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Analyzing the Lysergic Acid Amide Content Extracted from the Seeds of Argyreia nervosa via the Use of LC-MS

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by

Elisabeth Barone

An Abstract of a Project
in
Forensic Science

Submitted in Partial Fulfillment
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Abstract

Psychedelic plant use by humans can be traced back centuries to the times when early cultures used these plants in activities ranging from healing rituals to religious ceremonies. In recent years, several of these plants have reemerged on the drug scene and are currently being marketed as "legal highs" (since the plant seeds are *legal* to purchase through a variety of online vendors). With the growing number of internet forums where recreational drug users can write about their experiences and share them with others, it has become relatively simple for almost anyone to obtain the materials necessary to experience a legal high.

One active compound touted by recreational drug users is lysergic acid amide (LSA). LSA is known to exist naturally in the seeds of the *Rivea corymbosa, Ipomoea violacea (Morning Glory)*, and *Argyreia nervosa* (Hawaiian Baby Woodrose) species. Sharing a common chemical structure with the well-known psychedelic drug, lysergic acid diethylamide (LSD), LSA has also been known to produce hallucinogenic effects in its users. However, unlike LSD, which is one of the most potent psychedelic drugs ever discovered, LSA provides its users with a much weaker psychedelic experience. Frequent LSA users have also reported side effects such as nausea and fatigue. Currently, LSA is classified as a Schedule III drug in the United States, but seeds containing LSA remain legal for purchasing.[6]

The first aim of this project was to confirm the presence of LSA in seed samples of Hawaiian Baby Woodrose purchased over the internet. In order to do this, seed samples were first subjected to two extraction protocols: one mimicking a common street method and a second mimicking a procedure from a published research study. After obtaining the crude extracts from both extraction methods, extract samples were subjected to analysis via liquid chromatography-mass spectrometry. Through this technique, liquid chromatography was employed to separate the

various compounds present in each extract and mass spectrometry subsequently was employed to identify each compound by computing its protonated ion mass.

The second aim of this project was to evaluate the amount of LSA present in each extract. Ideally, a quantitative analysis would have been carried out using a pure LSA standard obtained from a reputable chemical vendor. However, due to the lack of availability of such a standard, a pseudo-quantitative approach was followed using an analytical grade standard of LSD, a drug very close in structure to that of LSA.

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Chapter 1: Introduction

1.1 Overview of Lysergic Acid Amide as a Psychedelic Drug

Humphry Osmond was the psychiatrist who first used the term "psychedelic" to describe mind-altering drugs during a conference in 1957 at the New York Academy of Sciences.[1, 2] But while he was the first to use the term, humans had already known of the effects of these drugs for several hundreds of years. Hallucinogenic plants were utilized by ancient societies for spiritual purposes and to treat a broad range of illnesses ranging from infections to inflammations.[3] The psychoactive compounds in these plants are still used today for religious ceremonies and recreational resources.[4] One of the main reasons humans are attracted to psychedelic substances is for their ability to provide mind-altering experiences.

Most people are familiar with the psychedelic drug lysergic acid diethylamide (LSD), but fewer people know about its structurally similar relative lysergic acid amide (LSA). LSA exists naturally in some plants, unlike LSD which is a semi-synthetic drug. It is present in 16 different types of Morning Glory seeds,[5] including the strains Heavenly Blue, Pearly Gates, and Ololiuqui. LSA has also been identified as the main alkaloid in the seeds of *Argyreia nervosa*, or Hawaiian Baby Woodrose (HBWR).[6] Like LSD, LSA is capable of being abused for its hallucinogenic properties. Moreover, LSA has been gaining notoriety around the world for many years due to those properties.

1.2 Overview of *Argyreia nervosa* Seeds

Like the Morning Glory, *Argyreia nervosa* plants are members of the flowering plant family Convolvulaceae, which includes a vast number of plant species.[7] Though native to India, *Argyreia nervosa* seeds grow in several tropical regions throughout the world.[8] *Argyreia nervosa*

is commonly referred to as Hawaiian Baby Woodrose (HBWR) due to how well the plant thrives in Hawaii's tropical climate. Figure 1 displays a photo of HBWR seeds next to a metric ruler.

A variety of ergot alkaloids and their stereoisomers have been identified in the seeds of HBWR. These include the compounds lysergic acid amide (LSA), ergometrine, lysergol, elymoclavine, setoclavine, and chanoclavine.[9] Due to the high LSA content contained within *Argyreia nervosa*, consumption of HBWR seeds has become a popular way for people to get high. LSA users will typically consume the seeds directly or prepare an alcoholic extract from the seed material.[10]

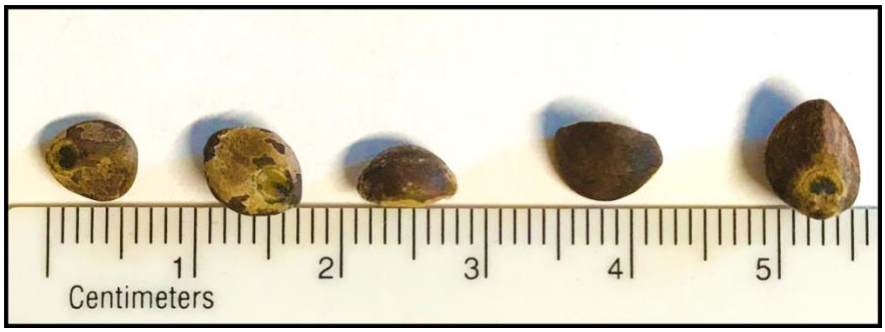

Figure 1. Hawaiian Baby Woodrose seeds shown with ruler.

1.3 The Chemical Foundations of LSA

1.3a Alkaloid Chemistry

In organic chemistry, LSA belongs to a class of compounds known as the ergot alkaloids. Ergot alkaloids comprise only one subdivision of the numerous alkaloidal groups known to man. In fact, several different classes of alkaloids have been identified, all of which have evolved into their own branches of research. For this reason, before discussing the specific chemistry and behavior of ergot alkaloids, it is critical to have a general understanding of what alkaloids are

and why these compounds have evoked such a profound interest among so many researchers today.

The term “alkaloid” was first introduced in the year 1819 by the German scientist Carl Friedrich Wilhelm Meissner, despite the concept having been known for centuries prior to then. Meissner noticed the tendency of certain natural products to act like bases, or *alkalis,* when they reacted. These newly termed alkaloids were described as bitter-tasting compounds that originated from plants.[11] Today, with the diversity of existing alkaloids, assigning a single definition to describe all alkaloids has been challenging for chemists. In the simplest of terms, alkaloids are a group of naturally occurring chemicals that primarily contain basic nitrogen atoms.[12] Alkaloids can be extracted from their natural plant sources by manipulating the appropriate reagents.[13]

Amino acids act as the building blocks for alkaloids, and this is evident in the chemical structure of most alkaloids. The characteristic nitrogen atom in alkaloids originates from an amino acid. In fact, the carbon skeleton of most amino acids used for the biosynthesis of alkaloids is retained in the final alkaloidal structure.

The overall basicity of alkaloids can be attributed to the presence of at least one nitrogen atom in their structure. Though some alkaloids exist as quaternary salts where a positive charge resides on the nitrogen atom, most alkaloids contain primary, secondary, or tertiary amine functional groups where a lone pair of electrons resides on the nitrogen atom and confers overall basicity to the molecule.[14] In this state, the alkaloid is considered trivalent and therefore electroneutral. Upon entering into a biological system, the amine portion of the alkaloid becomes protonated due to physiological pH values. This yields a tetravalent alkaloid with a positive charge.[11]

Alkaloids, while certainly found in large numbers in the plant kingdom, are not unique to this kingdom, and can indeed be found in microorganisms (e.g., bacteria and fungi), birds, amphibians, and mammals.[15] The role of alkaloids in the living organisms that produce them continues to be an active area of study. Alkaloids from one organism are typically toxic to another organism when consumed in large amounts.[12] For example, a study conducted by Thamhesl *et al*. reveals how cattle who feed on pasture grass containing ergot alkaloids are more likely to experience difficulties with reproduction, milk production, and weight gain.[16]

When alkaloids are used in very small amounts as forms of medicine, they exhibit a variety of healing properties.[13] When exposed to cells, either as the result of endogenous production or exogenous administration, the majority of alkaloids produce a physiological effect on the associated tissue and organism. Since some alkaloids share structural characteristics with common neurotransmitters, alkaloids have been known to interact with the nervous system.[17]

1.3b Ergot Alkaloid Chemistry

LSA and LSD are both compounds that are part of a large group of alkaloids known as the ergot alkaloids. Ergot is a parasitic fungus from the genus *Claviceps*,[18] which is made up of roughly 36 different species of fungi.[19] Ergot alkaloids have been identified in three families of plants and two orders of fungi.[20] They are known to grow on various grains and cereals. By definition, ergot alkaloids are secondary metabolites of the ergot fungus.[18] Despite their name, however, ergot alkaloids are produced by several families of higher plants in addition to fungi.[21] LSA and its isomer iso-lysergic acid amide (iso-LSA) were both successfully isolated from *Claviceps paspali* in 1960.[22]

All ergot alkaloids share a common structural core, which is made up of a tetracyclic ring system called ergoline. Figure 2 shows the molecular structure of ergoline.

