2022

Development of a Chromatographic Method to Authenticate Aspirin Brands

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DEVELOPMENT OF A CHROMATOGRAPHIC METHOD TO AUTHENTICATE ASPIRIN BRANDS

BY

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A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Chemistry

South Dakota State University

2022

THESIS ACCEPTANCE PAGE

Megan Guetzloff

This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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I would like to dedicate this thesis to my mother, Christine Justel Guetzloff (Richter), and my father, Thomas Fredrick Guetzloff, who were my inspiration to earn a Master of Science degree (M.S.). You both gave me unconditional love, support, encouragement, and guidance over the years that I will cherish forever. Not only are you my heroes, but your passion towards chemistry ignited the spark in me to lead in your footsteps and become curious to learn about the universe on a macro and micro level. Thank you for being the best parents that a daughter could ask for. I love you both forever and always. To my loving husband/best friend, Jonathan David Simms: I would like to thank you for your love, support, and patience you given me on this journey towards obtaining my master's degree. I also, want to thank you for believing in me when at times I did not believe in myself. You always know how to cheer me up on my good and bad days. Thank you for being my best friend, partner in crime, and soulmate. I love you *(always)* and cannot wait for what life has in store for us. To my family, thank you for your support and encouragement to get to this point in my life.

ACKNOWLEDGEMENTS

I would like to personally thank and acknowledge my advisor, Dr. Brian A. Logue, for all the guidance you have given me on my journey to receiving my M.S. degree. You taught me how to critical think throughout my research project and most of all, you believed in me when at times I did not. Thank you for not only being my principal investigator, but someone I can look up to in my years to come. I would also like to thank the LARGE group for the supporting me. I will cherish the memories that we have created together. I would like to also thank Dr. Saunders, Cami Fuglsby, and Janean Hanka for working with me on my research project. Your perspectives allowed this research project to take flight and I appreciate everything. I would lastly like to thank the South Dakota State University Chemistry and Biochemistry Department for providing me this wonderful opportunity to obtain my master's degree. I have made so many friends and memories here that I will treasure for my lifetime.

ABBREVIATIONS

GC-MS	Gas chromatography mass spectroscopy
LDA	Linear discriminate analysis
QDA	Quadratic discriminate analysis
IRMS	Isotope ratio mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LC	Liquid chromatography
MS	Mass spectrometry
PCA	Principal component analysis
ICP-MS	Inductively coupled plasma-mass spectrometry
MRM	Multiple Reaction Monitoring
CI	Chemical ionization
EI	Electron Ionization
CE	Collision energy
DP	Declustering potential
CXP	Cell exit potential
NIST	National Institute of Standards and Technology
DC	Direct current
RSD	Relative standard deviation
API	Active pharmaceutical ingredient
SVM	Support vector machines

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ABSTRACT

DEVELOPMENT OF A CHROMATOGRAPHIC METHOD TO AUTHENTICATE ASPIRIN BRANDS

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Counterfeit pharmaceuticals pose a threat to society that can include inaccurate amounts of the active pharmaceutical ingredient (API), no API, or containing off-target compounds. For example, there are many recent examples of counterfeit pharmaceuticals containing potentially lethal doses (> 2 mg) of fentanyl (i.e., a synthetic opioid). Current measures to combat illicit pharmaceuticals (e.g., unique packaging and product serialization) have merit, however with evolved technologies, counterfeiters can relatively easily simulate these measures and continue to distribute illicit pharmaceuticals. The only accurate way to definitively determine that a suspected counterfeit is, in fact, counterfeit is advanced chemical analysis. However, current methods of authentication via chemical analysis have disadvantages. Therefore, a general drug authentication method was developed to authenticate and correctly classify pharmaceuticals, specifically Bayer®, Walgreens©, and Premier Value® aspirin. Gas-chromatography mass-spectrometry (GC-MS) and liquid-chromatography tandem mass spectrometry (LC-MS/MS) were evaluated for analysis of aspirin. LC-MS/MS produced the most consistent analysis results. Additionally, three statistical techniques, linear discriminant analysis (LDA), quadratic discriminant analysis (QDA), and atypicality analysis, were evaluated for their usefulness in source attribution. LDA outperformed the other statistical treatments, with perfect classification of the training data set using LDA.

However, when applying the method to a set of double-blinded pills, all statistical treatments failed to correctly classify over 25% of the pills. Because this method of source attribution was inconsistent, further optimization of the method is needed before introducing unknown sources.

CHAPTER 1: INTRODUCTION

1.1. Significance

In recent years, counterfeit pharmaceuticals have produced an increasingly detrimental impact on human health worldwide. Overdose, underdose, or exposure to off-target chemicals (e.g. fentanyl), is much more likely than with authentic medicines, leading to up to 1 million deaths per year [1]. Because the tactics of counterfeiters are sophisticated, including manufacture of pharmaceuticals which are virtually indistinguishable from the original product, strategies to mitigate counterfeiting are diverse. Primary strategies to combat counterfeit pharmaceuticals include unique packaging, product serializing, and verification methods at different points in the supply chain [2]. For example, the Food and Drug Administration (FDA) produced a counterfeit detector device that uses ultraviolet and infrared light the scan drugs and packaging to help determine if the packaging of a drug is authentic [3]. However, even with current strategies, counterfeiting of pharmaceuticals has continued to increase at an alarming rate, fueled by advanced counterfeit technologies and heavy consumer demand for cheap medicine. While current strategies to combat counterfeiting each have merit, the most accurate is advanced chemical analysis, which allows attribution of a pharmaceutical to its source. Therefore, there is a critical need for improved analysis methodologies to authenticate suspected counterfeit drugs more easily and accurately.

1.2. Objective

The objective of this study was to develop the most accurate authentication protocol to identify the source of suspected counterfeit pharmaceuticals by using an accurate chemical analysis technique and advanced chemometric methods. In order to

accomplish the objective, two chromatographic methods were executed to analyze aspirin brands (Bayer®, Premier Value®, and Walgreens©): gas chromatography-mass spectrometry (GC-MS) and liquid chromatography tandem mass spectrometry (LC-M/MS). When comparing the two techniques used to establish chemical fingerprints of the various aspirin brands, LC-MS/MS yielded the most consistent results. Therefore, in the final method, LC-MS/MS was implemented, and linear discriminate analysis (LDA), quadratic discriminate analysis (QDA), and atypicality based measures were utilized to differentiate these fingerprints.

1.3. Counterfeit Drugs

1.3.1. The Impact of Counterfeit Pharmaceuticals

The World Health Organization (WHO) and the International Federation of Pharmaceutical Manufacturers & Associations (IFPMA) define counterfeit drugs those being falsely labeled, regarding their identity and/or origin, to deliberately deceive consumers [4]. According to Interpol, approximately 30% of all drugs sold globally are counterfeit [5]. Methods to counterfeit drugs can be sophisticated, such as the full-scale manufacturing of fake drugs, or simple, such as minor changes in packaging (e.g. altering the expiration date) [4]. Counterfeiters tend to focus on more expensive drugs, such as various chemotherapeutic drugs, antibiotics, vaccines, AIDS medicines, antivirals, and antianxiety drugs. Due to the high price of these drugs, people are tempted to search for drugs from a cheaper source, which creates a market for cheaper counterfeits. Consequently, the consumer may unknowingly take the counterfeit pharmaceuticals, which could lead to delayed or immediate health problems, including death [6].

The amount and type of counterfeit pharmaceuticals consumed in each country is independent from other countries, due to various factors. The majority of illicit drugs purchased in the U.S. originate from the web (e.g., dark web) [3]. The counterfeit drug problem in developing countries is more severe because there are fewer regulations, ineffective authority control, and loss of trust in the health care system [7]. In these countries, counterfeiters specifically target life-saving drugs such as anti-retroviral, antimalarials, and antibiotics [7]. For example, in 1995, 60,000 people were injected with a counterfeit meningitis vaccine in Niger, Africa [7].

Recently, the opioid epidemic has increased the risk for those consuming counterfeit pharmaceuticals. According to the DEA, 27% of confiscated counterfeit pills contain "potentially lethal doses of fentanyl" [8] Fentanyl is a powerful synthetic opioid used to treat patients with severe pain that is 50 to 100 times more potent than morphine. While fentanyl is a legally prescribed drug, it is highly addictive, and as little as 2 mg of fentanyl can be lethal to 95% of the population [8, 9]. Additionally, the U.S. Drug Enforcement Administration (DEA) issued a warning that the Mexican drugs cartels' manufacturing mass quantities of counterfeits containing fentanyl is "killing Americans" [8].

1.3.2. Current Strategies to Combat Counterfeit Pharmaceuticals

Counterfeit pharmaceuticals are highly profitable and only modest punishments are associated with convictions. Hence, counterfeiters are motivated to continue developing evolved counterfeiting methods. With counterfeiters developing advanced technology to produce fake medicines, it is difficult for

authorities to enforce anti-counterfeiting laws and almost impossible for consumers to identify counterfeits [2]. Luckily, there are strategies that can be implemented to combat counterfeit pharmaceuticals. Common strategies are listed in Table 1.1.

Table 1.1: Executing Strategies to Combat Counterfeit Pharmaceuticals. Counterfeiters are persistent to distribute illicit goods due to high profit margins, however, implementing strategic methods can reduce counterfeit products.

Strategies to Combat Counterfeit Drugs	**Justifications of Implementing Strategies**
Appoint a leader to examine the security measures of the supply chain [2].	To ensure that no illicit activity is occurring in the company [2].
Collaborate with regulatory agencies, international organizations, and law enforcement [2].	Most regulatory agencies already have policies put in place [2]. International organizations can assist with preventive schemes and current issues [2].
Develop Unique Packaging [2, 10]	Unique and custom packaging and custom can be distinguished and is more difficult to counterfeit (e.g. tamper evident packing) [10].
Utilize Product Serializing [2, 3]	FDA's Counterfeit Detectors and Truscan devices can be used to scan if the drugs are indeed fake [3]. Also, bar codes and quick response (QR) codes are other ways for verification [10].
Use verification methods at strategic points in the supply chain [2]	Due to the potential for a compromise in the supply chain at various points, a variety of assurance criteria needs to be established [2].

While these strategies can be employed to help prevent counterfeiting, the only truly accurate way to authenticate medications is the use of advanced chemical analysis for definitive attribution of a pharmaceutical products.

1.4. Current Methods for Source Attribution

1.4.1 Raman Spectroscopy

Raman spectroscopy is a non-destructive, fast, and portable chemical analysis technique that provides information based on chemical structure, phase and polymorphism, crystallinity, and molecular interactions. This information is obtained by irradiating the sample with light from a laser source and measuring light scattered by molecules in the sample. The wavelength of most scattered light is the same as the laser source, known as Rayleigh scattering, which does not provide useful information about the chemical makeup of the sample. However, some of the scattered light has a different wavelength than the laser source based on the interaction of the irradiated light with chemical bonds, as shown in Figure 1.1 [11]. This type of scattering is known as Raman Scattering.

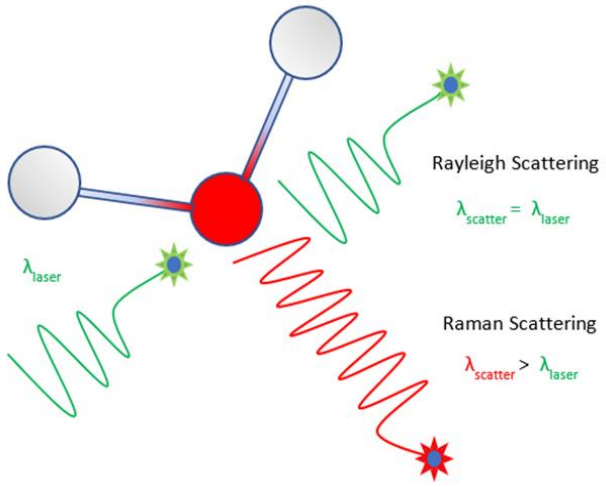


Figure 1.1 Schematic of Raman principle utilizing a H_2O molecule. H_2O is bombarded with irradiated light from a laser. The wavelength of the scattered light from the H_2O sample that is equivalent to that of the laser light is not analyzed, however, the difference of the excited and emitted light of the sample yields the vibrational spectrum [11, 12].

