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QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) Extraction – Gas Chromatography for the Analysis of Drugs

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BY

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DISSERTATION

Submitted to the Department of Chemistry and Biochemistry at Seton Hall University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 2015

We certify that we have read this dissertation and that in our opinion it is adequate to scientific scope and quality as a dissertation for the degree of Doctor of Philosophy.

APPROVED

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ABSTRACT

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) is an extraction technique developed by Anastassiades and co-workers initially for the extraction of veterinary drugs from animal tissue. Since its inception, it was discovered that this method is particularly adept for the extraction of polar and basic compounds, thus the majority of research previously performed using QuEChERS involves the extraction of pesticides from various matrices, especially fruits, vegetables, and other food products. Combining a liquid-liquid extraction (LLE) and a dispersive solid phase extraction (d-SPE) clean up, QuEChERS provides a clean sample for analysis by gas chromatography (GC) or liquid chromatography (LC) and is commonly used for samples with complex matrices.

The goal of this work was to expand the research that has been previously performed using QuEChERS, as reviewed in Chapter 1, particularly in the area of GC analysis by exploring both the fundamental chemistry involved in the method as well as expanding its applications. The first original portion of the research performed involved investigating the chemistry in QuEChERS using a model study, the extraction of caffeine from tea, and is detailed in Chapter 2. QuEChERS parameters have been optimized in the past but those optimized parameters have yet to be studied in depth to provide a deeper understanding of the chemistry involved in both the LLE and d-SPE portions of the method. Study parameters such as pH, salt amount and type, solvent amount and type, temperature, extraction kinetics, and the partition coefficient of the final optimized method were evaluated. The findings from studying these parameters were then applied to several original applications performed during the entirety of this research. Analytical

figures of merit were determined for the method during validation as was percent recovery. A percent recovery greater than 95% and an intra and interday %RSD less than 6% and 12%, respectively, illustrated a successful and reproducible extraction for caffeine from tea using QuEChERS.

The applications of QuEChERS investigated during the course of this research include: the extraction of glucocorticoids from water and herbal medicinal products using gas chromatography triple quadrupole mass spectrometry (GC-MS/MS) and high performance liquid chromatography (HPLC), the extraction of hormones from water using GC-MS/MS and GCxGC-TOFMS including a comparison of QuEChERS to solid phase microextraction (SPME) using GC-MS/MS, and finally the extraction of drugs of abuse from synthetic urine.

The first two QuEChERS applications involved original work for the analysis of two classes of steroids in herbal medicinal products (HMPs) via gas chromatography triple quadrupole mass spectrometry (GC-MS/MS). The first class of steroids investigated were 8 glucocorticoids studied for their possible adulteration in herbal medicines which target joints due to their ability to reduce inflammation. Three main QuEChERS parameters were optimized including pH, salt amount, and solvent type for these compounds in water. The optimized QuEChERS and GC-MS/MS methods were then applied to real herbal medicines for these 8 steroids. Method validation was performed including percent recovery and partition coefficients for each of these steroids from water. The second class of steroids investigated was 7 hormones including estrone and

estradiol. In this application, the optimized QuEChERS method from use with the glucocorticoids was used to investigate the presence of these compounds in herbal medicines as well. The analysis of both steroid classes in HMPs using GC-MS/MS has not been performed in the literature and thus is an addition to the work that has been performed using QuEChERS-GC. The method development for both sets of steroids can be found in Chapter 3.

A third and fourth application involved use of the 7 hormones from the previous study. In the third application discussed in Chapter 4, the QuEChERS method was briefly compared to an optimized solid phase microextraction (SPME) method in which the fiber type, sample preparation parameters, and SPME extraction time were optimized. Samples containing the hormones of interest were prepared using the same concentration for both the QuEChERS and SPME methods. The optimized methods were then applied to each sample and the resulting peak areas were compared to determine the extraction ability of these methods against each other. A comparison between QuEChERS and SPME has not been performed in the literature and is thus a third original contribution to the literature using QuEChERS.

The fourth application involved the pairing of the original current work using SPME and GC-MS/MS with the work performed by a former student using SPME and comprehensive two-dimensional gas chromatography time of flight mass spectrometry (GCxGC-TOFMS) for these hormones. A comparison of these two instruments is

included in Chapter 5 of this thesis as well as the resulting chromatograms for the analysis of these compounds.

A fifth application study using QuEChERS was performed using the first set of 8 glucocorticoids and LC. It was attempted to reproduce and improve a previously published high performance liquid chromatography (HPLC) method for the analysis of these 8 steroids to determine the method reproducibility and ruggedness. It was interesting to find that the gradient method reported was not reproducible and provided limited separation of the steroids on our instrumentation; however, an isocratic method achieved separation for all of the steroids of interest using both a similar length column as described by the published article as well as a shorter, more efficient column in which total elution time was reduced from 25 minutes to 10 minutes. The method optimization is discussed in detail in Chapter 6 of this thesis.

A final original application of QuEChERS involved the extraction of drugs of abuse from synthetic urine. There has been some work performed for the extraction of drugs of abuse from blood using QuEChERS with both LC and GC; however, the extraction of these drugs of abuse from urine using QuEChERS-GC has yet to be published. This study was performed using both GC-MS/MS and GC-MS-SIM and is being completed by an undergraduate student at Seton Hall, Leanne Mocniak, whom I was mentoring during my time as a graduate student. She will be finishing a comparison of the QuEChERS method for the extraction for these drugs from synthetic urine to both ionic liquid single drop microextraction (IL-SDME) and solid phase microextraction (SPME). She will also

be evaluating the effects of derivatization and non-derivatization on the recovery of the analytes of interest. Chapter 7 of this thesis discusses the method optimization completed thus far as well as a brief description of future work to be performed.

The end of Chapter 7 includes a brief look at future work that can be performed using QuEChERS as well as future uses of the method. The possible uses of QuEChERS-GC are truly unlimited as many of the analytes analyzed using QuEChERS-LC are amenable to QuEChERS-GC. Thus the applications of QuEChERS-GC can include those previously employed using LC as well as fields of study yet to be fully explored using QuEChERS in general including forensic samples.

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CHAPTER 1 – AN INTRODUCTION TO THE THEORY OF QUECHERS AND SEPARATION VIA GAS CHROMATOGRAPHY

This chapter briefly summarizes the various research performed in the literature using QuEChERS, focusing on areas using gas chromatography for instrumental analysis as well as the theory behind gas chromatography mass spectrometry. The theory of QuEChERS and method optimization are discussed and provide the knowledge necessary for future chapters in which the research performed will be discussed. As the analysis of pesticides dominates the literature for both liquid chromatography (LC) and gas chromatography (GC) with QuEChERS, the analysis of these compounds will be briefly explored in this chapter; however, particular attention will be paid to less common types of analysis and what will hopefully be the future of QuEChERS, including the analysis of drugs, organic contaminants, mycotoxins, and a few novel methods.

1. QuEChERS Introduction

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) offers several advantages over other extractions in that it is quickly, easily, and safely performed, as its name implies, through the use of non-halogenated solvents and simple methodology. Anastassiades developed the QuEChERS method while performing postdoctoral research under Lehotay. The method was originally designed for the extraction of veterinary drugs from animal tissues; however, its success with basic, polar compounds was quickly realized and the method was adopted for pesticide extraction in plant material [1].

Anastassiades and Lehotay presented the method in 2002 in Rome at the European Pesticide Residues Workshop, and the first publication of QuEChERS providing a detailed method was in 2003 by Anastassiades, Lehotay, Stajnbaher, and Schenck for pesticide residues [2]. Since its inception, QuEChERS has evolved to include buffering salts to increase recovery of analytes that are pH dependent in the AOAC 2007.01 method [3] as well as the use of various forms of buffering salts including citrates in the European Standard EN 15662 method in order to expand the working range of QuEChERS [4]. Not only have the methods evolved over the years, but also the matrices in which QuEChERS is applied have expanded. The various techniques of QuEChERS and gas chromatography and how it has evolved are the topics of discussion for this chapter.

Initially, QuEChERS demonstrated increased recovery and reproducibility compared to previous methods including classic multi-residue methods for analytes such as pesticides residues extracted from food products. QuEChERS has been used for pesticides to such an extent that there are now over 650 pesticides and metabolites present in the EURL-datapool webpage for validation data of QuEChERS methods [5]. QuEChERS is an ideal technique for extracting complex matrices, as it combines a liquid-liquid extraction (LLE) and a dispersive solid phase extraction (d-SPE), allowing for removal of matrix interferences and generating clean samples. Agricultural products can have quite complex matrices and thus QuEChERS is well suited to the extraction of analytes from these matrices. Pesticide, insecticide, fungicide, and herbicide extraction from food products, drinks, and soil using QuEChERS dominates the current literature for analysis

with both gas chromatography and liquid chromatography, with the latter being more prevalent [1], [6], [7], [8].

QuEChERS has been used for several compounds and matrices other than pesticide extraction from agricultural products. Research has been performed regarding the extraction of mycotoxins and organic contaminants, such as volatile organic compounds (VOCs) and polycyclic aromatic hydrocarbons (PAHs) in matrices including food and drink, animals, sewage and water treatment sludge, breast milk, and baby formula [9], [10], [11]. Another area of use for QuEChERS involves veterinary drug extraction from animal products, urine, and soil, though this area primarily uses liquid chromatography (LC) for analysis [12]. Pharmaceutical drugs such as steroids, hormones, and acetaminophen have been extracted from sewage and water treatment sludge, soil, dietary supplements, livestock, and biological matrices [13], [14]. Though the analysis of biological matrices has been explored with QuEChERS for these compounds, QuEChERS has yet to be fully investigated for the analysis of drugs of abuse in matrices such as urine, blood, and hair. The success of QuEChERS for the use of veterinary and pharmaceutical drug extraction from similar matrices indicates that QuEChERS-GC lends itself well to the analysis of drugs in forensic and pharmaceutical samples.

The aforementioned areas of QuEChERS applications be discussed, as well as the use of instrumental analysis after extraction, with emphasis on those that are most commonly used in analysis of forensic drug samples, gas chromatography. Most of the current literature involves analysis with liquid chromatography, especially the analysis of

veterinary drugs. Conversely, the field of forensics is primarily gas chromatography based and thus this chapter will mainly address the use of QuEChERS and gas chromatography for sample analysis.

1.1. QuEChERS Theory and Methodology

The QuEChERS method involves two main steps: a liquid-liquid extraction and a dispersive solid phase extraction clean up step. In the first step, an organic solvent such as acetonitrile (ACN) is used to extract an aqueous-based sample in which salts are used to separate the aqueous and acetonitrile phases, as they are miscible. In the second step, a d-SPE sorbent is added to bind unwanted compounds, providing a cleaner sample for analysis. As mentioned above there are three commonly used methods that have given rise to all of the current QuEChERS methods: the original method, the AOAC 2007.01 method, and finally the European Standard EN 15662 method [8]. The basic steps for each method are the same, a LLE between an organic phase and water with the use of salts for liquid-liquid partitioning. The sample is shaken then centrifuged and an aliquot of the organic extract is removed and subjected to a d-SPE clean up using magnesium sulfate ($MgSO_4$) and a sorbent that will bind matrix interferences such as primary secondary amine (PSA) as pictured in Figure 1-1. The sample can then be analyzed using GC or LC. The fundamental chemistry involved in the parameters of both the LLE and d-SPE steps including the effect of adding salts in the LLE as well as different solvents will be discussed below and differences between the three aforementioned methods will be outlined as seen in Figure 1-2.

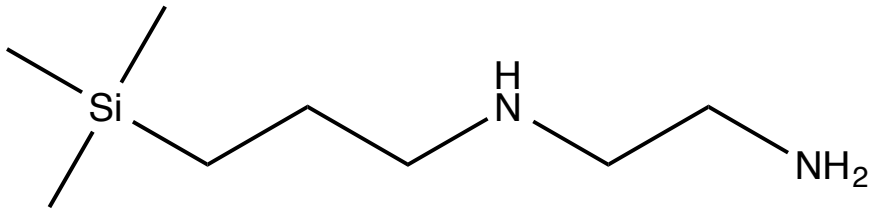


Figure 1-1. Structure of endcapped primary secondary amine (PSA) solid phase extraction (SPE) bulk packing.

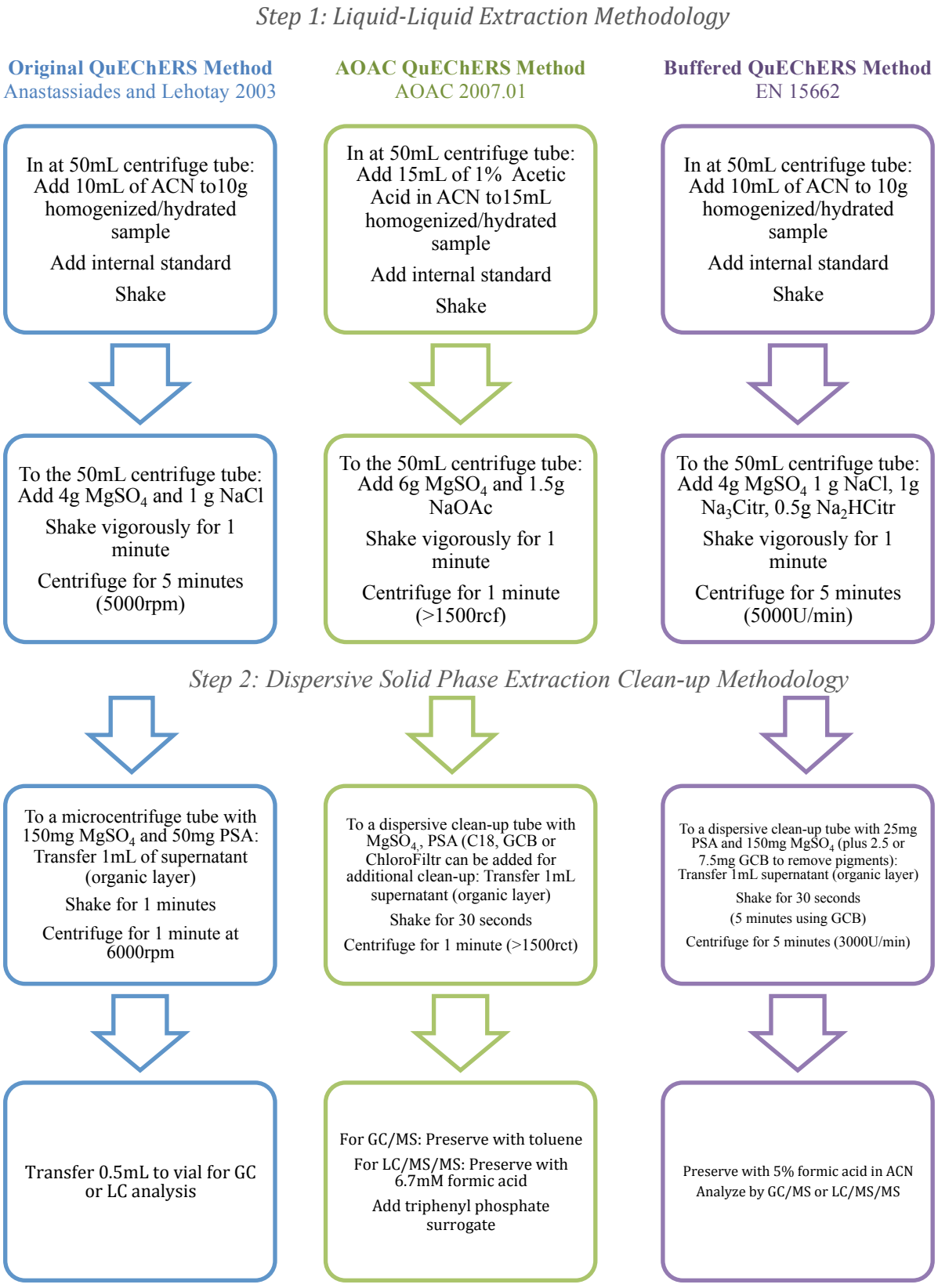


Figure 1-2. Methodology of the LLE and clean up step for the original, AOAC 2007.01, and EN 15662 QuEChERS methods. Adapted from The UCT Pesticide Residue Analysis QuEChERS Information Booklet [8].

During the LLE, acetonitrile, ethyl acetate, or acetone are the three organic solvents most commonly used during QuEChERS, as they are safer than chlorinated solvents. ACN is the most frequently used organic solvent of the three as it minimizes the amount of interferences extracted while also extracting a broad range of analytes. The LLE also includes the use of salts to drive the analyte of interest into the organic solvent as well as aid in phase separation. Sodium chloride (NaCl) and magnesium sulfate ($MgSO_4$) are used in the original and European methods, with the latter also using citrate buffering salts including sodium citrate dibasic sesquihydrate ($Na_2HCitr\text{-}1.5H_2O$) and sodium citrate tribasic dehydrate ($Na_3Citr\text{-}2H_2O$) [2], [4]. Salts aid in the partitioning of polar compounds by increasing the ionic strength that can result in salting out or salting in depending on the properties of the compound and solvents. Adding salt can increase the polarity of a solvent, thus increasing the solubility of the polar compounds in that solvent. The goal is to increase solubility into the organic layer. During the LLE, sodium chloride decreases the amount of polar interferences extracted, allowing for better selectivity for the compound of interest. Magnesium sulfate works to improve polar analyte recovery and aids in solvent partitioning during the LLE. Most publications cite $MgSO_4$ and NaCl in a 4:1 ratio; however, different salts such as $CaCl_2$ can be used which may better serve in the extraction process. Other salts and buffers can also be employed as seen in Figure 1-1, depending upon the analytes of interest. The AOAC method uses $MgSO_4$ and sodium acetate (NaAc) rather than NaCl as well as ACN with 1% acetic acid in order to buffer the system for base-sensitive problematic pesticides such as folpet, dichlofuanid, and pymetrozine [3]. It is important to understand the effects of these salts on the

partitioning process so one can choose the proper solvent and salts for the optimal extraction of the analyte(s).

The d-SPE step is similar in all three methods: a clean up sorbent such as primary secondary amine (PSA) removes polar matrix interferences such as sugars, fatty and organic acids, and some pigments, and $MgSO_4$ is added to act as a desiccate, removing any water transferred with the organic phase [2], [3], [4]. An aliquot of the liquid is then transferred to a vial for analysis. In the European method it is suggested to acidify the extract with formic acid once QuEChERS is complete in order to improve the storage conditions for base-sensitive pesticides [4]. The sorbent chosen can be optimized based upon the analytes of interest to provide the cleanest sample. Each sorbent removes specific interferences to provide a cleaner sample depending on the composition of the matrix. Some examples include: primary secondary amine (PSA) which decreases levels of organic acids and lipids based upon weak ion exchange, endcapped C18 where residual silanols of the sorbent have been reacted with reagent so they are no longer active which removes lipids and non-polar interferences, graphitized carbon black (GCB) which works to bind planar analytes and lower the recovery of pigments, and aminopropyl which is similar to PSA but has less affect on base sensitive analytes, providing higher recovery of those analytes. Dual phase sorbents that combine two or more of the aforementioned sorbents are also of interest, especially for specifying the removal of certain compounds. For example, a dual phase sorbent of GCB/PSA removes pigments while helping to retain planar analytes whereas GCB alone would remove the latter. The choice of sorbent would depend on the matrix of the sample as well as the

composition of the analytes of interest. The optimized result would provide high recovery and remove matrix interference peaks.

It should be noted that though the original publications for each of the three methods have specific amounts of reagents that were used, it is important to optimize each step in the QuEChERS method for the compounds under investigation as the optimal parameters may vary from those pesticides that were analyzed in the original publications. For instance, the solvent system, amounts and types of salts used, and choice of d-SPE sorbent should all be optimized, with the ultimate goal of increased recovery and removal of matrix interferences [1], [6], [7], [8].

For example, optimization of a QuEChERS method for the extraction of several basic drugs of abuse and their metabolites from whole blood samples was performed by Matsuta and co-workers [14]. In Matsuta's work, the dehydrating reagent, organic solvent, pH, and adsorbent type were optimized. In the first step of QuEChERS, inorganic salts are used to aid in layer separation between the organic and aqueous phase and drive the analyte into the organic phase. Here, $MgSO_4$ and Na_2SO_4 were examined due to their solubility and neutral pH in water. Here, $MgSO_4$ provided easier collection of the organic phase with an optimized amount of 100mg (larger amounts decreased the organic layer volume obtained). Four organic solvents were investigated: chloroform, chloroform-isopropyl alcohol (3:1 v/v), ethyl acetate, and acetonitrile. The two chloroform-based solvents formed emulsions consisting of an aqueous layer that persisted on the surface of the organic layer making recovery of the organic layer difficult, while

acetonitrile and ethyl acetate provided better phase separation. Acetonitrile provided the best recovery so it was used for further studies. The pH was adjusted to 5 and 9 by using 0.2% acetic acid in acetonitrile and 5mg of Na_2CO_3, respectively. Only one metabolite, a metabolite of zolpidem, showed significant pH effects, though an acidic pH (pH 5) provided better recoveries for all compounds than neutral or basic conditions as well as decreasing loss of volatile amine drugs present in the sample. The final optimized parameter was the adsorbent. The adsorbent was added prior to the LLE in order to scavenge cholesterol that was interfering in the gas chromatographic analysis with triazolam. Sorbents C_2, C_{18}, CH, and EC (Envi-Carb) were investigated and it was found that EC scavenged 90% of the cholesterol and was chosen as the optimal sorbent. A portion of this study that should be included in all QuEChERS studies for new applications was the comparison of the QuEChERS method to LLE or another traditional extraction method commonly used for these analytes and matrix. The LLE consisted of chloroform-isopropyl alcohol (3:1 v/v) at pH 4.5 and 9. QuEChERS was shown to provide better recoveries for all analytes over the conventional LLE method, thus demonstrating the utility of QuEChERS for this application [14].

It is well known that QuEChERS methods increase recoveries for very polar, basic pesticides: compounds that can be problematic when using other multi-residue methods. Average recoveries of 95% and a repeatability of less than 5% in conjunction with a rapid and cost effective method (less than 30min and $1 per sample) caused the method to become the most commonly used sample preparation method for pesticide residues worldwide. Now that more research has been performed, the use of QuEChERS has

expanded, allowing for its application to more areas providing an easier, safer, more cost effective sample preparation alternative for many different compounds.

Not only is it of interest in this current work to use the QuEChERS method for sample preparation, but it is also of interest to improve upon the most current QuEChERS method by investigating the theory as it pertains to such items as the kinetics and thermodynamics involved in the partitioning during the liquid extraction step between the analyte, solvents, and salts as well as the mechanism occurring in the d-SPE step in order to provide the most efficient extraction possible. This will involve understanding the theory involved in extraction equilibrium. The partition coefficient (K_c) involved during equilibrium is the distribution constant (K_D) and is defined as:

$$K_c = \frac{[A]_{solvent}}{[A]_{matrix}} \equiv K_D \qquad \text{(Equation 1-1)}$$

For an extraction, it is desired that the distribution constant be greater than one so as to have a majority of the analyte in the extraction solvent [15], [16]. The extraction is also effected by type of solvent, temperature, and pH used during the process. The pH of the aqueous sample phase must be adjusted to provide the non-ionized form of the analyte of interest in order to maximize extraction. Thus, in order to optimize K_c, several experiments will have to be performed in which the solvent, pH, and temperature of the extraction are optimized.

The temperature of the system is related to thermodynamics in that it affects the spontaneity of the process. The reaction quotient, Q, and Gibbs Free Energy, ΔG, are temperature dependent as seen below:

$$\Delta G = -RTlnQ \quad \text{(Equation 1-2)}$$

$$Q = exp\frac{\Delta G}{RT} \quad \text{(Equation 1-3)}$$

Here, Q is of interest as it reflects the completeness of an extraction, similar to K_D, and thus Equation 1-2 has been rearranged to Equation 1-3. The reaction quotient, Q, is inversely proportional to temperature as seen in Equation 1-3. As temperature decreases, Q will be greater than 1, indicating a larger concentration of the analyte of interest present in the extracting medium. This will provide a more negative ΔG indicating that the extraction is more favorable [16]. The affect of temperature on the extraction steps involved in the QuEChERS method will be explored in Chapter 2 for the extraction of caffeine from tea and water.

Kinetics is also involved in the extraction process. The application of agitation to the sample will provide faster kinetics resulting in a faster extraction since the amount of analyte dissolved over time is increased. Ultrasonication-assisted extraction uses ultrasonic vibrations for agitation resulting in a release of the analyte from the matrix into the extraction solvent. The solvent system must also be considered during an extraction in order to maximize the extraction. Solubility is affected by polarity, which can affect the Van der Waals interactions occurring during extraction. When the polarities of the

analyte and solvent are similar, this causes the strength of the Van der Waals interactions to dominate other intermolecular interactions such as dipole-dipole and hydrogen bonding, resulting in greater solubility [16]. All of these factors play a role in the extraction process and must be considered here. This will be accomplished through optimization of the various parameters as stated above. By evaluating the solvent type, pH, temperature, and agitation of the system, the results will provide information on the interactions occurring as well as the thermodynamic and kinetic properties of the system.

1.2. Current Applications of QuEChERS

QuEChERS offers several advantages over other extractions in that it is quickly, easily, and safely performed, as its name implies, through the use of non-halogenated solvents and simple methodology. QuEChERS also has demonstrated increased recovery and reproducibility compared to previous methods for analytes such as pesticides when extracted from food products. QuEChERS is an ideal technique when working with complex matrices as it combines a liquid-liquid extraction and a dispersive solid phase extraction (d-SPE), allowing for removal of matrix interferences, providing a clean sample. Agricultural products can have quite complex matrices and thus the extraction of analytes from this matrix is well suited to QuEChERS. Pesticide, insecticide, fungicide, and herbicide extraction from food products, drinks, and soil using QuEChERS dominates the current literature for both analysis with gas chromatography and liquid chromatography; with the latter being more prevalent [1], [2], [3], [4]. There has been a multitude of research performed on the use of QuEChERS for the extraction of

pesticides, fungicides, insecticides, and herbicides for various matrices [17]. A brief summary will be discussed in order to expose the reader to this area of QuEChERS, followed by a more in depth analysis of other applications of QuEChERS including both GC and LC analyses.

Pesticide residues are the primary interest as it pertains to QuEChERS and their detection in mostly food-based products including nutraceuticals due to its larger extraction range for pesticides in plant based matrices [17], [18]. One study of interest evaluated the use of the three aforementioned main QuEChERS methods, the original unbuffered method, the AOAC 2007.01 acetate buffering method, and the EN 1556 citrate buffering method. This study looked at the extraction of 32 pesticides in fruits and vegetables using both GC and LC/MS and found that, in general, the three methods provided similar results for all matrices resulting in an overall recovery of 98% and residual standard deviations less than 10%. It was found that the original unbuffered method did provide slightly lower recoveries for pH dependent pesticides with the acetate buffering AOAC 2007.01 method providing the most consistent and highest recovery for these compounds including pymetrozine and thiabendazole. The use of ethyl acetate was also investigated in this study and gave lower recoveries as compared to acetonitrile [19].

In comparing ultrasonic extraction (USE), pressurized liquid extraction (PLE), the European Norm Din 12393 method (acetone extraction with ethyl acetate and cyclohexane partitioning followed by gel permeation chromatography clean up), and QuEChERS, a study found that QuEChERS and PLE were the only two methods able to

recover all 24 pesticides using GC/MS. This study also determined that QuEChERS was the most efficient extraction, providing recoveries of 27.3-120.9% [20]. The effect of washing and cooking foods was evaluated for the removal of pesticides. During this study, 31 foods and 44 pesticides were monitored using QuEChERS and LC-MS/MS before washing, after washing, and after processing such as boiling. It was concluded that levels of the pesticides did decrease significantly or were eliminated after washing and cooking with the exception of green chilies. The level of acetamiprid actually increased during boiling and stir-frying of green chilies [21].

Herbicide, fungicide, and insecticide residue analysis in food products or soil is also commonly performed using QuEChERS. Various forms of the QuEChERS method have been investigated for the extraction of herbicides in polished rice, yogurt, milk, and soil [22-27]. In particular one study combined the liquid-liquid extraction and clean up step of QuEChERS in one step for the extraction of 5 herbicides in soil resulting in recoveries of 74.5-98.5% and RSD values form 3.2-11.8%. Baby food was analyzed for 10 fungicides using QuEChERS and LC-ion trap-MS/MS. The analysis of real samples showed the presence of some of the fungicides investigated, but at a level below 10μg/kg, the cutoff level designated by the European Commission Directives [24]. One final study evaluated the detection of insecticides in banana leaves used to feed cattle and hogs and was performed using QuEChERS and GC-MS/MS with 89-104% recoveries and less than 9.1% RSD [25].

The use of QuEChERS for the analysis for these compounds has been performed using both GC and LC, as seen from the brief summary above. These compounds contain many similar components and functional groups in compounds that have not been thoroughly investigated using QuEChERS, such as the analysis of drugs in forensic applications. QuEChERS has also been used for various compounds and matrices other than pesticide extraction from agricultural products. There has been some research performed regarding the extraction of mycotoxins and organic contaminants such as volatile organic compounds (VOCs) and polycyclic aromatic hydrocarbons (PAHs) in matrices including food and drink, animals, sewage and water treatment sludge, and breast milk/formula [9], [10], [11]. Another area of use for QuEChERS involves veterinary drug extraction from animal products, urine, and soil, though this area primarily uses liquid chromatography (LC) for analysis [12], [28]. Pharmaceutical drugs such as steroids, hormones, and acetaminophen have been extracted from sewage and water treatment sludge, soil, dietary supplements, livestock, and biological matrices [28], [29]. Though the analysis of biological matrices has been explored with QuEChERS for these compounds, QuEChERS has yet to be fully investigated for the analysis of drugs of abuse in matrices such as urine, blood, and hair. The success of QuEChERS for the use of veterinary and pharmaceutical drug extraction from these same matrices indicates that QuEChERS-GC would lend itself very well to analysis of drugs in forensic and pharmaceutical samples.

Not only will the aforementioned areas of QuEChERS application be discussed, but also will the use of instrumental analysis after extraction, with emphasis on gas

chromatographic analysis. Most of the current literature involves analysis with liquid chromatography, especially the analysis of veterinary drugs. There are many analytical techniques published using QuEChERS as the sample preparation method. Ultra high performance liquid chromatography-electrospray tandem mass spectrometry (UPLC/MS/MS) gas chromatography tandem mass spectrometry (GC/MS/MS), and GCxGC-TOFMS are examples of the more sophisticated systems used for the analysis of tea for pesticides, providing more sensitive and selective techniques [30]. QuEChERS has also been used for the extraction of ibuprophen and its metabolites from soil, followed by liquid chromatography with fluorescence detection [28]. It is the success of these instruments, in particular GC-MS/MS and GCxGC-TOFMS, with pesticide analysis in complex matrices that lends its use in this research [31].

1.3. QuEChERS and Gas Chromatography

There have been three main areas of study for the use of QuEChERS with GC excluding pesticide analysis: drug analysis, environmental studies concerning contaminants, and mycotoxins. These three areas will be addressed in turn below with emphasis on QuEChERS methodology, analytical figures of merit such as percent recovery and detection and quantitation limits, and connections of these areas to the application of QuEChERS to forensic samples.

1.3.1 Drugs

Various drugs have been extracted from biological matrices using QuEChERS including pharmaceuticals, antibacterial agents, and a few drugs of forensic interest [14], [31-35].

A summary of the results can be seen in Table 1-1. The work performed in this area in particular provides substantial evidence for the potential of QuEChERS-GC in drug analysis. However, this literature for QuEChERS is sparse for gas chromatography analysis and forensic-type samples.

All the research performed in this area with GC analysis included the same basic QuEChERS LLE method with the use of NaCl and $MgSO_4$ as extraction salts and ACN as the solvent with varying amounts of acetic acid depending upon the matrix. Among drug analysis methods, the d-SPE steps varied for the sorbent used, including primary secondary amine (PSA) for pharmaceuticals in whole blood [33] or graphitized carbon black (GCB) for benzodiazepines in blood and urine [34], and sometimes in combination including C_{18} and GCB for drugs of forensic interest [14], C_{18} and PSA for analysis of nevirapine [32], and PSA and Florisil for triclosan and methyltriclosan in fish roe and surimi [35]. The research performed with forensic drugs in whole blood did not use a d-SPE step but instead employed solid phase extraction (SPE) [14]. It would be useful to apply the entire QuEChERS procedure to blood and compare the results to those from SPE to determine if the use of loose sorbent increases efficiency at binding matrix interferences and increasing recovery.

In addition, a method similar to the d-SPE step in QuEChERS, matrix solid-phase dispersion (MSPD), has also been applied to drug residues including veterinary drugs, antibacterials, hormones, and drugs of abuse from biological matrices, including hair by GC-MS and the application of new sorbents such as multi-walled carbon nanotubes

Table 1-1. Summary of results from the literature involving QuEChERS and GC-MS for various drugs.

Drug(s) of Interest [reference]	Matrix	LLE	d-SPE	Recovery (%)	Instrument	LOD/LOQ
Nevirapine (NVP) [32]	Human Plasma	1.5mL NVP 1.5mL plasma 4.0mL ACN (0.1%HAc) 2.6g salts	Entire extract C_{18} and PSA 150mg $MgSO_4$	83%	GC-MS EI – full scan EI – SIM PCI – full scan PCI - SIM	LOD: 11.1-29.8µg/L LOQ: 16.5-66.7µg/L (Helium carrier gas PCI – SIM had the best sensitivity)
Drugs of forensic interest [33]	Whole blood	100µL whole blood 50mg NaCl 100mg $MgSO_4$ 500µL ACN (0.2% HAc)	N/A SPE was used with C_{18} and GCB	59-93%	GC-MS	LOD: 0.01-0.1µg/mL
40 pharmaceutical drugs [14]	Whole blood	1000µL whole blood 10µL I.S. 2000µL ACN 250mg NaCl 500mg $MgSO_4$	1000µL extract 25mg PSA 25mg $MgSO_4$	Above 80%	GC-MS	LOD: 5.6-17.2ng/mL LOQ: 11.3-39.0ng/mL (For 8 model analytes) Overall <20ng/mL
Benzodiazepines [34]	Blood and Urine	10mL sample 10mL ACN 1g NaCL 4g $MgSO_4$	1mL extract 0.025g GCB 0.150g $MgSO_4$	---	GC-MS	---
Triclosan (TCS) and Methyltriclosan (MTCS) [35]	Fish Roe and Surimi	50mg sample 1mL ACN 0.5mL H_2O 50mg NaCl 150mg $MgSO_4$	Entire extract 50mg PSA 50mg C_{18} 50mg Florisil 150mg $MgSO_4$ Derivatization necessary	97% or above	GC-MS	*TCS surimi; fish roe* LOD: 2.0ng/g; 4.0ng/g LOQ: 6.6ng/g; 13ng/g *MTCS surimi; fish roe* LOD: 2.1ng/g; 5.6ng/g LOQ: .72ng/g; 18ng/g

(MWCNTs) [36]. The success of this method as well as the aforementioned studies using QuEChERS support the viability of QuEChERS in the extraction of drugs of abuse from these matrices and in combination with GC-MS.

There are numerous QuEChERS methods that have been performed using LC and LC-MS for drugs that could be GC amenable as well. A few examples include the analysis of steroids such as beclomethasone, cortisone acetate, hydrocortisone, dexamethasone, and methylprednisolone in adulterated herbal medicinal products using HPLC [27]; the investigation of pharmaceuticals and hormones in sewage sludge using LC time-of-flight mass spectrometry (acetaminophen, androstenone, caffeine, codeine, diazepam, ibuprofen, ketoprofen, lorazepam, mestranol, oxycodone, prednisolone, and progesterone) [37]; banned veterinary drugs such as boldenone, alpha-testosterone, naproxen, and betamethasone in urine using LC/MS [12]; and the analysis of pharmaceuticals and plant toxins/secondary metabolites in herbal dietary supplements using LC/MS (amphetamine, phentermine, tadalafil, ephedrine, methamphetamine, and warfarin) [38]. All of these methods used GC-amenable compounds and thus could be analyzed using QuEChERS-GC in addition to QuEChERS-LC.

1.3.2. Novel Applications of QuEChERS for Drugs and Similar Compounds

The use of QuEChERS has been expanding recently to include novel applications for drug analysis and compounds with similar structural components including pesticides, herbicides, and fungicides, some of which can be seen summarized in Table 1-2 [18]. Methods for pesticides should be translatable to the analysis of drugs. The evaluation of

new sorbents for the d-SPE step is perhaps the most prevalent novel QuEChERS application and is the focus of this section.

Pesticides, pharmaceuticals, and personal care products (PCPs) have all been investigated using innovative sorbents such as chitin, zirconium dioxide, multi-walled carbon nanotubes (MWCNTs), and magnetic nanoparticles (MNPs) in both food and drink matrices [39], [40], [41], [42], [43]. Chitin is very abundant and is found in shrimp shell waste. It was used as the d-SPE sorbet in the investigation of contaminants in drinking water treatment sludge and demonstrated higher recoveries for pharmaceuticals than C_{18}, PSA, and C_{18}/PSA sorbents using LC analysis, though once again this method could be easily transferred to a QuEChERS-GC method as it analyzes GC amenable compounds (atrazine, bisphenol A, caffeine, ibuprophen, methylparaben and polyparaben) [39]. Another study was able to decrease matrix effects and fatty interferences in pesticide extraction from avocado and almonds by using a zirconium dioxide based sorbent [40].

Multi-walled carbon nanotubes (MWCNTs) is another sorbent that is of interest due to the large surface area and unique structure that allows increased adsorption ability compared to other sorbents. MWCNTs have been successfully implemented in SPE and this allowed for its transition into use as a d-SPE sorbent for QuEChERS for the extraction of pesticides from vegetables, fruits, and tea [41], [42]. A final sorbent which is new to QuEChERS is magnetic nanoparticles (MNPs) in conjunction with GCB and PSA where the supernatant of the extraction is collected using an external magnet in extracting pesticides from vegetables [43].

Table 1-2. Summary of results from the literature involving QuEChERS and GC-MS using novel sorbents for d-SPE clean-up.