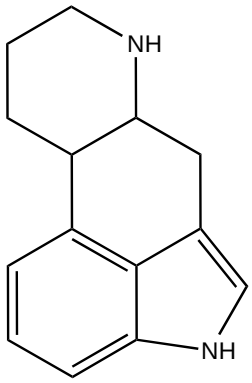

Figure 2. Molecular structure of ergoline.

Ergot alkaloids are subdivided into three groups based on their chemical structure: the clavine alkaloids, the ergopeptines, and the lysergic acid amides.[9] Clavine alkaloids, also called clavines, are distinguished by their ergoline ring system or one of its precursors.[20] Ergopeptines are composed of both lysergic acid and a peptide component. Ergotamine is an example of an ergopeptine because it contains both lysergic acid and an L-proline complex.[19] The lysergic acid amides, also referred to as the ergoamides,[20] contain lysergic acid and small amide substituents. Both LSA and LSD are classified as lysergic acid amides. Figure 3 shows the molecular structures of the three different classes of ergot alkaloids.

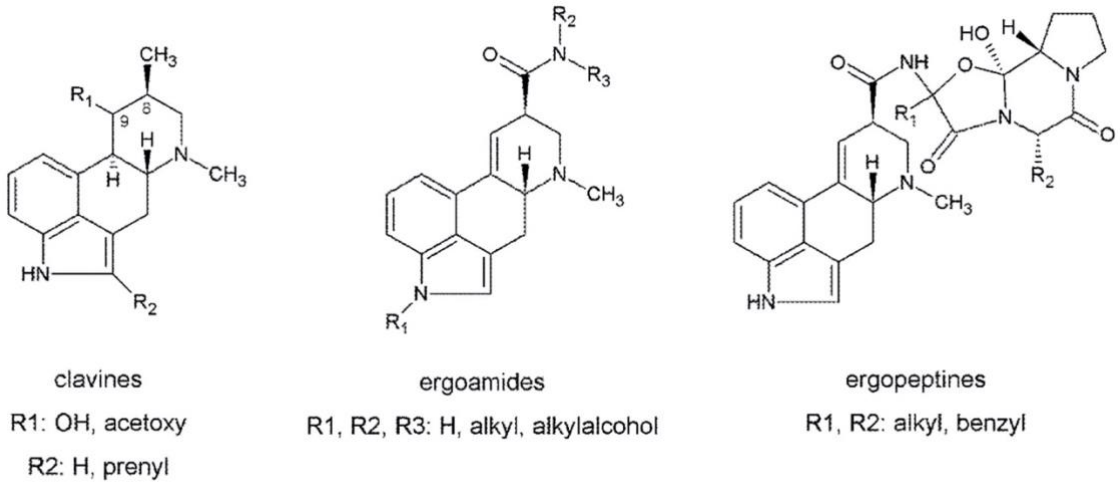

Figure 3. Molecular structures of the three ergot alkaloid classes. Adopted from Baldim *et al.*[23]

1.4 The Chemistry of LSD versus LSA

LSD was first synthesized in 1938 by Dr. Albert Hofmann, who was working on synthesizing a series of lysergic acid derivatives at the time. Initially, Hofmann subjected ergot alkaloids to alkaline hydrolysis in order to produce the compound lysergic acid. Lysergic acid is the common core present in all ergot alkaloids. Figure 4 shows the molecular structure of lysergic acid.

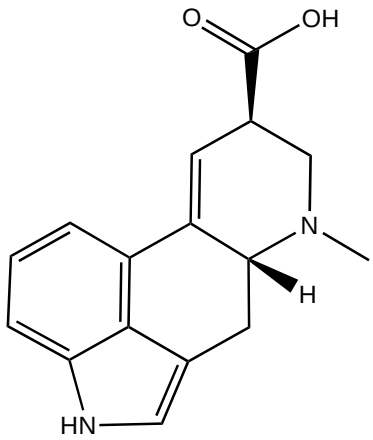

Figure 4. Molecular structure of lysergic acid.

Once lysergic acid was produced, Hofmann experimented with using various amines to form amide compounds. In this way, Hofmann created a series of lysergic acid amide derivatives. The 25th derivative produced in this series was the diethylamide of lysergic acid. This compound became known as LSD and was given the code name LSD-25 at the time.[24, 25] Figure 5 shows the molecular structure of LSD.

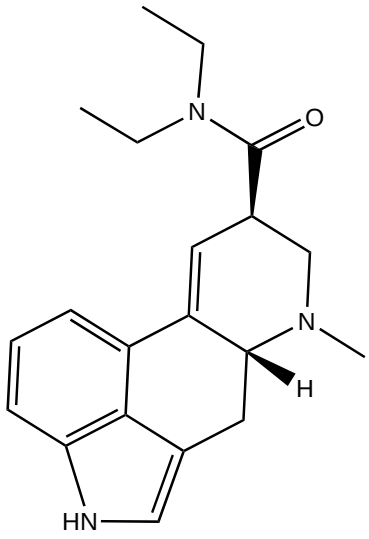

Figure 5. Molecular structure of lysergic acid diethylamide.

Unlike the semi-synthetic drug LSD, LSA is produced naturally by certain plants and fungi. LSA is structurally similar to LSD with the exception of its amide group. In LSD, the amide group has two ethyl groups connected to it. LSA is the simplest amide derivative of lysergic acid, having the two ethyl groups of LSD replaced by two hydrogens atoms. Figure 6 shows the molecular structure of LSA.

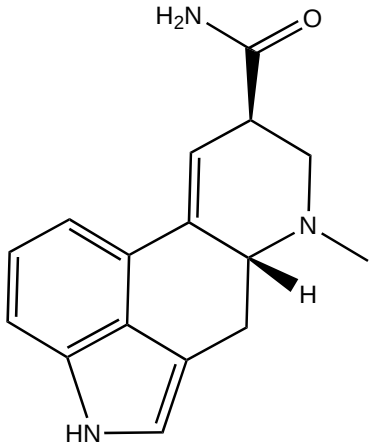

Figure 6. Molecular structure of lysergic acid amide.

1.5 The Physiological Effects of LSD

LSD is one of the most popular hallucinogenic drugs of all time. One of the reasons for this is due to the potency of a sample of pure LSD. Another hallucinogenic drug, mescaline, was discovered before LSD and reported to contain roughly 3-4 individual doses per gram. One gram of LSD, on the other hand, could represent around 10,000 individual doses.[24]

LSD was originally expected to act like an analeptic, or central nervous system stimulant. One of the reasons for this was the structural similarity between LSD and the popular analeptic drug nikethamide. When LSD was administered to animals, they became more energetic and excited. In pharmacological studies, LSD was demonstrated to show uterotonic activity. This was most likely due to the structural similarities between LSD and the oxytocic drugs ergometrine and methergine.[25]

The effects of LSD as a powerful human hallucinogen were demonstrated in a personal study by Albert Hofmann. According to his own records, Hofmann reported feelings of laughter, dizziness, and unrest after taking a 0.25 mg dose of LSD. This was followed by a period of time where he had difficulty concentrating and began to experience visual abnormalities. Several hours after taking the initial dose, Hofmann reported experiencing enhanced vision and sound. He also reported synesthesia where audible sounds provoked visual images in his head.[25]

In the 1950s and early 1960s, LSD was used as a treatment for neurosis, schizophrenia, psychopathy, and autism.[2] Though LSD initially showed promise as a treatment for several mental disorders, it was eventually replaced by other drugs better understood by doctors at the time.

