Spring 5-21-2022

Extending the application of Polyol-induced extraction for the analysis of Glucocorticoids and NSAIDs in water using UHPLC-MS/MS

Shipra Patel
shipra.patel@student.shu.edu

Extending the application of Polyol-induced extraction for the analysis of Glucocorticoids and NSAIDs in water using UHPLC-MS/MS

BY

Shipra Patel

Mentor: Nicholas H. Snow, Ph.D.

DISSERTATION

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

May, 2022

SETON HALL UNIVERSITY

COLLEGE OF ARTS & SCIENCES

DEPARTMENT OF CHEMISTRY & BIOCHEMISTRY

APPROVAL FOR SUCCESSFUL DEFENSE

Shipra Patel has successfully defended and made all required modifications to the text of the doctoral dissertation for the Ph.D. during this **Spring Semester 2022**.

DISSERTATION COMMITTEE
(sign and date next to your name)

Mentor:

Nicholas H. Snow, Ph.D. _____

Committee Member

Wyatt R. Murphy, Ph.D. _____

Committee Member

Yuri V. Kazakevich, Ph.D. _____

Chair

Stephen P. Kelty, Ph.D. _____

The mentor and any committee members who wish to review revisions will sign and date this document only when revisions have been completed. Return three signed original copies of this form to the Chemistry & Biochemistry Department Office: one will be placed in your file, one will be page 2 of the Departmental Copy of your dissertation and one will be page 2 of the Library Copy of your dissertation.

ACKNOWLEDGMENTS

I would like to thank my mentor Dr. Nicholas H. Snow, for his guidance and mentorship throughout my research work. His knowledge, support, patience, and motivation are invaluable to me and greatly appreciated. He always guided me to think as a scientist and gave a scientific perspective to the way I observe data. Dr Snow has encouraged me throughout my time at Seton Hall University, and his encouragement have made an impact that will stay with me throughout my life.

I would also like to thank Dr. Yuri Kazakevich and Dr. Wyatt Murphy for their feedback, questions, and insightful comments in my dissertation and research work. Their feedback taught me to think with a broad scientific prospective. In addition, I would like to thank Dr Stephen Kelty for serving on my matriculation committee and for providing guidance on time-to-time basis. I also would like to thank previous Seton Hall professors Dr. Joseph T. Maloy and Dr. Monika Raj for serving on my matriculation committee and for providing me their guidance.

I would like to extend a special thanks our present and former separation science group members at Seton Hall University. The opportunity to work together and learn from each other was irreplaceable. Feedback from the team and advice throughout my time at Seton Hall University truly helped me in my research work. I would like to express my appreciation to Perkin Elmer for the UHPLC-MS-MS system.

Most importantly, I would like to extend my greatest appreciation to everyone in my family for their support, care, love, and blessings. This research work and dissertation is dedication to my children, Praket and Nishtha.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABSTRACT

Polyol induced extraction (PIE) is an extraction technique developed and patented by Sowa Jr., Murphy, and Deshpande at Seton Hall University. PIE is an aqueous biphasic system of extraction, which is a technique that separates water from a mixture containing an organic liquid and water by adding a polyol to the mixture that leads to phase separation of aqueous and organic phase. This technique was originally discovered as a method to recycle acetonitrile during a production shortage in 2008. This application is used to extract water from organic liquid by adding polyol mass separating agent, which leads to phase separation. After a successful application of the technique for extraction of essential oils by DelMastro[1] and successful preliminary experiment results, it was decided to explore the potential of PIE as an extraction technique. The goal of this work was to demonstrate that PIE can be an alternative method for extraction of glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDS) which then can be analyzed using ultra high-performance liquid chromatography-triple quadrupole mass spectrometry (UHPLC-MS/MS). This research is categorized into these applications of PIE and one comparison application study.

Chapter 1 provides introduction to PIE, the basic theory of UHPLC-MS/MS, a brief discussion of tandem mass spectrometry and atmospheric pressure electrospray ionization. Although steroidal and non-steroidal drugs are prominently analyzed by liquid chromatography, gas chromatography is also popular due to the resolving power, high peak capacity and ability to separate steroid isomers. Due to low volatility, these analytes require derivatization by techniques such as hydrolysis or methylation after extraction, and before GC analysis. Liquid chromatography is a separation technique that separates compounds based on different degrees

of physiochemical interactions with a stationary phase and a mobile phase. After the separation, quantitation and identification of compounds is carried out via a detector connected to column outlet. The most common detectors used with liquid chromatography include ultraviolet (UV) spectroscopy and mass spectrometry. Tandem Mass spectrometry is very popular in trace analysis due to the higher selectivity, specific and sensitivity.

The first application involves the extraction of glucocorticoids from water into acetonitrile using glycerol as a phase separating agent. Glucocorticoids are a natural and synthetic type of steroids and are very essential in daily functioning of vertebrates. They have very intense anti-inflammatory and anti-immunosuppressive action. They are prescribed in large numbers by medical doctors and veterinarians. Extensive use of these compounds can lead to contamination of the environment. Many different techniques have been used in the analysis of glucocorticoids using several different extraction methods. Most common are solid-phase extraction, liquid-liquid phase extraction and QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe). Glucocorticoids have been studied widely in various matrices. PIE was used for the extraction and UHPLC-MS-MS was used for the analysis of glucocorticoids in water. The focus of this work was to demonstrate PIE as an effective technique that allows extraction of solutes from an aqueous matrix into polar organic solvent. In this work glycerol is used as the mass separating agent in the extraction of glucocorticoids from water into acetonitrile. Eight different glucocorticoids were extracted by PIE and analyzed by UHPLC-MS-MS. Percent recovery, linearity and accuracy were determined for each glucocorticoid. Extraction conditions were optimized, and extraction results were compared to extracted glucocorticoid standards. The glucocorticoids in this study were: beclomethasone, cortisone acetate, prednisone, hydrocortisone, prednisolone, dexamethasone, fludrocortisone acetate and methylprednisolone.

Prior to PIE each individual glucocorticoid was directly infused into MS for the tuning of MS condition. A multiple reaction monitoring transitions of selected precursor ion to selected product ions were optimized for each analyte for quantitative analysis. Optimization of liquid chromatography was carried out to determine chromatographic condition.

The second application in Chapter 3 is a comparison study of PIE to QuEChERS. QuEChERS was developed for the analysis of pesticides from a variety of different matrices, and it is widely used for extraction of a variety of different analytes. PIE is like QuEChERS in terms of use of organic solvent and a mass separating agent to generate phase separation of organic and aqueous phases which leads to extraction of analytes of interest into organic phase. To compare these two methods same eight glucocorticoids were subjected to QuEChERS technique and then analyzed using UHPLC-MS/MS. Method validation was carried out by evaluating accuracy, precision and extraction efficiency. Finally, both methods were compared in terms of extraction procedure.

The application described in Chapter 4 focuses on the extraction of non-steroidal anti-inflammatory drugs (NSAIDs) from water into acetonitrile using glycerol as a phase separating agent. NSAIDs have anti-inflammatory actions and are easily available over the counter medications. Residues of these drugs are also emerging pollutants in water that enter the environment during manufacturing process, improper disposal of drugs and through human and animal excretion. These drugs are also misused for suicidal overdose. Eight NSAIDS were subjected to PIE and then analyzed using UHPLC-MS/MS. Like the first application each individual NSAID was directly infused into the MS for the tuning of MS conditions. Multiple reaction monitoring transitions were optimized with the protonated molecular ion selected as the precursor. Optimization of liquid chromatography and extraction procedure was carried out to

determine final extraction method conditions. Method validation was performed, and accuracy, percent recovery, and precision were determined. Additionally synthetic urine samples were spiked with NSAIDS and then were subjected to PIE and analyzed using UHPLC-MS/MS. Finally, results obtained from urine extraction were discussed in terms of using PIE as an extraction method for various matrices.

In the last application of this research work PIE of glucocorticoids and their analysis using gas chromatography (GC) is described. The PIE method and sample preparation technique is described in detailed in this chapter. GCxGC-TOFMS was used in one dimension mode to carry out the analysis. Therefore, basic discussion of GC is described appropriately in this chapter. Seven glucocorticoids were analyzed except fludrocortisones acetate used in previous study of this research work. Glucocorticoids were analyzed without any derivatization process and method validation was performed, and accuracy, percent recovery, precision, and partition co-efficient determined.

CHAPTER 1 - AN INTRODUCTION TO THE THEORY OF EXTRACTION, PIE BACKGROUND AND SEPARATION VIA LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

1.1 Extraction Introduction

1.1.1 Classical extraction: Basic concept

Classical methods of extraction are liquid-liquid extraction (LLE), solid-liquid extraction, solid phase extraction, Soxhlet extraction, etc. Extraction techniques are based on the equilibrium between two phases either liquid-liquid or solid-liquid. This equilibrium can be given by the following equation 1-1:

$$[\boldsymbol{A}]\boldsymbol{matrix} \rightleftharpoons [\boldsymbol{A}]\boldsymbol{solvent} \qquad \text{Equation 1- 1}$$

The partition coefficient (K_c) involved during equilibrium is the distribution constant or equilibrium constant (K_D) and is defined as:

$$\boldsymbol{K_c} = \frac{[\boldsymbol{A}]_{\boldsymbol{solvent}}}{[\boldsymbol{A}]_{\boldsymbol{matrix}}} \equiv \boldsymbol{K_D} \qquad \text{Equation 1- 2}$$

For an extraction procedure, it is desired that K_D be greater than one to have the majority of the analyte extracted. If K_D is less than one most (but not all) of the analyte remains in the matrix and if $K_D >> 1$ analyte is extracted from the matrix by exhaustive extraction but there is always a definable amount in both phases.[1, 2] In typical liquid-liquid extraction an aqueous phase is mixed

with the organic phase and after the phase separation analytes are partitioned into both layers based on their affinity. Various factors affecting the extraction procedure are type of solvent used, extraction temperature and time as well as pH of the medium during extraction.

1.1.2 Aqueous two-phase systems (ATPS)

Aqueous two-phase systems (ATPS) are biphasic systems that can be used in LLE processes as water-organic solvent extraction systems. Generally, in biphasic systems both immiscible components are water based and two phases formed by mixing two polymers, one polymer and one salt or two salts at a critical concentration where water miscible solvent is separated from water. The formation of a two phase system is affected by temperature, ionic strength and pH.[3] ATPS have been used in the extraction, separation, recovery and purification of bio-molecules such as proteins,[4] enzymes,[5] and nucleic acids.[6] These systems have also been used for small molecules such as antibiotics,[7] antioxidants[8] and alkaloids.[9] Biphasic systems have been induced by polymer-salt, alcohol-salt, two polymers, sugars, ionic liquid-salt, ionic liquid-carbohydrate, ionic-liquid polymer and polyols.[10-14] Polyol induced phase separation is the most recent methodology of phase separation. The partitioning effect can be induced using PIE and it can be an alternative extraction method for small molecules that is potentially more cost effective, eco-friendly and yields higher recovery.

1.2 Polyol Induced Extraction (PIE): Background and theory

Components inducing phase separation are mass separating agents (MSA). As mentioned above these chemical species are added to a mixture of water based miscible solvent system to create an

immiscible solvent system via phase separation where one of the solvent separates in its pure form. This procedure of introducing a mass separating agent (MSA) into a solution system to create a two immiscible phase is a promising alternative for extraction. PIE was developed in response to the acetonitrile shortage in 2008 as a new way to recycle and separate it from water for future use. Use of salts and sugars as MSAs is a well-studied concept and it is commonly referred to as 'salting-out' and 'sugaring-out. Salting out and sugaring out techniques have been used in removal of water from organic solvents.[15-19] Applications of ionic liquids as azeotrope breakers for solvent recycling purposes has also been reviewed and published.[20] Extractive distillation and azeotropic distillation are also existing techniques to recycle organic solvents.[21] But purity of the solvents recycled using these techniques often fall below the desired level of the solvents to be reused. Using a polyol as an MSA, it was determined that acetonitrile can be separated in to upper organic-rich layer with purity high enough for reuse (>95%).[22]

These types of solvent recovery extraction methods have been applied into analyte extraction. Sugaring-out assisted liquid–liquid extraction method has been demonstrated by W. Tsai et al[23] in 2010 in combination with high performance liquid chromatography with fluorescence detection (HPLC-FL) for the extraction and determination of sulfonamides in honey. Their method demonstrated the use of sugar/water/ACN system where acetonitrile could be used as a phase to extract both the sulfonamides and the fluoresceine-derivatized sulfonamides. The most relevant application of PIE to this work is polyol-induced partitioning of essential oils in water/acetonitrile solvent mixtures by T. DelMastro et. al.,[24] which demonstrated a successful extraction system for essential oils based on acetonitrile/aqueous solvent systems and glycerol as the MSA. The partition coefficient and recovery results obtained from this method further prove the usefulness of this chemical process where heat, cost, and time are a concern.

Acetonitrile is a widely used organic solvent in synthesis, manufacturing, purity, quality control analysis and research and development of organic compounds used in pharmaceuticals, cosmetics, personal care products. Similarly, acetonitrile-water mixtures at various compositions are also widely used in many applications of liquid chromatography, solvent extraction, and organic synthesis. Acetonitrile is an aprotic and polar solvent which is miscible in water at any ratio due to the dipole-dipole interaction and hydrogen bonding with water molecule through the partially negative charged nitrogen. However, the hydrogen bonding is relatively weaker than hydrogen bonding between water molecules.[25] Figure 1-1 shows the hydrogen bonding between acetonitrile and water.[10] The intra-molecular bonding between acetonitrile molecules is weak, leaving the formation of clusters when mixed with water and acetonitrile clusters are surrounded by water molecules through both hydrogen bonding and dipole−dipole interaction.[25] Acetonitrile-water mixtures form binary azeotropes, which have a characteristic constant boiling point even though the individual characteristic of boiling points are different.[15]

Polyols are sugar alcohols or polyhydric alcohols occurring naturally or derived from sugars by the reduction of the aldehyde or ketone group to an alcohol group through chemical or biochemical process and they are chemically and heat stable compounds.[26] Most of the polyols except ethylene glycol are nontoxic, widely available, inexpensive, biodegradable and recyclable.[10] Ethylene glycol, propylene glycol and glycerol are liquids at room temperature, they are easily dissolve and/or are dispersed in organic liquids and other polyols such as erythritol, xylitol, sorbitol, mannitol and isomalt are solid at room temperature require time and heat to dissolve in an organic liquid.[10] The study about polyol induced extraction invention suggested that the amount of polyol needed to induce phase separation depends on the nature of

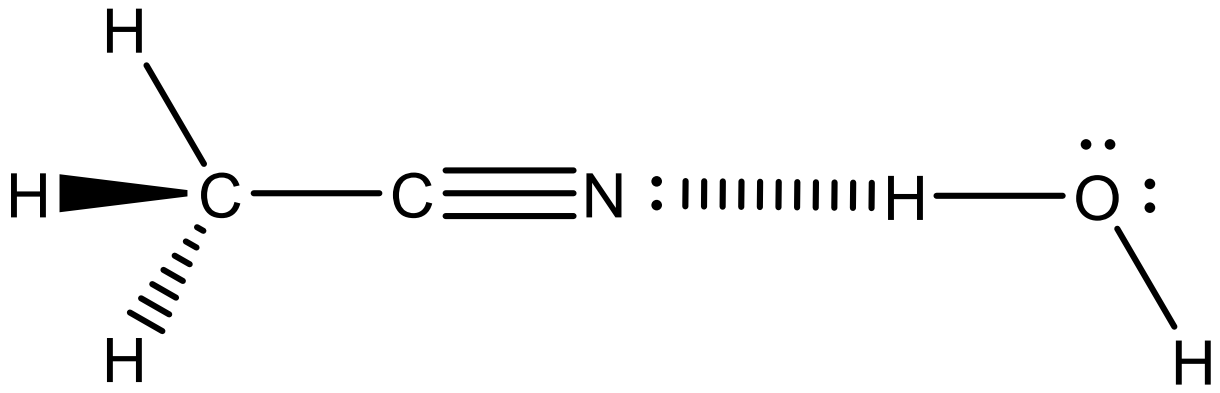


Figure 1- 1 Hydrogen bonding between acetonitrile and water.

the organic solvent, the percentage of water in the composition, total volume of the composition, specific polyol used as the extraction agent and for 10 mL volume of acetonitrile/water (50%v/v) at room temperature 21%wt/v glycerol gave maximum phase separation.[10] The chemical structure of glycerol is shown in Figure 1-2. Since Polyols have multiple hydroxyl groups, they can form an extensive hydrogen bond network with water and their high boiling points make them ideal mass separating agents.

The temperature of the glycerol induced phase separation system is related to thermodynamics in a way that it affects the spontaneity of the process. The reaction quotient or equilibrium constant, K_D and Gibbs free energy ΔG are temperature dependent as in equation 1-3.

$$\Delta \boldsymbol{G} = -\boldsymbol{RT} \ln \boldsymbol{K_D} \qquad \text{Equation 1- 3}$$

$$\boldsymbol{K_D} = \boldsymbol{e}^{\frac{\Delta \boldsymbol{G}}{\boldsymbol{RT}}} \qquad \text{Equation 1- 4}$$

In above equations K_D or reaction quotient refers to completeness of an extraction. Solving equation 3 for the natural log and taking an exponential expression in equation 4 shows that completeness of the process is inversely proportional to temperature meaning as temperature decreases, equilibrium will be greater than 1. The phase separation is influenced by decreasing the temperature which is indicating an exothermic phase separation process.

The objective of this research is to demonstrate that phase partitioning through PIE can be an alternative method for extraction of steroidal drugs, specifically glucocorticoids and non-steroidal anti-inflammatory drugs from water. When compounds of interest are suspended in

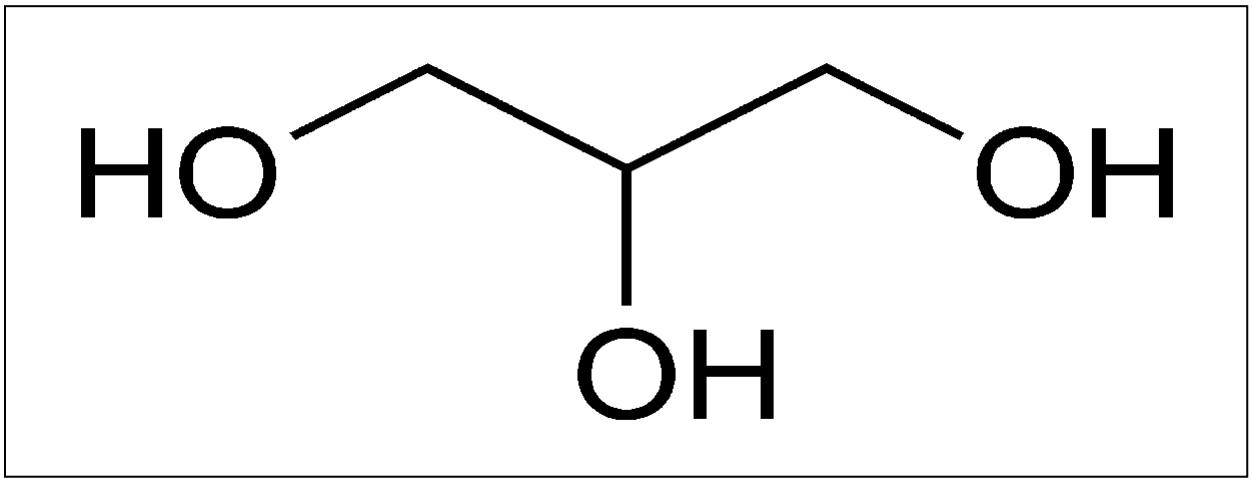


Figure 1- 2 Chemical structure of Glycerol.

miscible aqueous/organic solvent mixture, addition of a polyol induces a phase separation of an organic phase containing compound of interest and an aqueous phase containing water Polyol and matrix compounds. Analytes sensitive to temperature or harsh extraction solvents are extracted and determined without having analytes compromised. Figure 1-3 describes the basic concept of PIE in a graphical illustration. Water containing analytes is mixed with water miscible solvent acetonitrile and 2 mL polyol (glycerol) is added, this solution is mixed by vortexing and equilibration at 0°C leads to phase separation.

1.3 Basic theory of liquid chromatographic technique separation and analysis

1.3.1 Discussion of chromatographic systems

The basic function of a chromatographic technique is to separate, identify, and quantify the components of a sample. A general classification of chromatographic techniques can be seen in Figure 1- 4. In liquid chromatography, analytes or samples are dissolved in solvent or mobile phase which then travels through a stationary phase. In the stationary phase, analytes are separated based on their affinity towards either phase. Separated compounds are then identified and subsequently quantified using detector connected to outlet of the column. In liquid chromatography, a liquid solvent mobile phase is delivered into the system through a pump providing high pressure. A small amount of sample is introduced into the mobile phase flow through an injector then pumped through the column where it interacts with the stationary phase of the column. Depending on the analyte interactions with the stationary phase and analyte polarity in relation with stationary phase, liquid chromatography is further classified into various types such as normal phase, reversed phase, ion-exchange, and size-exclusion chromatography.

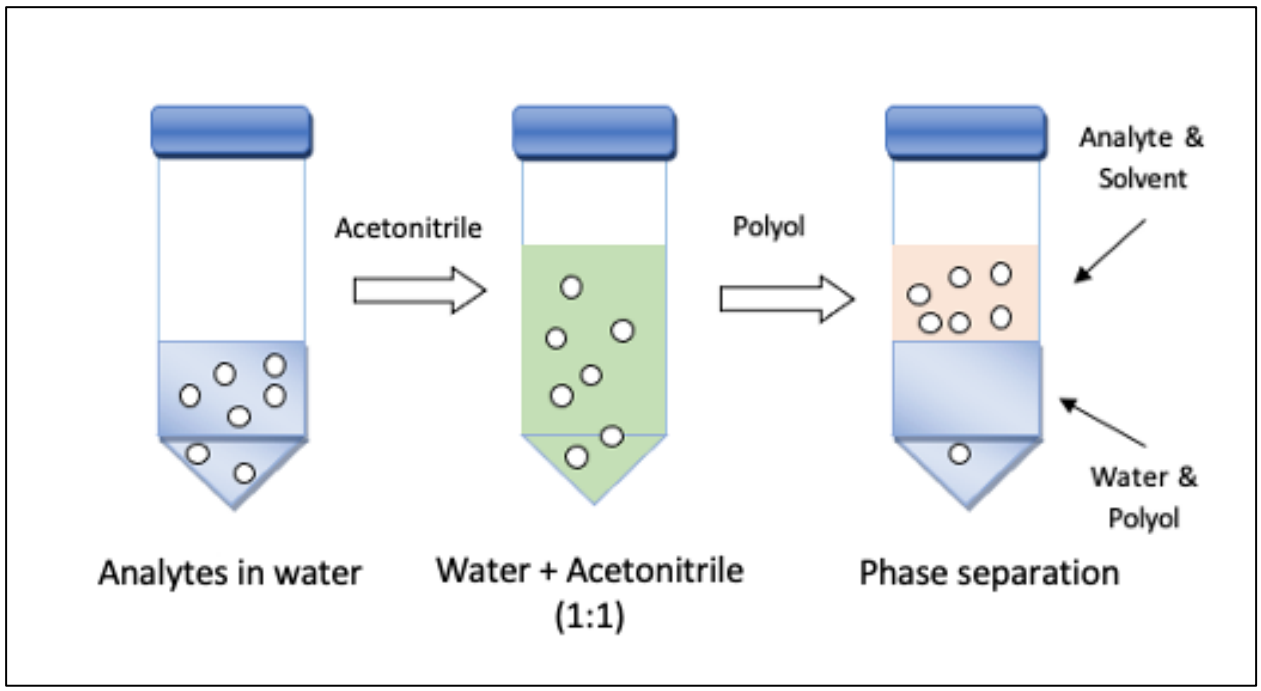


Figure 1- 3 Polyol induced extraction of analytes

.

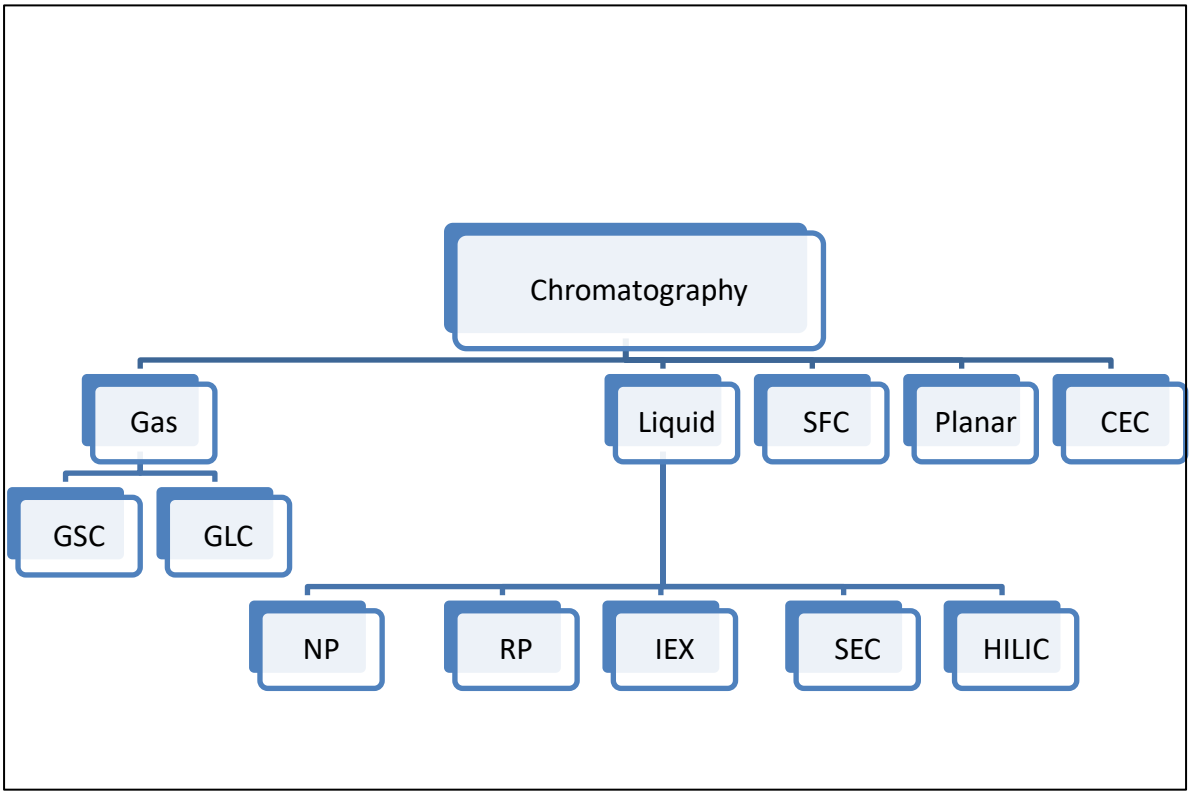


Figure 1- 4 General classification of chromatographic methods.[27]

In normal phase liquid chromatography, the stationary phase is polar, and the mobile phase is non-polar. In reversed phase, a non-polar stationary phase is facilitating hydrophobic analyte-stationary phase interactions and the mobile phase is polar. Ion-exchange chromatography is based on ionic interactions of the analyte with the charged stationary phase and the separation are based on the affinity of ionic analytes for the stationary phase surface. In SEC the elution process is based on the size of the analytes, larger molecules move faster through the column due to the lower chances of the analytes penetrating pores of the stationary phase. Common analytical techniques and their separation principle is described in Table 1-1. After the interactions with stationary phase, separated individual compounds enter the detector and signal is analyzed by the chromatographic data system. Various types of detection systems are available such as photodiode array (PDA), ultraviolet (UV), fluorescence, refractive index, electron light scattering (ELS), and mass spectrometry. A schematic diagram of a liquid chromatographic system is shown in Figure 1-5. The most common types of instrumentation in liquid chromatography are high performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography (UHPLC). A typical chromatographic system consists of a solvent reservoir, pump, injector, column, detector and data system.

1.3.2 LC Detectors

Upon separation and elution by LC, the analytes of interest can be detected using various detectors based on the properties of analytes and analytical conditions. In general, for optical detectors, when an eluted analyte passes through the detector a change in the optical property is observed and a chromatogram (signal vs time) is generated. Chromatograms provide the information necessary to identify and quantify substances based on the retention time and peak

Type of LC methodology	Separation principle
Reversed phase	• Polar mobile phase and non-polar stationary phase. • Separation and elution based on the analyte's polarity and affinity towards each phase. • Polar analytes elute faster, and non-polar analytes take longer.
Normal phase	• Polar stationary phase and non-polar mobile phase. • Separation and elution based on the analyte's polarity and affinity towards each phase.
Hydrophilic interaction liquid chromatography (HILIC)	• Similar to normal phase. • Water is added to the organic solvent to separate and elute polar molecules.
Ion Exchange chromatography	• Retention and separation of ions based on electrostatic affinity of the analytes for the stationary phase containing opposite charged functional group.
Size exclusion chromatography	• Stationary phase column filled with porous particles. • Smaller molecule travel slowly due to the penetration into the pores. • Larger molecules travel faster

Table 1 - 1 Type of liquid chromatography

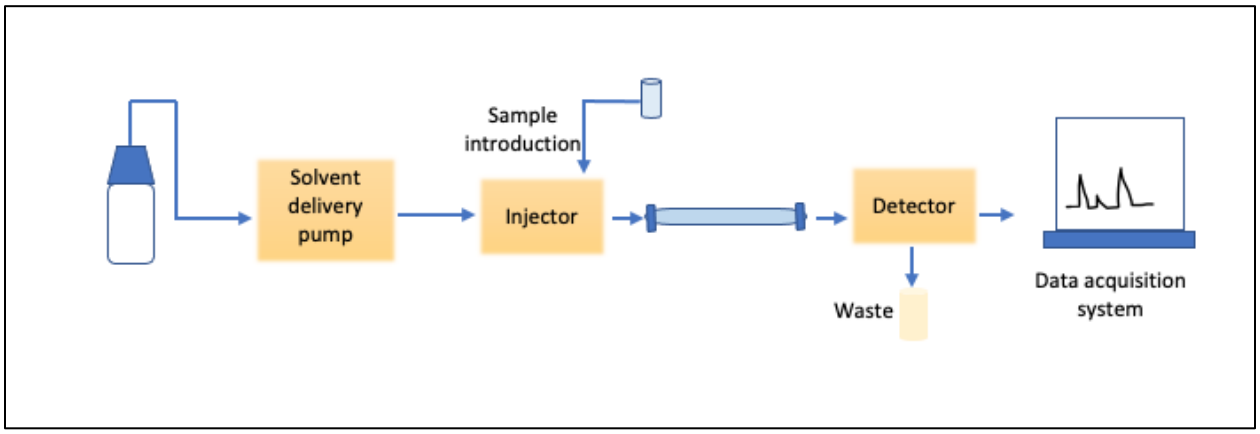


Figure 1- 5 diagram of the liquid chromatographic system

Detector	Compatible Analytes
Absorbance detectors- UV; PDA	Analytes absorbing radiation in UV-Vis range
Fluorescence detector	Fluorescent analytes
Evaporative light scattering detector (ELSD)	Universal: nonvolatile analytes
Refractive index	Universal
Conductivity	Cation, anion
Mass spectrometry	Universal

Table 1 - 2 List of LC detectors

area or intensity. Table 1-2 provides list of the liquid chromatography detectors and compatible analytes.

1.3.2.1 Absorbance detectors: UV and PDA

Absorbance detectors are the most popular detectors for liquid chromatography. Figures 1-6 and 1-7 shows schematic of an Ultraviolet-visible detector and PDA respectively. When a compound is exposed to the light source of the radiation of the wavelength range of 190 to 800nm, it absorbs light of specific wavelength. Upon absorption the electron transition occurs from the ground state to the excited state. The wavelength of the light absorbed is specific to the analyte and depends on the structure of the analyte. Absorbance is directly proportional to the concentration of the analyte and path length as shown in Figure 1-8.

1.3.2.2 Fluorescence detectors

This type of detection is like UV detection in the same range of electromagnetic radiation. Instead of absorbance it measures the fluorescence light emitted by fluorescent compounds.[33]

1.3.2.3 Evaporative light scattering detector

Any non-volatile compounds can be detected using this type of detection method. In this technique solvents and analytes eluted from column are sprayed by means of nebulizer and solvent is removed by evaporation then light from the source is directed towards analytes and the light scattered by analytes is detected.[3]

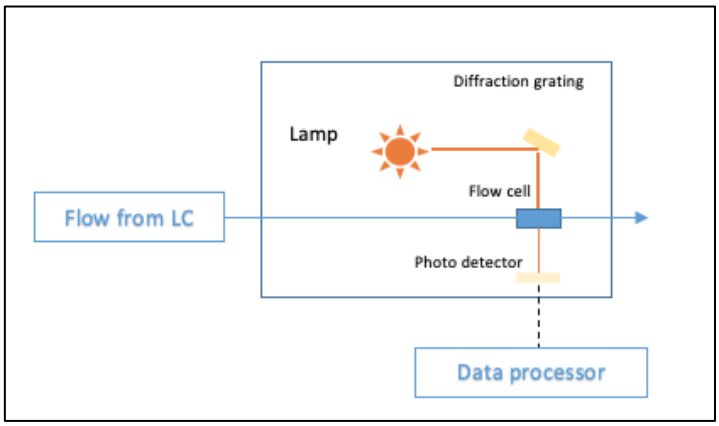


Figure 1- 6 Schematic diagram of UV detector

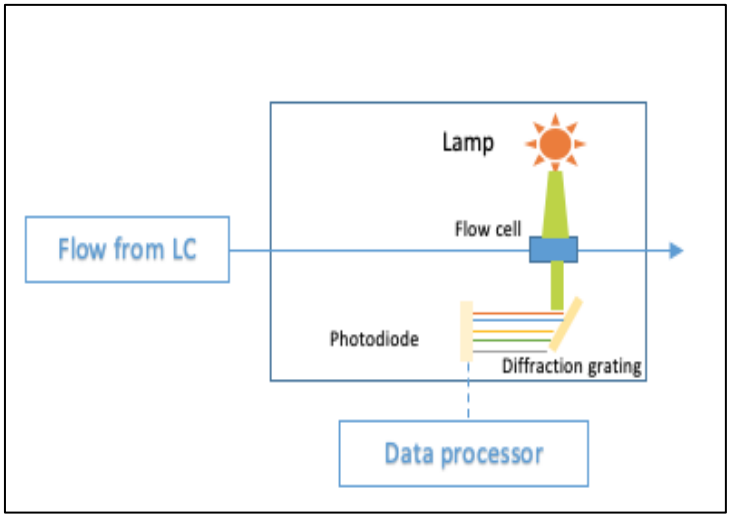


Figure 1- 7 Schematic diagram of PDA detector

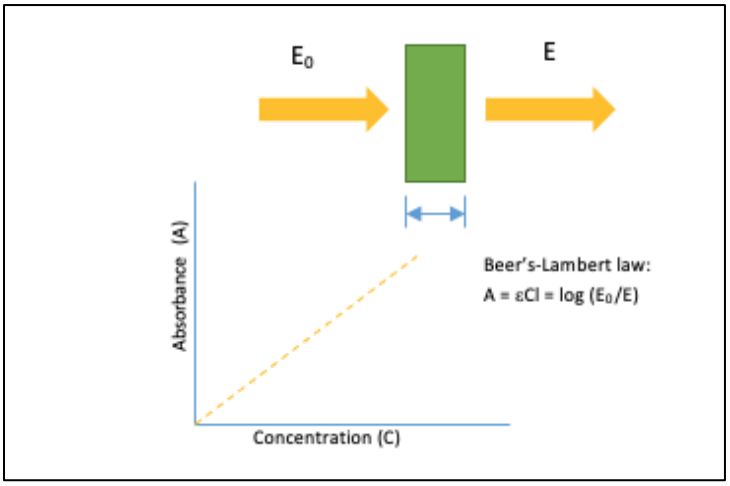


Figure 1- 8 Principle of UV-VIS detection

1.3.2.4 Refractive index detector

The refractive index detector can detect almost all compound having a different refractive index than that of the mobile phase. When analyte passes through the sample cell, the change in the light received is detected.[34]

1.3.2.5 Conductivity detector

This is a method of detecting ions in the solution by detecting the change in the electric current. This detector is highly sensitive and mainly used with ion chromatography.[34]

1.3.2.6 Mass spectrometry (MS) Detector

Detailed discussion on MS is to follow in this chapter in the section of liquid chromatography mass spectrometry because it is the primary type of detection method used in this research. In general, MS detector ionizes the sample compounds and separates ions based on their mass to charge ratio. It is a most discriminating detector of the highest sensitivity and selectivity. It is useful for structural elucidation. Ion intensities against mass to charge ratio can be used for quantification. It is also one of the more expensive and high maintenance detectors.

1.4 Ultra-high performance liquid chromatography (UHPLC)

This research work is utilizing the UHPLC system. Therefore, the differences and similarities

HPLC	UHPLC
Similarities	
Liquid chromatography	
Separate, Identify and quantify components	
Similar level of accuracy and precision	
Differences	
Up to 6000psi	Up to 15000psi
column particle size: 3 to 5µm diameter	column particle size: $\leq$2µm diameter
Simple applications: pharmaceuticals and impurities in pharmaceuticals	Higher sensitivity, better resolution, and faster analysis time: complex mixtures like dietary supplements, herbal, and biological samples

Table 1 - 3 HPLC v/s UHPLC summary Table

between HPLC and UHPLC is appropriate. Table 1-3 summarizes the similarities and differences between the two. UHPLC pumps operate at much higher pressure due to the smaller size of the column particles. This reduces the size of the column as well. Reduction in column size increases the efficiency of the separation and speed of the analysis. UHPLC column particle size is often 2µm diameter or less versus common 5 to 3 µm diameter for classical HPLC. Therefore, UHPLC columns produce narrow chromatographic peaks, high resolution and resulting high sensitivity. Higher resolution due to smaller particle size allows for analysis of complex mixtures and higher sensitivity allows analysis at lower level. Faster analysis through UHPLC leads to higher sample throughput with a reduction in solvent consumption and waste disposal. In this case, the choice of stationary phase becomes important, and the system hardware must be capable of allowing the column to deliver efficient chromatography. HPLC is a more robust, coast effective instrumentation and analysis remains unaffected by small variations in method parameters. On other hand UHPLC requires UHPLC or LCMS grade solvents and samples need to be filtered of particulates. Both HPLC and UHPLC are widely used applications. UHPLC is a recent development.

1.4.1 Chromatographic analyte retention, column efficiency, selectivity, and resolution

Retention factor, efficiency, resolution, and selectivity are commonly used parameters to describe chromatographic column, system, and separation.

1.4.1.1 Retention factor

Analyte retention in liquid chromatography involves many different molecular behaviors and interactions.[29] Retention factor (k) is the measure of the retention of a particular compound on a particular chromatographic system at given conditions calculated as:

$$\boldsymbol{k = \frac{V_R - V_0}{V_0} = \frac{t_R - t_0}{t_0}} \qquad \text{Equation 1- 5}$$

Where, V_R is the analyte retention volume, V_0 is the volume of the liquid phase in the chromatographic system, t_R is the analyte retention time, and t_0 is defined as the retention time of a non-retained analyte.[29] As the analyte travels through the column, analyte distribution equilibrium between the stationary phase and the mobile phase is the driving factor. Retention factor is also defined as the ratio of amount of analyte in stationary phase to amount of analyte in mobile phase.

$$\boldsymbol{k = \frac{C_s V_s}{C_m V_m}} \qquad \text{Equation 1- 6}$$

The amount of analyte is the Concentration (C) in the respective phase multiplied by the respective phase volume (V).

1.4.1.2 Efficiency

Efficiency is defined by band dispersion or broadening of an analyte band in the column while it travels through the column which is also called the number of theoretical plates and expressed as Equation 1-7.[29]

$$\boldsymbol{N = 16 \left(\frac{t_R}{w}\right)^2} \qquad \text{Equation 1- 7}$$

In this equation, t_R is the analyte retention time and *w* is the peak width at the baseline. The larger the theoretical plate number the large numbers of peaks can be separated. In a gas chromatography column efficiency is highly dependent on the flow rate but in liquid chromatography, variations of the flow rate do not affect column efficiency as much due to the higher viscosity of the mobile phase.

In liquid chromatography, uniformity and density of the column packing are the factors defining the efficiency of any column. There is no fundamental relationship between particle diameter and column efficiency but, with decrease in the particle size an increase of the efficiency can be expected due to the uniform flow inside and around the particles.[29] The general form of this dependency is the plate height theory and expressed as the Van Deemter equation in Equation 1-8.

$$\boldsymbol{H = A + \frac{B}{v} + Cv} \qquad \text{Equation 1- 8}$$

In above equation *v* is the linear flow velocity, and *A*, *B*, and *C* are constants for given column and mobile phase each term represents unique process contributing to band-broadening.

A—Multipath effect or eddy diffusion defines the ability of different molecules to travel through the porous medium of different path length. Eddy diffusion takes place due to presence of multiple channels of different length and diameter in porous structures. Particle size variation causes band broadening due to eddy diffusion. In liquid chromatography this term plays a major role in band dispersion because analytes can travel vial multiple paths through the column.[29]

B—Molecular diffusion, this term is inversely proportional to the flow rate. With slower flow rate analyte stays longer in the stationary phase and leading B term more time to broaden the peak. Similarly, higher flow rate will decrease the time spend in the column and less broadening due to molecular diffusion.[29]

C—Mass transfer, this term is the mass transfer of the analyte in the stationary phase or in the mobile phase. Quick analyte sorption and desorption will keep analyte molecules together leading to minimum band broadening. This term is proportional to the flow rate and effects in both stationary and mobile phases.[29]

Figure 1- 9 shows a schematic of the Van Deemter equation. Theoretically all dependencies of the column efficiency on the flow rate follow the theoretical Van Deemter curve and there is an optimum flow rate that can achieve the highest efficiency. Faster flow rates need resistance to higher backpressure. The latest developments involve utilizing smaller particles and higher flow rate. However, there is not much difference in the efficiency of the columns packed with smaller particles (<2μm) compared to conventional columns with 3 to 5 μm particles. Columns with smaller particles can achieve separation much faster reducing the analysis time. It must be noted that *N* will decrease as particle size increases (for particles ≥3 μm) and faster flow rates required longer columns to achieve the required theoretical plates.[29]

1.4.1.3 Selectivity (α)

Selectivity is defined by Equation 1-9, ratio of the retention factor of two different analytes. It can also be defined by the ability of the chromatographic system to differentiate or discriminate between two different analytes.

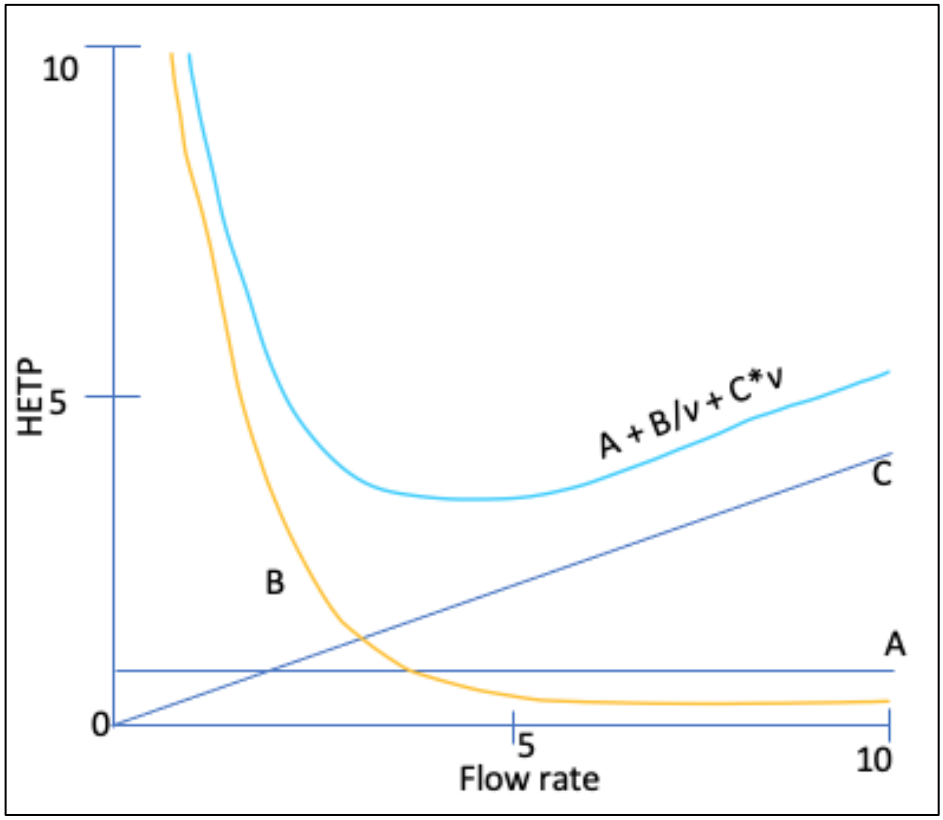


Figure 1- 9 Schematic of the Van deemter [29]

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$ Equation 1-9

Selectivity is dependent on the difference in the interaction of the analytes with the stationary phase. Eluent type and the eluent composition effects on the separation selectivity is a secondary factor.[30] As discussed earlier in types of chromatography different separation modes were classified based on the stationary phase and ways analytes interact with a stationary phase. This work involves the reversed phase mode of separation and the basic principle for reversed phase is hydrophobic interactions. For any reversed phase column, the way bonded groups are attached to the support, support chemistry, interactions with bonded groups significantly affect the retention of various analytes.[30] Beyond chromatographic separation, selectivity can also be achieved by choosing a specific detector based on the type of analytes. If each compound has a different molecular weight, chromatographic resolution of all components of interest may not be necessary. By using a mass spectrometer, analyte separation can be attained. However, a mass spectrometer will not be able to discriminate between isomers, in such cases a prior chromatographic resolution is very important.

1.4.1.4 Resolution

Resolution is defined as the ability of the stationary phase to resolve two analytes in two separate peaks also simplified as the difference in the retention time between two peaks in relation with peak width as described in equation 1-10.

$$R = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(w_2 + w_1)}$$ Equation 1- 10

The resolution of one analyte from another in a chromatographic separation is determined by three factors mentioned above: efficiency, selectivity, and retention. The resolution equation was suggested by Said[31] is described in equation 1-11. This equation suggests taking the average value.

$$\boldsymbol{R} = \left[\frac{\sqrt{N}}{2}\right]\left[\frac{\alpha-1}{\alpha+1}\right]\left[\frac{\bar{k}}{1+\bar{k}}\right] \qquad \text{Equation 1- 11}$$

N is the number of theoretical plates, α is selectivity, and $\bar{k}$ is an average retention factor of two closely eluting analytes. The contribution of each term for the resolution varies but increasing each will help achieving separation. The resolution equation is based on the separation of only selected pair of two components in a mixture of component and it gives the approximate values of resolution. This equation is good for conceptual discussion of above-mentioned factors in resolution, and not for rigorous calculations.

1.5 Basics of liquid chromatography – mass spectrometry (LC-MS)

LC-MS is a powerful technique of analysis and widely used in pharmaceutical, chemical, food, clinical, environmental, forensic industries.[35, 36] It is a widely used analytical technique for quantification, qualification, and structural elucidation. LC-MS brings higher selectivity and sensitivity by the combination of LC separation and MS detection. Co-eluting peaks from LC can be isolated and separated based on their mass. When LC is coupled to MS, MS detector ionizes the sample components using various ionization techniques, then resulting ions are separated in vacuum based on their mass to charge ratio and intensity of the ion is measured. A schematic diagram of LC-MS system is shown in Figure 1-10. LC-MS systems are useful in analyzing non-volatile components or samples that are not amenable to GC-MS. It is suitable for

the analysis of large molecules, small molecules, polar, thermally sensitive, non-volatile, charged molecules and analytes that cannot be modified by derivatization as well as compounds that do not have chromophores. MS can be coupled to various analytical instruments and several ionization methods are available. LC-MS with atmospheric pressure ionization is much more widely applied than any other method.[37] Figure 1-11 describes the applicability of common ionization techniques.[38] LC-MS requires the ionization to occur at atmospheric pressure, which is known as soft ionization. Examples of soft ionization techniques are atmospheric pressure photoionization (APPI), atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). Atmospheric pressure soft ionization importance and details are discussed later in this chapter. Matrix assisted laser desorption ionization (MALDI) is also a soft ionization method used for large molecules.[38]

1.5.1 Instrumentation of (LC-MS/MS)

The basic components of the LC-MS system are an LC system, and its components are described in Figure 1-10. The interface between LC and MS, ion source for ionization of the analytes, ion guide to transfer ions into MS, mass analyzer to separate ions based on the mass to charge ratio and finally a detector to detect ions comprise the detector. The ion guide, mass analyzer and detector are all under high vacuum to minimize collision. An atmospheric pressure interface provides the means of ionization for the formation of gaseous phase ions.