Analyte(s) of interest [reference]	**Matrix**	**LLE**	**d-SPE**	**Recovery (%)**	**Instrument**	**LOD/LOQ**
Pesticides, Pharmaceuticals, and Personal Care Products (PCPs) [39]	Drinking water treatment sludge	10g sludge 10mL ACN 100µL HAc 4g $MgSO_4$ 1g NaCl	2mL extract 50mg chitin 150mg $MgSO_4$	50-120%	LC-MS/MS	LOQ; 1-50µg/kg
170 Pesticides [40]	Avocado and almonds	10g avocado or 5g almond with 5mL H_2O 10mL ACN 1g NaCL 4g $MgSO_4$ 1g $C_6H_5Na_3O_7$-$2H_2O$ 0.5mg $C_6H_6Na_2O_7$-$1.5H_2O$	5mL extract 5mL H_2O 750mg $MgSO_4$ 175mg Z-Sep	*Avocado* 60-115% *Almond* 28-159%	GC-MS/MS	Avocado and Almond LOQ: 10 and 50µg/kg
30 Pesticides [41]	Vegetables and fruits	10g sample 10mL ACN 1g NaCL 4g $MgSO_4$	1mL extract 10mg MWCNTs 150mg $MgSO_4$ Filter	71-110%	GC-MS - SIM	LOD: 0.001-0.02mg/L LOQ: 0.003-0.05mg/L
78 Pesticides [42]	Tea	2g tea 10mL H_2O 10mL ACN 100µL I.S. 1g NaCl 4mg $MgSO_4$	6mL extract 6mg MWCNTs 150mg PSA 750mg $MgSO_4$	70-120%	GC-MS/MS	LOQ: 0.001-0.038mg/kg
Ten Pesticides [43]	Vegetables	10g sample 10mL ACN 1g NaCl 4g $MgSO_4$	0.5mL extract 10mg GCB 25mg PSA 30mg MNPs	69.9-125%	GC-MS	LOD: 0.39-8.6ng/g LOQ: 1.3-29.0ng/g

If these novel methods for QuEChERS-GC are successful for the analysis of pesticides that have many structural components such as multiple functional groups (benzene rings, amine groups, halogens, etc.) that drugs of abuse and pharmaceuticals possess, then it stands to reason that these methods would also have success in the analysis of drugs. All of the above sorbents can be applied to compounds other than pesticides, such as drugs of abuse in complex matrices. The success of a chitin sorbent for pharmaceutical drugs over previously used sorbents shows the extraction of drugs of abuse in forensic samples can be investigated using both the traditional QuEChERS method as well as these newer methods using novel d-SPE sorbents. Molecular imprinted polymers (MIPs) are another form of sorbent that could expand the use of QuEChERS methods by the ability to bind a particular compound of interest or a particular matrix interferent.

1.3.3. Organic Contaminants

Environmental contaminants, including volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), pesticides, and endocrine disrupters (EDs) are a major concern so detection of these compounds in various environmental matrices including soil, animal tissue, and agricultural products is of interest. These samples are complex and full of possible matrix interferences, making them amenable to QuEChERS. QuEChERS allows extraction of the compounds of interest with relatively high recoveries, providing a clean sample for analysis with GC-MS. Most of the literature in this area involves extractions with multi-class compounds, demonstrating the range of the QuEChERS method. Table 1-3 provides a summary of QuEChERS methods for the analysis of various organic contaminants. Representative methods are discussed below.

The extraction of VOCs such as trihalomethanes, benzenes, and xylenes from soil has been performed using QuEChERS with recoveries ranging from 62-94%. A difference in this method compared to those discussed thus far is the use of ethyl acetate (EtOAc) rather than ACN as the solvent during the extraction as well as no needed d-SPE step [44], [45], [46]. Though recoveries are greater than 60% for these analyses, it would be of interest to see if applying the d-SPE step would aid in increasing selectivity of desired analytes, which may allow for easier data interpretation with matrix interferences removed. For instance, when analyzing wine for haloanisoles such as 2,4,6-trichloroanisole, which is responsible for cork taint, the use of primary secondary amine (PSA) and calcium chloride ($CaCl_2$) rather than the typical $MgSO_4$ in the d-SPE step decreased extraction of sample interferences and matrix effects, improving the extraction results for the analytes of interest [47].

The analysis of four PAHs in smoked and non-smoked teas and tea infusions was performed and optimized using a dual organic solvent system of ACN:acetone (60:40) with recoveries of 74-89%; however, an SPE clean up step using a C_{18} cartridge was used rather than a d-SPE method [48]. Another use of a dual solvent system (ACN:THF) for OCP and PCB extraction from salmon tissue gave a 42-79% recovery for PCBs and 47-101% recovery for OCPs [49]. A zirconium dioxide sorbent was also used successfully for the extraction of organic contaminants including PCBs and PAHs from fish tissue [50]. These compounds are another class of analytes that have similar structure and functional groups to drugs, allowing for translation of the QuEChERS method from these contaminants to drugs of abuse.

Table 1-3. Summary of results from the literature involving QuEChERS and GC-MS for organic contaminants.

Analyte(s) of interest [reference]	Matrix	LLE	d-SPE	Recovery (%)	Instrument	LOD/LOQ
Trihalomethanes [44]	Soil	5g soil 3mL H_2O 2.5mL EtOAc 2g $MgSO_4$	N/A Organic extract analyzed directly	65-94%	GC-ECD	LOD: 6-659ng/kg LOQ: 17-1998ng/kg
Trihalomethanes, benzene, toluene, ethylbenzene, and xylenes [45]	Soil	5g soil 3mL H_2O 2.5mL EtOAc 2g $MgSO_4$	N/A Organic extract analyzed directly	66-76%	PTV-GC-MS	LOD: 0.2-15µg/kg LOQ: 0.5-45µg/kg
Chloroform, 1,2-dichlorobenzene, hexachlorobenzene [46]	Soil	2.5g soil 1.5mL H_2O 2.5mL EtOAc 1g $MgSO_4$	N/A Organic extract analyzed directly	62-93%	PTV-GC-µECD	LOD: 0.15-2.2µg/kg
2,4,6-trichloroanisole [47]	Wine	Toluene $MgSO_4$ and NaCl	$MgSO_4$, PSA, and $CaCl_2$	92-108%	GC-MS/MS GC-ToFMS	LOD: 8.3ng/L
4 PAHs benzo(a)anthracene, chrysene, benzo(b)fluoroanthene, and benzo(a)pyrene [48]	Smoked/non-smoked black teas and tea infusions	5g tea powder + 10mL H_2O or 10mL tea infusion 10mL ACN:Acetone (60:40) 4g $MgSO_4$ 1g NaCl 1g Na3Citr-$2H_2O$ 0.5g Na2HCitr-$1.5H_2O$	N/A SPE performed using C_{18} cartridge	Leaves: 74-83% Infusions: 81-89%	GC-MS/MS	Leaves LOD: 0.2-0.3µg/kg LOQ:0.3-0.6µg/kg Infusions LOD: 0.1µg/kg LOQ: 0.2-0.4µg/kg
OCPs and PCBs [49]	Fish tissue	Fish tissue 10mL H_2O 10mL solvent (ACN or ACN/THF 75/25) 4g $MgSO_4$ 1g NaCl 1g Na3Citr-$2H_2O$ 500mg Na2HCitr-$1.5H_2O$	6mL extract 1g $CaCl_2$ supernatant from $CaCl_2$ shake 900mg $MgSO_4$ 150mg PSA	*Tilapia* (ACN) 70-115% *Salmon* (ACN/THF) 42-79% (PCBs) 47-101% (OCPs)	PTV-GC-MS	LOQ Tilapia: 1-5ng/g Salmon: 2-10ng/g
Pesticides, PCBs, PAHs, PBDE, Flame Retardants [50]	Fish	10g sample 10mL ACN 4g $MgSO_4$ 1g NaCl	1mL extract 50mg Z-Sep	70-120%	LP-GC-MS/MS	LOD: 0.1-10ng/g
80 Environmental Contaminants [51]	Honeys, honeybees, and pollens	5g honey/bees 2g pollen 10mL H_2O (honey) 3mL H_2O (bees) 8mL H_2O (pollen) 3mL hexane (bees/pollen) 10mL ACN	6mL extract PSA (honey) PSA/C_{18} (honeybees and pollen)	60-120%	GC-ToFMS	LOD: 0.01-23.9ng/g LOQ: 3.0-70.4ng/g

		4g $MgSO_4$ 1gNaCl 1g Na3Citr-$2H_2O$ 500mg Na2HCitr-$1.5H_2O$				
Bisphenol A (BPA) and Bisphenol B (BPB) [52]	Canned fruits and vegetables	10g sample 100µL I.S. 5mL H_2O 10mL ACN 4g $MgSO_4$ 1g NaCl	1mL extract 5% K_2CO_3 (to pH 10) 50µL C_2Cl_4 30µL AA 3mL H_2O Derivatization necessary	>69%	GC-MS	BPA LOD: 0.3µg/kg BPB LOD: 0.6µg/kg
16 PAHs [53]	Rice	10g sample 10mL H_2O 10mL ACN (1% HAc) 6g $MgSO_4$ 1.5g NaOAc	1.5mL extract 150mg $MgSO_4$ 50mg PSA	70-106%	GC-EI-MS	LOQ: 1-5µg/kg
40 Endocrine Disruptors (EDs) [54]	Fish fillet	10g fish 10mL ACN (1% HAc) 2.0g NaCl 0.3g $MgSO_4$ 1.7g NaAc	3mL extract 450mg $MgSO_4$ 75mg PSA 375mg C_{18} Filter	70.1-120%	GC-MS/MS	LOD: 0.3-7.5µg/kg

Endocrine disruptors (EDs) are a classification of organic contaminant that is commonly investigated, especially in fish. Many endocrine disruptors are drugs and thus are another model for the use of QuEChERS-GC and drug analysis. Alkylphenols, polychlorinated biphenyls (PCBs), PAHs, bisphenol A (BPA), and pesticides are all considered EDs as they mimic endogenous hormones, interfering with the endocrine system, preventing the action of those hormones. The use of QuEChERS for this area involves the mostly citrate salts for analysis, though there are some which only use $MgSO_4$ and NaCl in the LLE step with comparable results [50]. Other sorbents have also been investigated such as zirconium based sorbents as well as the use of tetrahydrofuran (THF) along with ACN in the extraction method which increased the recovery of polychlorinated biphenyls in salmon [49]. Honeybees and pollen have also been investigated for the aforementioned environmental contaminants and for veterinary drug residues. Using hexanes and acetonitrile for the extraction combined with either a PSA sorbent (honey) or combined PSA/C_{18} sorbent (honeybees and pollen) provided a recovery of greater than 60%. It was found that using hexane along with ACN improved the method by removing lipids that can interfere with detection [51]. As the matrices become more complex, optimization of all parameters involved in the QuEChERS extraction becomes increasingly critical.

1.3.4. Mycotoxins

The study of mycotoxins via QuEChERS and GC has grown recently. Mycotoxins are toxigenic molds that contaminate food. Some analyses have been performed with GC, including both food and drink products. Sodium carbonate (Na_2CO_3) has been used in most of these methods to alkalinize the sample, aiding in extraction of the analyte(s), in

Table 1-4. Summary of results from the literature involving QuEChERS and GC-MS for various mycotoxins.

Mycotoxin [reference]	Matrix	LLE	d-SPE	Recovery (%)	Instrument	LOD/LOQ
Patulin [55]	Apple juice	5mL sample 10µg/mL patulin 15mL ACN $MgSO_4$:NaCl:Na_2CO_3 (4:1:0.5)	11.5mL extract 400mg PSA 1200mg $MgSO_4$ Derivatization necessary	79.9-87.9%	GC-MS	LOD: 0.4µg/mL LOQ: 1.3µg/mL
Multi-mycotoxin [56]	Wheat semolina	10mL sample 10mL ACN 1g NaCL 4g $MgSO_4$	1mL extract 0.025g GCB 0.150g $MgSO_4$ Derivatization necessary	74-124%	GC-MS/MS	LOQ: 1.25-10µg/kg
Multi-mycotoxin [57]	Popcorn	5g sample 10mL H_2O (20mL for popped sample) 5.0mL Na_2CO_3 10.0mL ACN 1g NaCl 4g $MgSO_4$	6mL extract 300mg C_{18} 900mg $MgSO_4$ Derivatization necessary	*Unpopped* 61-118% *Popped* 65-89%	GC-MS	LOD: 7-65µg/kg LOQ: 20-196µg/kg

contrast to most other QuEChERS methods as can be seen in Table 1-4. Analysis of mycotoxins via GC-MS also requires derivatization of the analyte after the clean up step and before injection [55], [56], [57]. None of the methods have attempted a pre-extraction derivatization, which may be of interest to try in future work.

2. Basic Theory of Gas Chromatographic Separations

2.1. Discussion of Mobile and Stationary Phases

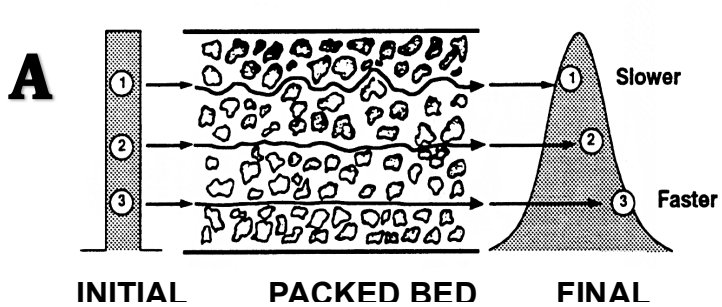
The purpose of any type of chromatography is to separate a mixture into its components. In gas chromatography (GC), this is achieved based upon an analyte's vapor pressure and partitioning between two phases, a mobile phase and stationary phase. The stationary phase is contained within a column. The column can be a packed column containing solid particles that aid in separating the sample, or a capillary column that has a liquid coating on the walls of the capillary tube. In this research, a capillary column was used during GC analysis. The choice of stationary phase depends upon the sample composition and goals of analysis. The principle involved in extractions of 'like dissolves like' is also applicable in chromatography. An analyte that is more polar will not be as attracted to a non-polar stationary phase and will thus not be retained as long as a non-polar compound and will elute faster. While traveling through the column, Van der Waals interactions occur between the analyte and stationary phase, causing the resulting separation. These interactions will determine the amount of analyte present in the stationary phase as compared to the mobile phase. For instance, if the analyte is polar and a non-polar stationary phase is used, the Van der Waals interactions will not be as

strong as if a non-polar analyte were being analyzed. This scenario would result in less interaction of the analyte with the stationary phase and a shorter retention time. The strength of these interactions will in turn affect the partition coefficient as will be further discussed in section 2.2. The most commonly used stationary phase for a wide range of analyses is a 5% diphenyl/95% dimethyl polysiloxane phase [15].

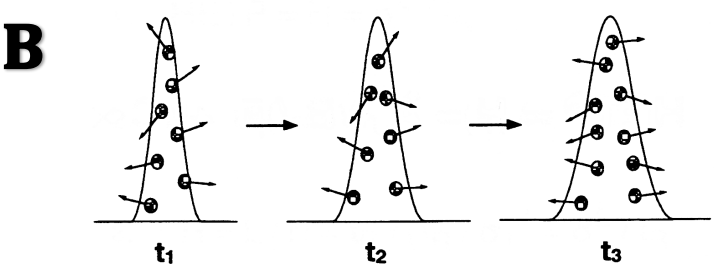
The mobile phase is an inert gas such as helium, hydrogen, or nitrogen, which carries the sample through the instrument, hence it is also known as a carrier gas. Effects of different gases have been studied and plotted in what is known as a van Deemter plot. This plot associates the kinetic and mass transfer effects of chromatography through the use of "rate theory" as developed by van Deemter for explaining band broadening of peaks in a chromatogram. The van Deemter equation illustrates the terms involved in band broadening.

$$H = A + \frac{B}{\bar{\mu}} + (C_s + C_m)\bar{\mu} \qquad \text{(Equation 1-4)}$$

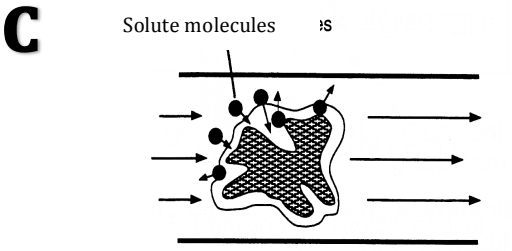
In the above equation, A is the eddy diffusion, or multi-path term. This term is of more importance in liquid chromatography and packed columns in which the analytes can take multiple paths through the column (see Figure 1-3A). Longitudinal diffusion is accounted for by the B term, and the mass transfer effects in both the stationary and mobile phases are seen in the C_s and C_m terms, respectively (Figures 1-3B and C). Linear velocity of the carrier gas, $\bar{\mu}$, can effect each term. An increase in carrier gas flow will decrease the occurrence of longitudinal diffusion; yet increase the mass transfer effects.



Eddy Diffusion through a packed column



Longitudinal Diffusion



Mass Transfer

Figure 1-3. The three terms in the Van Deemter equation. A: eddy diffusion; B: longitudinal diffusion; C: mass transfer. Adapted from Y. Kazakevich Separations Introduction Lecture Slides [59].

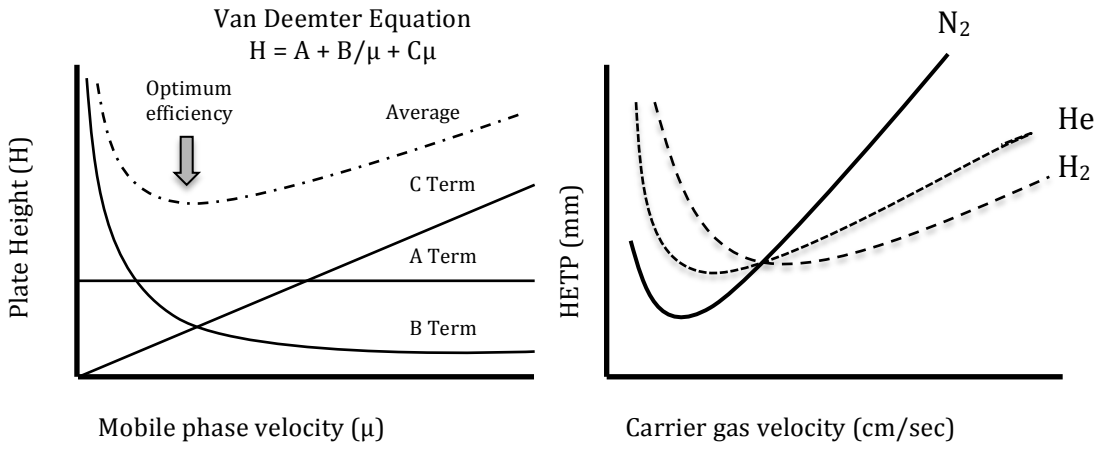


Figure 1-4. The three terms in a Van Deemter plot (left) and the various gases as how they appear in the Van Deemter plot (right).

This equation demonstrates why it is important to consider not only the linear velocity used but also the type of carrier gas. By plotting the resulting H values versus the linear velocity for various gases as in Figure 1-4, it can be seen how the velocity and gas type affect the chromatography. The optimal linear velocity providing the best column efficiency in which the variables of the van Deemter equation are minimized varies with different gases as well. The carrier gas used in this research was helium as it is safer to use than hydrogen, provides faster analyses than nitrogen, and has a greater use over a wider range of carrier gas flow rates as seen in Figure 1-4 [15], [16], [58].

2.2. Discussion of Analyte Retention and Sample Introduction

Analyte retention is best explained by first discussing the partitioning between the mobile phase (MP) and stationary phase (SP) providing a partition coefficient (K_c) as seen below.

$$[A]_{MP} \leftrightarrow [A]_{SP} \quad where \quad K_c = \frac{[A]_{MP}}{[A]_{SP}} \qquad \text{(Equation 1-5)}$$

The partition coefficient has a relationship to the retention factor of the analytes (k) using the phase ratio (β) which is equal to the volume of analyte in the mobile phase to the stationary phase and is determined using the capillary column's dimensions as seen in Equation 1-6 (r = the column's radius, d_f = stationary phase film thickness).

$$K_C = k\beta \quad where \quad \beta = \frac{r}{2d_f} \qquad \text{(Equation 1-6)}$$

The retention of the analytes is not only based upon the capillary column and stationary phase selected, but also the properties of the analyte including the partition coefficient which is affected by the analyte's boiling point, vapor pressure, and volatility [16].

Volatility is an important property of the sample under investigation. As the injection port for sample introduction is heated, it is imperative that the sample be volatile or semi-volatile to the extent that it can be vaporized and introduced into the carrier gas. The inlet employed in this research, and the most commonly used inlet for GC, is a split/splitless inlet. With this type of inlet, a sample can be introduced via liquid injection into a split or splitless liner. A liquid injection can result in solvent effects and an expansion of the sample volume due to the solvent being present and must be taken into account during method development and solvent type/amount used during the injection [15], [58].

As seen in Figure 1-5, the main difference between these two types of injections is the opening of the purge valve. As its name implies, during a split injection the purge valve is open for the entirety of the sample analysis allowing only a portion of the sample that is injected to be analyzed, splitting it. No dilution of the sample is necessary with this type of injection. The amount of sample analyzed is determined by the split ratio. This type of sample introduction is best used for samples that are considered dirty such as urine that may contain contaminants and non-volatile components to keep these compounds from entering the column. Splitting the sample means this form of analysis is

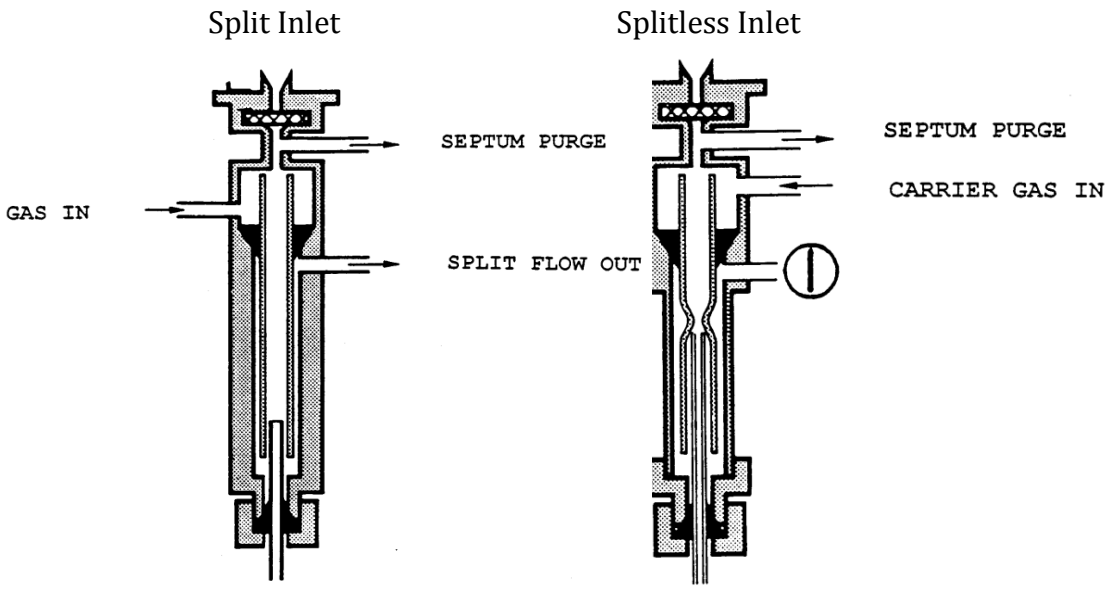


Figure 1-5. Split (left) and splitless (right) injection. Adapted from Y. Kazakevich GC Injectors Lecture Slides [60].

not as accurate as splitless injections for quantitation of samples and is not conducive for trace analysis [58].

It is for these main two disadvantages that splitless injections were used in this research. Splitless injections are well suited for trace analysis because the entire sample injected is analyzed by keeping the purge valve closed during sample introduction, increasing the sensitivity and reproducibility. This valve is then open after a certain amount of time to purge the inlet and remove any residual solvent. The sample stays in the inlet for a longer amount of time, increasing the possibility for unwanted interactions occurring in the inlet. This must be taken into consideration when using this type of injection as it can result in tailing if the purge valve time is not optimized. Another result of splitless injections is wider peak widths when analyzed using isothermal conditions. If temperature programming is employed with splitless injections and a cooler initial column temperature, this will focus the sample resulting in solvent focusing and sharper peaks through 'cold-trapping' due to the large temperature difference between the inlet and column [58].

3. GC Detectors: Mass Spectrometers

There are many types of detectors that can be coupled to a gas chromatograph; however, the primary one used in this research was a mass spectrometer. A mass spectrometer is composed of three main components: an ion source, a mass analyzer, and a detector. In the ion source, the sample is impacted with a beam of electrons (70eV) obtained from a

tungsten filament, exciting and ionizing the analyte molecules, causing fragmentation derived from the analyte's structure. The ion source used here was electron ionization (EI) as seen in Figure 1-6, which is a form of hard ionization meaning it produces more fragmentation than a softer technique such a chemical ionization [15].

In the mass analyzer, the ions that were created in the ion source are separated based on their mass-to-charge (m/z) ratio in quadrupole mass analyzers and by their kinetic energies in a time-of-flight mass analyzer. The types of mass analyzers used in this research include a quadrupole, triple quadrupole, and time of flight. The latter two will be discussed in greater detail in Chapters 3 and 5, respectively. A quadrupole, shown in Figure 1-7, is composed of four parallel rods at right angles to each other with alternating electrostatic charges and a magnetic field formed by a radio frequency surrounding the poles. The ions travel through the center of the poles, reaching the detector only if they are in the chosen mass range. The entire range of masses can be scanned or a selected number can be analyzed using selected ion monitoring (SIM). SIM confirms an analyte's identity as well as increases selectivity and the signal to noise ratio [16], [58].

The detector used in this research is an electron multiplier and is pictured in Figure 1-8. An electron multiplier uses dynodes to amplify the signal approximately 1 million times the original signal. The detector helps to establish the sensitivity and limits of detection and quantitation; however, the mass analyzer defines these parameters as well as the resolution and speed of analysis [15].

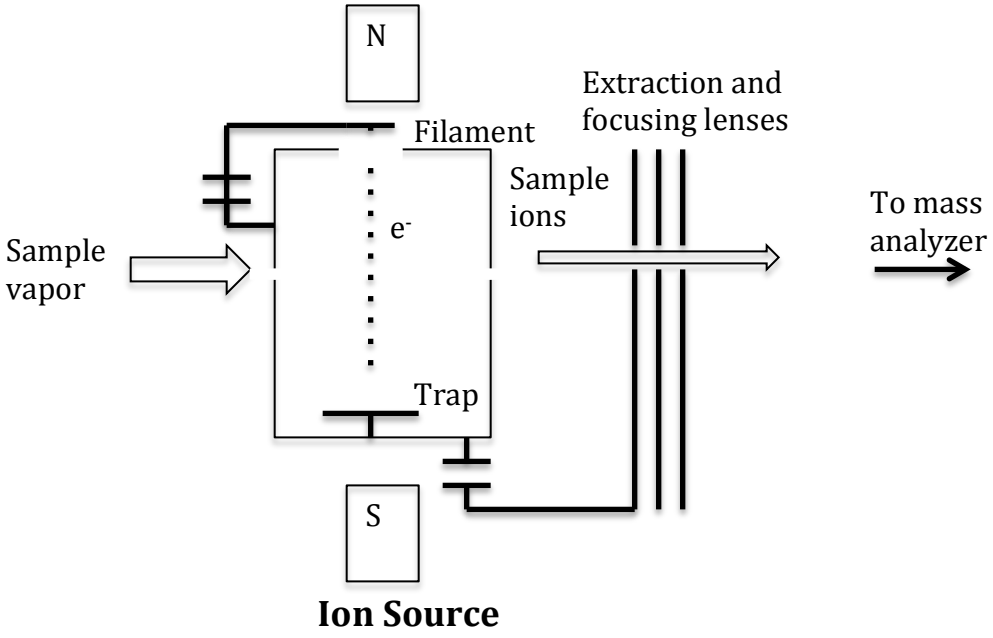


Figure 1-6. Schematic of an electron ionization (EI) source.

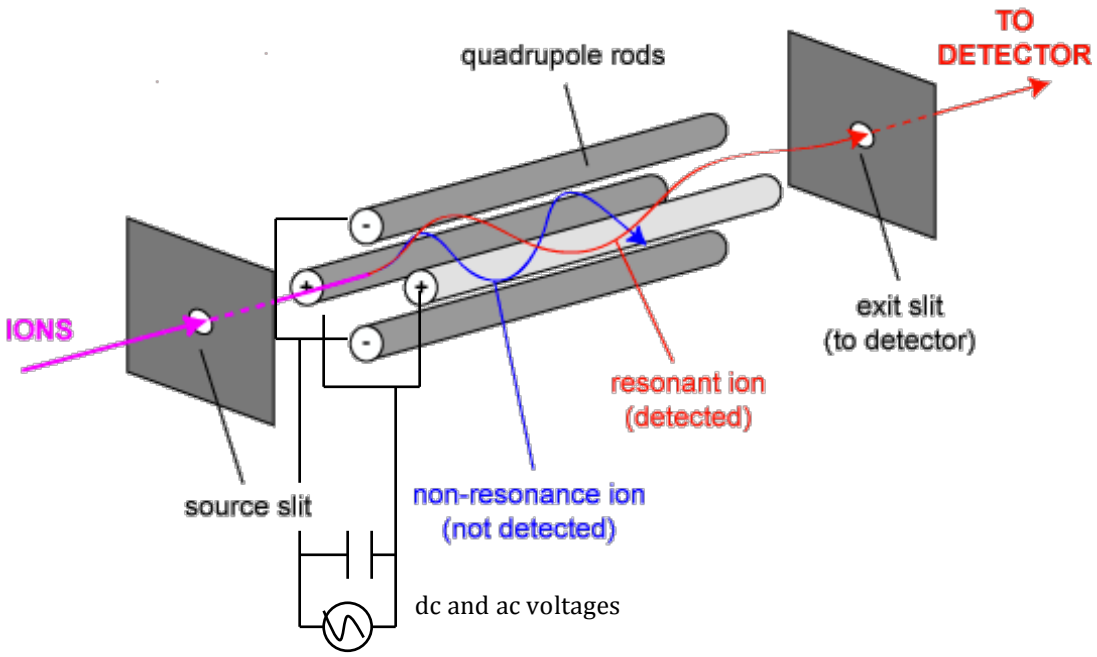


Figure 1-7. Schematic of a quadrupole mass analyzer. Adapted from P. Gates Gas Chromatography Mass Spectrometry (GC/MS): Figure 2 [61].

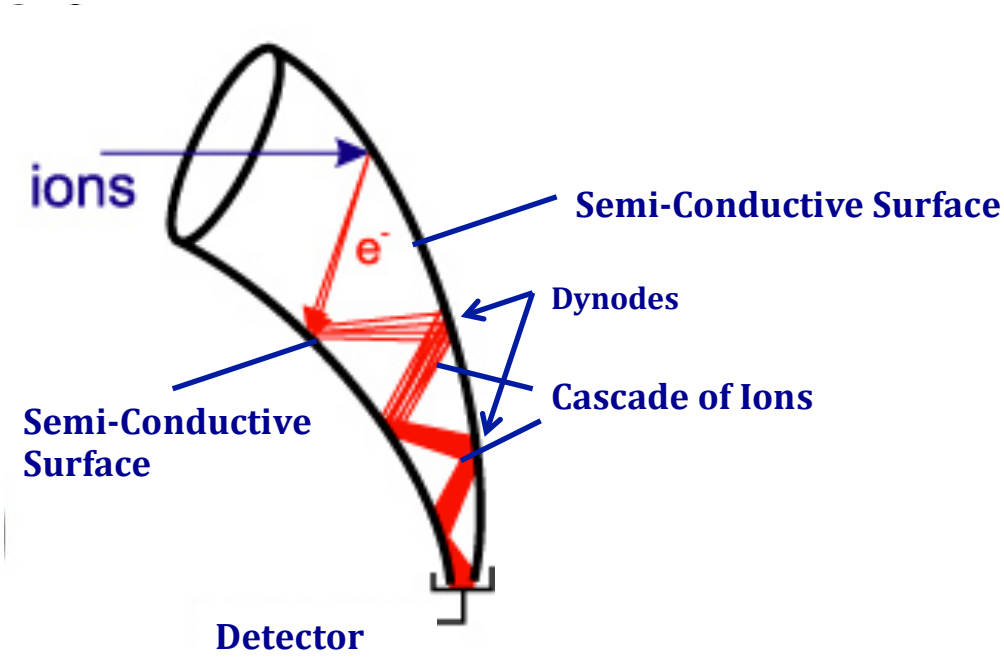


Figure 1-8. Schematic of a continuous dynode electron multiplier. Adapted from J. Benedikt, A. Hecimovic, D. Ellerweg, and A. von Keudell. J Phys D-Appl Phys. 45(50) (2012) [62].

4. Conclusions

Recently, the literature for QuEChERS has begun to include more research including biological matrices and analytes such as the extraction of pharmaceutical drugs from blood. This shift has paved the way for the future of QuEChERS applications to include forensic samples such as drugs of abuse in urine and blood. The constant modifications that have been made to the original three methods previously discussed allow for its growth for future applications and improvement to existing ones. One such modification involves the combination of the LLE and d-SPE portions into a single step. The analyses that have been applied to liquid chromatography for compounds that are also GC amenable, allow for an unlimited number of applications that QuEChERS can be used for including further integration into environmental chemistry, food chemistry, and forensic samples.

QuEChERS is a method that has multiple applications that have been explored as well as novel techniques and applications that have yet to be discovered. One such original study includes the investigation of the fundamental chemistry involved in the extraction parameters that are optimized. This, along with several novel applications of QuEChERS, will be discussed throughout the chapters of this dissertation. A caffeine study, Chapter 2, will address the fundamental chemistry of the extraction by optimizing various parameters and discussing the results at a chemical level including a look at the extraction kinetics, temperature effects, and partition coefficient of the extraction. Chapter 3 will look at an original application of QuEChERS for the extraction of steroids from water and herbal medicinal products using GC analysis rather than the more

common LC methods. This research uses QuEChERS, GC-MS/MS and GCxGC-TOFMS as well as SPME for a few of the steroids as discussed in Chapters 4 and 5. A methodology using HPLC and these steroids was also investigated, Chapter 6, as it was attempted to reproduce the method used in a research article in which steroids were extracted from water. The method used by the authors in the original work will be discussed in this chapter as well as the development of a new, more efficient method. Chapter 7 will address the novel use of QuEChERS in a forensic sample aspect involving the extraction of drugs of abuse from urine, a field which QuEChERS has yet to be used to its fullest potential and may possibly be a major direction of research for this extraction technique.

CHAPTER 2 – A FUNDAMENTAL STUDY FOR THE EXTRACTION OF CAFFEINE FROM TEA AND WATER USING QUECHERS

The extraction of caffeine from tea has long been an experiment used in classroom laboratories, but this extraction dates back to the Stone Age. According to a Mongolian legend, an Emperor was boiling water and some tea leaves accidentally fell into the hot water producing a fragrant and revitalizing drink. In the past, people would chew on seeds, bark, or leaves that contained caffeine and noticed ease in fatigue and elevated mood. It was only later discovered, perhaps by the Mongolian emperor if the legend is true, that steeping the leaves in hot water provided an increase in these effects [63]. Caffeine is a xanthine alkaloid that acts as a central nervous system stimulant and is one of the most widely consumed psychoactive substances today. It is estimated that in North America, approximately 90% of adults ingest caffeine daily, and 80% worldwide [63], [64]. In adults between the ages of 25 to 65, coffee and soda are the two primary sources of caffeine with a shift to coffee and tea for adults over 65. On average, a person will consume 106-170 mg of caffeine per day [64]. The amount of caffeine present varies depending on the type of tea, for instance herbal tea contains almost no caffeine in a tea bag whereas black tea contains 25-110 mg per tea bag [65].

There are many different types of extraction methods that can be used for the extraction of caffeine from tea, most commonly the use of dichloromethane or ethyl acetate. These methods can be as simple as a separatory funnel liquid-liquid extraction, or more complex by the addition of salts and clean up sorbents to the method as one would find

during a QuEChERS method [66]. In this study, caffeine and tea were used as a model analyte and matrix. Caffeine has a structure similar to other compounds, including drugs, and has been studied quite extensively. Tea has both qualities of a plant and food matrix making it ideal to investigate the use of QuEChERS and its ability to eliminate matrix interferences upon the extraction of caffeine.

Thus far in the literature, QuEChERS has been used primarily for the analysis of pesticides in food products as well as other uses including the extraction of drugs and environmental contaminants from various matrices. Though optimization of the method has been performed, the fundamental chemistry has yet to be explored and explained within the literature, thus this study seeks to describe the chemistry involved in QuEChERS in a greater detail. Not only were the parameters of the method optimized including salt amount and type (500mg $MgSO_4$:500mg NaCl) , but also the partition coefficient of the extraction was determined and extraction kinetics and temperature effects were investigated. This study resulted in a method with a percent recovery greater than 95%, an average partition coefficient of 2.1, and an intra and interday %RSD less than 6% and 12%, respectively. Overall, this method helped to evaluate the chemistry involved in the QuEChERS method, providing insight into its future uses as an extraction method.

1. Introduction

QuEChERS is an extraction technique that combines a liquid-liquid extraction (LLE) with a dispersive solid phase extraction (d-SPE) to remove matrix interferences. This method is attractive due to its ease of use, limited solvent use, and effectiveness in providing a clean sample [1], [2], [3], [4]. QuEChERS is especially known for its use in the determination of pesticides in many agricultural products such as fruits and vegetables as well as analyzing various matrices including soil, tea, biological fluids, and sewage sludge for pharmaceutical drugs, drugs of forensic interest, personal care products, and environmental contaminants to name a few [10-13], [34], [45], [49]. QuEChERS has been reported by multiple sources as a sample preparation technique in conjunction with liquid chromatography (LC); however, gas chromatography mass spectrometry (GC/MS) is not nearly as abundant, possibly due to the complexity of certain matrices and the large abundance of LC in fields that use this technique, especially for the analysis of pesticides. Also often lacking is the explanation of the resulting optimization parameters in terms of the fundamental chemistry involved in those steps of QuEChERS and how certain parameters such as extraction kinetics are influenced by these parameters and their optimization.

Two basic steps are involved in the QuEChERS method: extracting the aqueous sample via an organic solvent such as acetonitrile followed by the use of a dispersive solid phase extraction (d-SPE) sorbent as a clean up step. There are three commonly used methods: the original method, the AOAC method, and finally the European version, the latter two employing the use of buffers and various salts in the method. It is obvious that the

QuEChERS method used will depend upon the analytes of interest; however, each method would require optimization of items such as the solvent system, the amounts and types of salts present, and the choice of d-SPE sorbent [1], [3].