1.6 The Physiological Effects of LSA

In the years that followed his discovery of LSD, Albert Hofmann performed self-experimentation studies using the drugs LSA and its isomer iso-LSA. After taking approximately 0.5 mg of LSA, Hofmann reported feeling exhausted with a loss of psychomotor abilities. He also experienced nausea and sensitivity to noise. Upon self-administration of approximately 2.0 mg of iso-LSA, Hofmann reported feelings of tiredness and lack of emotion. He also described "a feeling of mental emptiness and of the unreality and complete meaninglessness of the outside world."[26] Based on these reports, it appeared that both LSA and its isomer iso-LSA produced different physiological responses than those associated with LSD. Hofmann believed LSA was characterized by a more depressive and narcotic component than LSD.[25]

More recent studies have shown that LSA, like LSD, binds to a serotonin receptor in the brain called 5-HT2a. LSA consumption has led to symptoms including euphoria, hallucinations, and anxiety.[8, 27] Recreational users of LSA have reported experiencing positive feelings including elevated mood[28] and laughter.[29] Negative effects of the drug have included symptoms of memory loss, depression, hypertension, fear, and vomiting.[8]

Since the physiological nature of LSA is still not fully understood, LSA poses threats to those experimenting with the drug. And even though LSA is known to be less potent than LSD, there have been reported instances of LSA consumption leading to intoxication[27] and even suicide.[30]

1.7 Forensic Issues Regarding LSA

Unlike LSD, which is classified as a Schedule I drug, LSA is classified as a Schedule III drug in the USA.[31, 32] This means that possession of LSA by an individual is not permitted unless

they have a prescription given by a medical practitioner. However, seeds which contain LSA, including HBWR seeds, are not considered illegal in the USA. For this reason, consuming HBWR seeds is sometimes described as a way to achieve a *legal* high.

In recent years, LSA has gained popularity in the world of forensic science. In addition to being used as a recreational drug, LSA is commonly employed as a precursor in the illegal manufacture of LSD.

LSA is an appealing drug to both users and illegal drug manufacturers because it is easy to obtain. Since they are not classified as a scheduled drug, HBWR seeds may be readily bought and sold legally in the USA. Moreover, extracts of LSA can be easily prepared from HBWR seeds with the readily available extraction procedures found on the internet and the purchase of common reagents from local utility stores.

For legal reasons, it is critical to examine how much LSA can be produced from HBWR seeds and publicly available extraction reagents. Only then will forensic scientists and law officials understand the potential risks of LSA.

1.8 Objective of Project

Once recreational drug users purchase seeds containing LSA online, they are able to extract the active drug components (including LSA) using relatively simple procedures. These extraction protocols are readily available through many online websites. Though such websites include a disclaimer stating that the extraction protocols are to be used only for educational purposes, it may be assumed that this is not always the case.

The first aim of this project was to confirm the presence of lysergic acid amide (LSA) in seed samples of Hawaiian Baby Woodrose (HBWR) purchased over the internet. In order to do

this, seed samples were first subjected to two extraction protocols: one mimicking a common street method and a second mimicking a procedure from a published research study. After obtaining the crude extracts from both extraction methods, extract samples were subjected to analysis via liquid chromatography-mass spectrometry (LC-MS). Through this technique, liquid chromatography worked to separate the various compounds present in each extract and mass spectrometry subsequently worked to identify each compound by computing its protonated ion mass.

The second aim of this project was to evaluate the amount of LSA present in each extract. Ideally, a quantitative analysis would have been carried out using a pure LSA standard obtained from a reputable chemical vendor. However, due to the lack of availability of such a standard, a pseudo-quantitative approach was followed using an analytical grade standard of LSD, a drug very close in structure to that of LSA.

Chapter 2: Liquid Chromatography-Mass Spectrometry (LC-MS) Background

2.1 Introduction to High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a popular analytical technique utilized every day in various scientific laboratories such as forensic labs, toxicology labs, pharmacology labs, and biomedical research labs. In simple terms, chromatography refers to a type of separation science, which works to separate mixtures into their component parts.[33] Chromatography methods, both simple and advanced, consist of a non-moving stationary phase and a mobile phase, which travels through the stationary phase. When the sample to be analyzed is introduced into the chromatography system, its components are either attracted to the stationary phase or the mobile phase. These opposing attractions cause sample constituents to move through the system at characteristic speeds, allowing for their separation. HPLC is a modern-day form of chromatography. Though an HPLC instrument is constructed of several modules and may look intimidating, it is an instrument that operates on the basic principles of science.[34]

Liquid chromatography was established by the Russian botanist Mikhail S. Tswett when he separated leaf pigments from plant extracts. He used a column with calcium carbonate and alumina as the stationary phase. By pouring the plant extract into the column, it entered into the stationary phase and moved slowly towards the bottom due to gravity. Tswett then added a pure solvent to act as the mobile phase by traveling down the column. Sample pigments were either attracted to the stationary phase or the mobile phase. Those more attracted to the mobile phase traveled more quickly down the column. Those with greater attraction to the stationary phase traveled down the column at a much slower speed. The end result was a separation of bands. This technique eventually became known as *column chromatography.*[34, 35]

HPLC is a technique which works on the same principles as column chromatography. However, instead of using gravity to pull the mobile phase solvent through the stationary phase

column, it uses high pressure and a pump.[36] And as one would expect, this allows for samples to be analyzed much more quickly.

There are two main forms of HPLC: *normal phase* HPLC (NP-HPLC) and *reverse phase* HPLC (RP-HPLC). NP-HPLC was demonstrated with Tswett's column chromatography work. It uses a polar stationary phase like silica, and a non-polar mobile phase like hexane. As a sample mixture separates into its components, the polar parts are retained more strongly on the column, while the less polar parts are more attracted to the moving solvent. The speed at which sample compounds travel through the column depends on several of their individual properties including their relative polarity.[34]

These days, RP-HPLC is much more popular than NP-HPLC, and it is the main form used in analytical research laboratories. RP-HPLC consists of a non-polar stationary phase and a polar mobile phase. Popular mobile phase solvents include methanol, acetonitrile, and water. These solvents can be combined in a variety of ways to achieve the optimal mobile phase. Researchers will typically test out several different mobile phases before deciding on the best one for a given project. Likewise, choice of stationary column is essential. Many stationary columns contain silica bonded to a hydrophobic carbon chain. Since silica contains oxygen atoms and is considered to be a polar molecule to some degree, a non-polar hydrocarbon chain can be bonded to it in order to create a non-polar stationary column. Many columns found in reverse phase systems contain C18 bonded silica, which is silica bonded to a chain of eighteen carbon atoms. Another name for this packing is ODS, or octadecylsilane.[37] In RP-HPLC, sample components partition themselves between the non-polar column and the polar mobile phase. Polar compounds are more attracted to the polar solvents based on the principle that *"like dissolves like."* Non-polar compounds prefer to associate more strongly with the hydrophobic column. Consequently, more polar analytes move through the chromatography system first, while less polar analytes emerge from the column later on.

Figure 7 shows a typical HPLC apparatus consisting of the following parts: a mobile phase reservoir, a column, a pump, and injector, a detector, and a data processing unit. The mobile phase reservoir houses the bottles of mobile phase solvents. It is critical to use ultra-pure solvents, or *HPLC-grade* solvents, when doing experiments with HPLC. The column holds the stationary phase, and the proper column choice is critical to the success of an experiment. Columns can vary in regard to particle size and hydrophobic properties.

The pump is critical to HPLC. The pump is used to push the mobile phase through the column at a constant *flow rate.* Since the polar mobile phase must be forced through the non-polar column at a steady pace, the pump cannot be influenced by any type of back pressure. Flow rates are another parameter which can be adjusted for the specific project. The injector, as one would expect, injects the liquid sample to be analyzed into the mobile phase. Since the mobile phase is moving at a steady pace thanks to the pump, the goal of the injector is to introduce the sample into this mobile phase without disturbing its programmed flow rate. Consequently, the injector must be able to withstand high pressure.[38] Typical injection volumes range from 1-10 microliters.

The most common detector encountered in HPLC is the ultra-violet (UV) detector. When a sample compound exits the column, it is hit with a beam of UV light. A detector then measures that compound's absorbance of the light. The higher the concentration of a particular compound, the greater its absorbance value. Lastly, a data analyzer, or computer, is essential to any HPLC instrument.[34] By processing retention times and absorbance values of sample components, the computer creates a characteristic chromatogram.

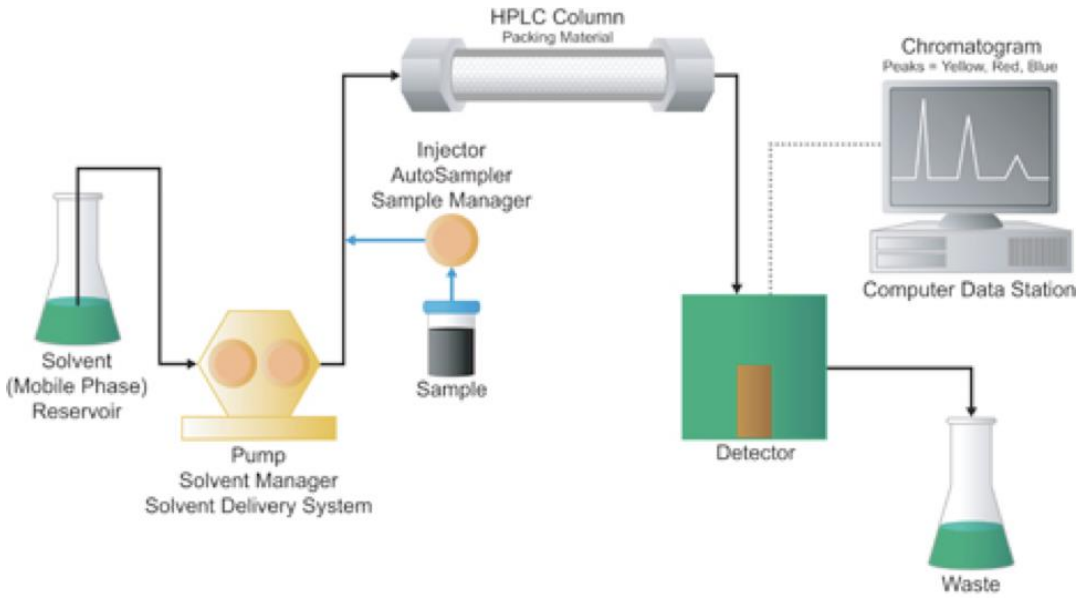

Figure 7. Schematics of HPLC. Adopted from Waters Company.