When inelastically scattered light is detected, a Raman spectrum (which has a unique chemical fingerprint) is obtained as combination of the intensity and wavelength of the scattered light. The wavelength of the scattered light corresponds to vibrational levels of a chemical bond, such as C-C, C=C, N-O, C-H, etc., or from a group of bonds in a chemical in the sample [11].

Raman spectroscopy has diverse applications. It has multiple advantages, such as the ability to provide qualitative and quantitative information, it is fast (i.e. on the order of seconds), organic and aqueous samples can be analyzed with minimal or no sample preparation, compounds can be identified without labeling, it is nondestructive, and it can be used to analyze most compounds in a variety of states of matter (solid, liquid, and gas) [13].

In respect to drug authentication, there are several useful methods utilizing Raman spectroscopy. Researchers at Queen's University (Belfast, UK) and Forensic Science Agency of Northern Ireland constructed Raman spectroscopic methods to differentiate between ecstasy (MDMA, 3,4-methylenedioxymethylamphetamine) and ecstasy analogues [14]. The researchers sampled 400 tablets from a group of over 50,000 tablets and were able to classify the tablets only based on the excipients (e.g. sorbitol, glucose, or cellulose) [14]. A further study with 1500 tablets was executed because the physical description coupled with active drug content did not fully characterize the 400 tablets due to tablet similarity. The 1500 tablets were analyzed as follows: (1) peak heights of the excipients band in the Raman spectrum were obtained (2) the ratios of peak heights were obtained (peak height of prominent MDMA peak/compared to the peak height of excipient) (3) analysis of variance (ANOVA) was used to determine variances within the

tablets [15]. In this study, the researchers were able to characterize the tablets; however, only two sets, out of the many sets tested, were considered identical [15]. In another study, Dégardin et al. [16] developed a Raman method for the analysis of medicinal counterfeits. The first step of the method consisted of identifying 31 types of genuine capsules and tablets and detecting their counterfeits by Raman spectroscopy [16]. The genuine spectra of the sample and Active Pharmaceutical Ingredient (API) peak detection were correlated together to determine legitimate from counterfeit samples [16]. The second step consisted of chemometric methods for chemical profiling purposes where 27 seizures of counterfeits were classified into 15 chemical classes [16].

Although Raman spectroscopy excels in certain situations, it has a number of disadvantages, including low sensitivity, difficulty in analyzing complex samples, and it is limited to surface analysis [17].

1.4.2 Isotope Ratio Mass Spectrometry

Isotope ratio mass spectrometry (IRMS) is precise and accurate technique used to measure isotopic abundances of a material. While natural isotope abundances are generally fixed, small changes in isotope ratios occur based on biological, chemical, and physical processes. The small differences in isotope ratios can be used to determine the source of a material. In preparation for IRMS analysis, samples are combusted to simple gases such as H_2, CO_2, N_2, and CO. These gases are analyzed via mass spectrometry to determine the ratio of stable isotopes, such as $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{18}O/^{16}O$, and using an internal standard (i.e., a well-characterized standard is needed to establish a known isotopic profile for determination purposes), the change in isotope abundances for a particular sample can be quantified [18].

The two most common instrumental designs used for gas source IRMS are continuous flow IRMS (CF-IRMS) and dual-inlet IRMS (DI-IRMS) [18, 19]. There are two types of elemental analyzers correlated to continuous flow systems such as elemental analyzer IRMS (EA-IRMS) and high temperature thermal conversion IRMS (HTC-IRMS). EA-IRMS is used for the analysis of carbon and nitrogen where and HTC-IRMS is used for the analysis of hydrogen and oxygen. For both types of elemental analyzers, the analysis steps are similar. First, the elemental analyzer is used to combust or thermally convert the analyte/material into gases. Next, the gases generated are introduced to the ion source at the mass spectrometer interface. Lastly, the gas molecules are ionized, mass filtered and detected in the mass spectrometer [18].

IRMS is an excellent technique for determining the source of a material since replicating the isotope ratios for specific chemicals in the material is extremely difficult. Therefore, it is particularly applicable to definitive authentication of pharmaceuticals. For example, in a study by Cristea et al.[20] analyzed six types of analgesics (from various manufactures and batches) were examined for differences in $\delta^{13}C$ for drug identification purposes [20]. The amount of $\delta^{13}C$ measured in each sample was determined with an Elemental Analyzer coupled with IRMS and in addition they used inductively coupled plasma-mass spectrometry (ICP-MS) to determine elemental impurities in the various manufacturers [20]. In their results, they saw differences between the carbon isotopic composition ($\delta^{13}C$) of ibuprofen, diclofenac, paracetamol, metamizole, ketoprofene and combinations containing paracetamol were between -32.9 and 22.6‰ (i.e. δ-values are typically multiplied by 1,000 and are denoted using ‰ or per mil) [20]. Linear discriminant analysis (LDA) was used to characterize the various pharmaceuticals by the

isotopic and elemental data where diclofenac was clearly separated from ibuprofen and paracetamol, but ibuprofen and paracetamol did not have the optimum separation [20]. In another study, Jasper et al. [21] tested the isotopic variability of carbon-, oxygen-, and hydrogen- in four types of analgesics: two types of acetaminophen and two types of aspirin [21]. Ratios of acetaminophen and aspirin were measured by continuous flow system (elemental analyzer) coupled with IRMS [21]. The results of the study indicated that the analgesic samples were isotopically heterogenous from batch to batch even though drugs are manufactured to a specific procedure [21].

Although IRMS is an excellent method for authentication, the instrument is sophisticated, costly, non-portable, and the instrument is prone to contamination. In addition, the determination of IRs for each sample is expensive [18].

1.4.3 Liquid Chromatography Tandem Mass Spectrometry

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a technique that can be used to separate molecules, such as proteins and complex peptides, using a mobile phase and stationary phase. A mixture of analytes are separated based on their interaction with the mobile and stationary phases (i.e., LC) and detected based on mass filtering (i.e., MS) [22, 23].

For LC, a mixture of analytes is separated based on their interactions with the mobile and stationary phase. In LC-MS (Figure 1.2), the sample mixture is first injected into the mobile phase where it passes through a chromatographic column under high pressure. Generally, a sample mixture is separated in the column into individual components based on polarity [23]. After the separated compound(s) exit the column, the compound(s) pass through an interface where the compounds are ionized using an ion

source. Then, the generated ions travel though the mass spectrometer and are separated based on their mass-to-charge ratio *(m/z)*, producing a spectrum comprised of the relative abundance of the resultant ions [24]. As shown in Figure 1.2, LC-MS consists of only one MS and LC-MS/MS uses a tandem MS configuration for detection, providing much more selectivity than an LC-MS, including the ability to identify structural isomers (e.g., L-lactic acid and D-lactic acid) [25, 26].

LC-MS/MS methods can be executed to analyze and authenticate pharmaceuticals. For instance, Pang et al. [27] developed an LC-MS/MS method to identify and quantitate the ten most commonly used synthetic antidiabetic drugs (e.g. gliquidone, glipizide, glibenclamide, glimepiride, rosiglitazone, repaglinide, metformin, phenformin, and tolbutamide) that are present in herbal remedies [27]. In this study, they concluded that the advertised all-natural herbal supplements actually consisted of some synthetic hypoglycemic drugs [27]. Another example of an LC-MS/MS method implemented for the analysis of pharmaceuticals was by Lee et al. [28]. In their study, they developed a method to extract and detect seven erectile dysfunction (ED) drugs (e.g. sildenafil, tadalafil, vardenafil, udenafil, mirodenafil, avanafil, and lodenafil carbonate) and their analogues in various suspected counterfeit drugs from 2009 to 2013 [28]. In 89 suspected counterfeit drugs and herbal medicines, they found that ED drugs were detected in 84 out of 89 drugs. Additionally, they determined that Viagra (sildenafil) was the most prevalent ED drugs in counterfeits (73.8%), followed by Cialis (tadalafil) (25.4%) [28].

LC-MS/MS is a sophisticated method for the analysis of various pharmaceuticals; however, its main disadvantages are that it is expensive, it is not amendable to polar compounds, and it consumes a large amount of organic solvent [22, 25, 26, 29].

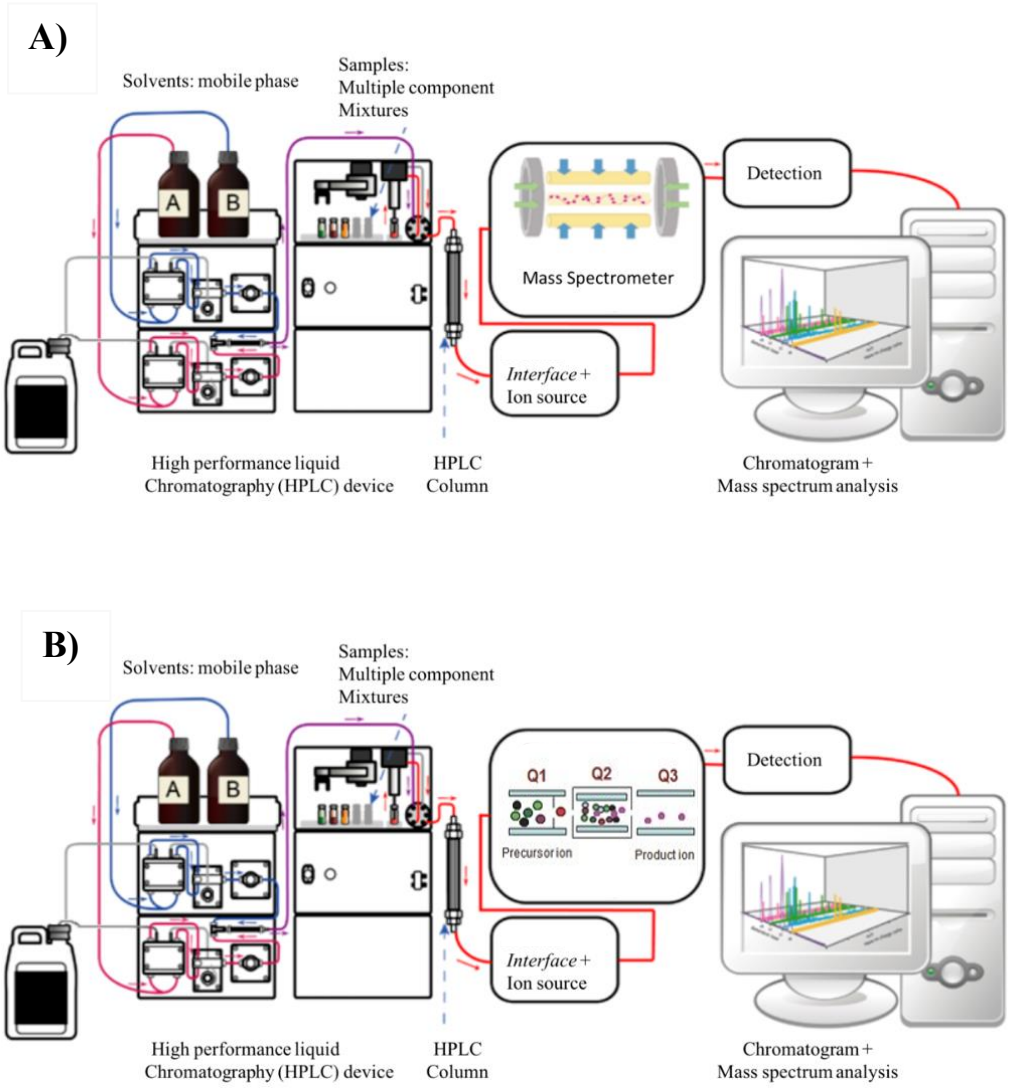


Figure 1.2 A) Basic schematic diagram of LC-MS. Sample is injected into the mobile phase (e.g., isocratic or gradient) where analytes are separated in the column. Partitioned sample is ionized, separated in the mass spectrometer by *m/z* ratio, and detected. B) Basic schematic diagram of LC-MS/MS. LC-MS/MS sample introduction is the same as LC-MS but differs once resultant ions are introduced to the MS. A specific precursor ion is selected where it passes through the first quadrupole (Q1). In the collision cell (Q2), the selected *m/z* ions are fragmented into product ions or daughter ions by collision. The third quadrupole (Q3) selects the products ions and traverse towards the detector [30-32].