The LLE step involves the use of salts to drive the analyte into the organic phase. Acetonitrile is most commonly used as a solvent due to its ability to minimize the amount of co-extractables while maintaining a large extraction range of desired analytes [1]. In the d-SPE clean up step, a sorbent is used to retain certain unwanted interferences in addition to a drying agent ($MgSO_4$). For instance, the sorbent primary secondary amine (PSA) will remove sugars, fatty and organic acids, and some pigments [3]. The ultimate goal after optimization includes high recovery and removal of matrix interference peaks. This study looks to improve upon current QuEChERS methods by investigating the theory as it pertains to the kinetics and effect of temperature on the partitioning steps. It is of interest to investigate the parameters optimized in the QuEChERS extraction of a model analyte, caffeine, from a model matrix, tea, as well as the use of GC-MS for instrumental analysis. The parameters studied include pH, type and amount of organic solvent, type and amount of salts, sonication time and temperature, and type of sorbent used during d-SPE.

2. Materials and Methods

2.1. Chemicals, Reagents, and Samples

Acetonitrile (ACN) was purchased from pharmco-AAPER (Kindermorgan, PA) and was reagent ACS grade. All salts used throughout the study as well as the caffeine standard were purchased from Sigma Aldrich (St. Louis, MO). QuEChERS tubes containing 150mg PSA and 50mg $MgSO_4$ were purchased from Restek (Bellefonte, PA) and 15mL PFTE centrifuge tubes were obtained from VWR International (Radnor, PA). The tea used was Wagh Bakri Masala Chai Tea Bags purchased from a Patel Brothers grocery store (Parsippany, NJ). Deionized water was used throughout the methodology.

2.2. Sample Preparation

The type of solvent, salts, temperature, and pH used during the QuEChERS process can affect the extraction of the analyte and thus need to be optimized. By evaluating these parameters in the following steps, the results can provide information on the interactions occurring as well as the thermodynamic and kinetic properties of the system. All optimization analyses were performed using tea to evaluate the effect of matrix interferences and determine the ability of the method to provide a clean sample. Also, for each optimization a standard dichloromethane (DCM) extraction was performed for comparison in which 1mL of DCM and 1mL of the aqueous tea sample was shaken in a GC vial and analyzed.

QuEChERS involves two main steps, a LLE step and a d-SPE clean up step. For this study, there was an additional step prior to the extraction involving the sonication of loose tea from a tea bag in deionized water (0.4g of tea for every 10mL of water), the time of which was optimized. Once sonication was complete, the liquid was separated from the loose tea and used as the aqueous sample in the LLE step of the method. During this process, the pH of the water used to soak the tea leaves was optimized using a sodium acetate buffer at both pH 7 and pH 8, the latter of which was prepared by adding 2M sodium hydroxide drop wise until a pH of 8 was obtained. These systems were compared to one in which no buffers were added at pH 6 (deionized water). The sonication time and extraction temperature (0°C, 100°C, and room temperature) were also investigated during the initial extraction where only the room temperature sample was subject to sonication. This was performed to evaluate both the extraction kinetics (sonication time) and temperature effects (sonication temperature).

The LLE portion of the method required the optimization of salt and solvent amount and type. In this study, various ratios of $MgSO_4$:NaCl were investigated including 1:1, 2:1, 3:1, and 4:1, with the later (500:500mg) providing the highest peak area for caffeine upon GC-MS analysis. Once the salts were placed in the centrifuge tube, 4mL of the aqueous sample and 2mL of ACN were added as opposed to 2mL of each solvent and the tube was vortexed for 1 minute and centrifuged for 3 minutes at 1,000rpm. The use of ethyl acetate, acetone, and ethanol/water mixtures were investigated as organic solvents as well. The extraction kinetics (sonication time) and temperature effects were investigated during the LLE portion of QuEChERS as with the initial sonication of loose tea.

From here, the top organic layer was transferred via Pasteur pipette to the QuEChERS tube containing 150mg PSA and 50mg $MgSO_4$. This tube was vortexed for 1 minute and centrifuged for 3 minutes at 8,000rpm. Once again, the extraction kinetics and temperature effects were studied during the d-SPE step as well. Once complete, the liquid was removed using a Pasteur pipette and transferred to a GC vial for analysis via direct liquid injection using GC-MS.

2.3. Instrumental Parameters

The instrumentation utilized for this study was an Agilent 6890 GC and 5973 MSD (Santa Clara, CA) as well as a CTC Analytics combiPAL (Zwingen, Switzerland). Splitless injection was used for all analyses, with selected ion monitoring being employed during method validation (ions 55, 67, 109, and 194 selected for the entirety of the run). The GC method consisted of the following parameters: inlet temperature of 250°C using a splitless liner, initial oven temperature of 40°C, oven ramp of 20°C/min to 220°C with a 5min hold (total time was 14min), and constant flow set to 1mL/min. The carrier gas was helium and a Restek RTX-5MS column was used (30m x 0.25mm x 0.25µm). For the MSD parameters, the thermal auxiliary was set to 220°C and the ion source was at 230°C.

2.4. Method validation

2.4.1. Calibration curve, linearity, and partition coefficient

Caffeine standards were prepared in deionized water ranging from 0.1ppb to 100ppm. These samples were then subjected to the optimized QuEChERS method as described in section 2.2 and then analyzed in triplicate using GC-MS. Limits of quantitation (LOQ) and detection (LOD) were assessed using the data analysis software where the S/N for the caffeine peak was 10 and 3, respectively. These results were also confirmed by evaluating the data by observation and repetition to confirm the software analysis results as well calculating the LOD and LOQ using the following equation:

$$LOD = \frac{3s_B}{m} \quad \text{(Equation 2-1)}$$

Where s_B is the standard deviation of the signal for 10 points from a blank sample and m is the slope of the calibration curve. For LOQ, the factor of 3 was changed to 10.

The calibration curve was prepared using samples with concentrations over approximately three orders of magnitude (8ppb to 1ppm), and the linearity was assessed over five orders of magnitude (8ppb to 100ppm). The R^2 value and equation of the line were obtained using Excel for both plots. Once the calibration curve was constructed, the equation of the line could be used to determine the partition coefficient using the following equation:

$$K = \frac{c_{organic}}{c_{aqueous}} \quad \text{(Equation 2-2)}$$

The variables used in equation 2-2 were calculated using the calibration curve and can be seen in section 3.4.1. For an extraction, it is desired that the partition coefficient be greater than one, where the majority of the analyte is in the solvent. The partition coefficient for three samples was determined at the LOQ (50ppb) and an average was reported.

2.4.2. Recovery, precision, accuracy

Three caffeine standard samples at the LOQ were analyzed and used in the determination of percent recovery by using the equation of the line from the calibration curve to find the concentration from the resulting peak area. Once determined, this value was divided by the LOQ concentration and multiplied by 100 to give percent recovery. An average value was reported. Percent error was determined for each of these three samples as well to assess accuracy of the method and an average value was reported. Five samples at the LOQ were performed and used in the determination of precision for both interday and intraday precision. The %RSD was calculated from these five samples for both days individually as well as collectively.

$$\%recovery = \left(\frac{experimentally\ determined\ concentration}{LOQ}\right)100 \quad \text{(Equation 2-3)}$$

$$Accuracy: \%error = \left(\frac{LOQ - experimentally\ determined\ concentr\square tion}{LOQ}\right)100 \quad \text{(Equation 2-4)}$$

$$Precision: \%RSD = \left(\frac{s}{\bar{x}}\right)100 \quad \text{(Equation 2-5)}$$

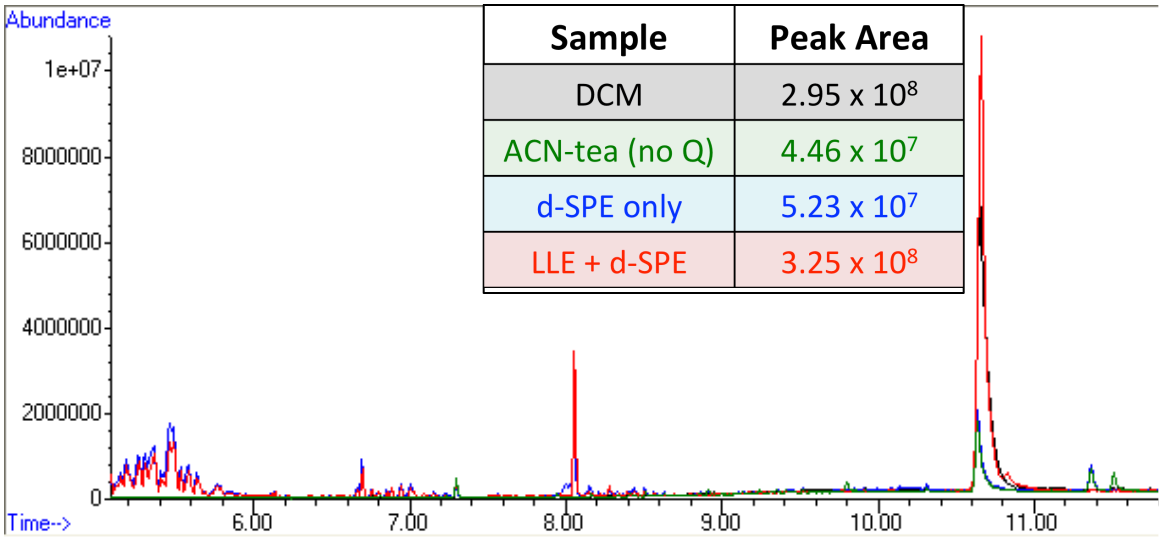
3. Results and discussion

3.1. Optimization of QuEChERS Extraction

Firstly, the peak area was used to evaluate the amount of caffeine extracted using QuEChERS as opposed to a simple organic solvent shake with the aqueous tea sample. The following were compared and are seen in Figure 2-1A: a 1mL DCM:1mL aqueous tea sample shake (black line in Figure 2-1A), the analysis of the tea sample using the QuEChERS method (red line in Figure 2-1A), the analysis of the sample using the LLE portion of the QuEChERS method with acetonitrile (green line in Figure 2-1A), and the analysis of the tea sample using only the d-SPE step from the QuEChERS method (blue line in Figure 2-1A). Only the full QuEChERS method ($3.25x10^8$) resulted in a peak area for caffeine greater than that of the DCM shake ($2.95x10^8$), as seen in Figure 2-1A. Figure 2-1B also shows the ability of QuEChERS to provide a cleaner sample.

3.1.1. Initial sonication of loose tea

Three items were optimized in the initial sonication of tea, the pH of the aqueous phase (deionized water), the sonication time, and the temperature. When evaluating pH, it was first determined to work at a pH below the pK_a of caffeine, closer to that of the pH of deionized water with no buffer added. Though the compound is ionized at this pH and the unionized form is usually desired as it will result in a better extraction into the organic phase, it was decided that the addition of a buffer could further complicate the system for the evaluation of the chemistry involved, thus a lower pH was used. In addition to working at a convenient pH, it was also desired to investigate the ability of the extraction and detector given this 'non-optimized' pH.



Sample	Peak Area
DCM	2.95 x 10^8
ACN-tea (no Q)	4.46 x 10^7
d-SPE only	5.23 x 10^7
LLE + d-SPE	3.25 x 10^8

Figure 2-1A. Overlaid chromatograms of a DCM-tea sample shake, LLE with ACN and salts, d-SPE with ACN and tea sample only, and the complete QuEChERS method.

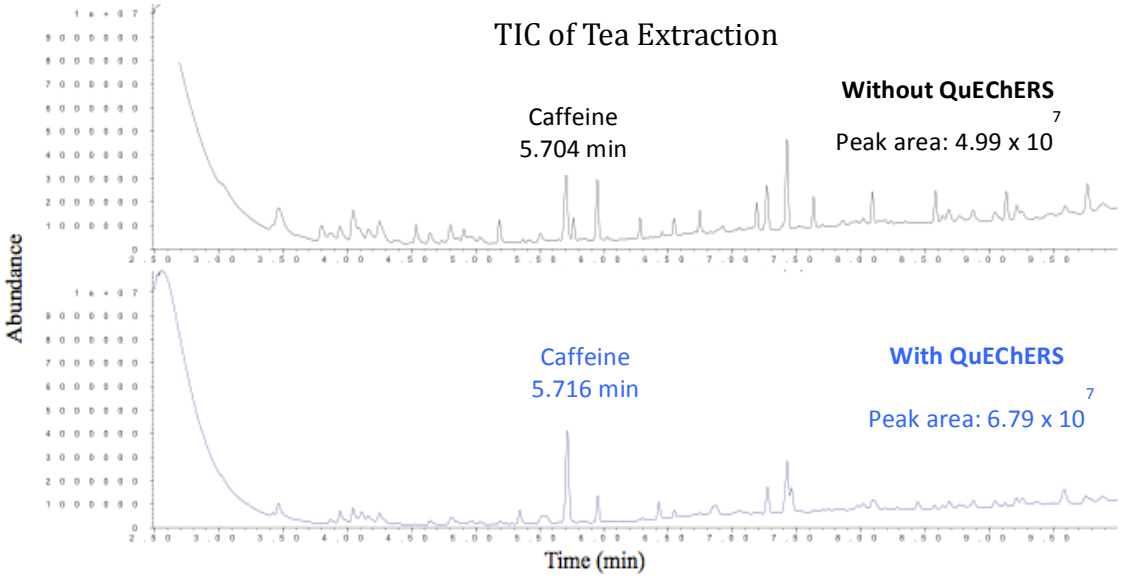


Figure 2-1B. Top: Chromatogram depicting the peak area of caffeine at 5.704min without QuEChERS. Bottom: Chromatogram depicting the increased peak area of caffeine at 5.716min with QuEChERS as well as decreased matrix interference peaks.

Through Figure 2-2, it can be seen that there was no significant increase in peak area or extraction stability upon the use of a buffer and thus it was deemed unnecessary.

Kinetics of this initial sonication was evaluated by determining the sonication time required to provide the most amount of caffeine extracted from the loose tea by the deionized water. A plateau was seen from 6-10 minutes indicating no further increase in peak area with time and thus it was determined that 8 minutes was the optimal time for the sonication. When peak area was plotted vs. time in Figure 2-3, a curved plot resulted indicating that the extraction does not follow a true zero order kinetic trend for the entirety of the extraction, as a linear trend would indicate a zero order extraction. Plotting Ln[A] vs. time also provided a curved line and thus the extraction is not first order either. This shows that the initial extraction of caffeine from the tea leaf is not a simple process as one may think. The caffeine is imbedded within the tea leaf, thus the water must penetrate the cuticle of the leaf, permeate the leaf and extract the caffeine, carrying it back across the cuticle of the leaf and into solution as seen in Figure 2-3.

The effect of temperature on the system was evaluated next through the use of three different temperatures and resulting caffeine peak area. Both hot (100°C) and cold (0°C) were investigated in addition to the use of a sonicated room temperature sample. It was seen when plotting the peak area of each temperature in Figure 2-4 that the use of hot water in the initial extraction, mimicking the act of steeping your tea, provided a substantially higher peak area for caffeine within the optimized 8 minutes. This was expected, as caffeine is more soluble in hot water, causing the extraction of more caffeine

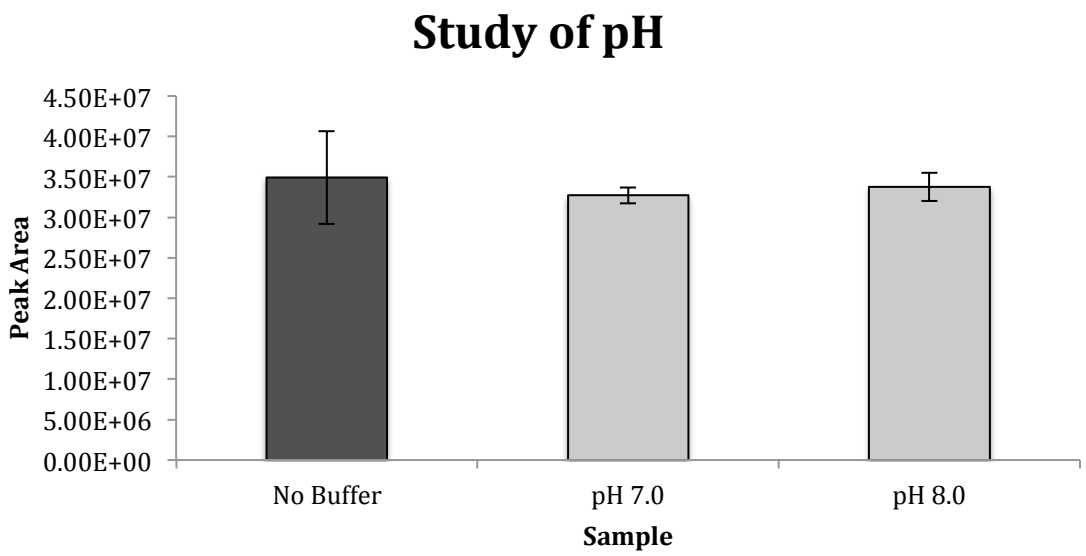


Figure 2-2. Optimization of the pH during the initial sonication of lose tea in deionized water.

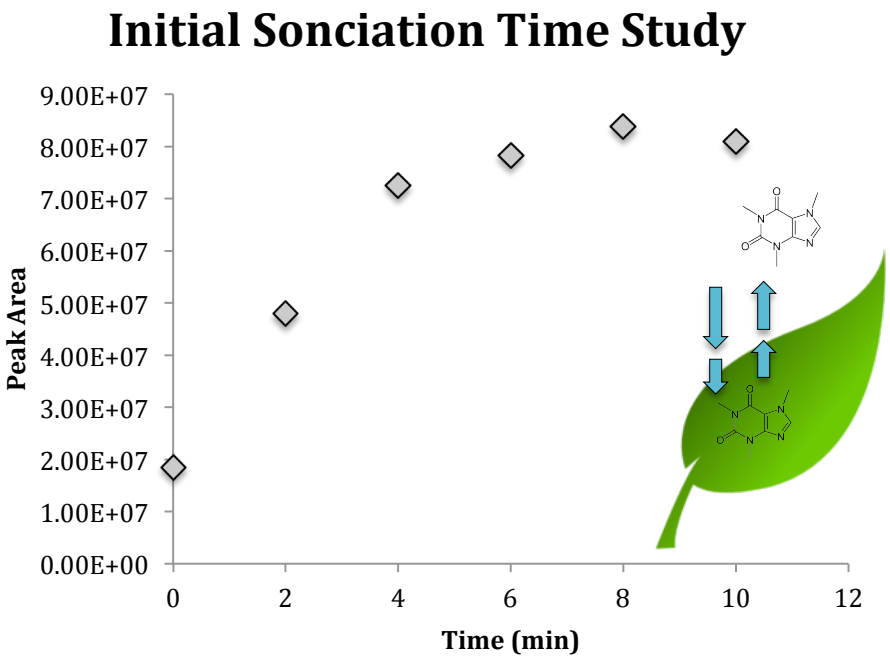


Figure 2-3. Investigation of the effect of time on the initial sonication time of loose tea in deionized water provided the resulting plot of peak area vs. time as a kinetic study. The process involved in extracting caffeine from the tea leaf using water is also shown.

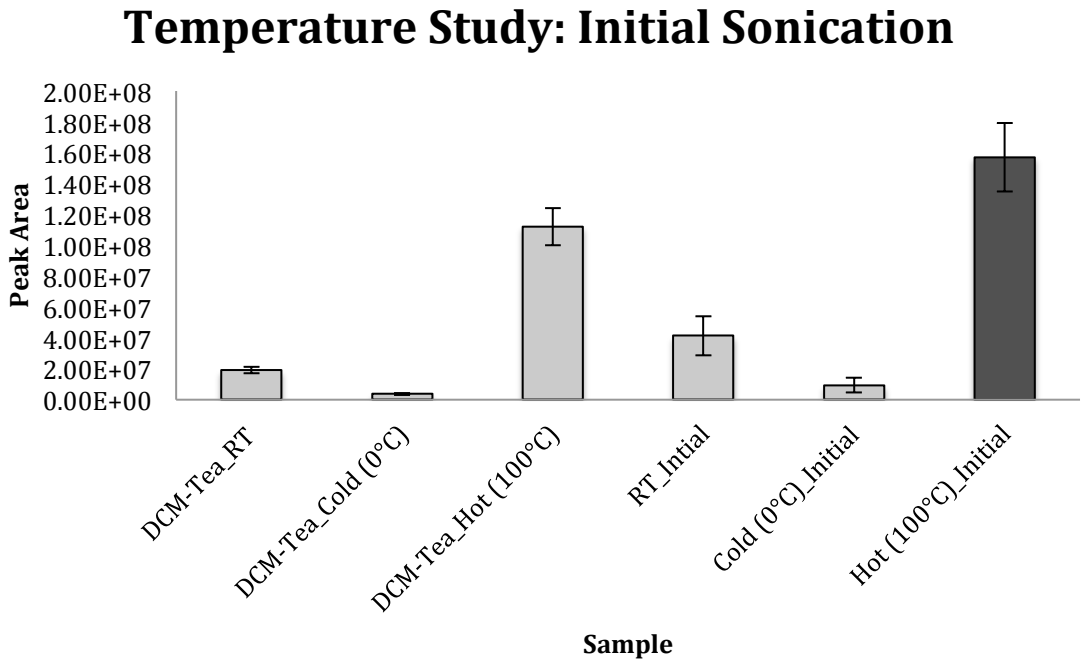


Figure 2-4. Investigation of the effect of temperature during the initial sonication of loose tea in deionized water as a thermodynamic study.

within 8 minutes. However, this also results in the extraction of more matrix interferences. These results were also mimicked when looking at a comparison to a DCM-shake control also exposed to the varying temperatures.

3.1.2. Liquid-Liquid Extraction (LLE)

The LLE includes the use of salts, typically $MgSO_4$ and NaCl as salting-out agents, the first of which aids in solvent partitioning between the aqueous and organic solvent as well as increasing polar analyte recovery. The second salt, NaCl, acts to decrease the amount of polar interferences. Both of these were evidenced in Figure 2-5 where it is seen that increasing the amount of $MgSO_4$ results in an increased amount of organic phase indicating a greater partitioning between the organic and aqueous phase. The addition of salts to the sample allows for the separation of the miscible organic solvent, in this case acetonitrile, and the aqueous sample. This leads to a more favored interaction between the water and the salts than any hydrogen bonds formed between the water and the analyte, thus allowing the analyte to be driven into the organic phase. Figure 2-5 also shows that the increased amount of NaCl results in less pigmentation of the extract. The fact that the sample becomes less colored as more NaCl is added also shows that NaCl adds selectivity to the extraction by decreasing the amount of co-extractables.

In this study, various ratios of $MgSO_4$:NaCl were investigated to optimize salt amount. It was determined that 1:1 and 2:1 ratios provided the best results, with 500mg $MgSO_4$:500mg NaCl providing the optimal peak area, seen in Figure 2-6. This is different from other commonly used methods which utilize a 4:1 ratio of $MgSO_4$:NaCl as

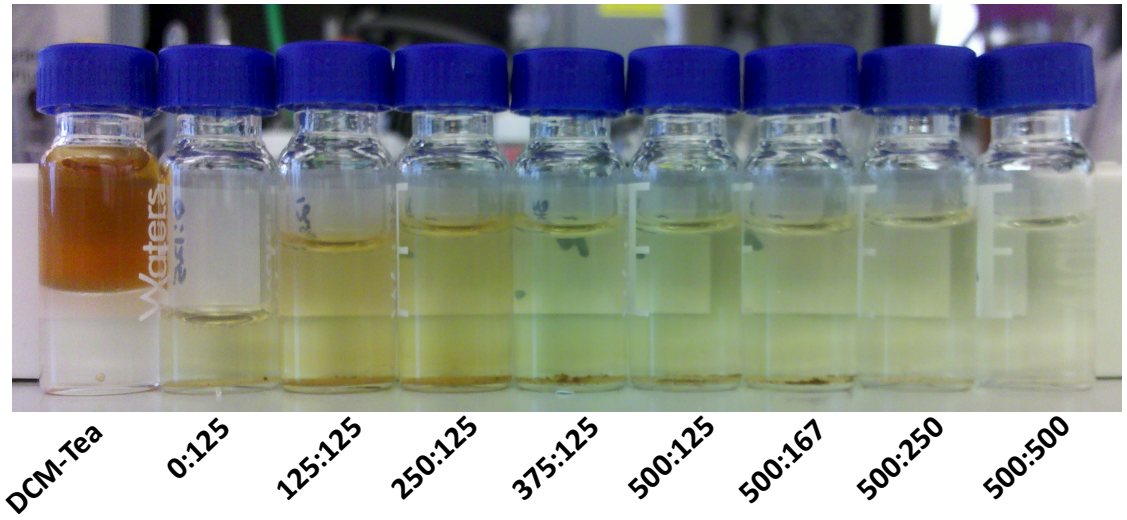


Figure 2-5. A visual example showing the effect of the salt ratio on the amount of organic solvent separated from the aqueous phase and the extraction of matrix interferences.

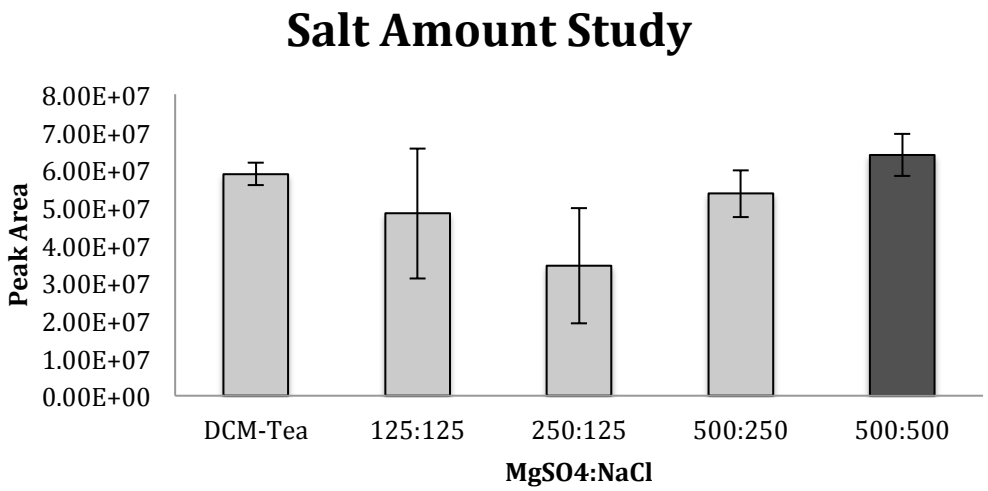


Figure 2-6. Optimization of salt ratio amount during the LLE step of the QuEChERS method

obtained from the original method parameters first established by Anastassiades and co-workers [3].

Optimization of salt type was also performed. NaCl is the standard salt used in addition to $MgSO_4$ due to its cost and availability; however, it was of interest to evaluate other salts to determine if there was a pattern to be observed on peak area depending on parameters such as size of atomic radius or electronegativity. Though NaCl did provide the best results, $MgCl_2$ provided similar results and could be used in the LLE extraction as well.

When peak area was plotted for each salt investigated (Figure 2-7), there was not much of a trend observed when evaluating the elements of each cation in the compound including atomic radii, electronegativity, and electron affinity. When looking at compound properties such as solubility, density, mass, melting point, and dipole moment, no observable trend was seen. It should be noted that the difference between the highest and lowest peak area was not a full order of magnitude (NaCl: 8.77×10^7; LiCl: 3.57×10^7), thus there may not be a great enough difference to observe a consistent trend as some of the standard deviations overlap each other. It would be of interest to investigate properties such as solvated radius, acidity, conductivity, and number of ions in solution. As caffeine is positively charged at pH 6, the number of chloride ions in particular could affect the ability of the extraction. It would be of interest to investigate these salts using the same molarity of each salt solution rather than the use of salt amount. This may help to normalize the data for the investigation of ionic strength as an affect on the process.

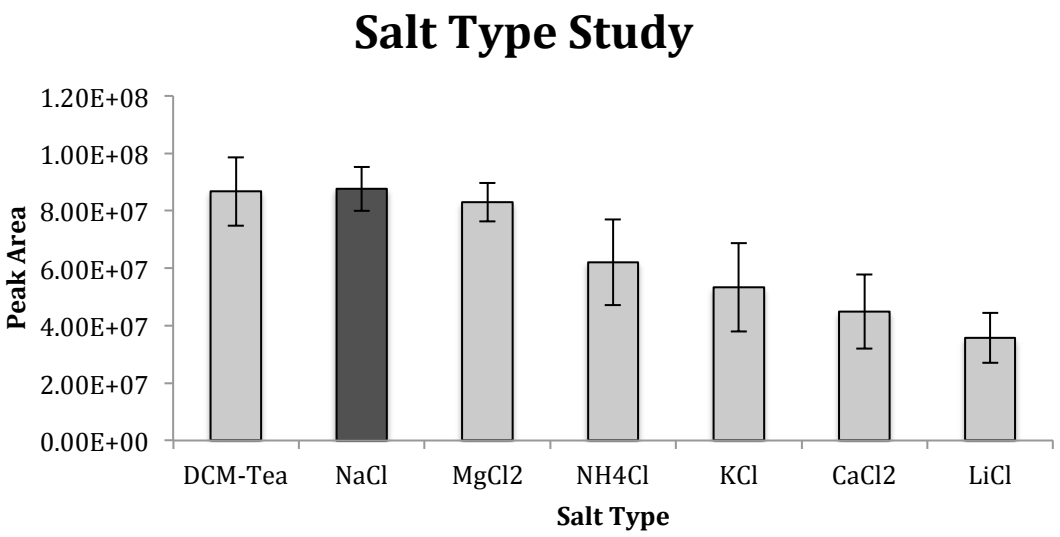


Figure 2-7. Optimization of the salt type during the LLE step of the QuEChERS method.

All of these parameters should be investigated in the future to further explore a trend in the data.

Solvent amount and type were optimized next and are pictured in Figures 2-8 and 2-9, respectively. Three different ratios of acetonitrile:aqueous tea sample were investigated and it was determined that a ratio of 1:2 (2mL ACN:4mL aqueous tea) provided a greater extraction of caffeine than a typical 1:1 ratio of organic solvent to aqueous sample. This was expected, as with an increase in aqueous sample amount there will be an increase in caffeine present to be extracted.

Finally, the solvent type was optimized between acetonitrile (ACN), acetone (ACE), ethyl acetate (EtOAc), ethanol (EtOH), and various ratios of EtOH:H_2O. It was found that all of the ethanol and ethanol:water samples were unable to be separated from the aqueous sample, thus only caffeine peak areas for ACE, EtOAc, and ACN were plotted. From Figure 2-9 it was determined that ACN had optimal results. Acetonitrile does not have as great an ability to form hydrogen bonds with water as other solvents such as ethanol, acetone, and ethyl acetate, and thus this allows for a greater separation between the aqueous and organic layer as opposed to the use of a solvent such as ethanol, which cannot be separated from water using this method. This fact also provided a more stable environment for the extraction of caffeine than other solvents most likely due to the lower vapor pressure of ACN as compared to ethyl acetate and acetone. The latter two solvents will evaporate more readily than ACN, causing inconsistent results in the amount of caffeine extracted.

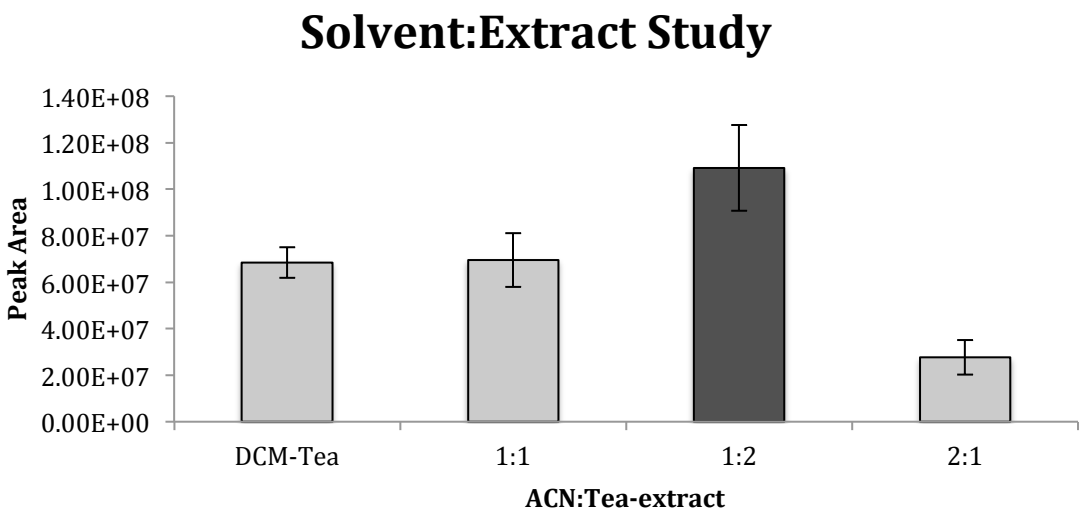


Figure 2-8. Optimization of the ratio of organic solvent to aqueous tea sample during the LLE step of the QuEChERS method.

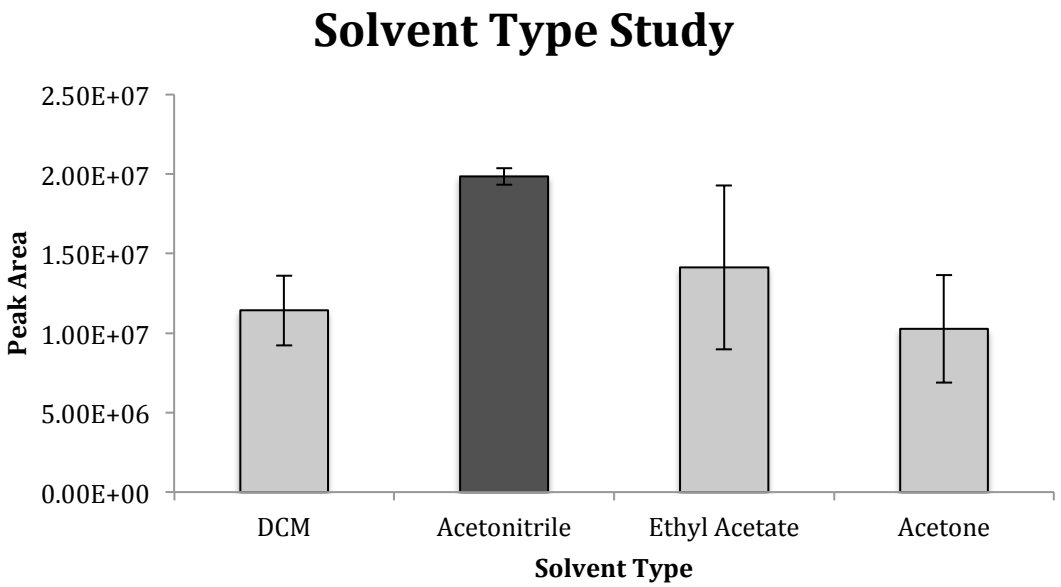


Figure 2-9. Optimization of the organic solvent type during the LLE step of the QuEChERS method.

3.1.3. Extraction kinetics: LLE and d-SPE sonication time

As aforementioned, the extraction kinetics was analyzed via optimization of the sonication time following a 1-minute vortexing of the sample. It was found that in the LLE sonication step, there were no significant changes throughout minutes 0-10 seen in Figure 2-10, indicating that the extraction is occurring primarily during the 1-minute vortexing period. This was also observed during the d-SPE step seen in Figure 2-11, where a negative effect was observed as time progressed indicating that the caffeine was binding to the sorbent as time continued during the sonication.

This hypothesis of caffeine binding was found to be true as a back extraction with ACN showed that there was an increased amount of caffeine recovered from the sorbents from the longer sonication times. When the peak area was plotted vs. time during the LLE sonication period, a slight upward slope was observed, with no curvature, indicating a zero order kinetics extraction; however, there is not enough of a change occurring to make any conclusions regarding the kinetics.

The same linear trend is true for the d-SPE step, though again it was difficult to make any conclusions regarding the kinetics, as the extraction was mostly complete during the vortexing step. One item that can be concluded from this study is that sonication is not necessary and in the instance of the d-SPE step, was actually negatively impacting the extraction of caffeine. Also, sonication can produce hot spots affecting the precision of the results and thus removing it from the method should help to improve the extraction overall.

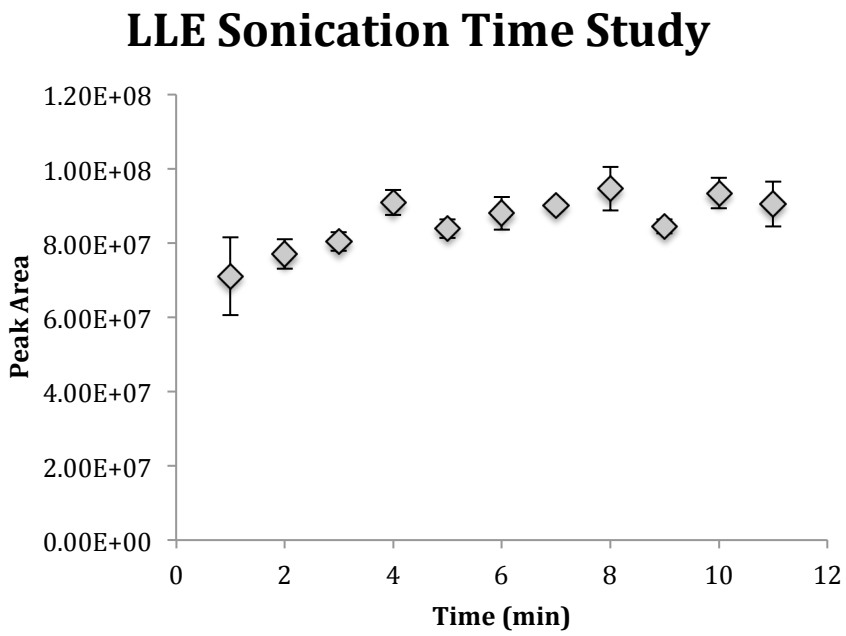


Figure 2-10. Investigation of the effect of time on the sonication time during the LLE step in the QuEChERS method provided the resulting plot of peak area vs. time as a kinetic study.