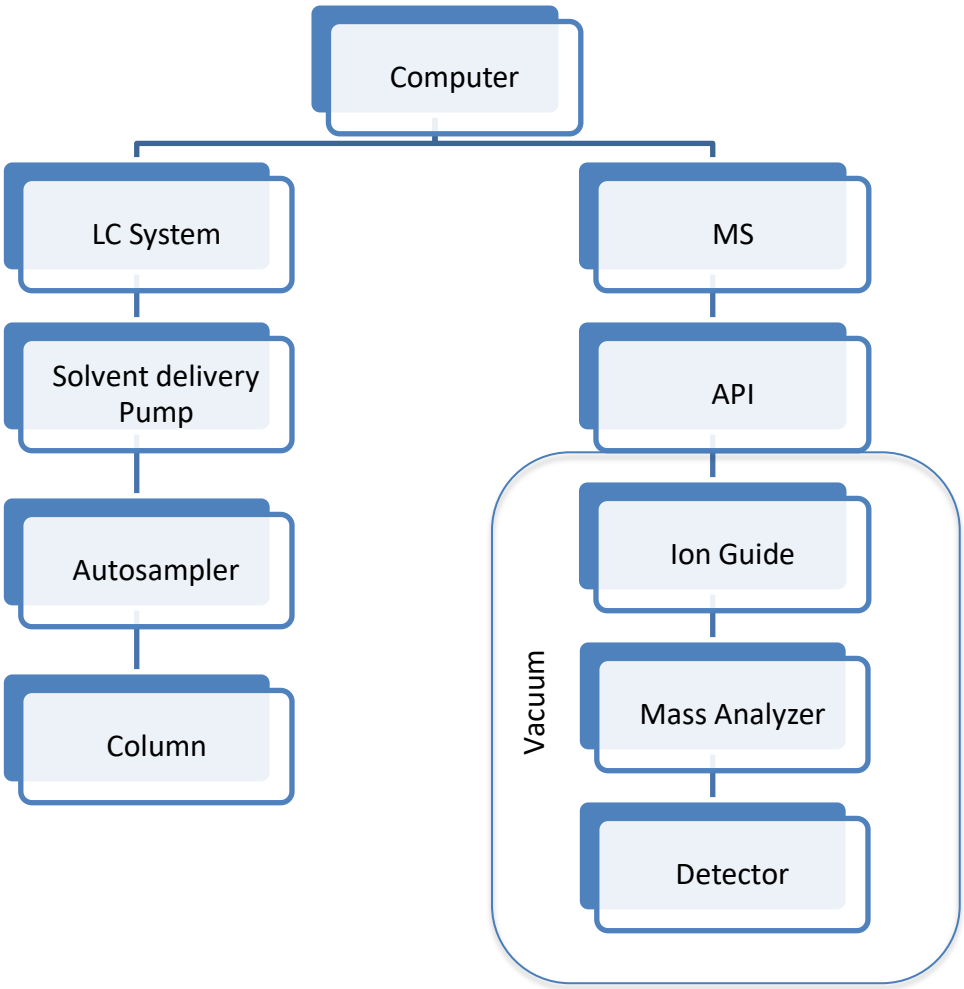


Figure 1- 10 Schematic diagram of LC-MS instrumentation.

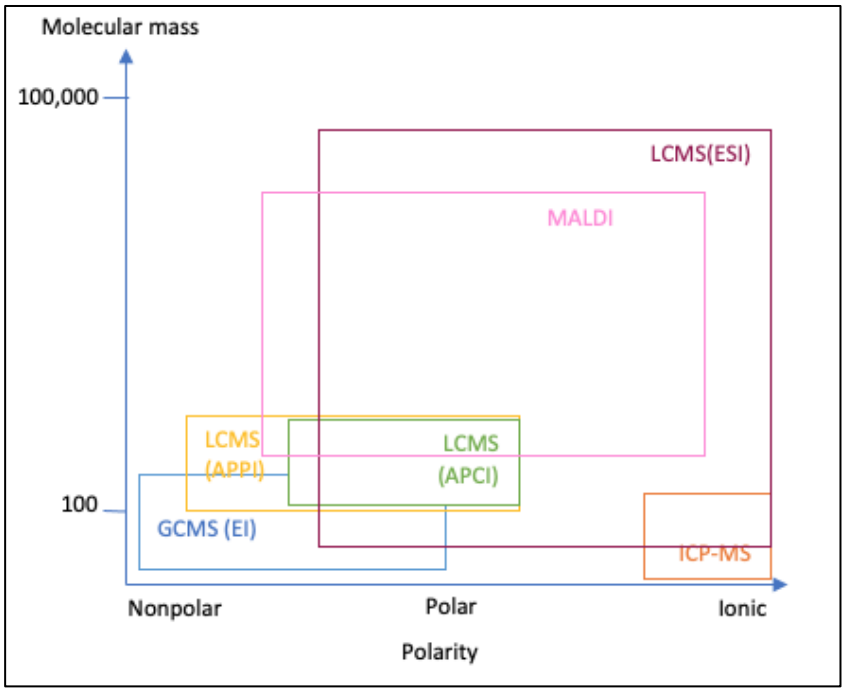


Figure 1- 11 Ionization techniques and range of application.

Adapted from: Comparison of LCMS and other techniques, Shimadzu, www.shimadzu.com[38]

APPI: Atmospheric pressure photoionization, APCI: Atmospheric pressure chemical ionization, ESI: Electrospray ionization, EI: Electron ionization, MALDI: Matrix assisted laser desorption/ionization, ICP-MS: Inductively coupled plasma-MS

1.5.1.1 Interface for LC-MS

It is very important to interface an ionization source with the chromatographic system, and ionization plays a critical role in the analysis. A major challenge in connecting both the systems is, interfacing high mobile phase flow from LC with the high vacuum requirements of MS. Various LC-MS interfaces have been developed to overcome this challenge.[39] A summary of ionization methods is listed in Table 1-4. In GC/MS eluent and analytes are in gaseous form therefore, they can be easily transferred to MS and compounds can be ionized. To understand why some ionization interface methods are not compatible with LC, the example of EI is used. EI is a hard ionization and is used in GC/MS. In EI, analytes are passed through a high energy electron beam and electron impact induces ionization and the high energy ionization causes the fragmentation of the analyte to generate smaller mass ions. The fragmentation process is reproducible at a given energy and EI spectra are used for the purpose of identifying analytes. EI operates at high vacuum, therefor it can be readily used with GC but not with LC. The LC flow is not suitable in high vacuum conditions. Moreover, heating of the metal filament of EI at atmospheric conditions destroys it. Therefore, it is very important to have an appropriate interface for the efficient transfer of the mobile phase to gas form and ionization of the analytes.[40] API acts as both the interface and ionization source. There are three API methods in common use, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). This research work involves ionization by ESI. Both APCI and APPI are discussed briefly, and ESI is covered with more detail.

Ionization method	Ionization agent	Application
Electron ionization	Electrons (~70 eV)	• Extensive fragmentation, reproducible, • large library base • Volatile/nonpolar molecules • Small molecules
APCI	Corona discharge/ gaseous ions	• Atmospheric pressure • Interface with LC • Large number of molecular ions • Low to moderately polar compounds
Desorption ionization	Energetic particles (atoms, ions), photons	• Large molecules • Difficult to interface to LC
ESI	Electrical/thermal/pneumatic energy	• Interface with LC • Atmospheric pressure, Multiple charged ions, Limited fragmentation • Small to large molecules • Not suitable for nonpolar molecules
APPI	Photon	• Atmospheric pressure • Minimum matrix suppression complementary to ESI and APCI • Nonpolar and neutral analytes

Table 1 - 4 Summary of ionization methods

1.5.1.2 Electrospray ionization (ESI)

Generally, in any API technique ions are generated by desolvation of the solvent. ESI is generated by applying a strong electric field to a liquid passing through a capillary tube. The electric field is obtained by applying a high voltage of 3 to 5 kV between the capillary tube. The electric field induces a charge accumulation at the liquid surface at the end of the capillary tube and highly charged droplets are formed.[41] ESI had been continuously developing since it first introduced in 1917.[39] A schematic representation of the equipment is described in Figure 1 -12. The ESI process involves three basic steps, nebulization and charging, desolvation, and ion evaporation. As shown in the Figure in the step of nebulization and charging, sample solution is sprayed at the tip of the capillary tube. The flow rate of the solvent in capillary is about 1 to 10 µl/min.[41] Sometimes the spraying is supported by the makeup gas flow which allows the use of higher flowrates.[49] Solvent is then passed through the electrospray needle at the tip of the capillary tube and high voltage is applied at tip of the spraying needle. This generates the charged droplets from the needle. Surface charge on this this droplet is of the same polarity as applied high voltage. To increase the compatibility with LC flow, nebulizer gas and drying gas flows outside of the capillary for faster solvent evaporation.[40] In the process of desolvation, charged droplets are repelled from the needle and attracted towards ion source. As the droplets travel through the space between the needle tip and ion source cone entrance drying gas of the heated nitrogen flows from opposite direction and solvent evaporation occurs and passing of the drying gas also removes any uncharged particles.[37] Finally in the ionization step, after solvent evaporation the droplet becomes small and electric field on the droplet surface increase and when the droplet become small enough and the charge force on the surface exceeds the liquid surface tension, a droplet is ripped apart and sample ions are generated into gaseous phase.[37, 50]

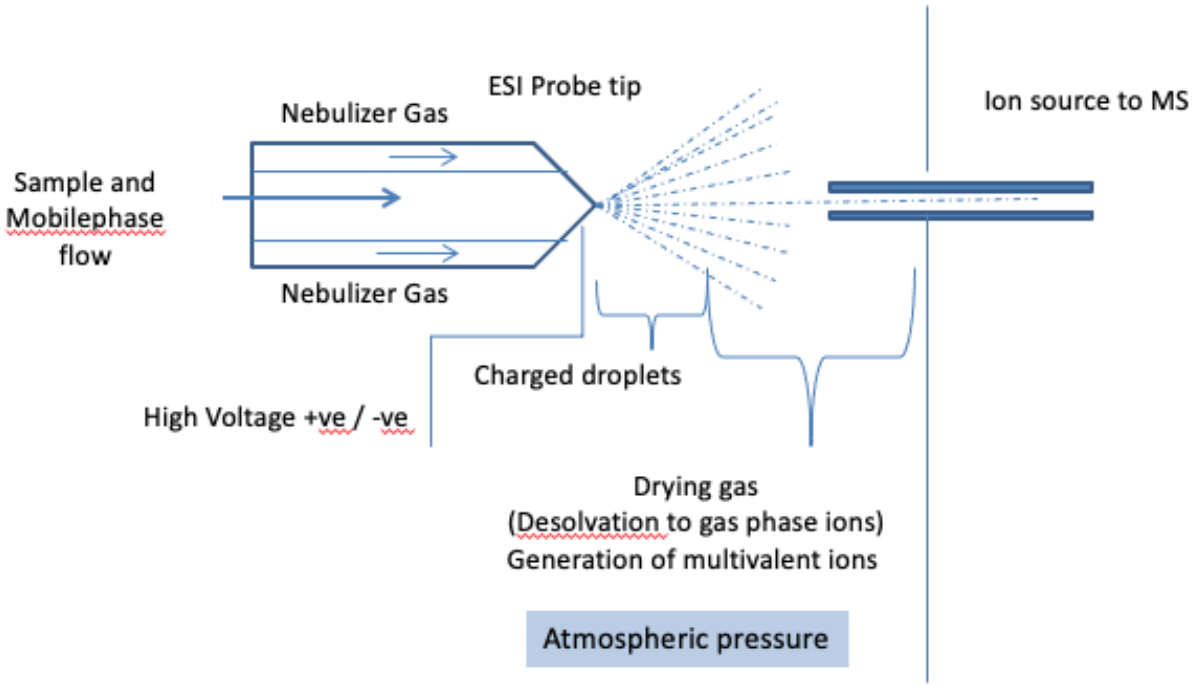


Figure 1- 12 Schematic representation of ESI

Figure 1- 13 is a magnified illustration of all three steps. It shows the positive mode of ionization; negatively charged ions are generated by choosing a negative voltage on the spraying needle. In this technique charged analytes are not necessarily ions and they are singly as well as multiply charged molecules. Multiply charged molecular ions are formed for analytes with several charge accepting functional groups. This feature makes the mass analysis of large biological molecules possible. Moreover, the sensitivity of ESI is related to concentration and not amount of sample introduced into the source based on this sensitivity of the ESI can be increased even higher by reducing or splitting the flow entering the source.[41]

1.5.1.3 Atmospheric pressure chemical ionization (APCI)

The interface design is similar to ESI, but the ionization process is similar to chemical ionization. This technique is applicable to polar and nonpolar analytes of moderate molecular weights. Figure 1- 14 illustrates the equipment and ionization in APCI. In this process major steps involved are evaporation/desolvation and ionization. In this method nebulization and evaporation takes place at a higher temperature in a vaporizer chamber. The heat in the chamber evaporates the solvents and small droplets are produced. In the process of ionization, corona discharge in APCI is a stream of electrons.[49] Gas phase solvent molecules are ionized by the discharge from the corona needle to generate stable reaction ions. Charge transfer from solvent ion to analyte molecule occurs, leading to ionization. APCI is a high energy process compared to ESI and does not form multiply charged ions. It is and suitable for nonionic compounds and nonionic compound with moderated molecular weight.[40, 41, 49]

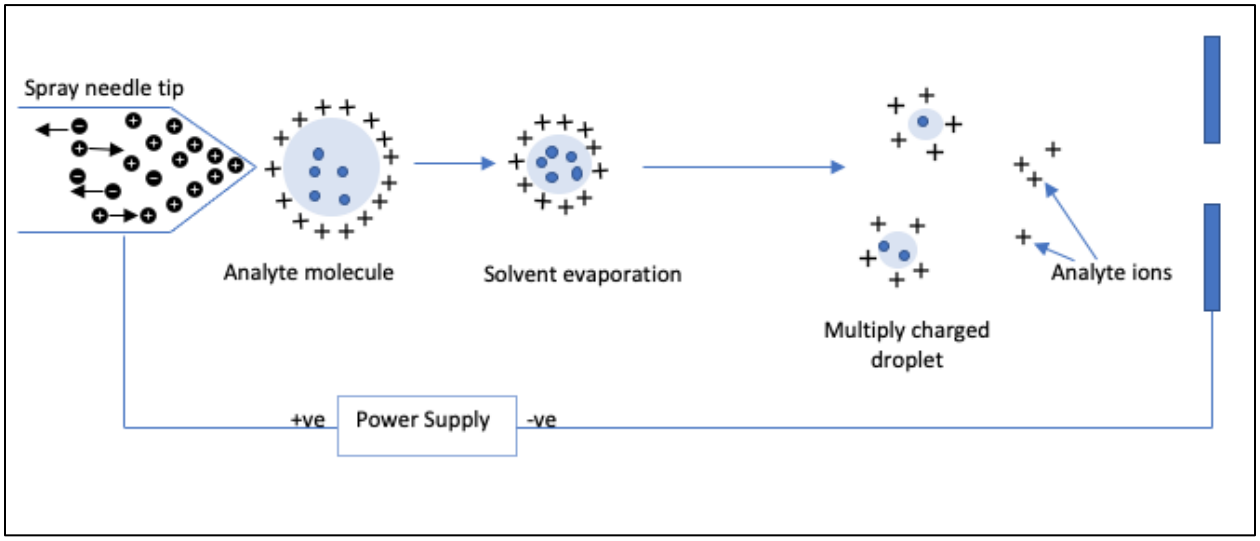


Figure 1- 13 magnified illustration of nebulization, desolvation and ionization.

(Adapted from: www.chm.bris.ac.uk[42])

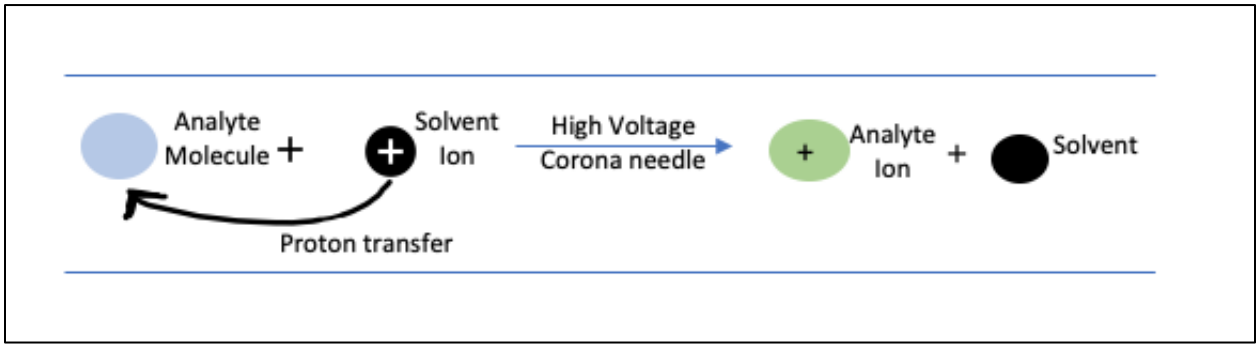


Figure 1- 14 Schematic diagram of APCI and ionization in APCI

(Adapted from: Fundamentals guide to LCMS, Shimadzu, (2019) chapter 2, 13-25.[40]

1.5.1.4 Atmospheric pressure photoionization (APPI)

APPI interface design is very similar to APCI. This technique uses the vacuum ultraviolet (VUV) radiation for ionization. The only difference in APPI to APCI is the use of VUV lamp instead of a corona discharge needle. As mentioned in the above ionization technique, there are three steps involved, nebulization, desolvation and ionization. In this process the nebulizer and heated chamber carry out the process of droplet formation and solvent evaporation. VUV light radiation photon has a sufficient energy to ionize most analytes. LC solvents have higher ionization potential and that will not be ionized. Protonated cations may be generated from the analyte ions ionized by photons due to the proton transfer from hydrogen in the solvent. Some analytes require higher ionization potential than that of the VUV photon. In such situation, ions

may not be produced. Such cases require dopants such as toluene and acetone to provide charge carriers for ionization of analytes.[39, 40]

Key factors aside from instrumentation affecting the efficiency and sensitivity of API are the flow rate of solvent inlet, type of solvent, pH of the mobile phase, additives used in the mobile phase, properties of the analyte, matrix of analytes. In LCMS, the preferred column internal diameter is preferred to be 2 mm. This allows flow rate of LC to be lower and at lower flow rate sensitivity is higher. pH and additives of the mobile phase can affect the sensitivity of any API, especially ESI. It has been well known that intensity of the ionization can be increased by adjusting the pH or adding the additives. For basic compounds adding an acidic reagent increases the production of positive ions, and desired mobile phase pH is 1 or 2 values lower than pKa of the analyte. On the other hand, for acidic compounds ionization can be increased by adding a

basic additive or having a pH of the mobile phase higher than the pKa of the analytes. Ionization of basic and acidic compounds are shown into equation 12 and 13 respectively.

$$\boldsymbol{BH + AH \rightarrow [B - H_2]^+ + A^-} \qquad \text{Equation 1- 12}$$

$$\boldsymbol{AH + B \rightarrow [A]^- + BH^+} \qquad \text{Equation 1- 13}$$

Ionization of the neutral compounds can be increased by adding a volatile salt that can influence an ionization in a positive way.[43, 44] However it has also been shown that some analytes ionization efficiencies are not affected by the pH of the mobile phase.[44] An excellent way to be compatible with LCMS is to use volatile solvents and when needed use volatile acids, bases, or buffers to adjust the pH. A relatively volatile ion pair reagent can also be used if required. Involatile salts are not recommended to use in LCMS because they can precipitate on the interface and can create contamination, potentially damaging to the needle.

1.5.2 Mass analyzer

Upon ionization in the interface ions enter the mass analyzer, which separate the ions based on their mass to charge ratio. There are various types of mass analyzers available separating ions according to mass to charge ration based on a distinct principle to each. All mass analyzers use electric and magnetic fields combined or alone; the manner such fields are used is the difference between different types of mass analyzer. The operating principle of the mass analyzer depends on interaction of charged particles with electrical or magnetic field.[39, 45] Mass analyzers are

classified based on how the ions are being introduced, either continuous or pulse mode. In continuous mode, the supply of ions enters the mass analyzer continuously while in pulse mode ions are accumulated and enter together at specific time points.[46] Commonly used mass analyzers are magnetic sector, quadrupole, time-of-flight (TOF), ion trap and Fourier transform ion cyclotron resonance. The combination of different MS is known as tandem/ hybrid MS or MS/MS. This type of multiple MS combination provides additional capabilities for structural elucidation. This research work used a quadrupole mass analyzer in the form of triple quadrupole MS/MS.

1.5.2.1 Quadrupole MS

The quadrupole mass analyzer as the name suggests, is made up of four cylindrical rods arranged parallel creating a hyperboloidal interior surface. Both direct current (D.C.) and high frequency alternating current (A.C.) are applied to the quadrupole. These currents are switched rapidly to move ions quickly and filter the ions with target m/z to pass through quadrupole and reach the detector by the combination of D.C. and A.C.. The quantity of ions that reach the detector is converted to a signal. Mass filters can be set to filter ions at specific m/z ratio or scan all m/z entering the analyzer through selected mass scanning range.[45, 47] Figure 1- 15 represents a schematic of a quadrupole mass analyzer. A continuous ion source generated in the ionization is moved towards the mass analyzer, and ions pass through opening and enter the quadrupole. Voltage of the same polarity is applied to the diagonally opposite poles and opposite voltage is applied to neighboring poles. When a certain condition of voltages is applied to the poles, ions with specific m/z ratio obtain a stable oscillation between the quadrupole rods to pass through and reach the detector.[45, 46] Oscillation of ions in quadrupole follows the Mathieu stability

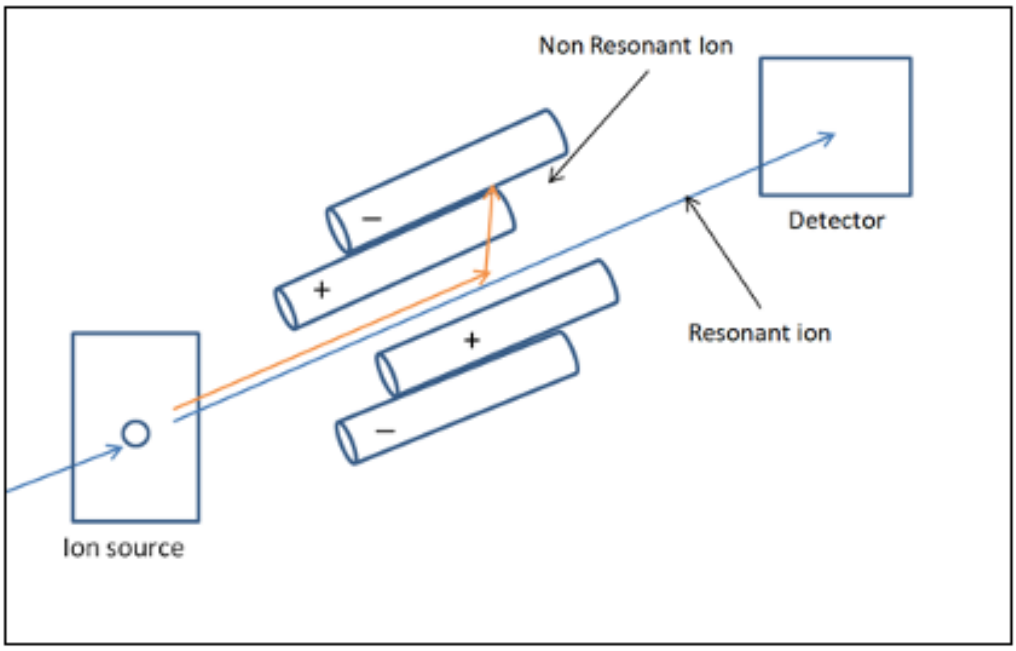


Figure 1- 15 Schematic of Quadrupole mass analyzer

diagram of the stable regions as shown in Figure 1- 16. The stable region is different for ions with different masses. When the combination of DC and AC is obtained for the stable region ions are detected in the order of small to large.[45,46] A single quadrupole works in two modes scan mode and selected ion monitoring mode (SIM). As shown in Figure 1- 17, scans mode scan the ions in the selected mass range with increasing voltage and mass spectra of all the ions in that range is obtained. SIM mode filters the ions at constant voltage and mass spectra including only specific ions is obtained.

1.5.2.2 Triple quadrupole (TQ) MS (MS/MS)

This is the most common and simple among tandem MS/MS system. There are three major components in this type of analyzer mainly three quadrupoles arranged in line. First and third quadrupole are MS1 and MS2 respectively and second quadrupole is the collision cell for collision induced dissociation. Triple quadrupole MS is a highly selective, specific and sensitive instrument for targeted quantitative analysis. Its high sensitivity is making this instrument a useful tool in trace analysis.[48, 49] Figure 1- 18 is a basic description of a triple quadrupole instrument. In Q1 or MS1 of any MS/MS system selection of a specific m/z ion (precursor ion) is done then collision induced dissociation or fragmentation takes place in the collision cell (Q2 or MS2) by a neutral gas like argon or nitrogen. Fragment ions are separated and recorded based on m/z in the third MS (MS3 or Q3). Multiple reaction monitoring (MRM) separates analytes in two stages making instrument more selective and highly sensitive. Figure 1- 19 is basic diagram of MRM, Detailed discussion of MRM to follow in chapter 2.

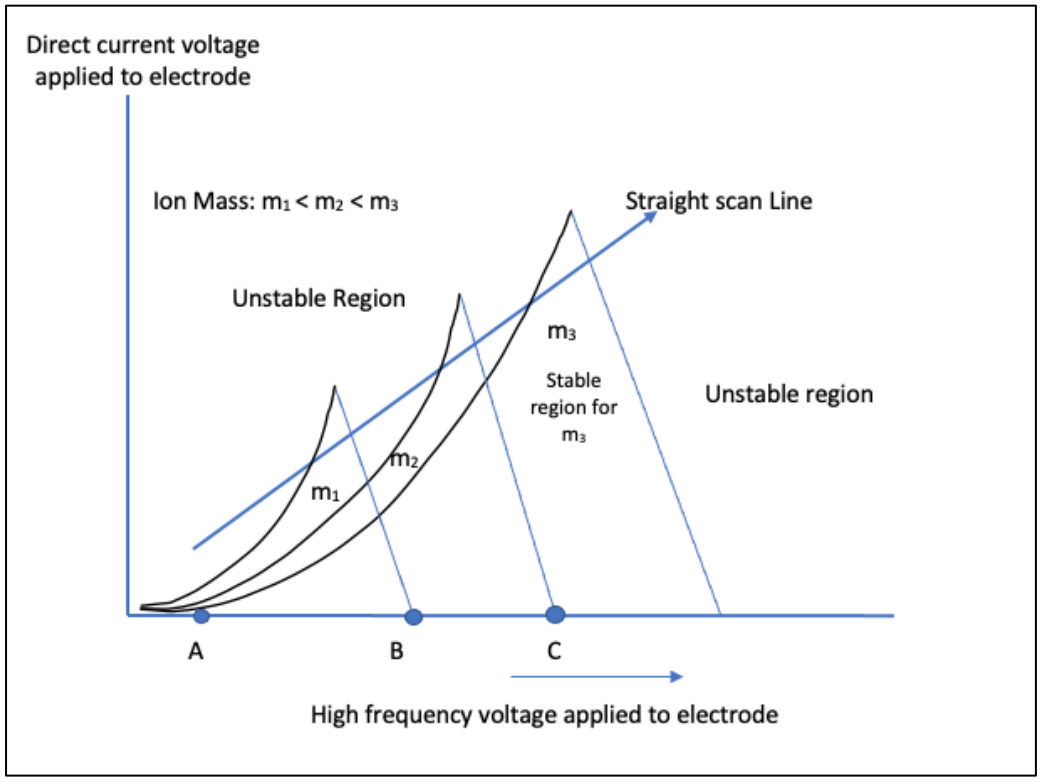


Figure 1- 16 Stability areas as a function of AC and DC voltage for ions with different masses.

(Adapted from: Fundamentals guide to LCMS, Shimadzu, (2019) chapter 3, 27-39.[46])

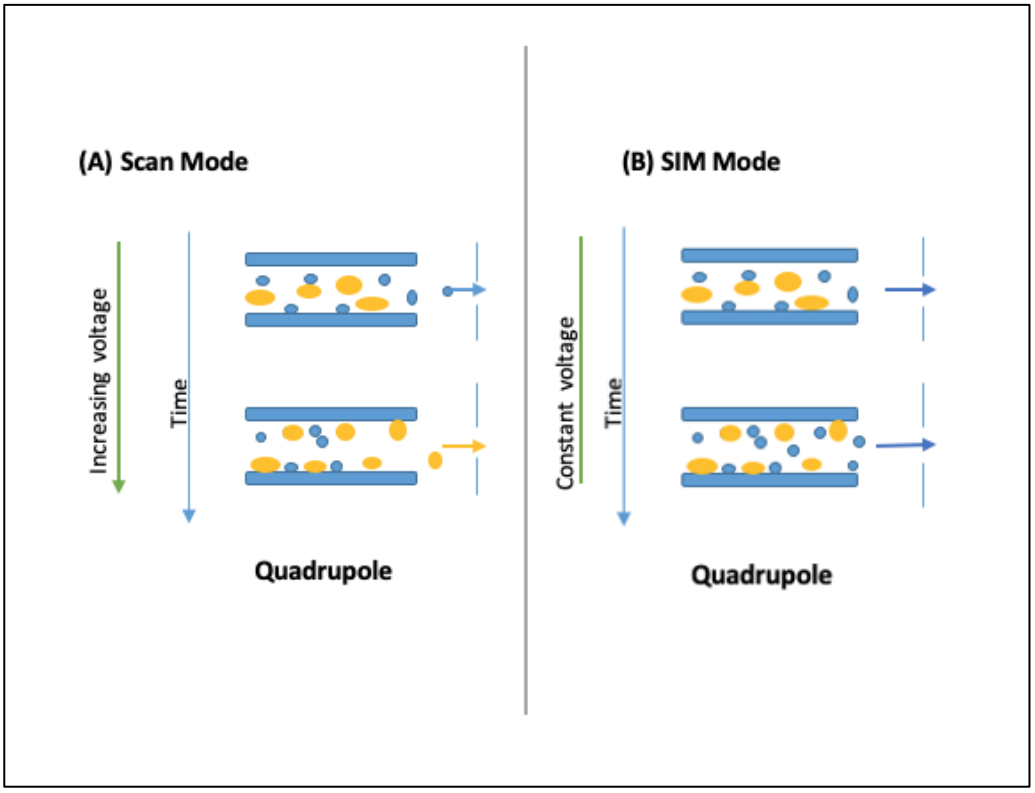


Figure 1- 17 Scan mode in single quadrupole.

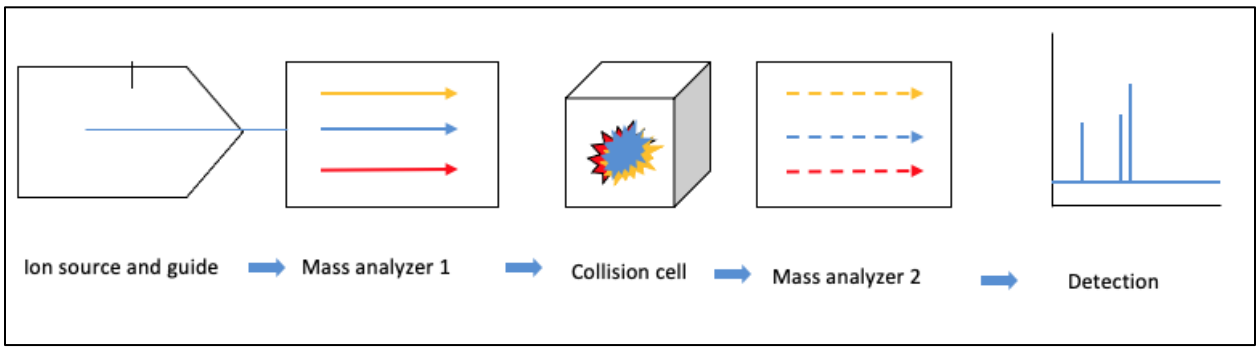


Figure 1- 18 Triple quadrupole (MS/MS) schematic diagram

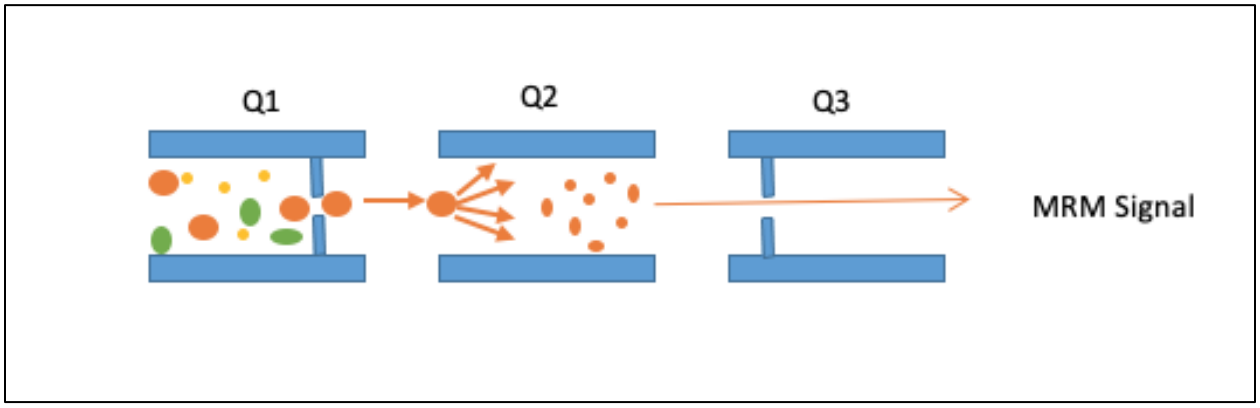


Figure 1- 19 Schematic of triple quadrupole multiple reaction monitoring (MRM).

Collision induced dissociation (CID) is the most common ion activation method in tandem MS.[51] In collision cell encounters between the precursor ion and a neutral gas causes the decomposition leading to fragmentation at much higher rate.[51] In the collision cell the kinetic energy is converted into an internal energy for the ion.[51] Collison processes are normally classified into one of two categories based on the kinetic or translation energy of the precursor ion – low energy collision and high energy collision. Low energy collisions in the range of 1 – 100 eV typically occur in organic ions of moderate mass are common in quadrupole and ion

trap MS.[51] When only RF voltage is applied, a quadrupole passes all ions within large range of mass-to-charge ratios and when both RF and DC voltage is applied the quadrupole separates ions of different mass to charge ratio. Various modes of scan are showed in Figure 1-20 which includes product ion scan, parent ion scan and multiple reaction monitoring (MRM). In MS/MS first parent ions are selected using a Q1 scan in full scan mode. Once parent ion is selected MS/MS is used to determine product ions of selected parent ion in product scan mode. In general, two or more fragments (Product ion transitions) are selected for a parent ion for MRM scan in MS/MS. Two or more set of MRM are selected for quantification and qualification for better selectivity and specificity.

1.6 Conclusion

There are only few applications in the literature for PIE including recovery of acetonitrile and partitioning of essential oil active component. The shift towards the green chemistry in the extraction processes has opened the way for the future of PIE application to include extraction of various type of drug substance in different matrices. There is an open-end scope of research and

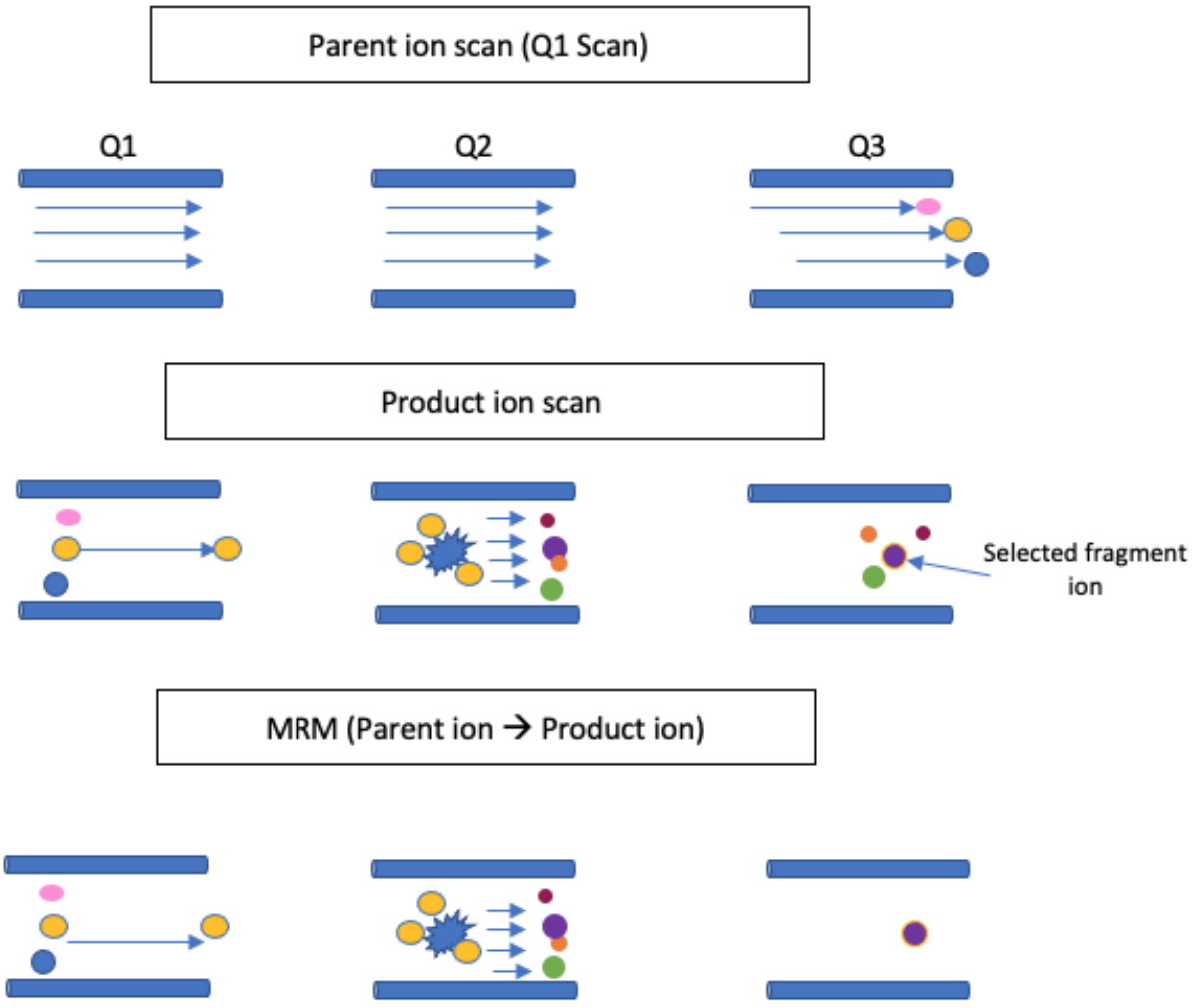


Figure 1- 20 Scan modes of MS-MS

growth for future applications. in this method. One such application involves utilizing PIE as a liquid-liquid extraction (LLE) of steroidal drugs and non-steroidal drugs from an aqueous matrix for the purpose of trace analysis. Analysis using PIE has been applied to GC, it can also be applied to LC.

PIE is the method that have not been explored and novel techniques and applications that have to be discovered. One such original study includes the application of PIE, will be discussed throughout the chapters of this dissertation. The study of glucocorticoid described in chapter 2 will address the application of PIE to the extraction of glucocorticoids from water by optimizing various parameters and discussing the results at chemical level. Moreover, the ionization and chromatography chemistries involve in LC-MS/MS analysis of glucocorticoids will also be considered. Chapter 3 will discuss the comparison of PIE with QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), a well-studied extraction method as well a discussion on comparison with other published methods. Chapter 4 will address another new application of PIE in the trace analysis of NSAIDs. PIE was used to extract NSAIDs from urine. PIE has yet to be used to its fullest potential in this type of analysis for non-biological and biological samples and may possibly be a major direction of research for this extraction technique.

CHAPTER 2 - ANALYSIS OF GLUCOCORTICOIDS IN WATER USING POLYOL INDUCED EXTRACTION WITH ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND TRIPLE QUADRUPOLE MASS SPECTROMETRY (PIE-UHPLC-MS-MS)

Glucocorticoids are potentially endocrine disrupting chemicals that have been widely detected in aquatic environments. Environmental occurrence and the potential harmful effects by these compounds are being recognized and have raised concerns. Several well-studied as well as new, simple, or tedious extraction techniques to extract glucocorticoids from various matrices and various liquid chromatography and gas chromatography techniques to determine glucocorticoids have been used and published.[52-72,] In this work Polyol-induced Extraction (PIE) was used for the extraction and UHPLC-MS-MS was used for the analysis of glucocorticoids in water. In PIE, a solvent mixture of acetonitrile and water can be separated by adding a polyol mass separating agent. PIE is used for the extraction of drug substance for the first time. This method can also be applied to determine glucocorticoids in in complex matrices such as herbal medications, urine, blood, food etc. This method can also be applied to different classes of compounds like drugs of abuse. This study demonstrates the unique selectivity of extraction using PIE, separation using ultra high-performance liquid chromatography and detection along with second dimension using triple quadrupole (LC-MS/MS). This work also highlights high sensitivity and selectivity of mass detector.

2.1 Introduction

Glucocorticoids are both natural and synthetic in origin. Natural glucocorticoids, such as cortisol and cortisone are produced by the adrenal cortex. Dexamethasone and prednisolone are very well-known synthetic glucocorticoids.[52] Glucocorticoids are involved in several essential daily functions of vertebrates under physiological conditions, such as the metabolism of carbohydrates, proteins and fats, water and electrolyte balance, immune response, growth, and reproduction. When used therapeutically, they have very intense anti-inflammatory and immunosuppressive actions and are used to treat asthma, rheumatic arthritis, allergies and other inflammatory diseases.[53, 54] Glucocorticoids are being prescribed in large numbers by medical doctors and veterinarians due to their wide range of therapeutic actions. Extensive use of these compounds can lead to contamination of the environment in various ways such as excretion of pharmaceutical residues in free form or derivatives, hospital discharge, and pharmaceutical industry waste.[55, 56] Studies of sewage treatment plants have showed low removal efficiencies of these compounds. The most frequently detected glucocorticoids in effluent were cortisol, cortisone, and prednisolone.[57, 58] Although the amount of exposure into the environment is probably very minor, the continuous exposure of these compounds over time may cause potential health risks to vertebrate animals. Long term exposure to hydrocortisone of fish have shown adverse effects on behavior and changes in immune response.[59, 60, 61] A long list of glucocorticoids has been found in illegal preparations and glucocorticoids can have severe side effects when used without medical supervision.[62]

Various liquid and gas chromatography-based techniques to determine glucocorticoids have been published. Among all LC-tandem mass spectrometry (LC-MS/MS) is the most used technique

due to high sensitivity and selectivity. Several other techniques for extracting glucocorticoids from various matrices have been published.[63-69] Published extraction methods are ranging from simple to tedious and most are labour-intensive. In general steroid analysis by GC is popular due to the resolving power, high peak capacity and ability to separate steroid isomers, but major drawback of gas chromatography is that it usually requires sample pre-treatment, chemical derivatization and/or enzymatic hydrolysis prior to any kind of steroid analysis.[70-73]

PIE requires very little solvent, and prior to this it has never been explored as technique of extraction for drug compounds. PIE allows separation of water from a composition containing a miscible organic liquid and water by adding a polyol to the mixture, leading to phase separation of aqueous and organic phases. The background and theory for PIE is discussed in detail in chapter 1. Although originally conceived for purifying acetonitrile during a shortage, we expected that some of the dissolved solutes in the acetonitrile-water mixture would transfer to acetonitrile layer, making PIE a potential analytical extraction technique as well. PIE can be an effective extraction method for analytes sensitive to temperature, harsh extraction solvents or analytes difficult to derivatize. The purpose of this work is to show PIE as an effective method of extraction of glucocorticoids and potentially for other drugs, from an aqueous matrix into a polar organic solvent, in this case, acetonitrile. PIE represents a potentially quick and simple means for analytically extracting drugs from water and other aqueous systems. This work demonstrates PIE of glucocorticoids from water into acetonitrile using glycerol as a mass separating agent and analysis with UHPLC-MS-MS for separation and detection.

UHPLC works on the same Principle as of HPLC with implementation of column particles size of less than 2.5μm.[74] Separation efficiency is increased by using smaller particle size as

recognized by Van Deemter equation and his model clarifies the relationship between the height equivalent of a theoretical plate and linear velocity and their dependance on diameter of particles of packed column.[74, 75, 76] There is a considerable increase in the resolution sensitivity and efficiency in lesser amount of time, with less amount of solvents.[74, 77] In this study, glucocorticoids are extracted from water using PIE and analyzed using UHPLC-MS/MS. UHPLC-MS/MS provides approach to multidimensional separation. Upon analysis of these parameters, this method can also be applied to determine the glucocorticoids in complex matrices such as biological samples, waste sediments, herbal medications (analysis of adulterants) as well as glucocorticoids concentration in drug formulations with easy sample preparation technique.

In this study, in UHPLC-MS/MS, the liquid chromatograph is connected to a triple quadrupole mass spectrometry. The triple quadrupole detector used in this study was a dual source mass detector containing both ESI and APCI with two separate probes. ESI in the positive ionization mode was taken as the source to investigate glucocorticoids extracted using PIE. ESI is a soft ionization technique producing large molecular protonated parent ions $[M + H]^+$. By changing the voltage lower or higher, lower or higher degrees of fragmentation are achieved. MS/MS involves the selection of a specific mass to charge (m/z) ion (precursor ion) in the first mass analyzer (Q1) followed by collision induced dissociation in the collision cell (Q2) filled with a neutral gas such as nitrogen. The fragment ions are the sorted according to their mass to charge ratio in second mass analyzer (Q3) and recorded by the detector. Multiple reaction monitoring separates ions in two stages, which makes the system more selective and sensitive at low level detection.

There are few challenges in glucocorticoids analysis using LC-MS/MS. Despite the large variety of new materials, solid-phase extraction based on silica-based nonpolar C18 remains the most widely used for steroids.[78] SPE is time consuming due to many steps involve which can also lead to higher cost. Use of PIE can be an alternative cost-effective option to attain extraction. Another challenge is the obtaining specificity of the technique in both chromatography and spectroscopy due to the structural similarity of glucocorticoids. Chromatographic specificity was obtained by using selected columns which contains superficially porous particles and the best possible gradient elution.

2.2 Materials and Methods

2.2.1 Chemicals, Reagents and Sample preparations

Beclomethasone, cortisone acetate, prednisone, hydrocortisone, prednisolone, dexamethasone, fludrocortisone acetate and methylprednisolone were purchased from Sigma-Aldrich (St Louis, MO) and used as purchased. The chemical structures and physical constants of the glucocorticoids under investigation in this work can be found in Table 2-1.

Glucocorticoid extracts and standards were analyzed using a Qsight 225MD UHPLC (Perkin Elmer, Shelton, CT) equipped with sampling module, solvent delivery module and column stability module, coupled to triple quadruple mass detector equipped with an electrospray ionization source. The system was operated by simplicity 3Q MD software (Perkin Elmer, Shelton, CT).