In order to appreciate the type of data generated by an HPLC instrument, a researcher should have a solid understanding of the *chromatogram*. Figure 8 illustrates some important parameters of a basic chromatogram. The x-axis represents time, and the y-axis represents the detector's response. The time of any sample run is determined beforehand. With ultraviolet-visible (UV-VIS) wavelength detectors, absorbance of a particular wavelength is measured. In Figure 8, the red dotted line to the left represents the time point of sample injection. The small peak to the right of the injection line represents the solvent front of the mobile phase and any compounds not attracted to the column. It makes sense that this is the first peak seen after injection because this material should have the shortest retention time. Later peaks represent sample compounds retained longer by the column. As a compound's polarity *decreases*, its time spent on the column *increases*. The retention time (t_R) of an analyte is measured from the time of injection to the time of peak apex.[39] Retention times are typically used in qualitative studies.

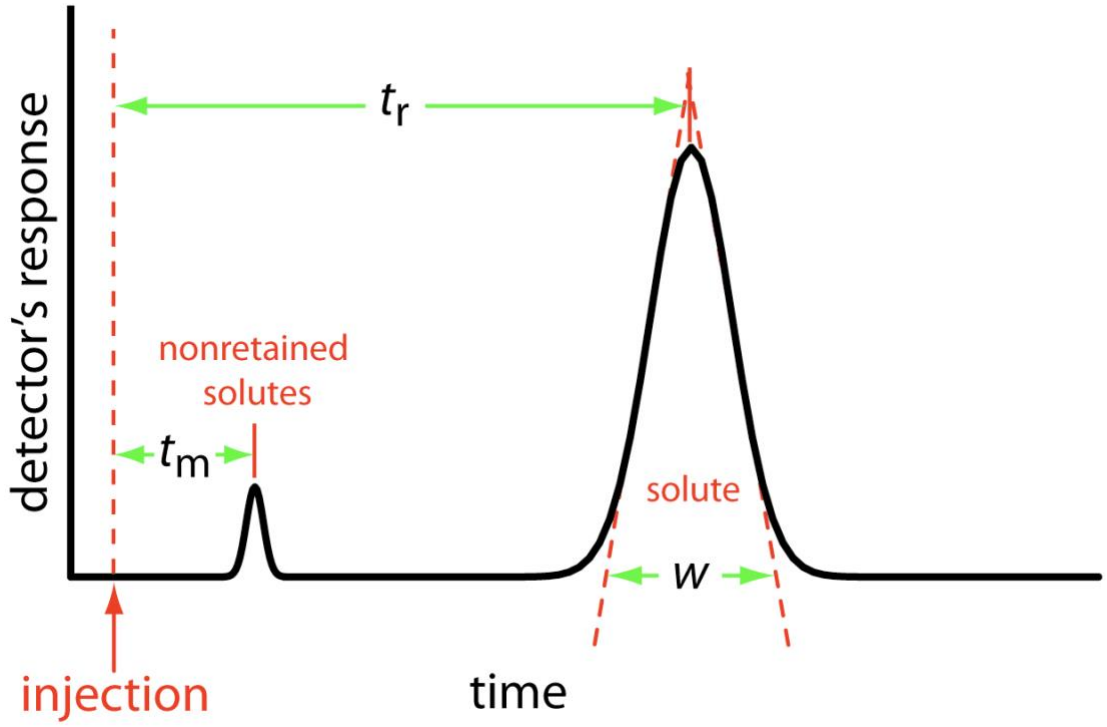

Figure 8. Schematics of basic chromatogram. Adopted from ChemWiki: The Dynamic Chemistry E-textbook, & Harvey, D. (n.d.).

HPLC instruments can be utilized for numerous jobs and projects. Qualitative studies focus on a compound's specific retention time. *What types of qualitative studies are commonly done with HPLC?* Depending on the nature of the scientific laboratory, the answer to this question will vary. In forensics, an obvious example includes drug analysis. Perhaps an unknown drug specimen from a crime scene is coming into the lab, and analyzers want to find out what's in the sample. If it's a white powder, *is it cocaine? Does it include any other active compounds?* These are the types of questions that forensic scientists will try to answer. A sample compound's retention time can be used for identification purposes. Sample chromatograms can be compared to those of known reference samples. Additionally, it is not uncommon for other techniques to be

used alongside HPLC in order to confirm results (e.g. mass spectrometry, which will be discussed in section 2.2).

Quantitative studies focus on a sample's peak height and peak area from its chromatogram. During a quantitative study, researchers want to determine how much of a particular analyte is present in a sample mixture. In order to do this, a series of standards must be obtained for calibration purposes.[39]

2.2 Introduction to Mass Spectrometry (MS)

Studies using HPLC can be greatly enhanced when the liquid chromatography (LC) portion of the instrument is used in conjunction with a mass spectrometer. In this type of set up, after sample analytes move through the chromatography column and are exposed to the LC detector, they are then injected into the mass spectrometer. Mass Spectrometry (MS) will identify organic compounds by measuring their mass to charge ratio (m/z). Here, "m" equals the mass number of ions, and "z" equals the fundamental ion charge. The unique mass spectrometer signals are a result of ionization and fragmentation reactions. Obtaining a mass spectrum requires manipulation by external electric and magnetic fields.

When MS converts analytes into ions, the ions are not stable. Some break apart into smaller parts. Fragmentation is extremely important in MS because different types of possible fragmentation patterns for parent molecules are possible. These fragments produce characteristic lines observed in the mass spectrum. Each line represents a different possible fragmented product. Data bases for mass spectra exist. Since it's unlikely for two compounds to have identical fragmentation patterns, MS can be quite definitive during analyte identification.

Chapter 3: Experimental Parameters

3.1 Reagents and Materials

Argyreia nervosa seeds, more commonly referred to as Hawaiian Baby Woodrose (HBWR) seeds, were purchased from an online vendor through the website E-Bay. Seeds were stored in a cool, dark cabinet prior to being used. A Certified Reference Material of Lysergic Acid Diethylamide (LSD) (1.0 mg/mL in 1 mL acetonitrile) was purchased from Cerillient® Corporation (Round Rock, TX, USA). Thin layer chromatography (TLC) glass plates (with dimensions of 2.5 x 7.5cm) coated with silica were purchased from EMD Millipore Corporation/Millipore Sigma (Billerica, MA, USA). For Extraction Method #1, Varnish Maker and Painter (VM & P) Naphtha (Klean-Strip Division of W.M. Barr & Company, Memphis, TN, USA) was purchased from a local Home Depot store. Everclear grain alcohol (Luxco, Inc., St. Louis, MO, USA) with an alcohol content of 95% by volume (190-proof) was purchased from a local liquor store. All other chemicals used in this research study, including those used for Extraction Method #2, were analytical reagent grade. All solvents used in this study were HPLC grade. HPLC water was prepared using a Milli-Q Plus (Millipore Corporation, Bedford, MA, USA) system.

3.2 External Standard

The external standard method was chosen for the quantitative analysis of this project. Ideally, the chemical chosen as the external standard would have been the compound of interest in this study, i.e., LSA. Certified reference samples of LSA were not available in the USA during the time this project was carried out. Instead, the LSA standard would have needed to be purchased from THC Pharm GmbH, a Pharmaceutical company in Frankfurt, Germany. Despite several attempts made by myself and another employee to obtain the LSA standard from THC

Pharm GmbH (See Appendix 1.), it was ultimately decided by a project advisor that the time and effort that would have been needed to assist the author with filling out the necessary paperwork was not warranted.