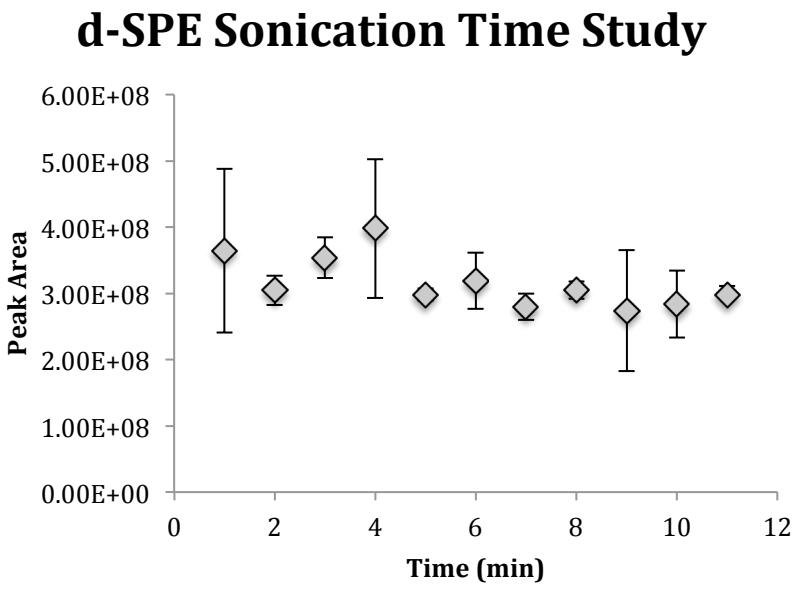


Figure 2-11. Investigation of the effect of time on the sonication time during the d-SPE step in the QuEChERS method provided the resulting plot of peak area vs. time as a kinetic study.

3.1.4. Extraction temperature: LLE and d-SPE sonication temperature

Not only were the kinetics of the extraction investigated, but the effect of temperature on the extraction was as well following the 1-minute vortex. Three temperatures were investigated for both the LLE and d-SPE steps: 0°C, 100°C, and room temperature (23°C). As the extraction was mostly complete after the vortexing step in both portions of QuEChERS, (see section 3.1.3), the optimization of the sonication temperature is not necessary but it was of interest to evaluate the effect of temperature on the system.

In an extraction, equilibrium will occur at a certain temperature. According to LeChatlier's principle, in an exothermic reaction if the temperature of the system is lowered, the extraction will release heat in order to achieve the temperature at which equilibrium will occur. This will cause an increase in the products during a reaction, or in the case of an extraction, an increase in the amount of analyte extracted. This can be seen in Figure 2-12 where the cold sample provided the greatest amount of caffeine extracted compared to the room temperature and heated sample, indicating that the extraction is exothermic and is favored, providing a $-\Delta H$.

For the d-SPE step, all three temperatures appeared to be fairly close to each other in terms of affecting caffeine peak area. Both a hot and room temperature environment produced very similar results with the colder temperature extracting slightly less. This also shows that the d-SPE step is exothermic and favored. The cold sample provided less caffeine remaining in solution; however, this indicates that the extraction of caffeine from the organic solvent to the solid sorbent occurs at a greater abundance than with the other

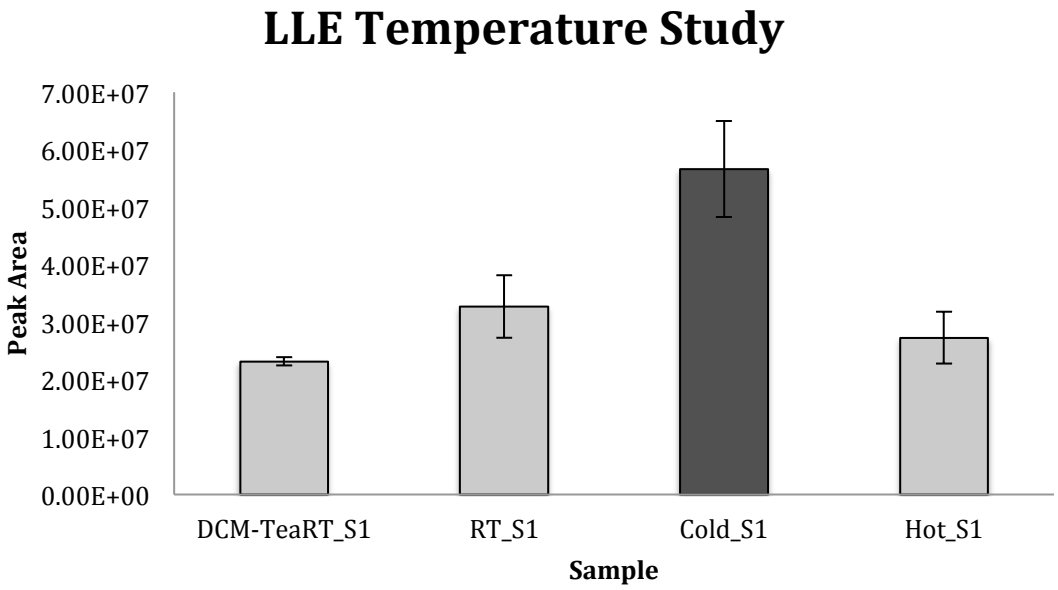


Figure 2-12. Investigation of the effect of temperature during the LLE step in the QuEChERS method.

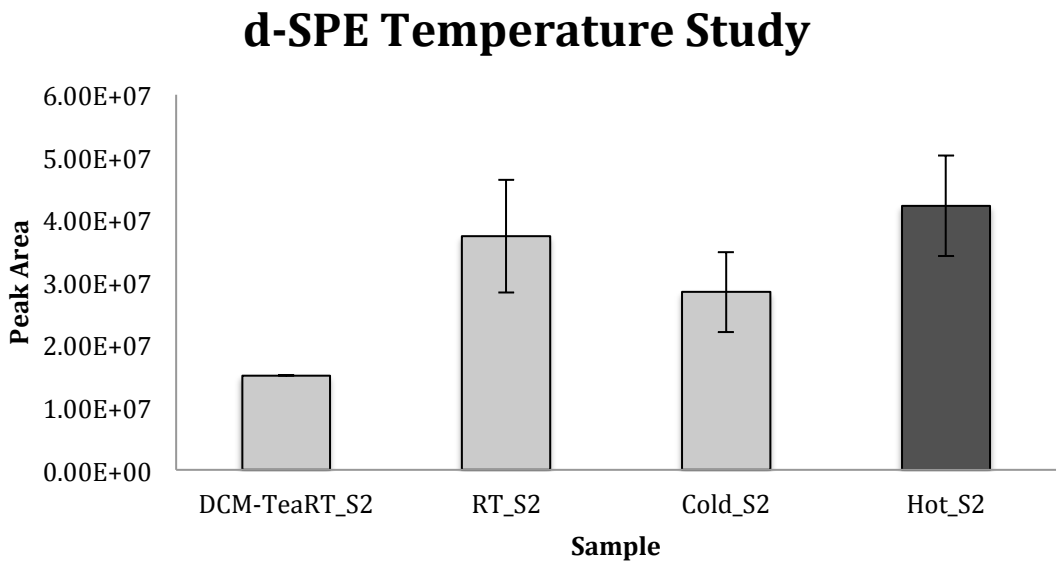


Figure 2-13. Investigation of the effect of temperature during the d-SPE step in the QuEChERS method.

two temperatures. Thus, the extraction is exothermic and favored, providing a -ΔH once again. Another reason for less caffeine remaining after the cold temperature study could be that cold temperature had a negative impact and promoted the binding of caffeine to the sorbent more so than a warmer temperature. Heat and a warmer environment in general could cause the molecules to be more active, whereas in a colder environment the molecules could be less active and thus more likely to bind to the sorbent as opposed to the more active molecules in the warmer system. Given this theory, the warmest system (100°C) would perform better than the other two, which can be seen in Figure 2-13 where the heated system performs slightly better than the room temperature and colder systems.

3.3. Comparison with LLE

It should be noted that with each optimization performed there was a comparison made to a simple DCM-tea sample shake. In each optimization, the QuEChERS extraction provided increased peak areas indicating its ability to perform better than or as equally well as the DCM-tea sample shake. The DCM-tea shake was plotted with each Figure during the above studies to illustrate the improved extraction ability when QuEChERS was used.

3.4. Validation

3.4.1. Calibration curve, linearity, and partition coefficient

Upon analysis caffeine standards of concentrations from 0.1ppb to 100ppm, a calibration curve from 8ppb to 1ppm was plotted as seen in Figure 2-14, providing an R^2 value of

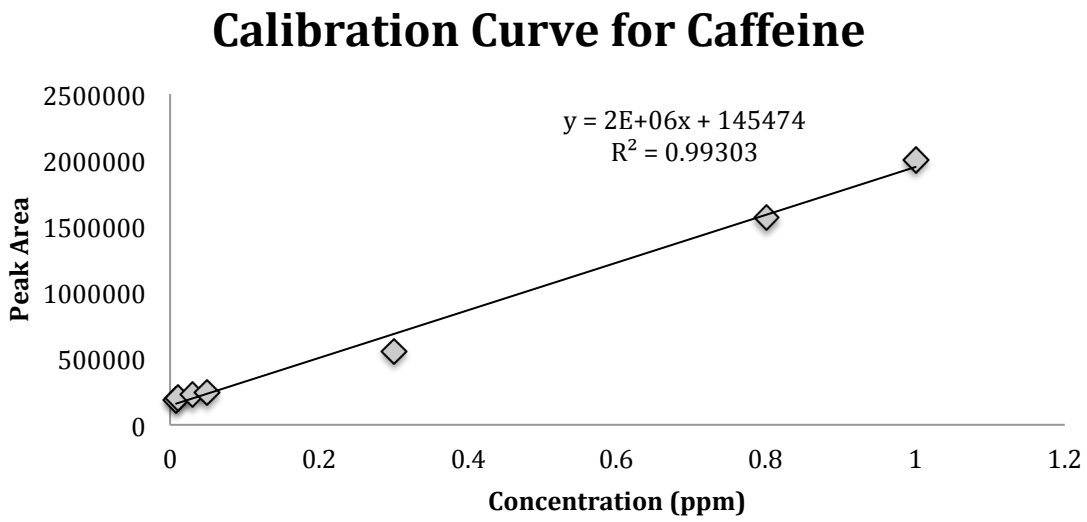


Figure 2-14. Calibration curve from the method validation of the optimized QuEChERS extraction.

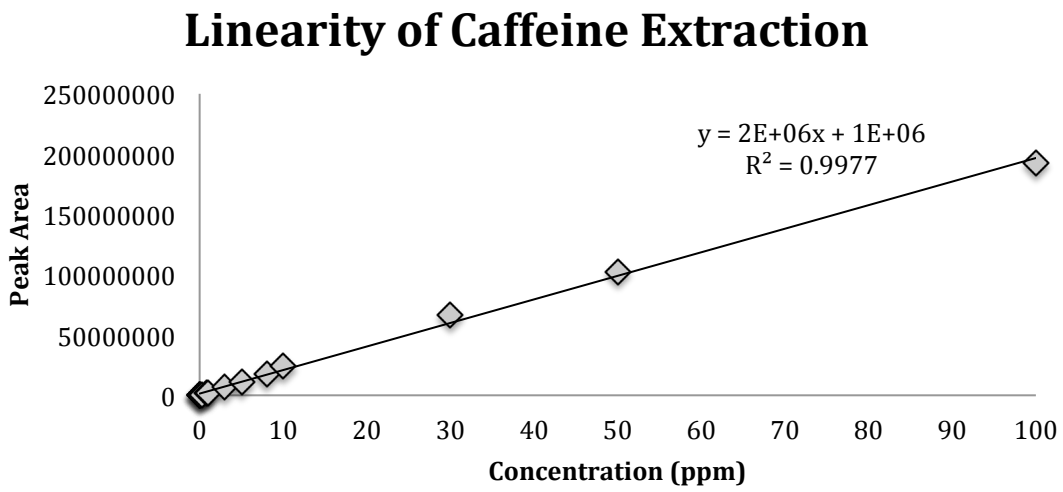


Figure 2-15. Linearity of caffeine extraction from the method validation of the optimized QuEChERS extraction.

0.993, an LOD of 8ppb (3S/N) and an LOQ of 50ppb (10S/N). It was determined that the linearity of the calibration curve could be extended over five orders of magnitude from 8ppb to 100ppm as shown in Figure 2-15, providing an R^2 value of 0.998. The LOD and LOQ determined using the software were also confirmed by using the following equation:

$$LOD = \frac{3s_B}{m} = \frac{3(4310.52)}{2x10^6} = 0.007 \quad \text{(Equation 2-6)}$$

The partition coefficient was determined using the equation of the line from the calibration curve ($y=2x10^6x+ 145474$) as seen in the set of equations below (Equations 2-7 to 2-12). The concentration used for determination of the partition coefficient was the LOQ, 50ppb (0.05ppm). This was performed three times yielding three partition coefficients ranging from 1.8 to 2.6 with an average of 2.1±0.4. In terms of the effect of the addition of salts to the system that are used during the QuEChERS method, it would be expected that the addition of salts to the system for a compound that is more soluble in water than acetonitrile would cause an increase in the partition coefficient as opposed to a compound that has less solubility in water. A partition coefficient greater than 1 supports the data found during the temperature study of a favorable extraction, as a partition coefficient greater than 1 indicates a $-\Delta G$. The temperature study showed an exothermic reaction that has a $-\Delta H$. The change in entropy would be minimal as the difference between water and acetonitrile is negligible for these purposes. A $-\Delta H$ and a small change in entropy would provide a $-\Delta G$, as supported by the calculated partition

Table 2-1. Summary table of important variables used to determine the partition coefficient in Equations 2-6 to 2-11.

Calibration Curve Line Equation	*y = 2E+06x + 145474*
Peak Area (0.05ppm sample)	241183
Calculated Concentration using Calibration Curve (c_{org})	0.048
Volume of Organic Phase (v_{org})	2mL (0.002L)
Volume of Aqueous Phase (v_{aq})	4mL (0.004L)

$$c_{org} = \frac{241183-145474}{2x10^6} = 0.048 \quad \text{(Equation 2-7)}$$

$$m_{org} = (c_{org})(v_{org}) = (0.048)(0.002L) = 9.57x10^{-5} \quad \text{(Equation 2-8)}$$

$$m_{aq(initial)} = (c_{aq})(v_{aq}) = (0.05ppm)(0.004L) = 2.0x10^{-4} \quad \text{(Equation 2-9)}$$

$$m_{aqu} = m_{aq(initial)} - m_{org} = 2.0x10^{-4} - 9.57x10^{-5} = 1.04x10^{-4} \quad \text{(Equation 2-10)}$$

$$c_{aq} = \frac{m_{aq}}{v_{aq}} = \frac{1.04x10^{-4}}{0.004L} = 0.026 \quad \text{(Equation 2-11)}$$

$$K = \frac{c_{organic}}{c_{aqueous}} = \frac{0.048}{0.026} = 1.84 \quad \text{(Equation 2-12)}$$

coefficient of 2.1. Using the calibration curve the average amount of caffeine present in a 4mL aliquot taken from a 20mL sample in which 0.8g of loose tea was sonicated (roughly 0.16g of tea/4mL) was determined. It was found that in this 4mL sample, after performing the QuEChERS optimization, that the average amount of caffeine present between three samples was approximately 171ppm. A tea bag contains approximately 1.95g of tea, thus in 1.95g of tea there would be an approximate caffeine concentration of 2,084ppm for the Wagh Bakri Masala Chai tea used. A summary of the LOD, LOQ, and partition coefficient data can be seen in section 3.4.2.

3.4.2. Recovery, precision, accuracy

Four samples were analyzed at the LOQ (50ppb) for determination of percent recovery and accuracy. To determine percent recovery, the concentration was found from the resulting peak area using the equation of the line for the calibration curve. This value was divided by 50ppb and multiplied by 100. These four samples gave an average percent recovery of 96.55% and an average percent error of 3.45% (accuracy). The intraday and interday precisions were determined using fives samples run at the LOQ (50ppb) and calculating the %RSD corresponding to each set of data. Day 1 yielded a precision of 3.70%, day 2 had a precision of 5.23%, and the precision between the two days (interday) was 11.35%.

Table 2-2. Summary of method validation results.

LOD (ppb)	LOQ (ppb)	Partition Coefficient	% Recovery (%; n=4)	Accuracy (%Error; n=4)	Intraday Precision (%RSD)	Interday Precision (%RSD)
8	50	2.1 ± 0.4	96.55	3.45	Day 1: 3.70 Day 2: 5.23	11.35

4. Conclusion

The extraction of caffeine has long been used whether it be for the steeping of tea or an experiment performed in a laboratory. Caffeine is a model analyte that has been studied many times and was chosen in this study for its similarities in structure and properties to other drugs that could be studied including pharmaceuticals and drugs of abuse using QuEChERS. The ability of QuEChERS to extract compounds such as pesticides from food products with high recoveries has been proven but the fundamental chemistry involved in each step of this method had not been evaluated in great detail.

This study was able to study several parameters for the QuEChERS method and evaluate them at a chemical level. The function of each salt used during the LLE step was clearly defined and the effect of these salts on the partition coefficient was discussed. It was also determined that acetonitrile not only has the ability to extract over a wide range of analytes, but that it also works as a better solvent due to its decreased ability of hydrogen bonding compared to other solvents that can be used in the QuEChERS method. Parameters such as extraction kinetics and effects of temperature on the extraction were evaluated in determining how time and temperature effect the extraction that occurs in both the LLE and d-SPE portion of the method. It was concluded that a majority of the extraction was complete after the 1-minute vortex and thus further sonication or shaking is not necessary, decreasing the time needed for sample preparation as most methods call for a 5-10 minute shaking.

It was seen that steeping the tea in hot water provided increased extraction of caffeine as expected, whereas during the LLE a colder environment seemed to provide the best result. The d-SPE step performed best at a room temperature or warmer environment as cold actually allowed for binding of the caffeine to the sorbent to occur. The optimization of these parameters allowed for a very efficient extraction of caffeine from tea using acetonitrile with a partition coefficient of 2.1, an average recovery of 96.6%, and precision values below 6% for intraday precision and 12% for interday precision. It was also determined that the average concentration of caffeine present in 1 tea bag (1.95g of tea) was 2,084ppm. The optimized method provided more than acceptable validation results, and each optimized parameter resulted in a greater amount of caffeine extracted than a simple DCM-tea sample shake as used for standard comparison.

The affect of increasing the pH of the aqueous phase used during the LLE was investigated using a 10ppm caffeine standard. A 0.2M potassium chloride solution and 0.2M sodium hydroxide solution were used to prepare a pH 13 buffer. This buffer was used to prepare a 10ppm caffeine standard and was compared to a pH 6, 10ppm caffeine standard in an aqueous solution with no buffer during the LLE. It was found that the peak area increased from 5.23×10^6 to 9.45×10^6 when using a pH 13 buffered aqueous solution during the LLE. Thus, it would be of interest in the future to perform further studies on the extraction using this buffered method and compare the results to those determined in this research.

CHAPTER 3 – EXTRACTION OF GLUCOCORTICOIDS AND HORMONES FROM WATER AND HERBAL MEDICINAL PRODUCTS USING QUECHERS VIA GC-MS/MS ANALYSIS

The use of herbal medicinal products (HMPs) as treatments has become more common as they are viewed as safe and natural; however, the adulteration of these medicines has become more frequent. Though the practice is prohibited, drugs such as steroids are added to these medicines in order to speed the healing process; thus an analytical method is necessary to identify the presence of these adulterants. It is also desired that this be a simple method in both sample preparation and data analysis, meaning that the resulting chromatogram be clean and easy to interpret. In the past, high-performance liquid chromatography (HPLC) with ultraviolet (UV) or mass spectrometry (MS) detection has been used to detect steroids in the herbal medicines. One study utilized the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method for sample preparation before HPLC analysis [29].

This study investigated the detection of 8 glucocorticoids and 7 hormones in herbal medicinal products by optimizing a QuEChERS extraction method for steroids from water and HMPs using gas chromatography triple quadrupole mass spectrometry (GC-MS/MS). The first 8 steroids were chosen for their ability to reduce inflammation. The detection of these 8 steroids in herbal medicines that target joint treatment was investigated using optimized QuEChERS and GC-MS/MS methods. It was determined

that a buffer was not necessary for use in this method, and that 500mg of $MgSO_4$ and NaCl as well as acetonitrile provided the best extraction of these steroids. The validation of this method for the glucocorticoids of interest provided precision, both interday and intraday, less than 9%, percent recoveries greater than 83%, and partition coefficients between 0.73 and 0.88. These partition coefficients were similar to those observed with the caffeine study in Chapter 2 as expected. The validated QuEChERS method was also used for the extraction of the 7 hormones from water as well as determination of the presence of these compounds in the herbal medicinal products analyzed previously.

1. Introduction

1.1. GC-MS/MS Instrumentation

The same basic principles discussed in Chapter 1 for gas chromatography and separation apply to triple quadrupole mass spectrometry as well. The main difference in this instrument is not the chromatographic separation, but the detector. In Chapter 1, a quadrupole mass spectrometer was discussed as coupled to gas chromatography (GC-MS). This instrumentation was used in the caffeine study from Chapter 2. For this application, a more complex detector, a triple quadrupole, also known as a tandem mass spectrometer, was used. The four parallel rods with alternating currents are still present as in a single quadrupole mass spectrometer; however, rather than a single quadrupole there are now multiple quadrupoles operated as a tandem mass spectrometer.

Figure 3-1 depicts how a triple quadrupole operates. There are three quadrupoles that compose the mass analyzer portion of a triple quadrupole, as its name indicates. Once the sample has undergone ionization, the ions enter the first quadrupole, or Q1, which acts as a mass filter that can scan an entire range of mass-to-charge ratios or be fixed for certain ions. This, in essence, performs as a single quadrupole would with the ion source and detector on either side, except here the quadrupole is followed by two more quadrupoles prior to the detector [67-70].

After Q1, these ions pass to Q2, a collision cell where an inert collision gas such as argon or nitrogen is used to cause further fragmentation of the ions in a process known as collision induced dissociation (CID). This is similar to the process that occurs in the ion source; however, this process takes place at a lower energy level thus the fragmentation caused is not as severe as can occur during electron ionization in the ion source.

Once complete, the ions travel to the third quadrupole, Q3, which acts as a second mass filter as in Q1. Once again, specific ions can be chosen to continue to the detector or the quadrupole can allow all ions to pass in full scan mode. This process is very similar to scan versus SIM mode in a quadrupole but with two mass filters rather than one. If ions are selected that are unique to a particular compound in both mass filters, this instrumentation has the ability to be very selective and specific to a certain compound. This instrument is also conducive for separating a mixture in which compounds co-elute and may not be able to be separated using a single quadrupole.

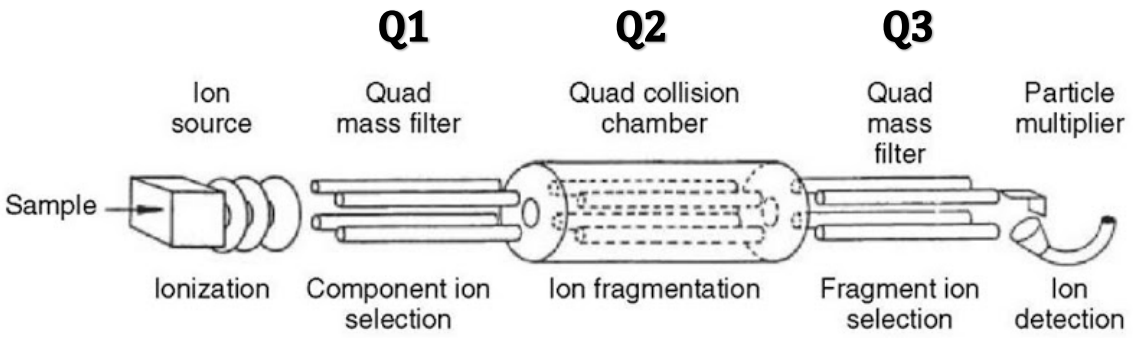


Figure 3-1. Schematic of a triple quadrupole mass spectrometer. Adapted from [71].

There are many operational modes for a triple quadrupole because there are two mass filters that can either be set in full scan mode or fixed for certain ions. When optimizing a multiple reaction monitoring (MRM) method, the first step is to perform a full scan of all the compounds in both mass filters. This allows for the determination of ions that are specific to each compound and are known as precursor ions. This process can be thought of as collecting a total ion chromatogram when using a single quadrupole mass spectrometer.

Once these ions are optimized for each compound, a product ion scan is performed. During a product ion scan, Q1 is fixed and Q3 is scanning in order to determine the resulting product ions that are produced from collision of the precursor ion in Q2 with the inert gas. The collision energy for each transition of precursor to product ion can be different and must be optimized in order to obtain optimal results. In the instrument used during this study, argon gas was used as the collision gas in the collision chamber.

In the final MRM method, both Q1 and Q3 are fixed for the determined precursor and product ions, respectively, and the collision energy has been optimized for each transition of precursor to product ion. A summary of a product ion scan and MRM scan can be seen in Figure 3-2.

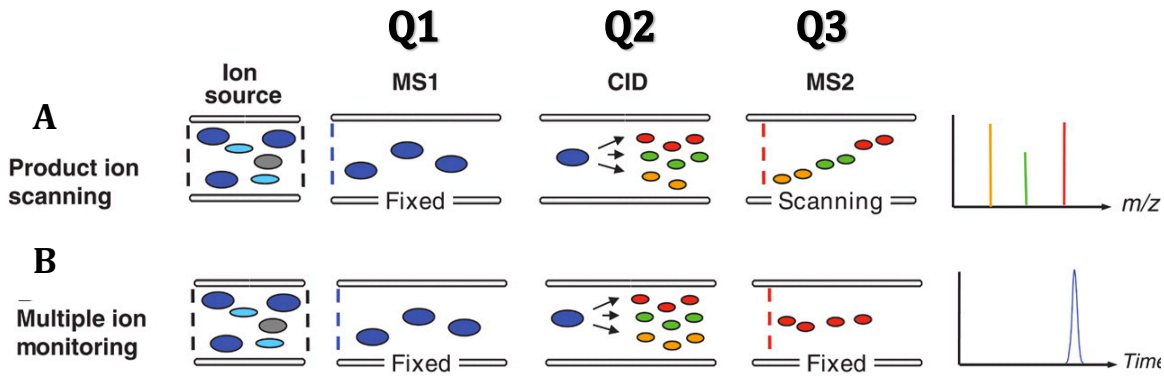


Figure 3-2. Schematic of two triple quadrupole mass spectrometer modes: product ion scan (A) and MRM scan (B). Adapted from [72].

In order to describe the optimization process in creating an MRM method, a step-by-step procedure is described below for one of the hormones of interest, prasterone, pictured in Figures 3-3 to 3-5. To begin, a full scan must be performed in order to obtain the retention time of the compound and a mass spectrum to choose a precursor ion. Once complete, an ion is chosen from the mass spectrum with a high abundance and relatively high molecular weight to insure fragmentation occurs during CID. If present, the molecular ion is the optimal precursor ion to choose. A product ion scan is then performed to determine product ions. Multiple precursor ions should be evaluated in separate product ion scans to determine the optimal precursor and product ions. The product ions are evaluated using product ion scans at differing collision energies. The collision energies for the product ions are chosen based on the base peak of the mass spectrum. When the base peak becomes an ion other than the precursor ion, then this ion should be chosen as a product ion at the collision energy that provides the highest abundance. Occasionally, a second ion may not become the base peak but is still at relatively high abundance. This instance is seen in Figure 3-4.

2. Materials and Methods

2.1. Chemicals, Reagents, and Samples

Acetonitrile (ACN) was purchased from pharmco-AAPER (Kindermorgan, PA) and was reagent ACS grade. All salts used throughout the study as well as the steroid standards were purchased from Sigma Aldrich (St. Louis, MO). QuEChERS tubes containing 150mg PSA and 50mg $MgSO_4$ were purchased from Restek (Bellefonte, PA) and 15mL

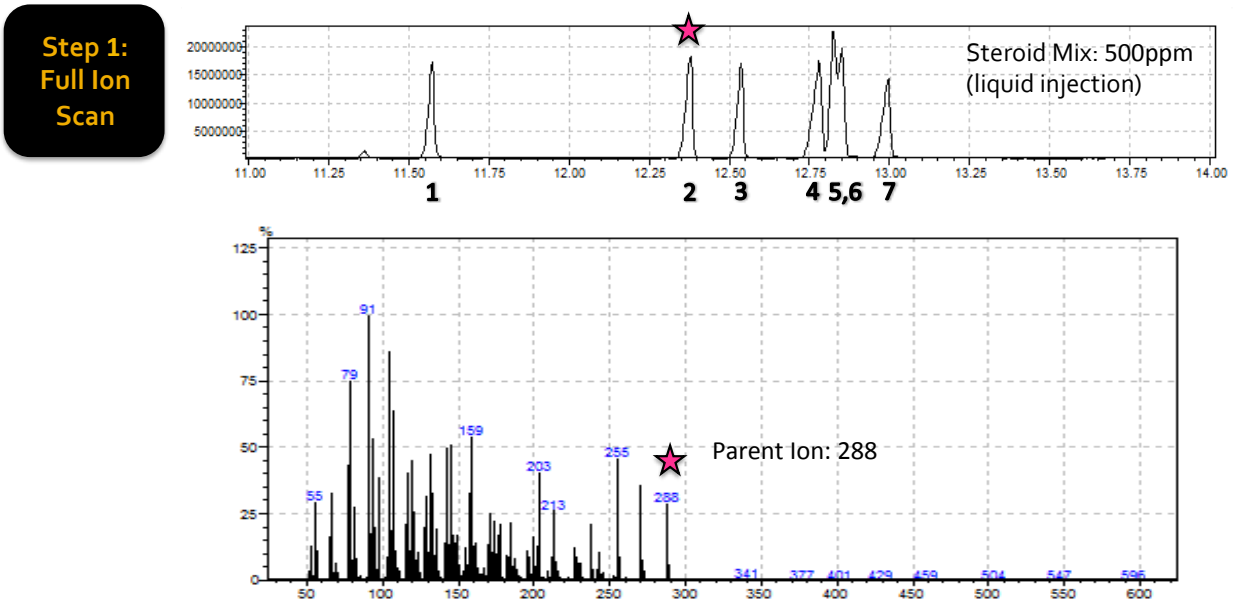


Figure 3-3. Step 1 in optimizing an MRM method. Top chromatogram: full scan of sample (star denotes prasterone). Bottom spectrum: the mass spectrum of the chosen precursor ion for prasterone.

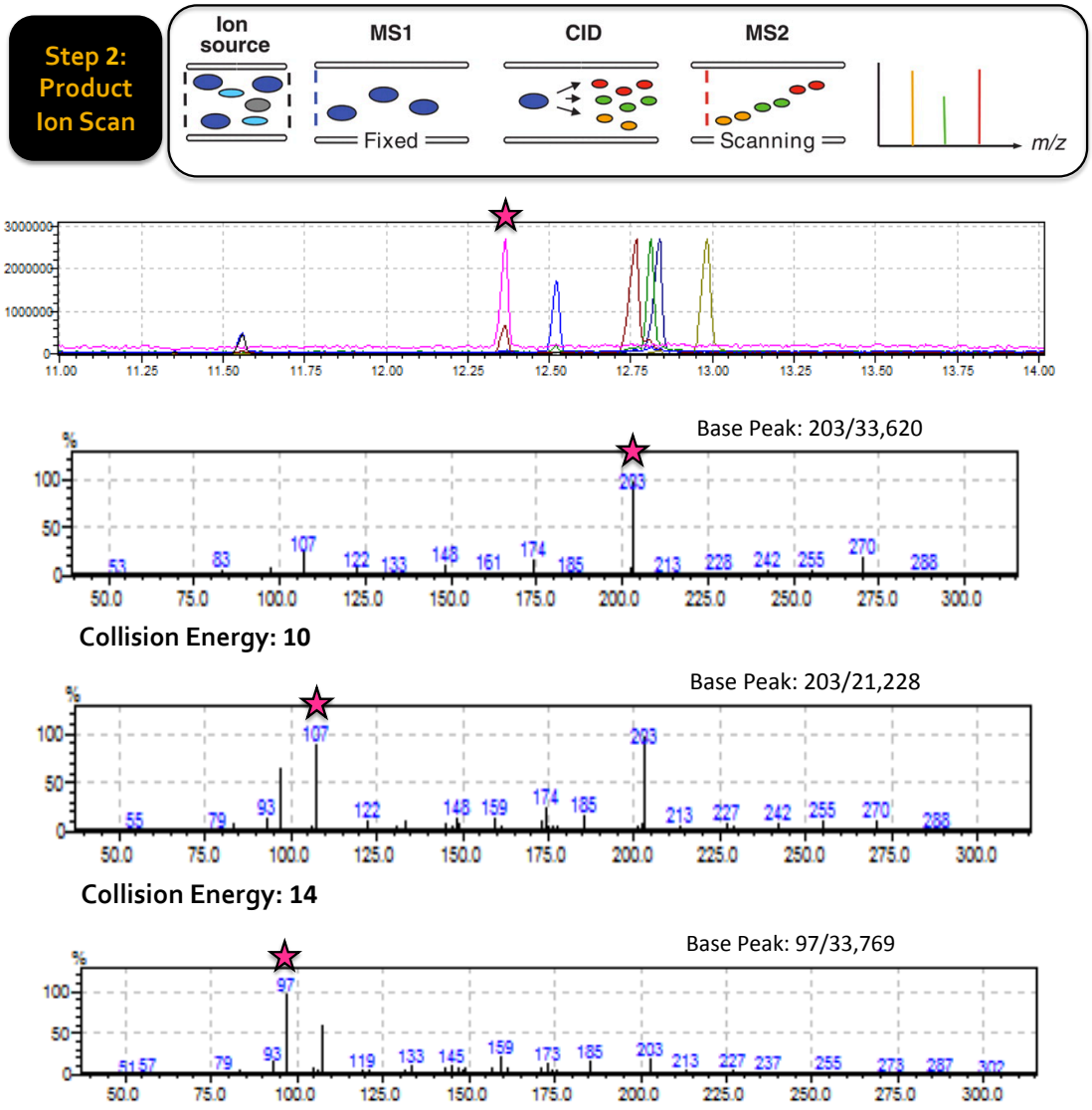
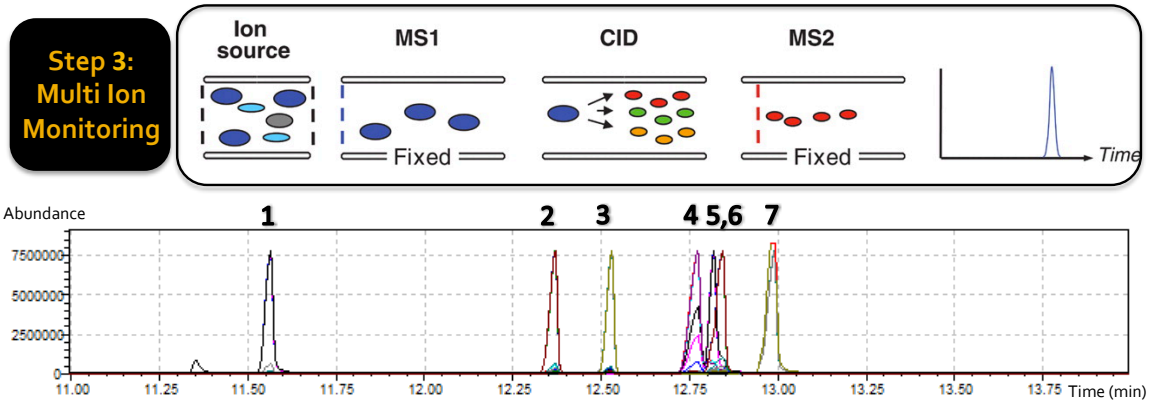


Figure 3-4. Step 2 in optimizing an MRM method. Top chromatogram: product ion scan of sample (star denotes prasterone). Bottom three spectra: the mass spectra for each of the chosen precursor ions at their respective collision energies. Note that spectrum 2 provides an example of the precursor ion not being the base peak for that collision energy.



Steroid	Precursor Ion	Product Ions	Collision Energy (eV)
1) Diethylstilbestrol (DES)	268	239; 145; 107	10; 18; 26
2) Prasterone	**288**	**203; 107; 97**	**10; 14; 20**
3) Methandriol	253	197; 155; 169	16; 26; 26
4) Estrone	270	185; 157; 172	14; 26; 20
5) Estradiol	272	185; 172; 213	8; 14; 16
6) Mesterolone	218	159; 105; 200	14; 26; 8
7) Boldenone	122	107; 77; 79	16; 26; 18

Figure 3-5. Step 3 in optimizing an MRM method. Top chromatogram: optimized MRM method for the sample. The table illustrates the parameters used in the optimized MRM method.

PFTE centrifuge tubes were obtained from VWR International (Radnor, PA). All herbal medicines were obtained from Auyurvedic Herbs Direct (Torrance, CA). Deionized water was used throughout the methodology.

2.2. Sample Preparation

The pH, salt amount, and type of solvent were all evaluated in the optimization of the QuEChERS method for the extraction of 8 glucocorticoids: beclomethasone, cortisone acetate, prednisone, hydrocortisone, prednisolone, fludrocortisone acetate, dexamethasone, and methylprednisolone. The optimization was performed using the glucocorticoids prepared in water at concentrations of 200ppm (hydrocortisone, prednisolone, dexamethasone, methylprednisolone) and 400ppm (beclomethasone, cortisone acetate, prednisone, fludrocortisone acetate). In addition to the glucocorticoids, these optimized conditions were used for the extraction of 7 hormones of interest from water at concentrations of 500ppm for each steroid (diethylstilbestrol, prasterone, methandriol, estrone, estradiol, mesterolone, and boldenone).

The QuEChERS extraction involves both a LLE step and d-SPE clean up step. During the LLE portion of the method, the pH, salt amount, and solvent type were optimized for the 8 glucocorticoids in Table 3-1. In this study, pH was investigated at pH 6 and 7, with the latter being achieved through the use of a phosphate buffer prepared using 0.2M monobasic potassium phosphate and 0.2M sodium hydroxide solution according to the

Table 3-1. Summary of glucocorticoids commonly used for adulteration in herbal medicines.

