the same parameters as previously mentioned. The resulting mass spectra were annotated according to their product label's listed ingredients, as shown in Figure 3.6, to assess validity, and were further compared against each sample's respective

duplicate sample for any observed variation between samples. Each ingredient was individually identified within the mass spectrum, and all ingredients were accounted for as listed, other than included extracts, not including the deer antler velvet extract, which do not have associated masses for identification.

Despite positively identifying each product's listed constituent according to their mass spectra literature values [109-112], when each sample was compared to its duplicate pair, significant variance was observed, as shown in Figure 3.7. This figure expresses that even though the Sample 2 and Sample 12 duplicate spectra both contain all their listed ingredients, sample 2's ingredients are at much higher concentrations within the 20-200 mass range than in sample 12. However, sample 12's higher mass ingredients are at higher concentrations, including both the deer velvet antler extract and the suspected adulterant, than in sample 2. This variance within the same product suggests that some consumers could be consuming much higher doses of these ingredients that could potentially become toxic, and other consumers are consuming such low doses of these ingredients that they aren't experiencing any results.

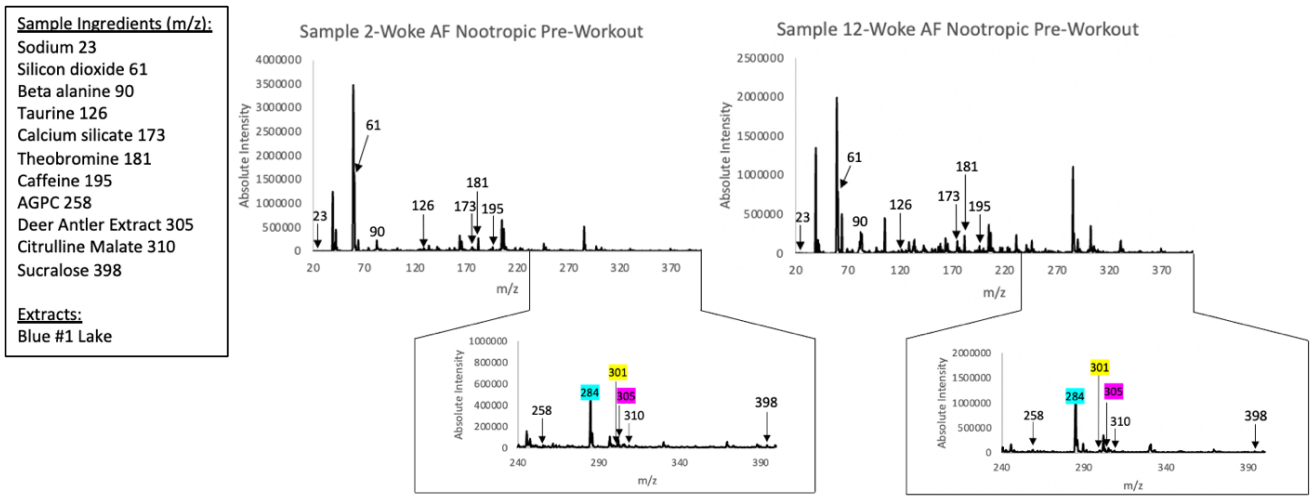

Figure 3.7: The mass spectra comparison of sample duplicate pairs 2 and 12 and their variance in ingredient concentration to the presence of an additional peak indicating steroid adulteration (yellow), the presence of a peak indicating the presence of deer antler extract (pink), and an overlapping peak of both the steroid adulterant and the deer antler velvet extract (blue).

In addition to observed concentration variance, when compared to deer antler velvet extract and methandienone, most of the samples did not contain additional peaks, except for Bucked Up. All six samples of Bucked Up products had positive peaks for their listed ingredients as well as the peaks associated with deer antler velvet extract. However, there were additional fragments unique to specifically methandienone. The presence of overlapping fragments of deer antler velvet and methandienone, shown in Figure 3.8, suggests that both compounds are sample constituents.

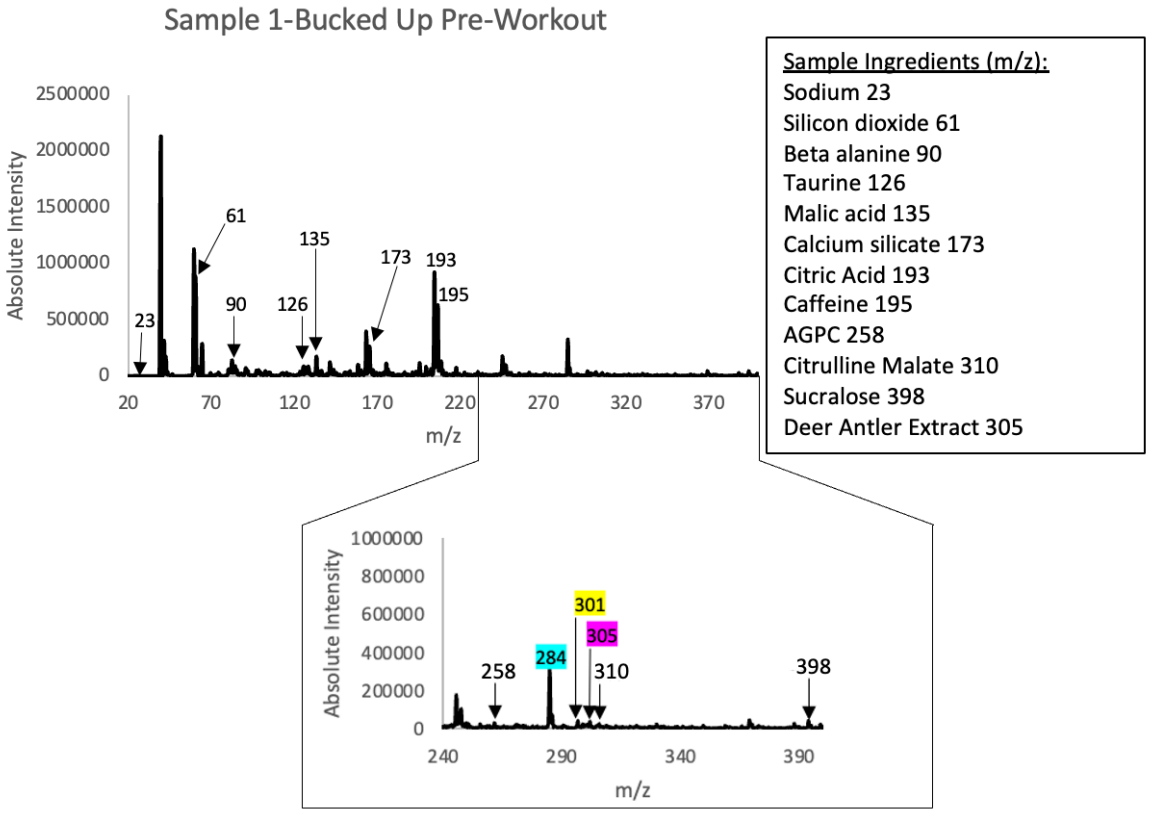

Figure 3.8: **Schematic showing the workflow of analyzing each sample's listed ingredients correlating to specific mass spectrum peaks in addition to the presence of an additional peak indicating steroid adulteration (yellow), the presence of a peak indicating the presence of deer antler extract (pink), and an overlapping peak of both the steroid adulterant and the deer antler velvet extract (blue).**

To determine which compound was responsible for producing peak 284, indicative of steroid adulteration, Collison-induced dissociation (CID) was performed on the deer antler velvet

extract and the methandienone. The deer antler extract CID mass spectrum, shown in Figure 3.9, did not include peak 284 in its fragmentation, indicating that methandienone includes peak 284 in its fragmentation pattern.

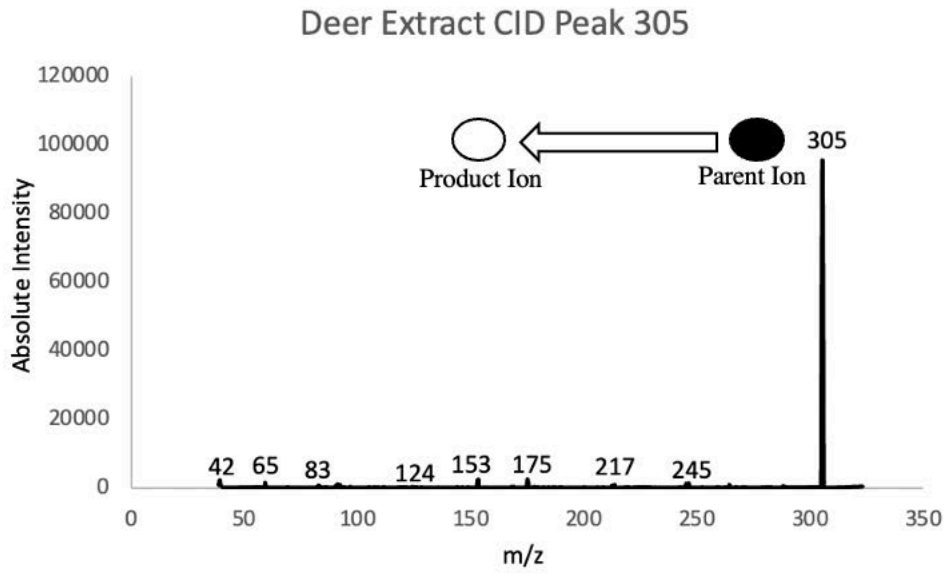

Figure 3.9: **The CID mass spectrum of deer antler velvet extract**

To confirm the inclusion of peak 284 in the fragmentation pattern of methandienone, CID was performed on the parent peak (301 m/z) as well as on peak of interest, 284, as shown in Figure 3.10. The produced spectral comparison confirmed the inclusion of fragment 284 resulting from thermal degradation of the methandienone and the loss of a water group.