With an LSA standard unavailable for use, another compound was chosen for the external standard method: LSD. As mentioned in Chapter 1, LSD is structurally similar to LSA. Though LSD replaces two hydrogens on the amide group of LSA with two ethyl groups, the remaining portions of the compounds are the same. Within the shared structural region of LSD and LSA exists the chromophore region. This region contains unsaturated groups which are capable of absorbing certain wavelengths of light. With LSD and LSA having nearly identical chromophore regions, it is probable they will produce similar absorption patterns when they are exposed to the same light source.

For this study, LC-MS with a variable wavelength detector was used to analyze samples. After sample analytes exit the chromatography column, they are exposed to a particular wavelength of light. If the sample absorbs the light, a peak is produced in the resulting chromatogram. A detector wavelength of 313 nm was chosen for this analysis. Both LSD and LSA produce strong absorption patterns when exposed to this wavelength of light. If LC-MS was used to analyze a mixture containing equal amounts of both LSD and LSA, the resulting chromatogram would likely show two distinct peaks similar in size. Having identical chromophore regions allows LSD and LSA to have very similar detector responses when exposed to a particular wavelength from the variable wavelength detector. However, since LSD and LSA are not identical compounds, they are expected to elute from the chromatography column at different times. Ultimately, a true quantitative analysis cannot be completed without a

certified reference standard of LSA. With LSD serving as the external standard, only a pseudo-quantitative study regarding the LSA content of seed extracts could be performed for this project.

3.3 Preparation of Standard Solutions

Five different concentrations of LSD standard were prepared by diluting the original LSD stock solution (1.0 mg/mL in 1 mL acetonitrile) using a 50:50 mixture of acetonitrile and 0.1% formic acid. The five different concentrations of LSD standard prepared were 0.625 μg/mL, 1.25 μg/mL, 2.50 μg/mL, 5.00 μg/mL, and 10.0 μg/mL. These standard solutions were kept at +15 °C and analyzed within less than 24 hours following their preparation.

3.4 Preparation of Sample Extracts

3.4a LSA Extraction Method #1: Street Method

To reenact one of the common street methods for preparing an LSA tincture, the following procedure was performed: 12 seeds (approximately 1.2 grams) were ground into a fine powder using a coffee grinder. Ground seed powder was transferred to a flask and submerged in naphtha for defatting. This solution was mixed vigorously for approximately 20 minutes and then filtered. Following the filtration, the naphtha was discarded, and the defatted seed matter was set aside for a 3-day period to dry out. Once the seed matter was completely dry, it was transferred to a new flask and submerged in approximately 60 mL of Everclear grain alcohol. The defatted seed residue and Everclear solution was mixed thoroughly over a 3-day period. After this time, a second filtration was performed. The resulting liquid tincture (approximately 50 mL) was filtered a final time before being analyzed by LC-MS. The remaining seed residue was discarded.

3.4b LSA Extraction Method #2: Traditional Method

A second method for extracting LSA from HBWR seeds is based on a published research study by Gröger.[40] For this method, 12 seeds (approximately 1.2 grams) were ground into a fine powder using a coffee grinder. Ground seeds were transferred to a flask and submerged in petroleum ether for defatting. The seeds and petroleum ether were mixed thoroughly for 5 hours. After this time, the seed residue was filtered out from the petroleum ether. The seed residue was then subjected to 3 separate washes. For each wash, the seed matter was mixed with a solution of acetone and tartaric acid for 1 hour and then filtered. The liquid filtrate from each wash was saved. After the third wash, the filtrates were combined and gently heated to expel the acetone. The resulting solution was then washed with ether 3 times and made basic by adding liquid ammonia until its pH was between 8 and 9. To extract the alkaloids from this basic solution, three washes were performed using dichloromethane. The dichloromethane portion from each wash was saved and combined together. This resulting extract (approximately 50 mL) was filtered a final time before being analyzed by LC-MS.

3.5 LC-MS Instrumentation

A high-performance liquid chromatograph (LC) was used in combination with a mass spectrometer (MS) for sample analysis. The LC system utilized was the UHPLC$^+$ focused Dionex* UltiMate 3000 Standard HPLC System, fully equipped with the Thermo Scientific Dionex RS Variable Wavelength Detector (VWD-3400RS) and the following parts from ThermoFisher Scientific: Autosampler (WPS-3000), Degasser (DG-1210), and Pump (HPG-3200SD). The chromatography column used was the Thermo Scientific Hypersil GOLD column (having dimensions of 50 mm x 2.1mm and a particle size (μ) of 1.9). The LC system was used

in conjunction with Thermo Scientific's Exactive™ Orbitrap Mass Spectrometer (MS). LC-MS software used included Chromeleon® 7 (LC) and Exactive Series 2.8 SP1 (MS), both of which were fully integrated through the control software package DCMS^{Link} with XCalibur® 3.1 (Thermo Fisher). Post-run data analysis was carried out using Thermo XCalibur Qual Browser.
**Now sold under the brand name Thermo Scientific*

3.6 LC-MS Method

To enhance separation efficiency during the LC portion of the analysis, a gradient elution program with two different solvents was employed. The ratio of these two solvents was adjusted throughout the duration of each sample run. The solvents used were acetonitrile (ACN) and 0.1% formic acid (0.1 FA). The flow rate was set to 0.3 mL/min. From 0 minutes to 0.5 minutes, the mobile phase consisted of 5% ACN and 95% 0.1 FA. From 0.5 minutes to 4 minutes, the ratio of the two solvents adjusted to a final composition of 30% ACN and 70% 0.1 FA. From 4 minutes to 6 minutes, the ratio of the two solvents adjusted to a final composition of 60% ACN and 40% 0.1 FA. From 6 minutes to 8 minutes, the ratio of the two solvents adjusted to a final composition of 90% ACN and 10% 0.1 FA. This composition remained constant for 1 minute. From 9 minutes to 11 minutes, the solvent ratio was adjusted back to its starting composition: 5% ACN and 95% 0.1 FA. This was followed by a 4-minute post run period for column equilibration. Additional parameters were also defined and set for this method. The chromatography column temperature was set to +40 °C. The autosampler temperature was set to +25 °C. The draw speed was 5.000 µL/s and the data collection rate was 2.5 Hz. The detection wavelength used to analyze samples was 313 nm. Injection volumes of 3.00 µL were repeated three times for both extract samples and LSD standards.

For the MS portion of the analysis, an orbitrap mass analyzer was used throughout the entire acquisition time of 11 minutes. The mode was set for positive polarity and ultra-high resolution. The ion source was positive electrospray ionization (ESI). The maximum injection time was 20 ms. The scanning range was set at 100.0 – 400.0 m/z.

Chapter 4: Results

4.1 LC-MS Results for External Standard

Figure 9 displays the chromatograms of the 5 dilutions of the LSD certified reference material. Each standard had an injection volume of 3.00 μL and was run 3 consecutive times using the method described in Chapter 3. The results from the 3 chromatograms at a detector wavelength of 313 nm were then averaged and plotted in Figure 9. As Figure 9 shows, each individual standard dilution shows a single peak at approximately 4.41 minutes. Corresponding mass spectra data for each of the LSD standard dilutions show base peaks with a m/z value of 324.199 ± 0.001. Since the exact mass of LSD is 323.199762 g/mol according to PubChem 2.1 (PubChem release 2019.06.18), the mass spectra data for the LSD standards supports the identification of LSD since all mass spectra were computed using a positive polarity mode. A m/z value of 324.199 ± 0.001 represents the EIC of $[M+H]^+$, or the protonated ion form of LSD. Figure 10 displays a mass spectrum computed by averaging the mass spectra results from the 5 LSD standards.