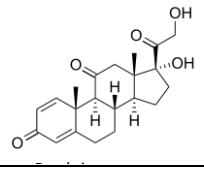
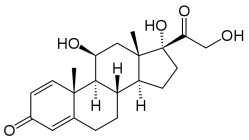
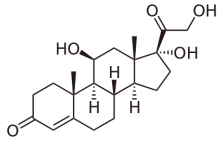
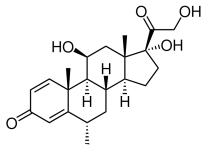
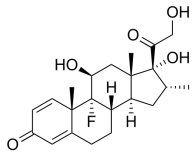
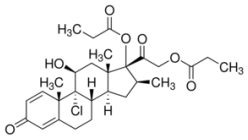
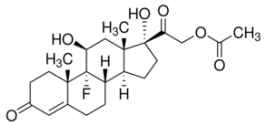
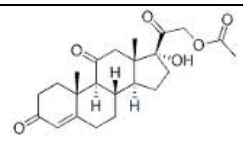
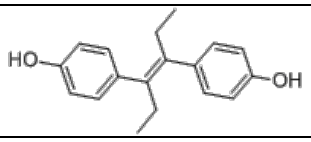
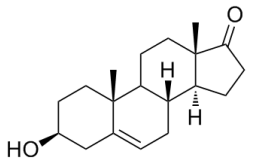
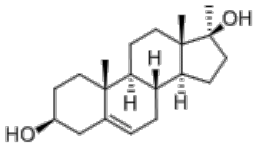
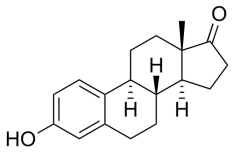
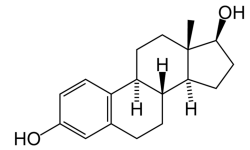
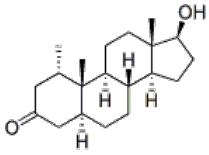
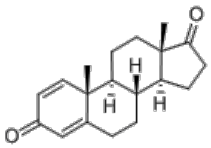
Steroid	Synonym	Classification	Structure
Prednisone	17,21-dihydroxypregna-1,4-diene-3,11,20-trione	Synthetic corticosteroid	
Prednisolone	1,4-Pregnadiene-11β,17α,21-triol-3,20-dione	Corticosteroid (active metabolite of prednisone)	
Hydrocortisone (cortisol)	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione,	Glucocorticoid	
Methylprednisolone	11β,17α,21-Trihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione	Synthetic glucocorticoid (variant of prednisolone)	
Dexamethasone	(11β,16α)-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	Synthetic glucocorticoid	
Beclomethasone	9-Chloro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione 17,21-dipropionate	Glucocorticoid (prodrug in free form)	
Fludrocortisone acetate	9α-Fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione acetate	Synthetic corticosteroid	
Cortisone acetate	17α,21-Dihydroxy-4-pregnene-3,11,20-trione 21-acetate	Glucocorticoid	

Table 3-2. Summary of hormones investigated for detection in herbal medicines.

Steroid	Synonym	Classification	Structure
Diethylstilbestrol (DES)	4,4'-(3E)- hex-3-ene-3,4-diyldiphenol	Synthetic non-steroidal estrogen	
Prasterone	(3S,8R,9S,10R,13S,14S)-3-hydroxy-10,13-dimethyl-1,2,3,4,7,8,9,11,12,14,15,16-dodecahydrocyclopenta[a]phenanthren-17-one	Steroid hormone	
Methandriol	3S,8S,9R,10R,13S,14R,17S)-10,13,17-trimethyl-1,2,3,4,7,8,9,11,12,14,15,16-dodecahydrocyclopenta[a]phenanthrene-3,17-diol	Anabolic steroid	
Estrone	(8R,9S,13S,14S)-3-hydroxy-13-methyl-6,7,8,9,11,12,13,14,15,16-decahydrocyclopenta[a]phenanthren- 17- one	Estrogenic hormone	
Estradiol	(17β)-estra-1,3,5(10)-triene-3,17-diol	Estrogenic hormone	
Mesterolone	1α-methyl-17β-hydroxy-5α-androstan-3-one	Anabolic steroid	
Boldenone	1,4-androstadiene-3,17-dione	Anabolic steroid	

U.S. Pharmacopia phosphate buffer preparation [73]. The optimized pH was 6.0, with the analytes in neutral form, and thus no buffer was used in this methodology. Various ratios of $MgSO_4$:NaCl were investigated including 4:1, 2:1, and 1:1, with 500mg $MgSO_4$ and 500mg NaCl providing optimal extraction of all eight steroids. The salts were placed in a 15mL centrifuge tube followed by 2mL of aqueous sample (a mixture of all 8 steroids) and 2mL of ACN. The organic solvent was investigated using acetone and ethyl acetate as well, with ACN and ethyl acetate providing comparable results. The samples were vortexed for 1-minute and centrifuged for 3-minutes at 1,000rpm. The top organic layer was removed using a Pasteur pipette and transferred to a QuEChERS tube (2mL centrifuge tube with 150mg PSA and 50mg $MgSO_4$). The samples were vortexed for 1-minute and centrifuged for 3-minutes at 8,000rpm. The liquid was then removed and transferred to a GC vial for analysis via splitless injection using GC-MS/MS. These optimized extraction parameters were used during the analysis of 7 hormones as well.

2.3. Instrumental Parameters

The instrumentation utilized for this study was a Shimadzu GC-MS/MS TQ8030 with an AOC-5000 Auto Injector (Santa Clara, CA). Splitless injection was used for all analyses of QuEChERS samples, with multiple reaction monitoring (MRM) being used during GC-MS/MS analysis. The separation and detection parameters for the 8 glucocorticoids and 7 hormones using GC-MS/MS are listed below in tables 3-3, 3-4, and 3-5.

Table 3-3. Method conditions used for GC-MS/MS analysis.

GC-MS/MS		
GC Parameters	*Oven Parameters*	*MS Parameters*
Column: RTX-5MS 15m, 0.25mm, 0.25µm	Initial Temperature: 150°C Hold 1 minute	EI Source: 250°C
Carrier Gas: Helium	15°C/minute Ramp to 300°C Hold 10 minutes	Transfer Line: 280°C
Column Flow: 0.98mL/min Linear Velocity: 51.0cm/sec		
Injection Mode: Splitless		
Inlet Temperature: 250°C		

Table 3-4. MRM method conditions for the 8 glucocorticoids.

Glucocorticoid	**Retention Time (min)**	**Precursor Ion**	**Product Ions**	**Collision Energy**
Beclomethasone	9.227	121	77; 91; 51	20; 13; 25
Cortisone Acetate	9.267	122	107; 77; 79	13; 25; 20
Prednisone	9.374	121	77; 91; 93	20; 13; 7
Hydrocortisone	9.931	163	148; 105; 145	20; 13; 11
Prednisolone	10.088	122	107; 77; 79	13; 25; 20
Fludrocortisone Acetate	10.144	121	77; 91; 93	20; 11; 7
Dexamethasone	10.269	160	145; 127; 115	11; 25; 25
Methylprednisolone	10.372	136	121; 77; 79	11; 25; 25

Table 3-5. MRM method conditions for the 7 hormones.

Hormone	**Retention Time (min)**	**Precursor Ion**	**Product Ions**	**Collision Energy**
Diethylstilbestrol	11.607	268	239; 145; 107	10; 18; 26
Prasterone	12.410	288	203; 107; 97	10; 14; 20
Methandriol	12.563	253	197; 155; 169	16; 26; 26
Estrone	12.820	270	185; 157; 172	14; 26; 20
Estradiol	12.847	272	185; 172; 213	8; 14; 16
Mesterolone	12.877	218	159; 105; 200	14; 26; 8
Boldenone	13.030	122	107; 77; 79	16; 26; 18

2.4. Method Validation

2.4.1. Calibration curve, linearity, and partition coefficient

Glucocorticoid mix standards containing beclomethasone, cortisone acetate, prednisone, hydrocortisone, prednisolone, fludrocortisone acetate, dexamethasone, and methylprednisolone were prepared in deionized water ranging from 10ppb to 500ppm. These samples were then subjected to the optimized QuEChERS method as described in section 2.2 and analyzed in triplicate using GC-MS/MS. Limits of quantitation (LOQ) and detection (LOD) were assessed using the data analysis software where the S/N for the each steroid peak were 10 and 3, respectively. The calibration curve was prepared using samples with concentrations ranging from 5 to 100ppm for all glucocorticoids but fludrocortisone acetate in which 200 to 500ppm concentrations were used. The R^2 value and equation of the line were obtained using Excel for all resulting calibration curves. Once the calibration curve was constructed, the equation of the line could be used to determine the partition coefficient using the following equation as derived in Table 2-1 in Chapter 2:

$$K = \frac{c_{organic}}{c_{aqueous}} \qquad \text{(Equation 3-1)}$$

The partition coefficient for three samples was determined at 50ppm for each steroid excluding fludrocortisone acetate in which 250ppm was used. An average was then taken of these three values.

2.4.2. Recovery, precision, accuracy

Three glucocorticoid mixture samples at 250ppm for fludrocortisone acetate and 50ppm for each of the remaining 7 steroids were prepared and used in the determination of percent recovery and accuracy by using the equation of the line from the calibration curve to find the concentration from the resulting peak area. Once determined, these values were divided by the appropriate concentration (250 or 50ppm) and multiplied by 100 to give percent recovery. An average value was reported. Percent error was determined for each of these three samples as well to assess accuracy of the method and an average value was reported. Six samples at 300ppm were performed and used in the determination of both interday and intraday precision. This high concentration was chosen in order to evaluate the precision of all steroids within the range of fludrocortisone acetate as this glucocorticoid was determined undetectable below 200ppm. The percent RSD was calculated from these six samples for both days individually as well as collectively. All three values were reported in Table 3-6.

$$\%recovery = \left(\frac{experimentally\ determined\ concentration}{LOQ}\right)100 \quad \text{(Equation 3-2)}$$

$$Accuracy: \%error = \left(\frac{LOQ - experimentally\ determined\ concentration}{LOQ}\right)100 \quad \text{(Equation 3-3)}$$

$$Precision: \%RSD = \left(\frac{s}{\bar{x}}\right)100 \quad \text{(Equation 3-4)}$$

2.4.3. Analysis of Real Samples: HMPs

Four herbal medicinal products were investigated for the presence of both the glucocorticoids and hormones using the optimized QuEChERS and MRM methods. These four HMPs encompassed two brands of Boswellia including both vegetarian

tablets as well as capsules, Yogaraj Guggulu tablets, and a revitalizing liquid tonic all targeting joint treatment. Any tablet products were ground and placed into a 10mL volumetric flask where deionized water was added to the mark. The contents of the capsule product were emptied into a 10mL volumetric flask as well with deionized water. For each of these products, 2mL of each were used in the optimized QuEChERS method, whereas 2mL of the liquid tonic was used with no dilution. The optimized QuEChERS method was applied and the sample was analyzed using the aforementioned GC-MS/MS parameters for both the glucocorticoids and hormones.

3. Results and Discussion

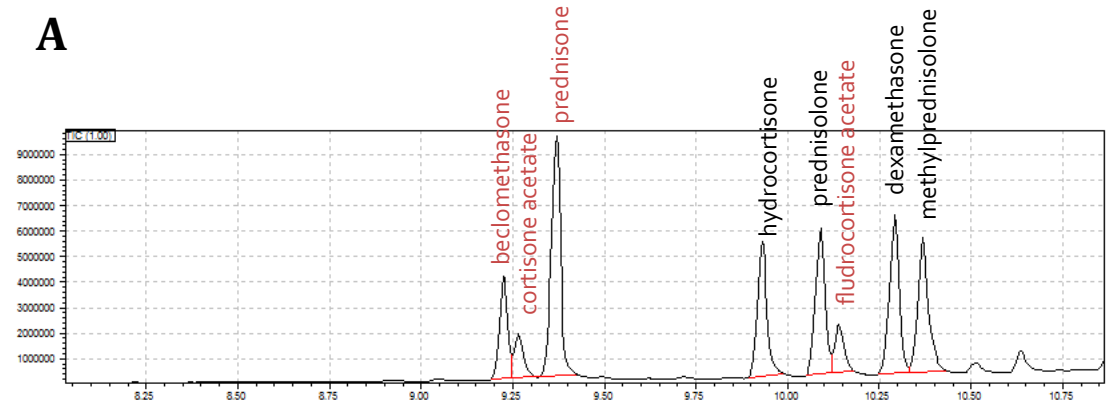
3.1. Optimization of MRM GC-MS/MS Method

In optimizing a multiple reaction method (MRM) for the analysis of the 8 glucocorticoids of interest (beclomethasone, cortisone acetate, prednisone, hydrocortisone, prednisolone, fludrocortisone acetate, dexamethasone, methylprednisolone) and the additional 7 hormones (DES, prasterone, methandriol, estrone, estradiol, methandriol, and boldenone: 500ppm) the first step taken was performing a full scan for all ions between m/z values of 40-500amu. Figures 3-6A and 3-8A depict the full scan chromatograms for both the glucocorticoids and hormones studied, respectively. The methods for both sets of compounds were optimized so as to achieve the best separation in the shortest time. The pairs with partial co-elution before optimizing this method were beclomethasone with cortisone acetate, and prednisolone with fludrocortisone acetate for the first 8 glucocorticoids, and estrone, estradiol, and methandriol for the second set of 7 hormones.

It was difficult to detect beclomethasone, cortisone acetate, prednisone, and fludrocortisone acetate, so their concentrations were increased to 400ppm in order to be better visualized for optimization purposes. Cortisone acetate, beclomethasone, and fludrocortisone acetate have more complicated structures including acetate groups which could be a reason for the difficulty in seeing their presence at lower concentrations using liquid injections as these groups are more prone to degradation in the inlet and possible adherence to the liner in the inlet though a deactivated liner was used throughout all research performed and was changed frequently.

A product ion scan was performed next, in which the ions for each steroid were examined from the mass spectrum for each analyte in the total ion scan. The ions with the highest molecular weight as well as relatively high abundance were chosen and tested. Those that provided the best sensitivity and selectivity for each compound were chosen and are depicted in Figures 3-6B and 3-8B for glucocorticoids and hormones, respectively.

Various collision energies ranging from 5-26eV were applied to each precursor ion to determine the best collision energy for each transition of a precursor ion to a product ion. Three transitions for each compound were chosen as seen in Figures 3-7 and 3-8C for the glucocorticoids and hormones, respectively. It can be seen here how an optimized MRM method can show clear separation of any compounds that were partially co-eluting in the full ion scan.



200ppm: hydrocortisone, prednisolone, dexamethasone, methylprednisolone

400ppm: beclomethasone, cortisone acetate, prednisone, fludrocortisone acetate

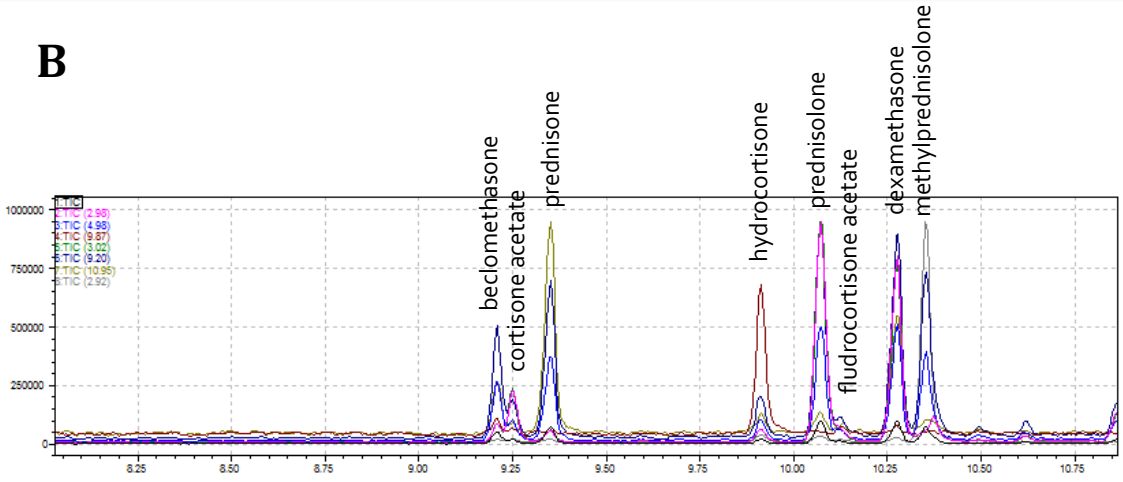
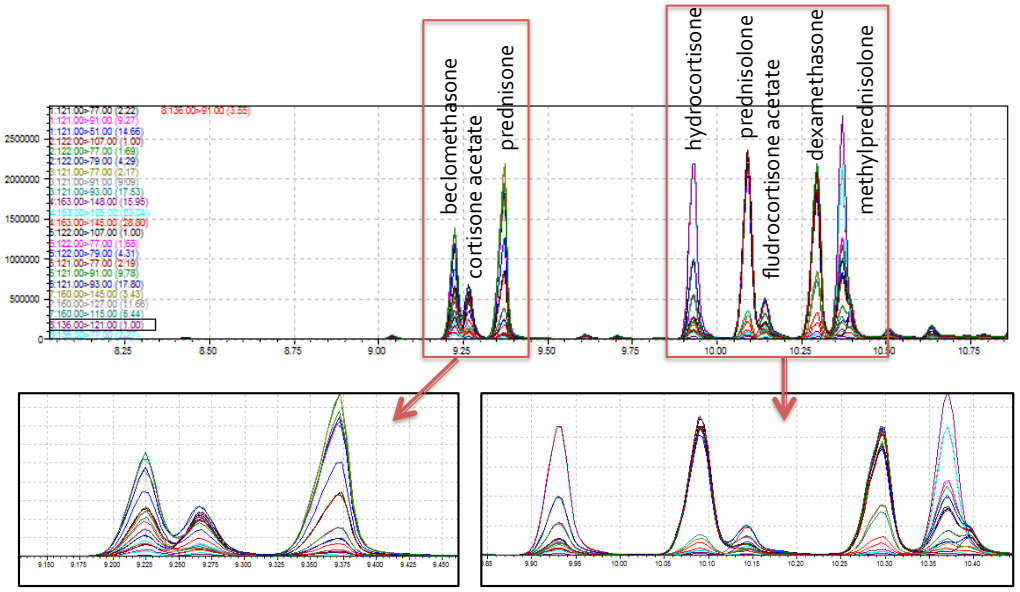
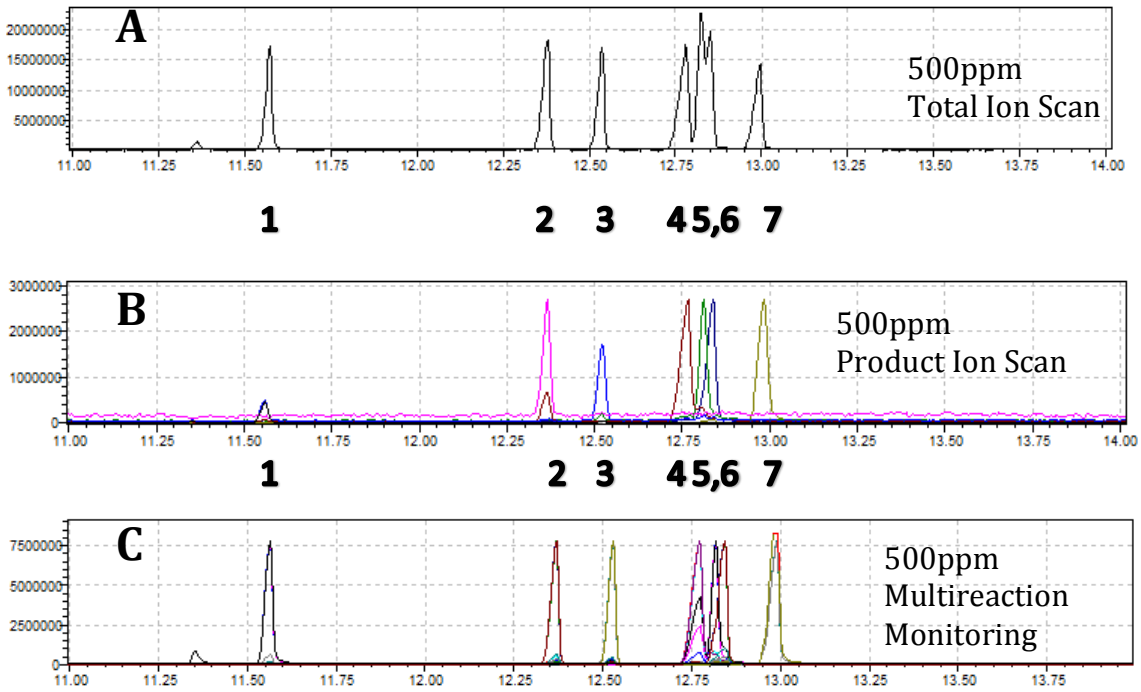


Figure 3-6. A: Full scan (total ion chromatogram) of the 8 glucocorticoids of interest. B: Product Ion Scan of the 8 glucocorticoids of interest.



Steroid	Retention Time (min)	Precursor Ion	Product Ions	Collision Energy
1) Beclomethasone	9.227	121	77; 91; 51	20; 13; 25
2) Cortisone Acetate	9.267	122	107; 77; 79	13; 25; 20
3) Prednisone	9.374	121	77; 91; 93	20; 13; 7
4) Hydrocortisone	9.931	163	148; 105; 145	20; 13; 11
5) Prednisolone	10.088	122	107; 77; 79	13; 25; 20
6) Fludrocortisone Acetate	10.144	121	77; 91; 93	20; 11; 7
7) Dexamethasone	10.296	160	145; 127; 115	11; 25; 25
8) Methylprednisolone	10.372	136	121; 77; 79	11; 25; 25

Figure 3-7. The MRM method parameters (bottom) used in the MRM chromatogram (top) for the 8 glucocorticoids of interest.



D

Steroid	Retention Time (min)	Precursor Ion	Product Ions	Collision Energy
1) DES	11.607	268	239; 145; 107	10; 18; 26
2) Prasterone	12.410	288	203; 107; 97	10; 14; 20
3) Methandriol	12.563	253	197; 155; 169	16; 26; 26
4) Estrone	12.820	270	185; 157; 172	14; 26; 20
5) Estradiol	12.847	272	185; 172; 213	8; 14; 16
6) Mesterolone	12.877	218	159; 105; 200	14; 26; 8
7) Boldenone	13.030	122	107; 77; 79	16; 26; 18

Figure 3-8. A: Full ion scan of the 7 hormones of interest. B: Product ion scan of the 7 hormones of interest. C: MRM of the 7 hormones of interest. D: The optimized MRM parameters for the 7 hormones of interest.

3.2. Optimization of QuEChERS Method

3.2.1. Optimization of pH for QuEChERS

The pK_a values of all steroids were above 10, thus it was determined to work at a pH below the pK_a values. Not only was this convenient pH as in the caffeine study, but also for these compounds, it was the pH at which the steroids were neutral as is desired for extraction into an organic phase. A previous study performed using QuEChERS for the extraction of a few of the steroids under investigation currently used no buffer and had success. Without buffer adjustment, the pH of the deionized water used to prepare the steroid standards was approximately 6. A phosphate buffer was used to adjust the system to a pH of 7. Drawing from the caffeine study, it was predicted that a buffer would not result in greater stability of the method as at a pH of 6 and 7, the compounds are all in the same ionization state. As expected, it was determined that there was no significant increase upon the use of a buffer and it was deemed unnecessary.

3.2.2. Liquid-Liquid extraction (LLE)

As described in chapter 2, the LLE includes the use of salts that aid in solvent partitioning and decreasing the amount of co-extractables. Based upon the results from the previous study, ratios of 4:1, 2:1, and 1:1 were investigated, and once again a 1:1 ratio with 500mg $MgSO_4$ and 500mg NaCl was the optimal salt amount allowing for the best extraction of all eight glucocorticoids. This is seen in Figure 3-10 upon plotting the resulting peak areas for each compound at the three different salt ratios investigated. This amount also provided the best reproducibility for the method represented by the

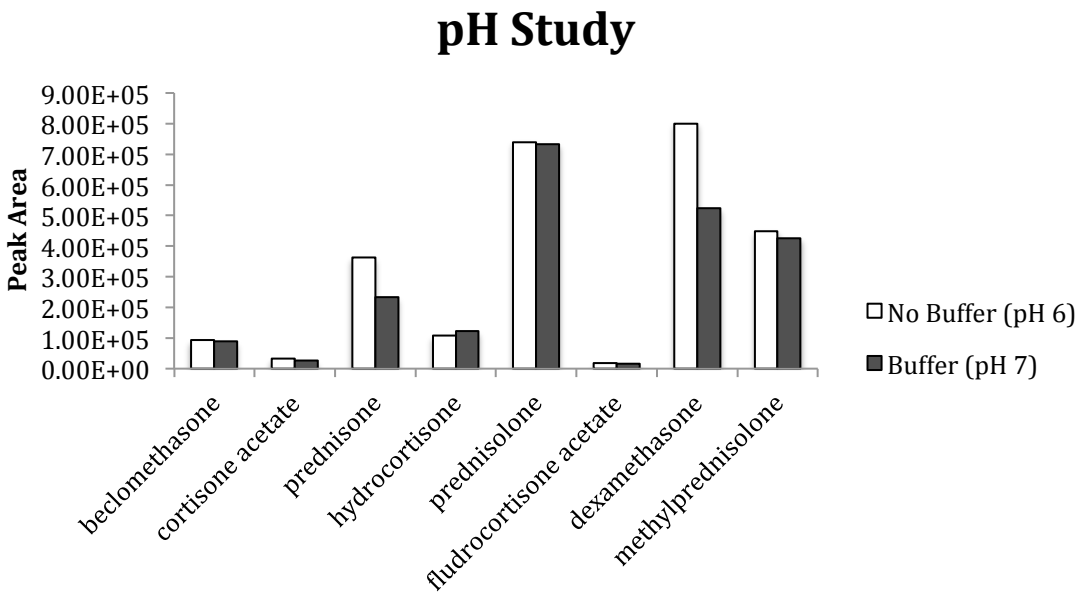


Figure 3-9. Optimization of the pH during the initial sonication of lose tea in deionized water.

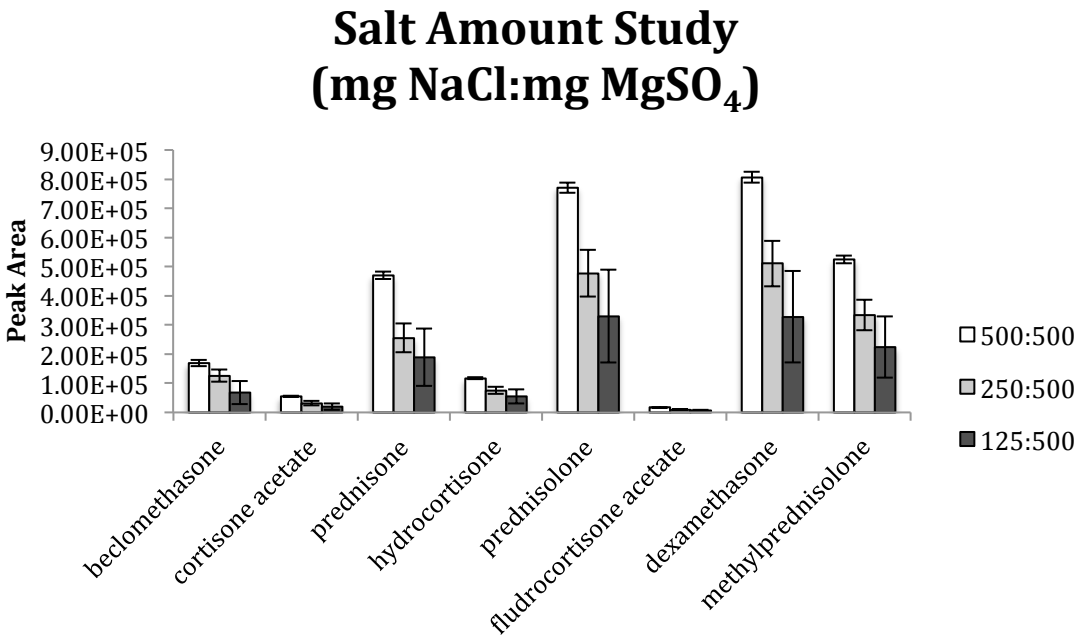


Figure 3-10. Optimization of salt ratio amount during the LLE step of the QuEChERS method

smaller error bars in Figure 3-10, most likely due to the principles in salting out and equilibrium. The lower salt amounts are not allowing for completion of the salting out effect, resulting in an inconsistent amount of analyte being driven into the organic phase. An increased amount of salt allows for salting out to occur completely in a consistent manner, resulting in a more reproducible extraction. The increased salt amount may result in a more consistent completion of the extraction equilibrium as well.

The final optimization parameter was solvent type. Acetonitrile (ACN), acetone (ACE), and ethyl acetate (EtOAc) were all investigated. As with the caffeine study, it was found that ACN provided the optimal results; however, ethyl acetate is fairly comparable and could be used during the extraction if desired. These results are consistent with those from the caffeine study in Chapter 2. The solubility data reported in the literature for the glucocorticoids investigated states sparingly soluble in water and acetone; however, there is not much data on ethyl acetate or acetonitrile. Given the results of this study, it can be deduced that these steroids have greater solubility in ethyl acetate and acetonitrile than acetone, hence the decreased peak areas and extraction ability using the latter solvent as seen in Figure 3-11.

3.3. Validation

3.3.1. Calibration curve, linearity, and partition coefficient

Upon analysis, glucocorticoid mix standards of concentrations from 10ppb to 500ppm resulted in calibration curves for each compound with limits of detection ranging from

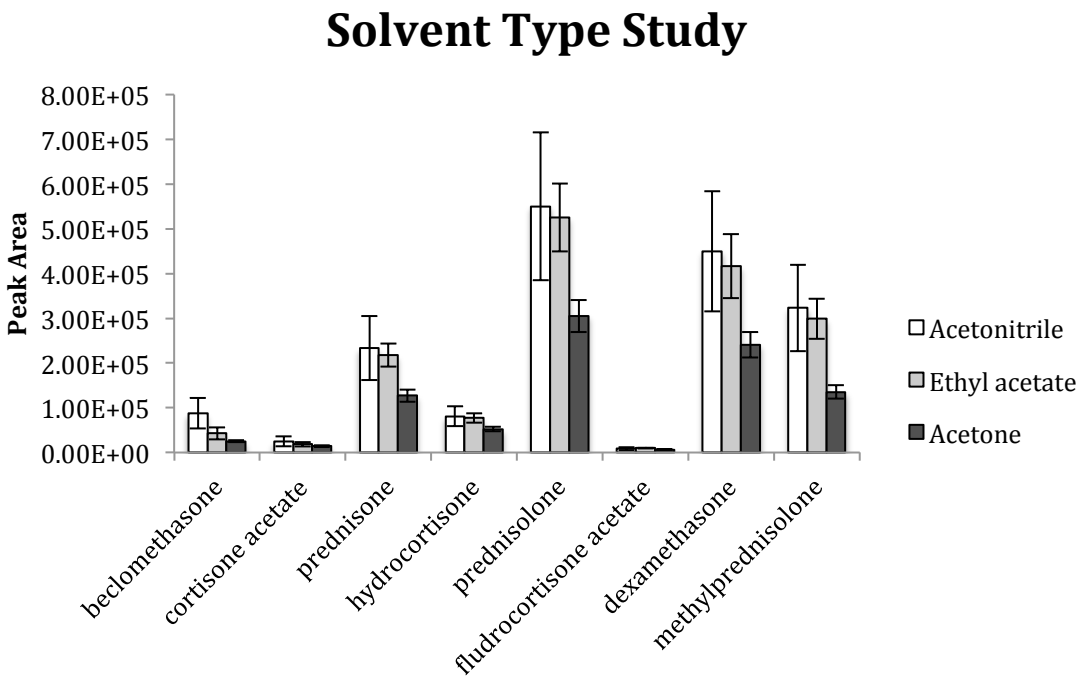


Figure 3-11. Optimization of the organic solvent type during the LLE step of the QuEChERS method.

5ppm to 450ppm depending on the steroid. The resulting LOD, LOQ, and R^2 values for each steroid are summarized in Table 3-6. In terms of the effect of the addition of salts to the system that are used during the QuEChERS method on the partition coefficient, it would be expected that the addition of salts to the system for a compound that is more soluble in water than acetonitrile would cause an increase in the partition coefficient as opposed to a compound who has less solubility in water. Beclomethasone, cortisone acetate, and fludrocortisone acetate were less soluble in water and would thus be less affected by the addition salts than the remaining 5 steroids. A summary of the LOD, LOQ, and partition coefficient data can be seen in section 3.3.2.

3.3.2. Recovery, precision, accuracy

The precision data was obtained by the analysis of 6 samples using the optimized QuEChERS and MRM methods. The percent RSD for these samples was performed for each of the 8 glucocorticoids, providing the precision results seen in Table 3-6. This was performed on two consecutive days, resulting in two interday precisions and one intraday precision. The percent recovery and accuracy (% error) were performed using 3 samples. The percent recovery and accuracy were calculated for each of these 3 samples and an average was taken. The average values for each glucocorticoid are seen in Table 3-6 along with the LOD, LOQ, R^2, and partition coefficient data.

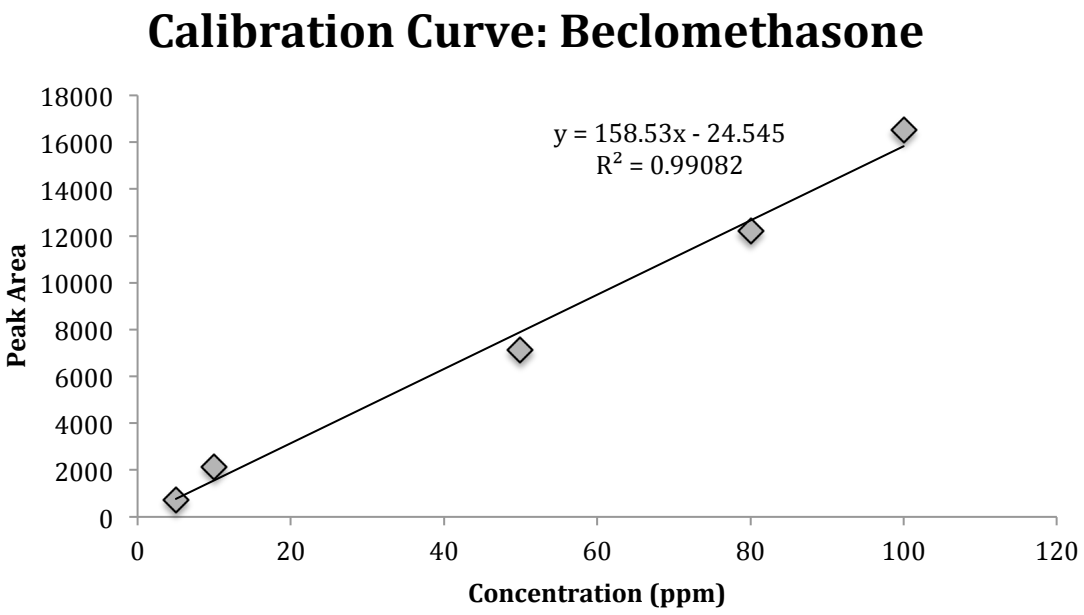


Figure 3-12. Calibration curve of beclomethasone from the method validation of the optimized QuEChERS extraction.

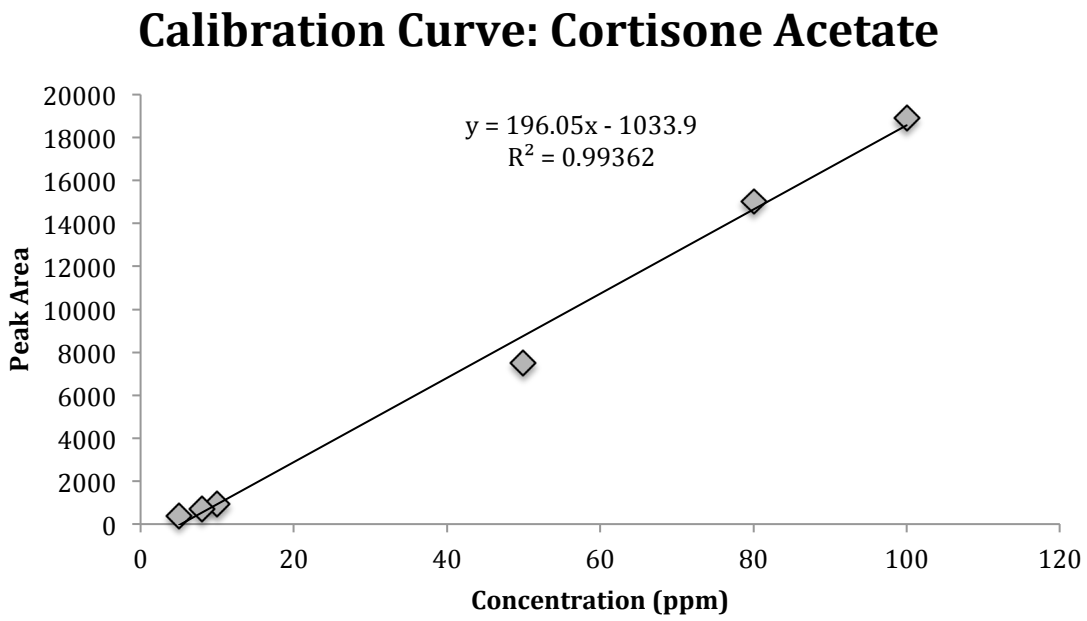


Figure 3-13. Calibration curve of cortisone acetate from the method validation of the optimized QuEChERS extraction.