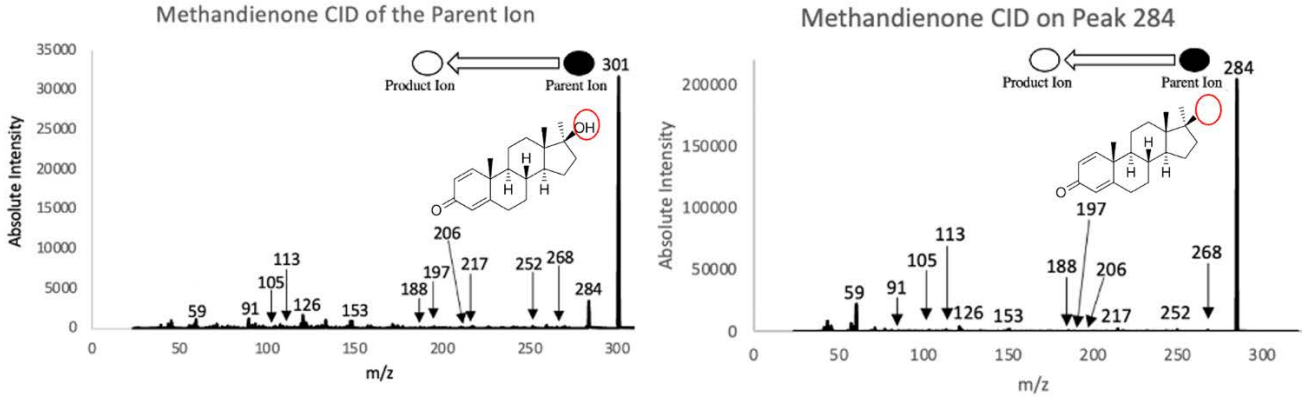

Figure 3.10: **The CID mass spectra showing the fragmentation patterns of the parent ion (left) and peak 284 (right) of methandienone**

The confirmed steroid adulterant peak present in the six Bucked Up samples was further analyzed by quantifying the amount of methandienone present. This was done by preparing standard solutions of the methandienone reference sample with concentrations of 0.1, 1, 10, and 50 ppm. These four solutions were run using the API 4000 Q Trap system following the same parameters as before. The absolute intensities of peak 284 associated with each steroid solution concentration was then used to construct a calibration curve shown in Figure 3.11.

(A)

Methandienone Concentration (ppm)	Peak 284 Intensity (10^5)
0.1	2.8
1	1.4
10	1.2
50	1.0

(B)

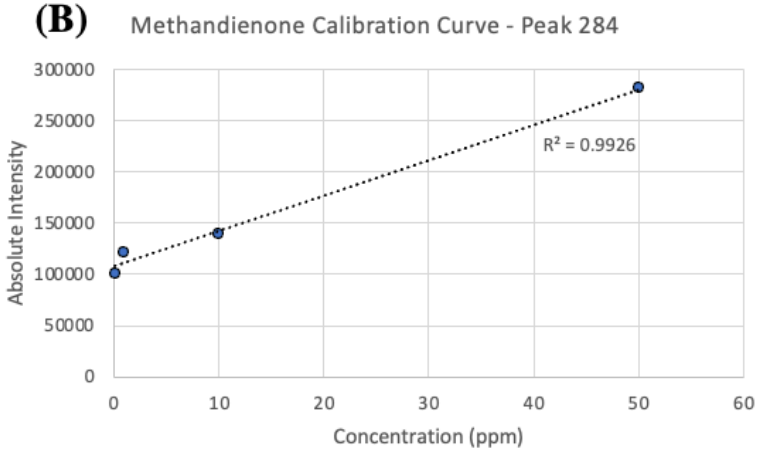

Figure 3.11: **The intensities of peak 284 associated with the methandienone standard solution concentrations (A) used to construct a calibration curve to quantify the amount of steroid adulterant present in Bucked Up samples (B)**

After establishing the associated concentrations with the absolute peak intensities of peak 284, the six Bucked Up samples, sample numbers 1, 2, 9, 12, 15, and 18 in Table 2.1, were prepared with final concentrations of 1ppm in 50:50 (v/v) chloroform/acetonitrile with 1% ammonium acetate. These sample analyzed using DIMS performed on the API 4000 Q Trap system using the same parameters as before. Their resulting intensities for peak 284 were plotted against the methandienone calibration curve to quantitate the amount of steroid adulterant present in each sample, as shown in Figure 3.12. Consistent with the variance observed between the ingredients in duplicate sample pairs, the identified concentration amounts of adulterated steroid significantly varied between duplicate sample pairs. Furthermore, samples 2 and 9 only

contained 1 ppm levels of methandienone, whereas sample 15 contained 48.2 ppm of steroid adulterant.

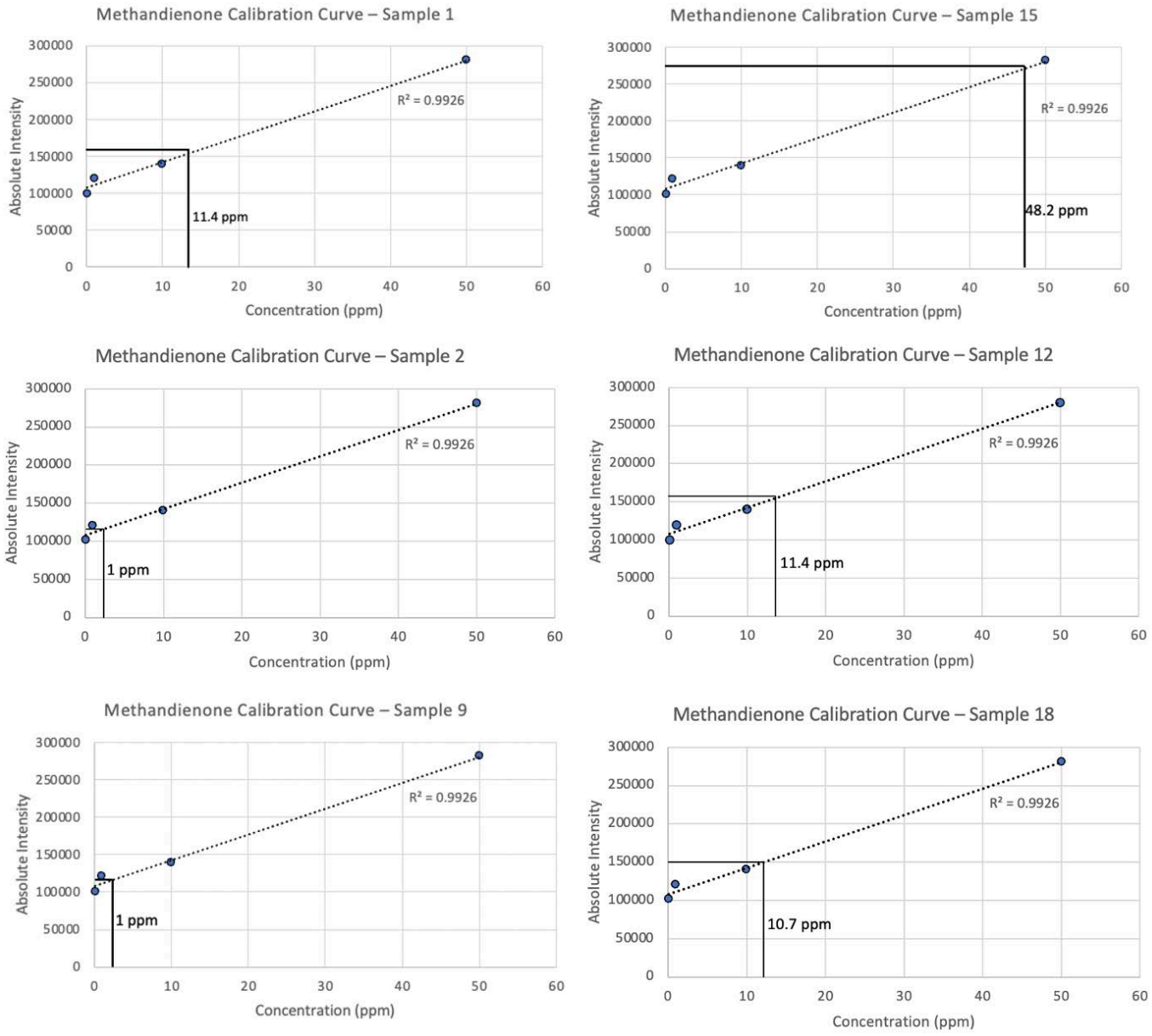

Figure 3.12: **The workflow and associated concentration values from quantitating each of the six Bucked Up samples using the methandienone calibration curve**

To identify if these concentrations levels were repeatable and consistent, larger sample amounts of each of the six Bucked Up samples were prepared while maintaining the original sample concentration. Two additional samples in the amounts of 100mg/mL and 1 gram/1000

mL were prepared in 50:50 (v/v) chloroform/acetonitrile with 1% ammonium acetate for each Bucked Up sample. The samples were then filtered using 0.45 μm PES syringe filters and diluted to a final concentration of 1 ppm. Each of the additional samples for each Bucked Up sample was analyzed using DIMS following the previous parameters, and assessed for variation, as shown in Figure 3.13. No concentration variance was observed in any of the samples where sample amount was altered. This confirmation indicates that the original quantitated concentration amounts are consistent and accurate.