1.4.4 Gas Chromatography Mass Spectrometry

In contrast to LC-MS/MS, gas chromatography mass spectrometry (GC-MS) is used for the analysis of semi-volatile or volatile compounds. Figure 1.3 is a schematic of a GC-MS. For GC-MS, a sample is first injected into a hot GC inlet to vaporize volatile components. The volatile components carried by an inert gas mobile phase, such as hydrogen, helium, or nitrogen. Components of the mixture are separated based on their boiling points and interaction with a stationary phase. They then travel towards the MS, where they are ionized and detected based on mass filtering [33].

The ion sources that are typically used in GC-MS are electron ionization (EI) and chemical ionization (CI). For EI, a beam of elections consisting of high energy (70 eV) ionizes the analyte producing mass fragments. CI is a softer ionization source that uses a reagent gas (e.g. methane) and uses less energy and produces less fragmentation than EI [34].

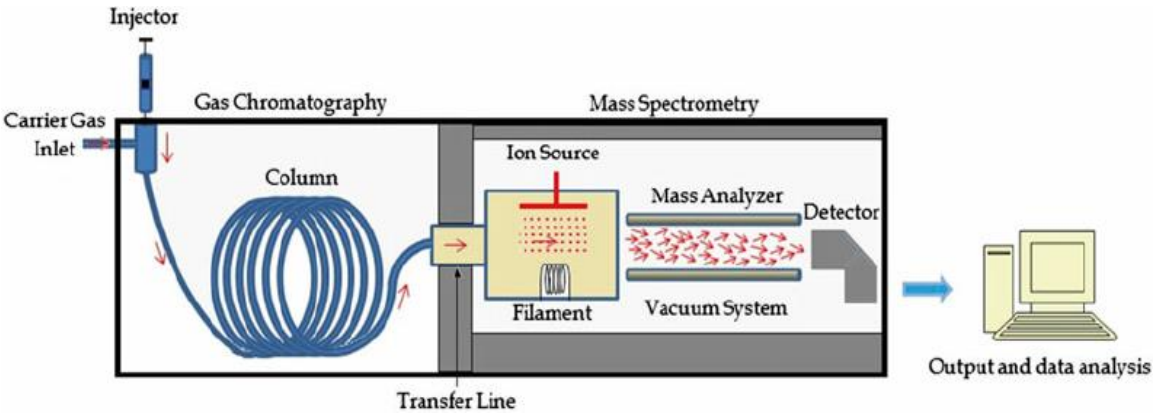


Figure 1.3 Schematic diagram of GC-MS. The injected sample is heated in the GC inlet where the mobile phase carriers the volatile analyte through the column. The separated analyte(s) are introduced into the ion source for the ionization process. The interferent ions are filtered out of the quadrupole based on DC and RF voltages and the selected ions are detected in the detector [35].

Following ionization, the ions produced enter the mass analyzer. The most common mass analyzers used in GC-MS are quadrupole and ion-trap [33]. Quadrupole mass spectrometers are cheap, robust, and simple to use, but offer low mass resolution and mass accuracy. Quadrupole mass filters consists of four metal rods that are connected to a direct current (DC) in permutation with RF voltages, which acts as a filter to allow specific m/z fragments to traverse to the detector [36, 37]. Ion trap mass spectrometers are set up similar to that of quadrupole mass filters, however, ions are not filtered like quadrupole mass spectrometers; instead, ion trap mass spectrometers use either electric or magnetic fields to "trap" the selected range of ions based on specific mass-to-charge ratios. There are a variety of designs for ion traps, such as a 3D ion trap (Paul ion traps), a linear ion trap (2D trap), an electrostatic ion trap (Orbitrap), or a magnetic field-based trap (ion cyclotron resonance). Ion trap mass spectrometers have several advantages such as high sensitivity, qualitative identification, and the capability for tandem MS. However, based on the limited size of the ion trap not all of the charged species can be quantitated and reduces the dynamic range [38, 39].

GC-MS is mainly used for the identification and quantification of organic compounds in complex matrices; however, non-volatile analytes can be analyzed via GC-MS with additional sample preparation techniques. With the help of various sample preparation techniques, GC-MS can analyze most compounds, including a variety of pharmaceuticals and potential pharmaceutical counterfeits. For example, Neves and Caldas [40] developed a GC-MS method that is suitable for the analysis of counterfeit and substandard anabolic steroids (tablet, aqueous suspension and oil solution forms) for analysis of individual pharmaceuticals (N= 345, with 328 medicines and 17 dietary

supplements) previously identified by the Brazilian Federal Police as potential counterfeits. They reported that 42% of the medicines, 28% of the tablets, 12% suspensions, and 65.2% oil solutions were counterfeit [40]. In another study, Foroughi et al [41] developed a method to detect undeclared active pharmaceutical ingredients in herbal medicines used as opioid substitution therapy. They analyzed 80 different herbal medicines by GC-MS. Most of the samples (96%) contained one active pharmaceutical, with diphenoxylate and tramadol identified in 90% and 67% of the samples, respectively. Other ingredients that were identified in the method were acetaminophen, codeine, sertraline, and fluoxetine [41]. While GC-MS is a useful technique for analyzing pharmaceuticals, it has its disadvantages, such as it is moderate expense, difficulty in development of a field portable device, and it is difficult to analyze polar and large molecular weight compounds [42].

1.5 Research Goal

There are limitations to current methods authentication techniques, including multiple sample preparation techniques for identifying chemical compositions of pharmaceuticals. Therefore, it is critical to develop the most simple, efficient, and accurate technique that is sensitive enough to identify chemical compositions and authenticate various drugs. Aspirin replicates were evaluated using GC-MS and LC-MS/MS to determine the most consistent method for analyzing various aspirin brands. After further evaluation, LC-MS/MS resulted in more precise and accurate results between pill replicates. In this study, LC-MS/MS and chemometrics were used to distinguish various aspirin brands by using multiple reaction monitoring method (MRM)

for the analysis of trace excipients, allowing application for authentication of commonly counterfeit drugs.

CHAPTER 2: DEVELOPMENT OF A CHROMATOGRAPHIC METHOD TO AUTHENTICATE ASPIRIN BRANDS

ABSTRACT

Aspirin is a nonsteroidal anti-inflammatory drug (NSAID) that is used to relieve pain, reduce fevers, and reduce inflammation. While aspirin is not commonly counterfeited, in 2013, French customs seized 1.2 million doses of counterfeit aspirin that originated from China and, in a separate incident, 26 people in 2018 were arrested for selling more than 1.49 million illicit drugs, including fake aspirin. Since aspirin is readily available from multiple manufacturers, a general authentication method was developed for aspirin that may allow authentication for counterfeit drugs. Aspirin pills from Premier Value®, Walgreens©, and Bayer® were used as known source objects. While gas-chromatography mass-spectrometry was evaluated, liquid-chromatography tandem mass-spectrometry (LC-MS/MS) produced more consistent results. Therefore, pills (N=3 per brand) were analyzed using a simple LC-MS/MS method to produce a chemical fingerprint. Three statistical techniques, linear discriminant analysis (LDA), quadratic discriminant analysis (QDA), and atypicality analysis, were applied to differentiate between aspirin brands. A double-blind study was executed to test the applicability of the LC-MS/MS method coupled with each statistical technique. Each aspirin pill utilized in the training set was perfectly classified using LDA, however, the double-blinded pills were only 25% correctly classified. While this technique shows promise, further development of the method is necessary to correctly classify unknown pills.

2.1 Introduction

It is estimated that counterfeit pharmaceuticals are responsible for up to 1 million deaths each year globally [1], creating a serious concern for public health officials, private companies, and consumers. Interpol estimates up to 30% of drugs sold worldwide are counterfeit [5], affecting the health of millions of people. The main strategies to combat counterfeit pharmaceuticals include unique packaging, product serialization, and product/packaging verification methods at strategic points in the supply chain [2]. Even with these measures, it is difficult to distinguish counterfeit from authentic drugs.

The most inexpensive, and least effective, way to identify counterfeit pharmaceuticals is by inspecting packaging, or the physical characteristics of the medicine (shape, color, etc.). This method is ineffective because counterfeiters regularly manufacture pharmaceuticals that are virtually indistinguishable from the original product. A more robust method of detecting counterfeits is chemical analysis of medicines. Chemical analysis techniques for drug authentication range from affordable, simplistic, and portable methods to extremely sophisticated laboratory-based techniques. While simple and sophisticated techniques are complementary, the only definitive way to authenticate pharmaceuticals is through advanced chemical analysis to attribute a chemical to its source. Due to increased sophistication of counterfeiting practices and the disadvantages of current techniques for authentication, there is a need for improved methods for authentication of drugs (i.e., verification of the drug matching the package description, confirmational analysis of ingredients listed on the label, and the storage conditions have been met) and potential source attribution (i.e., sufficient scientific results obtained from the source that can be used for identifying its origin).

Source attribution is best accomplished by identifying the chemical composition of pharmaceuticals. The most common analysis methods for source attribution of pharmaceuticals are Raman spectroscopy, isotope ratio mass-spectrometry (IRMS), liquid-chromatography tandem mass-spectroscopy (LC-MS/MS), and gas-chromatography mass-spectroscopy (GC-MS). While these techniques have proven to be effective for authentication of pharmaceuticals, they each have disadvantages. For example, in a study by Dégardin et al. [16] the authors developed a method to determine if pharmaceuticals were genuine or counterfeit using a combination of Raman spectroscopy and chemometrics. However, their method was complex and required multiple steps. For example, the first step consisted of identification of suspect samples as more likely genuine or counterfeit using Raman spectra treated with multiple statistical methods, including normalization, support vector machines (SVM) for classification, and an active pharmaceutical ingredient (API) correlation test. If suspected as counterfeit following this step, the drug was then classified using PCA and a distance measure to classify the counterfeits. If the drug was in an existing counterfeit class, then determination of the composition and a forensic investigation was executed. If the drug was considered a new counterfeit class, then interpretation of Raman spectra, infrared spectroscopy, and GC-MS was implemented to determine the chemical composition. Even with the multi-step method which utilized three analysis techniques and multiple chemometric techniques, the authors could not classify all 27 seizures of counterfeits into 15 separate PCA chemical classes due to the heterogenous nature of the illicit drugs [16]. In another study by Cristea et al. [20], the authors analyzed 38 pharmaceuticals from six pain relivers (e.g., ibuprofen and paracetamol) pharmaceutical classification by IRMS to

determine $\delta^{13}C$ isotopic compositions. Additionally, inductively coupled plasma-mass spectrometry (ICP-MS) was used for monitoring elemental impurities. Even with these two sophisticated analytical methods and linear discriminant analysis (LDA) for classification of pharmaceuticals, a clear separation for ibuprofen and acetaminophen was not produced [20]. Lee et al. [28] developed a quantitative LC-MS/MS method to determine the erectile dysfunction drugs and their analogues concentration in the counterfeit drugs. Out of the 89 counterfeit drugs and herbal medicines the author's analyzed, 73% of the secured drugs were adulterated with sildenafil where the concentration ranged from 21.0-947.5 mg/g. However, their sample preparation for 89 counterfeit drugs and herbal remedies (~0.5 g each) consumed a large amount of organic solvent for extraction (25 mL of methanol per sample) and the LC-MS/MS method was time consuming (i.e., a run time of 20 minutes per sample) [28]. Neves et al. [40] developed a quantitative GC-MS method determine the concentration of anabolic androgenic steroids in 345 counterfeit pharmaceuticals that were seized by the Brazilian Federal Police. In general, they found that counterfeits were adulterated or had no active ingredient. However, the GC-MS method was plagued by a large rise in baseline for testosterone cypionate (structural base of testosterone in addition of a cyclopentyl propionate group on C17β, MW 412.6 g/mol), nandrolone phenylpropionate (structural base of testosterone in addition to a phenylpropionate group on C17β, MW 406.8 g/mol), testosterone phenylpropionate (internal standard that has a structural base of testosterone in addition to a phenylpropionate group on C17β, MW 420.6 g/mol), and boldenone undecylenate (structural base of testosterone in addition to an alkenyl group on the C1

position and a undecylenate group on the C17β, MW 452.7 g/mol) which could produce inaccurate results for determining the illicit drugs [40].