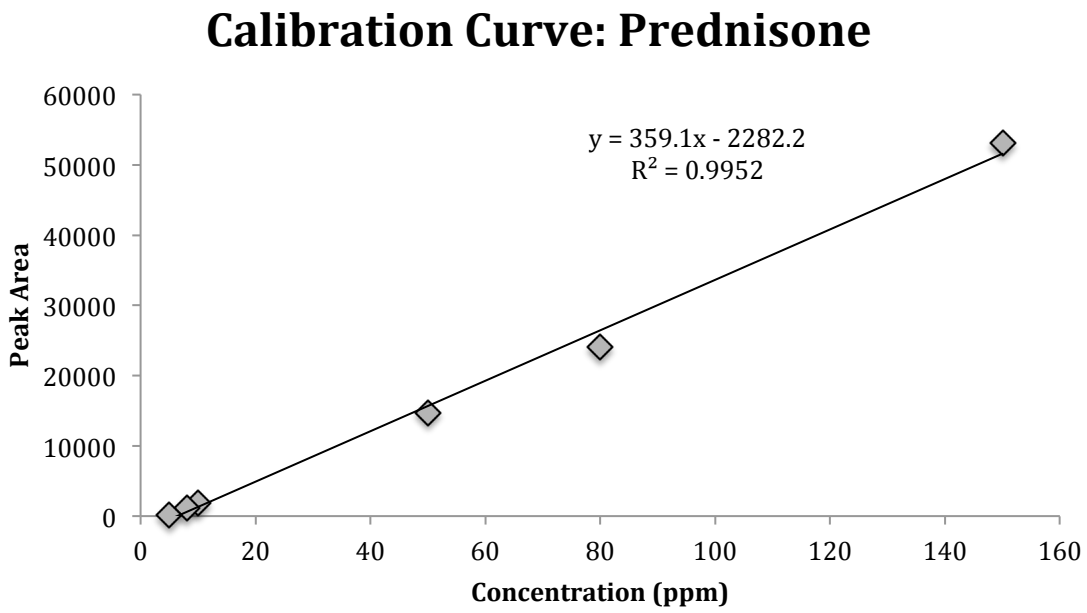


Figure 3-14. Calibration curve of prednisone from the method validation of the optimized QuEChERS extraction.

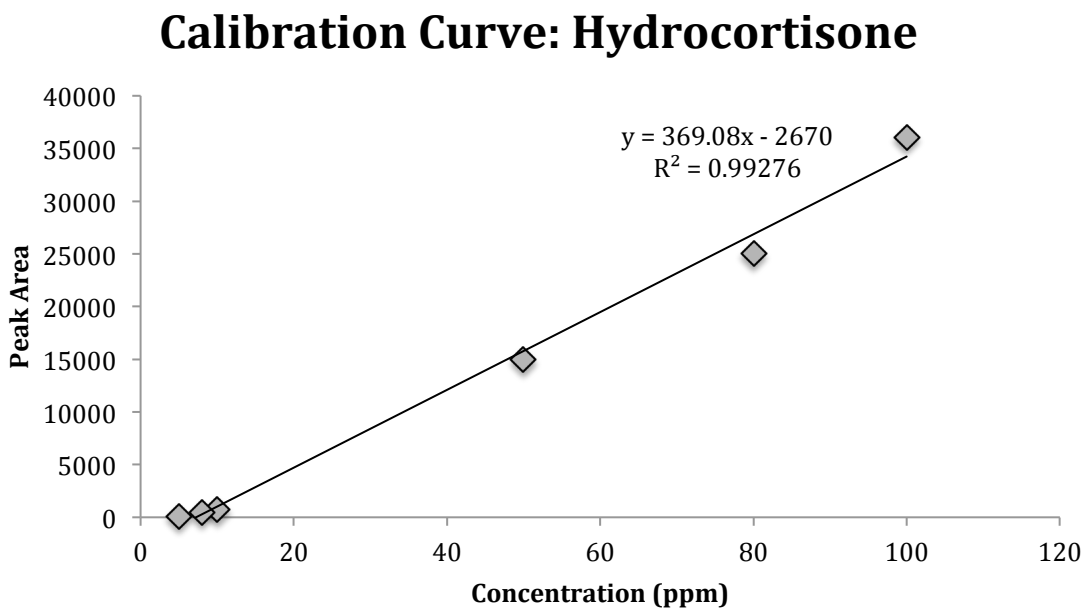


Figure 3-15. Calibration curve of hydrocortisone from the method validation of the optimized QuEChERS extraction.

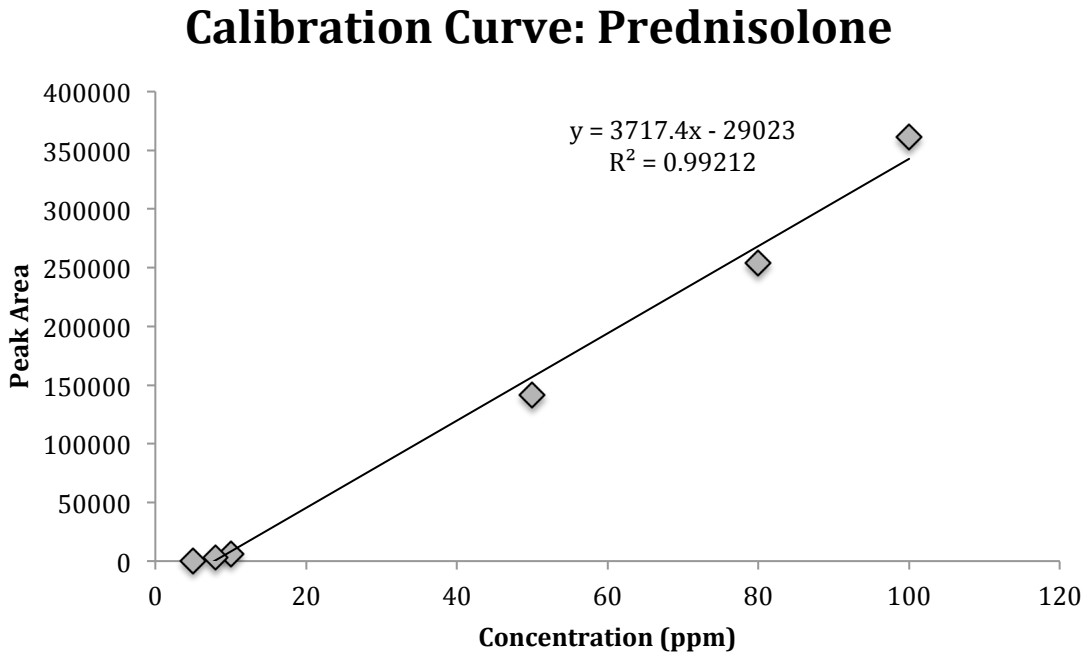


Figure 3-16. Calibration curve of prednisolone from the method validation of the optimized QuEChERS extraction.

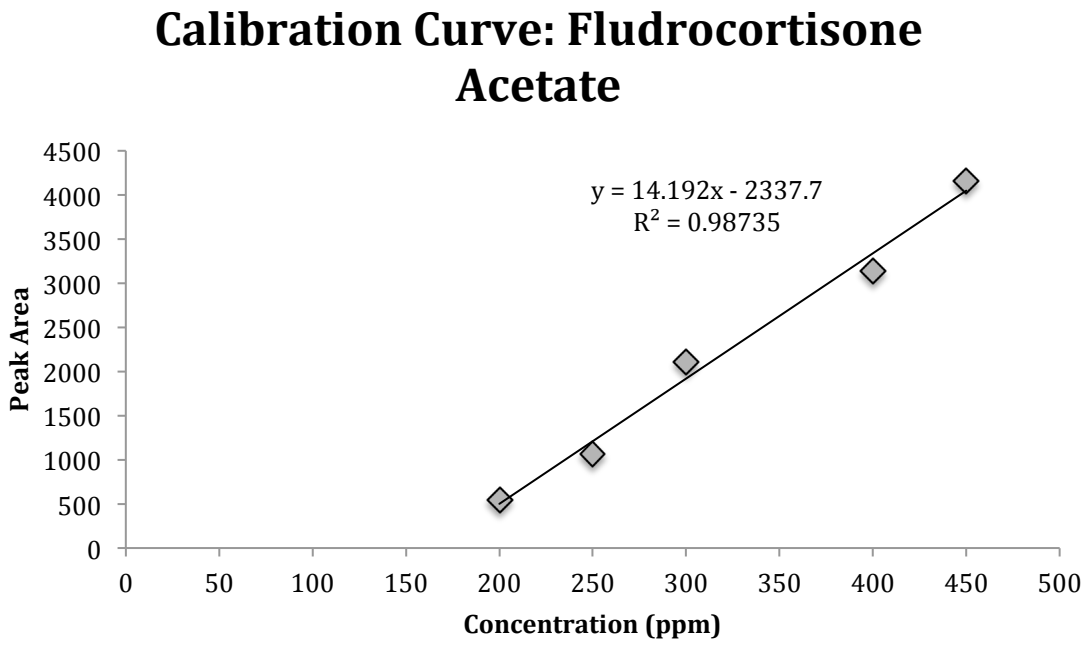


Figure 3-17. Calibration curve of fludrocortisone acetate from the method validation of the optimized QuEChERS extraction.

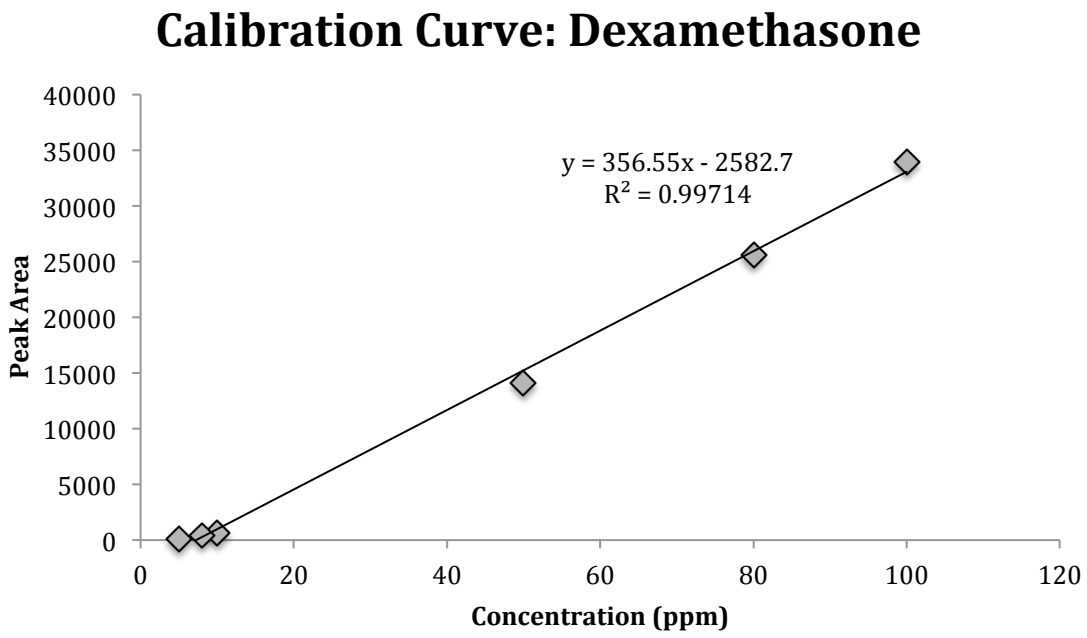


Figure 3-18. Calibration curve of dexamethasone from the method validation of the optimized QuEChERS extraction.

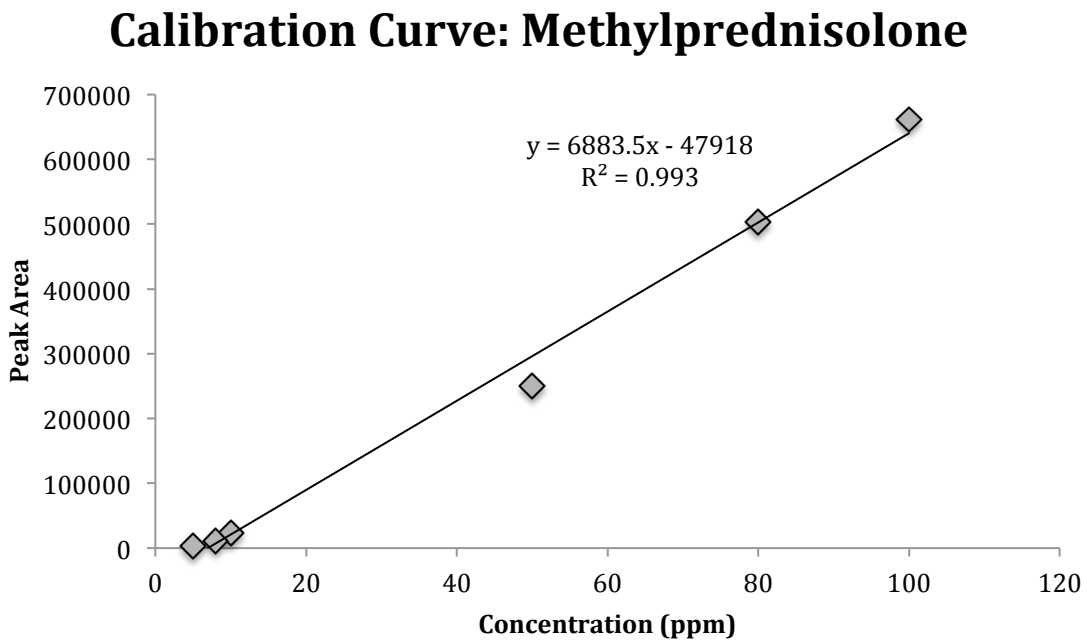


Figure 3-19. Calibration curve of methylprednisolone from the method validation of the optimized QuEChERS extraction.

Table 3-6. Summary of method validation results using GC-MS/MS.

Steroid (calibration range)	**LOD (ppm)**	**LOQ (ppm)**	**R^2**	**Recovery (%; n=3)**	**Accuracy (%Error; n=3)**	**Interday Precision (%RSD)**	**Intraday Precision (%RSD)**	**Partition Coefficient**
Beclomethasone (5 to 100ppm)	5	10	0.991	85.58	14.42	Day 1: 2.71 Day 2: 7.02	5.28	0.75 ± 0.06
Cortisone Acetate (5 to 100ppm)	5	10	0.994	83.87	16.13	Day 1: 4.90 Day 2: 6.47	7.75	0.72 ± 0.04
Prednisone (5 to 150ppm)	5	10	0.995	93.70	6.30	Day 1: 1.77 Day 2: 5.46	3.91	0.88 ± 0.02
Hydrocortisone (5 to 100ppm)	5	10	0.993	93.47	6.53	Day 1: 1.91 Day 2: 3.97	3.06	0.88 ± 0.04
Prednisolone (5 to 100ppm)	5	8	0.992	88.76	11.24	Day 1: 3.60 Day 2: 1.63	2.77	0.80 ± 0.04
Fludrocortisone Acetate (200 to 450ppm)	200	250	0.987	93.65	6.35	Day 1: 2.59 Day 2: 8.91	7.40	0.88 ± 0.03
Dexamethasone (5 to 100ppm)	5	10	0.997	90.71	9.29	Day 1: 4.82 Day 2: 1.60	4.89	0.83 ± 0.04
Methylprednisolone (5 to 100ppm)	5	8	0.993	84.14	15.86	Day 1: 3.68 Day 2: 1.98	3.04	0.73 ± 0.03

3.3.3. Analysis of Real Samples: HMPs

The four HMPs were analyzed for both glucocorticoids as well as hormones. This was performed using the optimized QuEChERS and MRM method as well as a Q3 Scan (total ion scan). The analysis results showed that neither glucocorticoids nor hormones were detected using the QuEChERS and MRM methods. Though it may be possible that these compounds are present in the samples and were simply not able to be detected. An extraction technique such as SPME that could provide lower detection limits may be able to detect if these compounds are present in the HMPs. Please see Chapter 4 for a discussion on the comparison of QuEChERS and SPME.

4. Conclusion

This study was successful in optimizing an instrumental method for the separation of the 8 glucocorticoids and 7 hormones of interest for GC-MS/MS analysis as well as optimization of the QuEChERS method. The knowledge gained from the previous caffeine study was employed here for optimization of salt amount as well as elimination of any sonication steps during the method. The resulting optimized QuEChERS and MRM methods provided successful validation with percent recoveries for all glucocorticoids greater than 83%, precision data both interday and intraday less than 9%, and partition coefficients between 0.72 and 0.88. Limits of detection for all steroids but fludrocortisone acetate were 5ppm with limits of quantitation at either 8 or 10ppm. Fludrocortisone acetate had much higher LOD and LOQ values of 200 and 250ppm, respectively.

Table 3-7. Summary of steroids found in studied HMPs.

Herbal Medicine Product	Steroid(s) Present
Brand 1 Vegetarian Tablet	None detected
Brand 2 Vegetarian Capsule	None detected
Brand 3 Tablet	None detected
Brand 4 Revitalizing Tonic	None detected

One reason for the higher detection limits for all of the compounds of interest involves the limitations of the QuEChERS method. This method involves an extraction into 2mL of organic solvent with no concentrating step. Thus, if 1ppm of sample is used during the extraction, then 2μg of sample is extracted into the organic solvent. Assuming no loss of sample throughout the entire extraction and injection processes, this means that only 20ng of sample is actually analyzed using a 1μL injection. Also, derivatization was not used during this process, which may have aided in preventing any loss of sample during the injection process due to active sites on the liner and inlet. Both of these items must be taken into consideration when analyzing the detection limit results for this extraction method in order to fairly evaluate its capabilities. This item is once again addressed in Chapter 4 during a discussion on SPME and QuEChERS detection abilities.

CHAPTER 4 – A COMPARISON OF SPME AND QUECHERS FOR THE EXTRACTION OF HORMONES FROM WATER

The previous chapters have discussed QuEChERS and the components of its extraction quite thoroughly; however, there are multiple extraction techniques that can be used for the extraction of hormones from water. Solid phase microextraction (SPME) is one such extraction method. This technique was investigated by a former student, Shilpi Chopra, for the extraction of hormones from water and will be briefly applied for a selection of those hormones using GC-MS/MS. The resulting SPME method was compared to the use of QuEChERS for the extraction of these hormones, a comparison that has yet to be performed in the literature. This comparison showed that SPME has sensitivities for the hormones of interest down to parts per trillion, whereas QuEChERS shows a part per million sensitivity as seen in Chapter 3. Reasons for this may be due to limitations of the QuEChERS method and is further discussed within this Chapter.

1. Introduction

1.1. Solid Phase Microextraction (SPME) Sample Preparation

As QuEChERS was discussed quite extensively in previous chapters, this section will concentrate on the theory involved in solid phase microextraction (SPME) and a comparison of these two extraction techniques. SPME is a solvent-less technique that was invented in 1990 by Pawliszyn [16]. This extraction is extremely versatile and can be performed for a multitude of analytes, using multiple techniques including headspace

and direct immersion. Both methods employ a fiber that has been coated with a polymer that is either suspended in the headspace above the sample in a SPME vial, or immersed directly in the sample itself. The latter tends to shorten the lifetime of a fiber; however, this was the method used for the analysis of the hormones of interest, as these compounds are semi-volatile and polar, making them more conducive to direct immersion SPME [74]. During extraction, the analytes adsorb onto the fiber and upon completion are thermally desorbed in the heated GC inlet as seen in Figure 4-1.

Both time and temperature will affect the rate of equilibrium, and it is imperative that both the extraction time and temperature are optimized to ensure that optimal extraction of all compounds is achieved. For instance, a 30-minute extraction may be optimal for lower molecular weight compounds in a sample, whereas a 60-minute extraction results in less lighter compounds due to the heavier molecules adsorbing and displacing the lighter analytes. In this case, perhaps a 45-minute extraction provides the best extraction in which all of the compounds are present at a satisfactory level. The temperature used during the extraction process should be optimized as well.

Once equilibrium has been reached, the fiber is placed into the heated GC inlet where the heat of the inlet causes the analytes to desorb from the fiber and be carried into the column for separation. Again, it is important to optimize the desorption time and temperature as these two factors are responsible for causing the analytes to transfer from the fiber to the inlet.

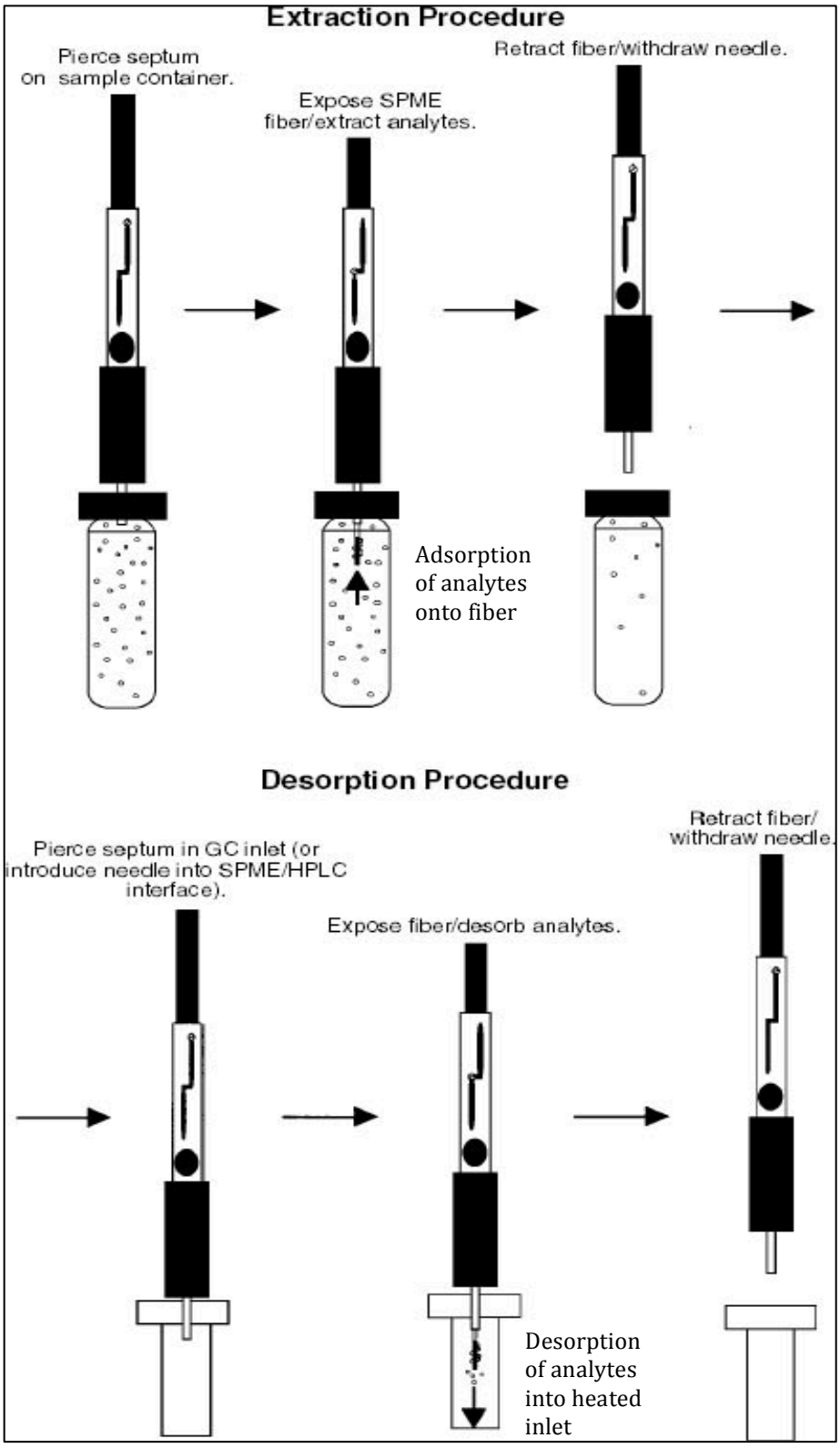


Figure 4-1. Schematic of the direct immersion SPME extraction (top) and desorption (bottom) processes. Adapted from S. Chopra [83].

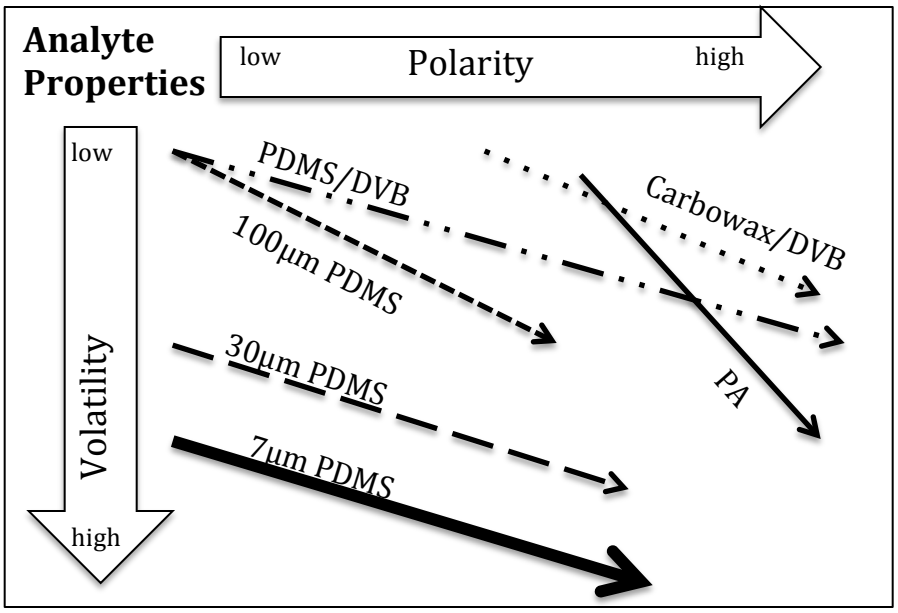


Figure 4-2. Guide for fiber coating selection using the polarity and volatility of analytes of interest.

In addition to factors such as agitation, temperature, and time, fiber characteristics will effect the resulting extraction too. There are many different polymers that can be chosen, and as with stationary phases in GC they are chosen based upon polarity and volatility of the analytes under investigation. If a sample is being investigated with only polar analytes present, then a fiber with polar properties such as polyacrylate (PA) should be used; however, if non-polar analytes are also present then a fiber including a wide range of polarity such as polydimethylsiloxane/divinyl benzene (PDMS/DVB) should be used. Both of these fibers and more can be seen in Figure 4-2. The thickness of the coating should also be taken into consideration. With direct immersion, typically a thinner coating polymer is used as this will prevent contamination of the fiber due to the fiber being immersed in the sample rather than exposed only to the headspace [16].

1.2. The Analysis of Hormones using SPME Sample Preparation

Solid phase microextraction (SPME) has been used for a wide range of applications including environmental, clinical, biological, food and flavor, pharmaceutical, and forensic samples [74]. Of particular interest to this study was the analysis of hormones using SPME and comparing the results to a QuEChERS extraction. In the past, non-volatile compounds such as steroids with polar functional groups were analyzed using direct immersion followed by on-fiber head space derivatization using bis-(trimethyl-silyl)trifluoroacetamide (BSTFA), forming TMS derivatives for GC-MS analysis [74-77].

Such work was performed by Snow and co-workers for the analysis of steroids from human serum as well as estrogens and anabolic steroids in both aqueous and biological

mediums, both studies using a carbowax/divinyl benzene (DVB) fiber with a 30 minute extraction at 60°C with on-fiber derivatization using BSTFA [75-80]. A PDMS/DVB fiber with a 60 minute extraction at 50°C was used for the evaluation of river water for steroid contamination in a third study by Snow; however, this study did not use any derivatization and was successful in extracting steroids from water with recovery ranges from 73-115% and LOD from 0.008-3.77µg/L [81]. Chopra and Snow also performed work without derivatization of steroid estrogens that were under investigation in the current study. Chopra's work involved the analysis of steroids using SPME and GCxGC-TOFMS as well as GC-MS/MS. The SPME conditions including the vial contents and use of a PDMS/DVB fiber used in their study were employed during the current work; however, the MRM method was re-optimized, as was the extraction time [82-83].

1.2. QuEChERS and SPME Sample Preparation

There are many advantages to both QuEChERS and SPME as compared to classic multi-residue methods. QuEChERS was invented as a quicker, cheaper, safer, more effective method alternative for a multi-residue sample preparation method. The amount of sample and solvent used is less in addition to the use of safer solvents. Simpler method steps are involved such as the use of a centrifuge and shaker as opposed to a filtration or ultra-turrax. Evaporation is also not a step necessary in QuEChERS and the d-SPE clean up used in QuEChERS is much less complicated than the clean up steps used in classic multi-residue methods (MRM). An outline of the differences between QuEChERS and classic MRM methods are listed in Table 4-1.

As aforementioned, SPME is a solvent-less technique, which QuEChERS is not. Thus the exposure to solvents and the cost for solvents is less; however, the cost of SPME fibers is high. SPME is also both an extraction and concentration method; therefore, the sensitivity of the method may be greater than QuEChERS as it does not include a concentration step. There is also an endless amount of combinations that can be used in QuEChERS between variations in solvent, salts used, and sorbents during the clean up step to optimize a method and make it applicable to a wide range of samples. SPME has limited fiber options that can be used for sample analysis though the use of fibers allows for direct analysis of a sample without additional extensive sample preparation. A comparison between SPME and QuEChERS is also included in Table 4-1.

The use of SPME for the extraction of hormones from water was investigated briefly using a previously optimized method by Chopra [83]. The use of various extraction times was investigated and the optimized SPME method was compared to the optimized QuEChERS method as these two methods have not been compared directly in the literature extensively. The resulting peak areas for each method were investigated for samples of same concentrations to determine the extraction ability of each comparatively.

2. Materials and Methods

2.1. Chemicals, Reagents, and Samples

Acetonitrile (ACN) was purchased from pharmco-AAPER (Kindermorgan, PA) and was reagent ACS grade. All salts used throughout the study as well as the steroid standards

Table 4-1. Comparison of the QuEChERS method to SPME and classical MRM methods.

SPME	QuEChERS	Classic Multi-residue Methods
Sample amount can vary	Smaller samples used	Macro-scale sample amount
Less complicated, simpler steps: - Single extraction step in a single vessel - Concentration technique as well - Sensitivity increased due to concentration - Limited by fiber type	Less complicated, simpler steps: - Single partitioning step in a single vessel - d-SPE clean-up - Centrifuge and shaker/vortex - Less error prone - Multitude of combinations possible	More complicated steps: - Multiple partitioning steps with multiple vessels - SPE, GPC clean-up - Filtration and ultra-turrax - More error prone
Solvent-less technique	Limited safer solvents used	Exposure to less safer solvents
Large method applicability	Larger extraction range	Limited extraction range

were purchased from Sigma Aldrich (St. Louis, MO). QuEChERS tubes containing 150mg PSA and 50mg $MgSO_4$ were purchased from Restek (Bellefonte, PA) and 15mL PFTE centrifuge tubes were obtained from VWR International (Radnor, PA). The PDMS/DVB fibers used during SPME analysis were obtained from Sigma Aldrich (Bellefonte, PA). Deionized water was used throughout the methodology.

2.2. SPME Sample Preparation

The 7 hormones investigated in Chapter 3 were also used in this study: diethylstilbestrol, prasterone, methandriol, estrone, estradiol, mesterolone, and boldenone. An optimized SPME method for hormones determined by Chopra was utilized in this research where the pH, salt amount, and water amount were previously determined [83]. A phosphate buffer was prepared at pH 8.0 in which 25µL was added to 2.15g NaCl and 8.5mL deionized water in a 10mL SPME vial. The extraction time was optimized using a sample containing 0.5ppm of the hormone mixture prepared in ethanol that was added to the vial and vortexed until mixed and the salt dissolved. The SPME parameters used for the extraction can be seen in Table 4-2.

2.2.1. Optimization of pH, salt amount, and extraction time for SPME

A previous study performed by Chopra used a 0.5M phosphate buffer. In this study, 100mL phosphate buffer was prepared by dissolving 0.36g sodium phosphate dibasic and 0.30g potassium phosphate monobasic in deionized water and brought to a pH of 8.0 using a 5M sodium hydroxide solution. The salt amount of 2.15g optimized by Chopra was used in this study [83]. As the GC-MS/MS used during Chopra's study was different

Table 4-2. SPME conditions used for GC-MS/MS analysis of hormones.

SPME Conditions		
Sample Preparation	*Fiber Type*	*Extraction/Desorption Parameters*
10mL SPME vial containing: 2.15g NaCl 8.5mL DI H_2O 0.25µL phosphate buffer (pH 8.0) Desired amount of hormone mix	PDMS/DVB (23 ga, d_f 65µm)	Incubation: 10 min at 55°C Extraction: 60 min at 55°C Desorption: 3 min at 250°C Post-Fiber Bake: 20 min

then that used in this study, the MRM parameters and extraction time were re-optimized and investigated at 15, 30, 45, and 60min using a 0.5ppm hormone standard mixture.

2.3. QuEChERS Sample Preparation

The hormones used in this study were also investigated using the optimized QuEChERS method described in Chapter 3 for the glucocorticoids analysis in which 500mg of NaCl and 500mg of $MgSO_4$ were placed into a 15mL centrifuge tube along with 2mL of aqueous sample and 2mL of acetonitrile. The sample was vortexed for 1-minute and centrifuged for 3-minutes at 1,000rpm. The top organic layer was transferred to a tube containing 50mg PSA and 150mg $MgSO_4$ that was then vortexed and centrifuged. The resulting liquid extract was transferred to a GC vial and analyzed.

2.3. SPME and QuEChERS Comparison

Both SPME and QuEChERS were performed for the purpose of comparing the resulting peak areas for the extraction of samples with the same concentrations. The following concentrations were analyzed using SPME and QuEChERS: 5, 1, 0.5, 0.05, and 0.005ppm, as well as 0.0005ppm for SPME.

2.4. Instrumental Parameters

The instrumentation utilized for this study was a Shimadzu GC-MS/MS TQ8030 with an AOC-5000 Auto Injector (Santa Clara, CA). Splitless liquid injection was used for all analyses of QuEChERS samples and SPME analysis also used splitless injection. Both

Table 4-3. Method conditions used for GC-MS/MS analysis of hormones.

GC-MS/MS		
GC Parameters	*Oven Parameters*	*MS Parameters*
Column: RTX-5MS 15m, 0.25mm, 0.25µm	Initial Temperature: 40°C Hold 1 minute	EI Source: 250°C
Carrier Gas: Helium	20°C/minute Ramp to 300°C Hold 3 minutes	Transfer Line: 250°C
Column Flow: 0.98mL/min Linear Velocity: 51.0cm/sec		
Injection Mode: Splitless		
Inlet Temperature: 250°C		

Table 4-4. MRM method conditions used for the 7 hormones of interest with GC-MS/MS analysis (quantitative ion transition in *italics*.

Hormone	**Retention Time (min)**	**Precursor Ion**	**Product Ions**	**Collision Energy**
Diethylstilbestrol	11.607	*268*	*239*; 145; 107	10; 18; 26
Prasterone	12.410	*288*	*203*; 107; 97	10; 14; 20
Methandriol	12.563	*253*	*197*; 155; 169	16; 26; 26
Estrone	12.820	*270*	*185*; 157; 172	14; 26; 20
Estradiol	12.847	*272*	*185*; 172; 213	8; 14; 16
Mesterolone	12.877	*218*	*159*; 105; 200	14; 26; 8
Boldenone	13.030	*122*	*107*; 77; 79	16; 26; 18

extraction methods used multiple reaction monitoring (MRM) previously optimized as described in Chapter 3. The separation and detection parameters for the instrument are listed in tables 4-3 and 4-4.

3. Results and Discussion

3.1. SPME Sample Preparation

As previously stated in section 2.2, the SPME conditions including pH and salt amount were previously optimized by Chopra; however, the extraction time was re-optimized in this study. It was found that 60 min provided the best extraction for all of the hormones of interest. This finding was congruent with that of Chopra. Figures 4-3 and 4-4 depict the optimization of the extraction time and a summary of the optimized MRM SPME extraction at 60min with a 0.5ppm sample, respectively.

3.2. QuEChERS Sample Preparation

It was found that the optimized parameters used for extraction of glucocorticoids was also successful for the extraction of the hormones of interest, as expected due to their similar structures and functional groups. Thus, the same parameters optimized in Chapter 3 were used during this study during QuEChERS.

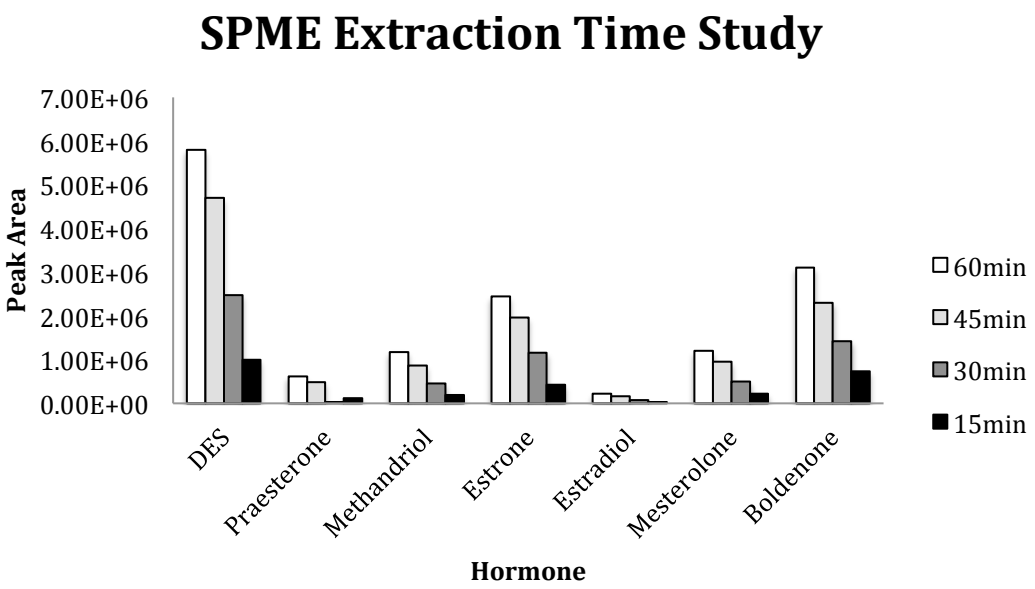
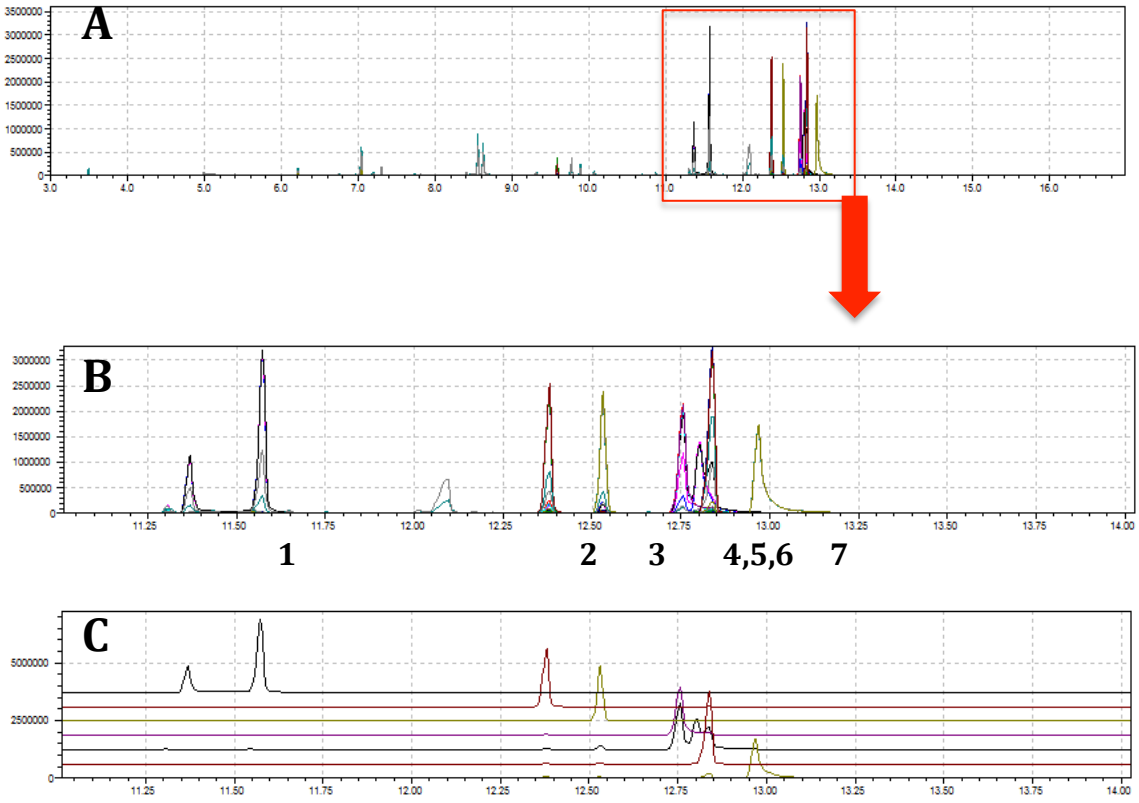
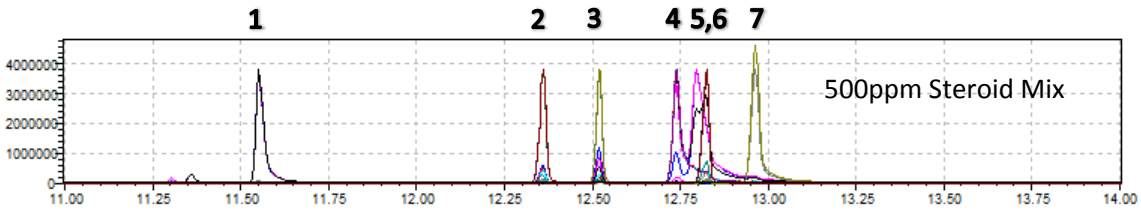


Figure 4-3. Optimization of SPME extraction time for the hormones of interest (0.5ppm).