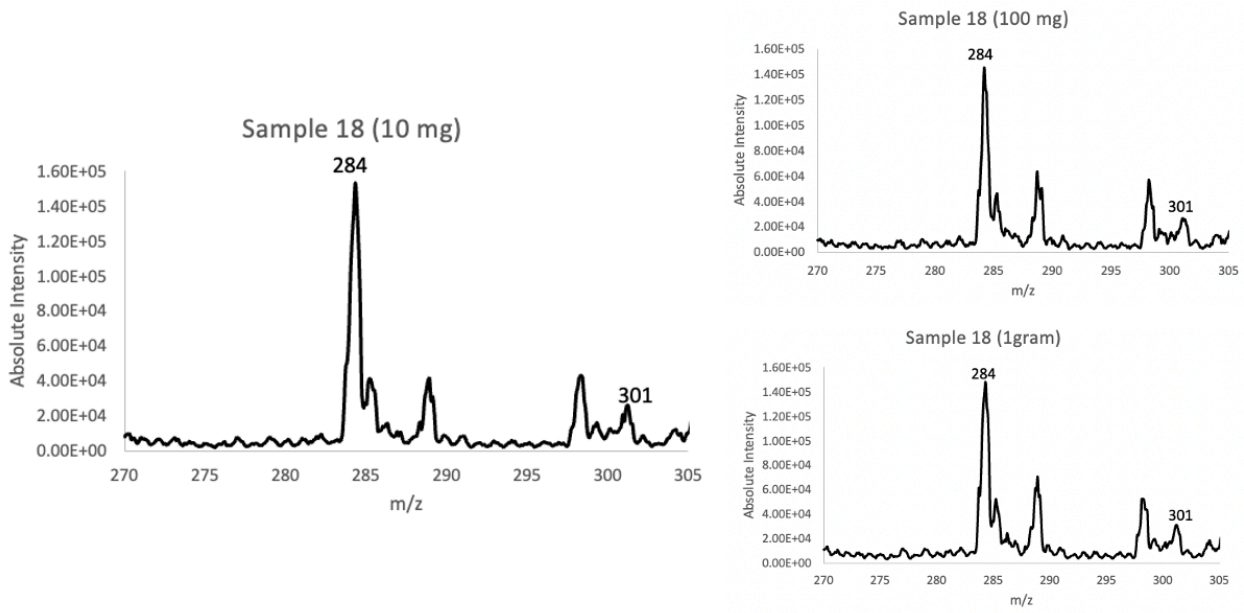

Figure 3.13: **Comparison between the mass spectra of prepared sample amounts of Bucked Up sample 18 altering in sample amount (10mg, 100 mg, and 1 gram) to confirm no variation in the adulterant steroid peak 284**

3.4 Discussion

By coupling ATR-FTIR and DIMS spectral analysis, the claimed ingredients present in a variety of nutraceutical samples could be identified and compared against their respective product ingredients to observe the degree of accuracy and variance. The mass spectra not only confirmed that the products did include all their listed ingredients, but they also indicated a significant degree of deviation in ingredient concentrations across all samples. This variance has

implications for consumer dosage to the degree that consumers may happen to purchase a product that may not contain high enough ingredient amounts to elicit therapeutic effects. Similarly, the same consumer may purchase the same product which happens to contain much higher ingredient amounts which poses a risk for toxicity.

This method also rapidly identified the presence of adulterated peaks in the samples produced by Bucked Up as well as other growth factors that resemble and produce the same effects as anabolic steroids. Furthermore, previous claims have historically accused Bucked Up of using non-proprietary combinations of growth hormones and steroids within their products. By coupling CID with DIMS, overlapping peaks of deer antler velvet extract, synthesized growth hormones, and anabolic steroids can be identified and differentiated within tainted samples. Furthermore, the concentrations of contaminants, specifically of steroids and steroid-alternatives, can be accurately quantified to indicate ineffective or potentially hazardous levels.

3.5 Conclusion

The application of spectral analysis using ATR-FTIR, and DIMS/MS exhibits a favorable approach for rapidly identifying the constituents of leniently regulated nutraceutical samples manufactured and distributed from various sources. ATR-FTIR is a non-destructive technique that allows for obvious ingredient alterations to be identified which can then be further analyzed using the rapid methodology of DIMS. This combined method has the potential to inform health-conscious, vulnerable consumers of the risks that they may be exposed to if they choose to utilize under-researched nutraceutical products for their dietary and health needs that are attained from fraudulent manufacturers.

3.6 References

[1] Dept. of Health and Human Services, Centers for Disease Control and Prevention,

National Center for Health Statistics. (2014). *Health, United States, 2013: With special feature on prescription drugs*.

[3] Manikandaselvi, S., V. Vadivel, and P. Brindha. "Nutraceuticals–An overview." *Int J Pharm Bio Sci* 7.1 (2016): 284-296.

[4] Iyen, Sandra, I., Ogofotha, Oghenekeno, G., & Odiba, J. (2019). Advances in nutraceuticals. *International Journal of Medicinal Plants and Natural Products*, *5*(2). https://doi.org/10.20431/2454-7999.0502002

[6] Nasri, H., Baradaran, A., Shirzad, H., & Rafieian-Kopaei, M. (2014, November 6). *New Concepts in nutraceuticals as alternative for pharmaceuticals*. International journal of preventive medicine. Retrieved September 28, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4336979/

[7] Williamson, E. M., Liu, X., & Izzo, A. A. (2019, December 4). *Trends in use, pharmacology, and clinical ... - wiley online library*. Trends in use, pharmacology, and clinical applications of emerging herbal nutraceuticals. Retrieved September 30, 2022, from https://bpspubs.onlinelibrary.wiley.com/doi/abs/10.1111/bph.14943

[8] Gil, F., Hernández, A. F., & Martín-Domingo, M. C. (2021, January 29). *Toxic contamination of Nutraceuticals and Food Ingredients*. Nutraceuticals (Second Edition). Retrieved September 30, 2022, from https://www.sciencedirect.com/science/article/pii/B9780128210383000689

[61] Pérez-Lavalle, L., Carrasco, E., & Valero Diaz, A. (2020). Microbiological criteria: Principles for their establishment and application in food quality and safety. *Italian Journal of Food Safety*, *9*(1), 8543. https://doi.org/10.4081/ijfs.2020.8543

[62] Food and Agriculture Organization of the United Nations, & World Health Organization. (2021). Microbiological risk assessment – guidelines for food. *Food and Agriculture Organization of the United Nations*, 288. https://doi.org/10.4060/cb5006en

[73] Premier Biosoft . (n.d.). *Mass Spectrometry*. Mass spectrometry: Introduction, principle of mass spectrometry, components of mass spectrometer, applications. Retrieved October 3, 2022, from http://www.premierbiosoft.com/tech_notes/mass-spectrometry.html

[74] El-Aneed, A., Cohen, A., & Banoub, J. (2009). Mass spectrometry, review of the basics: Electrospray, MALDI, and commonly used mass analyzers. *Applied Spectroscopy Reviews*, *44*(3), 210–230. https://doi.org/10.1080/05704920902717872

[94] Ng, L. M., & Simmons, R. (1999). Infrared Spectroscopy. *Analytical Chemistry*, *71*(12), 343–350. https://doi.org/10.1021/a1999908r

[95] Blum, M.-M., & John, H. (2011). Historical perspective and modern applications of attenuated total reflectance - fourier transform infrared spectroscopy (ATR-FTIR). *Drug Testing and Analysis*, *4*(3-4), 298–302. https://doi.org/10.1002/dta.374

[107] Cole, C. (2010). *Cut: A guide to adulterants, bulking agents and other contaminants found in illicit drugs*. Centre for Public Health, Faculty of Health and Applied Social Sciences, Liverpool John Moores University.

[108] Lichtenstein, A. H., & Russell, R. M. (2005). Essential nutrients: Food or supplements? *JAMA*, *294*(3), 351. https://doi.org/10.1001/jama.294.3.351

[109] Buck, K., Voehringer, P., & Ferger, B. (2009). Rapid analysis of GABA and glutamate in microdialysis samples using high performance liquid chromatography and Tandem Mass Spectrometry. *Journal of Neuroscience Methods*, *182*(1), 78–84. https://doi.org/10.1016/j.jneumeth.2009.05.018

[110] Desai, M. J., & Armstrong, D. W. (2004). Analysis of derivatized and underivatized theanine enantiomers by high-performance liquid chromatography/atmospheric pressure ionization-mass spectrometry. *Rapid Communications in Mass Spectrometry*, *18*(3), 251–256. https://doi.org/10.1002/rcm.1319

[111] Moein, M. M., El-Beqqali, A., Abdel-Rehim, A., Jeppsson-Dadoun, A., & Abdel-Rehim, M. (2014). Preparation of monolithic molecularly imprinted polymer sol–gel packed tips for high-throughput bioanalysis: Extraction and quantification of L-tyrosine in human plasma and urine samples utilizing liquid chromatography and Tandem Mass Spectrometry. *Journal of Chromatography B*, *967*, 168–173. https://doi.org/10.1016/j.jchromb.2014.07.029

[112] Wang, Y.-H., Mondal, G., Butawan, M., Bloomer, R. J., & Yates, C. R. (2020). Development of a liquid chromatography-tandem mass spectrometry (LC–MS/ms) method for characterizing caffeine, methylliberine, and theacrine pharmacokinetics in humans. *Journal of Chromatography B*, *1155*. https://doi.org/10.1016/j.jchromb.2020.122278

CHAPTER 4

ANALYSIS OF HEAVY METAL AND MICROBIAL CONTAMINATION IN NUTRACEUTICAL SUPPLEMENTS USING ICP-OES AND COLONY-PCR

4.1 Introduction

During the industrial manufacturing process of synthesizing chemical compounds, including both pharmaceuticals and alternative supplements, such as nutraceuticals, organic and inorganic impurities can be introduced through improper handling and storage conditions [8, 71]. These circumstances are typically avoided by enforcing laboratory protocols and establishing regulatory measures through quality control (QC) and quality assurance (QA). However, proper protocol can't be appropriately established if the compounds themselves have not been accurately defined. In the case of nutraceuticals, regulation is unclear due to its dual-nature of possessing both food-derivatives as well as pharmaceutical properties [3, 6]. This obscurity creates difficulty in classifying many of the compounds that fall within this category that are in high demand to be abundantly produced [7]. As popularity among health-conscious consumers grows for naturally sourced supplements for all their health needs, manufacturers are pressured for rapid production of product [5]. This time-sensitive demand to increase sales allows for already lenient QA and QC measures to be nearly disregarded without any concern for penalty [69, 70]. As more liberties are taken to supply more product, increased potential for contamination will go unaddressed and unpunished. Ultimately, consumers will be exposed to hazardous contaminants that are detrimental to an already vulnerable population [8].