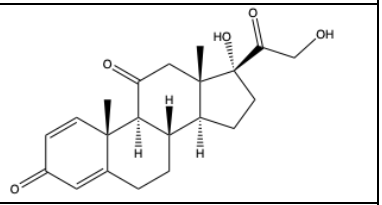
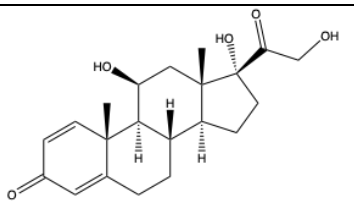
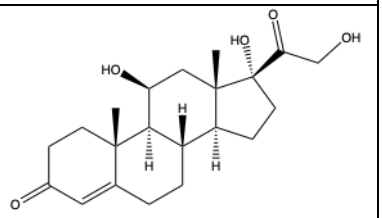
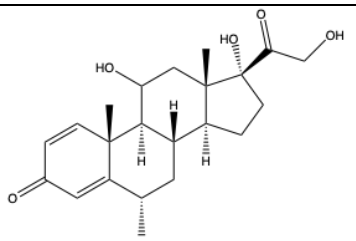
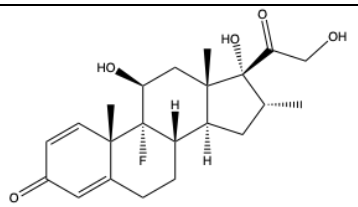
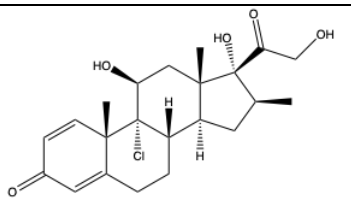
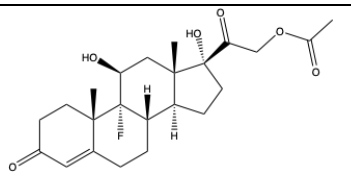
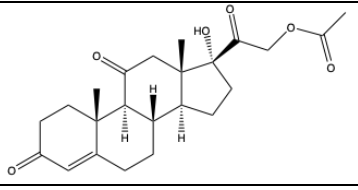
Steroid	Synonym	Structure
Prednisone	17,21-dihydrooxypregna-1,4-diene-3,11,20-trione	
Prednisolone	1,4-pregnadiene-11β,17α,21-triol-3,20-dione	
Hydrocortisone (Cortisol)	11β,17α,21-trihydroxypregn-4-ene-3,20-dione	
Methylprednisolone	11β,17α,21-trihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione	
Dexamethasone	(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione	
Beclomethasone	9-chloro-11β,17,21-trihydroxy -16β-methylpregna-1,4-diene-3,20-dione	
Fludrocortisone acetate	9α-fluoro-11β,17α,21-trihydroxy-4-pregnene-3,20-dione acetate	
Cortisone acetate	17α,21-dihydroxy-4-pregnene-3,11,20-trione 21-acetate	

Table 2- 1 Summary of glucocorticoids used in this study

2.2.2 Sample Preparation

Stock solutions of all compounds were prepared by dissolving them individually into methanol at concentration of 1 mg/mL. These solutions were used to prepare both extracted and non-extracted standards for the calibration and linearity curves as well as extraction optimization standards. PIE procedure steps were as follows: 5 mL of aqueous standard or sample was added to 15 mL, high-density polyethylene conical tubes with screw caps. Then 5 mL of acetonitrile was added into the tube and the tube was vortexed for 2 min. 2 mL glycerol was added for each extraction into the tube and the tube was vortexed again for 5 min. Tube was then equilibrated

equilibrated for 30 min at 0 °C. After the two phases separated, the volume of each phase was recorded, and the upper organic phase was recovered for analysis of glucocorticoids using UHPLC-MS/MS. Extraction temperature, time and amount of glycerol were optimized and the final optimized conditions were temperature 0 °C, time for extraction 30 minutes and amount of glycerol 2 mL. pH of the extraction has not been studied experimentally because these analytes do not show acid–base properties.[54] The effect of pH can be performed in future studies after confirming the efficiency of the extraction. Optimization of PIE was performed using a glucocorticoid standard prepared at a concentration level of 2000 ppb by equilibrating the extraction mixture at 10, 4, and 0° C with approximately 2 mL glycerol. The amount of polyol for extraction was evaluated using 10, 20, 30% glycerol and the extraction equilibrium time was optimized at 20, 30, 40 and 60 min. Outline for PIE procedure is described in Figure 2-1. Standards were prepared by spiking LCMS purity water over the concentration range of 25 to 5000 ppb. Calibration standards then were subjected to optimized PIE. Non-extracted standards were diluted into acetonitrile over concentration range of 25 to 2500ppb then analysed to

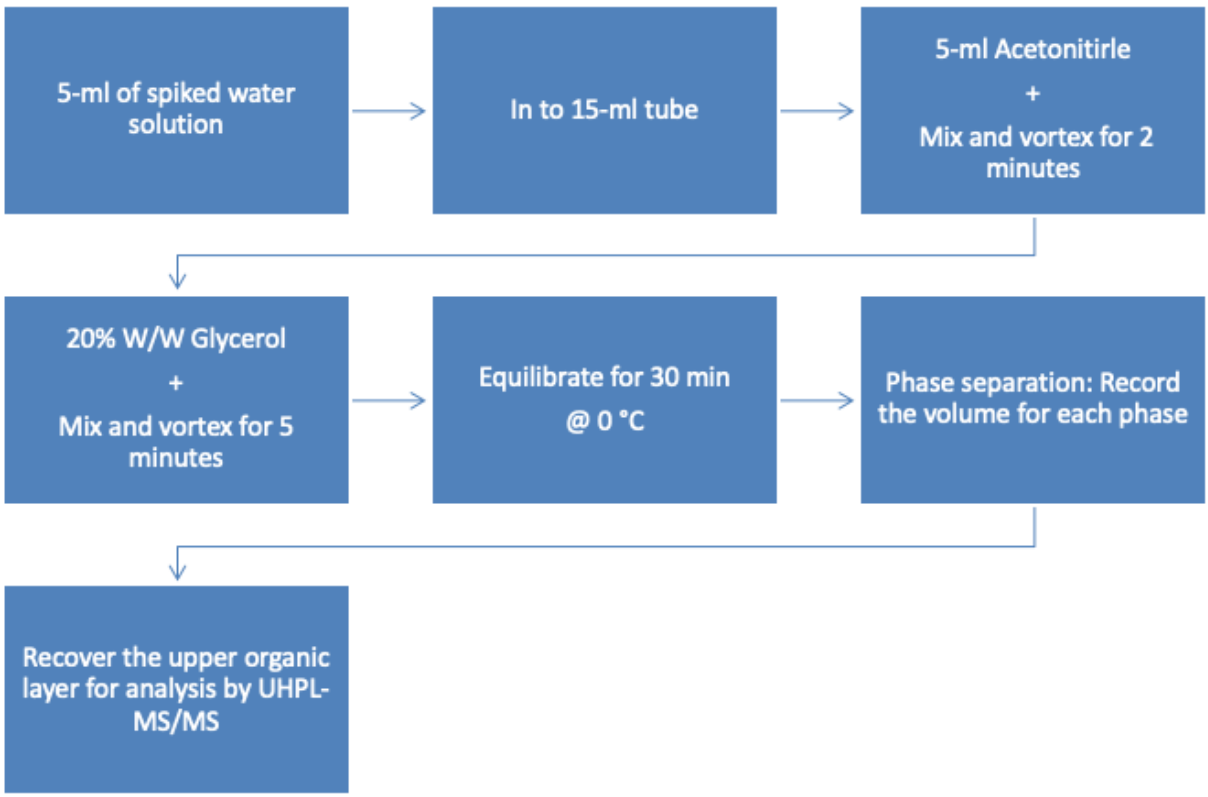


Figure 2- 1 Outline for polyol induced extraction

compare calibration curves and linearities with PIE standards. Samples were prepared in water for recovery, accuracy, and precision study at concentration of 1000 and 5000 ppb level.

2.2.3 Instrumental Parameters

Table 2-2 describes the basic outline of the analytical conditions used in this study. Glucocorticoids were qualified and quantified using a Perkin Elmer UHPLC Qsight 225MD. The

column used was a Brownlee SPP 2.7 µm; C_{18}; 2.1 x 50 mm column (Perkin Elmer, Shelton, CT) with 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. The mobile phase flow was kept at 0.4 mL/min. The gradient was started with 30% mobile phase B till 0.5 min then increase mobile phase B to 50% in 3 min and increase mobile phase B again to 80% in 3.5 min then hold for 1 min. The mobile phase was returned to the initial condition of 30% B at 5 min and equilibrated for 2 min. Column oven temperature was kept 35°C and injection volume was 1µL.

MS-MS detection was performed in positive ionization mode with the electrospray ionization voltage of 3000 V and source temperature at 250 °C. HISD temperature was set at 150 °C, drying gas was set at 120 °C and nebulizer at 200 °C. Detection was performed in multiple reaction monitoring (MRM) mode. Direct flow injection of a standard solution of each compound was used to find the optimum conditions for each compound in the ESI source and most suitable average conditions were selected for the analysis. Cone voltage and collision energies were optimized for each compound to obtain two MRM transitions, one for quantification and other for the qualification of each analyte.

LC Conditions			
Instrument	PerkinElmer UHPLC coupled to triplequad Qsight 225MD		
LC Column	PerkinElmer Brownlee SPP 2.7µm; C18; 2.1 x 50 mm		
Data analysis	Simplicity 3Q MD		
Mobile Phase A	0.1% HCOOH in Water		
Mobile Phase B	0.1% HCOOH in Acetonitrile		
Mobile Phase Gradient	Start at 0.4 ml/min with 30% mobile phase B hold for 0.5 min then increase B to 50% in 3 min and increase again to 80% in 3.5 min then hold for 1 min. Return to the initial condition at 5 min and equilibrated for 2 min.		
Column Oven Temperature	35 °C		
Injection Volume	1 µL		
MS Source Conditions			
ESI Voltage (Positive)	3000 V	Source Temperature	250 °C
Drying gas	120	HSID Temperature	150 °C
Nebulizer gas	200	Detection Mode	MRM

Table 2- 2 Analytical condition for glucocorticoids analysis

2.2.4 Method Validation and experimental Parameters

2.2.4.1 Calibration curve and linearity

Stock solutions at 1 mg/mL for all eight compounds were prepared in methanol and working standards for polyol induced extraction were prepared by diluting the stock solutions in water to produce concentration ranging from 25 ppb to 5000 ppb. Table 2-3 describes the standards and samples preparation. These standards were analysed in triplicate using LC-MS/MS. To prepare calibration curves for each analyte, non-extracted standards were prepared by direct dilution of the stock solutions in acetonitrile to produce concentrations ranging from 25 ppb to 2500 ppb. These standards were also analysed in triplicate and calibration curves were plotted for each for the comparison with extracted standards. The R^2 value and equation of the line were obtained using Excel for all calibration curves. Limit of quantitation (LOQ) and limit of detection (LOD) were assessed using the data generated for each peak. Once all data were compiled and calibration curves constructed, LOD and LOQ were determined for each peak by the classical IUPAC method and a propagation of errors method.

2.2.4.2 Recovery, precision, and accuracy

Four different samples at 1000ppb and 5000ppb were prepared by diluting stock solutions in tap water from South Orange, NJ, USA. Samples were then subjected to PIE and analyzed using UHPLC-MS/MS. All data generated was used in the determination of percent recovery and accuracy using the equation of the line from the calibration curve to find concentration from the peak area of each. Once determined, these values were divided by the appropriate concentration and multiplied by 100 to get percent recovery. An average of all four was reported as percent

Standard and Sample Preparation:	
Concentration Range	5000ppb to 25ppb
STD	PIE Extracted
Sample	Recovery and Accuracy: Spiked at 1000ppb and 5000ppb (n=4)
	Precision: Spiked at 1000ppb (n=6) for Day 1 and Day 2 Intraday
Extraction Procedure	5ml of Spiked water (aqueous); 5ml of acetonitrile; 20% (W/W) glycerol
	Vortex 5 min; extracted at 0 °C for 30 min
	After two phase separation volume of both phase recorded and upper organic layer recovered into LC vial for analysis.

Table 2- 3 Standard and Sample preparation outline

recovery for each substance. Accuracy was determined from percent recovery of an average value. Six separate samples were prepared as mentioned above at 1000 ppb for precision and analysed twice for the same day precision and analysed on second day for intraday precision. Precision was calculated as %RSD of all six. Equations needed to calculate necessary parameters are described below in equation 2-1, 2-2 and 2-3.

$$\%\text{Recovery} = \left(\frac{Experimentally\ determined\ concentration}{Theoretical\ concentration}\right) X\ 100 \qquad \text{Equation 2-1}$$

$$\text{Accuracy: } \%\text{error} = \left(\frac{|Theoretical\ concentration - experimental\ concentration|}{Theoretical\ concentration}\right) X\ 100 \qquad \text{Equation 2-2}$$

$$\text{Precision: } \%\text{RSD} = \left(\frac{s}{\bar{x}}\right) X\ 100 \qquad \text{Equation 2-3}$$

2.2.4.3 Analysis of Real Sample: Tap water

Water samples were investigated for the presence of glucocorticoids using the PIE and MRM methods. Several samples of environmental and tap waters collected from the environs of our laboratory. For samples 5 mL out of collected 100 mL was transferred to 15 mL HDPE tube then were subjected to the procedure of PIE and samples were analysed using the UHPLC-MS/MS parameter for glucocorticoids mentioned above.

2.3 Results and Discussion

2.3.1 Optimization of MRM

The tuning of the MS conditions for standards were performed by direct infusion of individual standard solutions of 1 ppm in acetonitrile using the syringe pump attached to MS. Direct

infusion in ESI-MS/MS is an approach for the determination of m/z of a precursor, product ion and MRM of a component prior to its analysis in a mixture. Each standard solution was infused at 30 µl/mL of flow rate to verify the pseudo molecular masses, $[M + H]^+$ in positive mode ionization. The ESI source parameters were then tuned for maximum intensity of $[M + H]^+$ ions of each analyte. The ESI source parameters were then tuned to accommodate LC flow rates by syringe infusion of each analyte. This was done by attaching a T-shaped connector into the LC flow to the ESI source. Figure 2-2 shows the instrument split infusion setup for the compound of interest. The LC flow rate was kept at the initial composition of the gradient. Multiple reaction monitoring (MRM) transitions were optimized with the protonated molecular ion selected as the precursor. The most abundant product ion was used for quantification and a second transition was selected for confirmation purposes. Finalized MRM parameters: entrance voltage, collision energy and collision cell lens are summarized in Table 2-4. Finalized MRM transitions are consistent with those seen in other studies. Previous studies have reported that glucocorticoids generate different precursor ions depending on the mobile phase, ionization mode and MS conditions.[58, 79-81] Source and hot surface induced desolvation (HSID) temperature along with voltage were optimized to generate maximum signal and best the suitable condition was selected to maximize ionization and minimize sodium or potassium adduct formation. HSID temperature is a compound dependent parameter: it was set at the optimized level best suitable for each compound. Drying gas and nebulizer gas flow parameters are dependent on LC flow rate, and they were optimized based on the range provided in the user manual of the system. Source temperature and ESI voltage played important roles in the ionization of glucocorticoids. Increasing the ESI source temperature above 250 °C was formed the sodium or potassium adducts with higher intensity than pseudo molecular ions for some

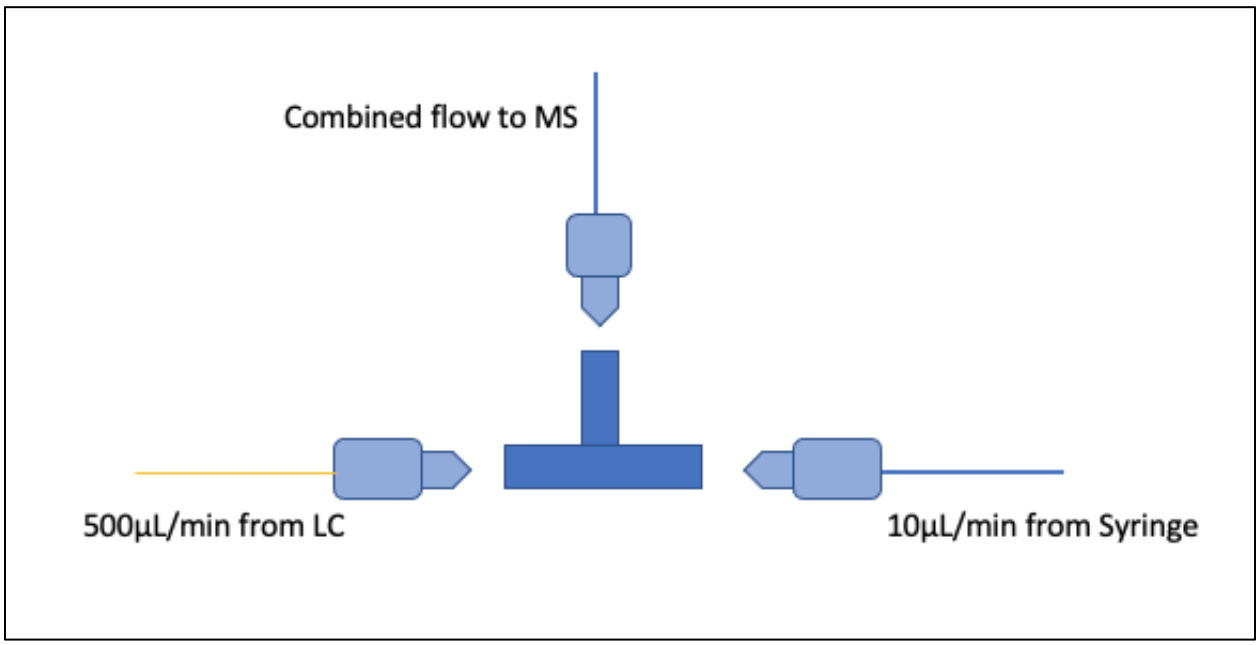


Figure 2- 2 Instrument split infusion setup for compound of interest.
Adapted from Qsight 225 MD UHPLC screening system user manual.[82]

Compound	MRM Transition		EV	CE	CCL2
	Q1 Mass	Q2 Mass			
Cortisone 21 acetate	402.80	162.90*	42	-35	-68
		342.80	36	-26	-72
Prednisolone	360.90	146.90*	25	-37	-68
		170.80	16	-39	-68
Beclomethasone	408.80	390.70*	19	-16	-60
		278.80	20	-27	-72
Dexamethasone	392.80	236.70*	22	-26	-68
		146.90	29	-42	-72
Fludrocortisone acetate	422.80	238.90*	48	-35	-76
		180.90	51	-40	-80
Hydrocortisone	362.80	120.90*	41	-34	-64
		326.80	34	-21	-60
Methylprednisolone	374.90	356.80*	16	-14	-56
		338.80	24	-15	-60
Prednisone	358.80	146.90	22	-45	-60
		340.70*	30	-16	-56

Table 2- 4 MRM transitions (* = Quantifier Ion)

analytes. An ESI voltage of +3000 V was best optimized voltage for glucocorticoids with none to very minimum adducts.

2.3.2 Optimization of chromatographic condition

These analytes show very minimum acidic or basic properties therefore extraction and chromatographic separation are not pH dependent except under extreme conditions. The several mobile phases used in UHPLC-MS/MS that include water, ACN or MeOH, acetic acid, 0.1% formic acid, ammonium hydroxide/ammonia solution, and ammonium acetate (10 mM).[74] Formic acid at a concentration of 0.1% in water and acetonitrile for mobile phase were selected based on the MS/MS compatibility and to avoid contamination to MS/MS. Two different C_{18} columns were studied for separation one with 2.7 μm diameter particles and other with 1.8 μm. Perkin Elmer C_{18} column with superficially porous particles of 2.7 μm and 2.1 mm inside diameter with 50 a mm length was selected out of the two due to better resolution. Column temperature had little effect on the selectivity or separation of these compounds. Column temperature was studied in the range of 25 to 40 °C for separation. Total ion chromatograms for the mixture are shown in Figures 2-3 and Figure 2-4. Prednisone, prednisolone, hydrocortisone eluted first, and these compounds have a common cortisol structure. Cortisone and fludrocortisone are in the form of acetate functional group on C_{21} which makes these compounds elute later, between 2.0 to 2.3 min. Beclomethasone, methylprednisolone, and dexamethasone have a methyl group in common, these compounds elute between 1.2 to 1.7 min. Structurally similar compounds prednisone, hydrocortisone and prednisolone were difficult to separate chromatographically as observed in literature. [58, 79-81] One of the most important advantages of using MS/MS for detection is the use of selective detection to separately quantify analytes that are not separated chromatographically.

MRM of quantifier ions for each compound are shown in Figure 2-5. Selective detection allows quantitation of each analyte without full chromatographic separation. Figure 2-6 and 2-7 shows MRM overlay for the quantifying ions for each glucocorticoid against time. Retention times are tabulated in Table 2-5.

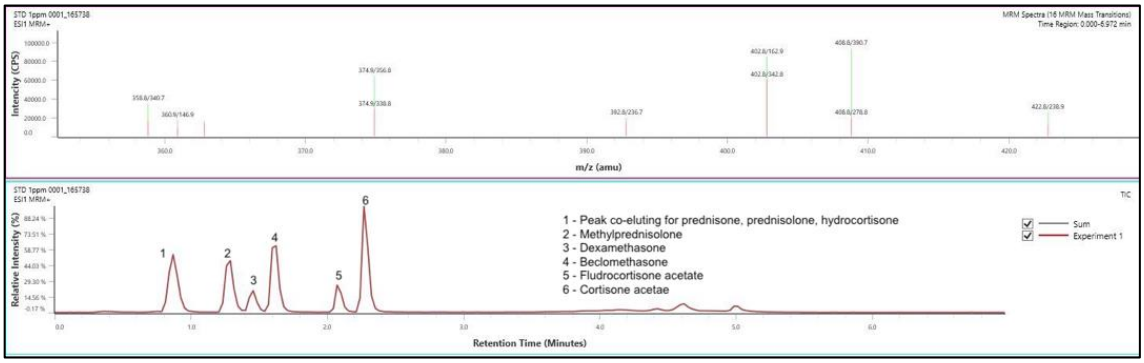


Figure 2- 3 Total ion chromatogram for PIE glucocorticoids standard at 1ug/mL

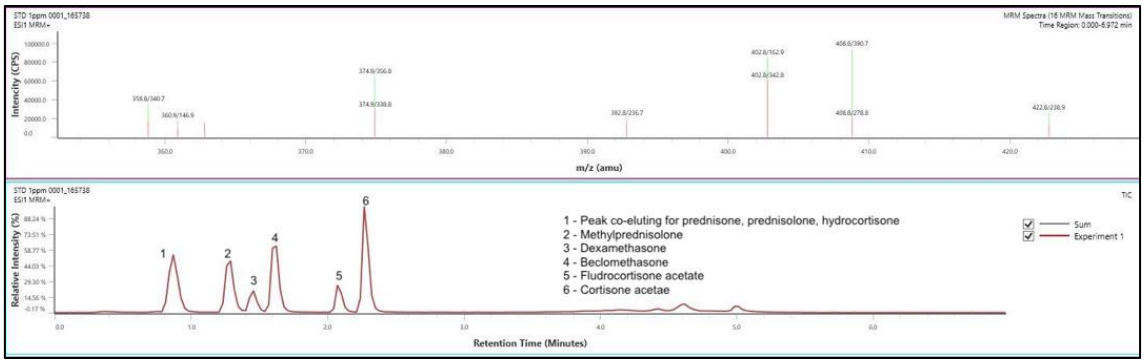


Figure 2- 4 Total ion chromatogram for PIE glucocorticoids sample at 1ug/mL

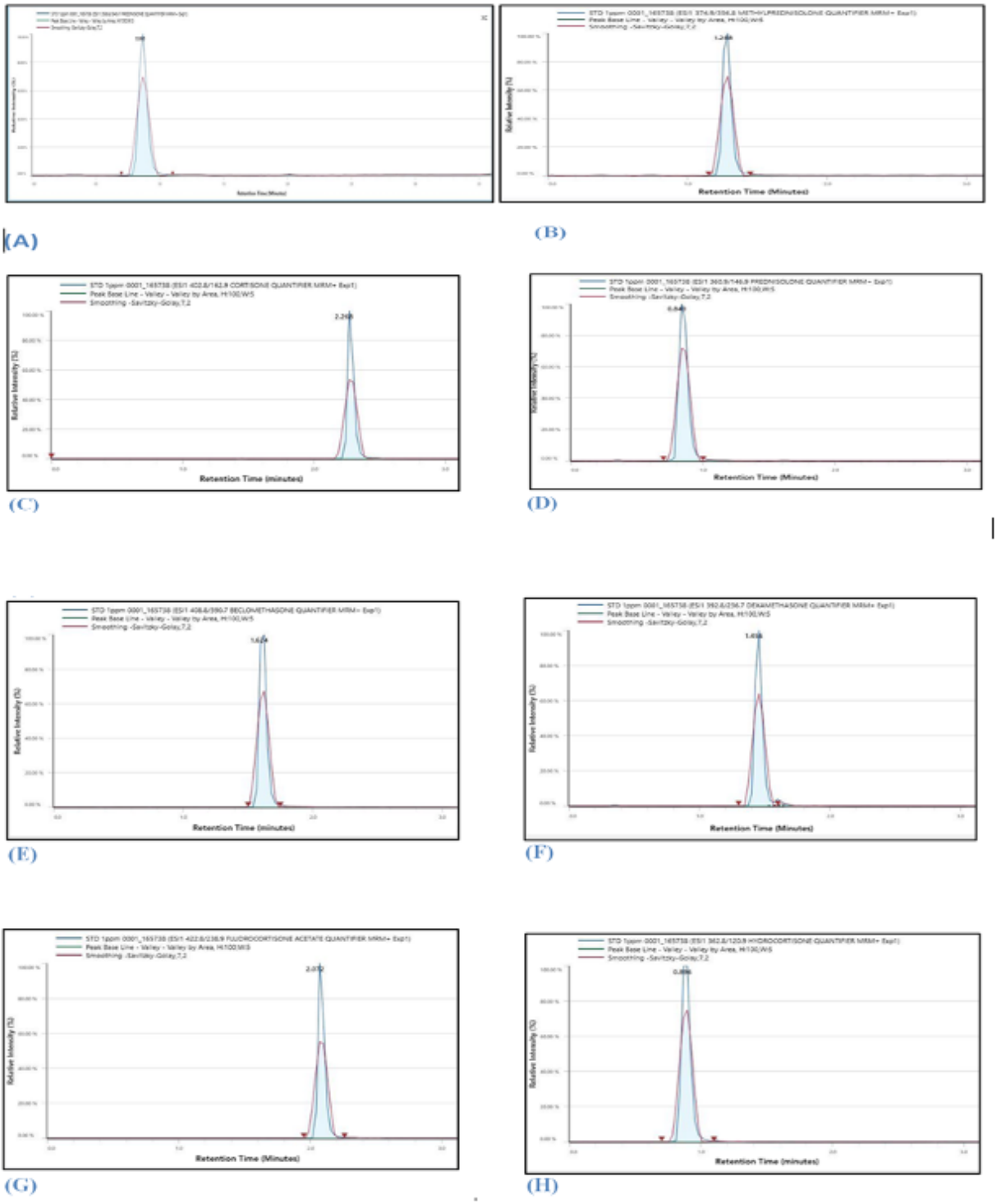


Figure 2- 5 MRM of quantifying ion for eight glucocorticoids (A) Methylprednisolone, (B) Prednisone, (C) Cortisone acetate, (D) Prednisolone, (E) Beclomethasone, (F) Dexamethasone, (G) Fludrocortisone acetate, (H) Hydrocortisone

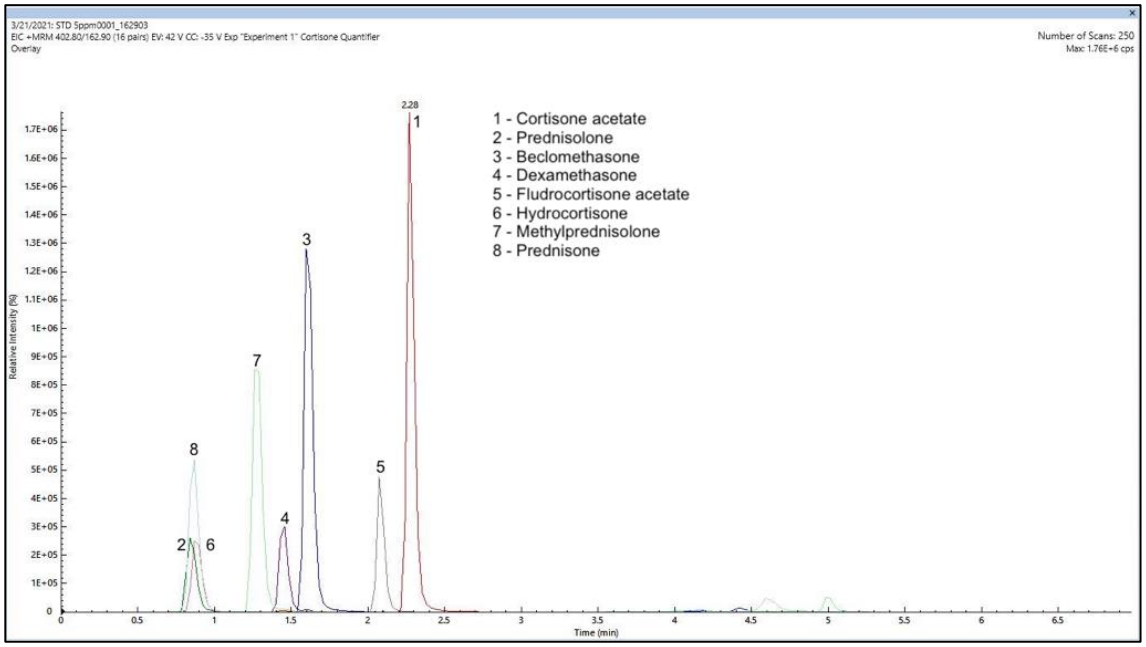


Figure 2- 7 MRM transition for PIE glucocorticoids Standard at 5 ug/mL

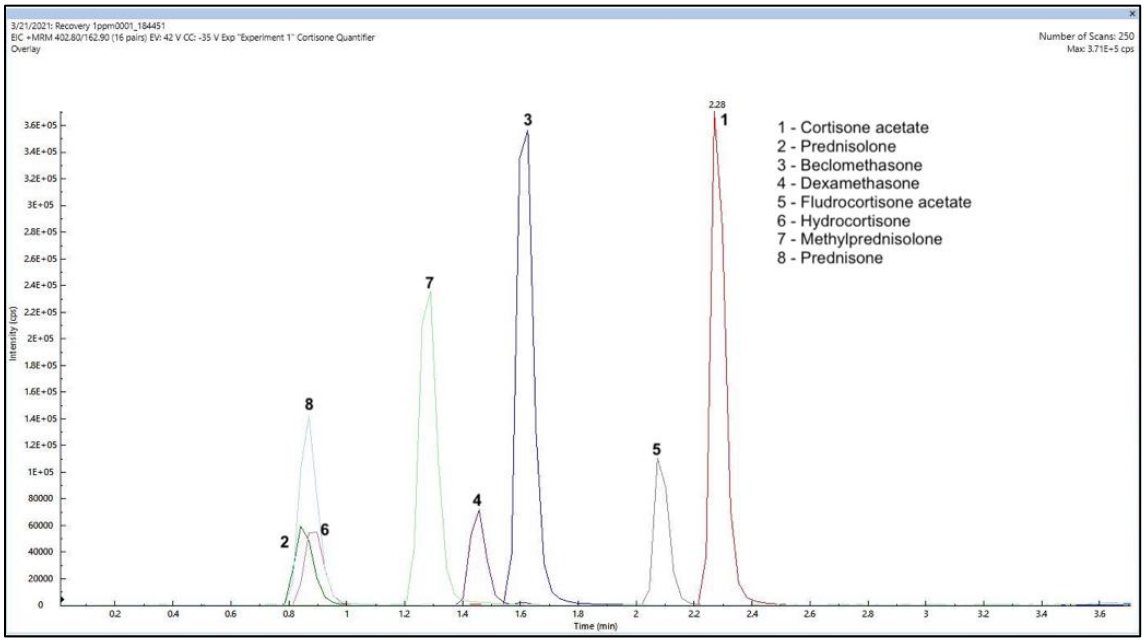


Figure 2- 6 MRM transition overlay for PIE glucocorticoids sample at 1 ug/mL

Steroid	RT (min)
Cortisone Acetate	2.268
Prednisolone	0.847
Beclomethasone	1.624
Dexamethasone	1.456
Fludrocortisone	2.072
Hydrocortisone	0.896
Methylprednisolone	1.288
Prednisone	0.868

Table 2- 5 Glucocorticoids Retention time summary

2.3.3 Polyol induced extraction

We are demonstrating that phase partitioning through polyol can be an alternative method for extraction of steroidal drugs, in this example, glucocorticoids. When solutes are dissolved in a miscible aqueous/organic solvent mixture, addition of a polyol induces a phase separation, with the organic phase ideally containing analytes of interest and the aqueous phase ideally containing water, polyol, and matrix compounds. Analytes sensitive to temperature or harsh extraction solvents can be extracted and determined without being compromised. Glucocorticoids are excellent candidates to evaluate this idea as they have been extracted from many matrices using a wide variety of extraction techniques. To evaluate PIE as an extraction process for glucocorticoids under this study, extraction conditions were studied and optimized for proper evaluation.

The experimental data obtained for extraction optimization of temperature, extraction time and amount of added glycerol are shown in Figures 2-8, 2-9, 2-10 and Table 2-6. Table 2-6 shows the final volumes of the mixture of 5 mL of acetonitrile and 5 mL of water with 2 mL of glycerol, following mixing and equilibration at three temperatures. The total volume of the liquids prior to mixing was 12 mL, but the final volume of the mixture was 11 mL, indicating that volume is not conserved, as is typical when mixing polar liquids. It is clearly seen that temperature has a dramatic impact on the final volume of the aqueous and organic phases. The mixing of acetonitrile and water is endothermic; therefore, separation of acetonitrile and water

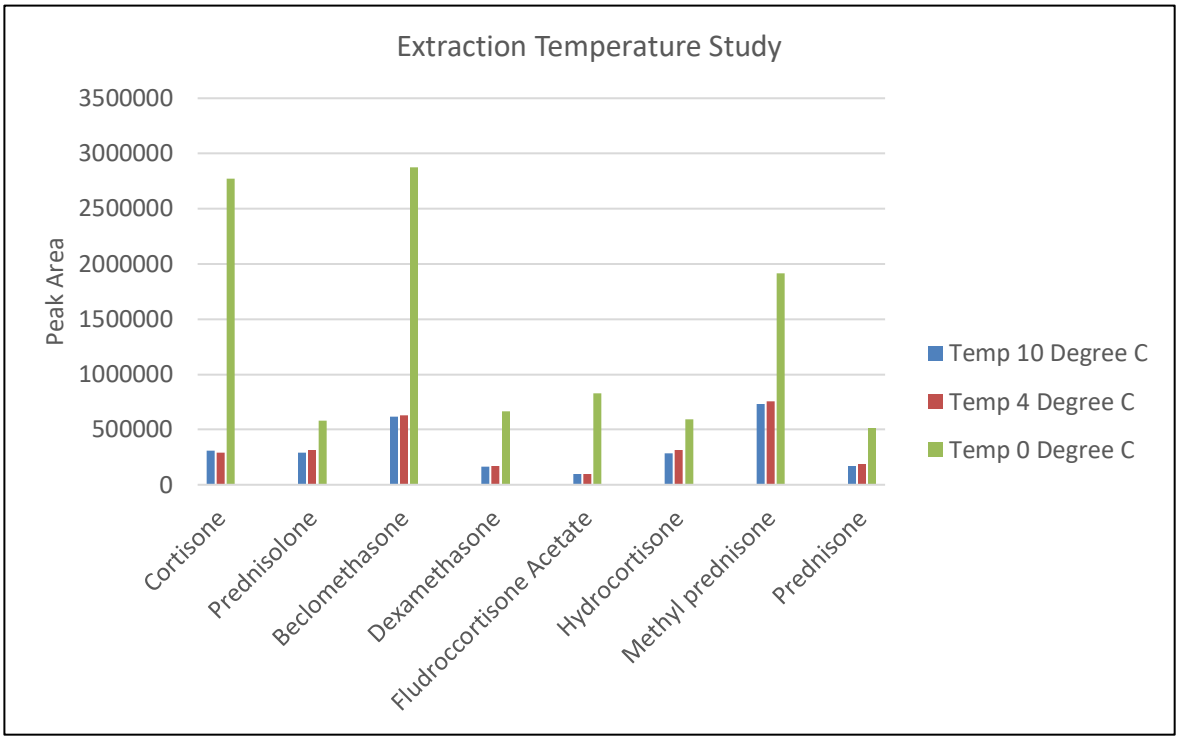


Figure 2- 8 Extraction optimization study: equilibration temperature v/s peak response

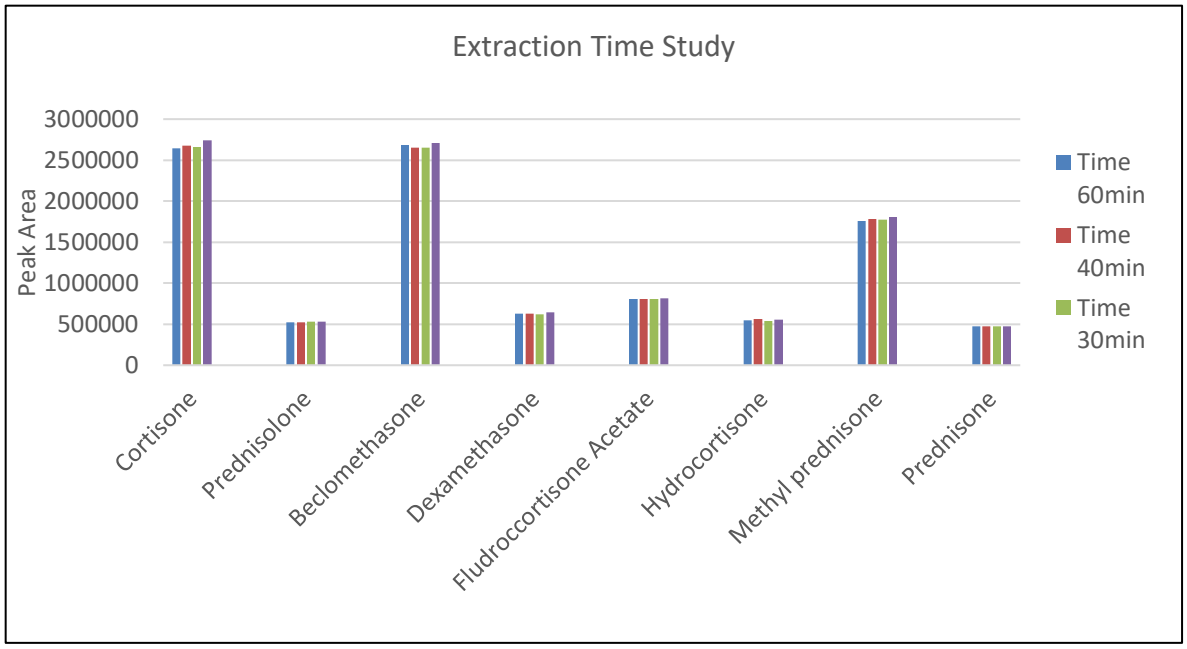


Figure 2- 9 Extraction optimization study: Extraction time v/s Peak response

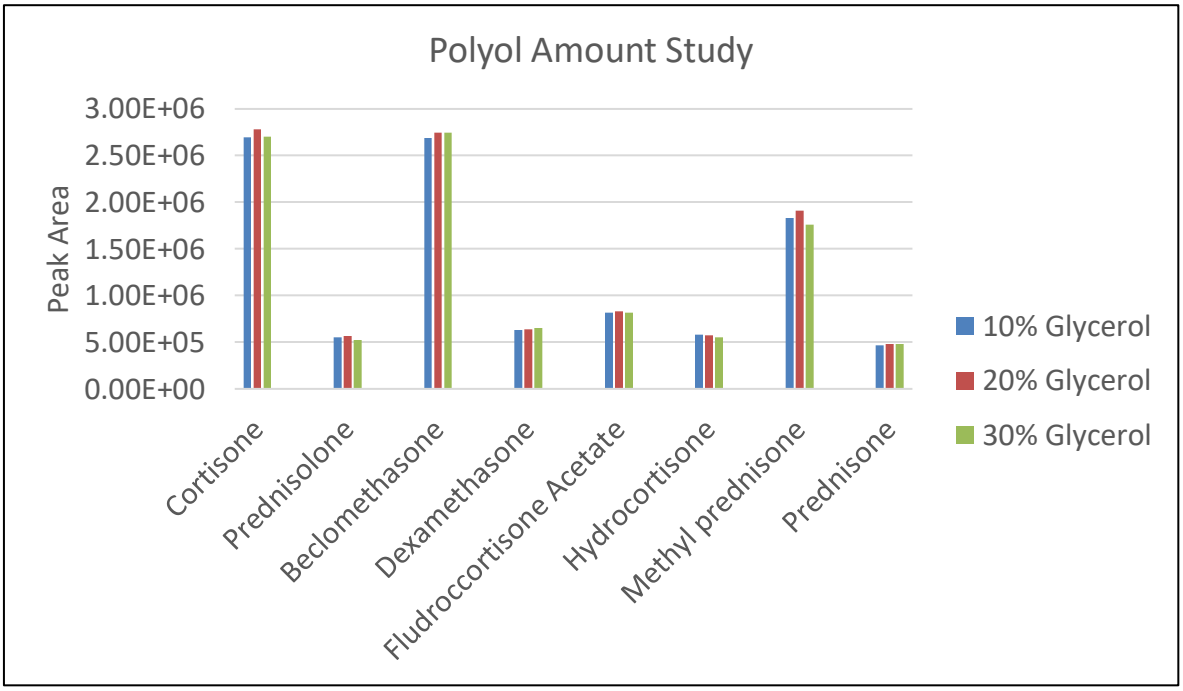


Figure 2- 10 Extraction optimization study: Amount of glycerol v/s peak response.

Temperature point	Volume of organic phase (ml)	Volume of aqueous phase (ml)	Phase ratio
0°C	3	8	0.38
4°C	2	9	0.22
10°C	1	10	0.10

Optimized extraction conditions:	
Extraction Temperature	0 °C
Amount of glycerol	20% (w/w)
Extraction time	30min

Table 2- 6 PIE phase separation volume and phase ration at three temperature point and summary of optimized extraction conditions.

is exothermic. This indicates that separation of acetonitrile and water will be more complete at lower temperatures. This is observed in the larger volume of the organic phase at 0 ^{o}C seen in Table 2-6. Figure 2-8 also shows the largest instrument response for all analytes at 0 ^{o}C. In PIE, lower temperatures are seen to favor both larger organic volume and recovery of analytes. Temperatures lower than zero degrees were not examined in this work, to keep the extractions in a simple ice bath. An extraction temperature of 0 ^{o}C was used for the quantitative studies.

Figures 2-9 and 2-10 show the effect of equilibration time and amount of polyol added to the mixture on peak response for the eight glucocorticoids. As seen in Figures 2-9 and 2-10, beyond 20 minutes of equilibration time and 10% (about 1 mL) of polyol added to the mixture, there is little effect on the peak responses. Liquid-liquid extraction techniques generally show rapid equilibration, and we would expect even more rapid equilibration when starting with fully miscible solvents. For the later studies, 30 min equilibration time and 2 mL of polyol were used as 30 minutes (longer equilibration) ensures that equilibrium is reached, and, in some cases, 2 mL of polyol did show slightly higher response. It should be noted that the extraction did not require a shaker, nor did it require monitoring during the extraction, so it was very time efficient.

While the extractions will be shown below to be reproducible, it is difficult to make clear statements about some measures such as recoveries and partition coefficients due to the complexity of an extraction system involving two polar, miscible solvents and a phase separating agent. Using the zero-degree example in Table 2-6, the original acetonitrile volume is 5 mL, and the final volume of the acetonitrile phase is 3 mL, and this phase may also contain some of the polyol as well. However, due to selective detection through MRM may not show any evidence of polyol or water in in the chromatogram. The remaining acetonitrile must therefore still be mixed

with the aqueous phase, so this partitioning is between acetonitrile and an acetonitrile-water-polyol mixture for which the solubility data that would allow ready determination of a partition coefficient would be unknown. This also make the determination of an acetonitrile-water partition coefficient, on which a true recovery determination would be based problematic. Although, in previous work of development of PIE by M. Deshpande[10] it was determined that PIE is capable of yielding acetonitrile recovery with a purity of 96% at -20 °C and 89% at 0 °C moreover, the composition of 89% of acetonitrile at 0 °C is not known.

2.3.4 Validation

2.3.4.1 Calibration and Recovery

Precision, accuracy, and linearity for this PIE-based analytical method were evaluated. Calibration curves were constructed for all analytes from 0.025 to 5 μg/mL (25ppb to 5000ppb) with standard concentration levels at 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 μg/mL. Correlation coefficients of $r^2 > 0.99$ for all analytes were obtained over these ranges. The standard curves are shown in Figures 2-11 to 2-18. As seen in the Figures each standard curve has very good linearity.

Table 2-7 shows analytical Figures of merit for the eight analytes using the optimized method. Six samples at 1 μg/mL (1000 ppb) were prepared to evaluate intra and inter day levels of precision. Four samples at the middle concentration of 1 μg/mL (1000 ppb) and high concentration 5 μg/mL (5000 ppb) were prepared estimate recoveries despite the stated challenges of this unique extraction system.