Aspirin is a common pain reliever that can relieve headaches, reduce swelling, lower fever, and decrease the risk of cardiovascular events. Because of the medicinal properties that aspirin provides, it is readily available and relatively cheap. Therefore, it is not counterfeited frequently as other drugs (e.g., fentanyl). Yet, there are still reports of aspirin being counterfeit. For example, in 2013 the French customs seized 1.2 million doses of counterfeit aspirin from China. Also in 2018, there was 26 people that were arrested for marketing 1.49 million fake drugs, including aspirin [43, 44]. Because aspirin is readily available from many manufactures, it is an excellent candidate to develop general authentication protocol for more commonly counterfeited drugs.

Due to limitations of current methods for drug authentication, there is a need for improved techniques to determine the authenticity of pharmaceuticals. Hence, the objective of this investigation was to evaluate the performance of GC-MS and LC-MS/MS for analysis of multiple aspirin brands coupled with assessment of multiple chemometric methods for their usefulness in source attribution of various aspirin brands. Development of a general methodology would potentially allow extension of the method to source attribution of more commonly counterfeited drugs.

2.2 Materials and Methods

2.2.1 Materials

The aspirin brands (325 mg) used to establish chemical fingerprints were Bayer®, Walgreens©, and Premier Value®, purchased from local stores in Brookings, SD, USA for Bayer®, and Premier Value® and Sioux Falls, SD, USA for Walgreens©. All aspirin

brands were stored in their respective containers at room temperature. For the double-blind study, expired aspirin brand Equate (325 mg) was purchased from a local store in Brookings, SD, USA and used. Phenyl Acetate ($C_8H_8O_2$, > 98%), methyl salicylate, ($C_8H_8O_3$, 99+%), 2-hydroxybenzoic acid ($C_7H_6O_3$, > 99.5%), acetylsalicylic acid ($C_9H_8O_4$, 98+%), and phenyl salicylate ($C_{13}H_{10}O_3$, > 98%) were purchased from Tokyo Chemical Industry (TCI) (Portland, OR, USA). Acetic acid, glacial (CH_3COOH) and methanol (CH_3OH, HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethyl acetate ($C_4H_8O_2$, ≥ 99.5%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenol (C_6H_6O, 99%) was purchased from Acros Organics (Fair Lawn, NJ, USA). Purified water was obtained from a water PRO PS polisher (Labconco, Kansas City, KS, USA) at a resistivity of 18.2 MΩ-cm

2.2.2 Standard Solutions

Stock solutions of acetic acid (5 mM) and methyl salicylate (5 mM) were prepared in 50 mL of water and stored at room temperature. 2-hydroxybenzoic acid (5 mM), phenyl salicylate (1 mM), and acetylsalicylic acid (5 mM) were prepared in 500 mL of purified water and stored at room temperature. These stock solutions were diluted with purified water to the desired concentrations for experiments. A stock solution of phenol (1 mM) was prepared in methanol and stored at 4 °C. Phenol was diluted with methanol to the desired concentration for individual experiments.

2.2.3. Aspirin Sample Preparation for GC-MS Analysis

Each aspirin pill (325 mg) was separately crushed in a clean mortar and pestle. A portion of crushed aspirin pill was weighed (20 mg for comprehensive GC-MS analysis via “Scan mode” and 13 mg for analysis via selected ion monitoring mode) and added to

a 15-mL centrifuge tube along with 5 mL of purified water. This solution was vortexed for 10 s to mix. Proceeding this step, the solution was added to a stainless steel ultrasonic bath (model 75D) and sonicated for 35 min at 55 °C. When completed, the extract was vortexed for 20 s to mix and subsequently filtered with a 0.45 µm nylon filter. An aliquot of this solution (500 µL, analyzed in triplicate) was transferred into a 4-mL vial and capped. Prior to analysis, samples were dried under N_2, the residue was reconstituted with 100 µL of methanol, and the solution was transferred into a 300-µL insert in a 2-mL capped vial for a GC-MS analysis.

2.2.3.2 Aspirin Sample Preparation for LC-MS/MS Analysis

Each aspirin pill (325 mg) was separately crushed in a clean mortar and pestle. A portion of crushed pill was weighed (1.8 mg) and added to a 15-mL centrifuge tube along with 10 mL of purified water. The 10 solution was vortexed for 10 s to mix. Proceeding this step, the solution was added to a stainless steel ultrasonic bath (model 75D) and sonicated for 35 min at 55 °C. The extract solution was then diluted from 1:10, vortexed at 3000 for 20 s to mix and filtered with a 0.2 µm polytetrafluorethylene (PTFE) syringe filter. For each study, triplicate samples (1.5 mL each) were transferred into a 2-mL capped vials for analysis via LC-MS/MS.

2.2.3.3 Double-Blind Study Sample Preparation

Three aspirin pills per brand (Bayer®, Walgreens©, Premier Value®, and expired Equate) were crushed in a clean mortar and pestle. The crushed pills were added to separate 20 mL scintillation vials. The vials were labeled with random letters by an independent individual; the sample key was recorded and kept isolated from anyone involved in this project. The labeled samples were delivered to another independent

individual and the sample labels were changed from letters to numbers. The sample key was recorded, and the double-blinded samples were given to project personnel for LC-MS/MS analysis.

2.2.4 Gas Chromatography-Mass Spectrometry

Each prepared sample was analyzed using an Agilent Technologies 6890N gas chromatograph and a 5975B inert XL electron ionization (EI)/chemical ionization (CI) mass selective detector (MSD) with a 7683 series injector. Samples were injected (1 µL injection volume) into an electronic pneumatics control (EPC) split/spitless inlet. The EPC inlet was set at 250 °C using spitless mode with a purge flow of 20 mL/min at 1 minute before introducing the analytes into the column. The GC oven temperature started at an initial temperature of 40 °C for 1 minute, it was increased at 5 °C/minute to 240 °C for 3 minutes, producing a total run time of 44 minutes. The excipients and active ingredients in aspirin were separated in a DB5-MS capillary column (30 m x 250 µm x 0.25 µm) with hydrogen as a carrier gas and a flow rate of 1.2 mL/min with a pressure of 10.67 psi. The MS source and MS quadrupole were set at 150 °C and 230 °C, respectively. Electron ionization (EI) was used as the ionization mode at 70 eV with an initial scan range of 40 – 400 m/z to identify compounds in the aspirin. After identification of compounds in the comprehensive scan of aspirin, one to three major ions were selected for each compound and added to a final selected ion monitoring (SIM) method for higher selectivity and sensitivity for analytes compared to scan mode. The SIM ions used (m/z) are as follows: acetic acid (45, 60), phenol (94), phenyl acetate (94, 136), salicylic acid, (92,138), methyl salicylate (92, 120, 152), methyl acetylsalicylate

(92, 120, 152), acetylsalicylate (92, 120, 180), phenyl salicylate (121, 214) and disalicylide (92, 120, 240).

2.2.5 Liquid Chromatography Tandem Mass Spectrometry

Analysis of prepared aspirin samples (10 µL injection volume) was carried out using LC separation with a Shimadzu HPLC (LC-20AD, Shimadzu Corp., Kyoto, Japan). The chromatographic separation was performed on an Agilent Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm). Mobile phase A was water (100%) and mobile phase B was methanol (100%). The chromatographic separation was achieved using gradient elution at a flow rate of 0.5 mL/min starting at 50% B and increased to 90% B over 4 minutes, held constant for 2 minutes, and decreased to 50% B over 4 minutes.

A tandem mass spectrometer (AB Sciex Q-Trap 5500 MS) with an electrospray ionization interface operating in negative polarity was used for detection. To determine which compounds were detected, a Q1 scan of a 9 mg/L aspirin solution of each brand (infused at a 10 µL/min flow rate) was performed (40-400 m/z). Nitrogen (20 psi) was used as both the curtain and nebulization gas. The ion spray voltage and source temperature were set at -4,500 V and 0 °C, respectively, with the ion source gas (GS1) pressure at 10 psi. After determining excipients present in the Q1 scan, multiple reaction monitoring (MRM) transitions were obtained and optimized (Table 2.1). All aspirin samples were analyzed in MRM mode. Chromatograms were acquired and analyzed with the Analyst software program.

Table 2.1: MRM transitions, optimized collision energies (CEs), declustering potential (DPs), and cell exit potentials (CXPs) for detecting Aspirin Brands by MS/MS analysis.

Compounds	Q1 (m/z)	Q3 (m/z)	Time (ms)	CE (V)	DP (V)	CXP (V)
Salicylic acid	136.5	92.9	100	-27.00	-19.00	-47.06
Methyl salicylate*	153.2	108.8	100	-20.18	-31.00	-0.940
Aspirin	178.7	93.0	100	-33.88	-34.00	-33.01
Aspirin (identification)	178.7	59.2	100	-103.93	-33.88	-33.00
Unknown	199.1	137.1	100	-18.12	-28.00	-33.12
Unknown	199.1	92.9	100	-42.98	-50.00	-33.03
Disalicylide	240.0	137.1	100	-26.08	-26.00	-18.08
Disalicylide	240.0	92.9	100	-50.97	-17.02	-33.03

*Only detected in Bayer® brand (inconsistently) and if used in chemometric method it would automatically classify the Bayer® pill.

2.2.4 Identification of Excipients and Active Ingredients in Aspirin

Compounds in the GC-MS comprehensive scan of aspirin that produced chromatographic peaks with a S/N of at least 3 compared to the purified water blank were examined with the National Institute of Standards and Technology (NIST) library (NIST/EPA/NIH Mass Spectral Library, Version 2.0d, 2005). Specific compounds were classified as follows: 0-40% = low probability, 41-70% = medium probability, 70-100% = high probability. After determining the highest probability of compounds in the aspirin scan, the experimental mass spectrum and known mass spectrum were compared for similarity. If the mass spectra were sufficiently similar, a standard of that compound was analyzed. The retention time of the compound in aspirin and the standard of that compound were required to have the same mass-spectra and elution time to consider the compound from aspirin definitively identified. The compounds that were identified via GC-MS were evaluated via LC-MS/MS for addition to that method.

2.2.5 Chemometrics

Three statistical methods were used when developing the method for the analysis of various aspirin brands. Linear discriminate analysis (LDA) and quadratic discriminate analysis (QDA) were used for classification of brands by determining a set of projections in the aspirin chromatograms that separate them into classes relative to within-class variation. LDA and QDA are similar, however, LDA uses a linear projection while QDA, uses a polynomial or non-linear projection. When using LDA and QDA, an observation is assigned to a known class that has the highest likelihood. If the observation has a low likelihood within that class, the observation does not belong to that class. In both the LDA and QDA methods, normality is assumed of each class and each method has their respective assumptions about variance [45]. When using LDA and QDA, no matter how low the likelihood of the observation is for any class, the methods require the observation to be assigned to a known class. For example, if an unknown aspirin pill was observed using the set of LDA and QDA functions, the unknown aspirin pill would have to be classified as either a Bayer®, Walgreens©, or Premier Value®, even if it does not belong to that class (e.g., expired Equate pill). Hence, an atypically discriminate rule was implemented to determine if in an unknown aspirin pill does not belong to the known aspirin pill brands and to double check the LDA and QDA class predictions.

Atypicality is the probability of observing a new sample that was randomly drawn from a class, where the likelihood of that sample being observed in that same class is greater than the likelihood of the observation in question. Atypicality is a modification of the Hotelling T^2 statistic, which has the relation to the F distribution, $F = mT^2$. The cumulative density function evaluated at the T^2 statistic can then be found; this value is

referred to as the atypicality value of an observation with respect to the known aspirin class/brand. Since this is a cumulative density function, the atypicality values range from zero to one. As the value approaches one, the more atypical the observation is to the known aspirin class. The smaller the atypicality value, the less atypical the observation is, and it can be claimed that the observation came from the known class. LDA, QDA, and atypicality were utilized to predict the pill brand based on the various compounds in aspirin (i.e., salicylic acid, aspirin, disalicylide, and unknown) chromatograms.

2.3 Results and Discussion

2.3.1 GC-MS Analysis of Aspirin

The traditional synthetic pathway for aspirin, shown in Figure 2.1, consists of the reaction of salicylic acid with an excess of acetic anhydride and in the presence of a small amount of acid to yield acetylsalicylic acid with acetic acid as a byproduct. However, when manufactured into a consumable pill form the aspirin tablets usually contain three main ingredients: the active ingredient (acetylsalicylic acid), corn starch, and a lubricant. While these are the main ingredients, other trace excipients are present that provide a chemical fingerprint which encodes information about the manufacturing/synthetic process.