Since nutraceuticals are naturally derived, contamination originating from the environment persists when these supplementary compounds are sourced. Inorganic contaminants, such as heavy metals, are ubiquitous in soil which then migrate into groundwater,

air, and the land, and eventually deposit and accumulate within plants and other organisms [10, 37]. These metals can result from natural erosion processes of the earth's layers, as well as artificial sources resulting from industrial production, daily automobile emissions, and combustion by-products [8, 9]. Both biologically essential and non-essential metals are produced within the environment and are capable of bioaccumulating. Essential metals, such as iron, zinc, and copper, are required in trace amounts to perform or contribute to vital biochemical functionality. Furthermore, total absence of these trace elements within the body results in irreversible damage to major organs [37]. On the other hand, when essential metals exceed their necessary minute amounts, they can quickly exert toxic effects by disrupting the same mechanisms that they are routinely involved in [38]. In addition to the presence of essential heavy metals, non-essential heavy metals also occur in the environment from both natural and artificial sources. Frequent interactions with cadmium, lead, and mercury within the environment exert their toxicity by readily interacting with essential metals within biological systems [20]. Other less observed but still prevalent non-essential metals, such as arsenic, produce lethal effects not only through disrupting these biochemical pathways, but by further inhibiting vital reactions that are imperative for life. These hazardous metals exert their toxicity and potential lethality typically through accumulating in vital organs and tissues upon ingestion. From there, these metals target specific biological systems to disrupt or inhibit normal functioning mechanisms. This ultimately intervenes with DNA repair mechanisms and affects cell proliferation through the generation of reactive oxygen species (ROS) which induces cell death via apoptosis. Depending on the targeted system, associated diseased states emerge from the resulting cellular and tissue damage as well as from overall metal accumulation within the organs [33-36]. In addition to inorganic heavy metal contamination, organic contamination can occur in

the form of microorganisms [61]. Opportunistic bacteria result from improper handling and storage conditions, as well as overall lack of implementing preventative measures to ensure a sterile manufacturing environment. Despite the commensal nature of essential microbes that play a vital role in gut microbiota, as well as producing primary metabolites such as amino acids, vitamins, and nucleotides, and secondary metabolites for antibiotics and drug therapies, opportunistic pathogens will take advantage of an immunocompromised host to proliferate [40-43].

When effective screening methods for both inorganic and organic contaminants are not established and further implemented during the manufacturing process, ignorant consumers become at risk to toxic levels of heavy metals as well as potential pathogens in the supplements responsible for enhancing their health [8]. Furthermore, these health-conscious individuals typically are seeking out these products for preventative and treatment options for currently existing diseased states [6]. Post-market recall actions are ineffective for these vulnerable consumers who already require intentional preventative actions to ensure that their health-concerns don't progress. Due to the ineffective QC and QA measures implemented that are responsible for ensuring consumers that they are purchasing and consuming a safe and effective product, updated paneling measures are required to properly assess the state of these products.

Generic panels are ineffective at identifying hazardous contaminants and require more intensive analytical techniques. To routinely monitor and quantify levels of heavy metals present in not only nutraceuticals, but also in any other pharmaceutical or dietary health products, instrumentation methods such as Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) can be utilized. This sensitive analytical technique can determine trace and ultra-trace levels of multiple elements withing different sample matrices. Furthermore, ICP-OES quantifies

elements present in samples by measuring excited atoms and ions at their characteristic wavelengths within a high-temperature plasma making it a highly specific multi-element analysis technique. Since this technique is compatible with diverse sample matrices, ICP-OES is highly effective for drug and pharmaceutical trace metal analysis [87-90]. Another method for analyzing contaminants, specifically micro-bacteria, is with Polymerase Chain Reaction (PCR). PCR is a rapid, high-throughout, high yield, automated technique that can screen bacterial colonies that have been cultured on selective media. This chain reaction cycles between three steps: denaturing double-stranded DNA, primer annealing, and primer extension. Each cycle is approximately 3-5 minutes and is typically repeated 30-40 times. The end of this cycling process results in the amplification of specific bacterial DNA sequences. The produced amplified target DNA sequences can then be visualized using a variety of staining techniques based on the amount of DNA that was able to be amplified and indicates the sequences of bacterial DNA present in the sample. This versatile technique has extensive diagnostic applications spanning across biological sciences and medicine, as well as implications for infectious disease identification [99-106].

4.2 Methods

The suspected adulterated nutraceutical samples listed in Table 2.1 were assessed for forms of contamination resulting from unregulated laboratory protocol. The samples were specifically evaluated for inorganic and organic contamination. Inorganic contamination was analyzed using ICP-OES to detect the presence of potential heavy metals. Similarly, potential organic contamination was assessed to indicate relative safety levels implemented or disregarded during the manufacturing process. To do this, the samples were cultured in multiple nutrient-rich media to observe any degree of microbial contamination. The cultured samples were then

analyzed using colony PCR, and the resulting DNA was visualized using Gel-electrophoresis.

4.2.1 ICP-OES Analysis

A stock solution of 2% nitric acid was created for metal dissolution and stabilization for trace metal analysis. Two metal standards were prepared which contained potential hazardous metals of interest present in the samples. One of the standard solutions was prepared in 1 ppm, 5 ppm, and 10 ppm solution concentrations, and consisted of beryllium, cadmium, chromium, mercury, lead, copper, zinc, nickel, and iron. The other standard solution consisted of arsenic and was prepared in 1 ppm, 5 ppm, and 10 ppm sample concentrations as well. A solution of 2% nitric acid was used as a blank solution. The samples produced by Bucked Up, samples 1, 2, 9, 12, 15, and 18, were prepared using 50 mg/mL concentrations in 2% nitric acid and were filtered using 0.45 μm PES syringe filters. The standard and sample solutions were then analyzed using an iCAP 7000 Series ICP-OES Duo to assess the presence of the ten heavy metals of interest. The resulting standard concentrations were then used to construct concentration curves for each metal standard to determine each the concentration of each sample's metal constituents.

4.2.2 Colony-PCR Analysis

Following the analysis of inorganic contaminants, organic contamination was assessed through qualitative culture methods to evaluate the presence of bacteria. One gram of each sample was dissolved in tryptic soy broth (TSB) for 24 hours and was incubated at 37℃ in a shaking incubator set at 230 rpm to cultivate a wide variety of potential microbial activity present in the samples. The cultures were then pipetted, streaked onto nutrient agar plates, and incubated at 37℃ for 24 hours to continue the cultivation of observed microbes and support the growth of various types of bacteria. The primer pairs specific to the bacterial DNA strains of interest, as shown in Table 4.2, were re-suspended with the specified amount of nuclease-free

water. The primers were then vortexed, centrifuged, and placed in a 37℃ water bath for 10 minutes. Each primer was diluted to a 5 μL/45 μL solution using nuclease-free water to create a working solution. Eleven primer-specific PCR reaction tubes were prepared by adding 60 μL of GoTaQ Master Mix, 3 μL of forward specific primer, 3 μL of reverse specific primer, and 54 μL of nuclease-free water. A negative control was prepared as well using only GoTaq and nuclease-free water. Colony picking was utilized by scooping a small sample amount of each cultured bacteria into each tube containing each bacterial-specific primer. The PCR reactions were performed in three temperature-specific groups using an Eppendorf Mastercycler Nexus PCR Thermal Cycler (Fisher Scientific, Waltham, MA). The first group's (Group A) reaction cycle was performed with an initial denaturation at 95℃ for 3 minutes, followed by 35 cycles at 95℃, 42℃, and 72℃ for 0.5 minutes, 0.5 minutes, and 1.5 minutes, respectively, with a final extension at 72℃ for 3 minutes. The second group (Group B) followed the following reaction cycle procedure: an initial denaturation at 95℃ for 3 minutes, followed by 35 cycles at 95℃, 45℃, and 72℃ for 0.5 minutes, 0.5 minutes, and 1.5 minutes, respectively, with a final extension at 72℃ for 3 minutes. The third group (Group C) followed the following reaction cycle procedure: an initial denaturation at 95℃ for 3 minutes, followed by 35 cycles at 95℃, 51℃, and 72℃ for 0.5 minutes, 0.5 minutes, and 1.5 minutes, respectively, with a final extension at 72℃ for 3 minutes. The final PCR products were visualized by loading the products into Agarose gel wells which were then covered with 1% tris-acetate-EDTA (TAE) and run using Gel-Electrophoresis. The resulting fragmentations were analyzed for positive bands indicating which bacterial DNA is present in each sample culture.

4.3 Results

Both heavy metal standard solutions, one containing beryllium, cadmium, chromium,

mercury, lead, copper, zinc, nickel, and iron, and the other containing arsenic, were prepared in increments of 1 ppm, 5 ppm, and 10 ppm, in 2% nitric acid in addition to a blank standard consisting of only 2% nitric acid. After running both standards using an ICP-OES, the resulting intensities associated with each standard concentration were constructed into a calibration curve for each metal standard, an example of iron and beryllium shown in Figure 4.1. This curve was utilized to identify the unknown concentrations of trace heavy metals potentially present in samples of interest. Additional information including the function (f(x)), the coefficient of determination (R^2), the background equivalent concentration (BEC), and the limits of detection (LoD) were provided for each curve as well.