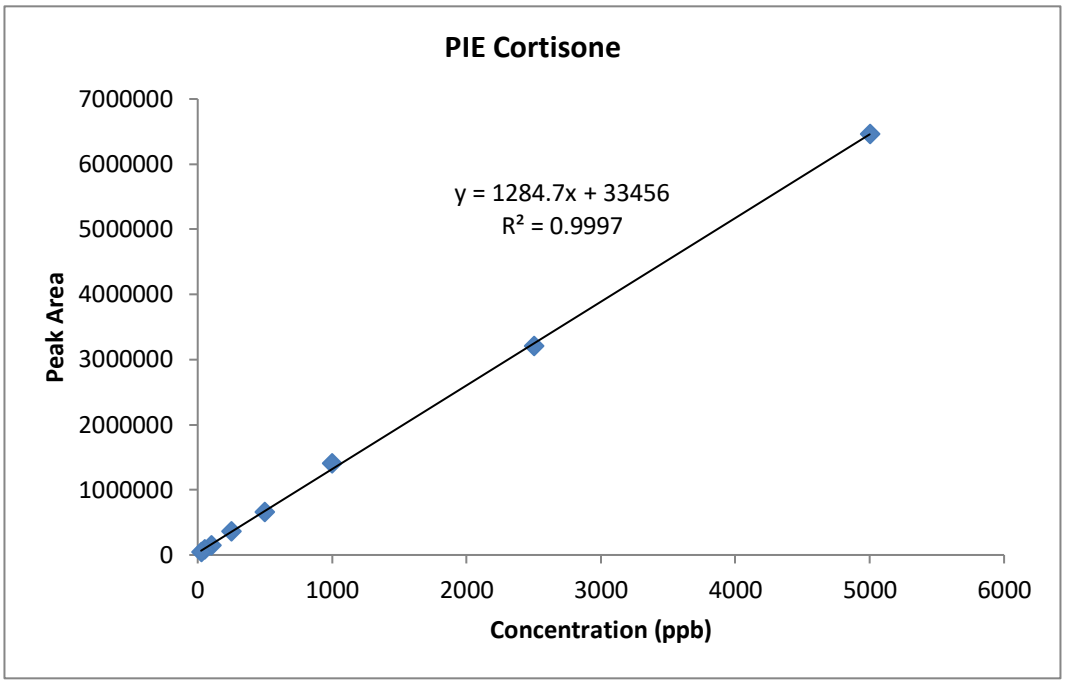


Figure 2- 11 Calibration curve for cortisone acetate (PIE)

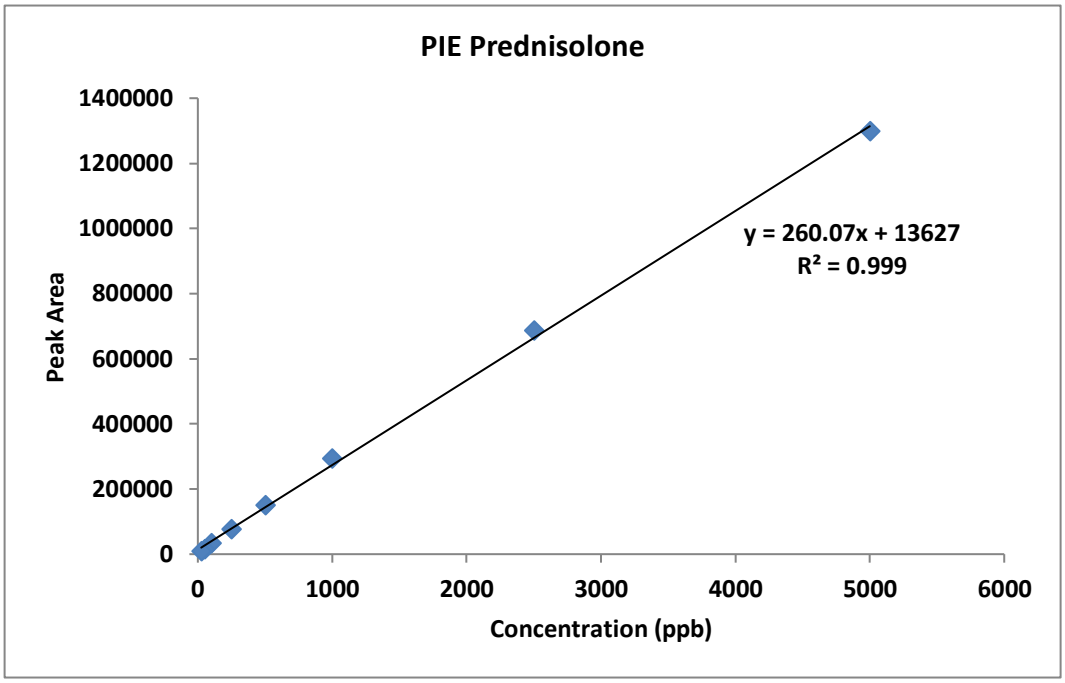


Figure 2- 12 Calibration curve for Prednisolone (PIE)

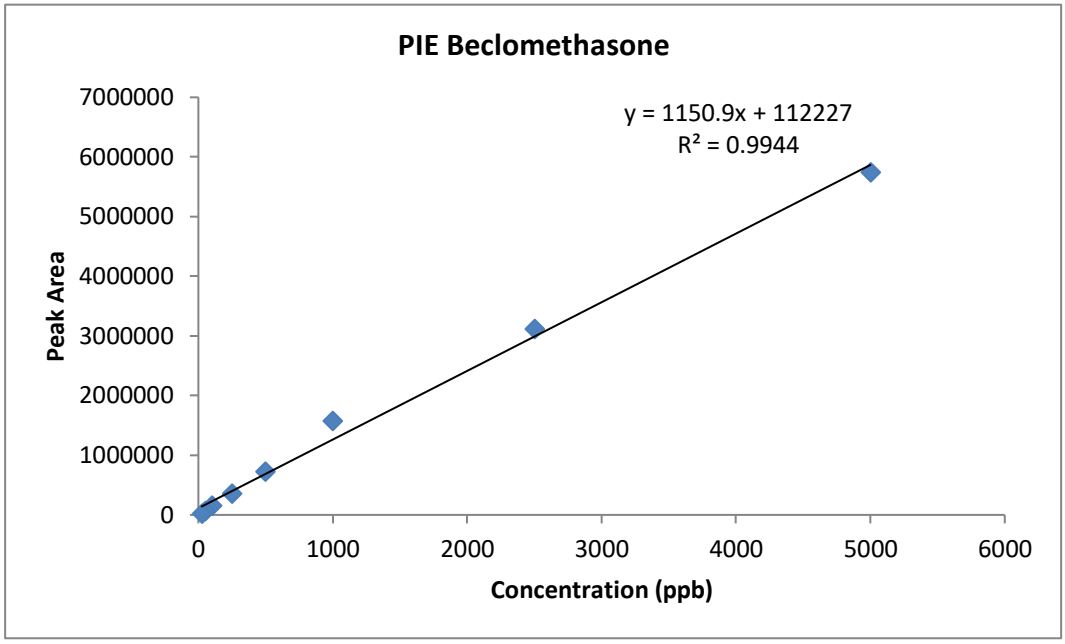


Figure 2- 13 Calibration curve for Beclomethasone (PIE)

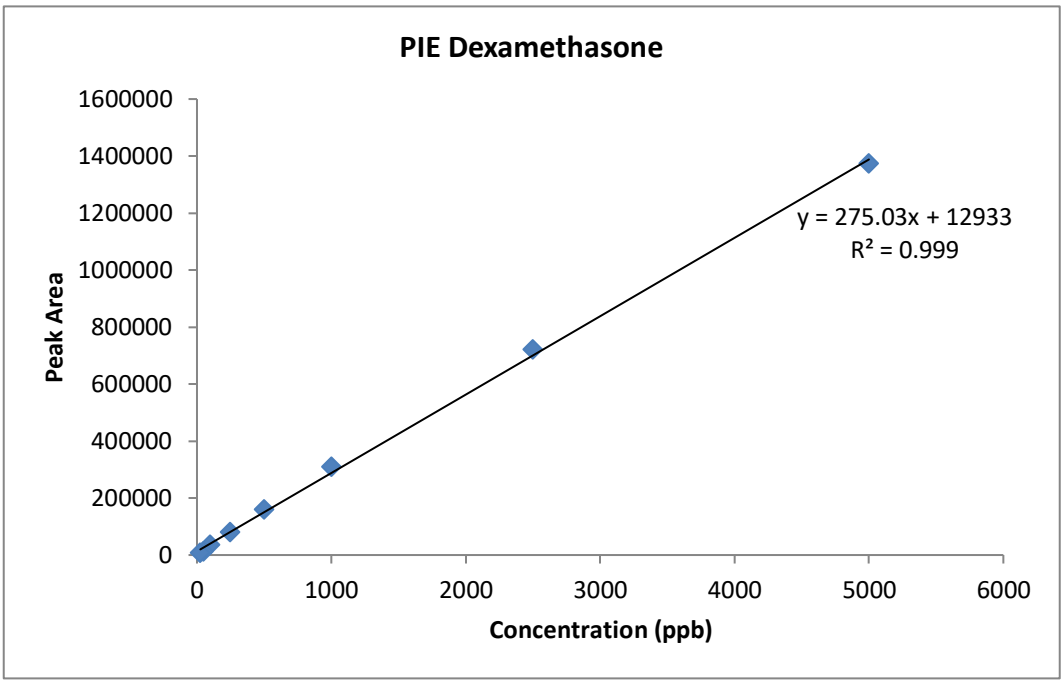


Figure 2- 14 Calibration curve for Dexamethasone (PIE)

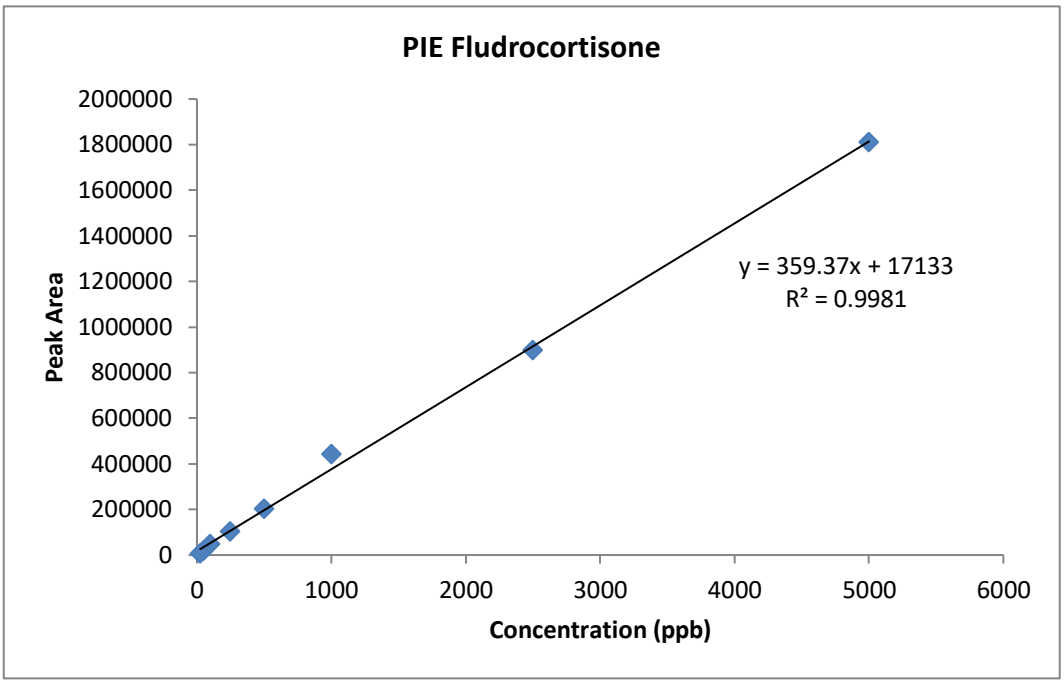


Figure 2- 15 Calibration curve for Fludrocortisone (PIE)

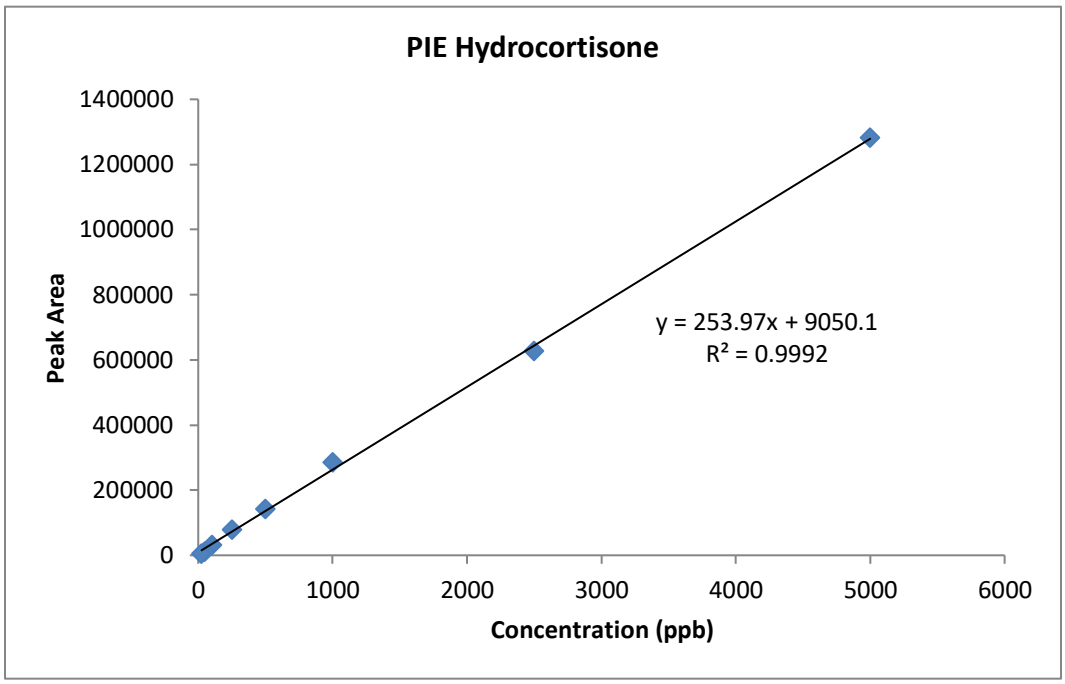


Figure 2- 16 Calibration curve for Hydrocortisone (PIE)

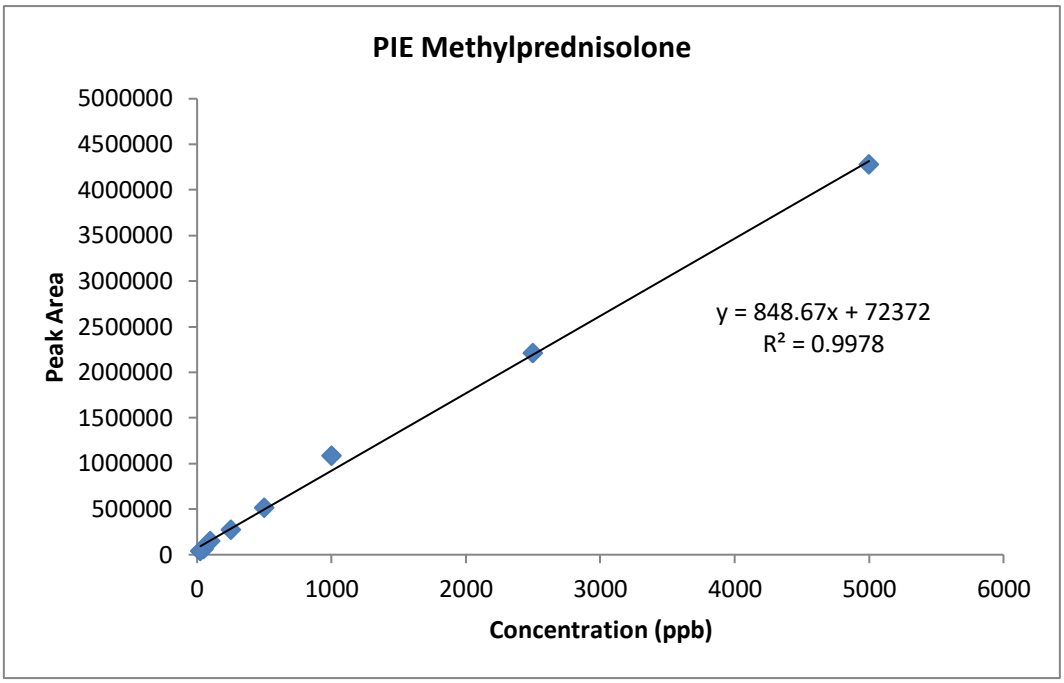


Figure 2- 17 Calibration curve for Methylprednisolone (PIE)

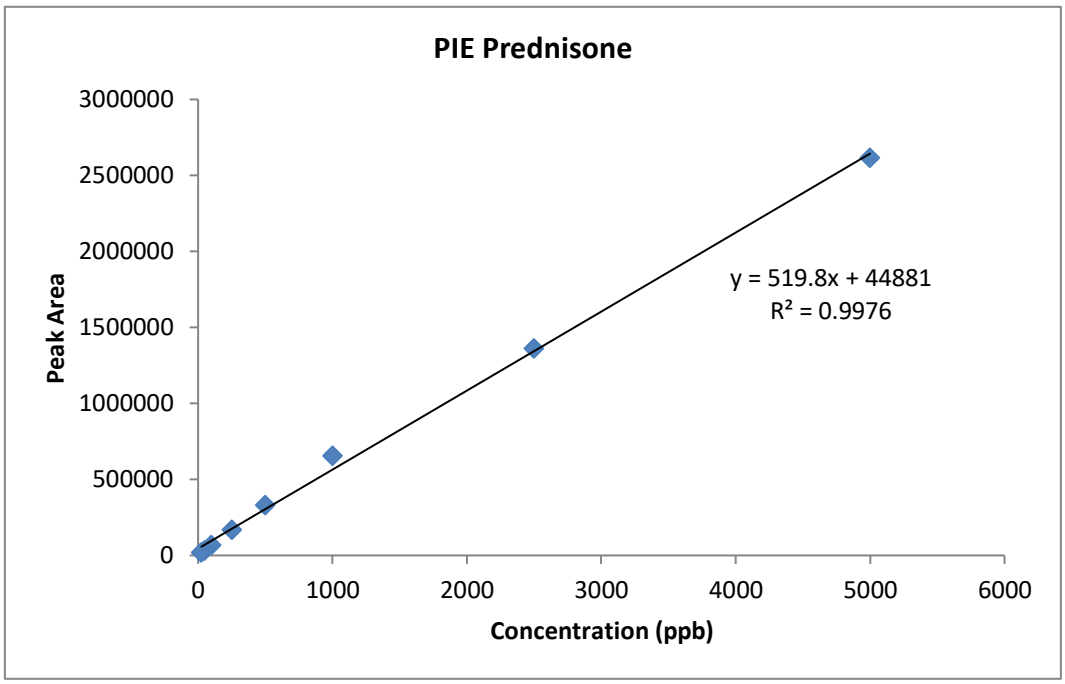


Figure 2- 18 Calibration curve for Prednisone (PIE)

Steroid (Calibration range)	R^2	Recovery (%; n=4)	Accuracy (% Error; n=4)	Precision (%RSD; n=6)	Intraday Precision (%RSD)
Cortisone Acetate (25 to 5000ppb)	0.999	1000ppb: 110.67 5000ppb: 99.33	1ppm: 10.67 5ppm: 0.67	Day 1(1): 1.82 Day 1(2): 1.18 Day 2: 1.96	1.93
Prednisolone (25 to 5000ppb)	0.999	1000ppb: 109.30 5000ppb: 100.03	1000ppb: 9.30 5000ppb: 0.025	Day 1(1): 2.05 Day 1(2): 1.62 Day 2: 2.01	1.85
Beclomethasone (25 to 5000ppb)	0.994	1000ppb: 132.40 5000ppb: 99.67	1000ppb: 32.40 5000ppb: 0.33	Day 1(1): 1.35 Day 1(2): 0.99 Day 2: 2.54	1.95
Dexamethasone (25 to 5000ppb)	0.999	1000ppb: 111.45 5000ppb: 100.00	1000ppb: 11.45 5000ppb: 0.00	Day 1(1): 1.27 Day 1(2): 1.57 Day 2: 3.31	2.29
Fludrocortisone (25 to 5000ppb)	0.998	1000ppb: 122.55 5000ppb: 99.81	1000ppb: 22.55 5000ppb: 0.19	Day 1(1): 0.81 Day 1(2): 1.78 Day 2: 2.69	1.93
Hydrocortisone (25 to 5000ppb)	0.999	1000ppb: 111.51 5000ppb: 101.39	1000ppb: 11.51 5000ppb: 1.39	Day 1(1): 1.93 Day 1(2): 2.21 Day 2: 2.02	2.05
Methylprednisolone (25 to 5000ppb)	0.997	1000ppb: 125.56 5000ppb: 100.95	1000ppb: 25.56 5000ppb: 0.95	Day 1(1): 1.56 Day 1(2): 1.68 Day 2: 2.40	1.84
Prednisone (25 to 5000ppb)	0.997	1000ppb: 123.95 5000ppb: 100.6	1000ppb: 23.95 5000ppb: 0.6	Day 1(1): 0.32 Day 1(2): 1.83 Day 2: 1.20	1.56

Table 2- 7 Glucocorticoids PIE summary

The data are summarized in Table 2-7. Percentage recoveries for all eight analytes at 1000 ppb concentration ranged from 110-130% and at 5000ppb concentration were all about 100%. Accuracy as percent error was determined for all compound from percent recoveries. Percent error for samples at 1000 ppb the calculated accuracy ranged from 9 to 30% and at 5000 ppb it was about 1% for all samples. For precision calculations, intra-day repeatability and inter-day reproducibility were expressed as relative standard deviation (% RSD) for each analyte, analysis were performed on two days. All the% RSD results for all compounds were found below 4%. For 1000ppb samples recoveries were well above 100% but less than 133%. As noted previously, traditional recovery calculations for this system may be difficult due to the multiple partitioning processes of both the acetonitrile/glycerol/water system and the analyte/acetonitrile/water system. These analyses were off from expected, which could be accounted for by the 89% pure acetonitrile. Moreover, MRM mode of detection has a blind spot to everything else except selected ions which makes it difficult to know the presence of polyol or water in upper organic layer. At the 5000 ppb level, recoveries were between 99 to 101 percent but not much should be read into this result. PIE provided adequate precision for quantitation and accuracy and recovery adequate for the rapid screening applications for which it might most likely be used.

2.3.4.2 Limit of detection

The limit of detection (LOD) and limit of quantification (LOQ) were determined for each analyte adopting a discussion from an article published in 1983 on limit of detection by Long and Winefordner.[83] The IUPAC definition of limit of detection is described in the equation 2-4.

$$C_L = \frac{ks_B}{m} \qquad \text{Equation 2-4}$$

C_L is limit of detection expressed as concentration, k = 3, s_B is the background signal standard deviation, m is the slope of the calibration curve. The classical IUPAC method for limit of detection calculation is simple, using the baseline noise (s_B) and the slope of the calibration curve (m), but it does not account for any noise in the calibration curve. It assumes that there is zero experimental uncertainty in the determination of the calibration curve, which we know is not true. In that 1983 article, Long and Winefordner recommended a propagation of errors-based approach, seen in Equation 2-5.

$$C_L = \frac{k\sqrt{\left[s_B^2 + s_i^2 + \left(\frac{i}{m}\right)^2 s_m^2\right]}}{m} \qquad \text{Equation 2-5}$$

Where, C_L is limit of detection expressed as concentration, k = 3, s_B is the background signal standard deviation, m is the slope of the calibration curve, i is the intercept of the calibration curve, s_i is error in the intercept and s_m is the error in the slope from the regression line. This method includes terms for uncertainty in the slope (s_m) and intercept (s_i) of the calibration curve but does not account noise in the calibration curve. The limit of detection values for both methods are calculated using equations and results are summarized in Table 2-8. The LOD values obtained by IUPAC method ranged from 0.3 ng/mL to 4 ng/mL and 40 ng/mL to 200 ng/mL for the propagation of error method. The values obtained by the propagation of errors method are significantly higher than the traditional IUPAC method mostly due to the uncertainty associated with the Y-intercept of the calibration. The regression calculation showed very minimal uncertainty associated with the slope. Since the propagation of errors-based calculation shown in Equation 2-5 includes terms for the uncertainty in the slope and y-intercept of the calibration curve, the limit of detection obtained by the propagation of error method was seen in some cases to be higher than the lowest calibration point on the calibration curve.

Steroid	LOD (ppb)	LOQ (ppb)	LOD by (ppb)
	IUPAC		Error of propagation method
Cortisone Acetate	0.3	1	44
Prednisolone	1	3	92
Beclomethasone	2	6	193
Dexamethasone	2	7	77
Fludrocortisone acetate	1	3	112
Hydrocortisone	4	14	84
Methylprednisolone	2	6	120
Prednisone	3	11	120

Table 2- 8 Limit of detection, quantification, and limit of detection by error of propagation method for glucocorticoids extracted by PIE.

If the calibration curve has a non-zero y-intercept, it is likely that the lowest points on the calibration curve would have much higher experimental uncertainties than higher points. Since there are few standard guidelines for the preparation of calibration curves, this illustrates the need for greater care in the determination of both calibration curves and limits of detection. It also illustrates the need and challenges involved in having the lowers concentration points of the calibration curve at or near the LOD and LOQ.

2.3.5 Analysis of real sample: Tap Water

Several samples of environmental and tap waters collected from the environs of our laboratory and were subjected to the PIE procedure. All the samples were negative for all glucocorticoids. Based on the calibration data, PIE-UHPLC-MS/MS as performed here appears to not include a sufficient concentration step for analysis of drugs in environmental water that may be present at sub-ppb levels. However, the method still shows potential for analysis of glucocorticoids at clinical (ppb to ppm) and pharmaceutical (ppm and higher) concentrations.

2.4 Conclusions

In this study, polyol induced extraction based on an acetonitrile-water mixture using glycerol as a mass separating agent was used to extract glucocorticoids from water. A mixture of glucocorticoids in water was partitioned into acetonitrile at 0 °C by addition of glycerol to the water/acetonitrile mixture. Intraday and inter-day precision results as percent RSD for all compounds were less than 4%, which shows the method to be reproducible and accurate. Limit of detection was calculated by the IUPAC and propagation of errors, with the propagation of

error method being more informative as it includes experimental uncertainty on the calibration curve. The LOD values obtained by IUPAC method ranged from 0.3 ng/mL to 4 ng/mL and 40 ng/mL to 200 ng/mL for the propagation of errors method. The LOQ values obtained by IUPAC method ranged from 1 ng/mL to 10 ng/mL. Limit of detection results generated by propagation of error method for this experiment gave significantly higher limit compared to IUPAC method, which was due to error in the intercept related to having a non-zero intercept.

CHAPTER 3 – EXTRACTION OF GLUCOCORTICOIDS via QuEChERS and PIE: A COMPARISION STUDY AND A COMPARISON STUDY WITH PUBLISHED METHODS

3.1 Introduction

This chapter discusses the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) extraction technique applied to the extraction of glucocorticoids from water and the methodology behind it. QuEChERS extraction experiments were performed and compared to PIE to evaluate PIE as an extraction procedure. Previously, in our group extraction parameters using QuEChERS were optimized by Schmidt for the extraction of glucocorticoids from water with analysis by gas chromatography.[84] In this work QuEChERS was performed with UHPLC MS/MS to evaluate PIE as an extraction technique. QuEChERS is widely used for various types of applications and many analytes. An abundant amount of research has been performed using this method as an extraction technique for many types of compounds such as pesticides, volatile organic compounds, polycyclic aromatic hydrocarbons (PAHs), veterinary drugs and pharmaceuticals, from wide variety of matrices such as food, animal, dietary supplements, sewage, and biological matrices.[85-89] Additionally this chapter discusses comparison of our PIE work with several published extraction methods for determination of glucocorticoids in water matrices. The proposed method of PIE provided comparable analytical qualities of analysis time, recovery, sensitivity and amount or type of organic solvent requirement.

3.1.1 QuEChERS Background

QuEChERS was developed by Anastassiades and Lehotay,[86] and was originally designed for extraction of veterinary drugs from animal tissue and then adopted for extraction of pesticides from botanical materials.[86] Over the years the QuEChERS method has evolved into two other methods, European standard EN 15662 method[89] and the AOAC 2007.0190 method.[90] These methods utilize buffer salts to increase the recovery of pH dependent analytes.

3.1.2 QuEChERS Theory and methodology

QuEChERS is a reliable sample preparation method for residue analysis. It is basically summarized in two steps: liquid-liquid extraction and dispersive solid phase extraction. There are three commonly used methods: the original method, the AOAC 2007.01 method and the European Standard EN 5662 Method. Basic steps for each method are similar: liquid-liquid extraction between an organic phase and water, with the aid of a salt, followed by shaking and centrifuging for liquid-liquid partitioning. After phase separation, the organic extract is removed and subjected to dispersive solid phase extraction (d-SPE) clean up using magnesium sulfate and a matrix binding sorbent such as a primary secondary amine (PSA), graphitized carbon black or end capped C_{18} silica. The sample can then be analysed using GC or LC. Figure 3-1 shows a flowchart of the steps in commonly used QuEChERS methods.

3.1.2.1 Liquid-liquid extraction

Acetonitrile, ethyl acetate or acetone are most used organic solvents. Acetonitrile is used most frequently due to a wide extraction range and minimum interference. In contrast with PIE, salts are used for phase separation but like PIE are the driving force for moving the analyte of interest

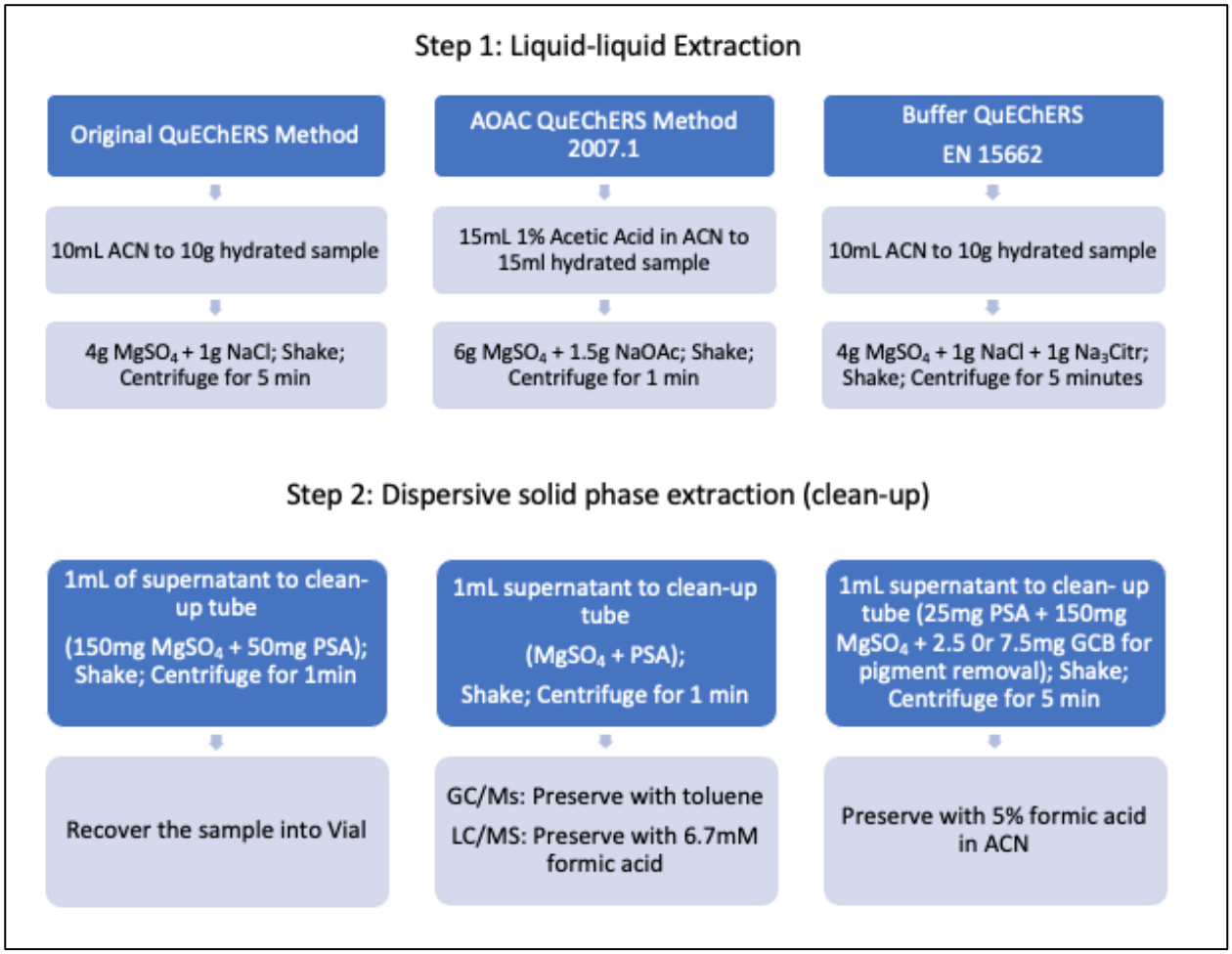


Figure 3- 1 Comparison of QuEChERS methods.

Adapted from M. Schmidt, QuEChERS Extraction – Gas Chromatography for the analysis of drugs, dissertation and theses, Seton Hall University, (2015).[84]

into the organic phase. Addition of salt leads to an increase in the ionic strength followed by salting out. It also increases the overall polarity of the solvent, which increases solubility of the analyte in the solvent being used. Sodium chloride and magnesium sulfate are used in the original and European method. Sodium chloride decreases the amount of polar interferences. Magnesium sulfate helps aid the recovery of polar analytes and improves the solvent partitioning in the liquid-liquid extraction. Typically, the sodium chloride to magnesium sulfate ratio is 4:1 but other salts and or ratio can be used depending on the analytes. The AOAC QuEChERS method uses $MgSO_4$ and sodium acetate in addition to acetonitrile with 1% acetic acid for base sensitive pesticide compounds.

3.1.2.2 d-Solid phase extraction (d-SPE)

This step is a clean-up step to remove matrix interferences and any residual water present in the organic phase d-SPE is not performed in PIE. Clean-up step of d-SPE is similar in all three methods described in Figure 3-1. A sorbent such as primary secondary amine (PSA) is added to remove matrix interference and $MgSO_4$ is then added to remove traces of water transferred with the phase separation.[89-90] Figures 3-2 and 3-3 show the examples of PSA and silica C_{18} sorbent respectively. The type of sorbent can be optimized depending on the analytes and each sorbent removes specific interferences. For examples, a PSA reduces the level of organic acids, fatty acids and sugars by means of weak ion exchange, end capped C_{18} removes lipid and non-polar interferences by non-reactive residual silanol. Graphitized carbon black (GCB) binds to planar analytes and reduces interference due to pigments. Aminopropyl is like PSA and improves the recovery for base sensitive analytes.

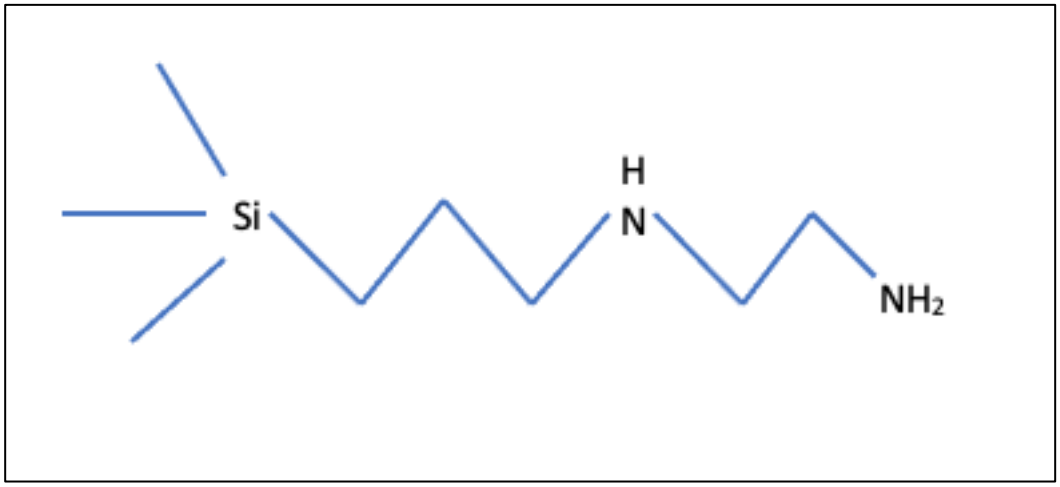


Figure 3- 2 Primary Secondary amine (PSA)

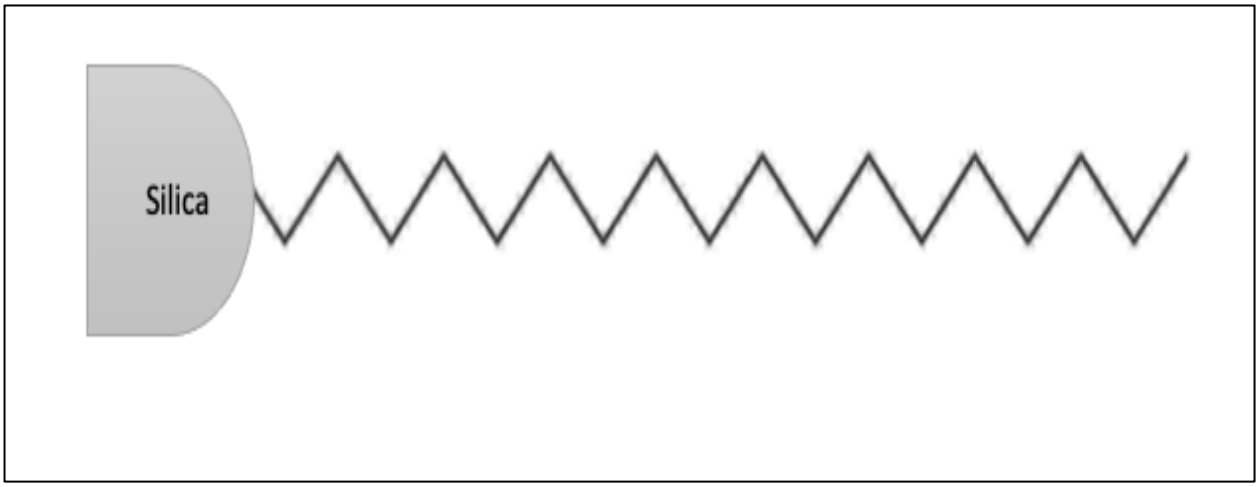


Figure 3- 3 C_{18} Silica d-SPE

Dual phase sorbents include combination of two or more sorbents to remove certain compounds and keeping certain analytes as is. The choice of sorbent would depend on the matrix of the sample as well as the composition of the analytes. In this chapter, PIE is evaluated in comparison to QuEChERS, since QuEChERS is a widely used and well-studied sample preparation for the extraction of various kinds of analytes. Both techniques are very similar as the phase separation is the key to extract analytes by inducing the partitioning of analytes into the organic phase. This similarity is an opportunity to compare both techniques. In previous studies[84] QuEChERS was used for glucocorticoids extraction from water and extracts were analyzed by GC-MS.

3.2 Materials and Methods

3.2.1 Chemicals, Reagent and Samples

Glucocorticoids: beclomethasone, cortisone acetate, prednisone, hydrocortisone, prednisolone, dexamethasone, fludrocortisone acetate and methylprednisolone were purchased from Sigma-Aldrich (St louis, MO) and used as purchased.

Reagents for QuEChERS and UHPLC-MS-MS analysis: HPLC grade water, acetonitrile and methanol were purchased from Fisher Scientific (Bridgewater, NJ), LC-MS grade formic acid was from RICCA (Arlington, TX), experiments were $MgSO_4$ obtained from Sigma-Aldrich (St Louis, MO), NaCl from Mallinckrodt chemicals (Phillipsburg, NJ) and Q Sep dSPE (150mg $MgSO_4$ + 50mg PSA) QuEChERS tubes from Restek (Bellfonte, PA). A Milli-Q Plus purification system, (Millipore, Milford, MA) was used to obtain Ultra-pure water in the

laboratory. Column used was optimized in chapter 2 PerkinElmer Brownlee SPP 2.7µm; C18; 2.1 x 50 mm (Hopkins MA).

Glucocorticoid extracts and standards were analyzed using a Qsight 225MD UHPLC (Perkin Elmer, Shelton, CT) equipped with a sampling module, a solvent delivery module and a column stability module, coupled to a triple quadruple mass detector equipped with an electrospray ionization source. The system was operated by simplicity 3Q MD software (Perkin Elmer, Shelton, CT).

3.2.2 Sample Preparation

Table 3-1 shows an outline of the QuEChERS extracted sample and standard preparation. Two samples at a middle concentration 1 µg/mL (1000 ppb) and a high concentration 5 µg/mL (5000 ppb) were prepared to evaluate the analyte recoveries and accuracy. Six samples at 1 µg/mL (1000 ppb) were prepared for precision. Calibration curves were constructed for all analytes from 0.025 to 5 µg/mL (25 ppb to 5000 ppb) at above mentioned points same as PIE.

All eight glucocorticoids' standards were prepared in water at same concentration range as PIE from 0.025 to 5 µg/mL (25 ppb to 5000 ppb), standard concentration levels at 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 µg/mL by diluting from the stock standard and then subjected to QuEChERS. 2 mL of water with standard was added to 15-mL, high-density polyethylene conical tubes with screw caps containing 500 mg of $MgSO_4$ and 500 mg of NaCl then 2 mL of

Standard and Sample Preparation:	
Concentration Range	5000ppb to 25ppb
STD	Extracted
Sample	Recovery and Accuracy: Spiked at 1000ppb and 5000ppb (n=2)
	Precision: Spiked at 1000ppb (n=6)
Extraction Procedure	500mg $MgSO_4$ + 500mg NaCl; 2ml Spiked water; 2ml acetonitrile.
	Vortex 5 min; centrifuge 5min at 3000 rpm
	After two phase separation volume of both phase recorded and upper organic layer recovered in to QuEChERS tube vortex for few minutes and filter into LC vial for analysis.

Table 3- 1 QuEChERS Preparation outline

acetonitrile were added and tube was subjected to vortex for 2-3 minutes and centrifuge for 5 minutes at 3000 RPM. After the liquid-liquid extraction two phases separated. The volume of each phase was recorded and the upper organic phase was recovered in a QuEChERS tube for d-SPE clean-up. The QuEChERS tube vortexed for 3 minutes and solution was recovered for analysis by UHPLC- MS/MS. The difference between PIE and QuEChERS is that, in PIE to the 1:1 aqueous and organic mixture 20% w/v glycerol is added, and the sample is vortexed following the mixing step. The sample is then equilibrated for 30 minutes at 0 °C.

3.2.3 Instrumental Parameters

Glucocorticoids were qualified and quantified using Perkin Elmer UHPLC Qsight 225MD. The UHPLC system was equipped with a sampling module, a solvent delivery module and a column stability module and was coupled to a triple quadruple mass detector equipped with an ESI source. The system was operated by simplicity 3Q MD software. Table 3-2 shows the instrumental parameter for both LC and MS as listed same for both PIE and QuEChERS. LC analysis was performed on a PerkinElmer Brownlee SPP 2.7µm; C_{18}; 2.1 x 50 mm column with 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. Instrumental conditions were kept same as PIE for all parameters. The mobile phase flow was kept at 0.4 mL/min. The gradient was started with 30% mobile phase B, hold for 0.5 min, then increase mobile phase B to 50% in 3 min, and increase to 80% in 3.5 min then hold for 1 min. The mobile phase was returned to the initial condition of 30% B at 5 min and equilibrated for 2 min. Column oven temperature was kept 35 °C and injection volume was 1 µL. MS-MS detection was performed in positive ionization mode with electrospray ionization voltage of 3000 V and source temperature 250 °C. HISD temperature was set at 150 °C, drying gas was set at

LC Conditions			
Instrument	PerkinElmer UHPLC coupled to triplequad Qsight 225MD		
LC Column	PerkinElmer Brownlee SPP 2.7µm; C18; 2.1 x 50 mm		
Data analysis	Simplicity 3Q MD		
Mobile Phase A	0.1% HCOOH in Water		
Mobile Phase B	0.1% HCOOH in Acetonitrile		
Mobile Phase Gradient	Start at 0.4 ml/min with 30% mobile phase B hold for 0.5 min then increase B to 50% in 3 min and increase again to 80% in 3.5 min then hold for 1 min. Return to the initial condition at 5 min and equilibrated for 2 min.		
Column Oven Temperature	35 °C		
Injection Volume	1 µL		
MS Source Conditions			
ESI Voltage (Positive)	3000 V	Source Temperature	250 °C
Drying gas	120	HSID Temperature	150 °C
Nebulizer gas	200	Detection Mode	MRM

Table 3- 2 Instrument parameters for glucocorticoids analysis

120 °C and nebulizer at 200 °C. Detection was performed in multiple reaction monitoring (MRM) mode. Direct flow injection of a standard solution of each compound was used to find the optimum conditions for each compound in the ESI source and most suitable average conditions were selected for the analysis. Cone voltage and collision energies were optimized for each compound to obtain two MRM transitions, one for quantification and other for the qualification of each analyte. Table 3-3 shows the MRM method condition and selected ion for 8 glucocorticoids. Figure 3- 4 shows the overlay of complete MRM of all selected ions. MRM of quantifier ions show spectroscopic separation of 8 glucocorticoids separation.

3.2.4 Experimental Parameters

3.2.4.1 Validation

Precision, recovery, accuracy, linearity was evaluated. Calibration curves were constructed for all analytes from 0.025 to 5 µg/mL (25 ppb to 5 000ppb) with standard concentration levels at 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 µg/mL. Correlation coefficients of r^2 analytes were obtained over these ranges. The LOD and LOQ were determined for each analyte adopting the IUPAC definition of limit of detection. The equation is described in the equation 3-1.

$$C_L = \frac{ks_B}{m} \qquad \text{Equation 3-1}$$

Where, k is 3 for LOD and 10 for LOQ.

3.3 Results and discussion

Compound	MRM Transition		EV	CE	CCL2
	Q1 Mass	Q2 Mass			
Cortisone 21 acetate	402.80	162.90*	42	-35	-68
		342.80	36	-26	-72
Prednisolone	360.90	146.90*	25	-37	-68
		170.80	16	-39	-68
Beclomethasone	408.80	390.70*	19	-16	-60
		278.80	20	-27	-72
Dexamethasone	392.80	236.70*	22	-26	-68
		146.90	29	-42	-72
Fludrocortisone acetate	422.80	238.90*	48	-35	-76
		180.90	51	-40	-80
Hydrocortisone	362.80	120.90*	41	-34	-64
		326.80	34	-21	-60
Methylprednisolone	374.90	356.80*	16	-14	-56
		338.80	24	-15	-60
Prednisone	358.80	146.90	22	-45	-60
		340.70*	30	-16	-56

Table 3- 3 MRM method condition for 8 glucocorticoids

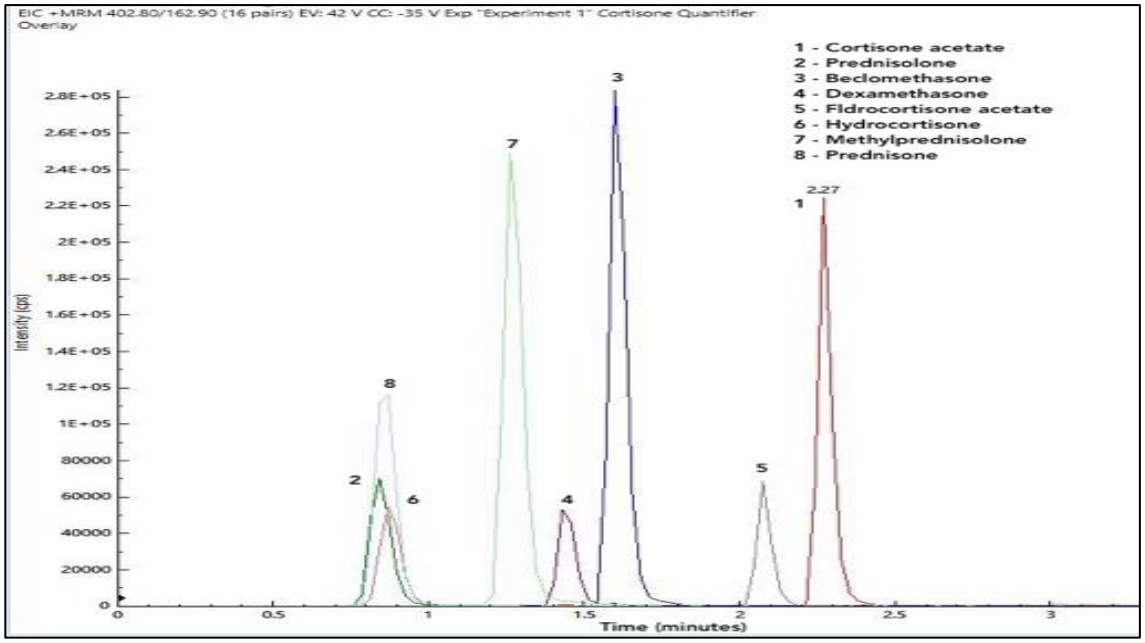


Figure 3- 4 MRM overlay of glucocorticoids standard quantifier ions by QuEChERS

3.3.1 Comparison of PIE and QuEChERS

3.3.1.1 Linearity and limit of detection (LOD)

Calibration curves were constructed for all analytes from 0.025 to 5 μg/mL (25ppb to 5000ppb) with standards prepared over this range Figure 3-5 to 3-12 show calibration curves of all 8 glucocorticoids standards. The equation of line was obtained for each glucocorticoid. Correlation coefficients of $r^2 > 0.99$ for all analytes were obtained over these ranges. Table 3-4 shows the comparison of linearity, LOD and signal to noise for QuEChERS and PIE. Results from both studies are comparable for each parameter. The LOD values obtained by the IUPAC method ranged from 0.3 ng/mL to 4 ng/mL for the PIE method and 1 ng/mL to 5 ng/mL for the QuEChERS method. Signal to noise for both QuEChERS and PIE were obtained from the simplicity software by selecting a background signal against peak in MRM of quantifier ions of 25ppb standard for each glucocorticoid. Tabulated data from simplicity software form selected background also gave the background standard deviation for the selected range that was used for the LOD calculations using IUPAC equations. Both methods gave comparable data for signal to noise.