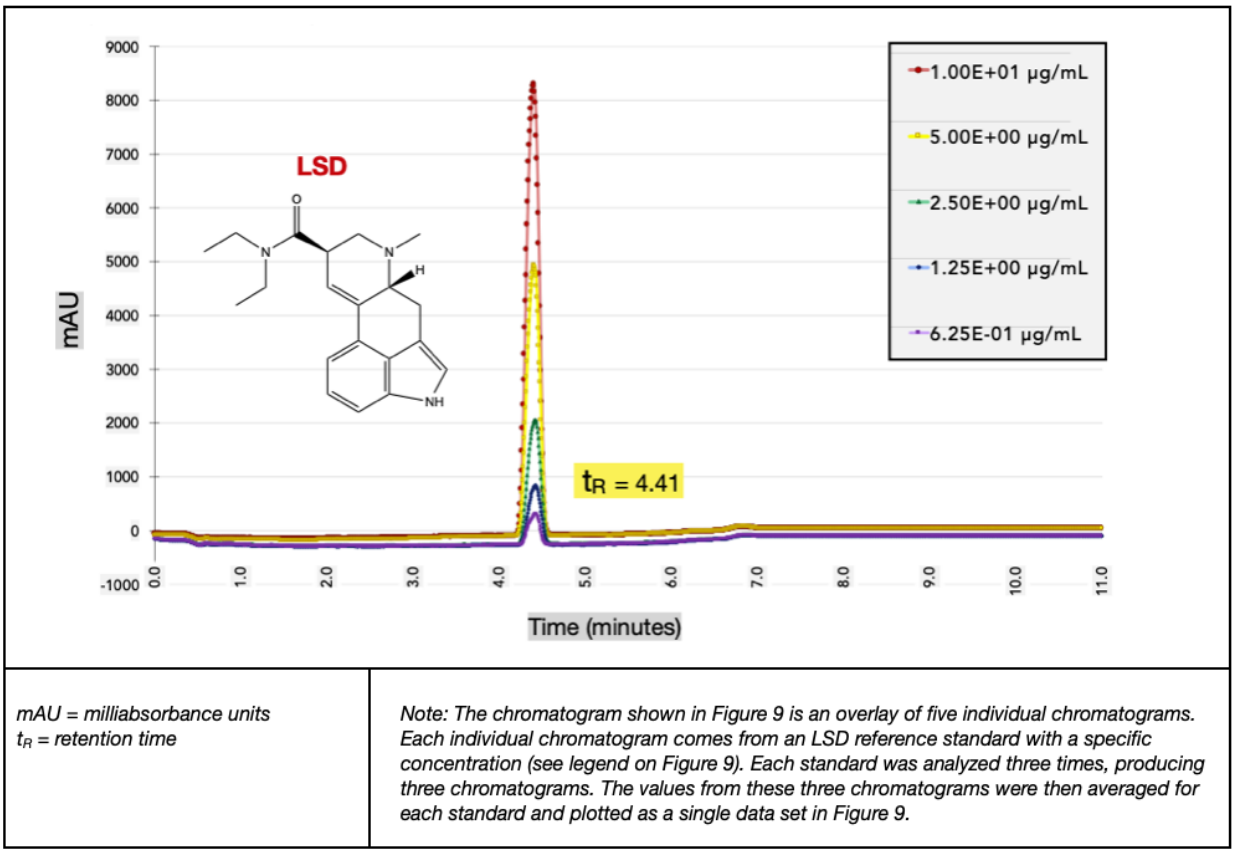

Figure 9. Chromatogram of LSD reference standard dilutions.

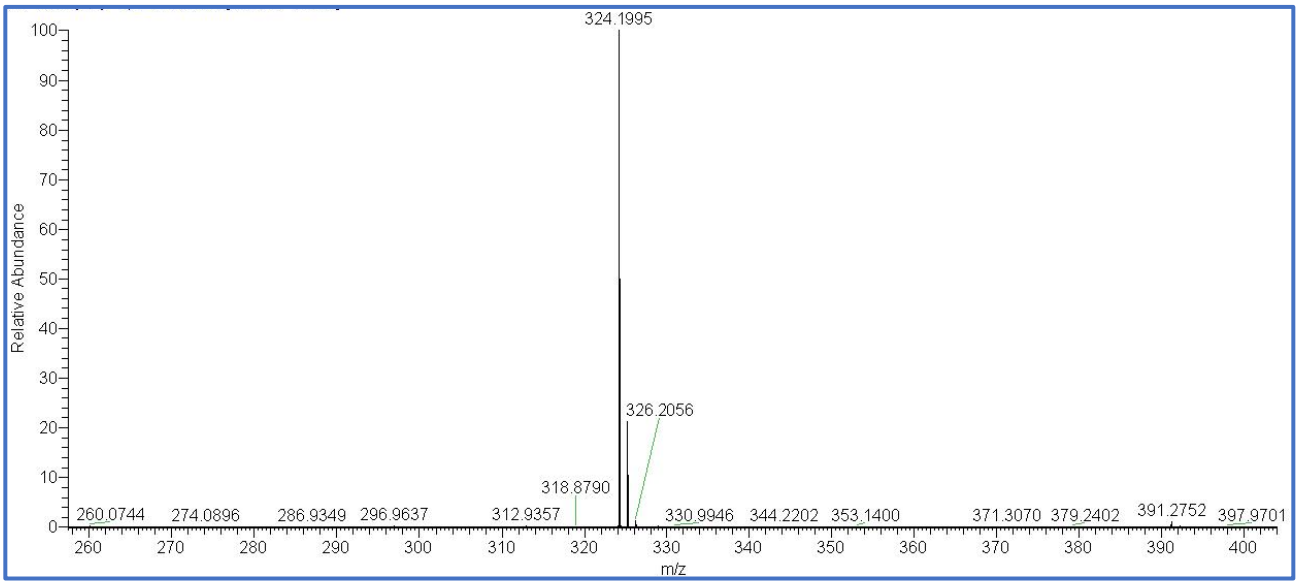

Figure 10. Mass Spectrum of LSD reference standard dilutions.

4.2 Calibration Curve

In order to establish the calibration curve shown in Figure 11, peak areas from each of the 3 chromatograms for every standard dilution were averaged and plotted against their corresponding standard concentration value. Method linearity was observed over a standard concentration range of 0.625 µg/mL to 10.0 µg/mL. Limit of detection (LOD) and limit of quantitation (LOQ) values were established from the calibration curve. The LOD was determined to be 0.86 µg/mL and the LOQ was determined to be 2.61 µg/mL. The correlation coefficient (R^2) value was determined to be 0.9924.

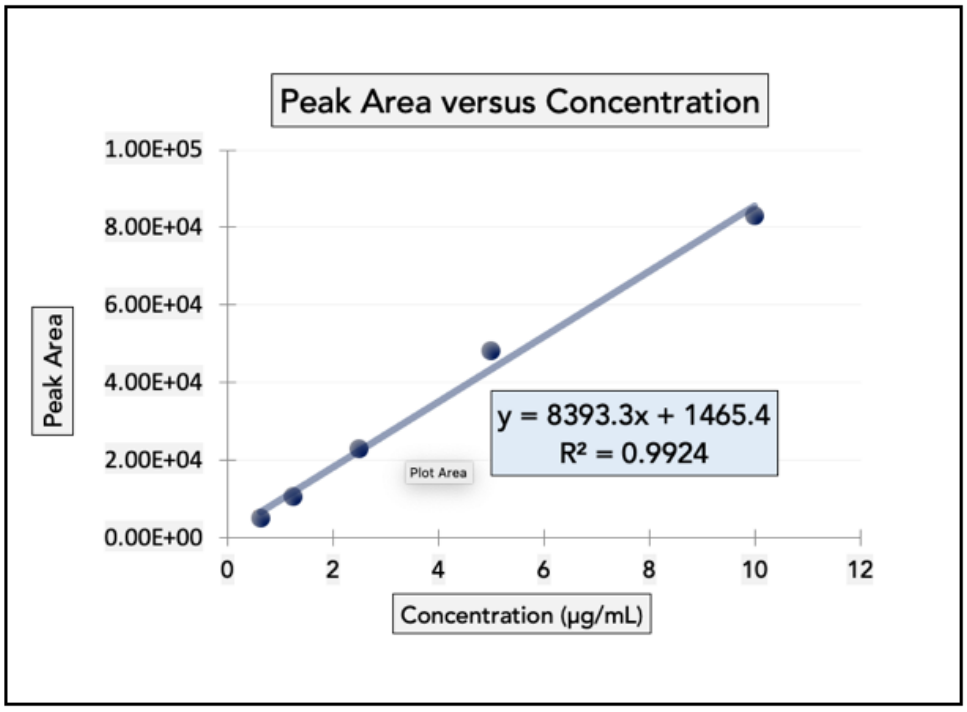

Figure 11. Calibration curve for LSD standard.

4.3 Thin Layer Chromatography Results

Prior to evaluating extract samples via LC-MS, thin layer chromatography (TLC) was performed on both extract samples. The mobile phase consisted of a 9:1 mixture of chloroform and methanol. Figure 12 displays the developed TLC plate. Extract #1, located in the far-left lane (I.), was obtained through extraction method #1 (street method) and extract #2, located in the middle lane (II.), was obtained through extraction method #2 (traditional method). In the third lane (III.), the LSD certified reference material was also spotted for comparison purposes. Approximately 1.5 µL of each sample was initially spotted on the plate. After the plate was developed and analyzed under a UV lamp, it was stained with Ehrlich's reagent. This reagent contains *p*-dimethylaminobenzaldehyde and reacts with indole derivatives including LSA and LSD. When applied to a developed TLC plate, Ehrlich's reagent stains the indole derivatives a purplish-blue color.[41] Figure 12 shows the developed TLC plate of the LSA sample extracts and the LSD reference standard. Both sample extracts (shown in lanes I. and II.) show more than one spot. This tells us that both extracts contain multiple compounds. Since the spots on the TLC plate turned purple once exposed to the Ehrlich's reagent, it is likely that each purple spot represents a compound containing an indole group. The two spots that resulted from Extract #1 (indicated as spots I-a. and I-b. on Figure 12) are likely the two most prominent ergot alkaloids in this extract due to their dark purple color. Spot I-c. represents another compound in Extract #1, however, due to its much lighter color, it is likely not present in as high of a concentration as spots I-a. or I-b. The TLC results for Extract #2 display a similar pattern to Extract #1. The two darkest spots (indicated as spots II-a. and II-b. on Figure 12) are likely the two most prominent ergot alkaloids in Extract #2. There is a third compound present in Extract #2 (indicated as spot

II-c. on Figure 12), which is likely present in a lower concentration than the compounds represented by spots II-a. and II-b.