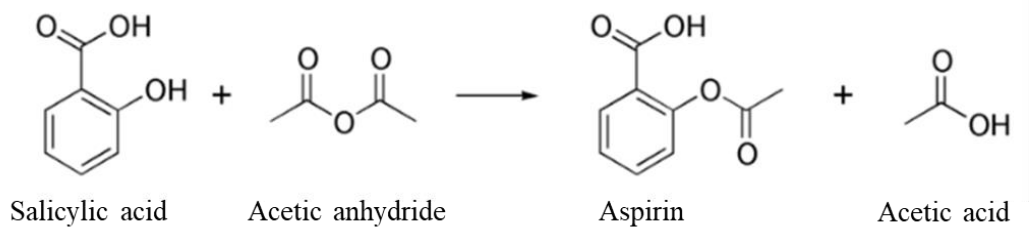


Figure 2.1 Reaction scheme of salicylic acid and acetic anhydride to yield aspirin and byproduct acetic acid.

To determine active ingredients and excipients in various brands of aspirin, the aspirin samples (Bayer®, Walgreens©, and Premier Value®) were simply prepared simply dissolving the compounds in aspirin in water under sonication, filtering, drying, and reconstituting the sample. The resulting solution was analyzed via GC-MS with a mass range of 40-400 m/z. An example of a GC-MS chromatogram produced via a comprehensive scan of the prepared Bayer® aspirin is shown in Figure 2.2. Once the compounds in each brand were established, one to three major ions were selected to add to the SIM method.

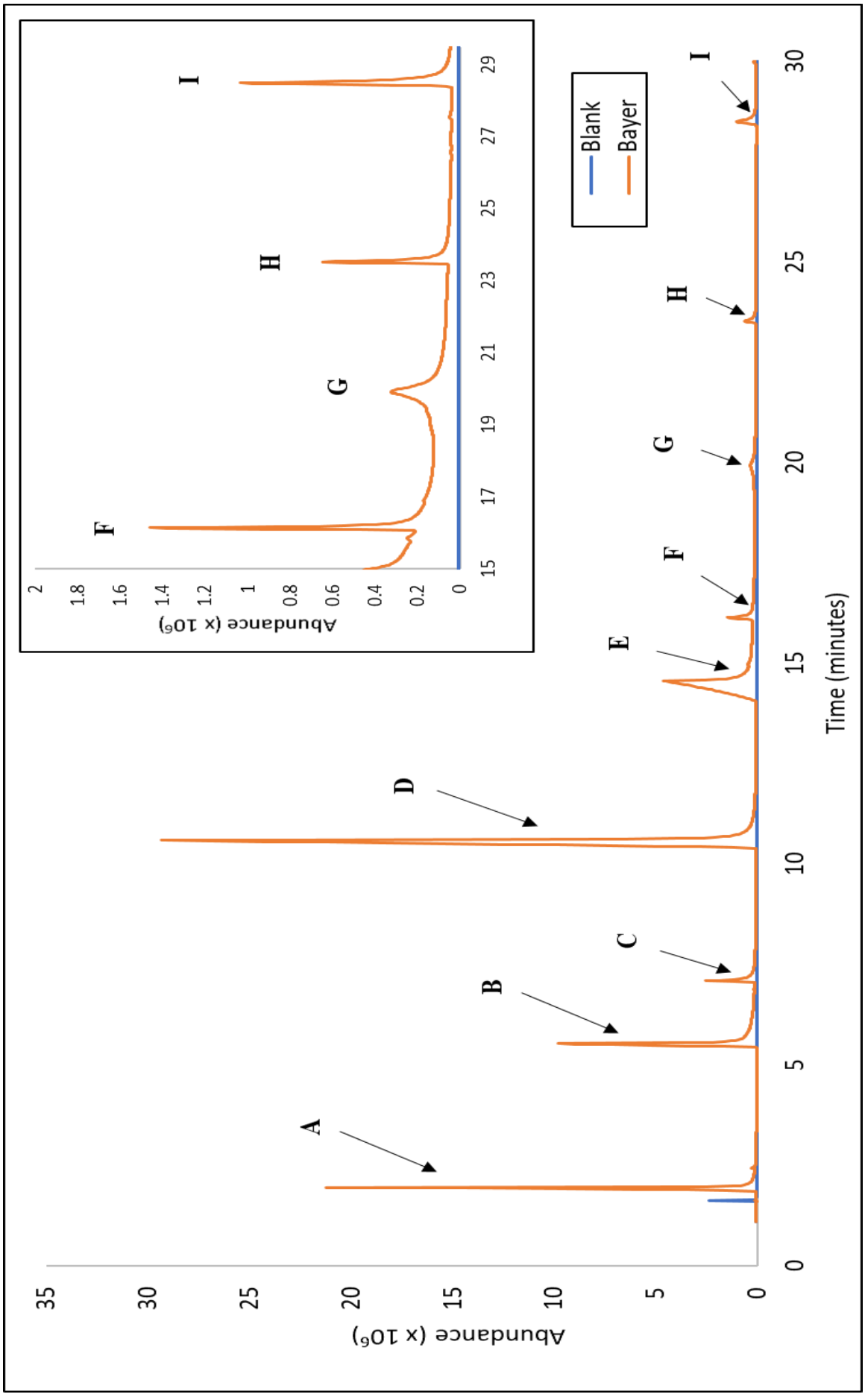


Figure 2.2 Total ion chromatogram of Bayer® aspirin (20mg/5 mL). Compounds detected in aspirin: (A), acetic acid, (B), phenol, (C), phenyl acetate, (D), methyl salicylate, (E), salicylic acid, (F), methyl acetylsalicylate, (G), acetylsalicylic acid, (H), phenyl salicylate, (I), disalicylide.

2.3.2 Compound Identification via GC-MS Analysis

Initial identification of compounds present in the aspirin pill was accomplished by comparing the MS of a compound eluting in the GC-MS chromatogram to the NIST library database spectrum. For example, the NIST library predicted that the compound eluting at approximately 7 min was phenyl acetate at 75.6% probability. For definitive identification, a standard of the suspected compound (e.g., phenyl acetate) was purchased and analyzed via the method described. For example, Figure 2.3 shows the process used to definitively identify methyl salicylate. The retention time and peak shape (Figure 2.3A), mass spectra compared to NIST database (Figure 2.3B), and mass spectra of the standard compared to the compound prepared from the aspirin pill (Figure 2.3C) from the methyl salicylate standard match the compound detected from aspirin. Of the suspected compounds extracted from aspirin, methyl acetylsalicylate (i.e., inconsistent detection in GC-MS) and disalicylide (i.e., not currently available for purchase), which had a probability of 62% and 81.2%, respectively, could not be definitively identified. After identifying the compounds in aspirin, major ions associated with assignable MS fragments were added to the final SIM method. Table 2.1 shows the seven compounds definitively identified and another two compounds with medium and high probability of identification (i.e., 41-100 %).

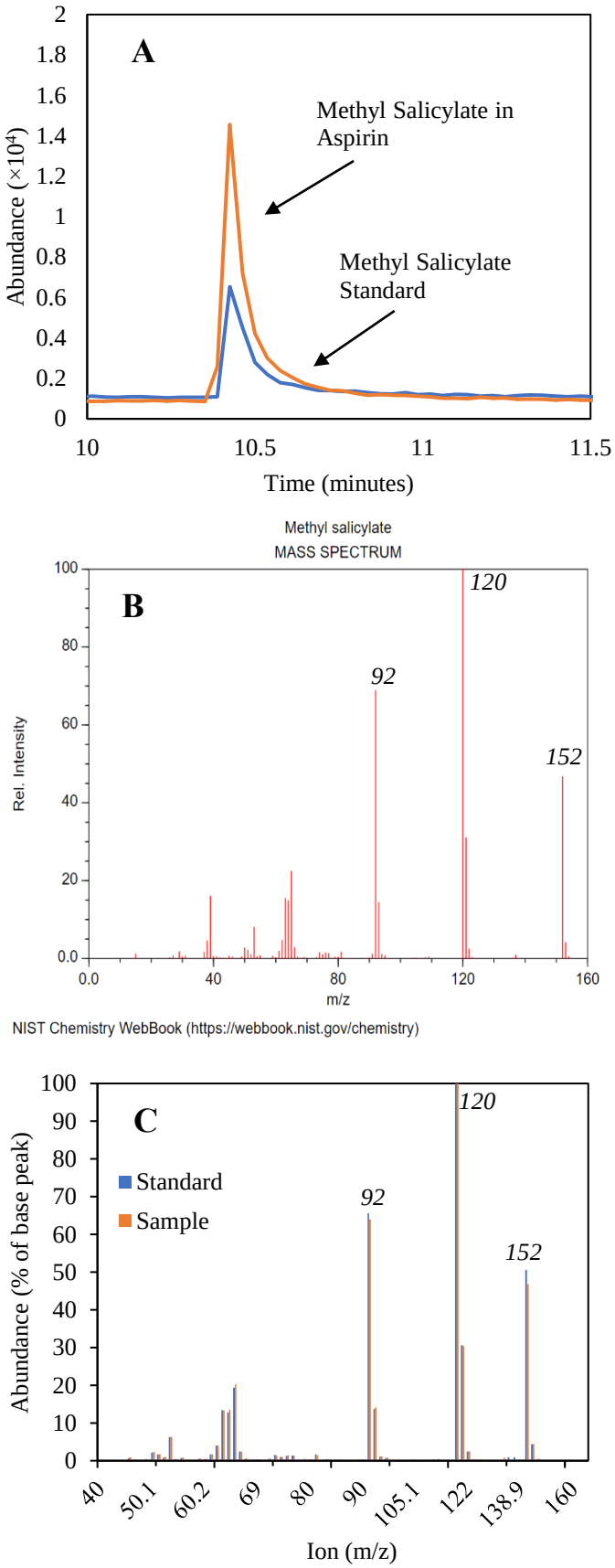
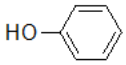
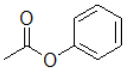
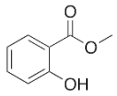
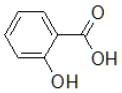
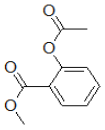
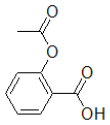
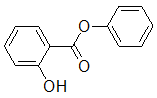
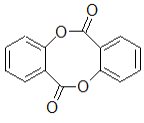


Figure 2.3 Example of definitively identified compound. A) GC-MS chromatogram of excipient methyl salicylate confirmed with its standard. B) NIST C) Mass spectrum of methyl salicylate in aspirin pill and methyl salicylate standard.

Table 2.2. Active ingredients and excipients detected in aspirin pills with physical properties and ions used for SIM detection.

Compound	Chemical Structure	Molecular Mass (g/mol)	Boiling Point (°C)	Ion (m/z)
Acetic acid		60.052	118	45; 60
Phenol		94.11	181.7	94
Phenyl acetate		136.1	196	94; 136
Methyl salicylate		152.14	220	92, 120, 152
Salicylic acid		138.121	211	92; 138
Methyl acetylsalicylate*		194.18	136	92; 120; 152
Acetylsalicylic acid		180.158	140	92; 120; 180
Phenyl salicylate		214.22	173	121; 214
Disalicylide*		240.211	N/A	92; 120; 240

*Tentatively identified based solely on the NIST library database with methyl acetylsalicylate and disalicylide having a 62% and 81.2% probability, respectively.