3.3.1.2 Recovery, accuracy, and precision

All data for the determination of the percent recovery and accuracy used the equation of the line from the calibration curve to find concentration from the peak area of each. Once determined, these values were divided by the appropriate concentration and multiplied by 100 to get percent recovery. An average of all four was reported as percent recovery of each. Accuracy was determined from the percent recovery of an average value. Six separate samples were prepared

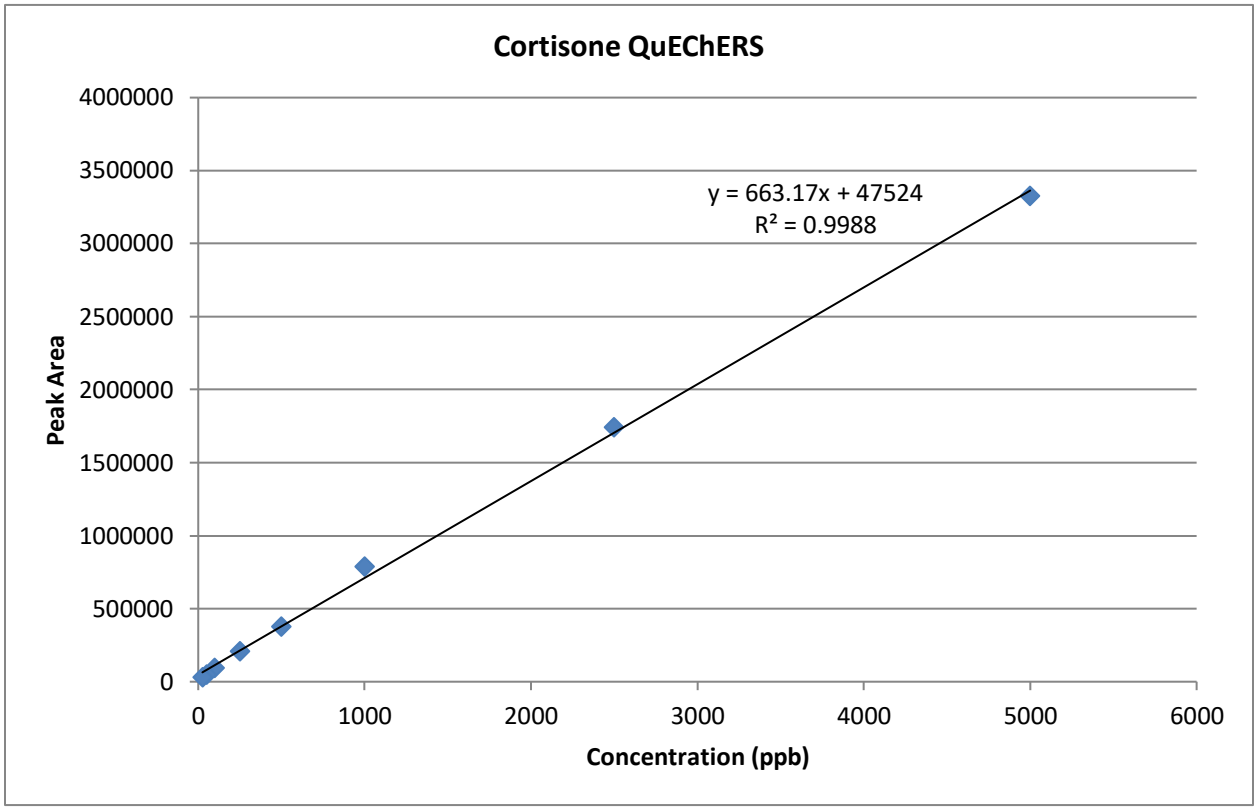


Figure 3- 5 Calibration curve for cortisone acetate (QuEChERS)

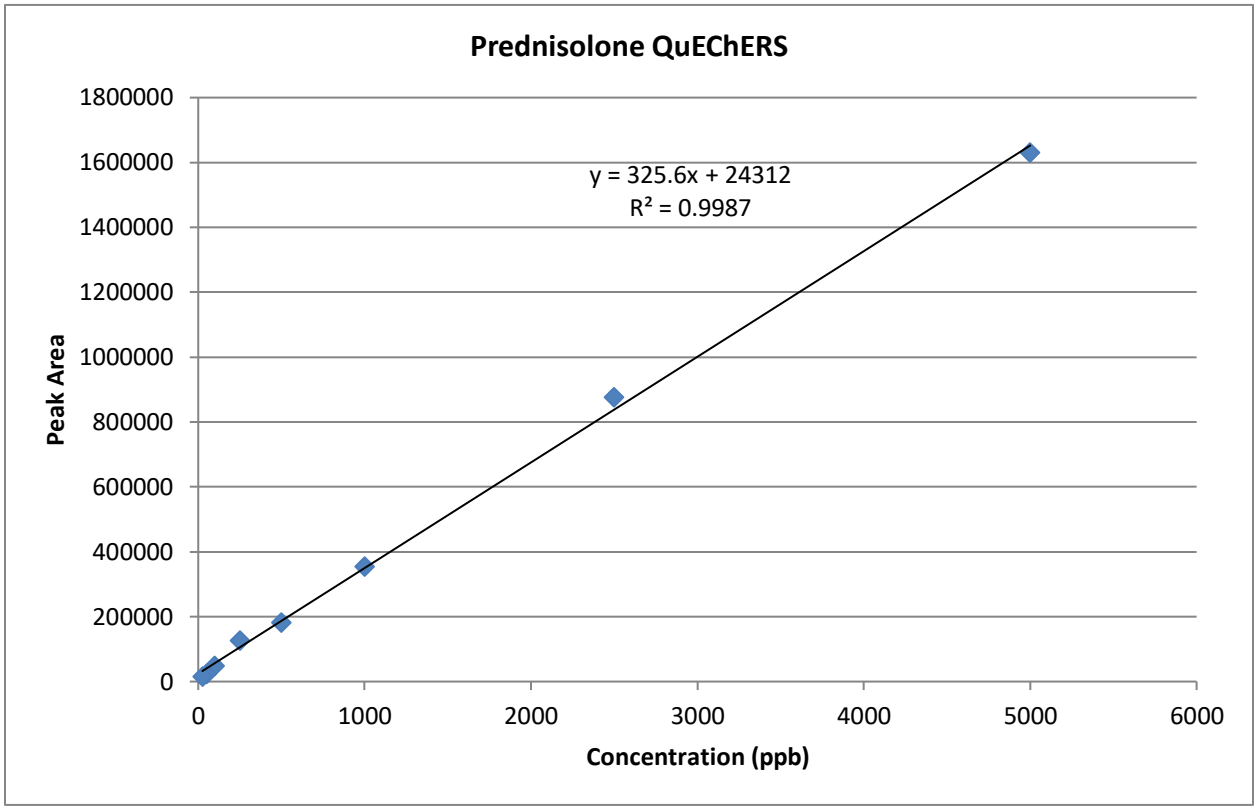


Figure 3- 6 Calibration curve for prednisolone (QuEChERS)

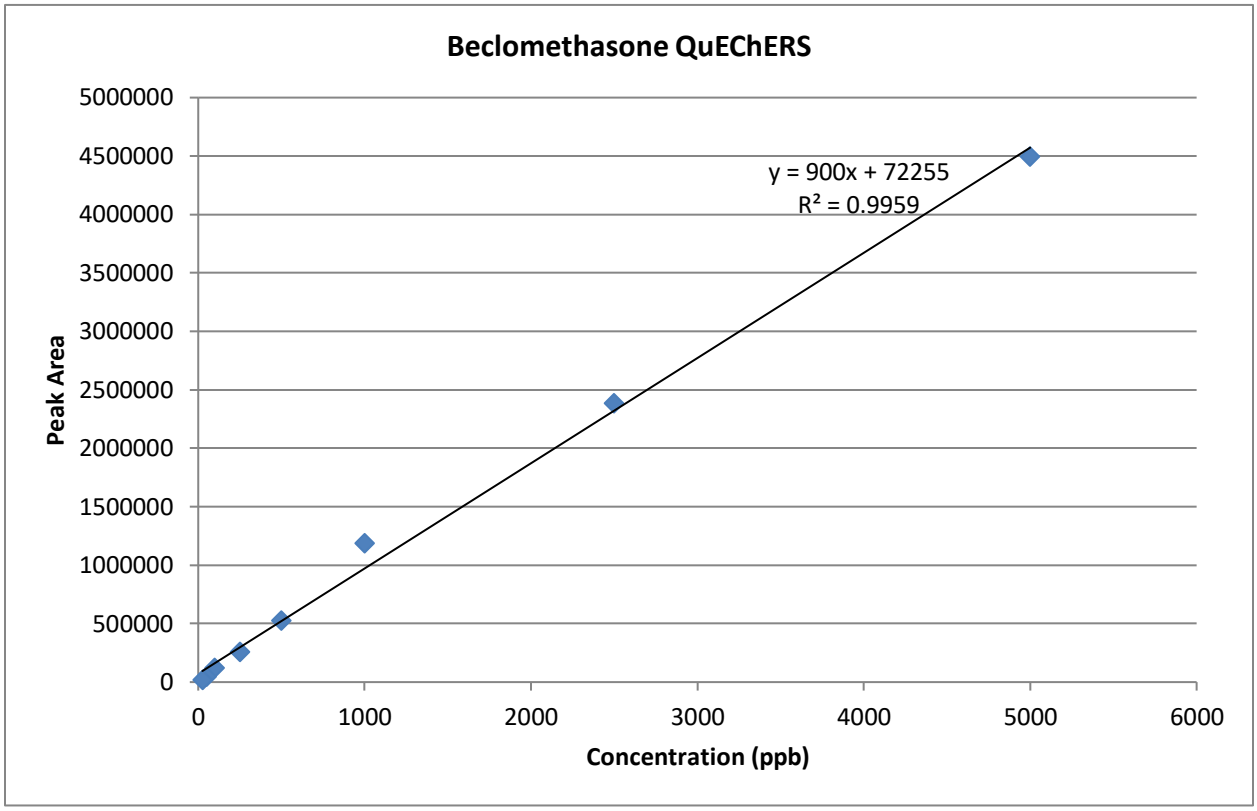


Figure 3- 7 Calibration curve for Beclomethasone (QuEChERS)

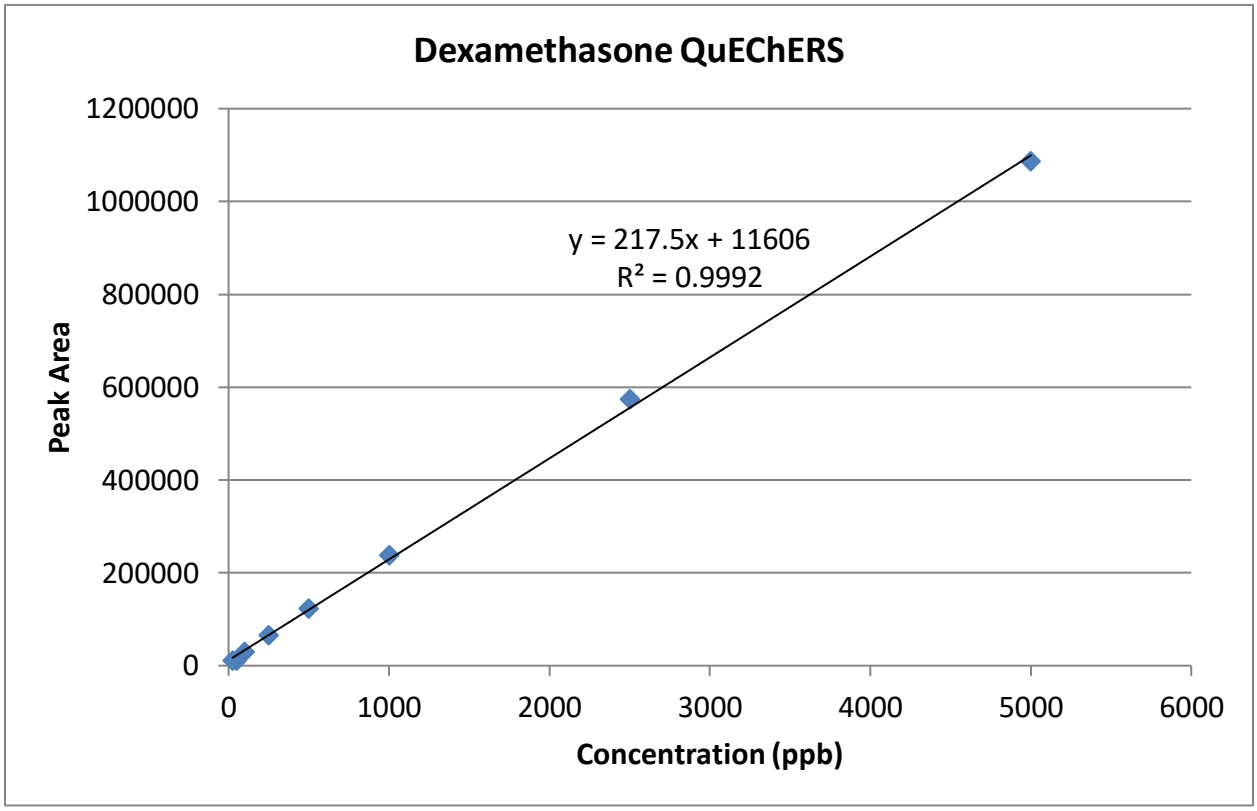


Figure 3- 8 Calibration curve for dexamethasone (QuEChERS)

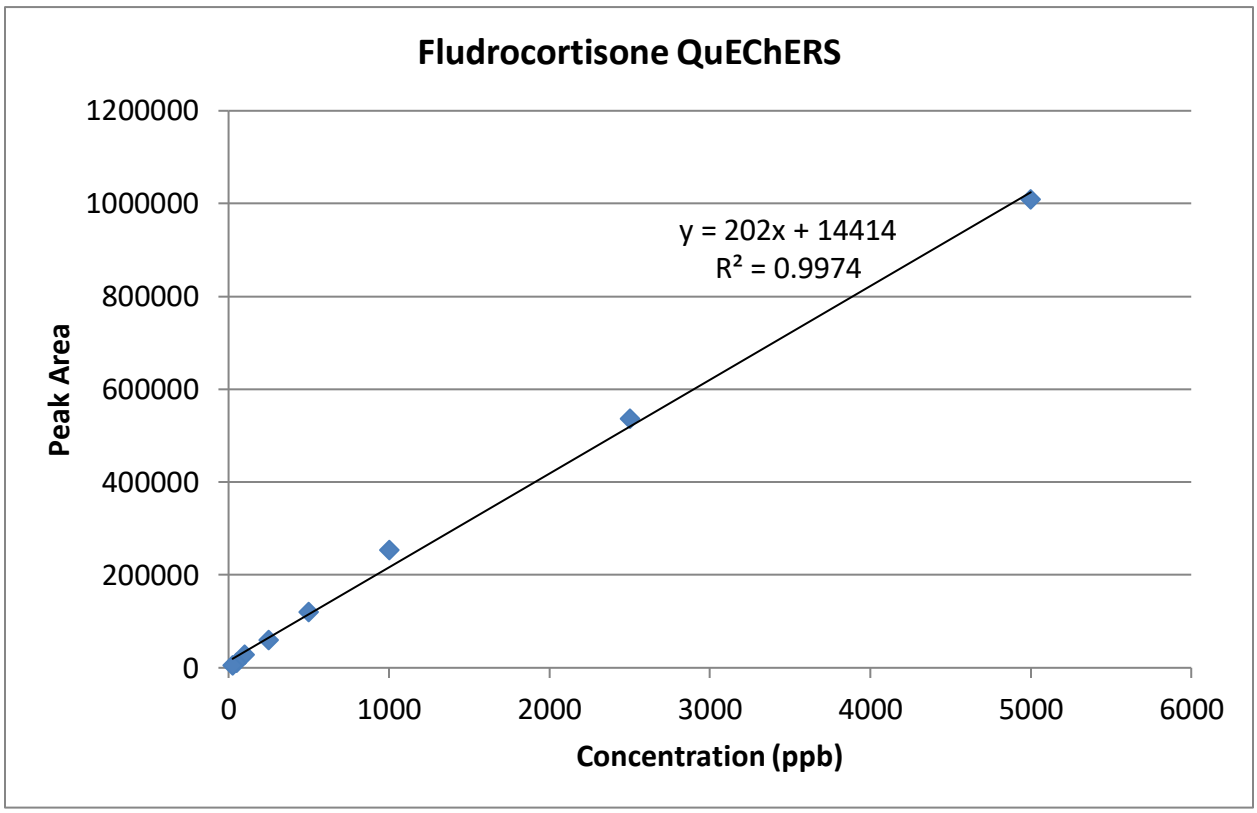


Figure 3- 9 Calibration curve for fludrocortisone (QuEChERS)

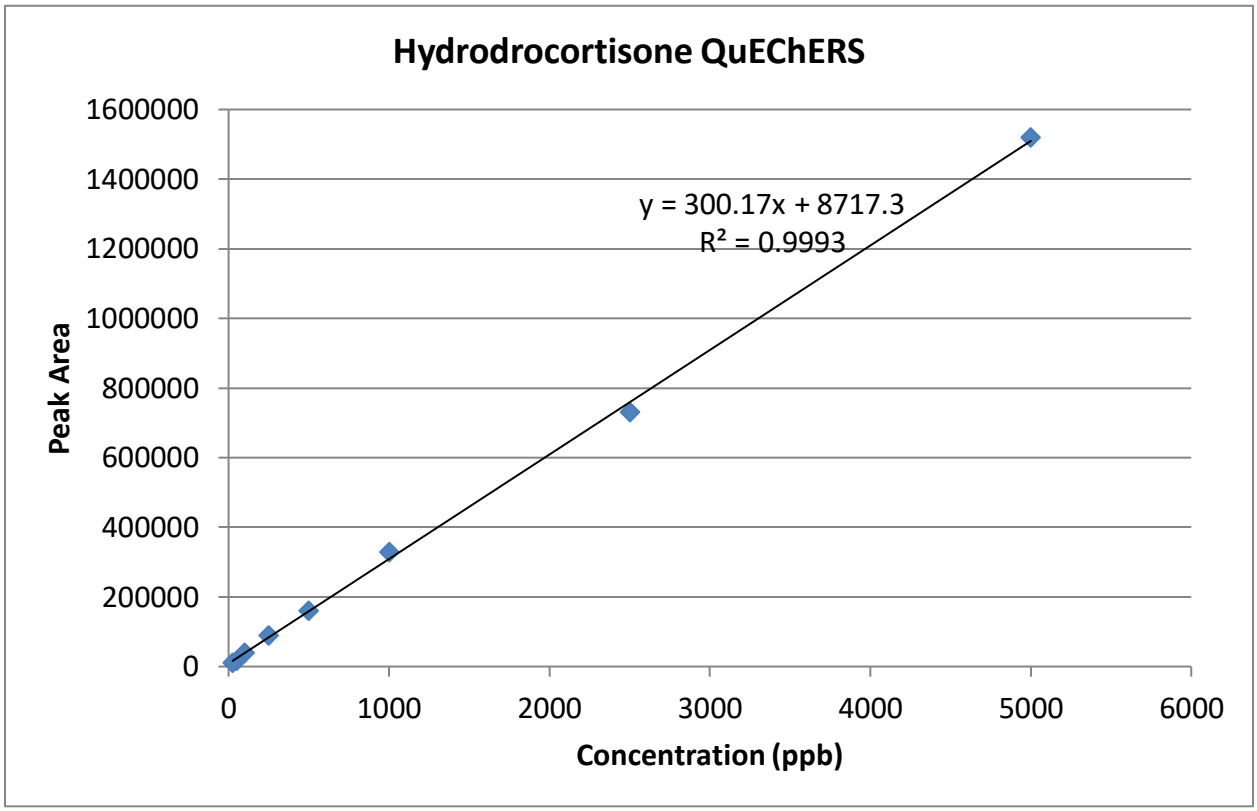


Figure 3- 10 Calibration curve for hydrocortisone (QuEChERS)

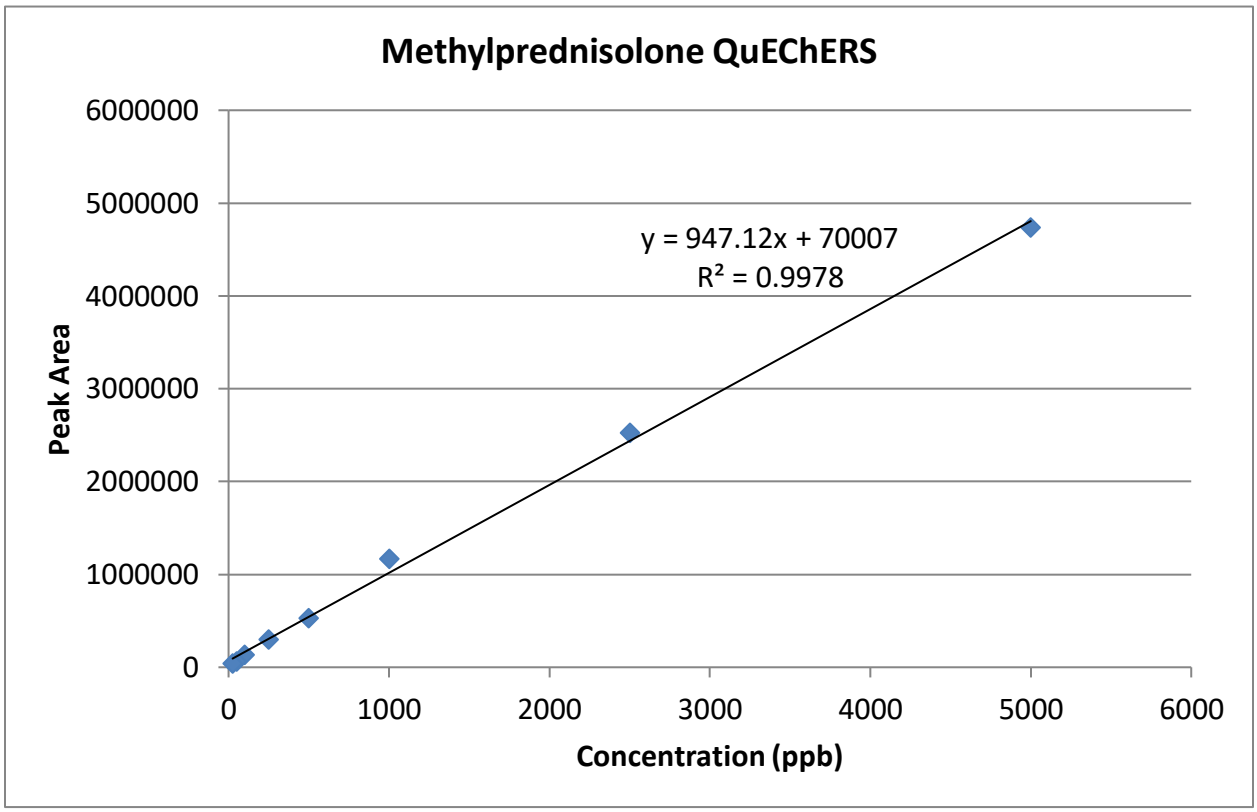


Figure 3- 11 Calibration curve for methylprednisolone (QuEChERS)

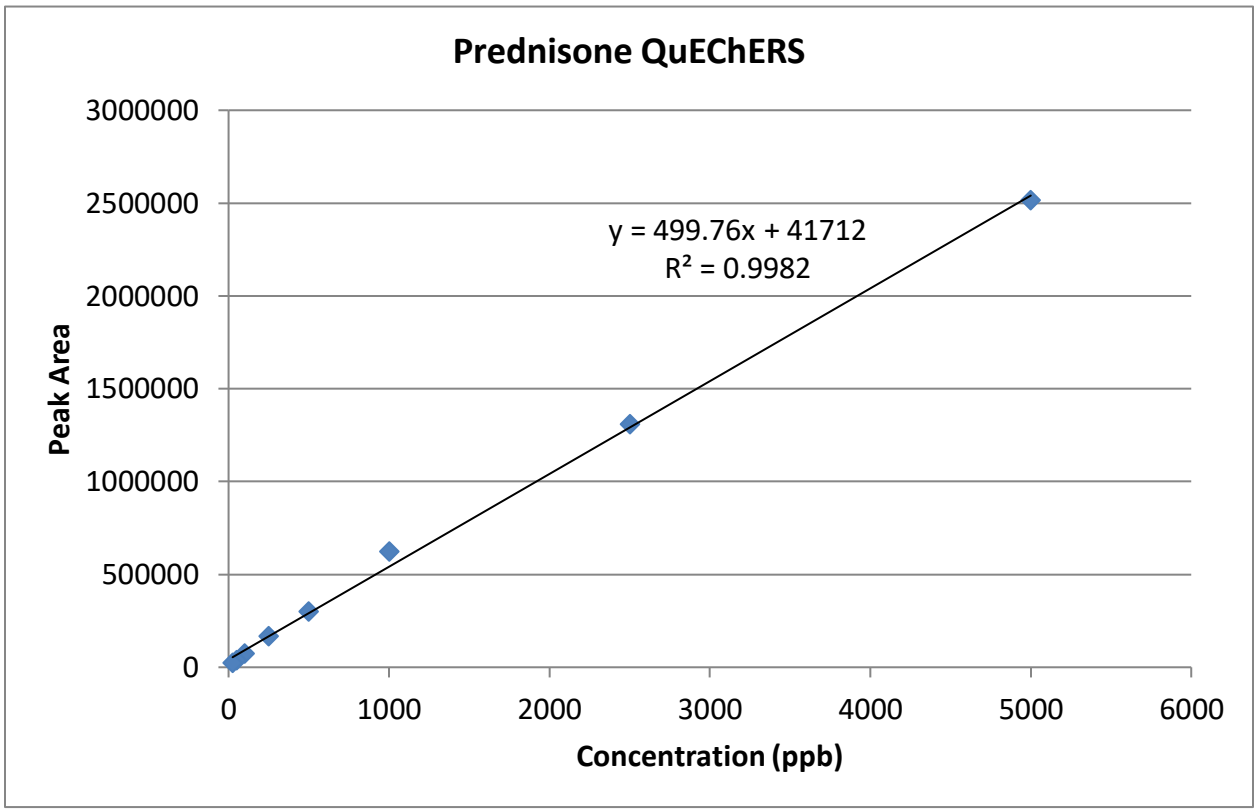


Figure 3- 12 Calibration curve for prednisone (QuEChERS)

Steroid (Calibration range 25 to 5000ppb)	PIE			QuEChERS		
	R^2	LOD (ppb)	Signal to noise	R^2	LOD (ppb)	Signal to noise
Cortisone Acetate	0.999	0.3	619	0.999	1ppb	260
Prednisolone	0.999	1	44	0.999	3ppb	60
Beclomethasone	0.994	2	19	0.996	2ppb	24
Dexamethasone	0.999	2	40	0.999	4ppb	16
Fludrocortisone acetate	0.998	1	44	0.997	5ppb	9
Hydrocortisone	0.999	2	29	0.999	3ppb	31
Methylprednisolone	0.998	2	38	0.998	2ppb	16
Prednisone	0.999	4	24	0.998	5ppb	17

Table 3- 4 Linearity, LOD and signal to noise comparison for PIE and QuEChERS

as mentioned above at 1000ppb for precision and analysed for the precision. Precision was calculated as %RSD of all six preparations. Equations needed to calculate necessary parameters are described below in equation 3-2, 3-3 and 3-4.

$$\%\text{Recovery} = \left(\frac{Experimentally\ determined\ concentration}{Theoretical\ concentration}\right) X\ 100 \qquad \text{Equation 3-2}$$

$$\text{Accuracy: \%error} = \left(\frac{|Theoretical\ concentration - experimental\ concentration|}{Theoretical\ concentration}\right) X\ 100 \qquad \text{Equation 3-3}$$

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

Recovery and accuracy data are summarized in Table 3-5 for both PIE and QuEChERS. Precision data for both methods are tabulated in Table 3-6. Recoveries for the analytes using QuEChERS were obtained between 103.8 to 126.3% for 1 μg/mL (1000 ppb) sample and 96.8 to 100.0% for 5 μg/mL (5000 ppb) sample. Accuracies for QuEChERS samples were calculated between 3.8 to 26.3% and 0.01 to 3.8% for 1 μg/mL and 5 μg/mL respectively. Recoveries for both methods are comparable also both methods have acceptable recovery and accuracy data. Precision as %RSD of six preparations were below 4.0% and 2.5% respectively for QuEChERS and PIE method. Obtained precision data show excellent reproducibility. Standard peak areas from QuEChERS and PIE were compared by plotting a bar graph for each analyte to compare area count with concentration as shown in Figures 3-13 to 3-20. Some components showed higher peak areas in PIE than in QuEChERS such as cortisone acetate and fludrocortisone acetate in Figure 3-13 and 3-17. Beclomethasone and dexamethasone, as shown in Figures 3-15 and 3-16 show higher peak areas in PIE but at a lower ratio than cortisone and fludrocortisone.

Steroid (Calibration range)	PIE		QuEChERS	
	Recovery (%; n=4)	Accuracy (% Error; n=4)	Recovery (%; n=4)	Accuracy (% Error; n=4)
Cortisone Acetate (25 to 5000ppb)	1000ppb: 110.67 5000ppb: 99.33	1ppm: 10.67 5ppm: 0.67	1000ppb: 115.85 5000ppb: 96.75	1000ppb: 15.85 5000ppb: 3.25
Prednisolone (25 to 5000ppb)	1000ppb: 109.30 5000ppb: 100.03	1000ppb: 9.30 5000ppb: 0.025	1000ppb: 103.77 5000ppb: 98.11	1000ppb: 3.77 5000ppb: 1.89
Beclomethasone (25 to 5000ppb)	1000ppb: 132.40 5000ppb: 99.67	1000ppb: 32.40 5000ppb: 0.33	1000ppb: 126.31 5000ppb: 98.72	1000ppb: 26.31 5000ppb: 1.23
Dexamethasone (25 to 5000ppb)	1000ppb: 111.45 5000ppb: 100.00	1000ppb: 11.45 5000ppb: 0.00	1000ppb: 105.74 5000ppb: 100.01	1000ppb: 5.74 5000ppb: 0.01
Fludrocortisone (25 to 5000ppb)	1000ppb: 122.55 5000ppb: 99.81	1000ppb: 22.55 5000ppb: 0.19	1000ppb: 123.34 5000ppb: 97.25	1000ppb: 23.34 5000ppb: 2.75
Hydrocortisone (25 to 5000ppb)	1000ppb: 111.51 5000ppb: 101.39	1000ppb: 11.51 5000ppb: 1.39	1000ppb: 103.81 5000ppb: 99.78	1000ppb: 3.81 5000ppb: 0.22
Methylprednisolone (25 to 5000ppb)	1000ppb: 125.56 5000ppb: 100.95	1000ppb: 25.56 5000ppb: 0.95	1000ppb: 118.83 5000ppb: 98.77	1000ppb: 18.83 5000ppb: 1.23
Prednisone (25 to 5000ppb)	1000ppb: 123.95 5000ppb: 100.6	1000ppb: 23.95 5000ppb: 0.6	1000ppb: 117.01 5000ppb: 98.06	1000ppb: 17.01 5000ppb: 1.94

Table 3- 5 Recovery, Accuracy comparison for PIE and QuEChERS

Steroid (Calibration range 25 to 5000ppb)	Precision (%RSD; n=6)	
	PIE	QuEChERS
Cortisone Acetate	1.93	2.34
Prednisolone	1.85	2.93
Beclomethasone	1.95	3.30
Dexamethasone	2.29	2.17
Fludrocortisone acetate	1.93	3.30
Hydrocortisone	2.05	3.48
Methylprednisolone	1.84	2.04
Prednisone	1.56	3.88

Table 3- 6 Precision comparison for PIE and QuEChERS

For prednisolone, hydrocortisone, and methylprednisolone in Figures 3-14, 3-18 and 3-19, the QuEChERS peak areas are higher than for PIE, but differences are small in comparison with cortisone and fludrocortisone. Prednisone in Figure 3-20 show areas very close to each other with PIE being slightly greater. Although it is unclear why there was a notable difference to no difference in peak areas for glucocorticoids between QuEChERS and PIE, these variations follow pattern in structurally similar compounds. Cortisone acetate and fludrocortisone acetate have an acetate group in common on C_{21}. Beclomethasone and dexamethasone are structurally similar except chlorine (Cl) and fluorine (F) on C_9 respectively. Hydrocortisone, prednisolone, prednisone, and methylprednisolone are similar in structure and for these analytes differences between peak areas for PIE and QuEChERS was smaller than for other compounds. These differences may have been possible due to phase separation ratio of both methods. The fact that in PIE after equilibration at 0 °C organic to aqueous phase ratio was 3 mL:8 mL (0.38) whereas for QuEChERS the phase ratio observed was 1.5 mL:2.5 mL (0.6). Therefore, depending upon the compound and its solubility some analytes may concentrate into organic phase. It should be noted that matrix interferences related to these similarities may have caused loss in the signal or spike in the signal in either method which may have led to the observed variation in the standards.

3.3.2 Comparison with various methods used for glucocorticoids analysis

Table 3-7 provides a comparison of this work with several published extraction methods for determination of glucocorticoids in water matrices. PIE provided comparable analytical qualities of analysis time, recovery, sensitivity and amount or type of organic solvent requirement. Most of the literature methods use the established methods of Solid Phase Extraction or QuEChERS.

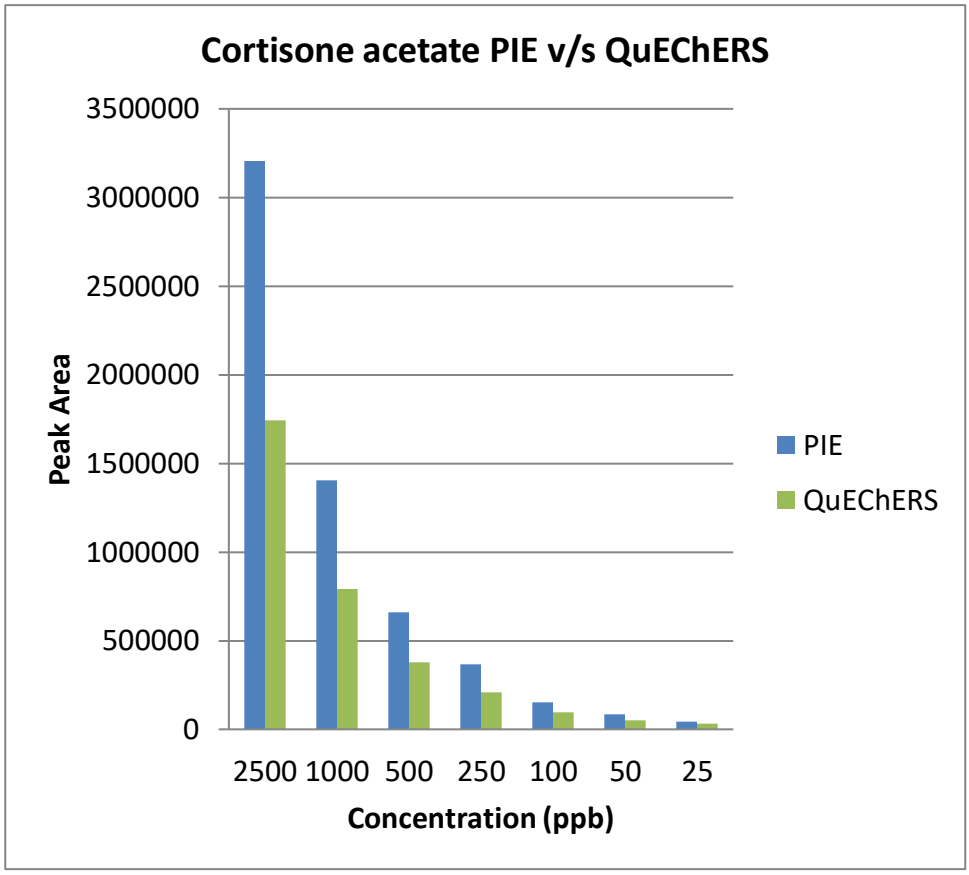


Figure 3- 13 PIE v/s QuEChERS: Cortisone

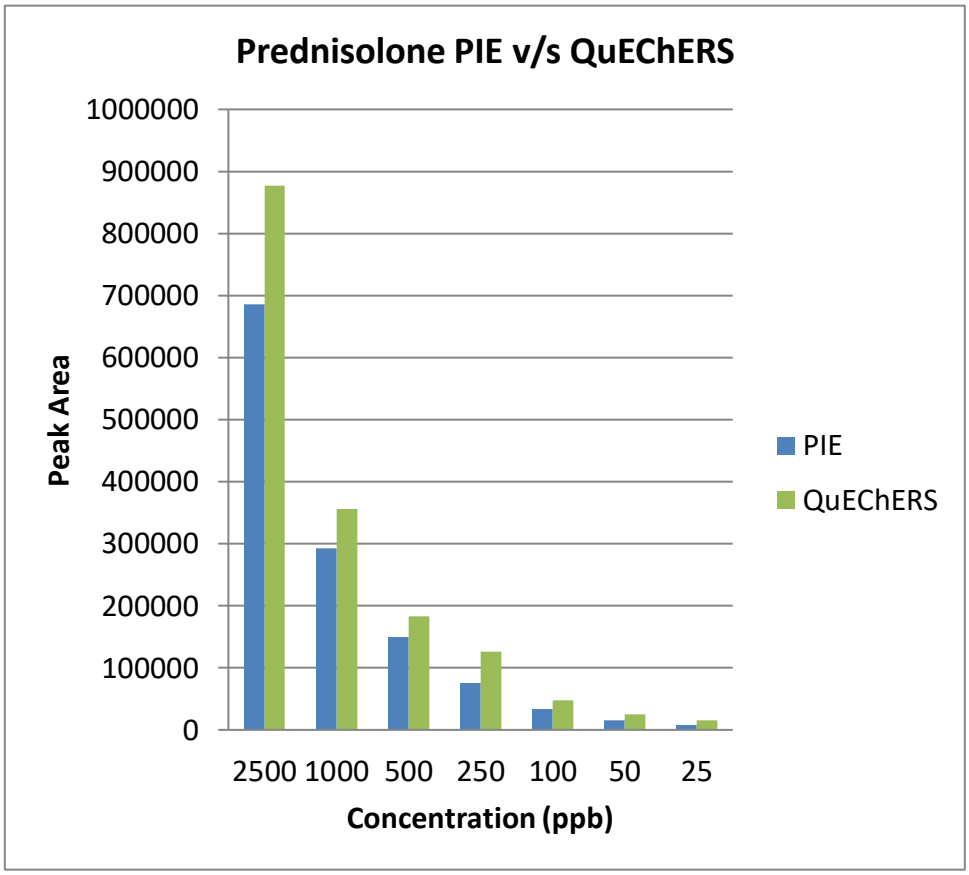


Figure 3- 14 PIE v/s QuEChERS: Prednisolone

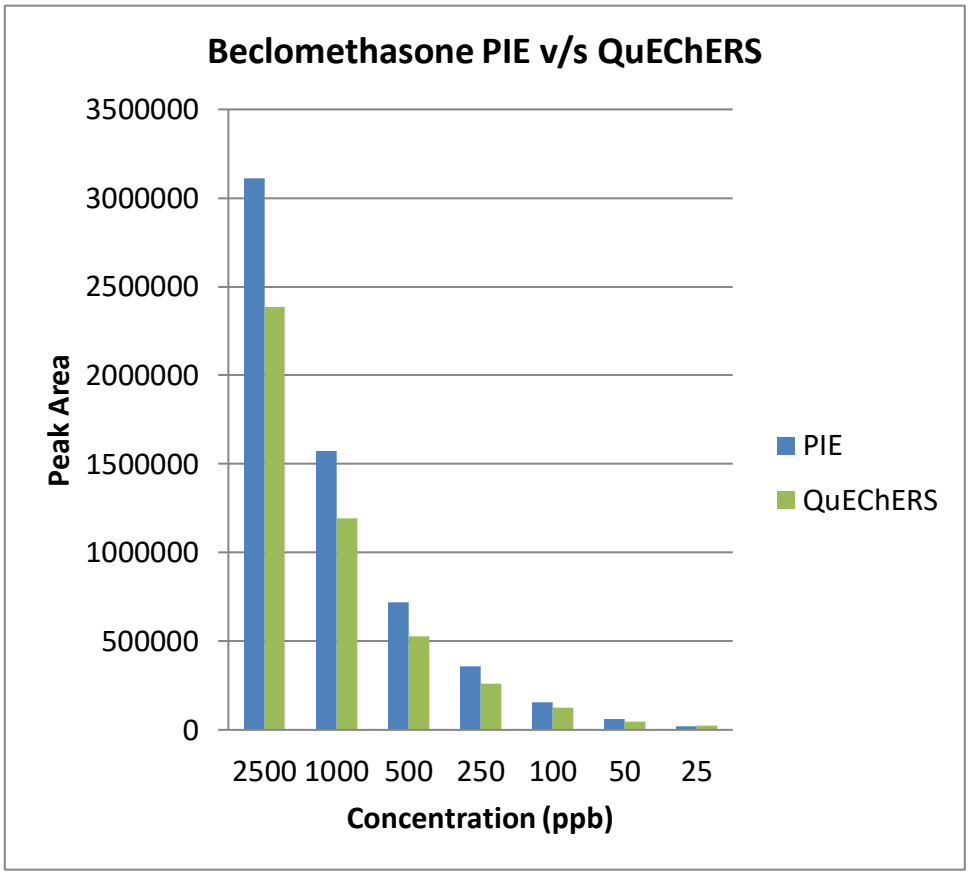


Figure 3- 15 PIE v/s QuEChERS: Beclomethasone

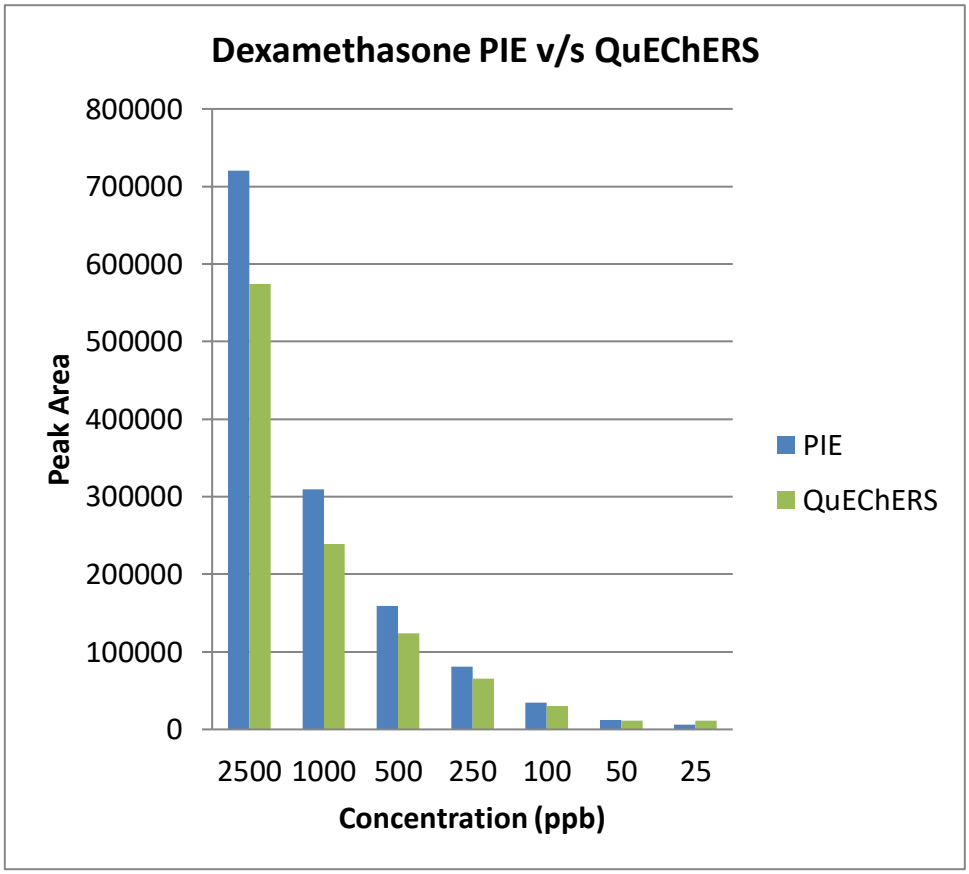


Figure 3- 16 PIE v/s QuEChERS: Dexamethasone

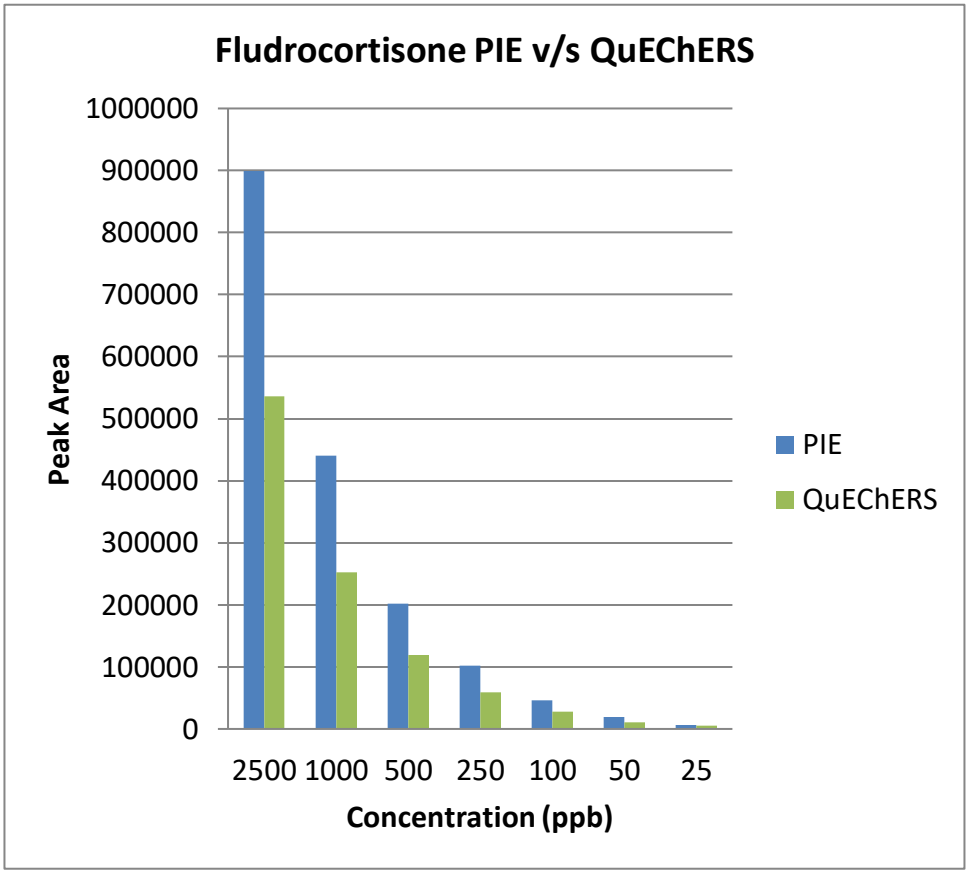


Figure 3- 17 PIE v/s QuEChERS: Fludrocortisone

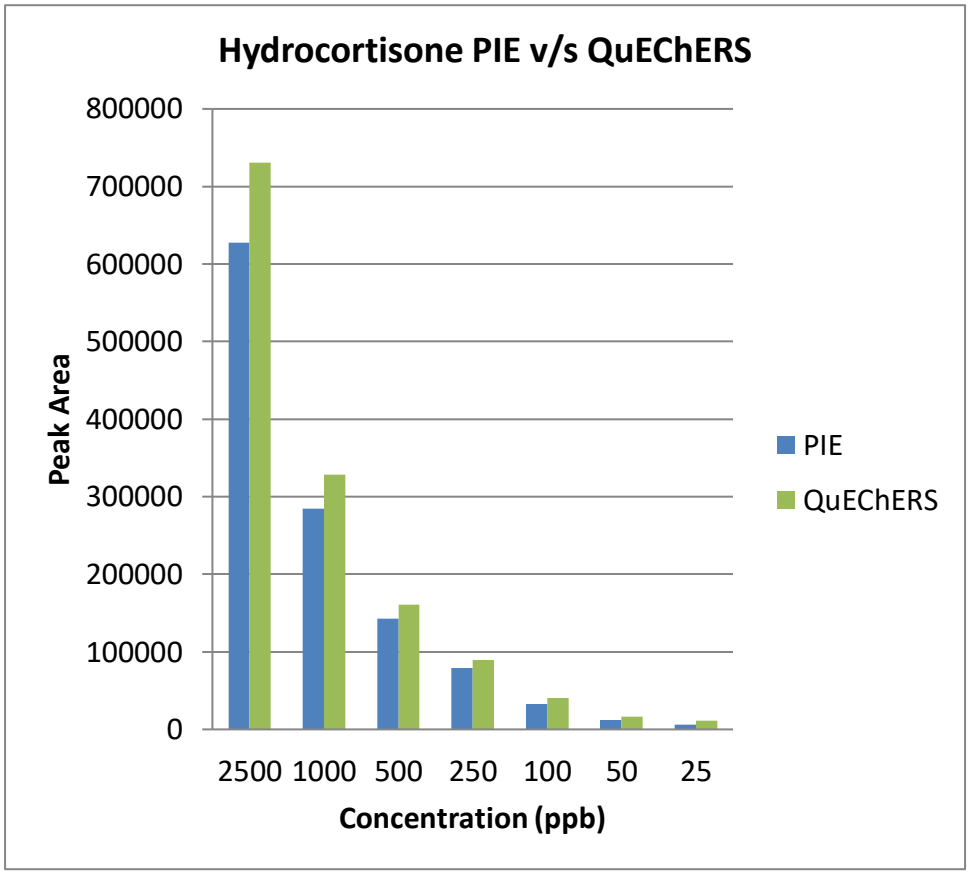


Figure 3- 18 PIE v/s QuEChERS: Hydrocortisone

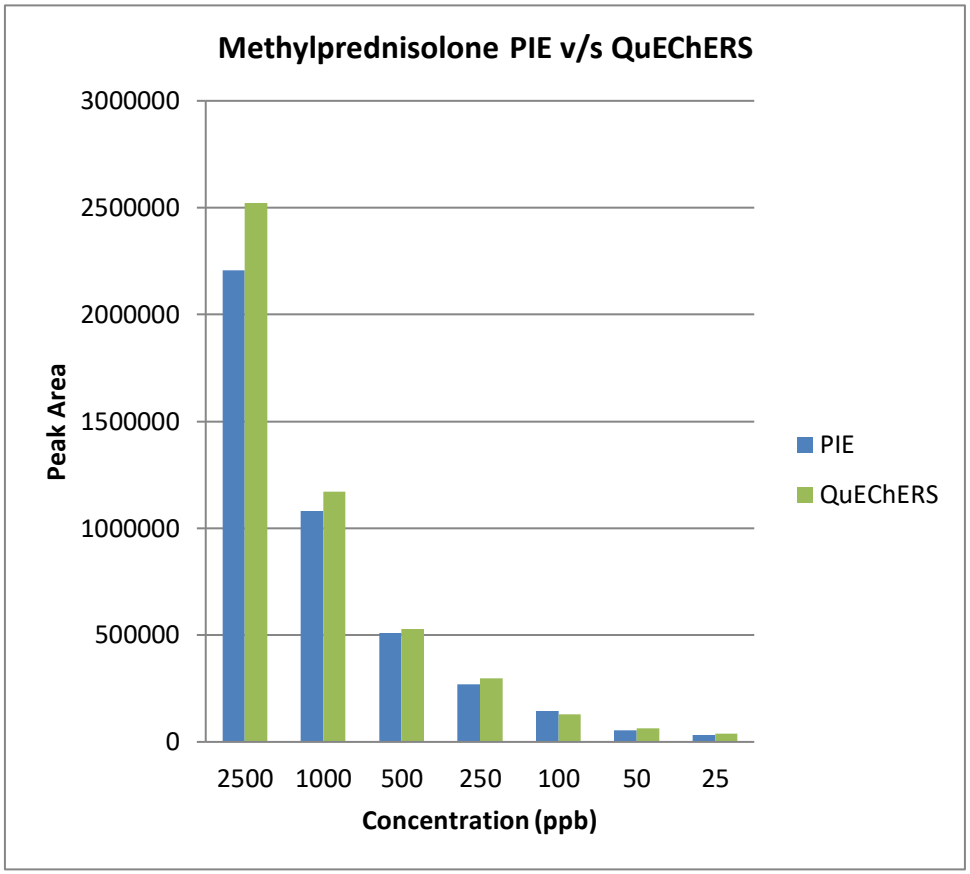


Figure 3- 19 PIE v/s QuEChERS: Methylprednisolone

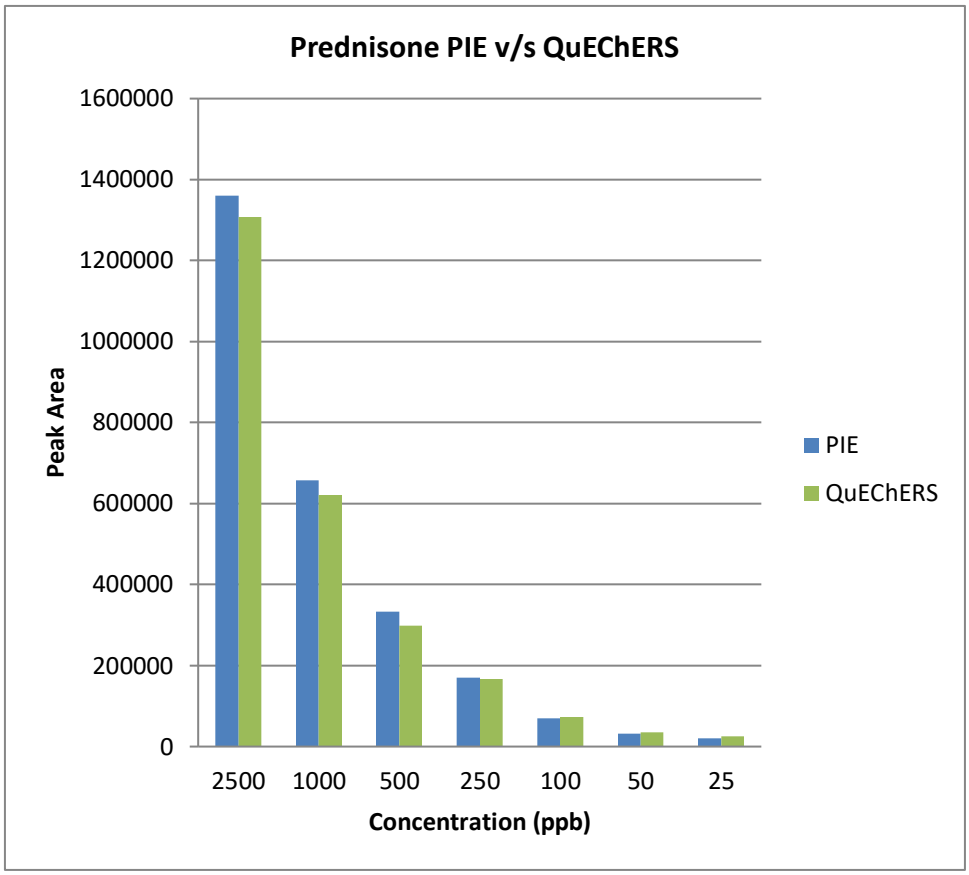


Figure 3- 20 PIE v/s QuEChERS: Prednisone

Extraction process [a]	Detection method	Solvent	Extraction time (min)	Recovery (%)	LOD (ppb)	ref
PIE	UHPLC-MS/MS	Acetonitrile	30	99-132	0.3-4	This work
SPE	RRLC-MS/MS	Methanol; Ethyl acetate	---	90.6-119.0	0.00001-0.00024	69
SPE	UHPLC-MS/MS	Methanol	>60	82-99	0.0005-0.02	58
SS-BVMME	HPLC-DAD	Butanol	<30	88-103	0.09925-2.428	91
LLE	LC-MS/MS	Ethyl acetate, DMSO	>24h	---	0.01-0.02	56
SDS-MBA-LPME	UHPLC-MS/MS	n-octanol	40	62.8-107.8	0.0331-0.166	92
IL-MBA-LPME	UHPLC-MS/MS	n-octanol	30	72.9-84.9	0.0128-0.0470	55
Modified QuECheRS	UHPLC-MS/MS	Ethyl acetate	---	70.3-117.1	0.01-0.98	93

[a] SS-BVMME: Supramolecular solvent based vortex-mixed microextraction; SDS-MBA-LPME: Surfactant(SDS) enhanced membrane bag assisted liquid phase microextraction; IL-MBA-LPME: Ionic liquid enhanced membrane bag assisted liquid phase microextraction.