In lane III on Figure 12, the reference standard LSD was spotted. Since this sample was a certified pure reference material purchased from a reputable vendor, it was expected to show a single spot on the developed TLC plate. As lane III on Figure 12 shows, only one spot is present (indicated as spot III-a on Figure 12). This single spot is indicative of a pure reference standard. Additionally, this single spot stained a deep purple when exposed to the Ehrlich's reagent. This makes sense since LSD is known to contain an indole group.

The R_f values computed for all spots on the TLC plate shown in Figure 12 are displayed in Table 1. LSD is less polar than LSA due to the two diethyl groups attached to its amide group. Since the TLC plate in Figure 11 consisted of a glass plate coated with silica as the polar stationary phase and the mobile phase consisted of less polar solvents, it makes sense that the LSD compound in lane III traveled further than any of the other components shown in lanes I and II. Though the spots in lanes I and II cannot be identified through TLC analysis, it is likely that each of them contains indole groups as indicated by their stained purple color. Additionally, if LSA is represented by one of the spots in lanes I or II, it makes sense that none of these spots traveled up the plate as high as the LSD standard in lane III. Since LSA is more polar than LSD, it would likely be more attracted to the polar TLC plate than the non-polar mobile phase used to develop the plate. This would result in any spot representing LSA having a lower R_f value than LSD. Moreover, LSD would have a higher R_f value than any spot representing LSA. Table 1 confirms that the compound expected to be LSD does have the highest R_f value out of all the spots analyzed.

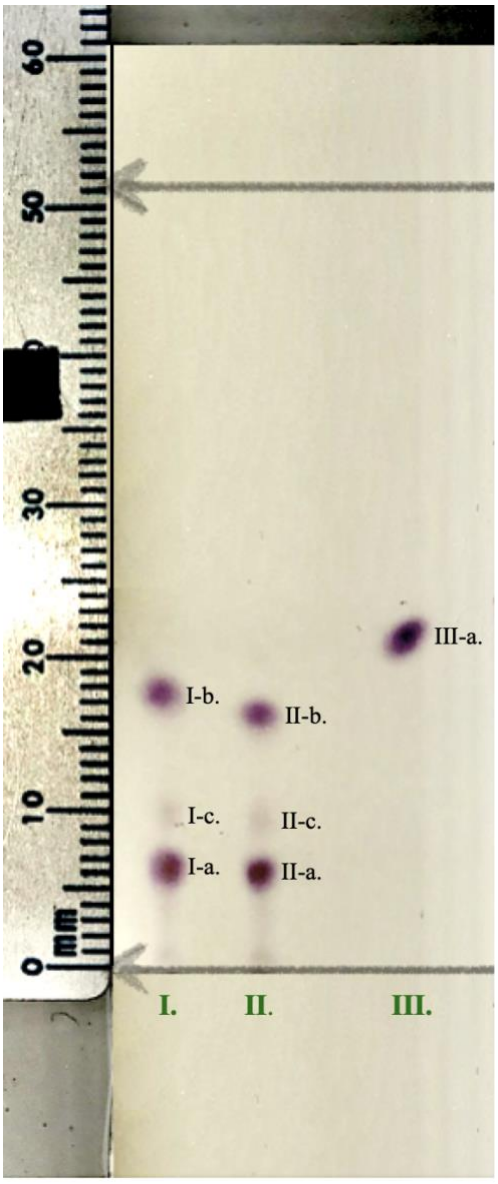

Figure 12. TLC plate stained with Ehrlich's Reagent. Lane I. represents Extract #1. Lane II. represents Extract #2. Lane III. represents the LSD reference standard.

Table 1. R_F values for TLC plate with Extract #1, Extract #2, and LSD standard shown in Figure 12.

Table 1.		
Solvent Front: 5.1 cm		
Spot	**Distance Traveled**	**R_F Value**
I-a.	0.6 cm	0.12
I-b.	1.7 cm	0.33
I-c.	1.0 cm	0.20
II-a.	0.6 cm	0.12
II-b.	1.6 cm	0.31
II-c.	0.9 cm	0.18
III-a.	2.1 cm	0.41

4.4 LC-MS Results for Sample Extracts

Figure 13 displays the resulting chromatograms for sample extracts #1 and #2. Peaks 1a and 2a both eluted with a retention time of approximately 2.31 minutes. The corresponding mass spectra for both peaks 1a and 2a show a m/z value of 268.137 ± 0.001. Since the exact mass of LSA is 267.137162 g/mol according to PubChem 2.1 (PubChem release 2019.06.18), peaks 1a and 2a potentially represent the LSA portion of both crude extracts. However, since iso-LSA has the same mass as LSA, it is also possible that peaks 1a and 2a represent this epimer or a combination of both LSA and iso-LSA. Additionally, as mentioned in section 4.3, LSA is more

polar than LSD. Since RP-HPLC was used for the initial step of analysis, LSA would be expected to elute faster than LSD during a sample run. This is because LSA would be more attracted to the polar mobile phase moving through the non-polar column. LSD, on the other hand, should be more attracted to the stationary column and display a longer retention time. Figures 12 and 13 support this result. LSD was shown to have a retention time of 4.41 minutes, while LSA was shown to have a shorter retention time of only 2.31 minutes.

Though both chromatograms in Figure 13 show their largest peaks eluting at 2.31 minutes, it is also evident from these chromatograms that neither sample extract contained only one compound. A minimum of two additional peaks are shown to elute shortly after the main peak in both extracts #1 and #2. This confirms that both extracts are composed of more than one compound. This is particularly important in regard to extract #1 because most people preparing this kind of alcoholic extract believe they are preparing a pure LSA extract safe to consume. However, as the results from both the TLC analysis and LC-MS analysis show, extract #1 contains more than one compound. Whether or not these additional compounds are safe for human consumption needs to be studied in much more detail.