2.3.3 Excipients Detected in Aspirin

The simple GC-MS method presented here was able to detect compounds other than the active ingredient in aspirin pills (Table 2.2). For example, one of the excipients identified in all three aspirin brands was phenol. Phenol is an organic compound that has been used to as a topical antiseptic to relieve itching and an oral analgesic to treat pharyngitis in products such as Chloraseptic [46]. Another excipient detected in aspirin was phenyl acetate, which has a sweet scent and is an odorant found in strawberries, passion fruit, and black tea. Phenyl acetate is a metabolite of phenylalanine with antineoplastic activity [47, 48]. The main active ingredient in aspirin is acetylsalicylic acid. This compound was detected along with other salicylates such as methyl salicylate, methyl acetylsalicylate, and phenyl salicylate. Methyl salicylate is naturally occurring in various species of plants, mainly wintergreens and is an external analgesic that can be used to relieve minor body aches, muscle, and joint pain [49, 50]. Phenyl salicylate is a mild analgesic used an active ingredient in some pharmaceuticals [51]. Disalicylide was found in the GC-MS analysis (although not definitively). While it is not likely specifically added to the aspirin or a byproduct of aspirin synthesis, Shulga et al. [52] determined that salsalate can convert disalicylide, and tri-salicylide in GC-MS at 150-280 °C. It is also possible that disalicylide may form via esterification of two salicylic acid molecules.

2.3.4 Evaluation of GC-MS and LC-MS/MS for Aspirin Authentication

To determine which analysis technique, GC-MS or LC-MS/MS, provided the most consistency (i.e., consistency of analysis is extremely important to allow statistical differentiation between pills with similar composition), pill replicates were evaluated using "pairwise scatter plots". Pairwise scatter plots visually show the distribution of single variables and the relationships between two variables within a dataset. Once the dataset is plotted, trends can be identified before implementing analysis techniques. Pairwise scatter plots are similar to line graphs in that the numerical variables in the dataset will be shared across the y-axes and x-axes. If the data points exhibit a trend that increases from the x axes to the y axes at a 45-degree angle then there is a positive correlation (i.e., a slope of 1 indicates a perfect positive correlation and a slope of -1 indicates a perfect negative correlation) [53]. To evaluate if analyzing samples in the GC-MS or LC-MS/MS provided the highest correlation within pill and between pill solutions, aspirin brands (N=7, with triplicate analysis) were analyzed and pairwise scatter plots were constructed. Because slight peak shifting was evident for each compound, the analyte peaks were shifted to the same retention time before comparing analytical techniques using pairwise scatter plots. Also, the noise was eliminated by comparing the limit of detection (LOD; 3*standard deviation + average) of the blank to the noise within the sample within a certain retention time. If the LOD of noise in the selected sample was lower than the LOD of the blank at the selected retention time range, then these "baseline" signals were removed. Examples of the chromatograms treated in this manner in preparation for pairwise scatter plots of the GC-MS and LC-MS/MS chromatographic peaks produced for two samples of Walgreens© aspirin are shown in Figure 2.4.

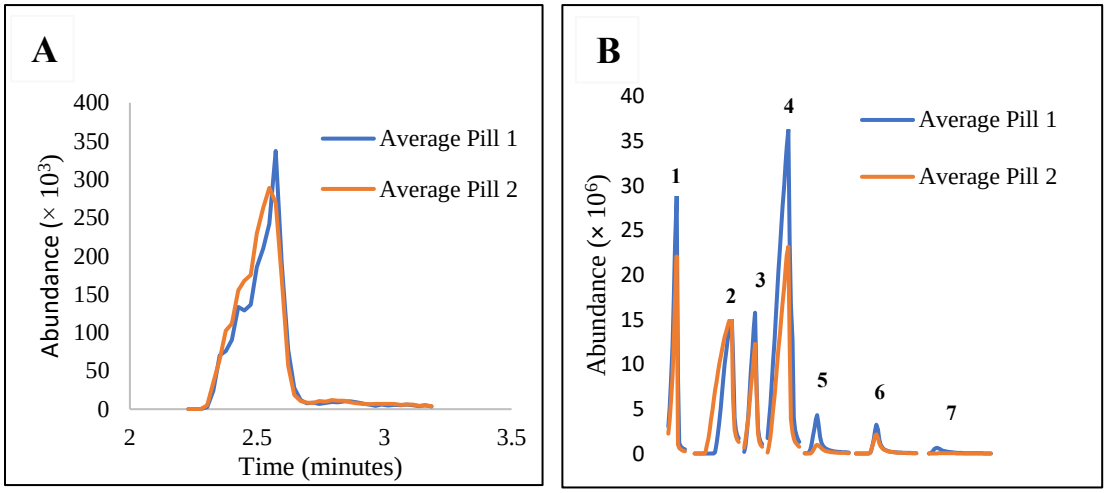


Figure 2.4 Data points across the Walgreens© chromatograms with the removal of noise based on the difference of blank and sample LODs were used for the pairwise scatter plots. A) Walgreen's (0.018g/L) chromatograms of two average pill replicates (triplicate analysis per pill); LC-MS/MS analysis; Salicylic acid, extracted ion Q1-136.5/Q3-92.9. B) Walgreen's (2.6 g/L) total ion chromatograms of two average pill replicates (triplicate analysis per pill); (1) acetic acid, (2) phenol, (3) phenyl acetate, (4) methyl salicylate, (5) methyl acetyl salicylate, (6) phenyl salicylate, (7) disalicylide (Note: salicylic acid and acetylsalicylic acid did not elute for all brands when diluting from 4 g/L to 2.6 g/L); GC-MS analysis

The pairwise scatterplots for two Walgreens© pills (each in triplicate) using the salicylic acid 136.5/92.9 transition from the LC-MS/MS method (i.e., the first number indicates the pill and the second denotes the analysis replicate for that individual pill) are shown in Figure 2.5. For these plots, the pill replicate in a column that intersects with another row are compared. For example, the plot outlined in blue compares Pill 1, replicate 1 and Pill 1, replicate 2 and the plot outlined in red compares Pill 1, replicate 1, to Pill 2, replicate 3. Replicates that have a strong correlation should generally produce points scattered randomly about a line with a slope of 1. Systematic deviation from the line, even if many points are near the line, indicates non-correlated data.

Evaluation of Figure 2.5A shows that correlation with some scatter is produced between replicates when using LC-MS/MS method. For example, comparing Pill 1.1 to

Pill 2.3 (i.e., the plot outlined in red), there is a relatively linear trend with generally random scatter about a line with a slope of 1. The linear trend between separate pill replicates can be interpreted that there is correlation between the two separate pill solutions and this correlation is relatively consistent for all replicates. Overall using LC-MS/MS analysis (i.e., salicylic acid, extracted ion Q1-136.5/Q3-92.9), the Walgreens© pill solutions within and between two pills have positive correlation with little, if any, systematically uncorrelated data. Conversely, in the pairwise scatter plots shown in Figure 2.5B, the plot outlined in blue compares Pill 1, replicate 1 to Pill 1, replicate 2 while many data points are perfectly correlated, there is obvious systematic deviation from this correlation. This is also evident when comparing Pill 1.1 to Pill 2.3 (plot outlined in red).

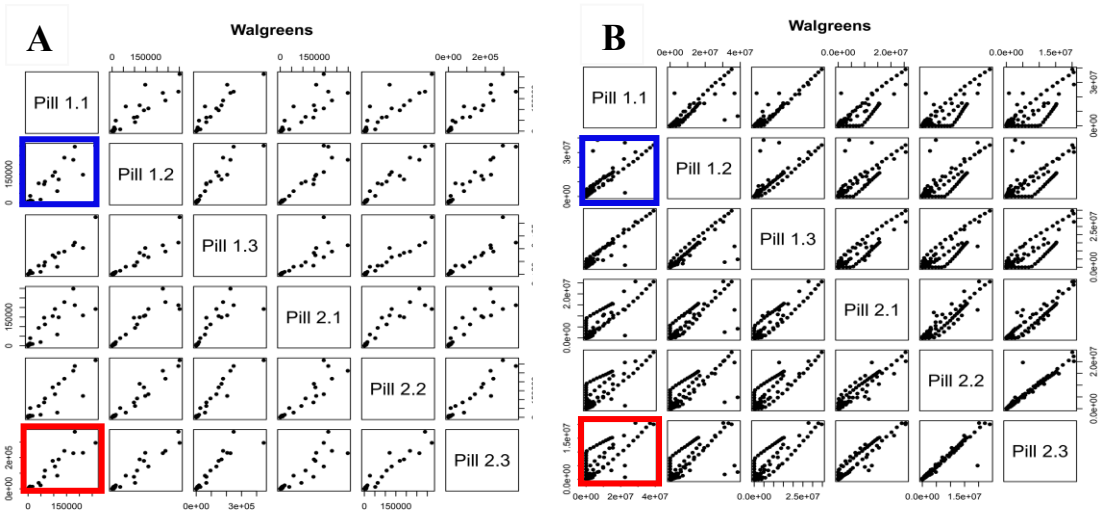


Figure 2.5 Pairwise scatter plots of two Walgreens© pills (triplicate analysis per pill). A) Two Walgreens© pills (triplicate analysis per pill) were analyzed using LC-MS/MS. Pill 1.1 is denoted as pill 1 replicate 1 and Pill 2.1 is denoted as pill 2 replicate 1. B) Two Walgreens© pills (triplicate analysis per pill) were analyzed using GC-MS. Pill 1.1 is denoted as pill 1 replicate 1 and Pill 2.1 is denoted as pill 2 replicate 1. Note: The pill replicate plot in a column is being compared to the pill replicate in the row that intersects.

As another measure of the consistency of the LC-MS/MS and GC-MS methods, the relative standard deviation (%RSD) of the peak area of the compounds identified were evaluated. The %RSD (i.e., standard deviation divided by mean*100%) is a qualitative measure of precision of the two methods for analysis of replicate pills and solutions. The compounds exhibited in the pill brands need to be precise within pill and between pills to ensure that the pill brands can be differentiated. In Table 2.3, when extracting for salicylic acid, aspirin, the unknown, and disalicylide MRM transitions, the Walgreens© Pill 1 and Walgreens© Pill 2 had %RSDs <15%. The lower the %RSDs (ideally <15%), the more precise salicylic acid is in the pill solution. In addition, when comparing compounds between two pills, the %RSDs again were <15%. Therefore, the amount of salicylic acid, aspirin, unknown, and disalicylide is precise in Walgreens© pill solutions homogenously and heterogeneously. However, when obtaining the peak area for the seven compounds in the GC-MS total-ion-chromatogram, shown in Table 2.4, the peak area precision of the seven compounds were relatively large, both within pill and between pills. Some compounds did produce acceptable %RSDs (i.e., <15%) when using GC-MS but multiple compounds produced >15% RSDs. For example, while phenol and methyl salicylate %RSDs were 10.91% and 8.34%, respectively for Pill 1, the %RSDs between pills were all >15% between pills.

Table 2.3: The relative standard deviation (%RSD) was obtained using peak area for within two separate Walgreens© pills (triplicate analysis per pill) and between two Walgreens© pills (triplicate analysis per pill) for the MRM transition compounds and was calculated to determine the precision of the pill solutions analyzed using the LC-MS/MS.

Walgreens© Pill Relative Standard Deviation (%RSD)

	Pill 1 (N=3)	Pill 2 (N=3)	Pill 1 and 2 (N=6)
Salicylic acid	10.74	4.36	7.94
Aspirin	6.02	4.04	8.46
Unknown	5.66	7.26	5.89
Disalicylide	6.40	8.14	8.14

Table 2.4: The relative standard deviation (%RSD) was obtained using peak area from the total-ion-chromatogram for within two separate Walgreens© pills (triplicate analysis per pill) and between two Walgreens© pills (triplicate analysis per pill) and was calculated to determine the precision of the pill solutions analyzed using the GC-MS.

Walgreens© Pill Relative Standard Deviation (%RSD)

	Pill 1 (N=3)	Pill 2 (N=3)	Pill 1 and 2 (N=6)
	%RSD	%RSD	%RSD
Acetic acid	6.62	19.02	23.04
Phenol	10.91	4.05	20.38
Phenyl acetate	10.22	20.37	17.00
Methyl salicylate	8.34	11.80	32.68
Methyl acetylsalicylate	12.21	10.09	57.37
Phenyl salicylate	31.30	37.43	32.19
Disalicylide	31.01	29.68	85.00

Since the pairwise scatter plots did not have a linear distribution and the pill replicates %RSDs was not <15% within pill and between separate pills when using the GC-MS analysis, it was not selected for further analyses and in developing the experimental design. Therefore, pill brands were analyzed in the LC-MS/MS and carried out to fulfill the objective.