Table 3- 7 Summary of methods used for the determination of glucocorticoids in water matrices

M. Schriks et al.[56] also reported a liquid-liquid extraction method using a very lengthy procedure using ethyl acetate and DMSO as an extractants. Microextraction procedures described in References 55 and 92 are also very quick, however the requirement of membrane assisted devices makes these methods complex and recoveries for some of the analytes are very low. PIE is a very simple and user-friendly procedure that is quicker and does not require toxic solvents or extraction reagents. Glycerol is a nontoxic, widely available, inexpensive, and biodegradable polyol. The PIE method shows the potential for user friendly routine analysis and opens the various possibilities of its application in analysis of different compounds.

3.3.3 Comparison of PIE standards with non-extracted standards

The extraction efficiency of the polyol induced extraction was explored by comparing the extracted standards directly to non-extracted standard prepared in acetonitrile. Standards were prepared by diluting stock standards into acetonitrile from the concentration range 0.025 μg/mL to 2.5 μg/mL for all analytes at 0.025, 0.05, 0.1, 0.25, 0.5, 1 and 2.5 μg/mL. Calibration curves were constructed for all compounds and correlation coefficients of $r^2 > 0.99$ were obtained for all analytes. Calibration curves for extracted and non-extracted standards were compared by plotting both on the same graph, comparison graphs are shown in Figure 3- 21. PIE sample prepared at 1 μg/mL (1000 ppb) was used to evaluate recovery against non-extracted standards. Obtained results are summarized in Table 6. Comparison of calibration curves on the same plot showed interesting data. For fludrocortisone acetate and cortisone acetate the extracted standard curve was plotting higher than non-extracted standards. For beclomethasone and dexamethasone, the extracted standards were approximately 1.2 times higher than non-extracted standards, so they are plotted very close to each other. For fludrocortisone acetate and cortisone acetate the

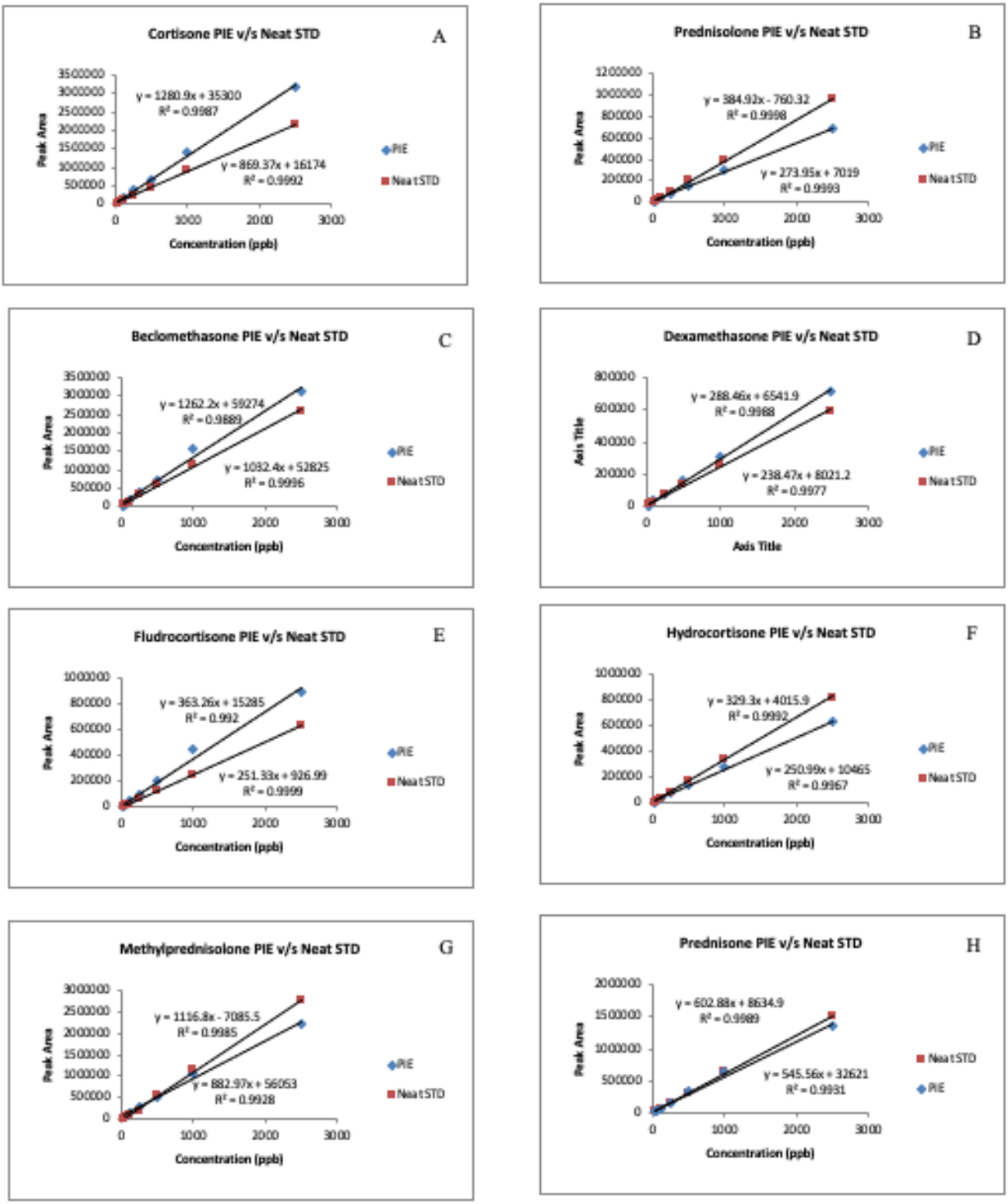


Figure 3- 21 Calibration curves plots on same graph for PIE and non-extracted standards (A) Cortisone acetate, (B) Prednisolone, (C) Beclomethasone, (D) Dexamethasone, (E) Fludrocortisone acetate, (F) Hydrocortisone, (G) Methylprednisolone, (H) Prednisone

extracted standards were approximately 1.7 and 1.5 times higher than non-extracted standards respectively making the curves far apart from each other.

Two scenarios can be concluded from this type of data. The first is error in sampling but having reproducible and repeatable results eliminate that option. The other is that some compounds are more soluble in acetonitrile and therefore getting concentrated into the organic layer during phase separation. Literature on partition coefficient and solubility of these compounds

showed a pattern which related the structure or functional group to the partitioning of these compounds. Data found in the literature about solubility in water and ether: water partition coefficient for these compounds and the obtained PIE results followed a similar pattern. Literature data and obtained data are tabulated in Table 3-8.[94-98] As shown in Table 3-8 it is important to note that compounds with low water solubility have a higher partition coefficient in organic phases and compounds with high water solubility have lower partition coefficients in organic phases, which is in alignment with the results obtained with PIE. Cortisone acetate and fludrocortisone acetate have an acetate group on 21 position which reduces the hydrogen bonding in the basic cortisol structure resulting in a higher partition coefficient. For steroids, a higher number of hydrogen bonds results in lower partition coefficient in the organic phase and reduction of hydrogen bonds leads to an increase in the partition coefficient. An aliphatic chain on a molecule also affects the partitioning of these compounds. Compounds with a straight chain have a higher partition coefficient and compounds with branched chain have a lower partition coefficient.[94] From all this available information it is expected that the compounds showing a higher calibration curve response than non-extracted standard might have been concentrated into the separated organic phase depending upon their solubility in acetonitrile.

Steroid	R^2 (Calibration range 25 to 2500ppb)	% Recovery against non-extracted standards (PIE experimental data)	Ether: water partition co-efficient[94]	Water solubility (mg/L)[98]
Prednisolone	1.000	77.29	0.053	223
Hydrocortisone	0.999	88.95	0.204	320
Methylprednisolone	0.999	103.99	0.537	120
Dexamethasone	0.998	131.14	0.588	89
Prednisone	0.999	113.38	0.600	115
Beclomethasone	0.999	151.68	—	—
Cortisone acetate	0.999	166.46	1.40	20
Fludrocortisone acetate	0.999	180.76	1.67	—

Table 3- 8 Summary of glucocorticoids against non-extracted standards, Ether: Water partition co-efficient and water solubility for glucocorticoids found in literature

3.4 Conclusions

In this study, PIE was compared to the widely known extraction technique QuEChERS as well as some published methods in the literature. From the results obtained, it is known that PIE is a comparable technique to QuEChERS, and other methods published in the literature. PIE can extract each glucocorticoid from the water into the organic phase in a very good amount. Mostly, in an extraction procedure, the organic phase is the phase of interest. Further investigations can be done by analyzing the bottom aqueous layer from both methods to study the actual partitioning of these analytes. PIE can be a comparable alternative extraction method for organic compounds.

CHAPTER 4- ANALYSIS OF NSAIDs IN WATER USING POLYOL INDUCED EXTRACTION WITH ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY AND TRIPLE QUADRUPOLE MASS SPECTROMETRY (PIE-UHPLC-MS-MS)

Residual analysis of non-steroidal anti-inflammatory (NSAIDs) drugs has gained interest for research for several years now. NSAIDs are widely manufactured and consumed pharmaceuticals all over the world due to their therapeutical actions. The presence of NSAIDs in the environment due to various factors such as losses during manufacturing process, improper disposal, and human and animal excretion makes them an emerging pollutant. Because of wide usage, highly polar nature and adverse effects associated with these drugs, monitoring them in the environment and the human body is reasonable. LC-MS is prominently used for the analysis of NSAIDs. For the sample treatment, liquid-liquid extraction and solid-phase extraction are used very commonly, but these methods are time consuming, often require large amount of solvents and may have recovery problems. In this work, polyol induced extraction (PIE) coupled to UHPLC-MS/MS were used to study NSAIDs, using both chromatographic and detection selectivity. PIE can also be applied to determine NSAIDs in complex matrices. Spiked urine control samples were analyzed using PIE.

4.1 Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are pharmaceuticals that have anti-inflammatory, analgesic, and antipyretic therapeutic actions.[97] Easily available over the counter (OTC) NSAIDs include the common medications ibuprofen, naproxen, and ketoprofen. NSAIDs are used to control pain and can be misused for suicidal overdose due to easy availability. Some

of the NSAIDs are characterized by endocrine disruption properties.[98] These widely used and manufactured pharmaceutical compounds are becoming a new environmental problem through excretion of drugs and their metabolites via human waste, improper disposal, and pharmaceutical manufacturing discharge.[99] Moreover NSAIDs are widely used in veterinary medicine for antibiotic therapy and commonly used for food producing animals.[101] Concentrations of these drugs in environmental matrices or water systems are very low, but continuous exposure to these substances may cause side effects such as ulcers in stomach, increased risk of heart attacks, intestinal and renal bleeding which can be harmful to human health.[97]

Table 4-1 shows the structures of eight NSAIDs used in this study which are, ibuprofen, naproxen, ketoprofen, tolfenamic acid, mefenamic acid, indomethacin, aceclofenac, and oxaprozin. NSAIDs analytes have a variety of functionalities such as aromatic rings with acidic, ketone, nitrogen, and chlorine groups.

Various analytical techniques have been reported for the determination of NSAIDs, NSAIDs metabolites and their degradation products, including visible spectroscopy, spectrofluorimetry, NMR, gas chromatography, liquid chromatography (LC), LC-MS, gas chromatography – mass spectrometry (MS), supercritical fluid chromatography – MS, and LC-MS/MS.[97] LC-MS/MS is the technique that satisfies sensitivity and selectivity requirements and is the most prominently utilized technique for analysis of these type of analytes.

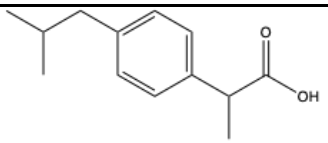
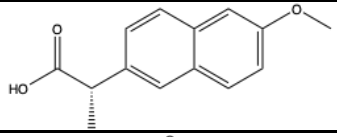
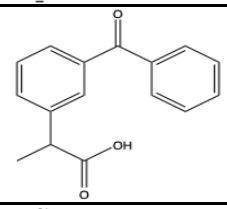
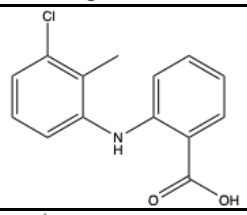
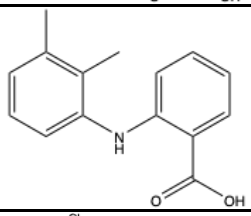
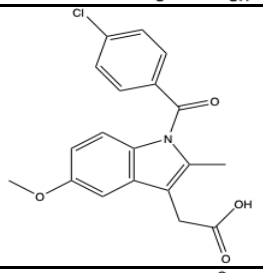
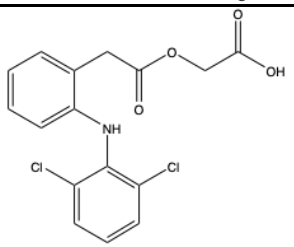
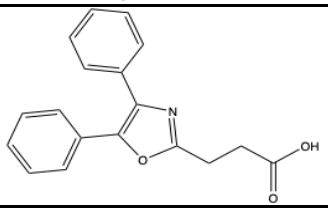
Name	Synonym	Structure
Ibuprofen	Benzeneacetic acid, α-methyl-4-(2-methylpropyl)-	
Naproxen	2-Naphthaleneacetic acid, 6-methoxy-α-methyl	
Ketoprofen	Benzeneacetic acid, 3-benzoyl-α-methyl-	
Tolfenamic acid	Benzoic acid, 2-[(3-chloro-2-methylphenyl)amino]-	
Mefenamic acid	Benzoic acid, 2-[(2,3- dimethylphenyl)amino]-	
Indomethacin	1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indole-3-acetic acid	
Aceclofenac	2-[2-[2-[(2,6-dichlorophenyl)amino]phenyl]acetyl]oxy acetic acid	
Oxaprozin	3-(4,5-Diphenyloxazol-2-yl)propanoic acid	

Table 4- 1 NSAIDs used in this study

Liquid-liquid extraction, solid phase extraction (SPE), online-SPE, solid phase micro-extraction has been employed for the analysis of NSAIDs from water.[97-100] These methods for sample treatment are very advantageous in removing impurities and sensitive for trace level analysis.[97] However, they can be time consuming, not readily available in some areas they use large amounts of organic solvents.

As discussed, in this dissertation PIE requires relatively little solvent, and can be an alternative cost-effective option for extraction. PIE is a liquid-liquid extraction which generates the phase separation by addition of glycerol to separate organic from aqueous liquid in a miscible aqueous/organic solvent mixture. Addition of a polyol induces a phase separation of an organic phase containing analytes and an aqueous phase containing water, polyol and matrix compounds. Prior to this work PIE has never been explored as technique of extraction for drug compounds. This phase partitioning through PIE can be an effective way of extraction for drugs and can be performed without having analytes compromised. In this study, NSAIDs are extracted from water using PIE and separated using UHPLC-MS/MS. UHPLC-MS/MS provides an approach to multidimensional separation. Upon analysis, this method can also be applied to determine the NSAIDs in complex matrices such as biological samples, waste sediments as well as NSAIDs concentration in drug formulations, with easy sample preparation.

In this study, in UHPLC-MS/MS, the liquid chromatograph is connected to a triple quadrupole mass spectrometer. Triple quadrupole used in this study was a dual source mass detector containing both ESI and APCI with two separate probes. The ESI probe in positive ionization mode was taken as the source to investigate NSAIDs extracted using PIE. ESI is a soft ionization technique which produces large molecular protonated parent ions $[M + H]^+$. By changing the

voltage lower or higher, lower, or higher degrees of fragmentation is achieved, and product ion or daughter ions are produced. MS/MS involves multiple reaction monitoring that separates ions in two stages, which makes the system more selective and sensitive at low level detection. The selection of a specific mass to charge (m/z) ion (precursor ion) occurs in the first mass analyzer (Q1) followed by collision induced dissociation in the collision cell (Q2) and then fragment ions are sorted according to their mass to charge ratio in second mass analyzer (Q3) and recorded by the detector.

Chromatographic separation is very critical in many analytical methods. However, tandem mass spectrometry is a forgiving detection technique where baseline separation of the analyte peaks may not be that necessary due to MRM. Proper selection of column and gradient help in achieving faster analysis, better peak shape, and higher sensitivity as well as reduced matrix interference.[101] NSAIDs compounds have a wide range of polarities with diverse physiochemical properties, and they are acidic in nature, therefore obtaining chromatographic specificity can be challenging for simultaneous analysis. Chromatographic specificity was obtained by using a column with superficially porous particles and the best possible gradient elution. In addition of water samples, spiked urine samples were evaluated with the same PIE conditions.

4.2 Materials and Methods

4.2.1 Chemicals, Reagents, and sample preparation

The NSAIDs used in this study included ibuprofen, naproxen, and ketoprofen were obtained from Sigma Aldrich (St. Louis, MO) and mefenamic acid, oxaprozin, tolfenamic acid, aceclofenac, and indomethacin were obtained from VWR (Randor, PA).

HPLC grade water, acetonitrile and methanol were purchased from Fisher Scientific (Bridgewater, NJ). Glycerol was purchased from Sigma-Aldrich (St Louis, MO). LC-MS grade formic acid was from RICCA (Arlington, TX). A Milli-Q Plus purification system, (Millipore, Milford, MA) was used to obtain Ultra-pure water in the laboratory. Running tap water from separation lab, Seton Hall University, NJ and MoniCheck synthetic urine control – Negative (non-spiked) was purchased from Branan Medical corporation (Irvine, CA) were utilized for real sample analysis.

Two columns were used to optimize the chromatographic separation - PerkinElmer Brownlee SPP 2.7µm; C18; 2.1 x 50 mm (Hopkins MA) and Agilent Rapid resolution HT; 1.8 µm; 2.1 x 50 mm; Zorbax SB C18 (Santa Clara, CA).

NSAIDs extracts and standards were analyzed using a Qsight 225MD UHPLC (Perkin Elmer, Shelton, CT) equipped with sampling module, solvent delivery module and column stability module, coupled to triple quadruple mass detector equipped with an electrospray ionization source. The system was operated by simplicity 3Q MD software (Perkin Elmer, Shelton, CT).

4.2.2 Sample preparation

Stock solutions of all compounds were prepared by dissolving them individually into methanol at a concentration of 1 mg/mL. Then a mixture of all eight analytes was prepared by spiking 500µl of each standard to 50 mL of Millipore water. Extracted standards for the calibration and linearity curves were prepared as follow. Into a 15 mL high-density polyethylene (HDPE) conical tube with screw cap 5 mL of spiked standard solution was added and mixed with 5 mL of acetonitrile. This mixture was mixed and vortexed for 2 minutes. 2mL of glycerol on was added and the mixture was mixed and vortexed for 5 minutes and equilibrated for the required time and temperature for phase separation. Extraction temperature, time and amount of glycerol were optimized in Chapter 2 and the same conditions were used here: 0 °C, 2 mL glycerol and 30 minutes equilibration. The pH of the extraction was not studied experimentally, but since these analytes are acidic in nature in future studies after validating the extraction application towards these compounds it can be optimized. After the two phases separated, volume of each phase was recorded, and the upper organic phase was recovered for analysis of NSAIDs using UHPLC-MS/MS. The overall outline for extraction procedure is described in chapter 2 in Figure 2-1.

PIE standards were prepared by spiking LCMS water over the concentration range of 25 to 5000ppb. Calibration standards then were subjected to optimized PIE. Non-extracted standards were diluted into acetonitrile over concentration range of 25 to 2500ppb then analyzed to compare calibration curve and linearity with PIE standards. Samples were prepared by spiking water for recovery, accuracy, and precision study at concentrations of 250 and 500ppb. Precision was performed on six separately prepared samples at 1000ppb. Real samples of tap water and negative synthetic urine samples were prepared by spiking at 1000ppb. Table 4-2 shows the summary for sample and standard preparation for NSAIDs.

4.2.3 Instrumental parameters

NSAIDs were qualified and quantified using Perkin Elmer UHPLC Qsight 225MD. LC analysis was performed on a PerkinElmer Brownlee SPP 2.7 μm; C18; 2.1 x 50 mm column with 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. The mobile phase flow was kept at 0.4 mL/min and the gradient was started with 10% mobile phase B hold for 0.5 min then increase mobile phase B to 90% in 5 min and hold for 1 minute then bring back to the initial condition at 6.5 min and finally equilibrate at initial conditions for 1.5 minutes. Column oven temperature was kept at 30 °C and the injection volume was 2 μL.

Mass detector mode for ionization was used as positive with an ESI voltage of 4000 V and a source temperature 300 °C. HISD temperature was set at 200 °C, drying gas was set at 120 °C and nebulizer at 200 °C. Detection was performed in multiple reaction monitoring (MRM) mode. A direct flow injection of a standard solution of each compound was used to find the optimum conditions for each compound in the ESI source and the most suitable average conditions were selected for the analysis. Cone voltage and collision energies were optimized for each compound to obtain two MRM transitions, one for quantification and other for qualification of the analytes. Table 4-3 describes the basic outline of the analytical conditions used in this study.

Standard and sample preparation	
Concentration Range	5000 ppb to 25ppb
STD	Extracted
Sample	Recovery and accuracy: Spiked at 250 ppb and 500ppb (n= 4)
	Precision: Spiked at 1000ppb (n=6)
Extraction procedure	5mL of spiked sample or std solution + 5mL acetonitrile + 2 mL glycerol mix + equilibrate 30 minutes at 0 °C
	After two phase separation volume of both phase recorded and upper organic layer was recovered into UHPLC vial for analysis.
Real Sample	Tap water: 1000ppb (n=2) Synthetic urine: 1000ppb (n=2)

Table 4- 2 Standard and sample summary for NSAIDs

LC conditions			
Instrument	PerkinElmer UHPLC coupled to triplequad Qsight 225MD		
LC Column	PerkinElmer Brownlee SPP 2.7µm; C18; 2.1 x 50 mm		
Data analysis	Simplicity 3Q MD		
Mobile Phase A	0.1% HCOOH in Water		
Mobile Phase B	0.1% HCOOH in Acetonitrile		
Mobile Phase Gradient	Start with 0.4ml/min flow at 10% mobile phase B hold for 0.5 min; then increase B to 90% in 5 min and hold for 1 min; then bring back to initial condition at 6.5min and finally equilibrate at initial condition for 1.5min.		
Column Oven Temperature	30 °C		
Injection Volume	2 µL		
MS source conditions			
ESI Voltage (Positive)	4000 V	Source Temperature	300 °C
Drying gas	120	HSID Temperature	200 °C
Nebulizer gas	200	Detection Mode	MRM

Table 4- 3 Analytical conditions for NSAIDs

4.2.4 Method validation and experimental parameter

4.2.4.1 Calibration curve and linearity

NSAIDs standards stock solutions at 1mg/mL for all eight compounds were prepared in methanol and working standards for polyol induced extraction were prepared by diluting the stock solutions in water by mixing each 500 µl into 50 mL and then further diluting to produce concentrations ranging from 25 ppb to 5000 ppb. These standards were analysed in triplicate using LC-MS/MS to prepare calibration curves for each analyte. The R^2 value and equation of the line were obtained using Excel for all calibration curves. The limit of quantitation (LOQ) and the limit of detection (LOD) were assessed using the data generated for each peak. Once all data were compiled and a calibration curve was constructed LOD and LOQ were determined for each peak by the IUPAC equation, shown as equation 2-4.

4.2.4.2 Recovery, accuracy, and precision

Four different samples at 250ppb and 500ppb were prepared by diluting stock solutions in Milli-Q pure de-ionized water. Samples were then subjected to PIE and analysed using UHPLC-MS/MS. All data generated were used in the determination of percent recovery and accuracy using the equation of the line from the calibration curve to find concentration from the peak area of each. Once determined, these values were divided by the appropriate concentration and multiplied by 100 to get the percent recovery. An average of all four was reported as percent recovery for each. Accuracy was determined from the percent recovery of an average value. Six separate samples were prepared as mentioned above at 1000ppb for precision and analysed twice for the same day precision and analysed on second day for intraday precision. Precision was

calculated as %RSD of all six preparations. Equations needed to calculate necessary parameters are described below in equation 4-1, 4-2 and 4-3.

$$\%\text{Recovery} = \left(\frac{Experimentally\ determined\ concentration}{Theoretical\ concentration}\right) X\ 100 \qquad \text{Equation 4-1}$$

$$\text{Accuracy: } \%\text{error} = \left(\frac{|Theoretical\ concentration - experimental\ concentration|}{Theoretical\ concentration}\right) X\ 100 \qquad \text{Equation 4-2}$$

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

4.2.4.3 Analysis of Real Sample: Tap water

Two tap water samples were investigated for the presence of NSAIDs sing the PIE and MRM methods. These two samples were from tap water from our lab in South Orange, NJ, USA. For both samples 5 mL out of collected 100 mL was transferred to 15 mL HDPE tube then were subjected to the procedure of PIE and samples were analysed using the UHPLC-MS/MS parameter for NSAIDs mentioned above. Moreover, two synthetic negative-urine samples were prepared similarly by spiking with NSAIDs. Due to the smaller volume of the urine sample PIE urine samples were prepared with 2:2 mL urine and acetonitrile by volume. Amount of glycerol was adjusted to 1.6 mL for urine sample. All the other parameters stayed the same.

4.3 Results and discussion

4.3.1 Optimization of MRM condition

The tuning of the MS conditions for standards was performed by direct infusion of individual standard solutions of 1ppm in acetonitrile using the syringe pump attached to MS. Direct infusion in ESI-MS/MS is an approach for the determination of m/z of a precursor, product ion and MRM of a component prior to its analysis in a mixture. Each standard solution was infused at 30 µl/min of flow rate to verify the pseudo molecular masses, $[M + H]^+$ in positive mode ionization. The ESI source parameters were then tuned for maximum intensity of $[M + H]^+$ ions of each analyte. The ESI source parameters were then tuned to accommodate the LC flow rates by syringe infusion of each analyte. The LC flow rate was kept at the initial composition of the gradient. Multiple reaction monitoring (MRM) transitions were optimized with the protonated molecular ion selected as the precursor. The most abundant product ion was used for quantification and a second transition was selected for confirmation purposes. Finalized MRM parameters including entrance voltage, collision energy and collision cell lens are summarized in Table 4-4. Source and hot surface induced desolation (HSID) temperature along with voltage were optimized to generate maximum signal and best the suitable conditions were selected to maximize ionization. Source temperature and ESI voltage played important role in the ionization of NSAIDs. Source temperature was optimized by gradually increasing up to 350 °C. It was founded that 300 °C gave optimum ionization without any adduct formation. Similarly, ESI voltage was also optimized by gradually increasing the voltage from 3000 V to 5000 V with 4000 V being the optimum for each analyte.

4.3.2 Optimization of chromatographic conditions

NSAIDs are acidic in nature and are highly polar therefore chromatographic separation might show a dependance on pH. However, in this study the optimization was done without use of any

Analyte	MRM Transitions		EV	CE	CCL2
	Q1	Q2			
Aceclofenac	353.70	214.70*	8	-31	-64
		249.60	11	-20	-60
Ibuprofen	206.90	160.80*	19	-21	-36
		118.80	21	-34	-48
Indomethacin	357.70	138.80*	24	-32	-48
		174.00	1	-17	-44
Ketoprofen	254.80	104.90	24	-34	-52
		208.80*	28	-20	-44
Mefenamic acid	241.90	223.80	21	-24	-48
		208.80*	23	-39	-84
Naproxen	230.90	184.70*	15	-22	-40
		169.80	15	-38	-60
Oxaprozin	293.80	275.70	28	-23	-56
		233.70*	29	-28	-56
Tolfenamic acid	261.80	243.70	21	-23	-48
		208.90*	24	-37	-72

Table 4- 4 MRM transition for NSAIDs (* = Quantifier ion)

buffer since LCMS requires a volatile buffer. The several mobile phases were used in UHPLC-MS/MS that includes many solvents such as water, ACN or MeOH, acetic acid, 0.1% formic acid, ammonium formate etc.[97-101] Formic acid at a concentration of 0.1% in water and acetonitrile for the mobile phase was selected to avoid any contamination to MS. Methanol in the mobile phase showed a higher pressure on column which was outside of the recommended pressure range for column. Two different C_{18} columns were studied for separation, one with 2.7 μm diameter particles and other with 1.8 μm. A Perkin Elmer C_{18} column with superficially porous particles of 2.7 μm and 2.1mm inside diameter with 50mm length was selected out of the two due to better resolution and peak shape. Column temperature had little effect on the selectivity or separation of these compounds. At 35 °C peaks were eluting little earlier and peak distortion was observed for some analytes while at 30 °C this issue was not observed. Total ion chromatograms for the mixture are shown in Figures 4-1 and Figure 4-2. Total ion chromatogram for the analytes shows co-elution of ketoprofen and naproxen around 3.1 minutes, indomethacin, aceclofenac and ibuprofen around 3.8 minutes. Spectroscopic separation was obtained through MRM transitions for qualifier and quantifier ions. MRM of quantifier ions for each compound are shown in Figure 4-3. As shown in the Figure each analyte has nice gaussian peak shape with complete separation due to MRM selection of procurer ion and daughter ions. Selective detection allows quantitation of each analyte without full chromatographic separation. Figure 4-4 shows MRM overlay for the quantifying ions for each NSAIDs. This Figure elaborates the MRM of quantifying ion over the chromatographic run time and shows each peak with during the run.

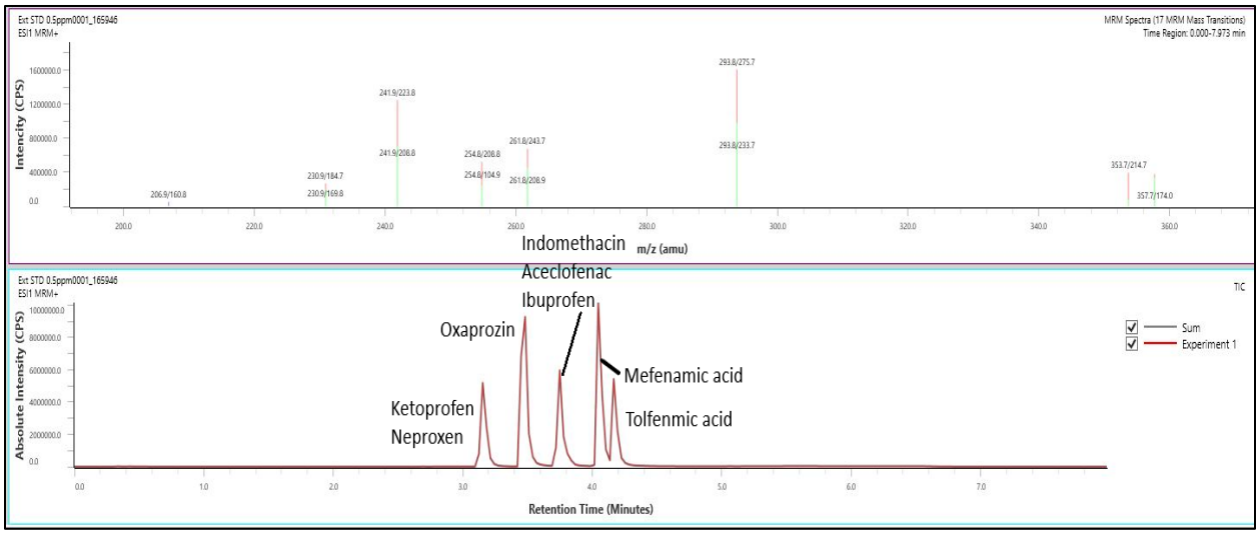


Figure 4- 2 Total ion chromatogram of NSAIDs STD (500ppb)

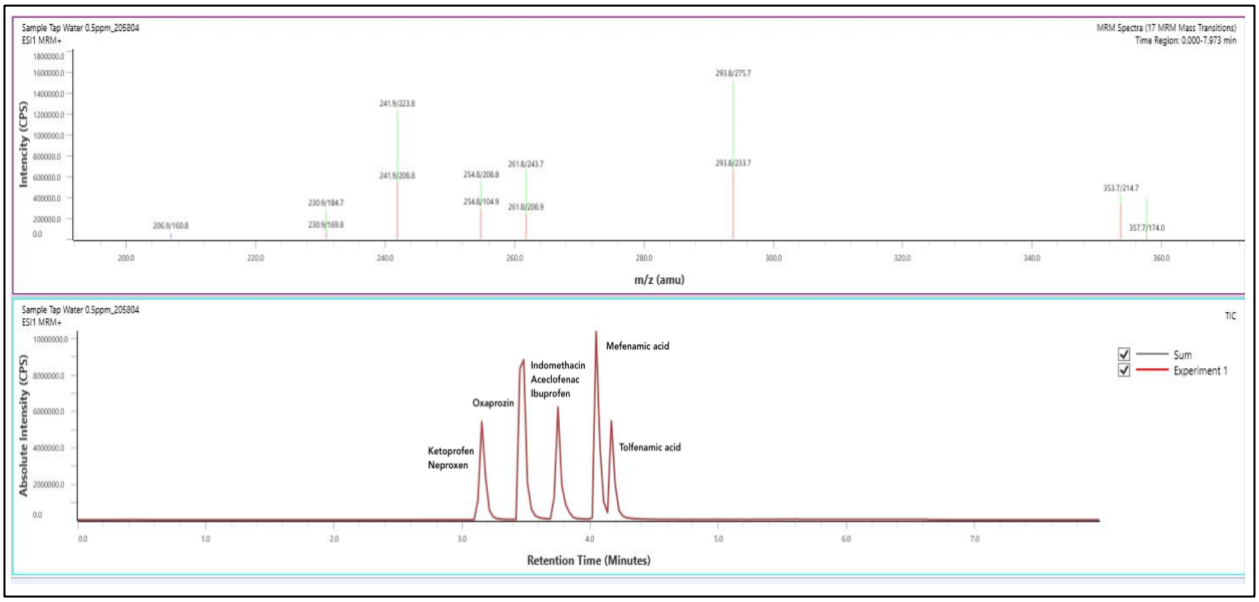


Figure 4- 1 Total ion chromatogram for NSAIDs Sample (500ppb)

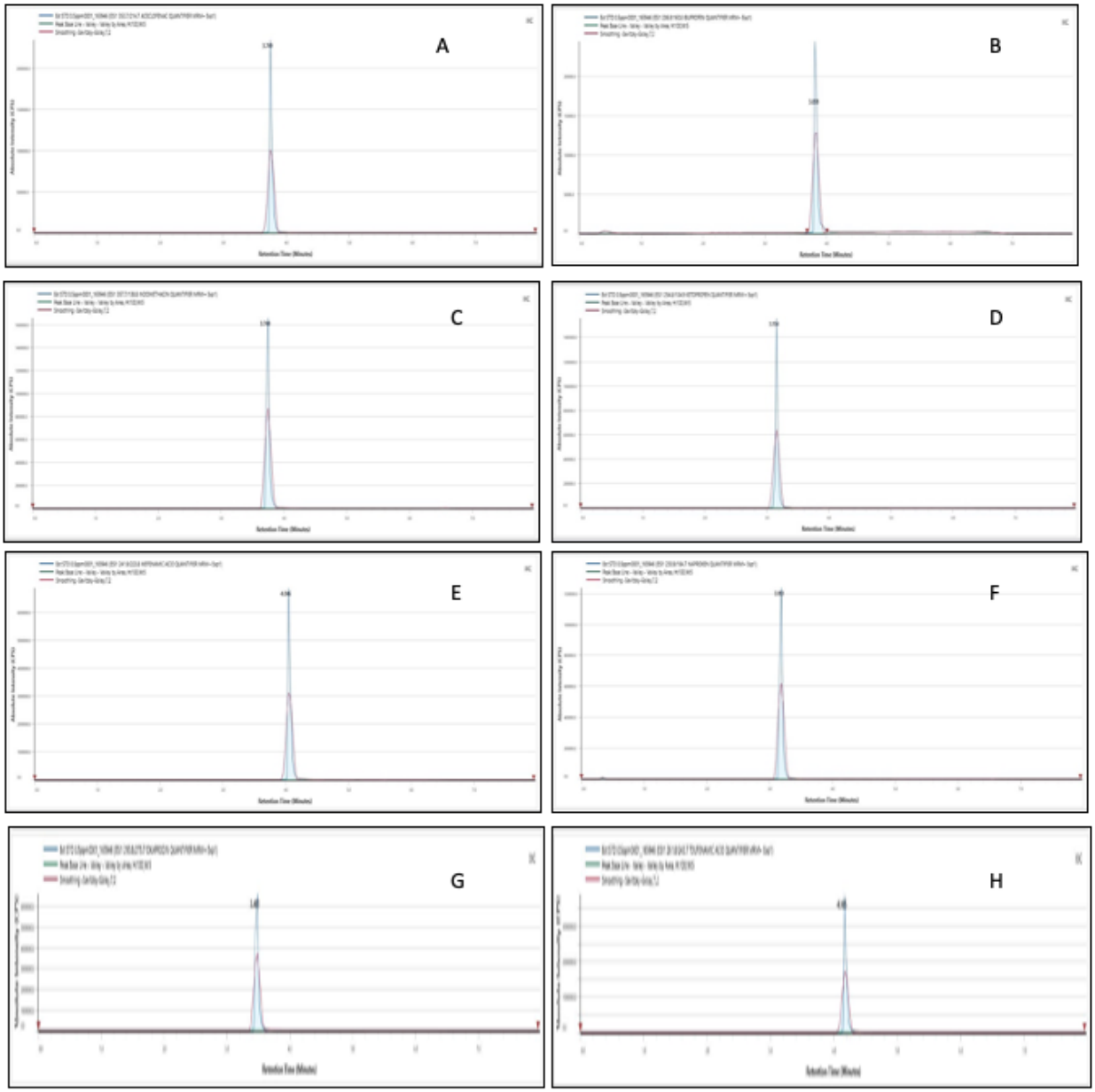


Figure 4- 3 MRM of quantifying ion for NSAIDs: (A) Aceclofenac, (B) Ibuprofen, (C) Indomethacin, (D) Ketoprofen, (E) Mefenamic acid, (F) Naproxen, (G) Oxaprozin, (H) Tolfenac

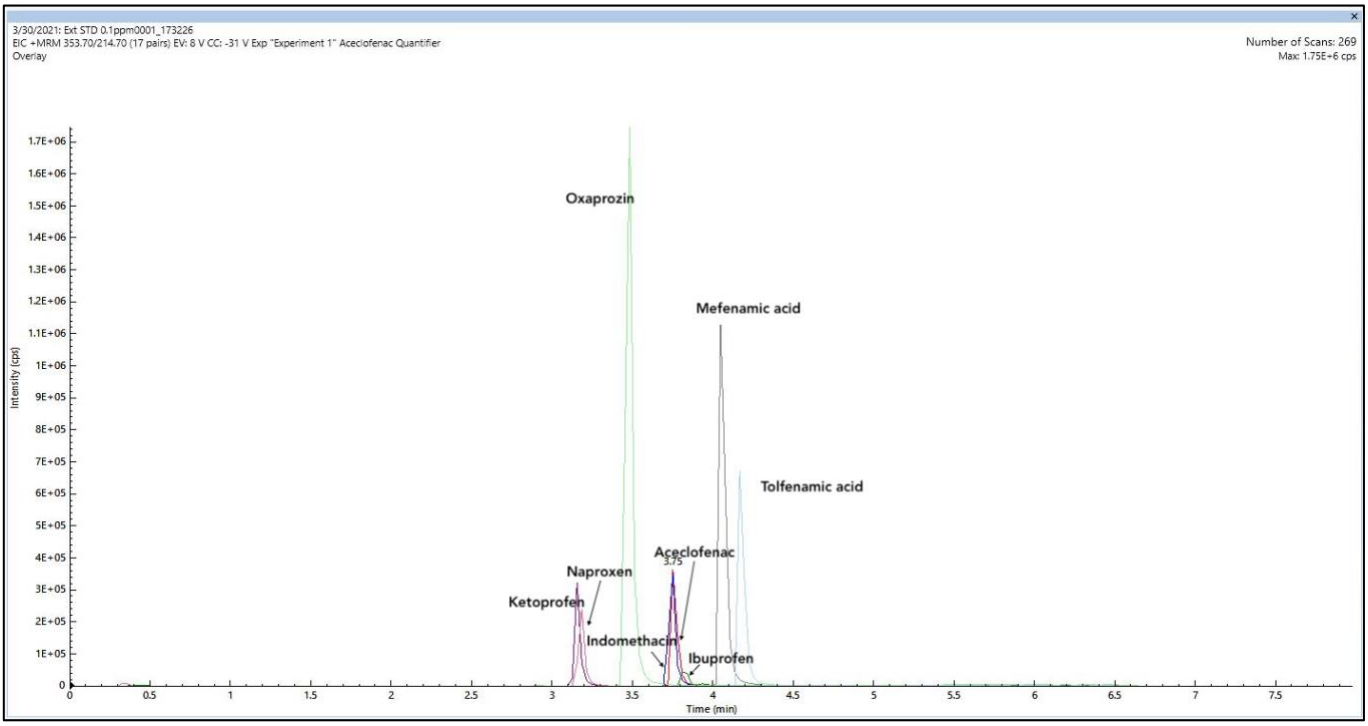


Figure 4- 4 MRM overlay for NSAIDs PIE STD 100ppb

Retention times for all eight NSAIDs are tabulated in Table 4-5. NSAIDs have similarities in chemical structure and properties. Tolfenamic acid (pKa 3.69) and mefenamic acid (pKa 3.73) have benzoic acid backbone in their structure. Only Cl group in tolfenamic acid replaces methyl group in mefenamic acid. They both elute later in the retention time range of 4.0 to 4.2 minutes. Propionic acid derivatives naproxen and ketoprofen eluted very close to each other with retention time 3.18 and 3.15 minutes respectively. Oxaprozin eluted soon after them around 3.5 minutes. Although ibuprofen is also a propionic acid derivative it co-eluted with aceclofenac and indomethacin at around 3.7 minutes.

4.3.3 Validation

4.3.3.1 Calibration and Linearity

Precision, recovery, accuracy, linearity were evaluated. Calibration curves were constructed for all analytes from 0.025 to 5 μg/mL (25ppb to 5000ppb) with standard concentration levels at 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 μg/mL. Correlation coefficients of $r^2 > 0.97$ for all analytes were obtained over these ranges. Standard curves are shown in Figure 4-6 to 4-13. Calibration curves looked curved for aceclofenac, indomethacin, ketoprofen, mefenamic acid, and oxaprozin. This type of bending in curve indicates possible overloading of the detector for higher concentration samples. For analytes with bending in calibration curve, co-relation co-efficient of r^2 was obtained > 0.97. Ibuprofen, naproxen and tolfenamic acid calibration curves appeared straighter and showed co-relation co-efficient of $r^2 > 0.99$. Linearity data along with LOD and LOQ data are tabulated in Table 4-6.