Hormone
1) Diethylstilbestrol
2) Prasterone
3) Methandriol
4) Estrone
5) Estradiol
6) Mesterolone
7) Boldenone

Figure 4-4. A: Chromatogram of hormones analyzed using the optimized MRM and SPME methods with a 60min extraction time and 0.5ppm concentration. B: Zoomed in portion of A including the hormones of interest. C: Chromatogram show separation of the hormones by quantitative ion transition (see Table 4-4).



Hormone
1) Diethylstilbestrol
2) Prasterone
3) Methandriol
4) Estrone
5) Estradiol
6) Mesterolone
7) Boldenone

Figure 4-5. Chromatogram of hormones analyzed using the optimized MRM and QuEChERS methods (500ppm).

3.3. SPME and QuEChERS Comparison

The 60-minute SPME extraction was performed using concentrations ranging from 5ppm to 500ppt. Three of the hormones were detected at the lowest concentration of 500ppt (prasterone, methandriol, mesterolone). The remaining hormones were detected at the following concentrations: DES at 1ppb, estrone and boldenone at 5ppb, and estradiol at 0.5ppm.

The QuEChERS extraction resulted in higher levels of detection in the ppm range; however, this is most likely due to the limitations of QuEChERS as compared to SPME. SPME has a concentration step that occurs during extraction that QuEChERS does not. The extraction for QuEChERS takes place in a 1:1 ratio of sample to solvent (2mL aqueous solvent:2mL acetonitrile) with a 1µL injection, resulting in a significant decrease in the amount of sample injected onto the column. If a 1ppm sample is used during extraction, this contains 1µg of sample in each mL, and assuming all 2µg are extracted from the aqueous layer to the organic layer, then only 20ng of sample will travel to the column in the 1µL that is injected. This would only be possible if the extraction was 100% efficient with no analyte loss in the inlet from the liquid injection. This amount is closer to the ppt levels that were observed with the SPME results and shows that these two techniques only appear to have very different detection levels due to the QuEChERS methodology.

Another item to keep in mind is the partition coefficient difference between these two methods. When performed by Chopra, partition coefficients on the order of 10,000 were

Table 4-5. Comparison of hormone detection using SPME and QuEChERS.

Hormone	SPME Lowest Detection	QuEChERS Lowest Detection
DES	1ppb	80ppm
Prasterone	500ppt	5ppm
Methandriol	500ppt	10ppm
Estrone	5ppb	80ppm
Estradiol	0.5ppm	80ppm
Mesterolone	500ppt	5ppm
Boldenone	5ppb	5ppm

observed when using SPME. This is significantly higher than when using QuEChERS as evidenced by both the caffeine and glucocorticoid studies. Thus once again limitations of the QuEChERS method are responsible for the difference between SPME and QuEChERS.

4. Conclusions

Though it may appear given the results from this study that SPME has much lower detection limits than QuEChERS for this set of compounds, this is not a fair direct comparison. One must take into account the limitations of QuEChERS including lack of a concentration step, difference in partition coefficients, larger extraction volume, and limited injection volume in order to make a more just comparison. If these parameters are evaluated it can be seen that the two methods are not that different in terms of detection limits.

It must also be considered that due to direct liquid injection, the compounds may be more prone to loss and solvent effects in the inlet during QuEChERS analysis. Derivatization could be performed to evaluate the effect on sensitivity. Another path for future work to improve sensitivity of QuEChERS is the use of a programed temperature vaporizing (PTV) inlet. This would allow for large volume injections of the extract, resulting in increased sensitivity. This has been performed in our lab using organic solvents for large volume injections with good peak shape and thus should be further studied for use with QuEChERS to improve sensitivity.

CHAPTER 5 – A DISCUSSION ON THE USE OF GC-MS/MS AND GCXGC-TOFMS FOR THE EXTRACTION OF STEROIDS FROM WATER USING SPME

1. Introduction

1.1. GCxGC-TOFMS Instrumentation

One of the main reasons for the use of this instrument for this particular study is to not only separate any co-eluting steroids via two-dimensional chromatography, but also to investigate any degradation products that may be occur during analysis. It is also predicted that the use of a more sensitive detector will allow for the detection of trace amounts of steroids that may be adulterated in herbal medicinal products. Multidimensional chromatography also allows for further separation of any matrix interferences from the analyte(s) of interest due to separation in two sequential columns with different stationary phase polarities as depicted in Figure 5-1. The use of two columns allows for an increase in the number of resolved peaks, also known as peak capacity. In single column chromatography, peak capacity is defined as:

$$n = \frac{\sqrt{N}}{2R} \ln\left(\frac{t_2}{t_1}\right) + 1 \qquad \text{(Equation 5-1)}$$

where n is peak capacity, R is adjacent peak resolution, N is column efficiency, t_1 is the start time, and t_2 is the end time; whereas in comprehensive two-dimensional

chromatography the equation multiplies the peak capacities of both columns [15-16], [84-87].

$$n_{GCxGC} = (n_{column\,1})(n_{column\,2}) \qquad \text{(Equation 5-2)}$$

Not only does this difference in polarity provide better separation of the components, but it also allows for orthogonal separation in that there are two separate mechanisms occurring simultaneously and independently with no interference. These two columns are connected by a deactivated fused silica pressfit. The first column is usually a non-polar stationary phase, for example polydimethylsiloxane, with typical capillary column dimensions (30 or 15m x 0.25mm x 0.25µm). The second dimension stationary phase is more polar than the first to aid in eliminating carry-over and for separating volatile analytes. The column is also shorter (1-2m), and has about half the internal diameter compared to the first column, resulting in a rapid separation with little analyte retention. The smaller secondary column dimensions help to focus the eluent onto the second column, providing narrower, sharper peaks that in turn increase the peak height providing easier detection and increasing sensitivity [15], [84-87]. The resulting chromatogram from multidimensional chromatography is known as a contour plot. Any co-elution occurring due to the complex matrix and/or multiple steroids of interest will also be solved by use of the GCxGC-TOFMS.

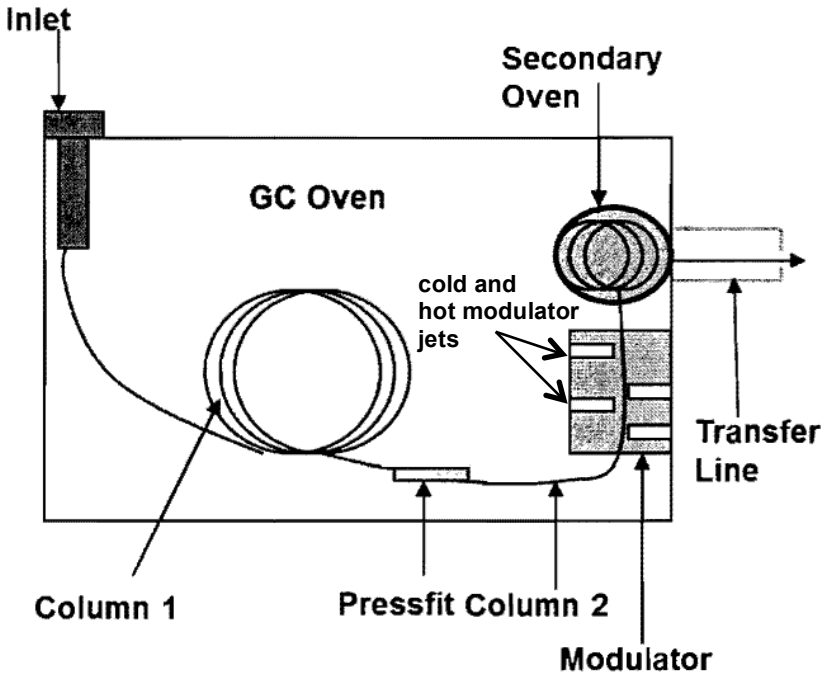


Figure 5-1. Schematic of the GC portion of the instrument used in this research. It can be seen that the second column is housed in a separate oven for heating and a modulator is between the two columns. Adapted from B.B. Barnes 2012 Dissertations Paper 1804 [16].

The two columns are connected by a pressfit composed of deactivated fused silica, after which there is a modulator. This portion of the instrument is responsible for peak modulation in which the eluent is focused onto the second column, allowing for no interferences to occur resulting in the ability for orthogonal separation. For this reason, the modulator is known as the heart of this instrument. There are three commonly used modulators: a thermal sweeper, Deans switch, and a cryotrap. The cryotrap modulator was present in the instrument used for this research and thus will be further discussed. In this type of modulator, alternating cold and hot jets of nitrogen gas help focus the eluent and keep the sample mobile to eliminate interferences. Two cold jets work to focus the eluent, splitting the peak providing fractions of the eluent, and two hot jets eliminate any interferences between the fractions by keeping the eluent mobile through the modulator. In order to preserve peak bandwidth and orthogonal separation, the hot jets tend to have shorter pulses. The hot jet pulse times and the total modulation time, or second dimension separation time, also effect retention and should be optimized depending on the complexity of the sample [16], [84-86]. A schematic of the cryotrap modulator can be seen in Figure 5-2.

Once separated via both columns, the eluent travels through a heated transfer line to the mass spectrometer. As discussed in Chapter 1, the mass spectrometer (MS) is composed of an ion source that ionizes and fragments the analytes, a mass analyzer, in this case a time of flight mass spectrometer (TOFMS), and a detector. The main difference from the discussion in Chapter 1 is the use of a TOFMS and thus the theory involved will be discussed briefly.

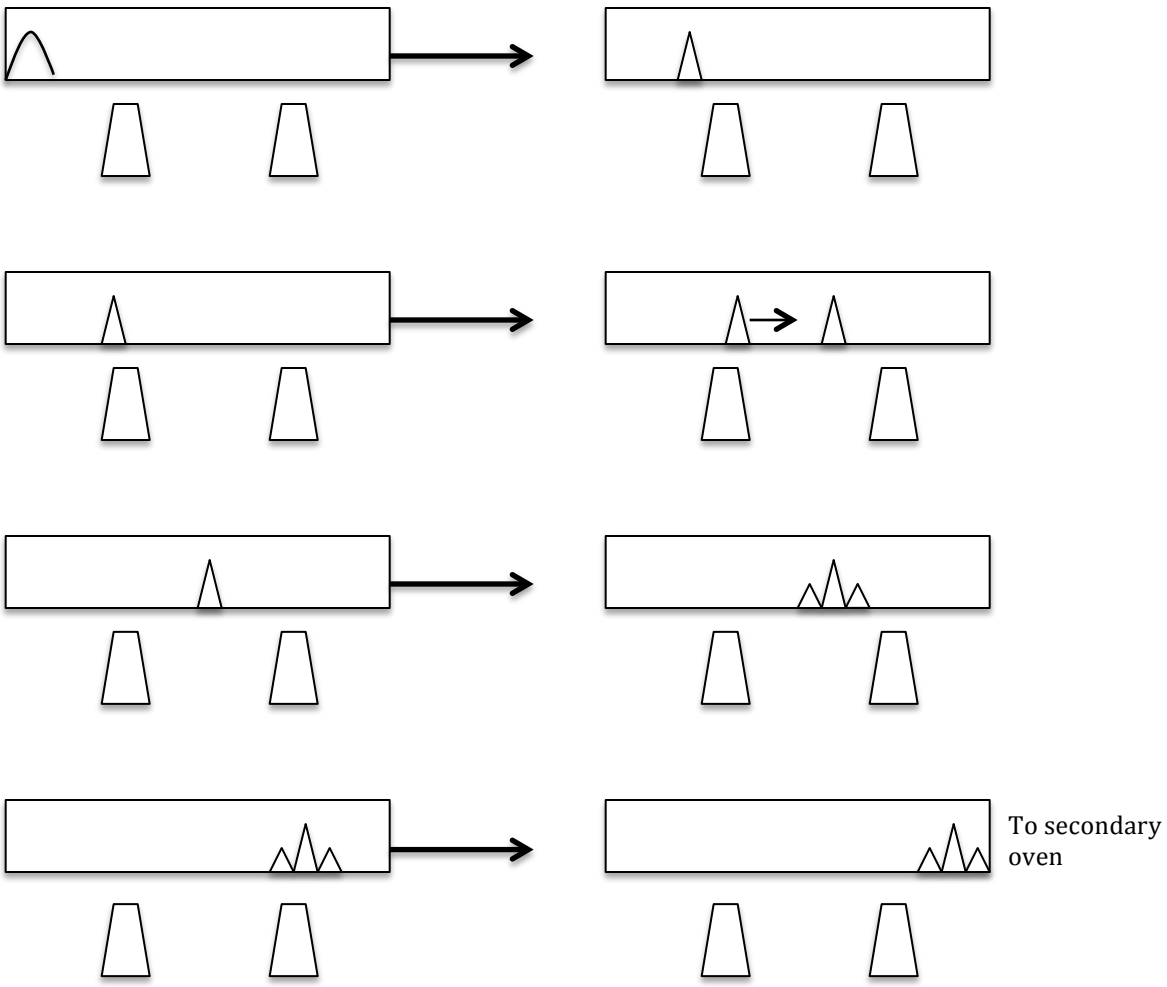


Figure 5-2. Schematic depicting the cryotrap peak modulation process. A) The first cold jet focuses a single component. B) The first hot jet moves the peak to the second cold jet. C) The second cold jet splits the peak of the component. D) The second hot jet moves the peak to the secondary oven.

The mass analyzer used in the instrument for this study was the TOFMS. In this mass analyzer ions are separated based on their kinetic energy (KE). It is assumed that the ion's potential energy and kinetic energy are equal which leads to the conclusion that velocity is inversely related to mass, thus smaller ions are faster as seen in the following set of equations [15-16].

$$Potential\ Energy = qV \qquad Kinetic\ Energy = \frac{1}{2}mv^2 \qquad \text{(Equation 5-3)}$$

Where q is the ion charge, V is the repeller accelerating potential, m is the mass of the ion, and v is the ion's velocity.

$$qV = \frac{1}{2}mv^2 \qquad where \qquad v = \sqrt{\frac{2Vq}{m}} \qquad \text{(Equation 5-4)}$$

$$velocity = \frac{distance}{time} \qquad therefore \qquad t = L\sqrt{\frac{m}{2Vq}} \qquad \text{(Equation 5-5)}$$

Where t is the time spent by the ion in the flight tube and L is the length of the flight tube.

Resolution is increased in TOFMS due to electrostatic plates that control the ion flow and maintain the distance between ions. There are also steering plates in the flight tube to control the path the ions take down the tube until they reach the reflectron at the end of the tube. This causes the ions to turn, doubling the path length traveled [6]. Leco's Pegasus 4D GCxGC-TOFMS was the instrument used in the research and can be viewed in Leco's Pegasus 4D GCxGC-TOFMS Brochure [89].

In order to obtain the desired results, GCxGC-TOFMS optimization of all necessary parameters must be performed. This includes not only the parameters normally optimized in GC/MS such as flow rate and oven ramps, but also parameters for the second column as well as the modulator. The method used was optimized by a former student, Shilpi Chopra, and any data reported using GCxGC-TOFMS is from her previous work and was not repeated [83]. All GC-MS/MS data was generated by the current research.

1.2. A Discussion on GC-MS/MS and GCxGC-TOFMS

The use of GC-MS/MS or GCxGC-TOFMS depends upon the goal of analysis. One instrument cannot be considered superior over the other, as their purposes are different, providing different outcomes. These instruments should be used in conjunction with each other to obtain all information possible. The main difference between these two instruments involves the method used to resolve any co-elution. GCxGC-TOFMS uses chromatography to chromatographically separate compounds as discussed in section 1.1; however, GC-MS/MS uses a detector to resolve co-elution as discussed in Chapter 4 by preparing a multiple reaction monitoring method (MRM). There are pros and cons to both instruments and thus the goal of analysis must be evaluated. For instance, GCxGC-TOFMS allows for separation of column and septum bleed from the peaks of interest but can result in two-dimensional tailing as well as the ability to see multiple degradation products. These degradation products can complicate the analysis of the resulting chromatogram; however, they can also be used to determine the origin of compounds that were analyzed which may be of interest. A summary table comparing various aspects of

Table 5-1. Comparison of certain characteristics of GC-MS/MS and GCxGC-TOFMS.

GC-MS/MS	**GCxGC-TOFMS**
Detector resolved co-elution	Chromatographic resolved co-elution
Column and septum bleed not separated from peaks of interest	Column and septum bleed separated from peaks of interest
Tailing only in 1D	Tailing in 2D
Sensitivity increased when using MRM	Multitude of degradation products

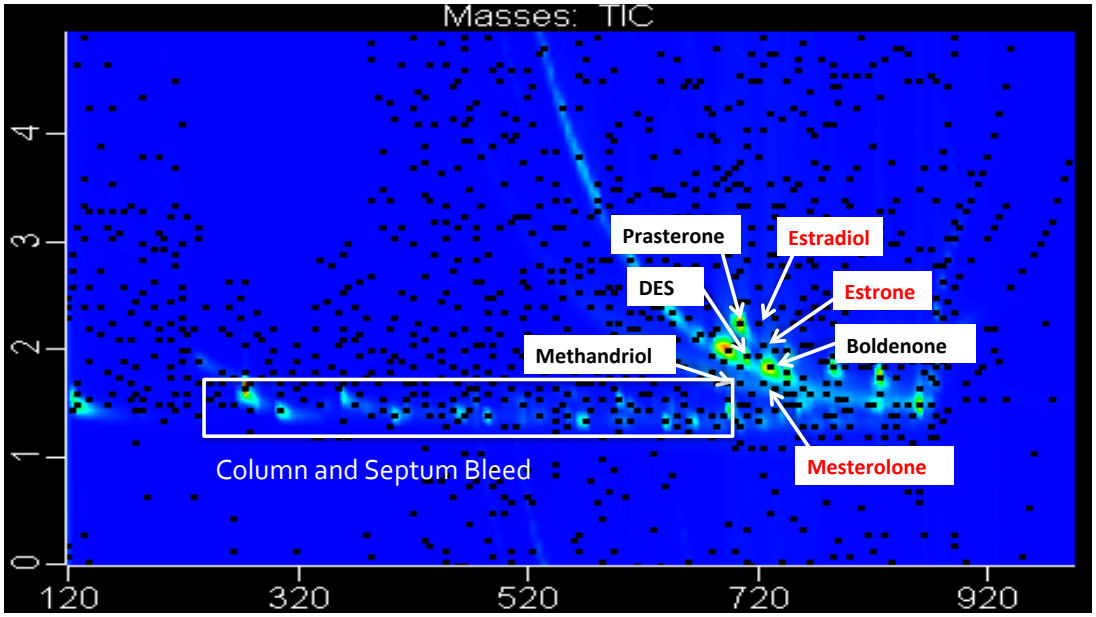


Figure 5-3. Total ion chromatogram of hormones resolved on GCXGC-TOFMS with SPME sample introduction showing the separation of column and septum bleed from the analytes of interest (0.03ppm). Reprinted with permission from Chopra S. Dissertation and Theses 2014 [83].

these two instruments can be seen in Table 5-1. An example of chromatographic resolution, separation of column and septum bleed from the analytes of interest, and presence of multiple peaks that include degradation products using GCxGC-TOFMS is depicted in Figure 5-3.

2. Materials and Methods

2.1. Chemicals, Reagents, and Samples

Ethanol was purchased from KOPTEC (King of Prussia, PA) and was 200 Proof. All salts used throughout the study as well as the hormone standards were purchased from Sigma Aldrich (St. Louis, MO). The PDMS/DVB fibers used for SPME analysis were obtained from Sigma Aldrich (Bellefonte, PA). Deionized water was used throughout the methodology.

2.2. SPME Sample Preparation

The 7 hormones investigated in Chapters 3 and 4 were also used in this study: diethylstilbestrol, prasterone, methandriol, estrone, estradiol, mesterolone, and boldenone. An optimized SPME method for steroids determined by Chopra was utilized in this research in where the pH, salt amount, and water amount were previously determined [80]. A phosphate buffer was prepared at pH 8.0 in which 25µL was added to 2.15g NaCl and 8.5mL deionized water in a 10mL SPME vial for the GC-MS/MS. The amounts were doubled for GCxGC-TOFMS where a 20mL vial was used. The extraction

time was optimized using GC-MS/MS as mentioned in Chapter 4, section 2.2. The SPME parameters used for the extraction can be seen in Table 5-2.

2.3. GCxGC-TOFMS and GC-MS/MS Instrument Comparison

In this study, the work performed by Chopra using GCxGC-TOFMS was compared to the work performed currently as outlined in Chapter 4 using GC-MS/MS for the extraction of hormones from water using SPME. The ability of both instruments to resolve co-elution as well as selectivity were compared using a 0.03ppm standard mixture for GCxGC-TOFMS and a 0.5ppm standard mixture for GC-MS/MS. The purpose of this study was not to evaluate sensitivity of the instruments, but simply to compare their separation ability.

2.4. Instrumental Parameters

The instrumentation utilized for this study was a Shimadzu GC-MS/MS TQ8030 with an AOC-5000 Auto Injector (Santa Clara, CA) and a LECO Pegasus 4D GCxGC-TOFMS with a Gerstel Auto Injector. Splitless injection was used for all SPME analyses. The previously optimized multiple reaction monitoring (MRM) method was used during GC-MS/MS analysis as described in Chapter 3. The separation and detection parameters for both instruments are listed below in tables 5-3 through 5-5.

Table 5-2. SPME method conditions used for GCxGC-TOFMS and GC-MS/MS analysis.

SPME Conditions			
Sample Preparation		*Fiber Type*	*Extraction/Desorption Parameters*
10mL SPME vial: 2.15g NaCl 8.5mL DI H_2O 0.25µL phosphate buffer (pH 8.0) Desired amount of hormone mix	20mL SPME vial: 4.3g NaCl 17mL DI H_2O 0.50µL phosphate buffer (pH 8.0) Desired amount of hormone mix	PDMS/DVB (23 ga, d_f 65µm)	Incubation: 10 min at 55°C Pre-Fiber Bake: 18min (GCxGC-TOFMS) Extraction: 60 min Desorption: 3 min Post-Fiber Bake: 20 min

Table 5-3. Method conditions used for GCxGC-TOFMS analysis by Chopra [83].

GCxGC-TOFMS		
GC Parameters	**Oven Parameters**	**MS Parameters**
Column: RTX-5MS 30m, 0.25m, 0.25µm	Primary Column: Initial Temperature: 40°C (hold 1 min) 20°C/minute Ramp to 200°C (hold 3 min)	EI Source: 230°C
Carrier Gas: Helium Column Flow: 1mL/min	Secondary Column: Initial Temperature: 46°C (hold 1 min) 20°C/minute Ramp to 250°C (hold 3 min)	Transfer Line: 250°C
Injection Mode: Splitless Inlet Temperature: 250°C	Modulator Parameters: Offset 35° to secondary column 0.90sec hot pulse time 1.60sec cool time between stages	Solvent Delay: 120 sec

Table 5-4. Method conditions used for GC-MS/MS analysis.

GC-MS/MS		
GC Parameters	*Oven Parameters*	*MS Parameters*
Column: RTX-5MS 15m, 0.25mm, 0.25µm	Initial Temperature: 40°C Hold 1 minute	EI Source: 250°C
Carrier Gas: Helium	20°C/minute Ramp to 300°C Hold 3 minutes	Transfer Line: 250°C
Column Flow: 0.98mL/min Linear Velocity: 51.0cm/sec		
Injection Mode: Splitless		
Inlet Temperature: 250°C		

Table 5-5. MRM method conditions used for GC-MS/MS analysis.

Hormone	**Retention Time (min)**	**Precursor Ion**	**Product Ions**	**Collision Energy**
Diethylstilbestrol	11.607	268	239; 145; 107	10; 18; 26
Prasterone	12.410	288	203; 107; 97	10; 14; 20
Methandriol	12.563	253	197; 155; 169	16; 26; 26
Estrone	12.820	270	185; 157; 172	14; 26; 20
Estradiol	12.847	272	185; 172; 213	8; 14; 16
Mesterolone	12.877	218	159; 105; 200	14; 26; 8
Boldenone	13.030	122	107; 77; 79	16; 26; 18

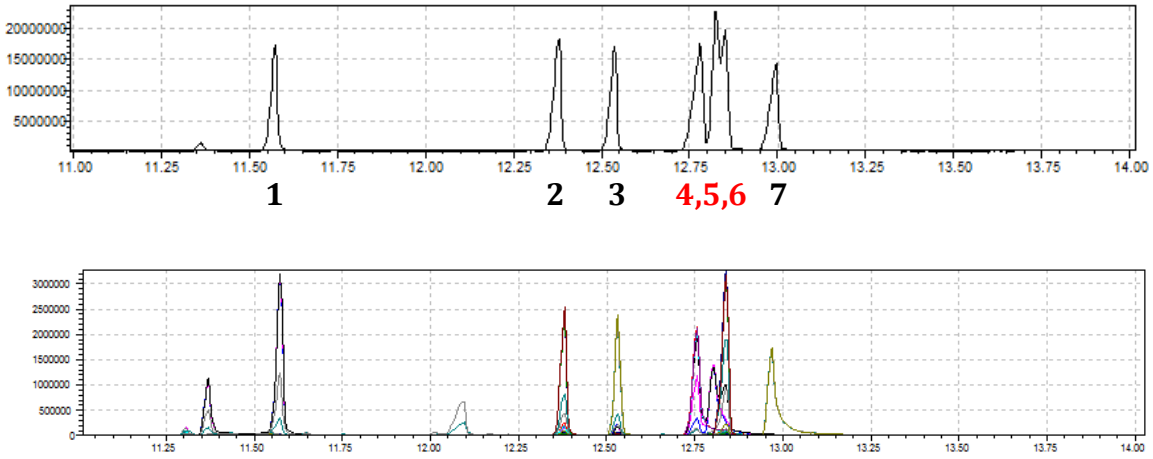
3. Results and Discussion

3.1. SPME Sample Preparation

The SPME conditions including pH, salt amount, and extraction time were previously optimized by Chopra using GCxGC-TOFMS and GC-MS/MS including 2.15g NaCl, 8.5mL DI H_2O, 25µL, and the desired amount of standard mixture in a 10mL vial with a 60 minute extraction time [83]. During this study, it was found that 60 minutes also provided the best extraction for all of the hormones of interest. Figures 5-4 and 5-5 depict the optimized SPME extraction at 60min with a 0.5ppm sample for GC-MS/MS analysis and a 0.03ppm for GCxGC-TOFMS, respectively.

2.3. GCxGC-TOFMS and GC-MS/MS Instrument Comparison

The analysis of these 7 hormones using both GC-MS/MS and GCxGC-TOFMS analysis provided a direct comparison of the resulting optimized separations. Figure 5-5b shows the separation using GC-MS/MS and SPME. The co-elution seen was resolved using the detector of the instrument and the optimized MRM method. As is seen in Figure 5-4a using a liquid injection, three of the compounds are co-eluting: Estrone, estradiol, and mesterolone. Rather than attempt to optimize the GC parameters further, the capabilities of the detector to resolve the co-eluted peaks using an MRM method was employed. Figure 5-4b shows the resolution of those three peaks using the MRM software by choosing ions specific to each compound at its specified retention time.



Hormone	Retention Time (min)
1) Diethylstilbestrol	11.607
2) Prasterone	12.410
3) Methandriol	12.563
4) Estrone	**12.820**
5) Estradiol	**12.847**
6) Mesterolone	**12.877**
7) Boldenone	13.030

Figure 5-4. A: Total ion chromatogram of the hormones of interest using GC-MS/MS and direct liquid injection (500ppm). B: Chromatogram of hormones resolved on GC-MS/MS using the optimized MRM method and SPME sample introduction (0.5ppm).

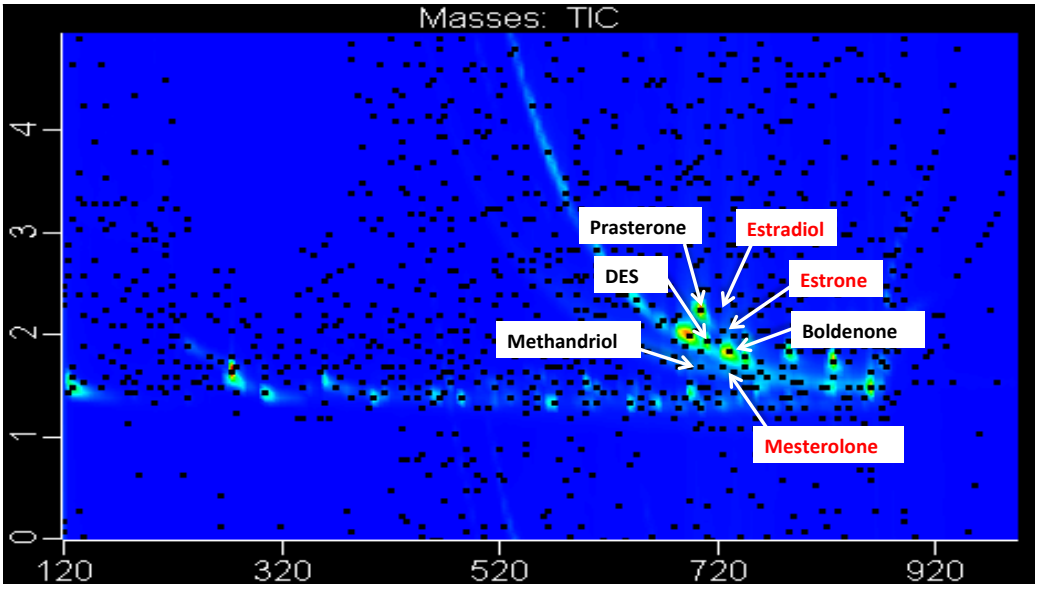


Figure 5-5. Total ion chromatogram of hormones resolved on GCXGC-TOFMS with SPME sample introduction (0.03ppm). The analytes that were co-eluting using single dimension GC are highlighted in red on the figure. Reprinted with permission from Chopra S. Dissertation and Theses 2014 [83].

The hormones were also separated using GCxGC-TOFMS using the two-dimensional chromatographic separation ability of the instrument. Figure 5-5 depicts the separation of all the compounds not only from one another but also from column and septum bleed as outlined by a white box. It can be seen that once again estrone, estradiol, and mesterolone are almost directly behind one another in the 2D separation space, thus in a single dimension separation they would co-elute as was seen during analysis with GC-MS/MS. However, the two-dimensional separation ability of the instrument was able to resolve this co-elution using chromatography rather than the detector as with GC-MS/MS. There is also a multitude of peaks seen in the separation space, some of which are impurities and degradation products that could allow for the collection of further data.

4. Conclusion

Comparing these two techniques during this study for the extraction of hormones from water using SPME illustrated the separation ability of each instrument. Though the methodology used to achieve this separation was different for each instrument, the overall goal of resolving co-elution was accomplished.

The use of a detector to achieve separation was demonstrated using an MRM method during analysis with GC-MS/MS in which three co-eluted peaks were resolved. Chromatographic separation was shown in which the two-dimensional separation ability of GCxGC-TOFMS provided resolution of these three co-eluted peaks as well. This study also showed the ways in which these techniques varied, such as separation of

column and septum bleed from the sample, demonstrating that these instruments should both be used for sample analysis in order to obtain as much information as possible. One instrument is not superior over the other as they have different strengths and weaknesses, thus it is in an analyst's best interest to utilize both in order to collect all information possible.

CHAPTER 6 – QUECHERS-HPLC FOR THE SEPARATION OF STEROIDS IN HERBAL MEDICINAL PRODUCTS

A previous study investigating the detection of steroids in herbal medicinal products was performed using high performance liquid chromatography (HPLC) and QuEChERS extraction. It was of interest to perform the conditions stated in this paper for both the extraction method as well as the HPLC conditions to see if the results and separation could be replicated. It was found that the parameters listed in the paper for both the extraction and HPLC method were not optimal for the work performed currently. The new optimized parameters will be discussed in this chapter.

1. Introduction

1.1. Adulteration of Herbal Medicinal Products (HMPs)

The detection of adulterants and contaminants in herbal medicinal products, or HMPs, is very important as the presence of these substances can cause adverse effects. In order for the safety of the consumer, a method must be developed for the detection of these possible adulterants. Common adulterants and contaminants include mold, fungi, pollens, dust, insects, rodents, microbes, parasites, toxins, toxic heavy metals, pesticides, and prescription drugs causing severe side effects including meningitis, organ failure, stroke, heavy metal poisoning, coma, and death. The most commonly adulterated HMPs are traditional Indian and Chinese herbal medicines [90-94].

1.2 QuEChERS and HMPs

QuEChERS has been used for the extraction of pesticides from many different matrices, including herbal medicinal products. The analysis of fresh herbs used in HMPs was performed using QuEChERS and GC-MS-SIM for the presence of pesticides and PAHs. It was found that acetonitrile provided the best extraction solvent, providing cleaner samples with increased recoveries of 71.6-116.9% and RSD values less than 15%. Pesticides were detected in real samples, with some pesticides present at levels greater than the maximum residue levels (MRL) stated by the European Commission [92]. Auyurvedic churna was analyzed for 200 pesticides in 28-minutes using GC-MS/MS. Dried leaves of herbs are used to make the churna so it is important to have a method of screening and detection for the presence of pesticides in order to assure product safety. Limits of detection were found as low as 2.5ng/g, which is below the Unani guidelines [93].

1.3. QuEChERS-HPLC for the Analysis of Steroid Adulteration in HMPs

There has been very little work in the literature performed for the analysis of steroids in HMPs using HPLC, and none using GC-MS analysis. Two studies were found that investigated steroid adulteration in HMPs using HPLC. One of these investigated dexamethasone adulteration in herbal medicines using QuEChERS and HPLC-UV analysis. The QuEChERS method used a 4:1 ratio of $MgSO_4$:NaCl (500mg:125mg) and acetonitrile with no d-SPE step. The sample was filtered and analyzed using HPLC with a 1% acetic acid and methanol mobile phase (40:60 ratio) with UV detection at 254nm.

Limits of detection and quantitation were determined to be 0.3ppm and 1.0ppm, respectively, with a percent RSD less than 10, and recoveries of 90-110% in both analysis of a standard and herbal pill obtained from Bangkok Thailand [94].

The specific article under investigation for the work outlined in this chapter was performed by Klinsunthorn and co-workers for the analysis of 9 glucocorticoids in HMPs using HPLC-UV. The authors optimized a QuEChERS method involving 500mg $MgSO_4$ and 125mg NaCl, 2mL ACN and 2mL liquid herbal medicine, 50mg of PSA used during d-SPE, and filtering of the final extract before HPLC analysis using a gradient method with an ACN and water based mobile phase and monitoring at 240nm using PDA UV-VIS detection. The method provided a 91-113% recovery, less than 4.6 and 3.2% intraday and interday precision, respectively, and limits of detection from 0.06-0.17ppm. The method was also able to detect steroids in 3 out of the 6 herbal medicines investigated: 1.6 and 8.8ppm dexamethasone and 0.43ppm prednisolone [29]. These parameters were different from those found during Chapter 3 in optimizing the extraction of glucocorticoids from water. The QuEChERS method optimized for the 8 glucocorticoids in Chapter 3 was used here in attempting to reproduce the HPLC method and results obtained by Klinsunthorn.

2. Materials and Methods

2.1. Chemicals, Reagents, and Samples

Acetonitrile (ACN) was purchased from pharmco-AAPER (Kindermorgan, PA) and was reagent ACS grade. All salts used throughout the study as well as the steroid standards were purchased from Sigma Aldrich (St. Louis, MO). QuEChERS tubes containing 150mg PSA and 50mg $MgSO_4$ were purchased from Restek (Bellefonte, PA) and 15mL PFTE centrifuge tubes were obtained from VWR International (Radnor, PA). Deionized water was used throughout the methodology.