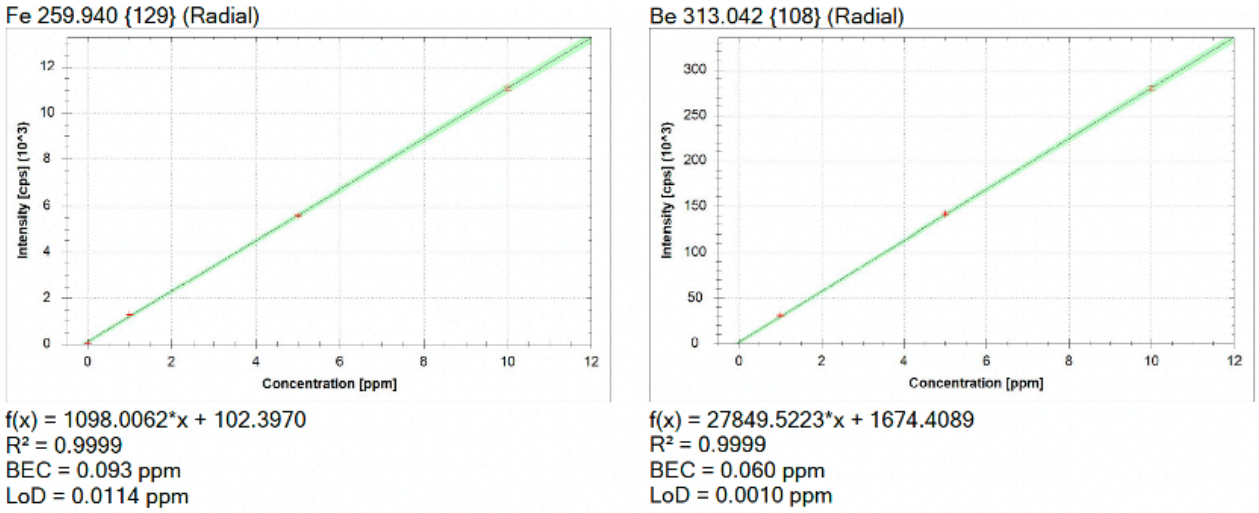

Figure 4.1: **The calibration curves of iron (left) and beryllium (right) constructed using a blank 2% nitric acid solution, a 1 ppm, 5 ppm, and 10 ppm solution containing all the heavy metals of interest other than arsenic, in addition to each curve's generated line function (f(x)), R^2 value, background equivalent concentration (BEC), and limits of detection (LoD) value.**

After constructing individual calibration curves for each metal of interest, 10 ppm solutions were prepared in 2% nitric acid for each sample and filtered using 0.45 μm PES syringe filters. Each of the six Bucked Up samples were run using the ICP-OES, and the concentration average of each metal standard present in the sample was listed, as shown in Table 4.1. The presence of hazardous heavy metals was not detected in any of the six samples except

for minute levels of iron in sample 15. However, this value was below 0.1 ppm which does not warrant concern for toxic effects.

	Concentration average	Concentration RSD	Concentration SD
Cr 283.563 {119} (Radial)	-0.028 ppm	20.8 %	0.0 ppm
Cd 228.802 {447} (Radial)	-0.070 ppm	3.1 %	0.0 ppm
Hg 184.950 {482} (Radial)	-0.110 ppm	2.3 %	0.0 ppm
Pb 220.353 {453} (Radial)	-0.078 ppm	0.5 %	0.0 ppm
Cu 324.754 {104} (Radial)	-0.090 ppm	4.5 %	0.0 ppm
Zn 213.856 {457} (Radial)	-0.070 ppm	3.1 %	0.0 ppm
Ni 221.647 {452} (Radial)	-0.084 ppm	0.8 %	0.0 ppm
Fe 259.940 {129} (Radial)	0.252 ppm	31.2 %	0.1 ppm
Be 313.042 {108} (Radial)	-0.060 ppm	0.2 %	0.0 ppm

Table 4.1: **The average concentration (SD) of each heavy metal of interest present in sample 15 in addition to the relative standard deviation (RSD) between the results.**

After analyzing inorganic contaminant levels, samples 1-20, in addition to the deer antler velvet powder and extract, designated as sample 21, and 22, respectively, were assessed for any indication of bacterial contamination. Each sample was cultured for 24 hours in glass tubes containing TSB, as shown in Figure 4.2.

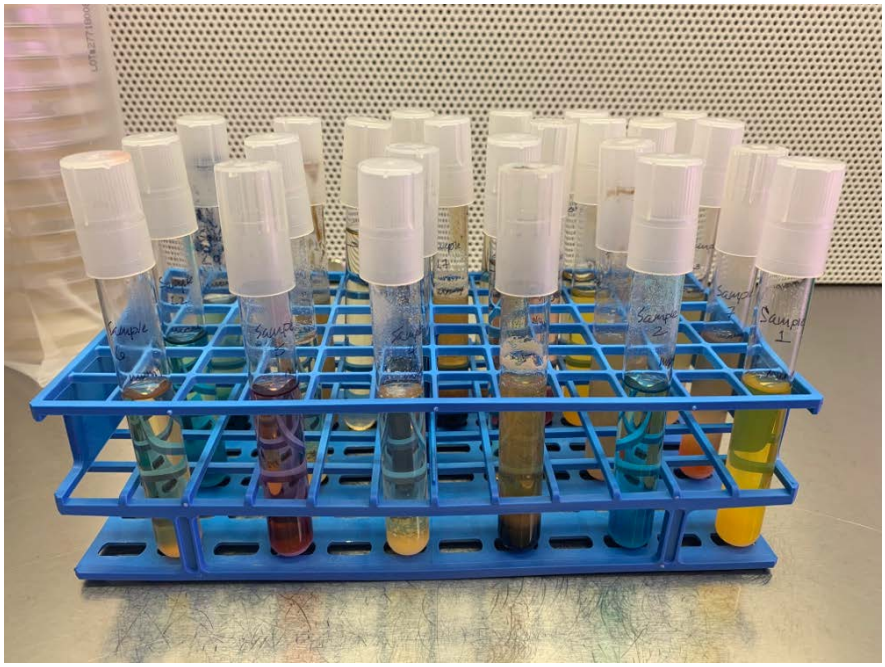

Figure 4.2: **Samples 1-20 after being cultured in tryptic soy broth (TSB) for 24 hours in a shaking incubator set at 230 rpm and 37℃**

Aliquots from the TSB cultures were then streaked onto nutrient agar (NA) plates and were cultured at 37℃ for 24 hours. No bacterial growth was observed in sample 2, 3, 4, 5, 6, 9, 10, 12, 15, 16, 17, 18, and 22 plates after 24 hours. Some potential growth was observed on sample 1 and 11 after one week of continued culturing. At this point, however, incubation had continued for too long and sample 1, 7, 8, and 11 plates were no longer viable. The remaining five sample plates that presented bacterial growth are shown in Figure 4.3.

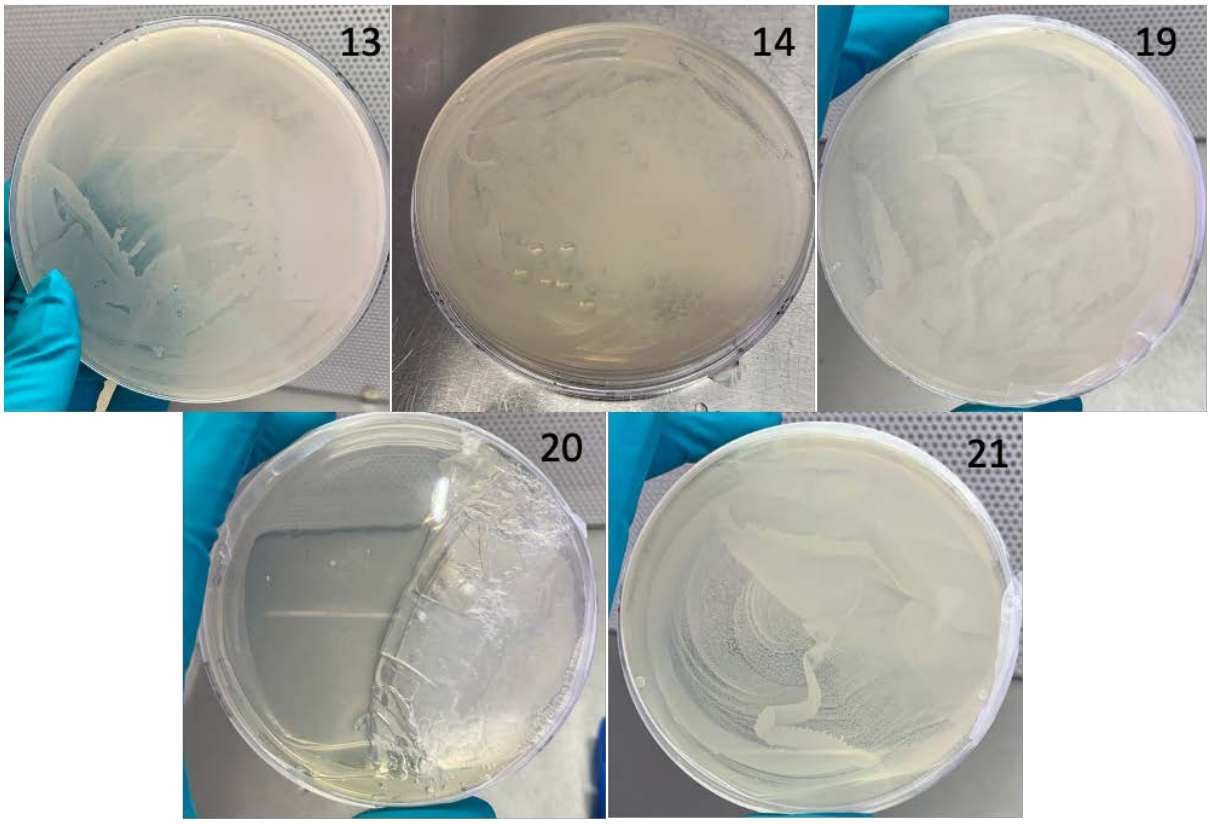

Figure 4.3: **The five viable culture plates expressing bacterial growth from samples 13 (Total War), 14 (MucleTech), 19 (Extenze), 20 (Performix), and 21 (Deer Antler Velvet Powder)**

Before conducting PCR analysis, these five plates were designated new plate numbers for further analytical reference, as shown in Table 4.2. For each sample plate, eleven different primer pairs specific to different bacterial DNA strains of interest, shown in Table 4.3, were prepared for PCR analysis.