Analyte (Calibration range: 25 to 5000ppb)	Retention time (t_R) (min)
Aceclofenac	3.778
Ibuprofen	3.838
Indomethacin	3.749
Ketoprofen	3.154
Mefenamic Acid	4.076
Naproxen	3.183
Oxaprozin	3.481
Tolfenamic Acid	4.165

Table 4- 5 Retention time summary for NSAIDs

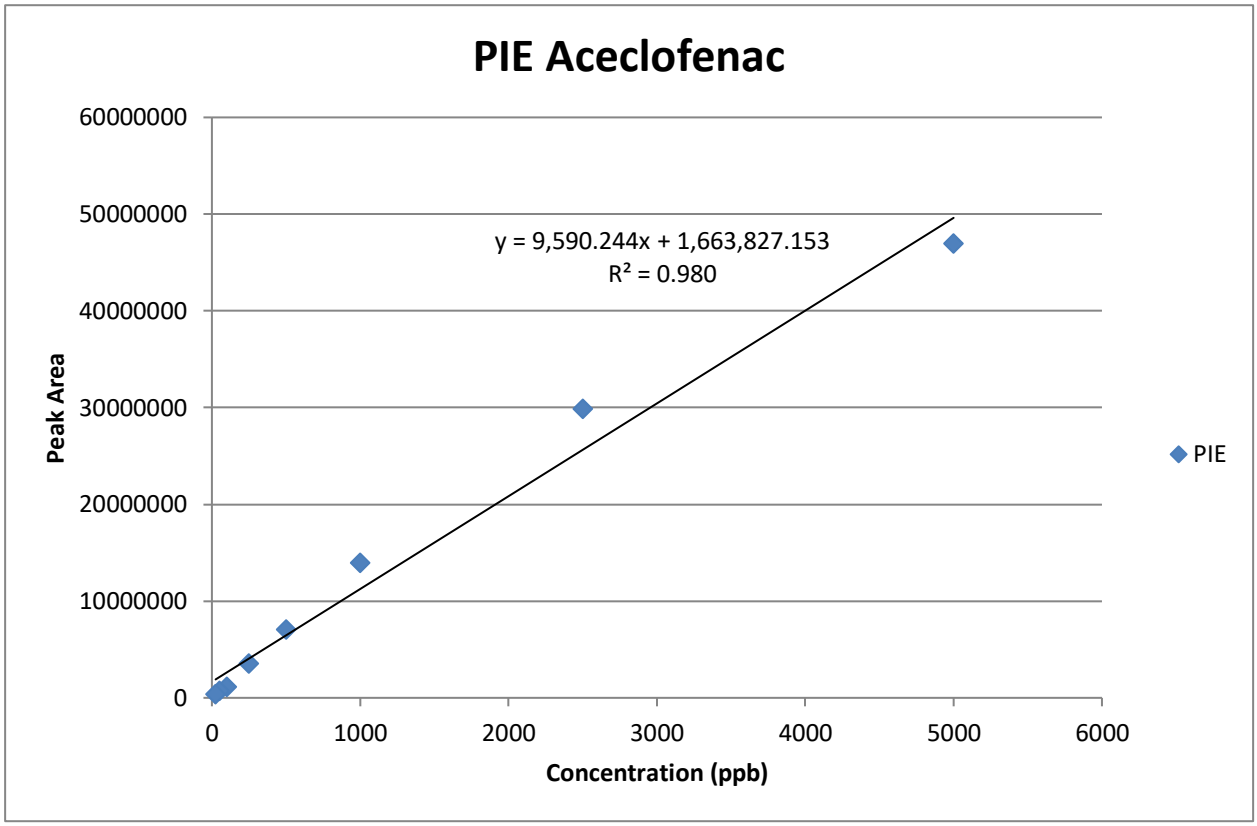


Figure 4- 5 Calibration curve: Aceclofenac (PIE)

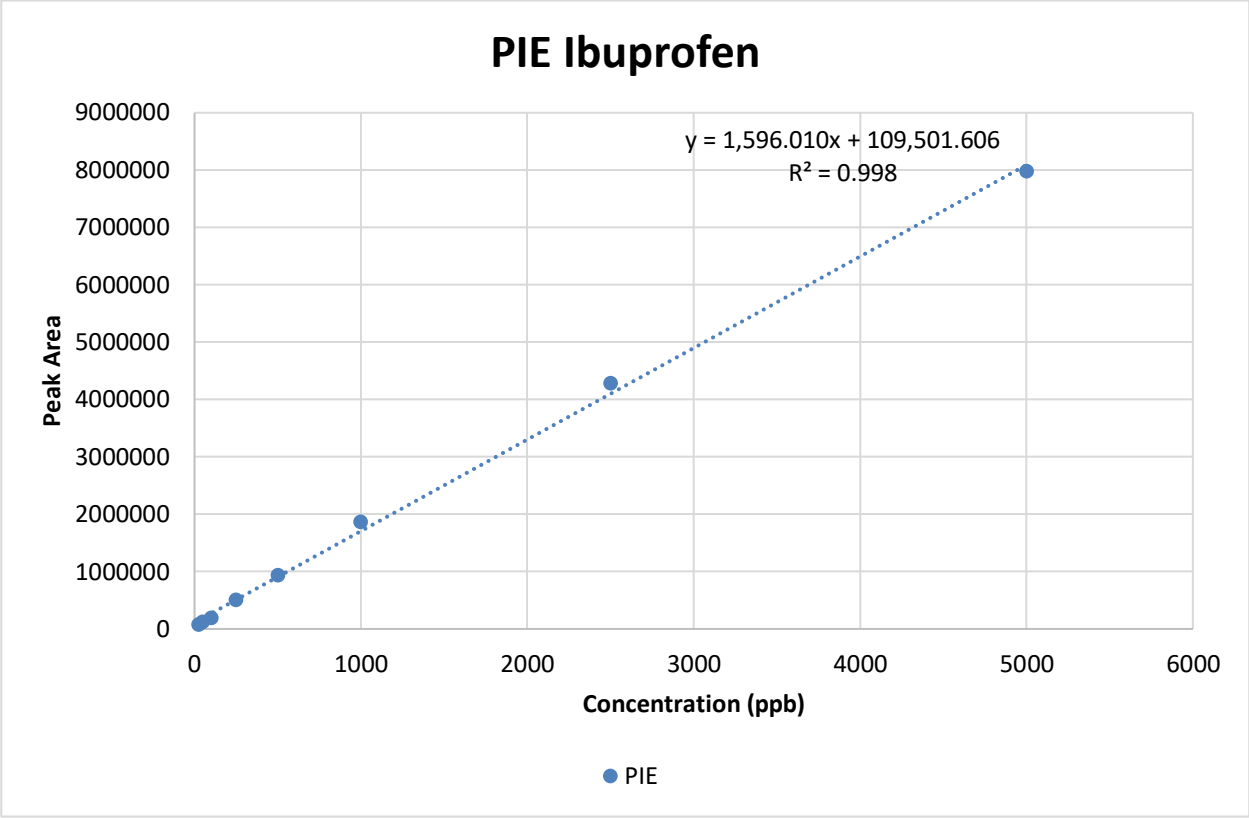


Figure 4- 6 Calibration curve: Ibuprofen (PIE)

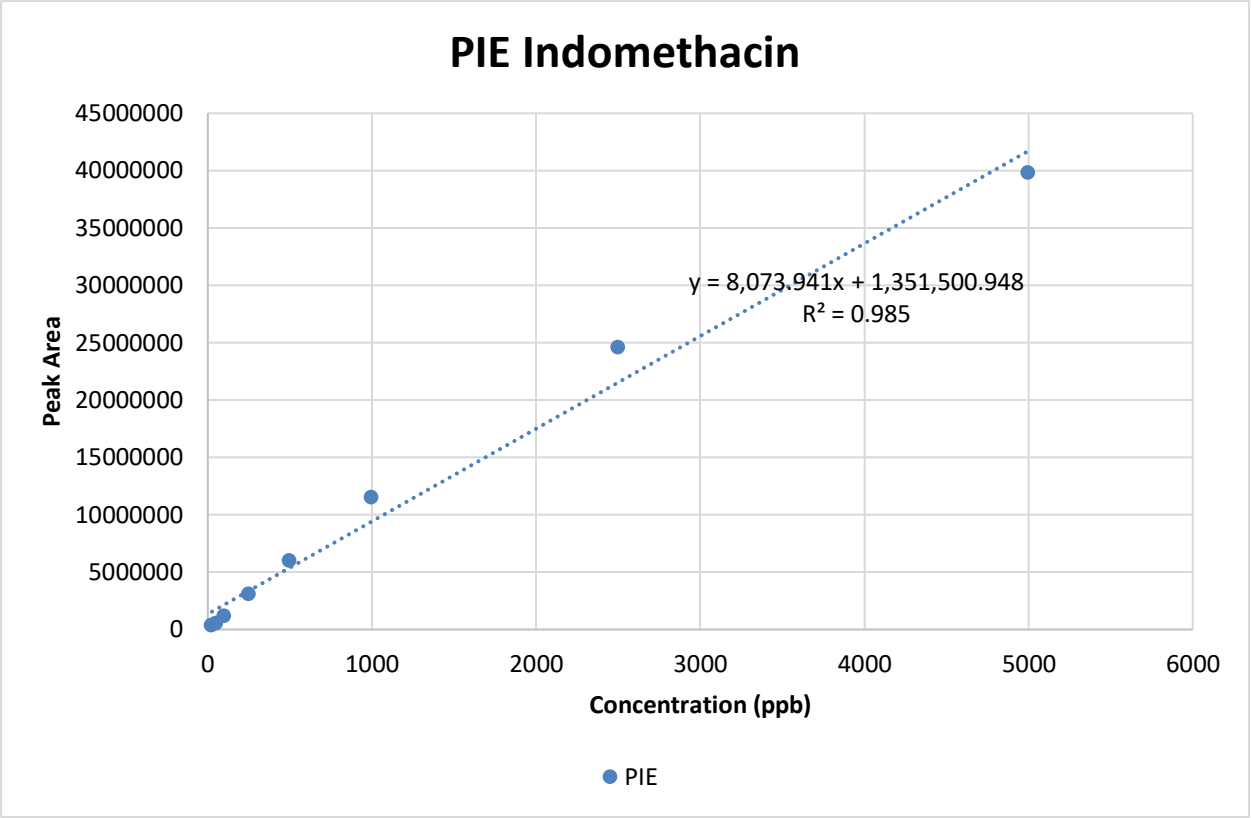


Figure 4- 7 Calibration curve: Indomethacin (PIE)

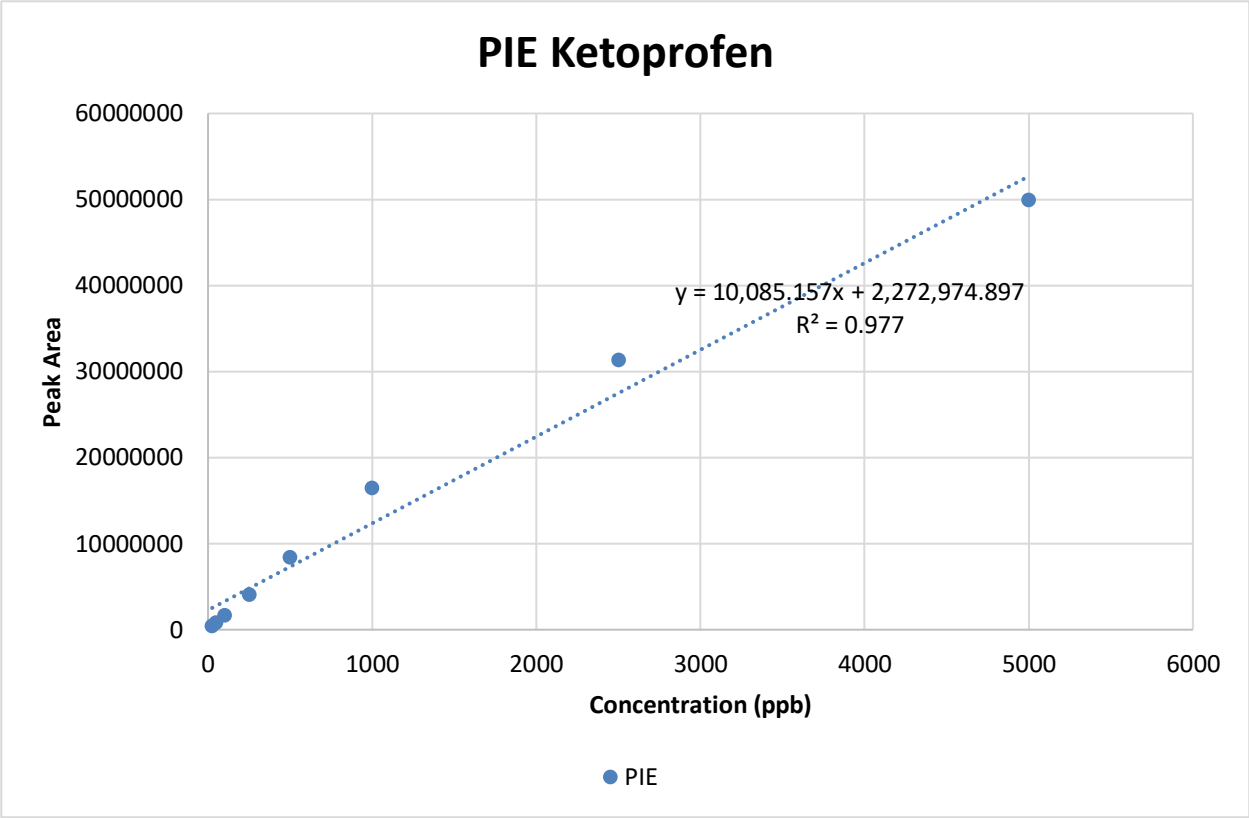


Figure 4- 8 Calibration curve for Ketoprofen (PIE)

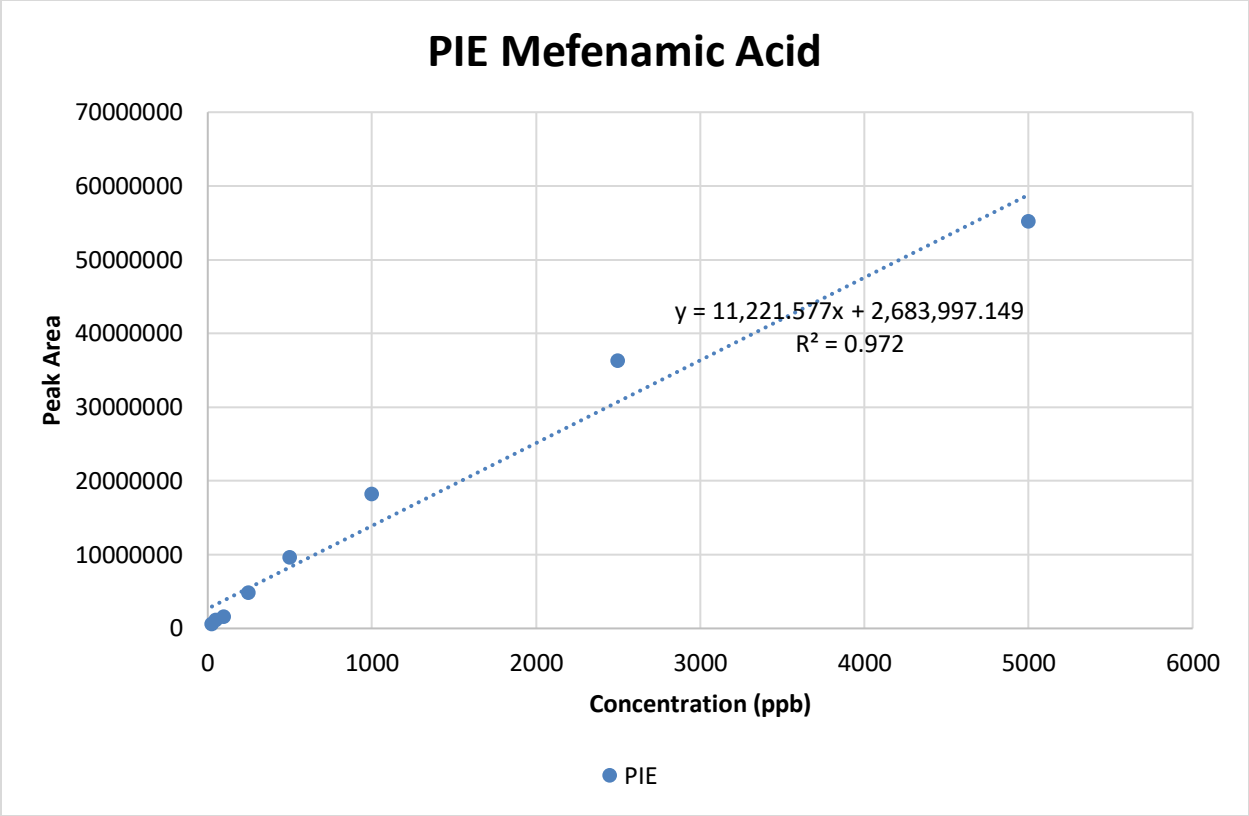


Figure 4- 9 Calibration curve: Mefenamic acid (PIE)

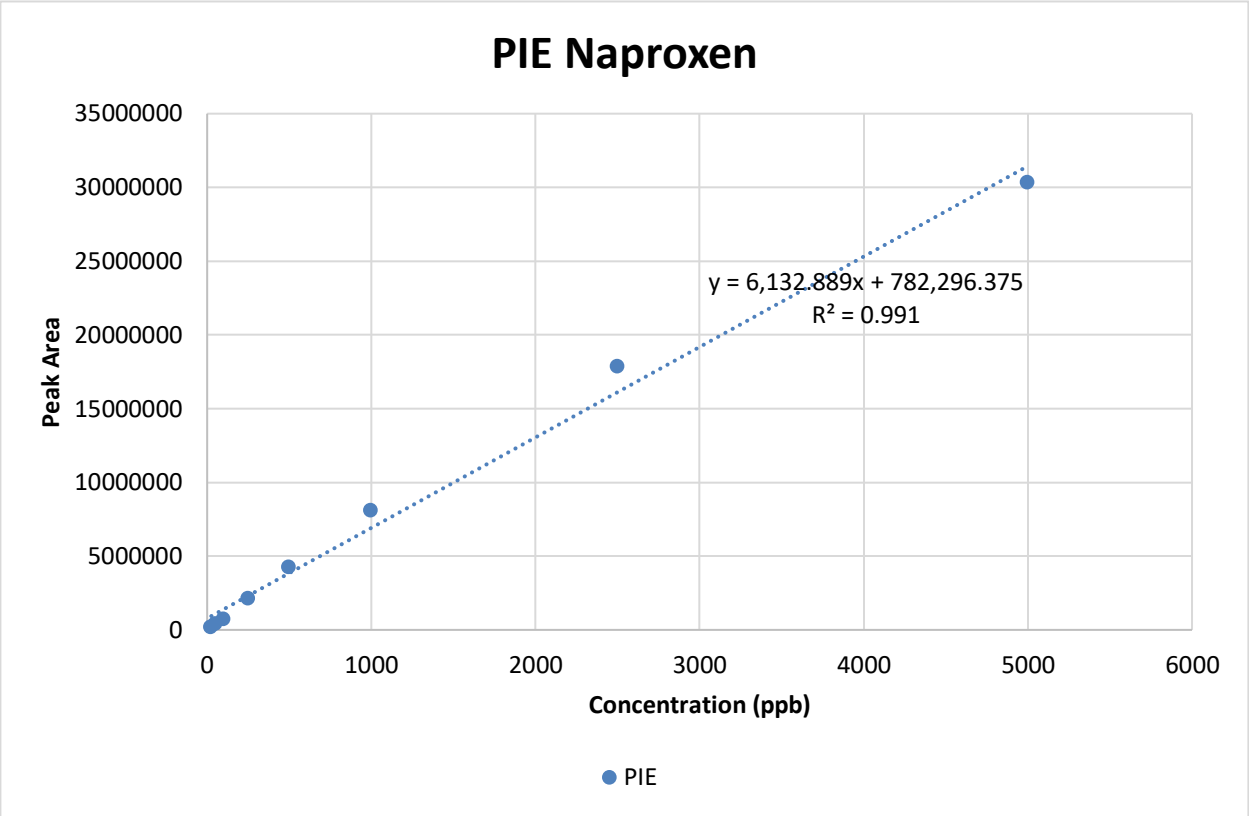


Figure 4- 10 Calibration curve: Naproxen (PIE)

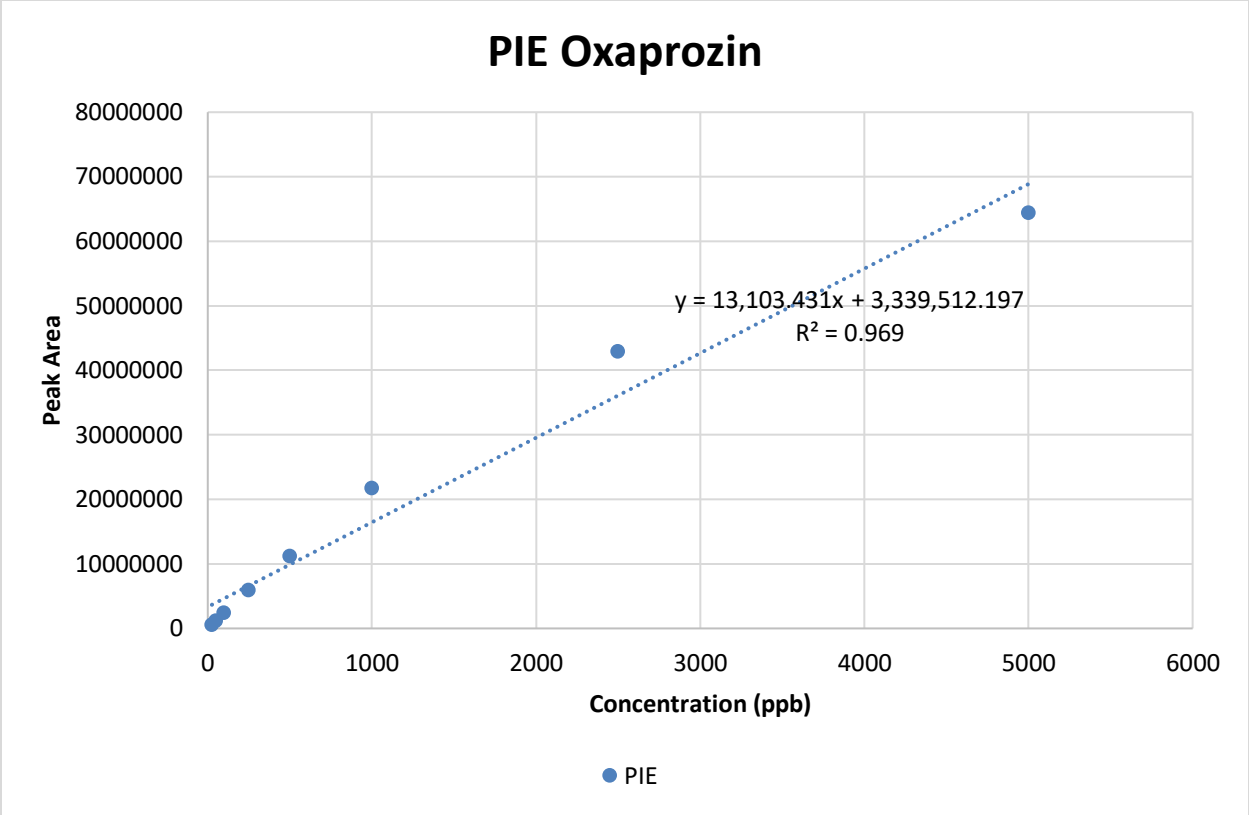


Figure 4- 11 Calibration curve: Oxaprozin (PIE)

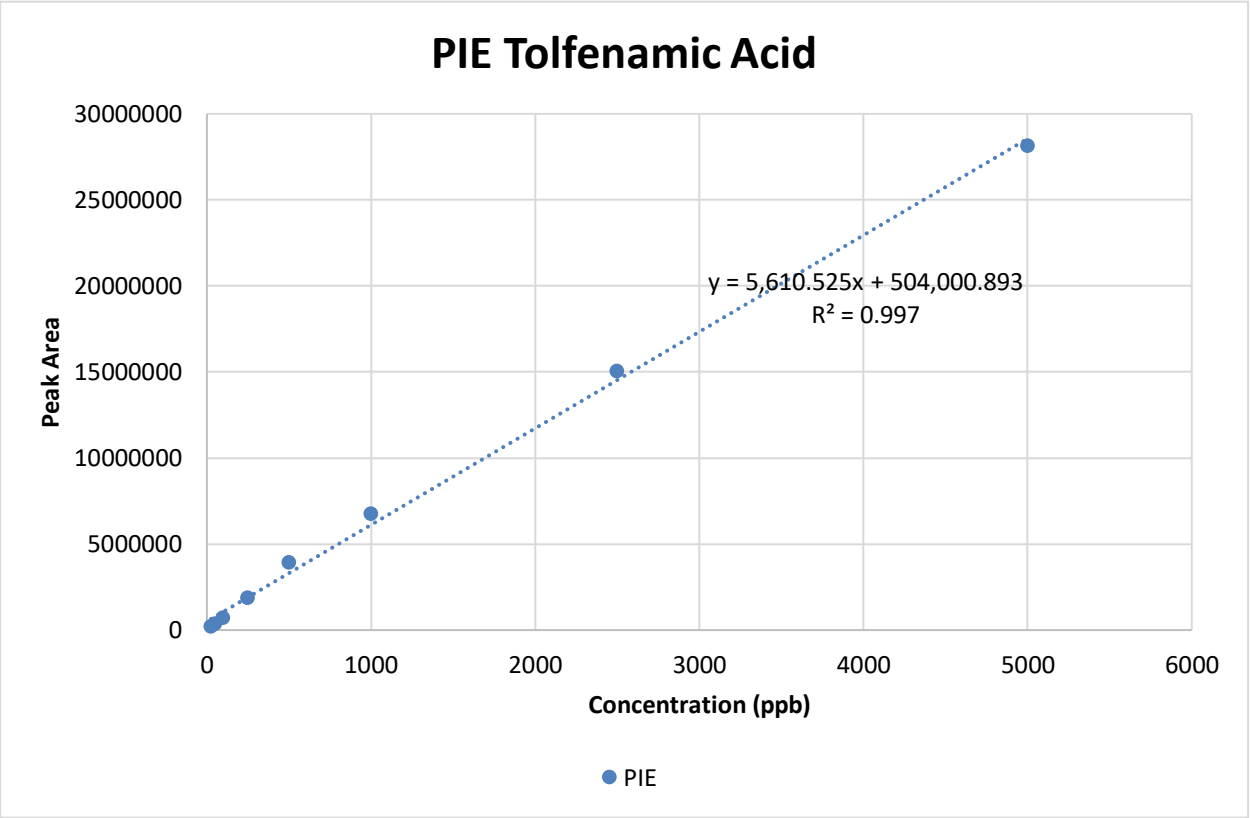


Figure 4- 12 Calibration curve: Tolfenamic acid (PIE)

NSAIDS	R^2	LOD	LOQ
Aceclofenac	0.980	0.08ppb	0.3ppb
Ibuprofen	0.998	4ppb	13ppb
Indomethacin	0.985	0.4ppb	1ppb
Ketoprofen	0.977	0.3ppb	1ppb
Mefenamic Acid	0.972	0.2ppb	1ppb
Naproxen	0.991	0.3ppb	1ppb
Oxaprozin	0.970	0.6ppb	2ppb
Tolfenamic Acid	0.997	0.4ppb	1ppb

Table 4- 6 Calibration data for NSAIDs PIE STDs

4.3.3.2 Limit of detection and limit of quantitation

The LOD and LOQ were determined for each analyte adopting the IUPAC definition of limit of detection. The equation is described in the equation 4-4. Where, k = 3 for LOD and k = 10 for LOQ. s_B is standard deviation of blank.

$$C_L = \frac{ks_B}{m} \qquad \text{Equation 4-4}$$

For the LOD and LOQ calculations background signals from blank were used to find standard deviation of blank and peak height curve for each analyte was plotted for the slope of the calibration curve. Obtained LOD and LOQ data are tabulated in Table 4-6.

4.3.3.3 Accuracy, recovery, and precision

Six samples at 1 μg/mL (1000 ppb) were prepared to evaluate intra- and inter-day levels of precision. Four samples at the middle concentration 0.5 μg/mL (500 ppb) and low concentration 0.25 μg/mL (250 ppb) were prepared to evaluate the efficiency of the analyte recoveries. Calculated data are summarized in Table 4-7. Percentage recoveries for all eight analytes at 250 ppb concentration ranged from 70 to 103% and at 500 ppb concentration ranged from 99 to 123%. Accuracy was determined as% error for all compound from percent recoveries. % Error for samples at 250 ppb the calculated accuracy ranged from 1.54 to 29.97% and at 500 ppb it ranged from 1.26 to 23.09%. For precision calculations, intra-day repeatability and inter-day reproducibility were expressed as relative standard deviation (%RSD) for each analyte. Precision analysis was performed on separate two days. All the% RSD results for all compounds were found below 6.5%. For 1000ppb samples recoveries are well above 100%.

NSAIDS	Recovery (%; n=4)	Accuracy (% Error; n=4)	Precision (1000ppb) (%RSD; n=6)	Inter-day Precision (%RSD)
Aceclofenac	250ppb: 80 500ppb: 108	250ppb: 19.77 500ppb: 7.79	Day 1(1): 0.63 Day 1(2): 2.05 Day 2: 1.65	5.15
Ibuprofen	250ppb: 102 500ppb: 99	250ppb:1.54 500ppb: 1.26	Day 1(1): 1.33 Day 1(2): 2.00 Day 2: 2.56	4.18
Indomethacin	250ppb: 82 500ppb: 114	250ppb: 17.65 500ppb: 14.25	Day 1(1): 1.23 Day 1(2): 2.10 Day 2: 1.57	4.98
Ketoprofen	250ppb: 70 500ppb: 123	250ppb: 29.97 500ppb: 23.09	Day 1(1): 1.87 Day 1(2): 2.51 Day 2: 2.22	4.12
Mefenamic Acid	250ppb: 87 500ppb: 121	250ppb: 13.1 500ppb: 20.70	Day 1(1): 1.92 Day 1(2): 1.48 Day 2: 2.05	5.97
Naproxen	250ppb: 86 500ppb: 114	250ppb: 14.03 500ppb: 14.19	Day 1(1): 1.53 Day 1(2): 2.58 Day 2: 2.08	6.36
Oxaprozin	250ppb: 82 500ppb: 116	250ppb: 18.19 500ppb: 15.76	Day 1(1): 1.56 Day 1(2): 0.72 Day 2: 1.60	4.01
Tolfenamic Acid	250ppb: 103 500ppb: 117	250ppb: 3.19 500ppb: 17.12	Day 1(1): 1.46 Day 1(2): 1.68 Day 2: 1.73	5.98

Table 4- 7 Result summary for NSAIDs extraction by PIE

4.3.3.4 Analysis of real sample

No peaks were observed in the analysis of real samples of non-spiked water and urine sample. For the analysis of spiked samples, the results are tabulated in Table 4-8. All spiked tap water sample gave satisfying recoveries. Results obtained for spiked urine samples shows recovery ranging from 5.56 to 59.44%. Figure 4-13 shows the MRM overlay of urine sample. These recoveries show that PIE can be an alternative method for matrices other than water also. Urine sample analyzed here were not optimized for pH and were performed to verify phase partitioning. Obtained data prove that if pH conditions are optimized for urine matrix it may give better outcome. NSAIDs have carboxylic acid groups and are weak acids in nature. The pKa's of the NSAIDs are listed in the Table 4-8 and are between 3.4 to 4.5. pKa values of NSAIDs indicate that lower pH values of about 3 would increase the proportion of ionizable NSAID that is in the neutral form. Although considering obtained data for the current extraction system with higher pH indicate that NSAIDs could be extracted at physiological pH without further preparation. If the extraction goal is to maximize signal response urine sample matrix can be studied further to understand the ionization of these analytes. This also indicates that extraction is possible for urine-like samples at non-optimum conditions.

Analytes (NSAIDs)	Spiked Urine sample %recovery (1ppm)	pKa	Spiked Tap water sample % Recovery (1ppm)	Spiked Tap water Sample % Recovery (0.5ppm)
Aceclofenac	29.46	3.44	121.87	120.65
Ibuprofen	25.99	4.41	101.79	110.50
Indomethacin	22.79	4.50	120.69	122.94
Ketoprofen	5.56	4.45	144.05	129.41
Mefenamic acid	59.44	3.73	136.13	125.57
Naproxen	14.00	4.84	121.30	121.10
Oxaprozin	12.69	4.28	135.17	130.57
Tolfenamic acid	58.51	3.69	109.30	121.66

Table 4- 8 Summary of spiked real sample analysis

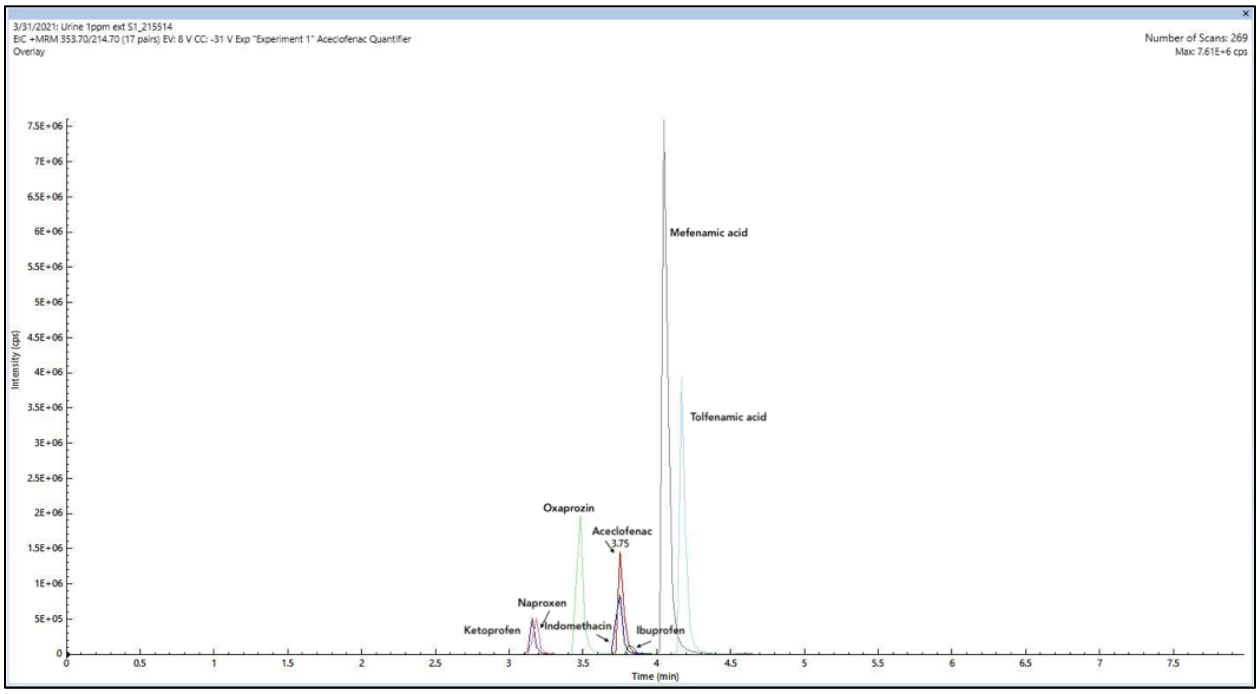


Figure 4- 13 MRM overlay of urine Sample

4.4 Conclusion

In this study, polyol induced extraction based on acetonitrile/aqueous mixtures using glycerol as a mass separating agent was used to extract NSAIDs from water. A mixture of NSAIDs in water was partitioned into acetonitrile at 0 °C by addition of glycerol to the water/acetonitrile mixture. All eight NSAIDs showed acceptable percent recovery and accuracy. Intra-day and inter-day precision results as% RSD for all compounds were less than 6.5%, which shows the method to be reproducible and accurate. Limits of detection were calculated by IUPAC method. LOD values obtained by the IUPAC method ranged from 0.08 ng/mL to 4 ng/mL The obtained data set demonstrates that PIE can be a potential extraction technique to be investigated further. Analysis of real samples as spiked tap water and spiked urine sample was performed. Obtained urine sample recoveries for each analytes opens the door for future application of PIE. Urine samples were analysed without pH modification. PIE shows the potential to extract NSAIDs from urine if PIE is optimized with pH conditions because NSAIDs are acidic in nature.

CHAPER 5 – DISCUSSION OF THE USE OF GC-MS FOR THE EXTRACTION OF GLUCOCORTICOIDS FROM WATER USING POLYOL INDUCED EXTRACTION

Steroid analysis by GC is popular due to the resolving power, high peak capacity and ability to separate steroid isomers. Moreover GC-MS has the advantage of higher reproducibility of spectra due to electron impact (EI) ionization and large library databases for robust identification. Previously, extraction of glucocorticoids using QuEChERS and GC-MS/MS has been studied by Schmidt.[84] Steroid analysis by GC requires sample pretreatment, chemical derivatization and/or enzymatic hydrolysis prior to analysis due to their low volatility of these molecules. Chapters 2 and 3 of this dissertation discuss the application of PIE in the extraction of glucocorticoids; this chapter is an extension of this idea to use PIE with gas chromatography analysis.

PIE shows a potential, simple and ecofriendly approach for the determination of glucocorticoids. The main purpose of this work is to show PIE as an alternative method of extraction for glucocorticoids with analysis by GC-TOFMS. This work demonstrates extraction and analysis of glucocorticoids by gas chromatography without any pre-treatment or chemical derivatization, with a significant recovery. The GC-TOFMS system used in this study was a comprehensive two-dimensional GC (GCXGC) coupled to a time-of-flight mass spectrometer. GCXGC is capable of a secondary separation by using secondary column with a different polarity than that of the primary column. However, this work only focused on a one-dimensional separation because the analytes showed appropriate separation in one dimension.

This study investigated the detection of seven glucocorticoids out of eight glucocorticoids studied in Chapters 2 and 3. Fludrocortisone acetate was not detected in initial screening therefore it was omitted from the study in this chapter. These analytes showed acceptable recoveries by PIE. The validated PIE method was used for extraction of the seven glucocorticoids in this study. In this study, the validation of this method for the glucocorticoids of interest gave results for precision, percent recoveries and partition co-efficient for each analyte.

5.1 Introduction

5.1.1 Basic theory of Gas Chromatography

5.1.1.1 Mobile and stationary phases

The driving force of separation in gas chromatography is based on the boiling point or vapor pressure of the analyte and partitioning of analyte between stationary and mobile phase.

The stationary phase on the GC column is of two types in gas chromatography. One is packed column where the column contains solid particles and the other is capillary column where column comprises a liquid coating on the inner wall of the capillary tube. Capillary column was used in this study. Choice of phase bed varies based on the analytes.

The most important characteristic required of the GC mobile phase is inertness. Most commonly used carrier gases are helium, hydrogen, and nitrogen. The effects of different carrier gases on separation are studied and plotted in plot known as Van Deemter plot. This plot explains the band broadening of peaks by associating kinetic and mass transfer effects to the rate theory. Van

Deemter plot theory is explained by equation 5-1. Helium was used as the carrier gas in this work.

$$H = A + \frac{B}{\overline{\mu}} + (C_s + C_m)\,\overline{\mu} \qquad \text{Equation 5-1}$$

In equation 5-1, The A term is eddy diffusion, which represents the analyte diffusion or path through packed column bed. The term B is longitudinal diffusion, C_s and C_m refer to mass transfer effects in the mobile and stationary phase. The term $\overline{\mu}$ refers to linear velocity of the carrier gas, and linear velocity of the carrier gas can affect each term. Each term is discussed in detail in Chapter 1. Figure 5-1 explains the equation in detail, it describes that type of carrier gas also play an important role besides linear velocity. Different carrier gases have different optimal velocities which provides best column efficiency. Figure 5-2 explains the term of Van Deemter equation.

5.1.1.3 Sample introduction

Injection port is the part through which samples are introduced into GC. Molecules to be analyzed must be volatile or semi-volatile because upon introduction it must be vaporized and introduced into carrier gas. To serve this purpose injection the port is heated and volatility of the analyte plays an important role in this step. There are several types of sample introduction techniques. In this study, split/splitless inlet in splitless mode is used. Figure 5-3 is the representation of schematic for the split and splitless injection port. The difference between split and splitless is the opening of the purge valve. The purge valve is open completely during the sample analysis allowing the injected portion of the sample to split.

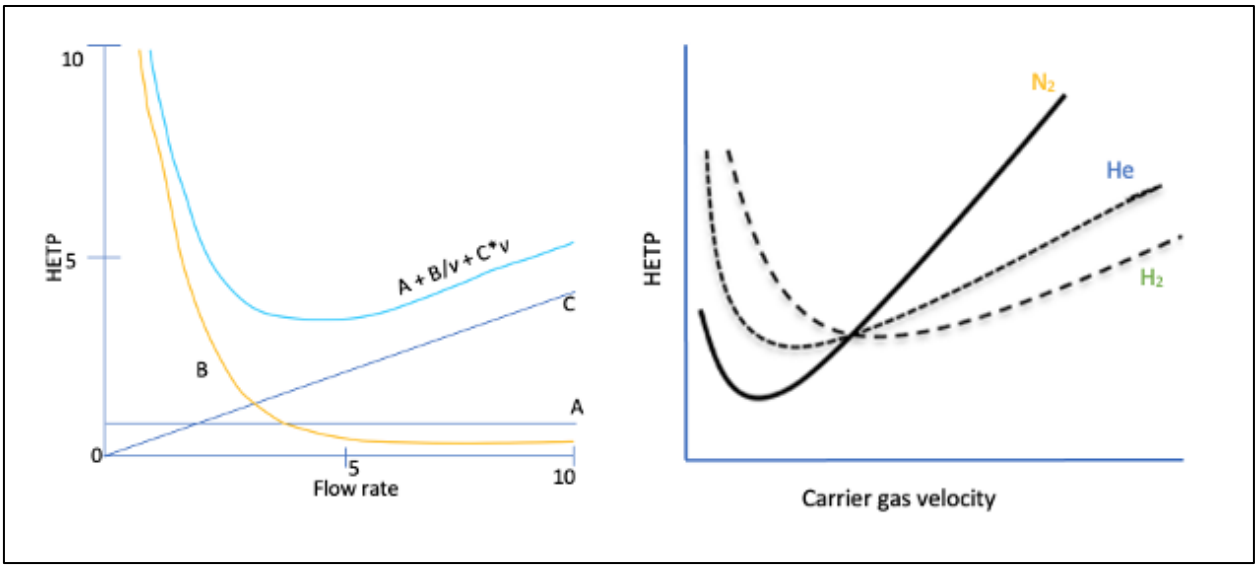


Figure 5- 1 The Van deemter plot and carrier gases in the Van deemter plot

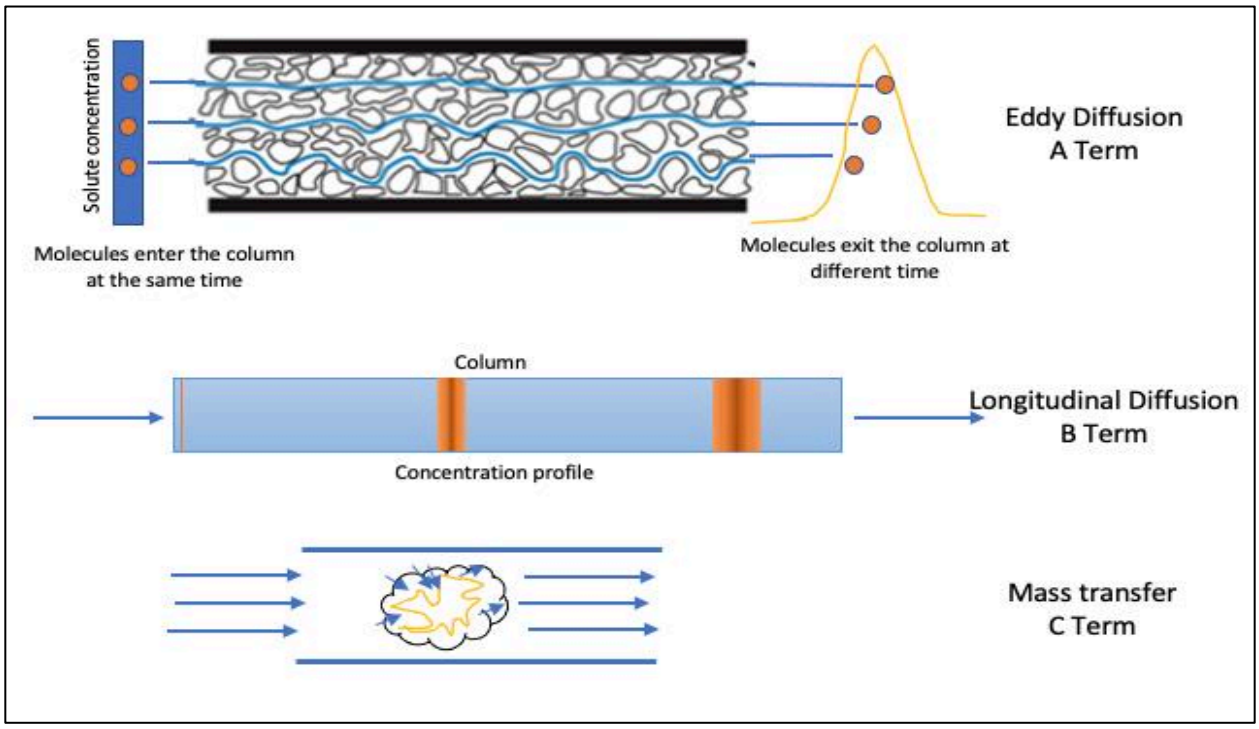


Figure 5- 2 A, B, and C terms for Van Deemter equation

Split analysis is used to protect column from getting contaminated and preventing the overloading of the column. Splitless injection is used for the trace analysis of the samples or where analyte concentration is very low.

5.1.2 GC Detectors: time-of-flight MS (TOFMS)

Analytes separated and eluted from the column are directed into detectors to be detected. In GC, analytes are vaporized and are in the gaseous form when arrive at the detector, therefore fast detection system with higher sensitivity, specificity, and accuracy is imperative. There are several types of detectors that can be coupled to GC. The choice of the detector depends on the requirement of the analysis and type of analytes. In this study, GC coupled to time-of-flight MS (TOFMS) was used. For mass spectrometry detection analytes need to be thermally stable and volatile. MS operates under high vacuum (10^{-5} to 10^{-7} torr) to prevent molecule collision. MS is a highly sensitive and universal detector; it is used for both quantification and qualification purposes. MS provides structural and molecular information and ionization, or fragmentation pattern that are highly reproducible. Like any MS used in LC, MS used in GC have very similar components except the interface. For the GC-MS interface, atmospheric pressure is not required. Mass spectrometer has three main components: ionization source, mass analyzer, and a detector. The types of components utilized in this study are discussed in the following sections.

5.1.2.1 Ionization source

The MS used in this study was equipped with an electron impact (EI) ionization source. EI is a hard ionization technique in which, analytes are bombarded with beam of electrons at 70 eV from a tungsten filament. Excitation and ionization of the analyte molecules occurs causing it to

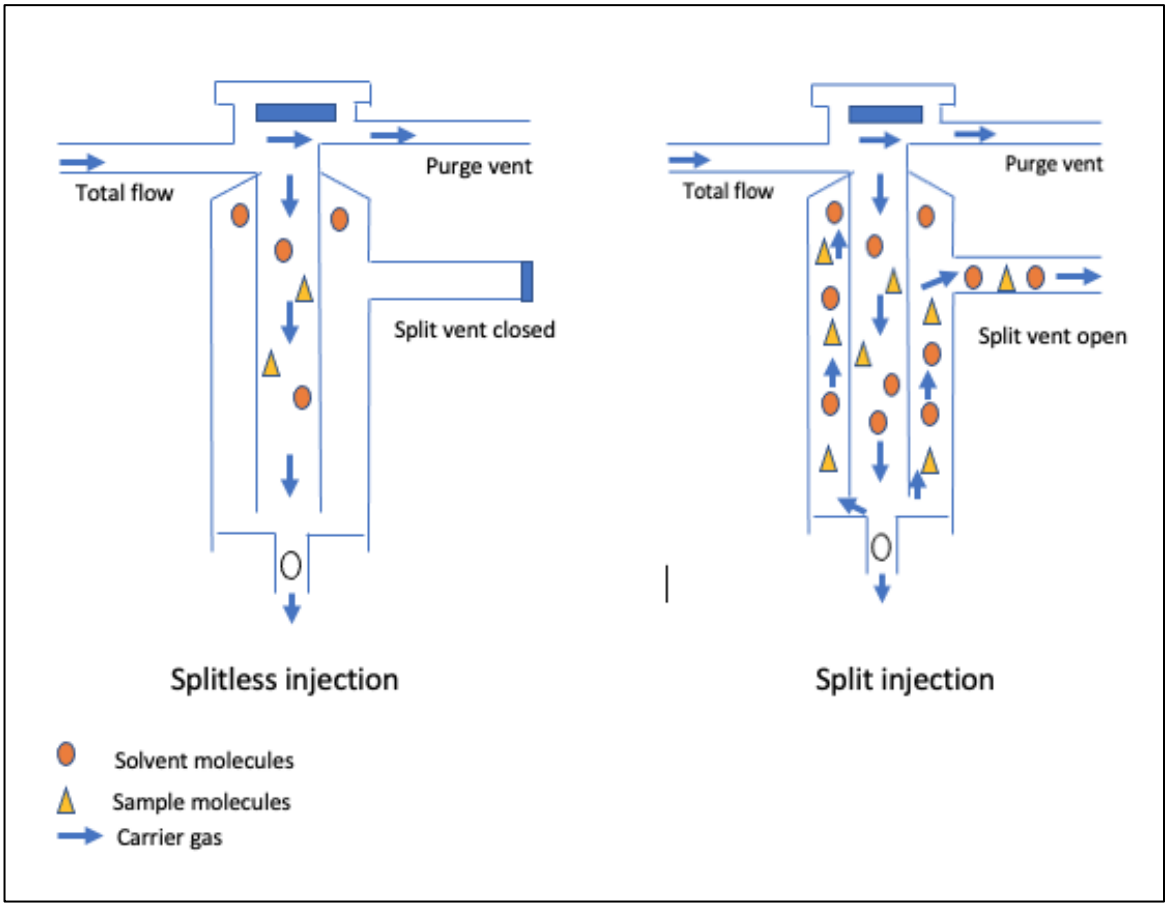


Figure 5- 3 Split and splitless inlets

fragment due to high energy electron impact.[41] Ion source is heated and under vacuum to provide vaporized ions. Figure 5-4 shows the schematic of EI source, as shown in the Figure analytes in vapor form eluting from column enter the source and ionized and further fragmented by electron beam impact and positively charged ions are focused to exit the source to enter the mass analyzer.

5.1.2.2 Mass analyzer: Time of flight mass spectrometer

Upon ionization by the into ion source, charged ions are moved into the mass analyzer. Ions are separated based on the mass to charge ratio by means of electric field in the mass analyzer. As discussed earlier in chapter 1, there are several types of mass analyzers such as quadrupole, time of flight mass analyzer, ion trap mass analyzer. In the Studies covered under this chapter the time of flight (TOF) mass analyzer was used which is discussed in this section.

Figure 5-5 shows the schematic of a time-of-flight mass analyzer. In TOF-MS analytes are analyzed based on the kinetic energy and the time it takes to travel the fixed distance. All ions entering the mass analyzer have potential energy, which is accelerated by the repelled voltage and converted into kinetic energy. All ions attain the same kinetic energy, but ions with smaller mass to charge ratios travel faster than that of the ions with larger mass to charge ratios. The length of the flight tube is typically 1 meter in TOF and following equation 5-5 and 5-6 show relation between kinetic energy and mass.[45]

$$Kinetic\ energy\ (KE) = {}^{1}/_{2}\ mv^{2} \qquad \text{Equation 5-5}$$

$$Potential\ energy = qV \qquad \text{Equation 5-6}$$

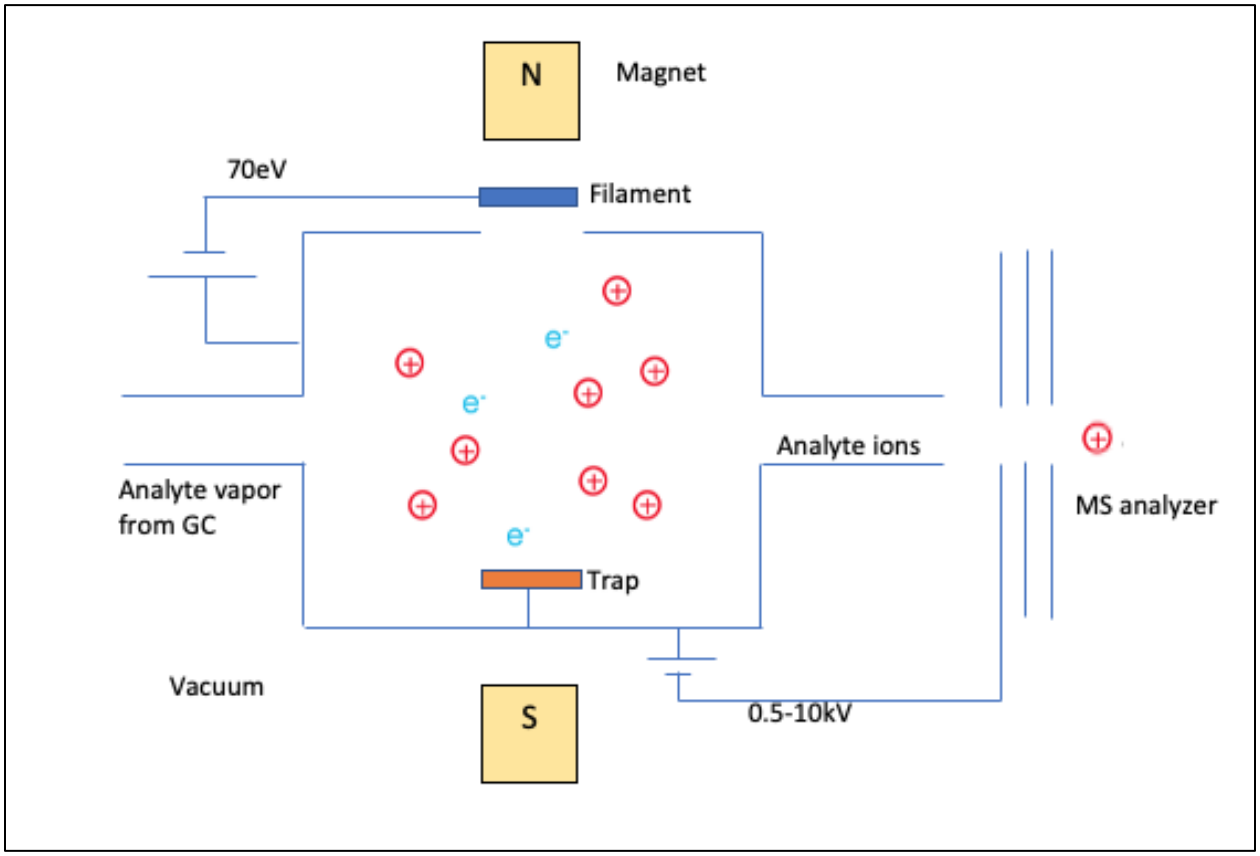


Figure 5- 4 Electron ionization (EI) source.