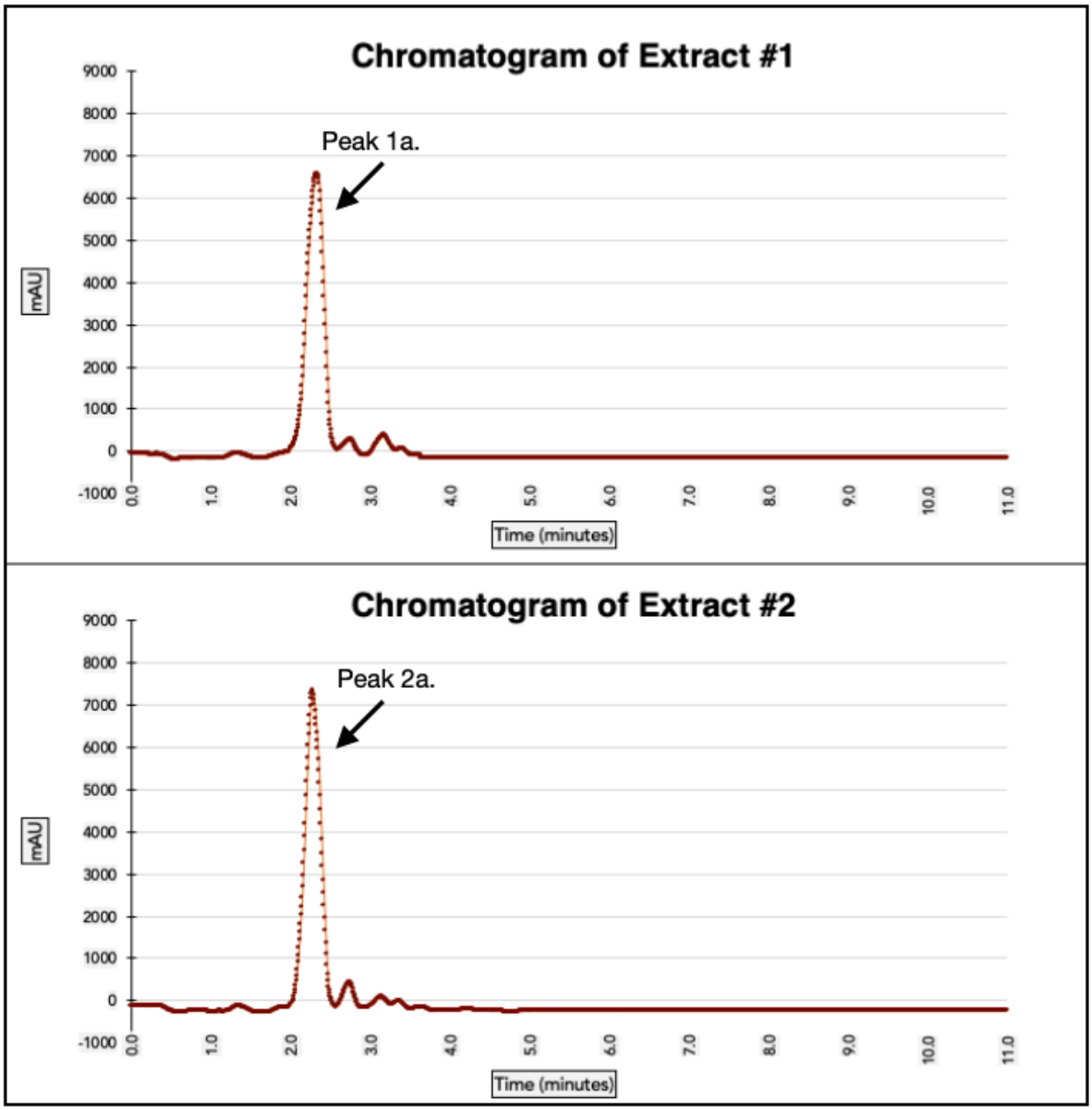

Figure 13. Chromatograms for sample extracts #1 and #2.

Figure 11 was used for the pseudo-quantitative part of this study. If it is assumed that peaks 1a and 2a in Figure 13 represent LSA, then the areas of peaks 1a and 2a correspond to potential LSA concentrations. In order to calculate these concentration values, the peak area values for

peaks 1a and 2a were substituted into the trendline equation from Figure 11. The resulting concentration values were then adjusted to account for the mass difference between LSD and LSA. In order to do this, the units were first converted into units of grams per liter (g/L). Then, the molar mass of LSD (323.4 g/mol) was used to convert the masses from grams into moles. The resulting molar quantities were then converted into their respective LSA masses using the molar mass of LSA (267.3 g/mol). The final concentrations calculated for peaks 1a and 2a were 6.4 µg/mL and 6.7 µg/mL, respectively.

Since the alcoholic extract produced from extraction method #1 resulted in a final sample volume of approximately 50 mL, a person consuming this extract would potentially be consuming close to 320 µg, or 0.32 mg of LSA. Approximately 500 µg, or 0.50 mg is considered to be a strong dose of LSA,[42] but this can vary depending on the person. Consuming 0.32 mg of LSA has the potential to produce moderate to strong side effects in the person taking the drug. This dosage may even be a dangerous amount for certain individuals.

The average mass of a single HBWR seed is approximately 100 mg, or 0.1 g.[42] When performing extraction #1, 12 seeds were used to prepare the final extract. The initial dry seed weight was reported as 1.2011 g. The amount of LSA in the final extract, 320 µg, represents 0.03% of the dry weight of the seeds.

Chapter 5: Discussion

Figure 12 and Table 1 correspond to the TLC data for sample Extract #1, sample Extract #2, and the certified LSD reference standard. To develop the TLC plate shown in Figure 12, a mobile phase consisting of a 9:1 ratio of chloroform to methanol was used. Use of this mobile phase along with the use of Ehrlich's reagent to develop the plate was adopted from a similar research study by Sewell. In the study by Sewell, HBWR seeds were ground up in lemon juice using a mortar and pestle, dried out, put into a tea bag, and submerged in hot water to create a consumable LSA drink. This final liquid was subjected to TLC analysis. The TLC plate from the Sewell study shows two prominent dark purple spots, just like those displayed in Figure 12 (spots I-a and I-b for Extract #1 and spots II-a and II-b for Extract #2), along with several other faint purple spots. The Sewell study claims that the lower dark spot represents LSA while the higher dark spot represents iso-LSA. The R_F values calculated by Sewell were 0.18 for LSA and 0.33 for iso-LSA. If we compare the retention times from the Sewell study with those displayed in Table 1 for Extract #1, spot I-b (which is also the higher dark purple spot of the extract) also has a R_F of 0.33 and could potentially represent iso-LSA. The lower dark spot (spot I-a) for Extract #1 looks the most similar to the lower dark spot in the Sewell study. However, spot I-a is reported to have a R_F of 0.12, which is lower than 0.18. Spot I-c has a R_F of 0.20 which is closer to the reported value for LSA, but spot I-c is also much fainter than both spot I-a for Extract #1 and the lower dark purple spot in the Sewell study. For Extract #2, TLC results from Figure 12 and Table 1 show that spot II-c has a R_F of 0.18, which matches the R_F for LSA in the Sewell study. However, unlike the LSA spot in the Sewell study, spot II-c appears much fainter in color. Spot II-a bears a much stronger resemblance to the LSA spot from the Sewell study, but the R_F for spot II-a is only 0.12. Spot II-b has a R_F of 0.31, and although this value is slightly below the

0.33 R_F value for iso-LSA mentioned in the Sewell study, spot II-b also appears as a dark purple spot. One potential reason why the R_F values from Table 1 differ from the R_F values in the Sewell study could be due to differences in the relative amounts of each substance prepared. There could also be some differences between the TLC plates used in the Sewell study and the TLC plates used for this project.

Figures 11 and 13 were used to calculate the average percentage of LSA in HBWR seeds by weight. This value came out to be roughly 0.03%. This value is lower than previously published values. In 1970, a publication by Miller et al. reported that LSA accounts for 0.04% of the weight of HBWR seeds.[6] This value was based on a previous publication by Hylin & Watson from 1965. Using TLC and colorimetric analysis, Hylin & Watson determined that alkaloids represent approximately 0.3% of the dry weight of HBWR seeds. LSA accounts for an eighth of this total alkaloid percentage, which is 0.04% of the dry weight of HBWR seeds.[43] A later publication by Chao & Marderosian appeared in 1973 and reported that LSA accounts for 0.14% of the dry weight of HBWR seeds.[44] There are a few possible reasons why our final mass percent of LSA in HBWR seeds (0.03%) differs from the percentages stated in earlier publications. Differences in results may be due to seed age and handling. Additionally, the pseudo-quantitation performed in this study was done using LC-MS instrumentation. Earlier publications performed quantitative studies using TLC methods and colorimetric analysis.

One of the largest complications during the course of this project's research was the inability to obtain a pure reference standard of LSA. A pure LSA standard would have confirmed the retention time of LSA in sample extracts using the exact same instrumentation and methods described throughout this paper. Since a pure LSA standard could not be acquired, a certified reference material of LSD was chosen as the next best option. Using LSD as a reference standard

only allowed for a "pseudo-quantitative" analysis of LSA in sample extracts. This technique assumes that the ultraviolet spectra of LSA and LSD are nearly identical. According to published records[45], the maximum absorption wavelength for LSA is 313 nm in aqueous acid form and 309 nm in aqueous alkali form, while the maximum absorption wavelength for LSD is 315 nm in aqueous acid form and 310 nm in aqueous alkali form.

In addition to the research performed for this project, similar studies have been performed that cited difficulties in obtaining a pure LSA reference standard and the subsequent need to adopt an alternative method for sample analysis. A study by Tagliabracci *et al.* used LSD as a reference standard for a gas chromatography-mass spectrometry (GC-MS) analysis of LSA in *Ipomea Violacea* seeds purchased from the internet.[46] Another study, performed by Deveaux *et al*., used an LSD standard for an LC-MS/MS analysis of the LSA concentration in *Ipomea tricolor*.[47] Like *Argyreia nervosa*, *Ipomea Violacea* and *Ipomea tricolor* are both plants belonging to the Convolvulaceae family. In a publication by Klinke *et al.*, the authors also cited a lack of LSA standard availability. However, rather than using a different standard structurally similar to LSA, Klinke *et al.* synthesized a stock solution of LSA from ergotamine.[27]

Chapter 6: Conclusion

Due to the ever-growing popularity of the internet as a means of obtaining novel psychoactive substances, it is critical that both analytical chemists and forensic scientists continue to pursue studies regarding drugs such as LSA. A simple internet search for preparing consumable LSA extracts results in many procedures that are similar to the one performed in this study (Extraction Method #1). Almost all of the non-published methods found on the internet claim that the end product is a "pure LSA extract," but that is far from the truth. LSA likely represents a portion of the extract, but it is not the only compound present. As Figures 12 and 13 show, the extracts prepared in this project contain more than just a single component. This means that if someone were to consume one of these extracts, they would be consuming other chemicals in addition to LSA. Until in-depth pharmacological studies can be performed on all of compounds in these extracts, they remain unsafe for human consumption.

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