Original Sample Number	New Plate Number
Sample 13 - Total War Pre-Workout	1
Sample 14 – MuscleTech Shatter Pre-Workout	2
Sample 19 - Fast Acting Extenze Maximum Release Male Enhancement	3
Sample 20 – Performix SST v2X Extreme Thermogenic Fat Burner Weight Loss Supplement	4
Sample 21 – Deer Antler Velvet Powder	5

Table 4.2: **The new designated plate numbers for the five remaining viable plates analyzed using colony-PCR**

Bacterial Primer Name	Forward (F) and Reverse (R) Primer Sequence	Annealing Temperature (℃)	PCR Product Size (bp)
Universal Primer Pair	F: 5'AGAGTTTGATCMTGGCTCAG3' R: 5'TACGGYACCTTGTTACGACTT3'	46.8	1500
S. hominis	F: 5'GTTCGATAGTGAAAGATGGCTC3' R: 5'GGAAACTTCTATCTCTAGAAGG3'	43.4	833-852
S. warneri	F: 5'GGTTCAATAGTGAAAGGCGGC3' R: 5'GGAAGACTCTATCTCTAGAGC3'	41.1	833-852
S. epidermidis	F: 5'TCTCTTTTAATTTCATCTTCAATTCCATAG3' R: 5'AAACACAATTACAGTCTGTTATCCATATC3'	54	174
P. aeruginosa	F: 5'ATGGAAATGCTGAAATTCGGC3' R: 5'CTTCTTCAGCTCGACGCGACG3'	55	504
B. anthracis	F: 5'AATCGTAATATTAAACTGACG3' R: 5'CCTTCATACGTGTGAATGTTG3'	40.5	244
B. cereus	F: 5'ATTGGTGACACCGATCAAACA3' R: 5'TCATACGTATGGATGTTATTC3'	41	364
B. subtilis	F: 5'CAGTCAGGAAATGCGTACGTC CTT3' T: 5'CAAGGTAATGCTCCAGGCATTGCT3'	57.2	1027
S. aureus	F: 5'GCGATTGATGGTGATACGGTT3' R: 5'AGCCAAGCCTTGACGAACTAA AGC3'	55	280
E. coli	F: 5'AAAACGGCAAGAAAAAGCAG3' R: 5'ACGCGTGGTTACAGTCTTGCG3'	50.7	147
S. enterica	F: 5'ATCGCCACGTTCGGGCAATTC3' R: 5'ACGGTTCCTTTGACGGTGCGAT3'	55	275

Table 4.3: **The eleven bacterial primer pairs with their associated sequences, temperature-specific annealing temperature, and PCR product size used for colony-PCR analysis in sample plates 1-5**

Due to the varying annealing temperatures of certain enzymatic primer pairs, the primers were split into three different groups based on temperature specificity. Group A was run at an annealing temperature of 42℃, and included primers for the Universal Primer Pair, *Bacillus cereus*, and *Bacillus anthracis*. Group B was run at an annealing temperature of 45℃, and included primers for *Salmonella warneri*, *Salmonella epidermidis*, *Salmonella hominis*, and *Escherichia coli*. Group C was run at an annealing temperature of 51℃, and included primers for

Bacillus subtilis, *Salmonella enterica*, *Salmonella aureus*, and *Salmonella aeruginosa*. After preparing the PCR reaction tubes containing the individual primer pairs per sample plate, a small amount of bacterial sample was directly scooped into its respective reaction tubes. Each temperature-specific group was placed in its designated PCR Thermal Cycler, and PCR was performed according to the cycle parameters previously mentioned. The PCR products were then pipetted next to a 1 kb DNA ladder into 1% agarose gel wells covered with 1x TAE buffer and were analyzed using gel-electrophoresis. The three gels containing the bacterial DNA fragmentation bands were visualized using a gel imager and are shown in Figures 4.4, 4.5, and 4.6, respectively.

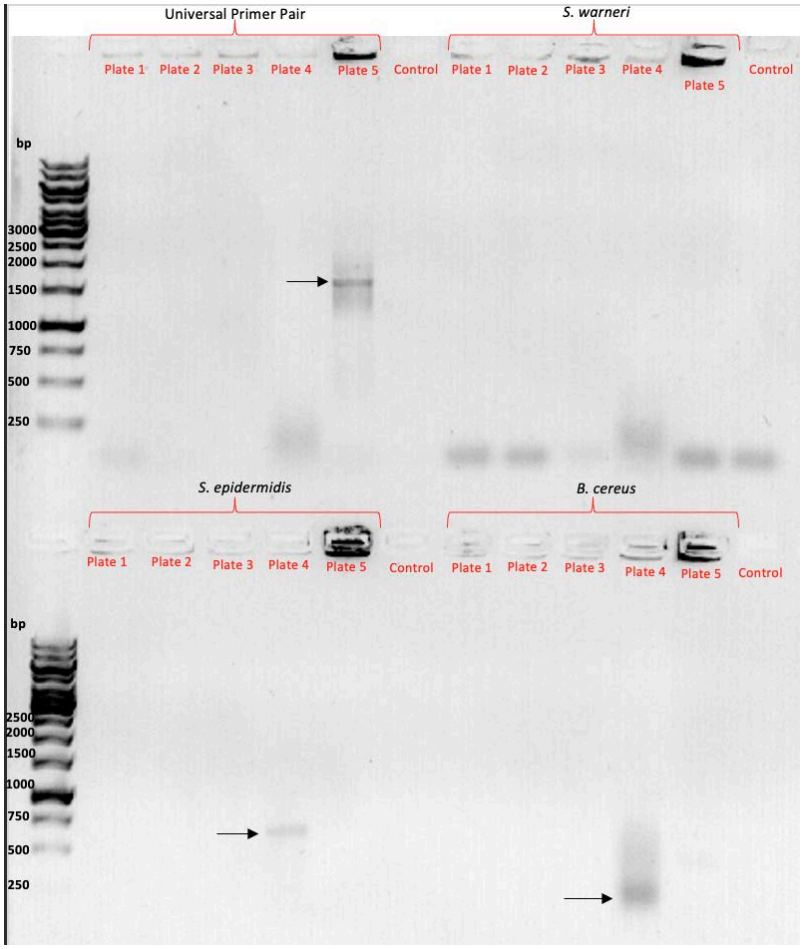

Figure 4.4: **The bacterial DNA fragmentation bands produced from Gel 1 after performing agarose gel-electrophoresis on plates 1-5 using the Universal Primer Pair, *S. warneri*, *S. epidermidis*, and *B. cereus* bacterial DNA primers**

Gel 1 assessed plates 1-5 for positive bands indicating the presence of *Salmonella warneri*, *Salmonella epidermidis*, and *Bacillus cereus*. Plate 5 produced a positive band at 1500 bp indicating the presence of the Universal Primer Pair. Plate 4 produced positive bands for *S. epidermidis* and *B. cereus* at 600 and 250 bp, respectively. Plates 1-5 did not produce positive bands for *S. warneri*.

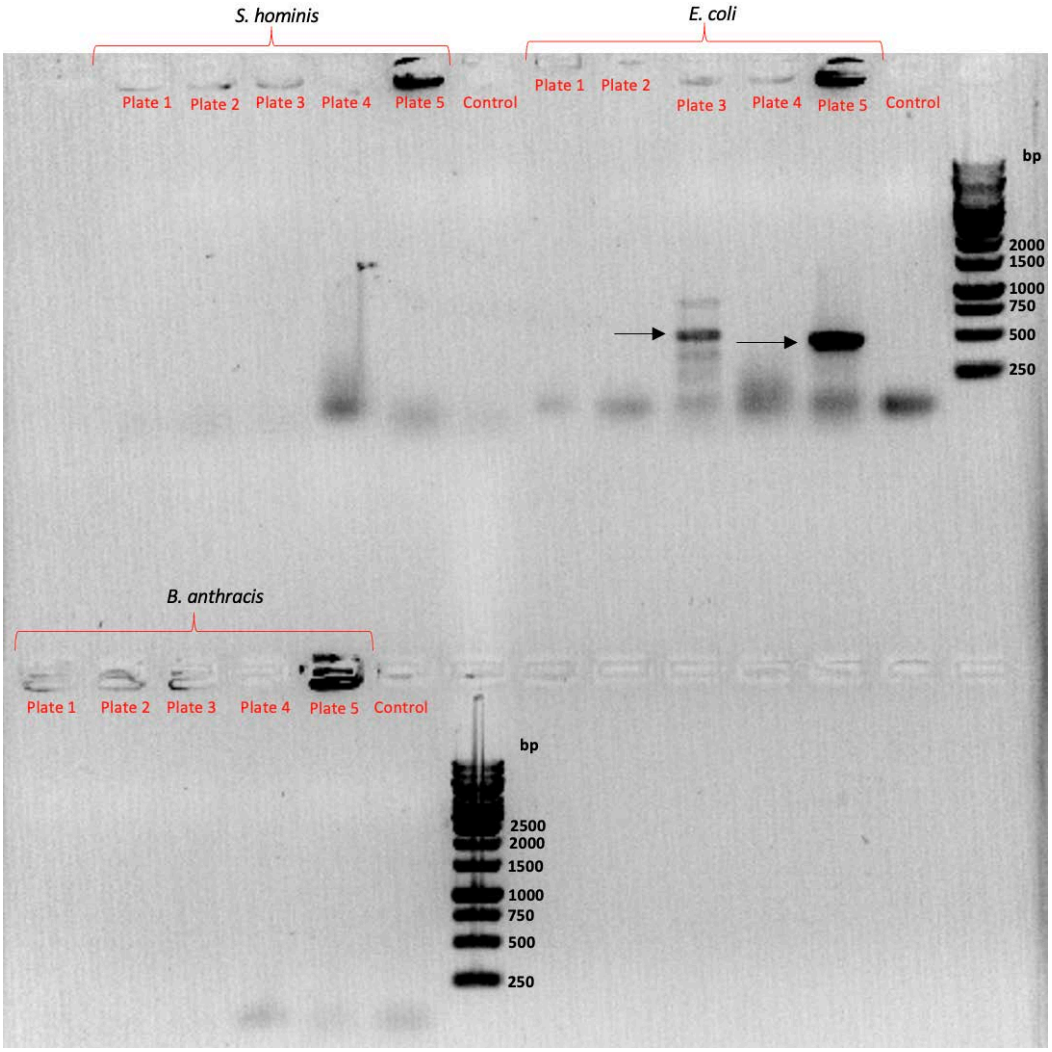

Figure 4.5: **The bacterial DNA fragmentation bands produced from Gel 2 after performing agarose gel-electrophoresis on plates 1-5 using *S. hominis*, *E. coli*, and *B. anthracis* bacterial DNA primers**

Gel 2 assessed plates 1-5 for positive bands indicating the presence of *Salmonella hominis*, *Escherichia coli*, and *Bacillus anthracis*. No positive bands indicated the presence of *S.*

hominis or *B. anthracis*. However, plates 3 and 5 positively identified the presence of *E. coli* expressed by a positive band each at 500bp.