2.2. QuEChERS Sample Preparation

The pH, salt amount, and type of solvent were all evaluated in the optimization of the QuEChERS method for the extraction of 8 steroids: beclomethasone, cortisone acetate, prednisone, hydrocortisone, prednisolone, fludrocortisone acetate, dexamethasone, and methylprednisolone as described in Chapter 3 using GC-MS/MS. One difference between the current study and a previous study by Klinsunthorn and co-workers involved the use of prednisone rather than betamethasone [29]. The pH, salt amount, and organic solvent were optimized using GC-MS/MS and can be seen in detail in Chapter 3. These parameters were investigated by Klinsunthorn and were compared to what was found during the optimization performed in Chapter 3. Both QuEChERS parameters are summarized in Table 6-1.

Table 6-1. QuEChERS parameters optimized during both studies with optimal conditions in **bold**.

QuEChERS Parameters	Klinsunthorn and co-workers [29]	Current Study
pH	Not mentioned	**6.0** and 7.0
Solvents	**Acetonitrile** Ethyl Acetate Acetone	**Acetonitrile** Ethyl Acetate Acetone
Salt amounts during LLE	**500mg $MgSO_4$, 125mg NaCl** Other total salt amount investigated: 1000mg, 750mg, 625mg, and 500mg	500mg $MgSO_4$, 125mg NaCl 500mg $MgSO_4$, 250mg NaCl **500mg $MgSO_4$, 500mg NaCl**
Sorbents during d-SPE	**PSA**, C18, alumina, GCB, HLB	**PSA**

2.3. Instrumental Parameters

The instrumentation utilized for this study was an HP1100 Series HPLC (G1312A Bin Pump, G1313A ALS, G1316A ColComp) with a UV-VIS diode array detector (G1315A DAD). A 1µL injection of a sample containing all 8 steroids was used for all analyses. Three different columns were investigated including a Luna(2) C18 column (250mm, 4.6mm, 5µm), a Halo C18 column (100mm, 4.6mm, 2.7µm), and an Ascentis Express C18 column (50mm, 4.6mm, 2.7µm). The first column was used to mimic the original conditions of Klinsunthorn and co-workers. The latter two were chosen for their more efficient column length and poroshell column packing that is proven to increase efficiency and resolution [95]. The separation and detection parameters for the current and previously performed study are listed below in table 6-1. The main difference being the use of an isocratic method in the current study versus a gradient method as well as the use of a shorter, more efficient column resulting in a shorter elution time and more efficient overall analysis.

2.4. Analysis of Real Samples

The four herbal medicinal products investigated during Chapter 3 for the presence of glucocorticoids were used here for determining the presence of glucocorticoids using the optimized HPLC method with the Luna(2) column. As in Chapter 3, any tablet products were ground and placed into a 10mL volumetric flask where deionized water was added to the mark. The contents of the capsule product were emptied into a 10mL volumetric flask as well with deionized water. For each of these products, 2mL of each were used in the optimized QuEChERS method, whereas 2mL of the liquid tonic was used with no

dilution. The optimized QuEChERS method was applied and the sample was analyzed using the HPLC parameters listed in Table 6-2.

3. Results and discussion

2.2. QuEChERS Sample Preparation

The QuEChERS parameters used by Klinsunthorn were compared to those optimized in this study [29]. During the LLE, two of the parameters between the methods were the same including solvent type and amount (2mL acetonitrile) and sample amount (2mL). The salt amount differed between the two methods. The commonly used ratio of $MgSO_4$:NaCl during the LLE step for QuEChERS is 4:1 as used in Klinsunthorn's study; however, the current study found that a ratio of 1:1 with 500mg of each provided better phase separation and analyte extraction as noted in Chapter 2 for the extraction of caffeine from tea as well as in Chapter 3 for the extraction of these 8 glucocorticoids from water. During the d-SPE step, PSA was used during the current and previous study. Klinsunthorn investigated other sorbents as well and observed interference peaks that persisted when using C18, alumina, and HLB (Hydrophilic-Lipophilic-Balanced) [29]. The use of the optimized QuEChERS method was successful in the extraction of these steroids from water using HPLC analysis. For detailed QuEChERS parameters please see Chapter 3.

Table 6-2. Method conditions used for HPLC analysis for the current and previous study.

	Current Study	**Conditions Used By: Klinsunthorn and co-workers**
Mobile Phase Conditions	ACN :Water **isocratic** elution 35% ACN	ACN:Water **gradient** elution 33:67 (0-10 min) 50:50 (10-20min)
Column	1: Luna (2) C18 column 250mm x 4.6mm x 5µm *Elution time 25 min* 2: Halo C18 column 100mm x 4.6mm x 2.7µm *Elution time 10 min* 3: Ascentis Express C18 50mm x 4.6mm x 2.7µm *Elution time 10 min*	Hypersil BDS C18 column 300mm x 4.6mm x 5µm *Elution time 20 min*
UV-VIS Conditions	PDA UV-VIS Scanning 200-400nm (monitor at 240nm)	DAD UV-VIS Scanning 200-400nm (monitor at 240nm)

3.1. Optimization of HPLC Method

This study attempted to replicate the results obtained by Klinsunthorn for the separation of glucocorticoids using HPLC as well as attempting to further optimize the method. Optimization of the HPLC method for the 8 steroids of interest began by first using a column of similar nature to that used by Klinsunthorn which was a C18 column, 300mm, 4.6mm, 5µm. The column used in the current study was a Luna(2) C18 column, 250mm, 4.6mm, 5µm. The gradient method used by Klinsunthorn listed in Table 6-2 was used and resulted in very little separation of the steroid mixture as seen in Figure 6-1. Various gradient methods were attempted with no complete resolution of the sample. Isocratic methods were then attempted ranging from 65% organic phase to 25% organic phase. A final isocratic method of 35% acetonitrile:65% water provided separation of all compounds of interest, shown in Figure 6-2. One item to note is the added compound, prednisone, co-eluted with hydrocortisone and caused some added difficulty in method optimization that may not have been present if the original mixture used by Klinsunthorn had been employed. The isocratic method was 25min compared to the 20-minute gradient method.

The method was further optimized using a shorter, more efficient Halo C18 column, 100mm, 4.6mm, 2.7µm. This resulted in a less than 10-minute separation with the 35% isocratic method as seen in Figure 6-3, cutting the method time in half. Again, there was limited separation between prednisone and hydrocortisone; however, this method would have been sufficient using the steroid mixture by Klinsunthorn. The 50mm Ascentis Express C18 column provided separation in under 5 minutes as illustrated in Figure 6-4;

Table 6-3. Summary of retention times for each steroid from Klinsunthorn and co-workers' study as well as the current study.

	Retention Time (min)			
Glucocorticoid	**Gradient Method** Klinsunthorn	**Isocratic Method** Luna(2)	**Isocratic Method** Halo	**Isocratic Method** Ascentis Express
Prednisolone	6.81	6.600	1.660	**0.956**
Hydrocortisone	7.13	6.954	**1.718**	**0.956**
Prednisone	---	7.098	**1.718**	**0.956**
Methylprednisolone	10.01	9.780	2.767	1.318
Betamethasone	10.73	---	---	---
Dexamethasone	11.20	11.519	3.305	1.546
Beclomethasone	12.59	13.596	3.983	1.792
Fludrocortisone Acetate	17.32	22.338	6.576	2.864
Cortisone Acetate	18.86	26.430	8.264	3.379

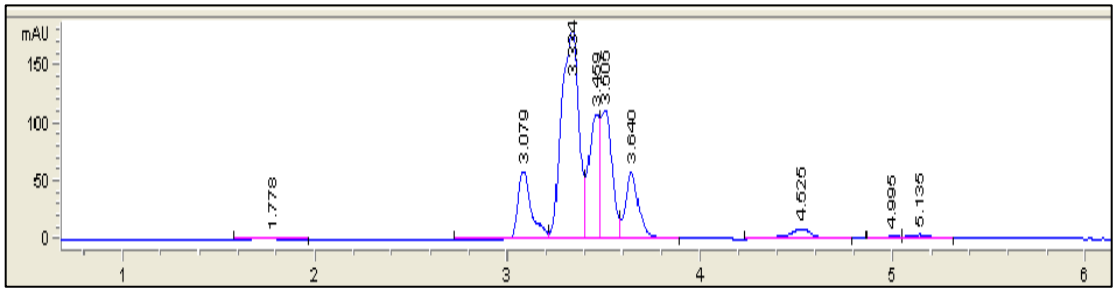
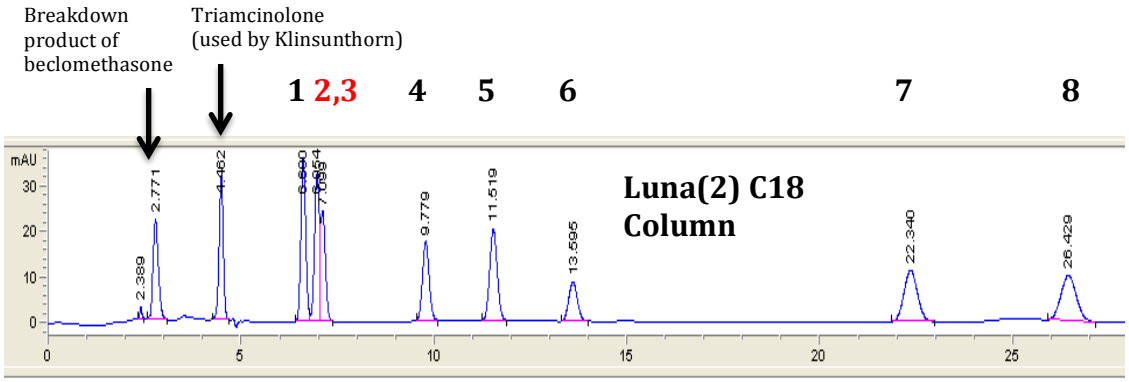


Figure 6-1. Resulting chromatogram in using the gradient method reported by Klinsunthorn.



Steroid	Retention Time (min)
1) Prednisolone	6.600
2) Hydrocortisone	**6.954**
3) Prednisone	**7.098**
4) Methylprednisolone	9.780
5) Dexamethasone	11.519
6) Beclomethasone	13.596
7) Fludrocortisone Acetate	22.338
8) Cortisone Acetate	26.430

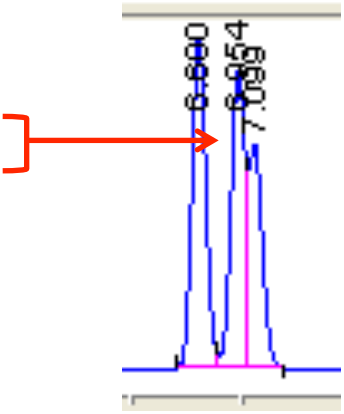
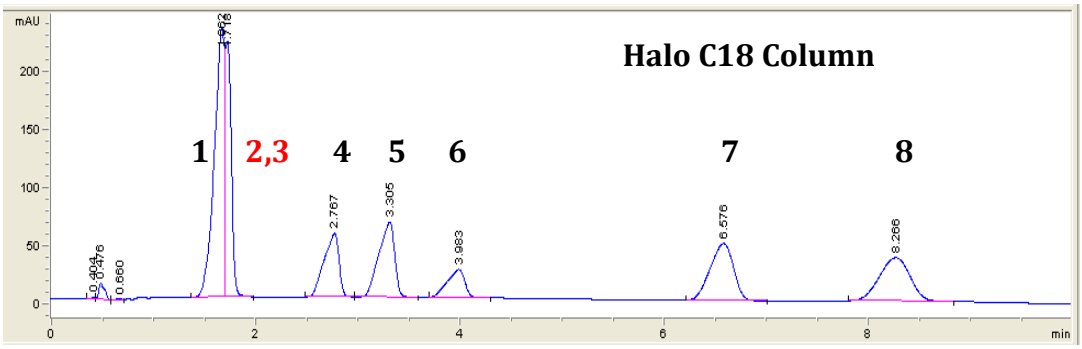
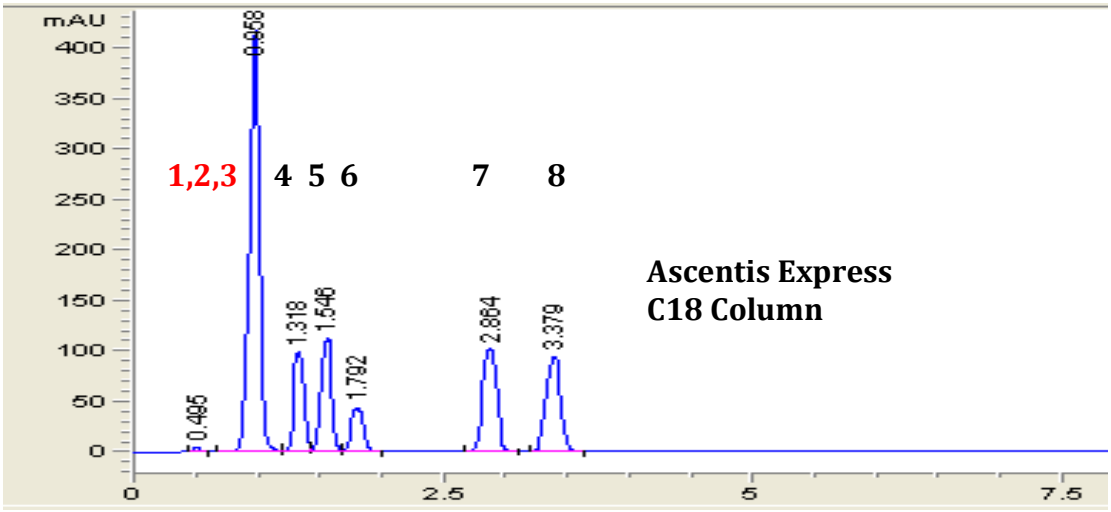


Figure 6-2. Resulting chromatogram and retention times using the isocratic method and Luna(2) C18 column in the current study.



Steroid	Retention Time (min)
1) Prednisolone	1.660
2) Hydrocortisone	**1.718**
3) Prednisone	**1.718**
4) Methylprednisolone	2.767
5) Dexamethasone	---
6) Beclomethasone	3.305
7) Fludrocortisone Acetate	3.983
8) Cortisone Acetate	6.576

Figure 6-3. Resulting chromatogram and retention times using the isocratic method and Halo C18 column in the current study.



Steroid	Retention Time (min)
1) Prednisolone	**0.956**
2) Hydrocortisone	**0.956**
3) Prednisone	**0.956**
4) Methylprednisolone	1.318
5) Dexamethasone	---
6) Beclomethasone	1.546
7) Fludrocortisone Acetate	1.792
8) Cortisone Acetate	2.864

Figure 6-4. Resulting chromatogram and retention times using the isocratic method and Ascentis Express C18 column in the current study.

however, not only were prednisone and hydrocortisone unresolved, but prednisolone was now eluting with these two analytes as well providing a method that would not have provided separation using Klinsunthorn's steroids.

3.2. Analysis of Real Samples: HMPs

The four HMPs were analyzed for glucocorticoid adulteration using the Luna(2) column and isocratic HPLC method conditions. The analysis results showed that none of the glucocorticoids were detected, which is congruent with the findings in Chapter 3 using GC-MS/MS analysis. Though, as concluded in Chapter 3, it may be possible that these compounds are present in the samples and were simply not able to be detected using the method and instrument employed. An instrument with lower detection limits and better selectivity for the analytes of interest may be needed such as LC/MS in order to detect if these compounds are truly present in the HMPs studied.

4. Conclusion

This study was successful in providing an isocratic method for the separation of the 8 steroids of interest. It was also determined that the method developed by Klinsunthorn was not a rugged or reproducible method [29]. The use of an isocratic method is desirable, as fundamental parameters such as theoretical plate height are only truly accurate when using an isocratic method. Though there was some separation difficulty from the added prednisone, the 7 other steroids investigated by Klinsunthorn were easily separated with the isocratic method using both a 250mm and 100mm C18 column,

resulting in a faster, more efficient separation, improving upon the method by Klinsunthorn. Though the co-eluting peaks could not be separated using the UV-VIS, if LC/MS were used for sample analysis, the separation of these compounds would be much more likely due to the fact that they have different mass spectra as opposed to their UV-VIS spectra, which are very similar.

Some future work to perform following this study would be to improve upon the separation achieved so that all 8 steroids can be separated using a shorter, more efficient column. This could be accomplished by evaluating different columns as well as different solvent systems for the mobile phase in an attempt to increase the separation. Once optimized, validation could be performed on the method and limits of detection could be compared to those found by Klinsunthorn. Various HMPs could also be analyzed using this method to determine if steroids are present as adulterants in the products.

CHAPTER 7 – QUECHERS FOR THE ANALYSIS OF DRUGS OF ABUSE IN SYNTHETIC URINE

QuEChERS has been used for the extraction of various compounds including pharmaceutical products and veterinary drugs from biological matrices with analysis via GC and LC successfully. Its success in other fields in providing a clean sample for analysis elucidates the viability of QuEChERS for the field of forensic drug analysis. This is an area of research which must be explored further as past literature for the extraction of similar compounds from biological matrices implies that QuEChERS would be extremely successful in forensics in providing a quick, easy, cheap, effective, rugged, and safe method for the analysis of forensic drug samples.

This study was performed to demonstrate proof of concept for the use of QuEChERS in the extraction of drugs of abuse from biological matrices such as urine. The study was successful in the detection of 3 drugs of abuse using GC-MS/MS and 6 drugs of abuse using GC-MS-SIM with no derivatization. Derivatizing the drugs in order to increase recovery during GC analysis could increase the number of drugs detected as well as decrease the detection limits during analysis.

1. Introduction

1.1. QuEChERS and Forensic Analysis of Drugs of Abuse

The primary focus of QuEChERS since its discovery has been the analysis of pesticides in food products. This method is adept at extracting compounds from complex matrices, thus it could be applied to a much larger range of compounds and matrices. This method has already been used for the extraction of pharmaceutical drugs from biological matrices and so it could be very useful to forensic samples for the extraction of drugs of abuse from matrices such as blood and urine [14]. This study looks to expand the uses of QuEChERS with a proof of concept study for the extraction of drugs of abuse from synthetic urine using GC-MS/MS and GC-MS-SIM analysis. GC-MS remains the primary instrumentation for forensic drugs of abuse analysis, although LC-MS based methods are gaining importance. Interestingly, QuEChERS has been primarily used with LC for drug analysis so far in the literature; however, this does not limit its potential with GC, as the compounds investigated are amenable to GC as well.

Studies that have used LC analysis for compounds which could be analyzed using GC as well include the analysis of urine, blood plasma, hair, and tissue samples for the extraction of lipids, banned veterinary drugs, and phenylethanolamine A using QuEChERS [96-98]. The extraction of lipids from both blood and urine had a percent recovery around 90% as well as minimized matrix effects due to clean up with C_{18} d-SPE sorbent [96]. Eighty-seven banned veterinary drugs such as steroid hormones, β-agonists, and tranquilizers were extracted from bovine urine with percent recoveies greater than 60% using three different QuEChERS methods which extracted 92-100% of

the desired compounds. Each of these QuEChERS methods performed better than a dilution extraction and two SPE methods in which only 32-70% of the desired compounds were extracted, demonstrating QuEChERS as a successful compliance monitoring tool for these substances [97]. Finally, the analysis of seven different matrices including animal hair, tissue, and feeds for phenylethanolamine A, a β-agonist that can be used illegally as a growth promoter in livestock, was performed using QuEChERS with an average percent recovery of 95.4-108.9% [98].

All of these applications of QuEChERS for the analysis of drugs in biological samples using LC can be applied to forensic samples using GC analysis. This study aims to broaden the application of QuEChERS-GC by extracting several drugs of abuse from synthetic urine.

2. Materials and Methods

2.1. Chemicals, Reagents, and Samples

Methanol was purchased from pharmco-AAPER (Kindermorgan, PA) and was reagent ACS grade. All salts used throughout the study as well as the drug standards were purchased from Sigma Aldrich (St. Louis, MO). The drug standards used were 1mg/mL and prepared in methanol. These standards were diluted to 100ppm and combined in a mixture for analysis and optimization of an MRM method. QuEChERS tubes containing 150mg PSA and 50mg $MgSO_4$ were purchased from Restek (Bellefonte, PA) and 15mL PFTE centrifuge tubes were obtained from VWR International (Radnor, PA).

MoniCheck synthetic urine was purchased from Branan Medical Corporation (Irvine, CA). The urine purchased was both negative and positive, with the positive urine containing the following drugs: amphetamine (3,000ng/mL), methamphetamine (3,000ng/mL), secobarbital (900ng/mL), phencyclidine (75ng/mL), methadone (900ng/mL), nortriptyline (3,000ng/mL), oxazepam (900ng/mL), morphine (6,000ng/mL), and benzoylecgonine (900ng/mL). Deionized water was used throughout the methodology.

2.2. Sample Preparation

The pH, salt amount, and type of solvent were all previously optimized in Chapter 3 using steroids and are summarized in Figure 7-1. These optimized parameters were used during this study for the extraction of drugs of abuse from synthetic urine.

2.3. Instrumental Parameters

The instrumentation utilized for this study was a Shimadzu GC-MS/MS TQ8030 with an AOC-5000 Auto Injector (Santa Clara, CA) and an Agilent 6890 GC and 5973 MSD (Santa Clara, CA) and CTC Analytics combiPAL (Zwingen, Switzerland). Splitless liquid injection was used for all analyses of non-extracted and QuEChERS samples, with multiple reaction monitoring (MRM) being used during GC-MS/MS analysis and selected ion monitoring during GC-MS analysis. The separation and detection parameters for both instruments are listed below in Tables 7-1 to 7-4.

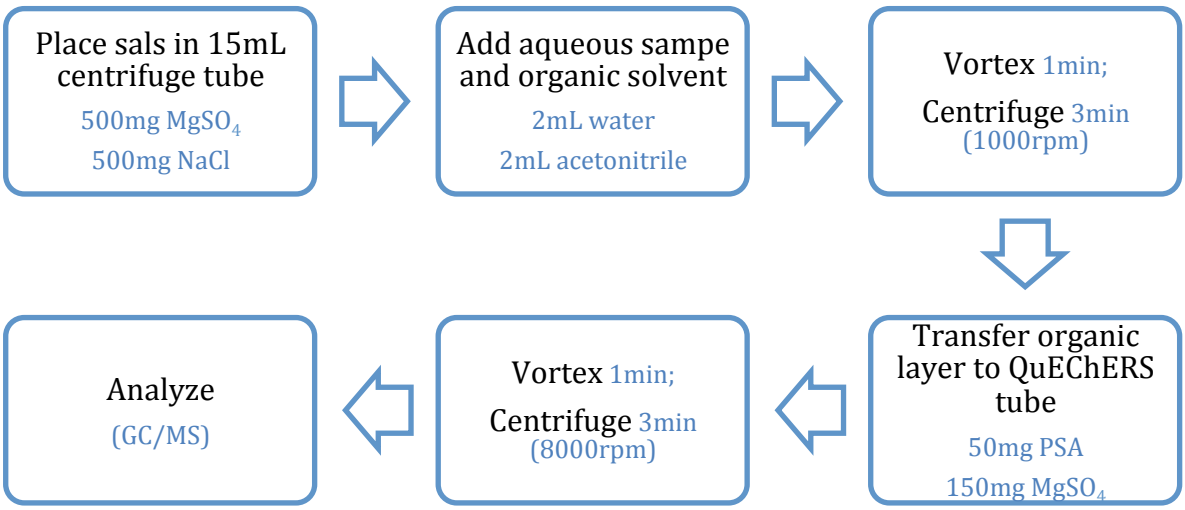


Figure 7-1. Summary of QuEChERS conditions used for analysis of synthetic urine samples.

Table 7-1. Method conditions used for GC-MS/MS analysis.

GC-MS/MS		
GC Parameters	*Oven Parameters*	*MS Parameters*
Column: RTX-5MS 15m, 0.25mm, 0.25µm	Initial Temperature: 60°C Hold 1 minute	EI Source: 250°C
Carrier Gas: Helium	20°C/minute Ramp to 300°C Hold 10 minutes	Transfer Line: 280°C
Column Flow: 0.98mL/min Linear Velocity: 51.0cm/sec		
Injection Mode: Splitless		
Inlet Temperature: 250°C		

Table 7-2. MRM method conditions for the drugs of interest

Drug of Abuse	**Retention Time (min)**	**Precursor Ion**	**Product Ions**	**Collision Energy**
Amphetamine	3.683	65	51; 63	10; 10
Methamphetamine	4.107	65	51; 63	10; 10
Secobarbital	8.110	167	124; 78; 106	10; 20; 18
Phencyclidine	8.597	200	117; 84; 115	18; 12; 24
Methadone	9.843	72	56; 57	24; 18
Nortriptyline*	10.157	202	*Successful with GC-MS-SIM (precursor ion used)	---
Oxazepam**	10.727	267	**Unsuccessful	---
Morphine**	11.190	285	**Unsuccessful	---
Benzoylecgonine**	11.780	82	**Unsuccessful	---

Table 7-3. Method conditions used for GC-MS analysis.

GC-MS		
GC Parameters	*Oven Parameters*	*MS Parameters*
Column: RTX-5MS 30m, 0.25mm, 0.25µm	Initial Temperature: 60°C Hold 1 minute	EI Source: 250°C
Carrier Gas: Helium	20°C/minute Ramp to 300°C Hold 10 minutes	Transfer Line: 280°C
Column Flow: 1.0mL/min		
Injection Mode: Splitless		
Inlet Temperature: 250°C		

Table 7-4. SIM method conditions for the drugs of interest

Glucocorticoid	**SIM Scan Time (min)**	**SIM Ion Parameters**
Amphetamine	3 to 7	**65;** 51; 63
Methamphetamine	3 to 7	**65;** 51; 63
Secobarbital	7 to 12	**124;** 78; 106
Phencyclidine	7 to 12	**117;** 84; 115
Methadone	7 to 12	**72;** 56; 57
Nortriptyline*	7 to 12	**202**
Oxazepam**	12 to 18	**267**
Morphine**	12 to 18	**285**
Benzoylecgonine**	12 to 18	**82**

3. Results and Discussion

3.1. Optimization of MRM GC-MS/MS method

In optimizing a multiple reaction method (MRM) for the analysis of the drugs of interest (amphetamine, methamphetamine, secobarbital, phencyclidine, methadone, nortriptyline, oxazepam, morphine, and benzoylecgonine), it was found that while all drugs were detected using full scan during analysis individually, when analyzed as a 100ppm mixture only amphetamine, methamphetamine, secobarbital, phencyclidine, and methadone were detected using GC-MS/MS. Nortriptyline was also detected when using the GC-MS-SIM method.

One reason for the compound drop out could be in combining the drugs, interactions occurred, causing degradation of these compounds, resulting in no detection. Those that were not detected in a mixture contained hydroxyl groups, whereas the other compounds did not. This will be studied further in future work to be performed by Leanne Mocniak by investigating different combinations of the various drugs of abuse to determine what is causing the loss of these three compounds. A 10ppm mixture was analyzed as well to determine if perhaps the 100ppm mixture was overloading the instrument, yet these drugs remained undetected at this lower concentration as well. Morphine and oxazepam had fairly substantial peaks during analysis alone and yet are completely absent from the mixture when analyzed, supporting the hypothesis that some drug interaction is occurring with these two particular drugs, causing them not to be seen on the chromatogram. The peak for benzoylecgonine is very small when run alone and thus is most likely overshadowed by the other drugs to the point where it is not detectable when run in the

mixture. This problem could be remedied by derivatization of the compounds to reduce the chances of any unwanted interaction with either the other drugs or the inlet or column that could be causing the absence of these drugs during analysis of the drug mixture.

Once the total ion scan was performed and ions for each of the detected drugs were selected, a product ion scan was performed to determine the product ions as well as the optimal collision energy. These parameters are listed in the instrumental parameters section of this chapter in Tables 7-2 and 7-4. The resulting chromatograms for the analysis of a 100ppm drug mixture are depicted in Figure 7-2 using GC-MS/MS and Figure 7-3 using GC-MS-SIM.

3.2. Analysis of Synthetic Urine Samples

The optimized QuEChERS method was applied to the positive urine sample containing the 9 drugs of abuse and negative urine samples. Secobarbital, phencyclidine, and methadone were detected in positive urine using the optimized MRM method. These three compounds were also detected in positive urine using the GC-MS-SIM method along with amphetamine, methamphetamine, and nortriptyline. Though the peak for secobarbital is very small in the GC-MS-SIM analysis and is difficult to see in Figure 7-5, it is present. Amphetamine and methamphetamine were difficult to detect when GC-MS/MS was used as seen in Figure 7-4. It is hypothesized that this is due to the increased sensitivity of the instrument detecting more compounds from the synthetic urine around the retention times of amphetamine and methamphetamine that make the determination of their presence difficult.

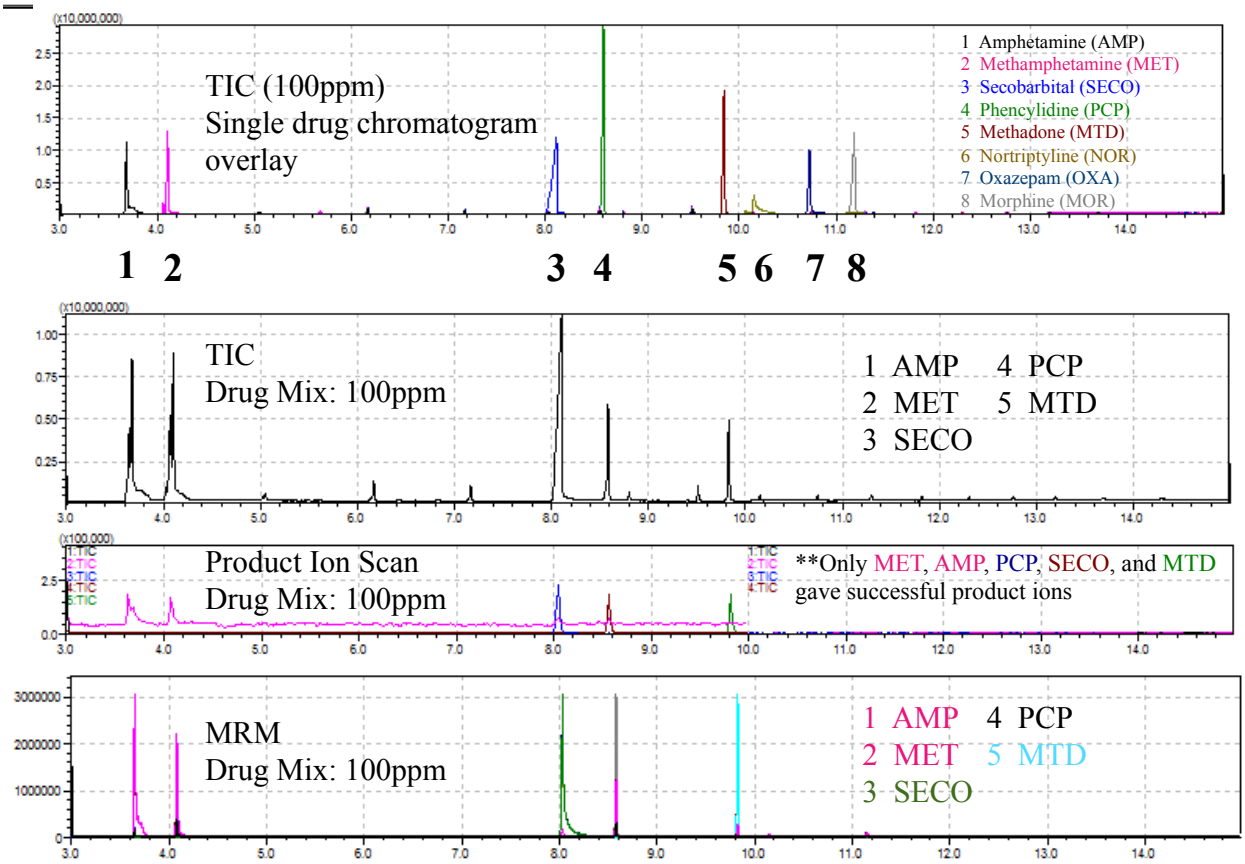


Figure 7-2. From top to bottom: Chromatograms of a total ion scan, product ion scan, and optimized MRM method for the drugs of interest (100ppm standard mixture).

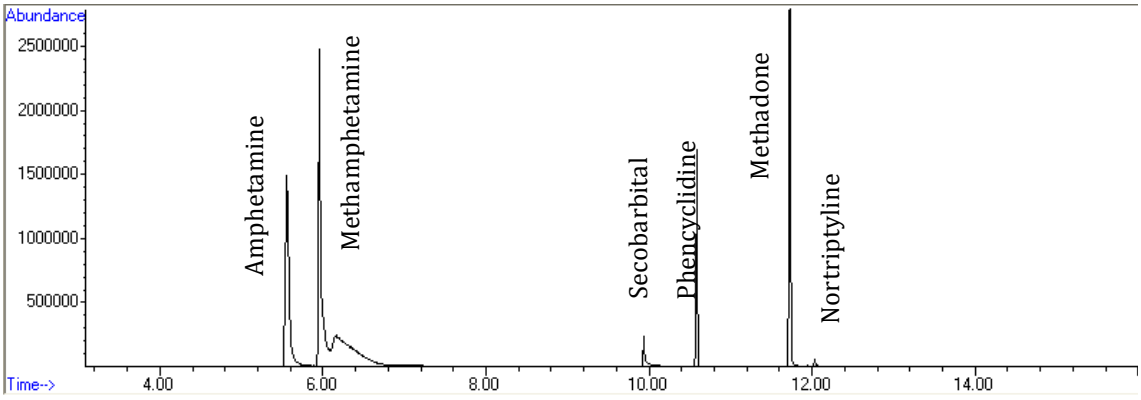


Figure 7-3. Chromatogram using GC-MS-SIM analysis of the drugs of interest (100ppm standard mixture).

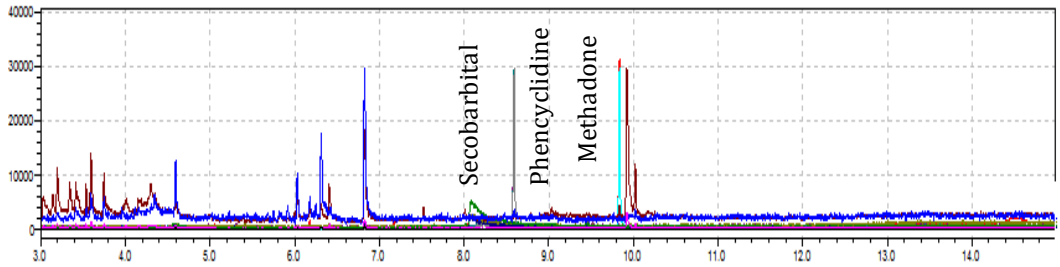


Figure 7-4. Chromatogram using GC-MS/MS analysis of the positive urine sample.

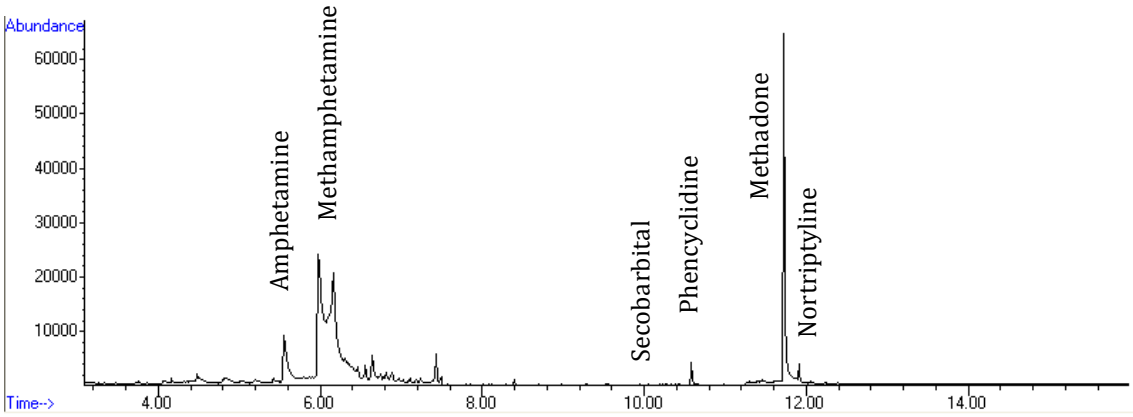


Figure 7-5. Chromatogram using GC-MS-SIM analysis of the positive urine sample.

4. Conclusions

This data illustrates the capacity for QuEChERS to be applied to forensic drug samples and shows proof of concept. QuEChERS was successful in the extraction and detection of multiple drugs of abuse from synthetic urine. It is hypothesized that derivatizing the sample before performing GC-MS analysis will increase the number of drugs extracted. As future work, in addition to investigating the effect of derivatization, the optimized QuEChERS method will be compared to other extraction methods such as SPME and ionic liquid single drop microextraction (IL-SDME).

5. Future Work for QuEChERS

QuEChERS is a method that has not been used to its fullest potential. The possible applications of this method are numerous and should be further investigated in the literature, especially its use for forensic and drug samples. This method, though it has proven to be extremely successful for the extraction of pesticides from various matrices, can be as successful for the extraction of drugs of abuse from biological matrices. Given the work performed throughout this thesis, QuEChERS is amenable to many different types of compounds not only for LC analysis, but GC as well. As GC instrumentation improves, the need for derivatization is not necessary and is evidenced by the analysis of steroids during this work that are normally derivatized in order for detection. However, for those that do require derivatization for improved sensitivity and detection, this technique can be coupled with QuEChERS as it was with SPME. The derivatization step could be included within the QuEChERS method so as to avoid transferring the sample

between vessels, causing further complication of the method and possible loss of any analytes.

Another area of interest that was mentioned briefly in Chapter 5 would be the use of QuEChERS-GC-PTV for using large volume injections of the sample rather than 1µL injections that may cause some loss in sensitivity. The QuEChERS extraction, though on a smaller scale than most other classic multi-residue methods, still uses 2mL of sample and organic solvent, resulting in a fairly large volume of extract as compared to that of SPME which concentrates the sample during extraction, leading to increased sensitivity. The use of a PTV inlet for large volume injections may allow the ability to overcome any sensitivity issues encountered with QuEChERS as compared to a technique such as SPME and further eliminate the need for derivatization of certain compounds. This is definitely an area of research that should be explored for QuEChERS. QuEChERS has limitless applications in terms of analyte extraction from complex matrices. It is up to scientists to explore those fields and expand the use of QuEChERS to improve current extraction methods.

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