2.3.5 Preparation of LC-MS/MS Data for Statistical Analysis

Known sources of Bayer®, Premier Value®, and Walgreens© aspirin needed to be established before introducing unknown source objects. To do this, pills per brand were analyzed on three separate days to account for day-to-day variation and to get enough data accumulation before introducing an unknown source. Extracted ions of aspirin (178.7/93), salicylic acid (136.5/92.9), disalicylide (240/137.1), and an unknown (199.1/137) were used for establishing the chemical fingerprint of the known source brands and the chromatograms were used to for chemometric studies. Before using the chromatograms for further statistical analyses, the noise was eliminated by comparing the limit of detection (LOD; 3*standard deviation + average) of the blank to the noise within the sample within a certain retention time. Once establishing the retention times ranges, those retention times were set for all future analyses. If peaks shifted out of the set time range, the peak with the respective transition was manually shifted to be in that set retention time range to prevent misclassification of brands. After shifting peaks to the same retention time with each respective transition, LDA, QDA, and atypicality were applied to the pills (i.e., seven pills for each brand with triplicate analysis for each prepared solution from individual pills).

2.3.5.1 Chemometric Analysis of LC-MS/MS Chromatograms

The normalized chromatograms for Bayer®, Walgreens©, and Premier Value® aspirin brands were used for LDA, QDA, and atypicality analysis. When observing the total correct percentage classification across three days and between brands, shown in Figure 2.5, LDA produced the highest correct classification percentage with 79.4%. Atypicality analysis produced the second highest correct classification with 74.6%. While

this provides useful information regarding the number of pills that were predicted correctly across days, it does not focus on the total correct classification within-pill. Therefore, the correct classification percentage for within-brand analysis was calculated and is presented in Table 2.6. Premier Value® had the highest percentage of being classified correctly for LDA, QDA, and atypically chemometric methods with percentage values of 90.5%, 90.5%, and 85.7%, respectively. Walgreens© had the second highest percentage of being classified correctly with LDA, QDA, and atypicality percentage values of 76.2%, 66.7%, and 76.2%, respectively.

LDA had the highest total correct percentage for predicting across and within classes. A visual representation of the LDA analysis across three different days is shown in Figure 2.6. There is separation between classes within a day, however, there is not a distinct classification trend across days. When plotting the first linear discriminant (LD1) and the second linear discriminant (LD2) for Day 1 analysis (Figure 2.6A), there is clear separation between the various brands. For Day 2 and Day 3, shown in Figure 2.6B and 2.6C, respectively, the separation between classes is not as distinct. The maximum separation for the known pill brands was mainly accomplished using LD1 for Day 2, while pill brands analyzed on Day 3 produced separation in both LD1 and LD2. Across all three days, Premier Value® is clearly separated from Bayer® and Walgreens©. Although there was not a similar classification trend across the three days, there is a relatively nice class separation in the LDA plots within a day, especially for Premier Value®. When looking at the Bayer® brand misclassification trends, it was most commonly misclassified as Walgreens©. Therefore, an investigation was executed to determine if Bayer® and Walgreens© were produced at the same manufacture. A

Walgreens© bottle spokesperson stated their aspirin was manufactured at LNK International Inc. whereas Bayer® as pills are manufactured by Bayer® HealthCare Pharmaceuticals, LLC.

Table 2.5: The total correct percentage of pill brand classification across all brands using LDA, QDA, and atypicality chemometric methods.

	Day 1			Day 2			Day 3			
Method	Bayer®	Walgreens©	Premier Value®	Bayer®	Walgreens©	Premier Value®	Bayer®	Walgreens©	Premier Value®	**Total Correct (%)**
LDA	7/7	6/7	6/7	5/7	5/7	6/7	3/7	5/7	7/7	***79.4%***
QDA	5/7	5/7	7/7	4/7	5/7	5/7	2/7	4/7	6/7	***68.3%***
Atypicality	6/7	5/7	7/7	4/7	6/7	5/7	3/7	5/7	6/7	***74.6%***

Table 2.6: The total correct percentage of within-brand classification using LDA, QDA and atypicality chemometric methods.

	Within-Brand Correct Classification Percent Day 1-3		
	LDA	QDA	Atypicality
Bayer®	15/21 (71.4%)	11/21 (52.3%)	13/21 (61.9%)
Walgreens©	16/21 (76.2%)	14/21 (66.7%)	16/21 (76.2%)
Premier Value®	19/21 (90.5%)	19/21 (90.5%)	18/21 (85.7%)

Atypicality had the second highest effectiveness for correctly predicting across and within classes. The smaller the atypicality value, the more likely the sample belongs to that class. When using atypicality, as shown in Table 2.5, it is clear when a pill is likely from Premier Value® due to the large differences in atypicality values between the respective known brands and the low value in the Premier Value® classification. Atypicality was used as another method to check the accuracy of LDA and QDA, when LDA and QDA assumptions were not met, and as a discriminant function that the

atypicality value is not above some threshold (i.e., to determine if an observation is not likely to belong to that class). Atypicality is also useful when an unknown aspirin brand is introduced to the known aspirin brands. Because it tests the discriminating power of the method, where the unknown brand should have a high atypicality value for all known classes indicating that is does not belong to a known class.

Because there is adequate separation of LDA classification parameters between pill brands within each day, but the classification is not stable between different days, shown in Figure 2.6, the known and unknown pill brands should be analyzed on the same day. Therefore, to test the applicability of the LC-MS/MS method coupled with the three chemometric methods, "unknown" aspirin pills were analyzed with all samples analyzed on the same day.

Table 2.7: Sample atypicality values for analyzing pill brands on Day 1. The atypicality values that are bolded in black had the lowest atypicality values and the values that are bolded black and have an asterisk were predicted incorrectly.

True Brand	Predicted Brand	Atyp Bayer	Atyp PV	Atyp WG
Bayer	Bayer	**0.68**	0.99	0.96
Bayer	Bayer	**0.97**	0.99	0.97
Bayer	WG	0.99	1.00	**0.87***
PV	PV	1.00	**0.79**	1.00
PV	PV	1.00	**0.83**	1.00
PV	PV	1.00	**0.26**	1.00
WG	PV	0.99	**0.86***	1.00
WG	PV	0.99	**0.44***	0.60
WG	WG	0.99	0.89	**0.06**

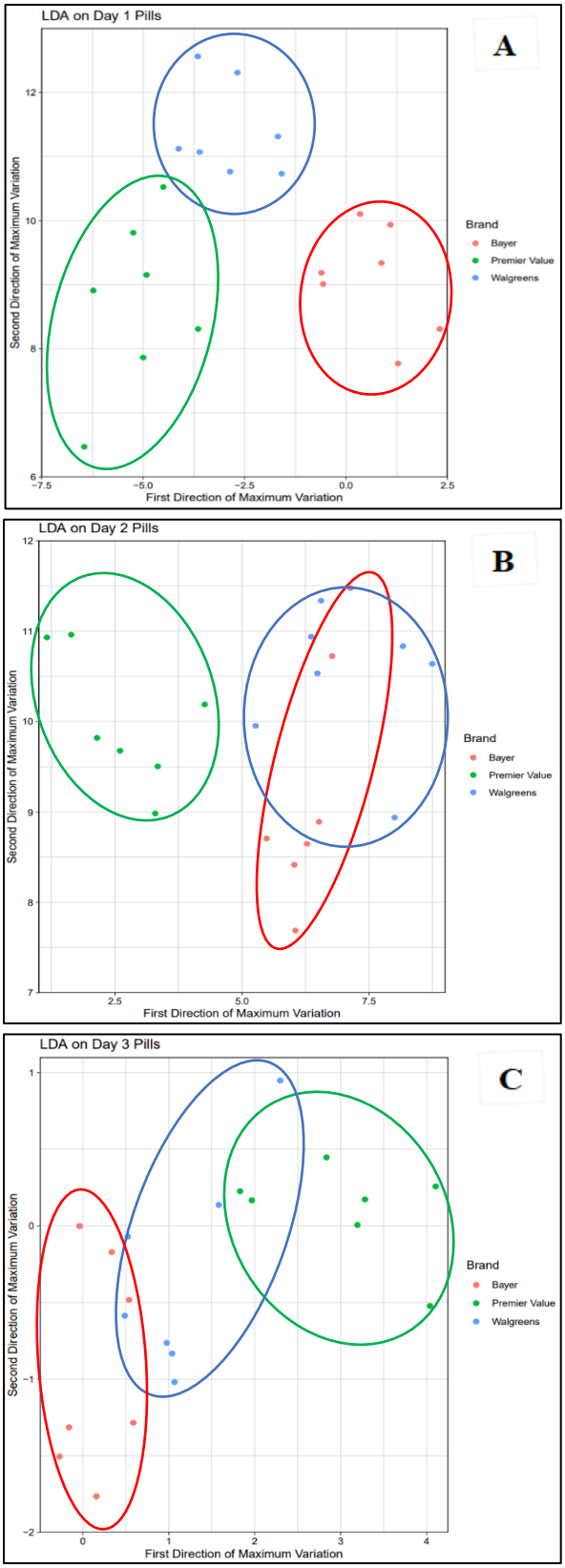


Figure 2.6 A) LDA plot of pill brands Bayer®, Premier Value®, and Walgreens© for Day 1 of the three-day study. B) LDA plot of pill brands Bayer®, Premier Value®, and Walgreens© for Day 2 of the three-day study. C) LDA plot of the pill brands Bayer®, Premier Value®, and Walgreens© for Day 3 of the three-day study.

2.3.6 Classification of Unknown Aspirin Brands

To test the applicability of the method for an "unknown", a double-blind study was implemented using an expired Equate pill brand, which was used to simulate a "counterfeit" aspirin pill. Samples (N=5) of all known and unknown brands of aspirin were double blinded for analysis. Since there was separation between aspirin pill brands within each day of analysis, but it was not consistent between days, replicates (N=7) of the known brands were analyzed on the same day as the double-blinded pills to train the statistical methods. Following analysis, retention time ranges were kept the same as the previous studies and the peaks from the double-blind and training/control samples were shifted for each respective transition but were randomized in Excel so that systematic bias would be prevented. After shifting the data for both the double-blind and training/control samples to the same retention times, LDA, QDA, and atypicality analysis were applied to the 7 (N=3) pills per brand for the known aspirin brands and to the 5 (N=3) pills per brand for the double-blinded pills. The three chemometric methods were evaluated to determine if the aspirin pill brands from the double-blinded study would classify to the respective brand and if the expired Equate would be differentiated from the "known" aspirin brands using Atypicality.

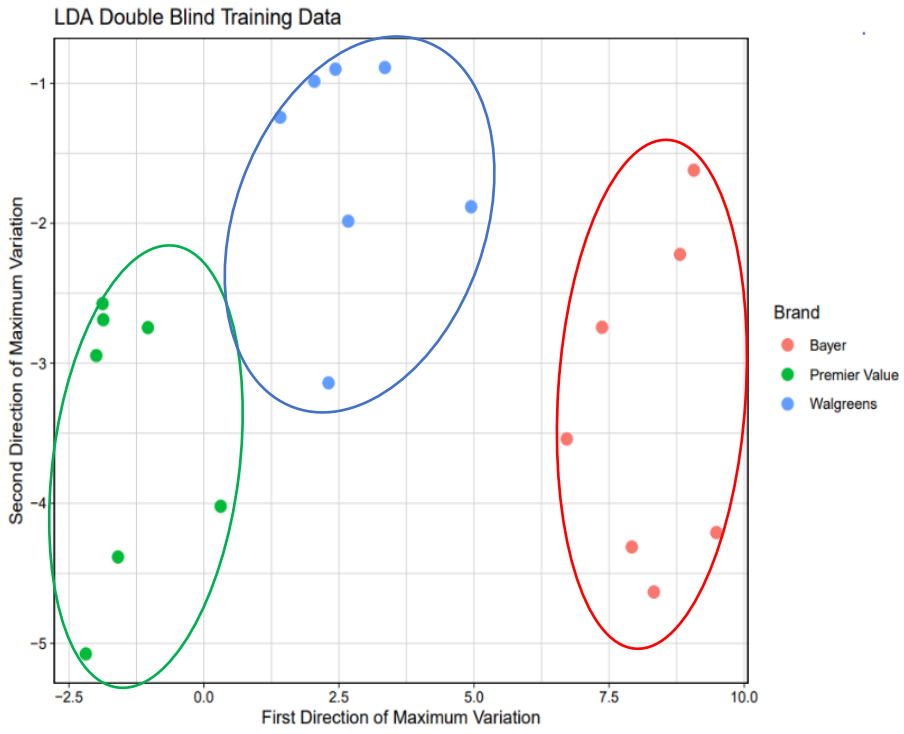
Before examining the double-blinded pills classification predictions using the three chemometric methods, the control aspirin pills were evaluated. The known aspirin pills had perfect classification using LDA (Table 2.8). In the LDA plot shown in Figure 2.7, the maximum separation for the training pill brands was mainly accomplished using LD1, while Walgreens© was mainly separated using LD2. Atypicality had the second highest correct percentage in pill classification with 85.7% for each aspirin brand. Furthermore, QDA correctly classified Bayer® pills at 85.7%, however, Walgreens© and

Premier Value® were only 57.1% correctly classified. LDA produced excellent classification for all known samples.