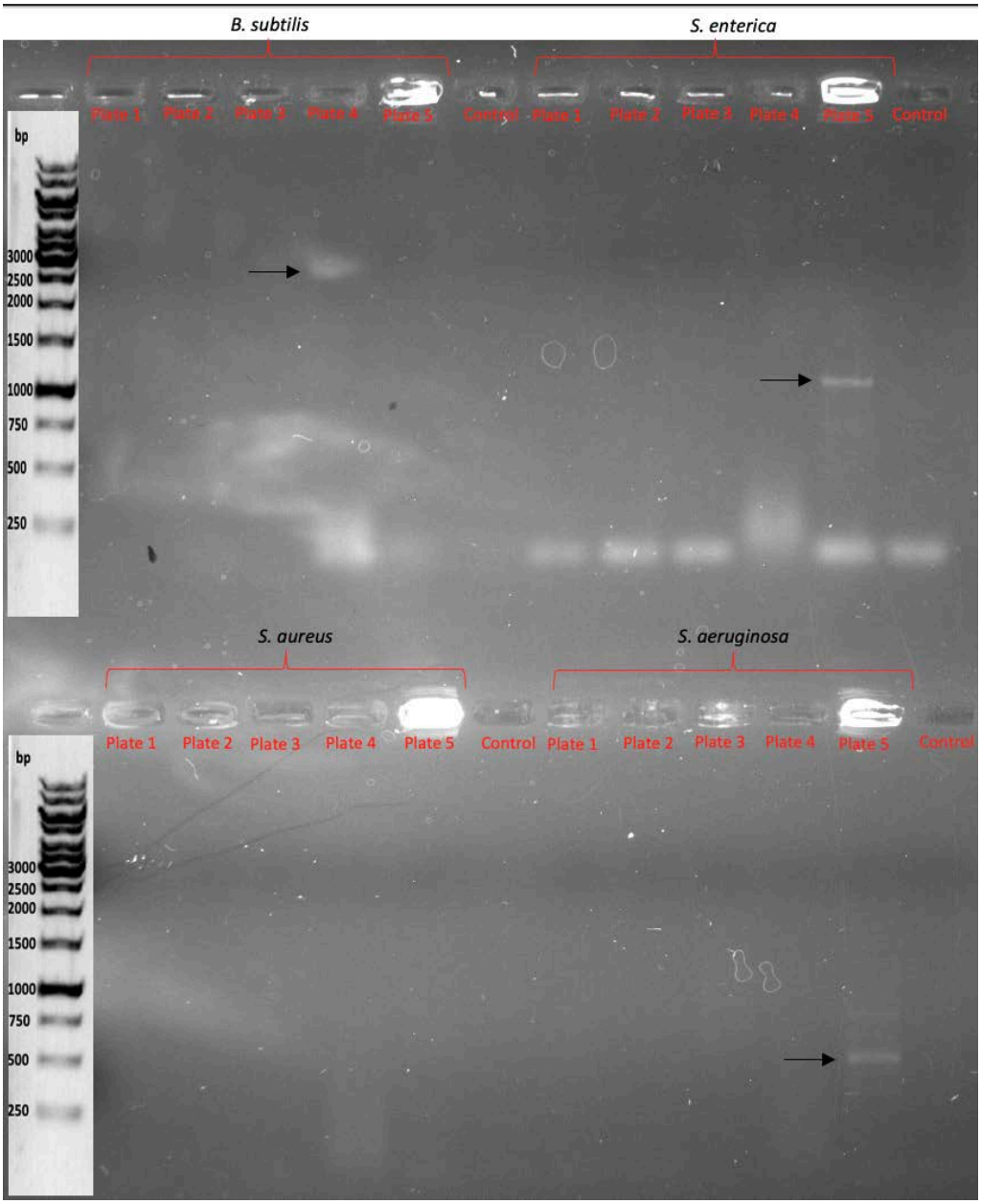

Figure 4.6: **The bacterial DNA fragmentation bands produced from Gel 3 after performing agarose gel-electrophoresis on plates 1-5 using *B. subtilis, S. enterica, S. aureus,* and *S. aeruginosa* bacterial DNA primers**

Gel 3 assessed plates 1-5 for positive bands indicating the presence of *Bacillus subtilis*, *Salmonella enterica*, *Salmonella aureus,* and *Salmonella aeruginosa*. Plate 4 presented a positive

band for *B. subtilis* at 3000bp, and plate 5 presented a positive band for *S. enterica* between 1,000-1,500 bp, as well as a positive band indicating the presence of *S. aeruginosa* at 500 bp.

4.4 Discussion

Despite the lack of significant heavy metal levels detected in the Bucked Up samples, ICP-OES demonstrates potential for not only monitoring multiple heavy metal levels in nutraceutical samples, but also identifying hazardous elements indicative of sub-par laboratory standards. By identifying candidate heavy metals within contaminated samples, specific countries associated with known heavy metal contaminants can be identified as the source of these tampered products. Furthermore, the presence of these heavy metals could have implications for emerging diseased states in humans, as well as bioaccumulation in plants and aquatic life ultimately leading to biomagnification within various ecosystems.

Additionally, colony-PCR displayed the ability of rapidly screening samples for microbial activity. A variety of bacteria were detected specifically in plates 3, 4, and 5, indicating the presence of *S. epidermidis, B. cereus, E. coli, B. subtilis, S. enterica, and S. aeruginosa.* This information is vital in determining associated implications for the safety of human health based on the types of bacteria detected, as well as the associated diseased states that could potentially be observed resulting from pathogenesis. Furthermore, the emphasis of implementing appropriate preventative measures is requisite to avoid potential injury of immunocompromised consumers relying on these products to prevent, treat, or enhance their health concerns.

Even though some individuals may experience immune sensitivity to unintended contaminants and lower levels of sample adulteration, most consumers exhibit robust immune responses. The majority of identified microbial activity and heavy metal deposition at these

minimal levels succumb to the ruthless acidity of the stomach and are quickly eliminated to prevent toxicity. Additionally, the mucous membrane lines the body's organs with lubrication to act as a protective barrier against hazardous particulates and invasive pathogens. Due to the inherent protective mechanisms of the body, low levels of careless contamination will not pose as a significant threat to human health and safety. However, these considerations can become vital in individuals with pre-existing diseased states as well as accounting for potential contraindications.

4.5 Conclusion

Versatile methods such as ICP-OES and PCR are capable of analyzing heavy metals and bacteria ubiquitous in the environment. By utilizing these techniques specifically for nutraceutical sample analysis, various contaminants prone to depositing in these supplementary products can be identified and potentially sourced. Furthermore, contaminant detection from these techniques will be able to establish appropriate paneling and protocol for the storage, manufacturing, and distribution of products categorized under the diverse class of nutraceuticals.

4.6 References

[3] Manikandaselvi, S., V. Vadivel, and P. Brindha. "Nutraceuticals–An overview." *Int J Pharm Bio Sci* 7.1 (2016): 284-296.

[5] Espín, J. C., García-Conesa, M. T., & Tomás-Barberán, F. A. (2007). Nutraceuticals: Facts and fiction. *Phytochemistry*, *68*(22-24), 2986–3008. https://doi.org/10.1016/j.phytochem.2007.09.014

[6] Nasri, H., Baradaran, A., Shirzad, H., & Rafieian-Kopaei, M. (2014, November 6). *New Concepts in nutraceuticals as alternative for pharmaceuticals*. International journal of preventive medicine. Retrieved September 28, 2022, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4336979/

[7] Williamson, E. M., Liu, X., & Izzo, A. A. (2019, December 4). *Trends in use, pharmacology, and clinical ... - wiley online library*. Trends in use, pharmacology, and

clinical applications of emerging herbal nutraceuticals. Retrieved September 30, 2022, from https://bpspubs.onlinelibrary.wiley.com/doi/abs/10.1111/bph.14943

[8] Gil, F., Hernández, A. F., & Martín-Domingo, M. C. (2021, January 29). *Toxic contamination of Nutraceuticals and Food Ingredients*. Nutraceuticals (Second Edition). Retrieved September 30, 2022, from https://www.sciencedirect.com/science/article/pii/B9780128210383000689

[9] Filipiak-Szok, A., Kurzawa, M., & Szłyk, E. (2014, November 4). *Determination of toxic metals by ICP-MS in Asiatic and European medicinal plants and dietary supplements*. Journal of Trace Elements in Medicine and Biology. Retrieved September 30, 2022, from https://www.sciencedirect.com/science/article/pii/S0946672X14001886

[10] Wu, W., Wu, P., Yang, F., Sun, D.-ling, Zhang, D.-X., & Zhou, Y.-K. (2018, February 20). *Assessment of heavy metal pollution and human health risks in urban soils around an electronics manufacturing facility*. Assessment of heavy metal pollution and human health risks in urban soils around an electronics manufacturing facility Author links open overlay panel. Retrieved September 30, 2022, from https://www.sciencedirect.com/science/article/pii/S0048969718305825

[20] Goyer, R. A. (1997). Toxic and essential metal interactions. *Annual Review of Nutrition, 17*(1), 37–50. https://doi.org/10.1146/annurev.nutr.17.1.37

[31] The Toxicology of Mercury Larry A. Broussard, PhD, DABCC, FACB,1Catherine A. Hammett-Stabler, PhD, DABCC, FACB,2Ruth E. Winecker, PhD,2,3 Jeri D. Ropero-Miller, PhD

[32] Farina, M., Rocha, J. B. T., & Aschner, M. (2011). Mechanisms of methylmercury-induced neurotoxicity: Evidence from experimental studies. *Life Sciences*, *89*(15-16), 555–563. https://doi.org/10.1016/j.lfs.2011.05.019

[33] Hughes, M. F. (2002). Arsenic toxicity and potential mechanisms of action. *Toxicology Letters*, *133*(1), 1–16. https://doi.org/10.1016/s0378-4274(02)00084-x

[34] Ratnaike, R. N. (2003). Acute and chronic arsenic toxicity. *Postgraduate Medical Journal*, *79*(933), 391–396. https://doi.org/10.1136/pmj.79.933.391

[35] Kaur, T., Singh, A. P., & Goel, R. K. (2011). Mechanisms pertaining to arsenic toxicity. *Toxicology International*, *18*(2), 87. https://doi.org/10.4103/0971-6580.84258

[36] Jomova, K., Jenisova, Z., Feszterova, M., Baros, S., Liska, J., Hudecova, D., Rhodes, C. J., & Valko, M. (2011). Arsenic: Toxicity, oxidative stress and human disease. *Journal of Applied Toxicology*. https://doi.org/10.1002/jat.1649

[38] Borzelleca, J. F. (2000). Paracelsus: Herald of modern toxicology. *Toxicological Sciences, 53*(1), 2–4. https://doi.org/10.1093/toxsci/53.1.2

[39] Ali, H., Khan, E., & Ilahi, I. (2019). Environmental chemistry and ecotoxicology of hazardous heavy metals: Environmental persistence, toxicity, and bioaccumulation. *Journal of Chemistry*, *2019*, 1–14. https://doi.org/10.1155/2019/6730305

[40] Demain, A. L., & Sanchez, S. (2009). Microbial Drug Discovery: 80 years of progress. *The Journal of Antibiotics*, *40*(19), 5–16. https://doi.org/10.1002/chin.200919215

[41] Hevia, A., Delgado, S., Sánchez, B., & Margolles, A. (2015). Molecular players involved in the interaction between beneficial bacteria and the immune system. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.01285

[59] Nataro, J. P., & Kaper, J. B. (1998). Diarrheagenic Escherichia coli. *Clinical Microbiology Reviews*, *11*(1), 142–201. https://doi.org/10.1128/cmr.11.1.142

[67] Agency for Toxic Substances and Disease Registry. (2019, July 2). *Lead (PB) toxicity: What are U.S. standards for lead levels?* Environmental Health and Medicine Education. Retrieved October 3, 2022, from https://www.atsdr.cdc.gov/csem/leadtoxicity/safety_standards.html

[68] Mensah, E., Kyei-Baffour, N., Ofori, E., & Obeng, G. (n.d.). Influence of human activities and land use on heavy metal concentrations in irrigated vegetables in Ghana and their health implications. *Appropriate Technologies for Environmental Protection in the Developing World*, 9–14. https://doi.org/10.1007/978-1-4020-9139-1_2

[69] U.S. Department of Health and Human Services. (n.d.). *Office of dietary supplements - dietary supplement health and education act of 1994*. NIH Office of Dietary Supplements. Retrieved September 30, 2022, from https://ods.od.nih.gov/about/dshea_wording.aspx

[85] Lübbert, C., & Peukert, W. (2021). Characterization of electrospray drop size distributions by mobility-classified mass spectrometry: Implications for ion clustering in solution and Ion Formation Pathways. *Analytical Chemistry*, *93*(38), 12862–12871. https://doi.org/10.1021/acs.analchem.1c00727

[86] Konermann, L., Metwally, H., Duez, Q., & Peters, I. (2019). Charging and supercharging of proteins for mass spectrometry: Recent insights into the mechanisms of electrospray ionization. *The Analyst*, *144*(21), 6157–6171. https://doi.org/10.1039/c9an01201j

[87] Ammann, A. A. (2007). Inductively coupled plasma mass spectrometry (ICP MS): A versatile tool. *Journal of Mass Spectrometry*, *42*(4), 419–427. https://doi.org/10.1002/jms.1206

[88] Sneddon, J., & Vincent, M. D. (2008). ICP-oes and ICP-MS for the determination of metals: Application to oysters. *Analytical Letters*, *41*(8), 1291–1303. https://doi.org/10.1080/00032710802013991

[97] Rossini, J. (2020, August 15). *FTIR: Hardware*. Chemistry LibreTexts. Retrieved October 3, 2022, from

https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_(Analytical_Chemistry)/Instrumental_Analysis/Spectrometer/How_an_FTIR_instrument_works/FTIR%3A_Hardware#:~:text=Michaleson%20Interferometer-,History,of%20the%20wavelengths%20of%20light.

[98] Bunaciu, A. A., Aboul-Enein, H. Y., & Fleschin, S. (2010). Application of fourier transform infrared spectrophotometry in pharmaceutical drugs analysis. *Applied Spectroscopy Reviews*, *45*(3), 206–219. https://doi.org/10.1080/00387011003601044

[99] Joshi, M., & Deshpande, J. D. (2011). POLYMERASE CHAIN REACTION: METHODS, PRINCIPLES AND APPLICATION. International Journal of Biomedical Research, 2(1). https://doi.org/10.7439/IJBR.V2I1.83

[100] Templeton, N. S. (1992). The polymerase chain reaction history methods, and applications. *Diagnostic Molecular Pathology*, *1*(1), 58–72. https://doi.org/10.1097/00019606-199203000-00008

[101] Schochetman, G., Ou, C.-Y., & Jones, W. K. (1988). Polymerase Chain Reaction. *The Journal of Infectious Diseases*, *158*(6), 1154–1157. http://www.jstor.org/stable/30137034

[102] Schochetman, G., Ou, C.-Y., & Jones, W. K. (1988). Polymerase chain reaction. *Journal of Infectious Diseases*, *158*(6), 1154–1157. https://doi.org/10.1093/infdis/158.6.1154

[103] Marmiroli, N., & Maestri, E. (2007). Polymerase chain reaction (PCR). *Food Toxicants Analysis*, 147–187. https://doi.org/10.1016/b978-044452843-8/50007-9

[104] Rajalakshmi, S. (2017). *Different types of PCR techniques and its applications*. International Journal of Pharmaceutical, Chemical and Biological Sciences. Retrieved October 3, 2022, from https://www.scribd.com/document/553020306/Different-Types-of-Pcr-Techniques-and-Its-Applications

[105] Bergkessel, M., & Guthrie, C. (2013). Colony PCR. *Methods in enzymology*, *529*, 299–309. https://doi.org/10.1016/B978-0-12-418687-3.00025-2

[106] Woodman, M. E. (2008). Direct PCR of intact bacteria (colony PCR). *Current Protocols in Microbiology*, *9*(1). https://doi.org/10.1002/9780471729259.mca03ds9

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 Nutraceutical Extract Analysis and Continued Research

The proposed method of coupling ATR-FTIR and DIMS is capable of effectively generating spectral analysis that can accurately identify the constituents of various nutraceutical supplement samples, as well as associated variance within the same product. This approach provides a baseline assessment to gauge the level of validity within these under-researched and leniently regulated products. By continuing to utilize these analytical methods, more niche areas within this broad category can be investigated to establish legitimate therapeutic doses, toxicity ranges, and identify any contraindications that may be associated. This information is vital to inform and reassure health-vulnerable consumers that their course of disease prevention or treatment is researched, and that appropriate parameters have been implemented regarding their products to maintain their safety. Additionally, these parameters are essential in identifying hazardous manufacturing procedures practiced by illegitimate brands responsible for consumer injury.

Not only can this method be applied to products already on the market, but these techniques can also be utilized to initiate research on under-researched plant extracts commonly used in “natural” supplements. Even though other countries that have gravitated toward eastern medicine have used these ingredients for generations, the actual mechanism of action and the physical properties of these extracts are unknown. By establishing this information, not only health-care workers, but also the general population, can become better educated as to how to properly implement these natural ingredients into disease prevention and treatment.

Lastly, implementation of ICP-OES and Colony-PCR requires continued testing to

establish effective parameters for identifying potential metal contaminants and microbial activity. These analytical techniques have the potential to indicate hazardous lab standards and protocols being used by analyzing resulting products. Once these parameters have been established to successfully identify inorganic and organic contaminants, quantitation can be implemented to designate appropriate levels of contamination present. This information will ultimately set standards for manufacturing and distribution nutraceutical products that meet the health and safety of the public.

5.2 Nutraceutical Database

While the need for intentional research, reliable data, and structure develops over time in the category of nutraceuticals, preliminary information can be compiled into an accessible database. The intention for this non-regulatory database is to promote public access to information pertaining to the health of under-researched consumer products. This information will help to inform individuals of general observations associated with products of interest, and ultimately guide consumers to the most appropriate preventive, treatment, or enhancement products to fit their health needs, with consideration of potential contraindications, associated adverse effects, and dosing levels.