Table 2.8: The total correct percentage of with-in-brand classification using LDA, QDA and atypicality chemometric methods for the known training/control pills that were analyzed on the same day as the double-blinded pills.

Training/Control 7 (N=3) Pills			
	LDA	QDA	Atypicality
Bayer®	7/7 (100%)	6/7 (85.7%)	6/7 (85.7%)
Walgreens©	7/7 (100%)	4/7 (57.1%)	6/7 (85.7%)
Premier Value®	7/7 (100%)	4/7 (57.1%)	6/7 (85.7%)

Figure 2.7 LDA plot of pill brands Bayer®, Premier Value®, and Walgreens© for the training/control pills where there was perfect class prediction.



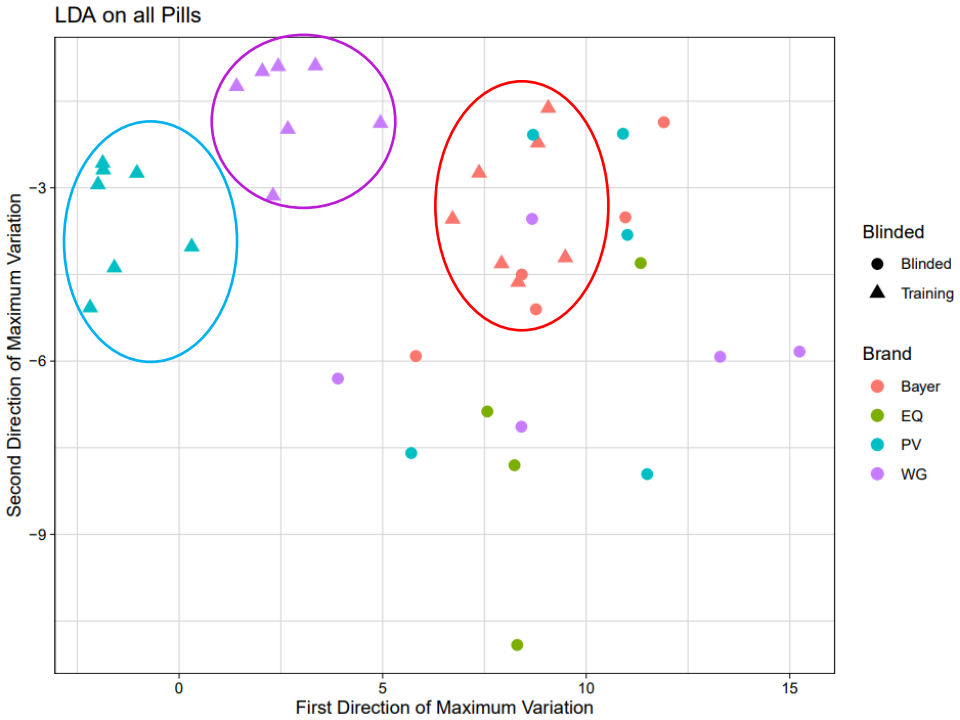
After observing the training/control aspirin samples classification trends, the double-blinded chemometrics class predictions were evaluated to determine whether the predictions were correctly classified. When using LDA to classify the double-blinded pills, as shown in Table 2.9, all pills were predicted to belong to Bayer®. When analyzing atypicality to classify the double-blinded pills, the class predictions were mainly Bayer® as well. LDA had the best classifications with 25% correct classification when compared the unblinded pills.

Table 2.9: The double-blinded pills were predicted using the three chemometric methods LDA, QDA, and Atypicality, but LDA and Atypicality predictions were noted. The four peak transitions that were used in the Day 1-3 study were used in developing the chemometric methods and classifying the pills brands. After classification predictions, the pills were unblinded to determine what pills were correctly classified.

Unblinded Pills	***LDA Predictions***	***Atypicality Predictions***
Equate 1	Bayer®	Bayer®
Premier Value® 1	Bayer®	Bayer®
Walgreens© 1	Bayer®	Bayer®
Equate 2	Bayer®	Bayer®
Equate 3	Bayer®	Bayer®
Equate 4	Bayer®	Bayer®
Equate 5	Bayer®	Bayer®
Bayer® 1	Bayer®	Bayer®
Premier Value® 2	Bayer®	Walgreens©
Walgreens© 2	Bayer®	Bayer®
Premier Value® 3	Bayer®	Bayer®
Bayer® 2	Bayer®	Bayer®
Bayer® 3	Bayer®	Bayer®
Walgreens© 3	Bayer®	Bayer®
Walgreens© 4	Bayer®	Bayer®
Walgreens© 5	Bayer®	Bayer®
Bayer® 4	Bayer®	Walgreens©
Premier Value® 4	Bayer®	Bayer®
Premier Value® 5	Bayer®	Walgreens©
Bayer® 5	Bayer®	Walgreens©

The training pills and the unblinded pills classification trends were evaluated in the LDA plot shown in Figure 2.8. In the LDA plot, the training pills had a distinct classification trend for Bayer®, Premier Value®, and Walgreens©. However, when plotting the unblinded pills on the same LDA plot, there is a not a distinct classification trend as the training pills where the pills are scattered towards the right side of the first maximum direction. After evaluating the class predictions in Table 2.9 and the classification trends of the training pills and unblinded pills on the LDA plot in Figure 2.8, LDA, QDA, and atypicality analysis were applied to two transitions 136.5/92.9 (i.e., salicylic acid) and 178.7/93 (i.e., acetylsalicylic acid) to determine if more pill brands could be correctly classified in the double-blinded study.

Figure 2.8 LDA plot of pill brands Bayer®, Premier Value®, and Walgreens© for the training/control pills compared the unblinded pills.



When using two peak transitions instead of four transitions for the three chemometric methods, as shown in Table 2.10, produced different classifications for the double blinded pills. Atypicality had the highest correct classification with 15% out of the twenty double-blinded pills where pills Walgreens© 1, Bayer® 2, and Bayer® 5 were correctly classified. Training/control data was classified perfectly using LDA, however, the pill classification was not ideal for the double-blind study and atypicality analysis was unable to be used to isolate the expired Equate pill.

To evaluate the reason for the misclassification of the double-blinded pills, the average area under the peak for each replicate per pill brand was obtained, followed by a final average of the averaged replicates; then, the final average of the pill replicates was used to compare the four transitions based on the time the known manufactures were ran on the instrument. The final average peak area per pill for the four MRM transitions in the known pill brands decreased over time. Instrument sensitivity could play a role in the pill classification. This could suggest that the samples need to be analyzed on the LC-MS/MS in a random order versus orientating the samples based on batch sequences. Also, an internal standard could be implemented to correct for loss of sample and instrument sensitivity.

Table 2.10: The double-blinded pills were predicted using the three chemometric methods LDA, QDA, and Atypicality. Two peak transitions 136.5/92.9 and 178.9/93 were used to see if there were other classification trends versus using the four MRM transition. After classification predictions, the pills were unblinded to determine what pills were correctly classified.

Unblinded Pills	***LDA Predictions***	***QDA Predictions***	***Atypicality Predictions***
Equate 1	Walgreens©	Walgreens©	Bayer®
Premier Value® 1	Walgreens©	Walgreens©	Walgreens©
Walgreens© 1	Walgreens©	Walgreens©	Walgreens©
Equate 2	Premier Value®	Premier Value®	Premier Value®
Equate 3	Premier Value®	Premier Value®	Premier Value®
Equate 4	Premier Value®	Premier Value®	Premier Value®
Equate 5	Bayer®	Bayer®	Bayer®
Bayer® 1	Walgreens©	Walgreens©	Premier Value®
Premier Value® 2	Walgreens©	Walgreens©	Walgreens©
Walgreens© 2	Premier Value®	Premier Value®	Premier Value®
Premier Value® 3	Walgreens©	Walgreens©	Bayer®
Bayer® 2	Walgreens©	Walgreens©	Bayer®
Bayer® 3	Walgreens©	Walgreens©	Walgreens©
Walgreens© 3	Premier Value®	Premier Value®	Premier Value®
Walgreens© 4	Bayer®	Bayer®	Bayer®
Walgreens© 5	Bayer®	Bayer®	Bayer®
Bayer® 4	Walgreens©	Walgreens©	Walgreens©
Premier Value® 4	Walgreens©	Walgreens©	Bayer®
Premier Value® 5	Walgreens©	Walgreens©	Walgreens©
Bayer® 5	Bayer®	Bayer®	Bayer®

2.4 Conclusion

The %RSDs for within-pill and between-pills was <15% when preparing and analyzing the known aspirin brands on the LC-MS/MS, whereas the %RSDs for between-pill analysis on the GC-MS was >15%. In addition, the pairwise plots for the pills analyzed on the LC-MS/MS had a higher correlation when comparing chromatograms versus pills analyzed on the GC-MS. The discriminating power of the three chemometric method varied, but LDA generally had the highest percentage of correct pill classification with atypicality analysis being second highest. The chemometrics that were applicated to

the known brands Walgreens©, Bayer®, and Premier Value® had discriminating power within a day, however, it was not constant between days. In the double-blinded study, the known pill training set was 100% correctly classified by LDA, and atypicality had 85.7% correct classification. However, when using the three chemometric techniques for the double-blinded data, atypicality analysis was unable to be used to isolate the expired Equate pills due to inconsistent peaks are between the training data and double-blinded pills. Due to variation of the training and double-blinded pills, it suggests that an internal standard(s) should be implemented to account for loss of sample, matrix effects, or instrument sensitivity.

3. CHAPTER 3: CONCLUSIONS AND FUTURE WORK

3.1. Conclusions

In this study, known source pill brands Walgreens©, Premier Value®, and Bayer® were compared to an "unknown" source pill brand using three chemometric methods: LDA, QDA, and atypicality. Before determining what analytical technique was best to provide a consistent chemical fingerprint for each pill brand manufacturer, the compounds were identified with the GC-MS detected 9 compounds versus 4 compounds on the LC-MS/MS. Active pharmaceuticals and excipients were initially identified from GC-MS data via the NIST library and seven of these compounds were definitively identified using commercially available standards. LC-MS/MS and GC-MS were then investigated to determine which would be best-suited for classifying Walgreens©, Premier Value®, and Bayer® aspirin pill brands. The %RSDs for two Walgreens© pills for salicylic acid (extracted ion 136.5/92.9) had <15% for within and between pill samples whereas % RSDs was >15% when comparing two pill samples. To accumulate enough data for the various pill brands, 7 pills per brand (each analyzed in triplicate) were evaluated over three separate days using LC-MS/MS coupled with three chemometric methods: LDA, QDA, and atypicality. Overall, the discriminating power of the chemometrics was good within-day, but not between days. Because there was within day chemometric separation, a double-blind study was executed to test the applicability of the method. LDA had perfect discriminating power for pill classification of known manufactures, however, it was unable to correctly classify the blinded pill brands. Also, atypicality analysis was unable to differentiate the "unknown" (i.e., expired Equate) it

from the known source pills. Due to peak area variation in the known and blinded data, an internal standard is likely necessary for this technique.

3.2 Future Work

Known aspirin pill brands Walgreens©, Bayer®, and Premier Value® chemical fingerprint is not consistent. The peak area of the known pills and double-blinded pills varied based on the time it was analyzed. This suggests that an internal standard is essential for reducing these variations. The internal standards acetylsalicylic acid-d4 and salicylic acid-d4 seem promising for optimization of the method. After optimization, the method should be further assessed.

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