In above equations, m is the mass and v is the velocity of the ion. q is charge and V is accelerating potential. Above two equations are combined into equation 5-7 and then it is solved for time, in velocity which is distance divided by time in equation 5-8 and equation 5-9 is obtained.

$$qV = {}^{1}/_{2}\, mv^2 \qquad \text{Equation 5-7}$$

$$v = L/t \qquad \text{Equation 5-8}$$

$$t = L\sqrt{\frac{m}{2Vq}} \qquad \text{Equation 5-9}$$

In equation 5-9, t is the time an ion spends in the flight tube and L is the length of the tube. The time each ion take into flight tube is respective to their masses. TOF mass analyzer is sensitive and fast.

5.1.2.3 Detector

Ions separated by the mass analyzer enters the detector to be detected. The detector used in this study is an electron multiplier. An electron multiplier detector uses the phenomena of secondary electron emission. When a charged ion strikes a surface, more and more secondary electrons are generated from atoms in the surface layer until all generated electrons reach end of the dynode to produce a signal.

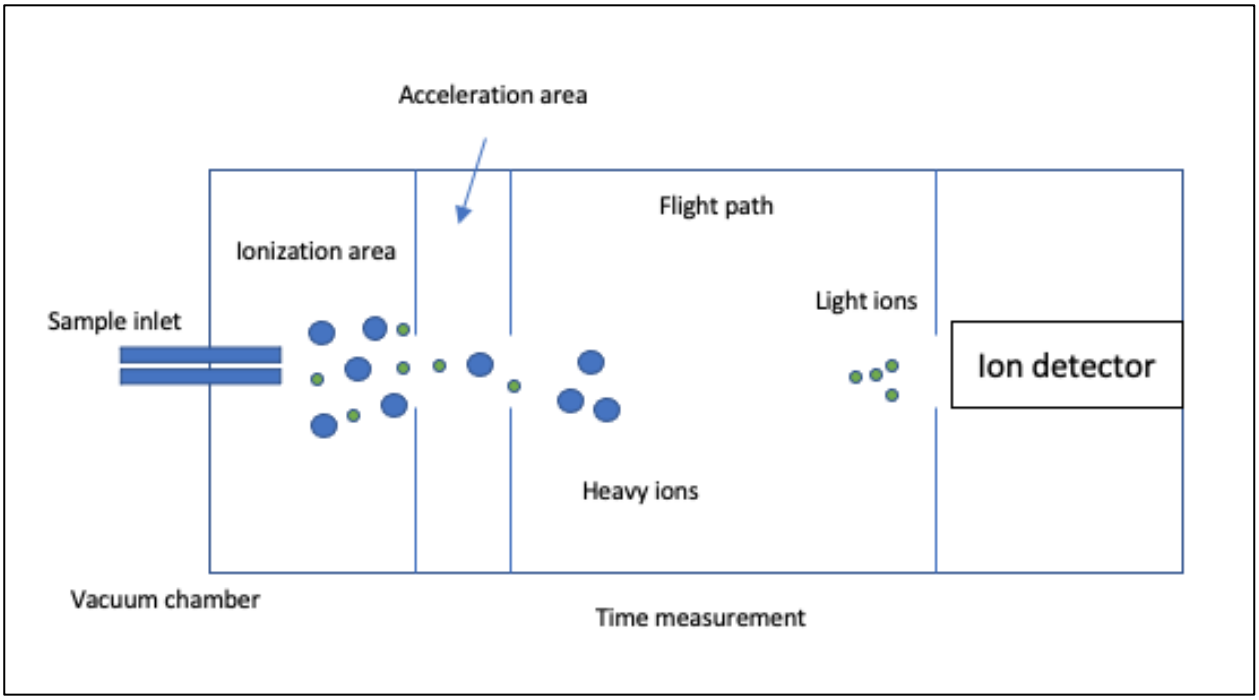


Figure 5- 5 Schematic of time-of-flight mass analyzer

Adapted from: Dr. Deepak, Mass discrimination and analysis in mass spectrometry, lab-training.com[103]

5.2 Materials and methods

5.2.1 Chemicals, reagents, and samples

Glucocorticoids, beclomethasone, cortisone acetate, prednisone, hydrocortisone, prednisolone, dexamethasone, and methylprednisolone were purchased from Sigma-Aldrich (St Louis, MO) and used as purchased.

Reagents for PIE such as HPLC grade water, acetonitrile and methanol were purchased from Fisher Scientific (Bridgewater, NJ). Glycerol purity of >99.5% was purchased from Sigma-Aldrich (St Leuis, MO). A Milli-Q Plus purification system, (Millipore, Milford, MA) was used to obtain Ultra-pure water in the laboratory.

5.2.2 PIE Sample preparation

PIE procedure steps were followed as, 5 mL of aqueous standard or sample was added to 15-mL, high-density polyethylene conical tubes with screw caps. Then 5 mL of acetonitrile was added into the tube and the tube was vortexed for 2 min. 2 mL glycerol was added for each extraction into the tube and the tube was vortexed again for 5 min. Tubes were then equilibrated for 30 min at 0° temperature. After the two phases separated, the volume of each phase was recorded, and the upper organic phase was recovered for analysis of glucocorticoids into GC vial for analysis via splitless injection using GC-TOFMS. A total seven glucocorticoids - beclomethasone, cortisone acetate, prednisone, hydrocortisone, prednisolone, dexamethasone, and methylprednisolone were evaluated in the study of the polyol induced extraction.

5.2.3 Instrumentation

The instrument utilized for this study was a LECO (St. Joseph, MI) Pegasus 4D GCxGC-TOFMS with a Gerstel (Columbia, MD) Autosampler. The instrument was utilized in one dimensional mode and all standards and samples were injected 1µl as splitless. The split purge was opened after 2 minutes. The GC analysis was performed on RTX-5MS 15m x 0.25mm x 0.25µm column using helium as a carrier gas. Column flow was kept constant at 1.0 mL/min and the inlet temperature set at 250 °C. The temperature program was kept at an initial temperature of 150 °C held for 1 minute; 15 °C/minute ramp to 300 °C and hold for 10 minutes with detector parameters as following: EI source set at 250 °C and transfer line set at 280 °C.

5.2.4 Experimental parameters

5.2.4.1 Calibration and linearity

Stock solutions at 1 mg/mL for all seven glucocorticoids were prepared in methanol and the working standards for PIE were prepared by diluting the stock solutions in water to produce concentration ranging 25 ppm to 200 ppm for beclomethasone, prednisolone, prednisone, methylprednisolone, dexamethasone, hydrocortisone and 100 ppm to 500 ppm for cortisone acetate. These standards were analyzed in triplicate and calibration curve for each was prepared. The R^2 value and equation of the line were obtained using Excel for all calibration curves. Limit of quantitation (LOQ) and limit of detection (LOD) were assessed using the data generated for each peak. Once all data were compiled and calibration curves were constructed LOD and LOQ were determined for each peak.

5.2.4.2 Recovery, precision, accuracy, and partition coefficient

Three different glucocorticoid mixture samples at 100ppm for cortisone acetate, and 50ppm for each of the remaining compounds were prepared by diluting into water. Samples were then subjected to the PIE method as discussed above and analyzed in duplicate using GC. All data generated were used in the determination of percent recovery, precision, accuracy, and partition coefficient using the equation of the line from the calibration curve to find concentration from the peak area of each. Once determined, these values were divided by the appropriate concentration and multiplied by 100 to get percent recovery. An average of all three was reported as percent recovery. Accuracy was determined from percent recovery of an average value. Precision was calculated as %RSD of all three preparations.

$$\%Recovery = \left(\frac{Experimentally\ determined\ concentraton}{Initial\ Concentration}\right)100 \qquad \text{Equation 5-10}$$

$$Accuracy: \%error = \left(\frac{100-\%\ Recovery}{100}\right)100 \qquad \text{Equation 5-11}$$

Once all concentrations were obtained partition coefficients were estimated by using the following equations.

$$C_{org} = \frac{Peak\ response-c}{m} \qquad \text{Equation 5-12}$$

Where, m = slop and c = intercept from calibration curve line equation.

$$m_{org} = (C_{org})(v_{org})$$

Equation 5-13

$$m_{aq(initial)} = (c_{aq})(v_{aq})$$ Equation 5-14

$$m_{aqu\,(final)} = m_{aq(initial)} - m_{org}$$ Equation 5-15

$$c_{\,aq} = \frac{m_{aq}}{v_{aq}}$$ Equation 5-16

$$Where, m = mole, aq = aqueous, org = organic, c = concentration, v = volume$$

$$K = \frac{C_{Organic}}{C_{aqueous}}$$ Equation 5-17

5.3 Results and discussion

Each analyte was prepared at 1 mg/mL and further diluted to appropriate concentrations by mixing each individual standard solution. Each glucocorticoid was injected separately first to identify the retention time and fragmentation pattern. Once each analyte was identified it was verified by searching into inbuilt libraries supplied by the manufacturer. Table 5-1 summaries the retention time and fragmentation ions for each glucocorticoid analyzed. In electron ionization high energy electron beam removes valance electrons to produce radical cations. Because of the high energy radical cation quickly breaks into smaller fragments containing cations and neutrals. Only cations and unfragmented molecular ions are detected in the detector. In the situation where molecular ions are not stable, the molecular ion peak known as the parent peak is not observed.

Glucocorticoid	Retention time (min)	Ion
Beclomethasone	10.483	121; 77; 91
Cortisone acetate	10.710	122; 107; 77; 79
Prednisone	10.802	121; 77; 91; 93
Hydrocortisone	11.283	163; 147; 121; 105; 145
Prednisolone	11.466	122; 107; 77; 79
Dexamethasone	11.750	160; 145; 127; 115
Methylprednisolone	11.843	136; 121; 77; 79

Table 5- 1 Identification summary of glucocorticoids

The most abundant fragment ions in the spectra corresponding the analyte are listed. Fragment ions related to groups show the similarity in the structures for glucocorticoids.

Chromatograms of glucocorticoids are shown in Figure 5-6 and 5-7 for standard and spiked sample respectively. No peaks were observed or detected for non-spiked water samples. It was difficult to detect cortisone acetate and fludrocortisone acetate, so their concentrations were increased to visualize them better. Cortisone acetate and fludrocortisone acetate have more complicated structures including acetate group which could be a reason for the lower to no response using liquid injections as these groups are more prone to degradation in the inlet. Fludrocortisone acetate was omitted from this study for that reason. Although beclomethasone and dexamethasone have a methyl group in common, beclomethasone has the lowest melting point among all glucocorticoids in this study. Elution order for closely eluting peaks of analytes, hydrocortisone, prednisolone, dexamethasone, and methyl prednisolone can be explained by understanding their chemical structures. Hydrocortisone is also known as cortisol which is the backbone of glucocorticoids and prednisolone is which like hydrocortisone. Dexamethasone is fluorinated at C_9 position, Methylprednisolone is a prednisolone derivative, and both compounds are structurally similar to hydrocortisone and prednisolone. Conditions were optimized to achieve the best possible separation. There was a partial co-elution between dexamethasone and methylprednisolone. The partial co-elution can be solved in running the method in 2D mode. Calibration curves for each compound with a line of equation and R^2 values are shown in Figure 5-8 and are summarized in Table 5-2. R^2 values for all compounds were calculated and found >0.99. However, it was noted that lines were linear in the range, but the intercepts were non-zero. This may have been occurred due to non-linear behavior near zero or at lower concentrations or odd signal response generated by the instrument.

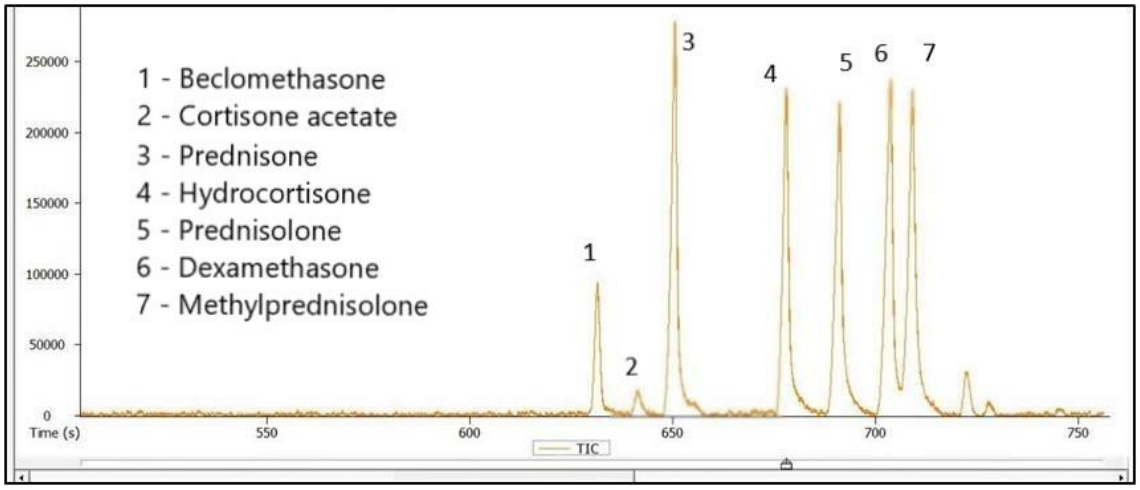


Figure 5- 6 Chromatogram for all seven PIE glucocorticoids standard

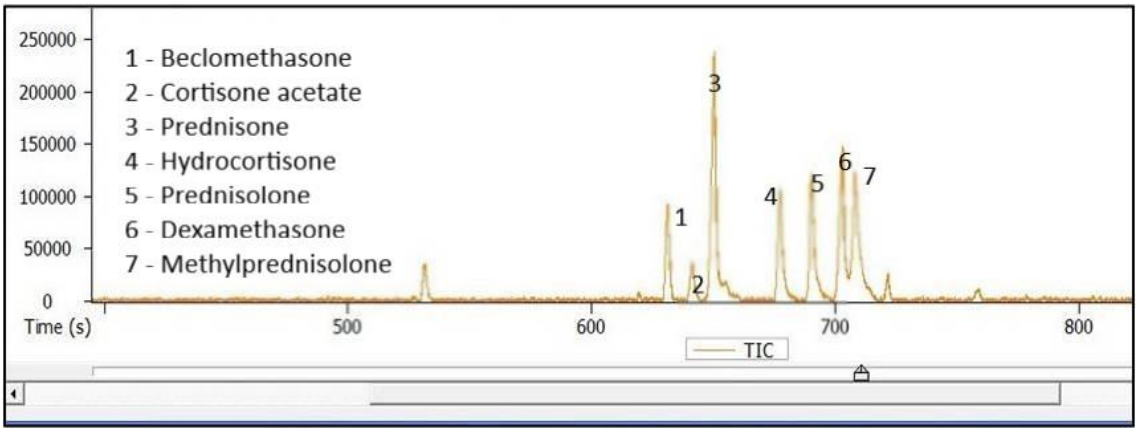


Figure 5- 7 Chromatogram for PIE glucocorticoids Spiked sample

50pppm for all glucocorticoids except 100ppm for cortisone acetate

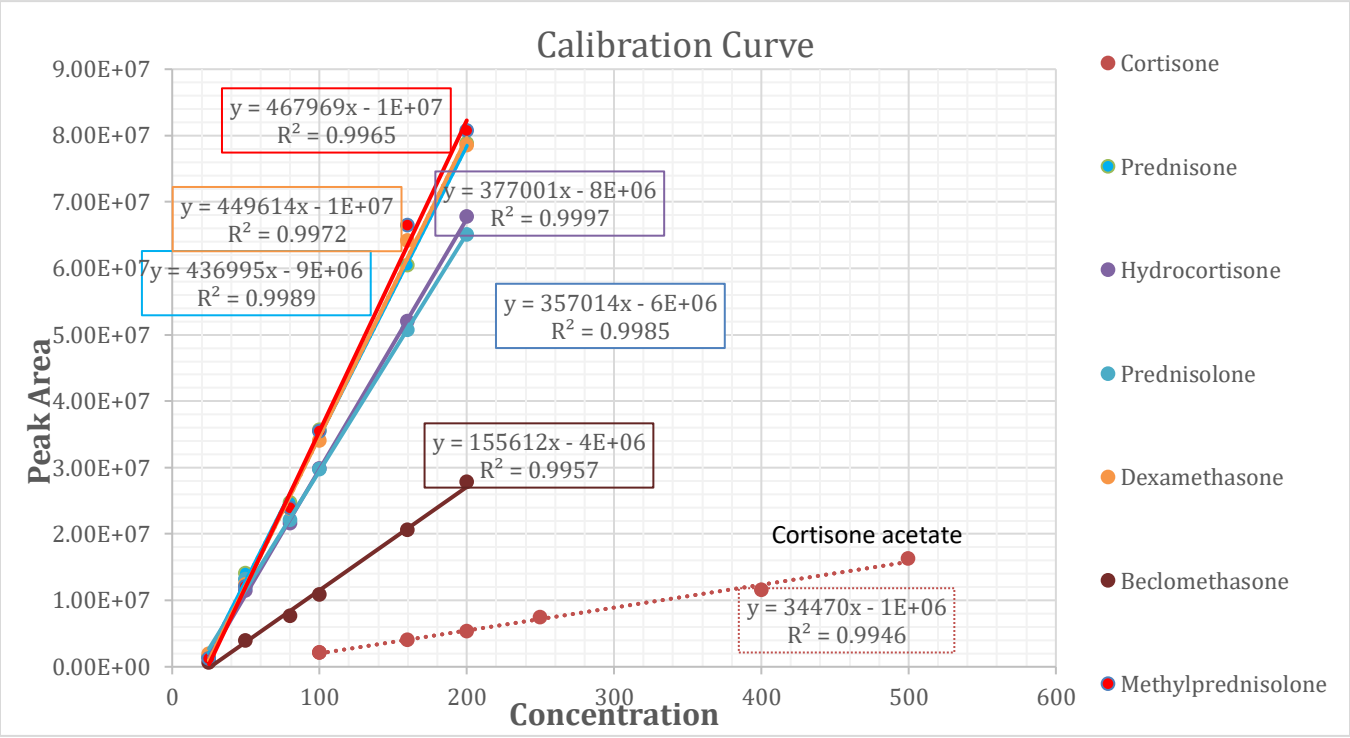


Figure 5- 8 Calibration curves of glucocorticoids PIE standards

Glucocorticoids	Calibration ppm	LOD (ppm)	LOQ (ppm)	R^2	Conc Level ppm	%Recovery (n=3)	Accuracy % error	Precision %RSD	Partition Coefficient
Beclomethasone	200-25	12	39	0.996	50	92	8.1	1.3	3.28
Cortisone Acetate	500-100	37	100	0.995	100	97	2.9	2.3	3.76
Prednisone	200-25	6	20	0.999	50	95	5.4	3.7	3.51
Hydrocortisone	200-25	3	11	0.999	50	94	6.2	3.2	3.44
Prednisolone	200-25	7	23	0.999	50	96	3.7	1.5	3.66
Dexamethasone	200-25	10	31	0.997	50	97	3.2	3.8	3.71
Methylprednisolone	200-25	10	35	0.997	50	96	4.5	1.9	3.58

Table 5- 2 Result summary of glucocorticoids extraction form water using PIE

All seven glucocorticoids calibration curves are plotted on the same graph. Compounds having similar response showed similar slope of the calibration curve. The beclomethasone signal response was lower compared to other analytes. The cortisone acetate calibration curve was in the range of 100 to 500ppm due to lower detection of the analyte. LOD and LOQ were calculated for each glucocorticoid and are compiled in Table 5-2. Calculated LOD for all analytes ranged from 3ppm to 37ppm and LOQ from 11ppm to 100ppm. All standards were analyzed in triplicate, %RSD for each compound was less than 4.0%. LOD and LOQ were calculated using the equation 5-18.

Where, k = 3 for LOD and k = 10 for LOQ. In this case, LOD and LOQ calculations were based off the standard deviation of the y intercept in the calibration curve, considering the observed uncertainty on calibration curves Figure 5-8.

$$C_L = \frac{ks_{y-int}}{m} \qquad \text{Equation 5-18}$$

To test PIE as an alternative method of extraction, experimental parameters for all 7 glucocorticoids were studied. Obtained experimental data are described above in Table 5-2. Phase separation was observed in all samples following the addition of glycerol. As explained in Chapter 2, initial volumes of the mixture of 5 mL of acetonitrile and 5 mL of water with 2 mL of glycerol, followed by mixing and equilibration at 0 °C. The total volume of the liquids prior to mixing was 12 mL, but the final volume of the mixture was 11 mL with 3 mL of organic phase and 8 mL of aqueous phase, indicating that volume is nearly but not fully conserved, as is typical when mixing polar liquids. It is clearly seen that temperature has a dramatic impact on the final volume of the aqueous and organic phases. Recorded volumes were used in the calculation for partition coefficient and percent recovery of all seven glucocorticoids. Obtained results shows

partition coefficients >1 for all seven compounds. Results show the trend as higher the partition coefficient higher the % recovery. Partition coefficients for all analytes were obtained around 3 and these results may explain the calibration curve difference and %recovery of glucocorticoids against non-extracted standards in Chapter 3 for some analytes. Cortisone acetate, beclomethasone and dexamethasone showed higher curve difference and% recovery in comparison to non-extracted standard similarly, cortisone acetate and dexamethasone showed highest partition coefficients of 3.76 and 3.71 respectively. However, the behavior of beclomethasone showing lowest partition coefficient is not conclusive and appropriate determination of partition co-efficient would need the analysis of the separated aqueous phase as well.

Percent recoveries were obtained in the range from 92 to 97%. For all seven glucocorticoids the partition coefficient at 0 °C were >1. The percent RSD for three different samples was performed for each glucocorticoid as a precision and summarized between 1.3 to 3.8%. Accuracy as % error was also performed on all samples for each glucocorticoid with the average values ranging between 2.9 to 8.1%.

Several samples of environmental and tap waters collected from the environs of our laboratory and were subjected to the PIE procedure. All the samples were negative for all glucocorticoids. Based on the calibration data, PIE-GC-TOFMS as performed here appears to not include a sufficient concentration step for analysis of drugs in environmental water that may be present at sub-ppb levels. However, the method still shows potential for the analysis of glucocorticoids at clinical (ppm) and pharmaceutical (ppm and higher) concentrations. The method was optimized to achieve best separation possible.

5.4 Conclusion

In this study, polyol induced extraction based on acetonitrile/aqueous mixture and using glycerol as mass separating agent is summarized. A novel approach to use PIE as an extraction technique for steroids is a first step in using this technique as an ecofriendly technique in many ways. All glucocorticoids present in the water-acetonitrile mixture were partitioned into organic phase at 0 °C followed by addition of glycerol. All seven glucocorticoids have partition coefficients >1 and percent recoveries >90%. At lower temperature higher the phase separation leads to higher partition coefficient and higher percent recovery.

CHAPTER 6 – OVERALL CONCLUSION AND FUTURE WORK FOR PIE

6.1 Overall conclusion

At a conclusion of this work, polyol induced extraction (PIE) has showed an amazing potential to be an extraction technique for drugs, especially glucocorticoids and NSAIDs. Chapter 1 discussed a basic introduction to extraction methods, chromatographic methods, and mass spectrometry. Detailed theory and methodology of PIE is also explained to better understand the procedure.

In Chapter 2, PIE was used to extract glucocorticoids from water and analyzed by UHPLC-MS/MS. Glycerol was used as a mass separating agent to induce phase separation in aqueous-organic mixtures (1:1). PIE of glucocorticoids was evaluated in terms of extraction condition optimization, where 0 °C, 30 minutes equilibration time and 2 mL amount of glycerol were determined to be optimized conditions for this study. Phase separation with respect to temperature showed that PIE is spontaneous process and exothermic reaction. A mixture of glucocorticoids in water was partitioned into acetonitrile at 0° C by addition of glycerol to the water/acetonitrile mixture. All eight glucocorticoids showed acceptable percent recovery, and accuracy. Intraday and inter-day precision results as% RSD for all compounds were less than 2.5%, which shows the method being reproducible and accurate.

In Chapter 3, PIE is compared to QuEChERS. Moreover, other literature methods to extract glucocorticoids were also compared with PIE data in terms of recoveries, LOD and time needed to perform the method as well as types of organic solvents used in those literature methods. PIE

glucocorticoids standard peak areas were also compared to non-extracted standards prepared over the same range and calibration curves were compared. From the results obtained, it is seen that PIE is a comparable technique to QuEChERS, and other methods published in the literature. PIE can extract each glucocorticoid from the water into the organic phase in a very good amount. Mostly, in an extraction procedure, the organic phase is only considered to be the phase of interest. All the reagents used in PIE can potentially be recycled. PIE is very simple method with minimum steps to follow. All these great points are leading to conclude PIE as cost effective and user-friendly extraction technique.

In Chapter 4, PIE was evaluated for the extraction of NSAIDs from water. Upon analysis of water samples, PIE was used to extract NSAIDs from urine samples. From the experiments performed, it was concluded that PIE can be a potential extraction technique for various kinds of drug classes as well as different matrices, with proper optimization. PIE of urine samples showed equivalent phase separation ratio despite the lower volume content. Without any pH optimization, urine samples showed recoveries in the range from 6 to 60% depending upon the analyte.

In Chapter 5, glucocorticoids were extracted using PIE and analyzed by GC-TOFMS. The obtained data are very reasonable for the extraction. And it can be concluded that PIE is the extraction method that can be compatible with both liquid and gas chromatography.

6.2 Future work for PIE

The dynamics of PIE as a liquid-liquid extraction have not been explored previously. The possible future applications of this technique are varied and should be studied further. PIE can be an alternative for the extraction of drugs of abuse in biological samples. The work performed in this thesis using PIE is applicable to many types of drug compounds. PIE can also be studied for non-drug molecules as well. This method is compatible with both liquid and gas chromatography, and it can be an alternative for the QuEChERS method. As GC instrumentation is improving, the necessity to derivatize drugs is lessening, and it was seen in this work by analyzing steroidal drugs using GC without any derivatization. For ionizable analytes PIE can be pH optimized for better recovery if needed depending upon the type of analytes and the sample matrix. Apart from biological samples PIE may also be extended to trace analysis in food samples.

Another area of interest that can be mentioned here would be the use of PIE in a larger scale of waste removal plant or water treatment plant. In this application PIE can be used on very big scale to remove drug or other small molecules from the waste sediments or for removal of impurities organic impurities from wastewater in water treatment plants. On an opposite note, PIE may also be developed for microextraction. PIE in this work utilized 5 mL of each both aqueous and organic solvent, but if the volumes are reduced equally PIE can be performed on a micro level as well to study samples that are very small in size or quantity. Exploring those possible application of PIE may provide an eco-friendly alternative extraction method in the times of green chemistry.

REFERENCES

1. Wells, M. Sample preparation techniques in analytical chemistry, John Wiley and sons: New York, 2003.
2. Roberts, R. M.; Gilbert, J. C.; Rodewald, L.B.; Wingrove, A. S. Modern experimental organic chemistry, 4th ed., Saunders College, 1985.
3. Pereira, J. F. B.; Coutinho, J. A. P. Liquid phase extraction, Ch 5 Aqueous two-phase systems, Elsevier Inc, 2020, 157-182.
4. Asenjo, J. A.; Andrews, B. A. Aqueous two-phase systems for protein separation: a perspective. *J. Chromatography A,* 2011, 1218, 8826-8835.
5. Barbosa, M.P.; Souza, R. L.; Fricks, A. T.; Zanin, G.M.; Soares, C. M. F.; Lima, A. S. Purification of lipase produced by a new source of bacillus in submerged fermentation an using aqueous two-phase system. *J. Chromatography B,* 2011, 879, 3853-3858.
6. Luechau, F.; Ling, T. C.; Lyddiatt, A. Primary capture of high molecular weight nucleic acids using aqueous two-phase systems. *Sep. purif. technol*, 2009, 66, 202-207.
7. Marques, C. F. C.; Mourão, T.; Neves, C. M. S. S.; Lima, A. S.; Boal-Palheiros, I.; Coutinho, J. A. P.; Freire, M.G. Aqueous biphasic systems composed of ionic liquids and sodium carbonate as enhanced routes for the extraction of tetracycline. *Biotechnol. Prog.,* 2013, 29, 645-654.
8. Reis, I. A. O.; Santos, S. B.; Santos, L. A.; Oliveira, N.; Freire, M. G.; Pereira, J. F. B.; Ventura, S. P. M.; Coutinho, J. A. P.; Soares, C. M. F.; Lima, A. S. Increased significance of food wastes: selective recovery of added-value compounds. *Food chem*., 2012, 135, 2453-2461.
9. Passos, H.; Trindade, M. P.; Vaz, T. S. M.; Costa, L. P.; Freire, M. G.; Coutinho, J. A. P. The impact of sel-aggregation on the extraction of biomolecules in ionic-liquid based aqueous two-phase systems. *Sep. Purif. Technol*., 2013, 108, 174-180.
10. Sowa, J. R.; Murphy, W. R. Jr.; Deshpande, M. Polyol-induced extraction of water from organic liquids. U.S. Patent US 20140263050 A1, Sep 18, 2014.
11. Grolbmann, C.; Tintiger, R.; Zhu, J.; Maurer, G. Aqueous two-phase systems of poly (ethylene glycol) and dextran- experimental results and modeling of thermodynamic properties. *Fluid phase equilib*., 1995, 106, 111-138.

12. Zhao, X.; Xie, X.; Yan, Y. Liquid-liquid equilibrium of aqueous two-phase systems containing poly (propylene glycol) and salt ((NH4)2SO4, MgSO4, KCl, and KAc): experiment and correlation. *Thermochim. Acta*., 2011, 516, 46-51.
13. Freire, M. G.; Cláudio, A. F. M.; Araújo J. M. M.; Coutinho, J. A. P.; Marrucho, I. M.; Lopes, J. N. C.; Rebelo, L. P. N. Aqueous-biphasic systems: A boost brought about by using ionic liquids. *Chem. Soc. Rev*., 2012, 41, 4966-4995.
14. Wang, B.; Ezejias, T.; Feng, H.; Blaschek, H. Sugaring-out: a novel phase separation and extraction system. *Chem. Eng. Sci.,* 2008, 63, 2595-2600.
15. Renard, J. A.; Oberg, A. G. Ternary systems: Water-Acetonitrile-Salts. *Journal of Chemical and Engineering Data*, 1965, 10, 152-155.
16. Renard, J. A.; Heichelheim, H. R. Ternary systems: Water-Acetonitrile-Salts. *Journal of Chemical and Engineering Data*, 1967, 12, 33-36.
17. Tabata, M.; Kumamoto, M.; Nishimto, J. Chemical Properties of Water-Miscible Solvents Separated Salting-out and Their Application to Solvent Extraction. *Analytical sciences*, 1994, 10, 383-388.
18. Wand, B.; Feng, H.; Ezeji, T.; Blaschek, H.; Sugaring-out separation of acetonitrile from its aqueous solution. *Chem. Eng. Technol*., 2008, 31, 1869-1874.
19. Dhamole, P.B.; Mahajan, P.; Feng, H. Phase separation conditions for sugaring out in acetonitrile-water systems, *J. Chem. Eng. Data*, 2010, 55, 3803-3806.
20. Periero, A. B.; Araujo, J.M.; Esperanca, I.M.; Marrucho, I. M.; Rebelo, L. P. N. Ionic liquids in separations of azeotropic systems - A review. *J. Chem. Thermodynamics*, 2010, 46, 2–28.
21. Waltermann, T.; Grueters, T, Muenchrath D.; Skiborowski, M. Efficient optimization-based design of energy-integrated azeotropic distillation processes. *Computers and chemical engineering*, 2020, 133, 106676.
22. Deshpande, M. Polyol induced extraction of water from organic solvents. Dissertation, Seton Hall University, 2014, 39-75.
23. Tsai, W.; Chuang, H.; Chen, H.; Wu, Y.; Cheng, S.; Huang, T.; Application of sugaring-out extraction for the determination of sulfonamides in honey by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. A,* 2010, 1217, 7812–7815.

24. DelMastro, T.; Snow, N. H.; Murphy, W. R.; Sowa, J. R. Polyol-induced partitioning of essential oils in water/acetonitrile solvent mixtures. *Journal of liquid chromatography & related technologies*, 2017, 40-7, 376–383.
25. Takamuku, T.; Tabata, M.; Yamaguchi, A.; Nishimoto, J.; Kumamoto, M.; Wakita, H.; Yamaguchi, T. Liquid Structure of Acetonitrile–Water by X-ray Diffraction and Infrared Spectroscopy. *J. Phys. Chem. B*, 1998, 102, 8880–8888.
26. Embuscado, M. E. Optimizing sweet taste in foods, Polyols, Chapter 8, McCormick & Company, Inc., USA, 2006, 153-174.
27. Kazakevich, Y.; LoBrutto, R. HPLC for pharmaceutical scientists, HPLC Theory and practice, Chapter 1. John wiley and sons inc., 2007.
28. Czaplicki, S. Chromatography in Bioactivity Analysis of Compounds. *Column Chromatography*, 2013, doi:10.5772/55620.
29. Kazakevich, Y.; LoBrutto, R. HPLC for pharmaceutical scientists, HPLC Theory and practice, John wiley and sons inc., 2007.
30. Schug, K. A. The Role of Selectivity in Liquid Chromatography Method Development. Pure chromatography, The Restek® Advantage, 2015.1, restek.com, (accessed Jan-25, 2022).
31. Said, A. S. Resolution, separability, and conditions for fraction collection in preparative gas chromatography. *J. Gas Chromatogr.,* 1964, 2, 60–71.
32. Swartz, M. HPLC detectors: A brief review. *Journal of liquid chromatography and related technologies*, 2010, 33, 1130-1150.
33. Ashrani, S.; Goyal, A.; Vaishnav, R. HPLC detectors, their types and use: A review Organic and medicinal chemistry. *International journal*, 2018,6, 5: 555700.
34. HPLC basic course, section 8, principle, and feature of various detection methods. Hitachi, www.hitachi-hightech.com/global/products/science/tech/ana/lc/basic/course8.htmL (accessed Jan-25, 2022).
35. Pitt, J. J.; Principles and applications of liquid chromatography mass spectrometry in clinical biochemistry. *Clin. Biochem. Rev.*, 2018, 30 (1), 19-34.
36. Nikalje, P. A.; Ramesh, G. Liquid chromatography mass spectrometry and it applications: A brief review. *Arc Org Inorg Chem Sci*, 2018, 1 (1), 26-34.

37. Basics of LC/MS, A primer, Agilent technologies. https://www.agilent.com/cs/library/support/documents/a05296.pdf (accessed Jan-26, 2022).
38. Comparison of LCMS and other techniques, Shimadzu https://www.shimadzu.com/an/service-support/technical-support/analysis-basics/fundamental/comparison_of_lcms.htmL (accessed on Jan-26, 2022)
39. Kazakevich, Y.; LoBrutto, R. HPLC for pharmaceutical scientists, LC/MS: Theory, instrumentation and applications to small molecules and practice, John wiley and sons inc., 2007.
40. Fundamentals guide to LCMS, LCMS primer, chapter 2, Shimadzu, 2019, 13-25.
41. Hoffmann, E.; stroobant, V. Mass spectrometry principles and applications, chapter 1, ion sources, 3rd edition, Wiley publications, 2007.
42. Gates, P. Electrospray ionization (ESI): Figure 2, University of Bristol, http://www.chm.bris.ac.uk/ms/esi-ionisation.xhtmL (accessed Jan - 26, 2022).
43. Liigand, P.; Heering, A.; Kaupmees, K.; Leito, I.; Girod, M.; Antoine, R.; Kurve, A. The evolution of electrospray generated droplets is not affected by ionization mode. *Journal of The American Society for Mass Spectrometry*, 2017, 28 (10), 2124 - 2131.
44. Liigand, J.; Laaniste, A.; Kurve, A. pH effects on electrospray ionization efficiency. *Journal of the American society of mass spectrometry*, 2017, 28, 461-469.
45. Hoffmann, E.; stroobant, V. Mass spectrometry principles and applications, chapter 2, Mass analyzer, 3rd edition, Wiley publications, 2007.
46. Fundamentals guide to LCMS, LCMS primer, Chapter 3, Shimadzu, 2019, 27-39.
47. Dass, C. Fundamentals of contemporary mass spectrometry, John Wiley and sons, New York, 2007.
48. Harvey, D. Mass spectrometry, overview. *Encyclopedia of analytical science*, 2005, 3 (6), 337-344.
49. Blasco, C. Pico, Y. Liquid chromatography-mass spectrometry, Food toxicants analysis, chapter 14, Elsevier, 2007.
50. Ho, C. S.; Lam, C. W.; Chan, M. H.; Cheung, R. C.; Law, L. K.; Lit, L. C.; Ng, K. F.; Suen, M. W.; Tai, H. L.; Electrospray ionization mass spectrometry: principles and clinical applications. *The Clinical biochemist. Reviews*, 2003, 24 (1), 3–12.

51. Sleno, L.; Volmer, D. A. Ion activation methods for tandem mass spectrometry. *Journal of mass spectrometry*, 2004, 39 (10), 1091-1112.
52. Nozaki, O. Steroid analysis for medical diagnosis. *J. Chromatogr A,* 2001, 935, 267–278.
53. Timmermans, S.; Souffriau J.; Libert, C. A general introduction to glucocorticoid biology. *Front. Immunology*, 2019, 10, 1545.
54. Gong, J.; Lin, C.; Xiong, X.; Chen, D.; Chen, Y.; Zhou, Y.; Wu, C.; Du, Y. Occurrence, distribution, and potential risks of environmental corticosteroids in surface waters from the Pearl River Delta, South China. *Environmental pollution*, 2019, 251, 102-109.
55. Goh, S. X. L.; Goh, H. A.; Lee, H. K. Automation of ionic liquid enhanced membrane bag-assisted-liquid-phase micro extraction with liquid chromatography-tandem mass spectrometry for determination of glucocorticoids in water. *Analytica Chimica Acta*., 2018, 1035, 77-86.
56. Schriks, M.; van Leerdam, J. A.; van der Linden, S. C.; van der Burg B.; van Wezel, A. P.; de Voogt, P. High-Resolution mass spectrometric identification and quantification of glucocorticoid compounds in various wastewaters in The Netherlands. *Environ. Sci. Technol.,* 2010, 44, 4766-4774.
57. Shen, X.; Chang, H.; Sun, Y.; Wan, Y. Determination and occurrence of natural and synthetic glucocorticoids in surface water. *Envirno. International*, 2020, 134, 105278.
58. Herrero, P.; Borrull, F.; Pocurull, E.; Marce, R. M. Determination of glucocorticoids in sewage and river waters by ultra-high performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A*, 2012, 1224, 19-26.
59. Goha, S. X. L.; Duarah, A.; Zhang, L.; Snyder, S. A.; Lee, H. K. Online solid phase extraction with liquid chromatography–tandem mass spectrometry for determination of estrogens and glucocorticoids in water. *Journal of Chromatography A*, 2016, 1465, 9–19.
60. Stavreva, D. A.; George, A. A.; Klausmeyer, P.; Varticovski, L.; Sack, D.; Voss, T. C.; Schiltz, R. L.; Blazer, V. S.; Iwanowicz, L. R.; Hager, G. L. Prevalent Glucocorticoid and Androgen Activity in US Water Sources. *Scientific Reports*, 2012, 2, Article 937.
61. Kugathas, S.; Sumpter, J. P. Synthetic glucocorticoids in the environment: first results on their potential impacts on fish. *Environ. Sci. Technol*., 2011, 45, 2377–2383.

62. Jin, P.; Liang, X.; Wu, X.; He, X.; Kuang, Y.; Hu, X. Screening and quantification of 18 glucocorticoid adulterants from herbal pharmaceuticals and health foods by HPLC and confirmed by LC-Q-TOF-MS/MS. *food additives & contaminants*, 2018, 35 (1) (A), 10–19.

63. Pedrouzo, M.; Borrull, F.; Marce, R. M.; Pocurull, E. Ultra-high-performance liquid chromatography–tandem mass spectrometry for determining the presence of eleven personal care products in surface and wastewaters. *J.Chromatogr A*, 2009, 1216, 6994–7000.

64. Postigo, C.; Lopez de Alda, M.J.; Barcelo, D.; Fully automated determination in the low nanogram per liter level of different classes of drugs of abuse in sewage water by on-line solid-phase extraction-liquid chromatography–electrospray–tandem mass spectrometry. *Anal. Chem.*, 2008, 80, 3123–3134.

65. Rodriguez-Mozaz, S.; Lopez de Alda, M. J.; D. Barceló, Picogram per liter level determination of estrogens in natural waters and waterworks by a fully automated on-line solid-phase extraction–liquid chromatography–electrospray tandem mass spectrometry method. *Anal.Chem*., 2004, 76, 6998–7006.

66. Tolgyesi, A.; Verebey, Z.; Sharma, V. K.; Kovacsics, L.; Fekete, J. Simultaneous determination of corticosteroids, androgens, and progesterone in river water by liquid chromatography–tandem mass spectrometry. *Chemosphere,* 2010, 78, 972–979.

67. Ciofi, L.; Fibbi, D.; Chiuminatto, U.; Coppini, E.; Checchini, L.; Del Bubba, M. Fully-automated on-line solid phase extraction coupled to high-performance liquid chromatography–tandem mass spectrometric analysis at sub-ng/L levels of selected estrogens in surface water and wastewater. *J. Chromatogr. A,* 2013, 1283, 53–61.

68. Guo, F.; Liu, Q.; Qu, G. B.; Song, S. J.; Sun, J. T.; Shi, J. B.; Jiang, G. B. Simultaneous determination of five estrogens and four androgens in water samples by online solid-phase extraction coupled with high-performance liquid chromatography–tandem mass spectrometry. *J. Chromatogr. A,* 2013, 1281, 9–18.

69. Liu, S.; Ying, G. G.; Zhao, J. L.; Chen, F.; Yang, B.; Zhou, L. J.; Lai, H. J. Trace analysis of 28 steroids in surface water, wastewater, and sludge samples by rapid resolution liquid chromatography–electrospray ionization tandem mass spectrometry. *J. Chromatogr. A*, 2011, 1218, 1367–1378.

70. Poho, P.; Scholz, K.; Karkkainen, N.; Haapala M.; Raikkonen, H.; Kostiainen, R.; Vaikkinen, A. Analysis of steroid in urine by gas chromatography-capillary photoionization-tanbem mass spectrometry. *J. chromatography A*, 2019, 1598, 175-182.
71. Amendola L.; Garribba, F.; Botre, F. Determination of endogenous and synthetic glucocorticoids in human urine by gas chromatography-mass spectrometry following microwave-assisted derivatization. *Analytica chemical acta*, 2003, 489, 233-243.
72. Delahaut, Ph.; Jacquemin, P.; Colemonts, Y.; Dubois, M.; De Graeve, J.; Deluyker, H. Quantitative determination of several synthetic corticosteroids by gas chromatography-mass spectrometry after purification by immunoaffinity chromatography. *J. chromatography B,* 1997, 696 (2), 203-215.
73. Rivero-Marabe, J. J.; Maynar-Marino, J. I.; Garcia-de-Tiedra, M. P.; Galan-Martin, A. M.; Caballero-Loscos, M. J.; Maynar-Marino, M. Determination of natural corticosteroids in urine samples from sportsmen. *J. chromatography B,* 2001, 761, 77-84.
74. Rathod, R. H.; Chaudhari, S. R.; Patil, A. S. et al. Ultra-high performance liquid chromatography- MS/MS in practice: Analysis of drugs and pharmaceutical formulations. *Futur J Pharm Sci*, 2019, 5, 6.
75. Walter, T. H.; Andrews, R. W.; Recent innovations in UHPLC columns and instrumentation. *Trends in analytical chemistry*, 2014, 63, 14-20.
76. Novakova, L.; Matysova, L.; Solich, P.; Advantages of application of UHPLC in pharmaceutical analysis. *Talanta*, 2006, 68 (3), 908-918.
77. Chawala, G.; Ranjan, C. Principle, instrumentation and applications of UHPLC: A novel technique of liquid chromatography. *Open Chemistry journal*, 2016, 3, 1-16.
78. Shackleton, C.; Pozo, O. J.; Marcos, J., GC/MS in recent years has defined the normal and clinically disordered steroidome: Will it soon be surpassed by LC/Tandem MS in this role?, *Journal of the endocrine society*, 2018, 2 (8), 974-996.
79. Asati, A.; Satyanarayana, G. N. V.; Patel, D. K. Vortex-assisted surfactant-enhanced emulsification microextraction combined with LC–MS/MS for the determination of glucocorticoids in water with the aid of experimental design. *Analytical bioanalytical chem*, 2017, 409, 2905- 2918.

80. Taylor, R. L.; Grebe, S. K.; Singh, R. J. Quantitative, highly sensitive liquid chromatography–tandem mass spectrometry method for detection of synthetic corticosteroids. *Clinical chemistry*, 2004, 50 (12), 2345-2352.
81. Ehling, S.; Reddy, T. M.; Liquid chromatography - mass sspectrometry method for the quantitative determination of residues of selected veterinary hormones in powdered ingredients derived from bovine milk. *journal of agricultural and food chemistry*, 2013, 61, 11782-11791.
82. QSight 225MD UHPLC screening system user manual, PerkinElmer, 2019.
83. Long, G. L.; Winefordner, J.D. Limit of detection – a closer look at the IUPAC definition. *Analytical chemistry*, 1983, 55, 712-724.
84. Schmidt, M.; QuEChERS Extraction – Gas Chromatography for the analysis of drugs, Dissertations and theses, Seton Hall University, (2015).
85. Leon, N.; Roca, M.; Igulada, C.; Martins, C. P. B.; Pastor, A.; Yusa. V. Wide range screeining of banned veterinary drugs in urine by UHPLC coupled to high resolution mass spectrometry. *J. Chromatogr. A*, 2012, 1258, 55-65.
86. Gonzalez Curbelo, M. A.; Rodriguez, B. S.; Herrera, A. V.; Salamo, G.; Borges, J. H.; Rodriguez Delgado, M. A. Evalution and application of the QuEChERS method. *TrAC.*, 2015, 17, 169-185.
87. Cerqueira, M. B. R.; Guilherme, J. R.; Caldas, S. S.; Martins, M. L.; Zanella, R.; Primel, E. G. Evaluation of the QuEChERS method for the extraction of pharmaceuticals and person care products from drinking water treatment sludge with determination by UHPLC-ESI-MS-MS. *Chemosphere*, 2014, 107, 74-82.
88. Matsuta, S.; Nakanishi, K.; Miki, A.; Zaitsu, K.; Shima, N.; Kamata, T.; Nishioka, H.; Katagi, M.; Tatsuno, M.; Tsuboi, K.; Tsuchihashi, H.; Suzuki, K. Development of a simple one-pot extraction method for various drugs and metabolites of forensic interest in bold by modifying the QuEChERS method. *Forensic Sci. Int*., 2013, 232, 40-50.
89. British Standard EN 15662:2008, Food of plant origin – Determination of pesticide residue using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE-QuEChERS-method, CEN, (2008) 4.

90. Lehotay, S. J.; Mastovska, K.; Lightfield, A. R. Use of buffering and other means to improve results of problematic in a fast and easy method for residue analysis of fruits and vegeTables. *J AOAC Int*., 2003, 88 (2), 615.
91. Qui, H.; Qui, X.; Zhao, J.; Liu, M.; Yang, Y. Supramolecular solvent-based vortex-mixed microextraction: determination of glucocorticoids in water samples. *J. Chromatogr. A*, 2013, 1311, 11-20.
92. Goh, S. X. L.; Chong, B. H. D.; Lee, H. K. Fully automated water sampleing-surfactant-enhanced membrane bag liquid phase microextraction-ultrahigh performance liquid chromatography-mass spectrometry. *Analytical chemistry*, 2020, 92, 5362-5369.
93. Yang, M.; Ma, Y.; Gui, W.; Ren, Y.; Zhu, G.; Liu, Y. Determination of 26 endocrine disrupting chemicals in fish and water using modified QuEChERS combined with Solid-phase extraction and UHPLC-MS/MS. *Anal. Methods*, 2015, 7, 8418-8431.
94. Harget, A. J.; Ellis, D. S. Classification of steroid partition coefficients by a pattern recognition technique. *Croatica chemical acta*, 1989, 62 (3), 449-458.
95. Flynn, G. L. Structural approach to portioning: estimation of steroid partition coefficient based upon molecular constitution. *Journal of pharmaceutical sciences*, 1971, 60 (3), 345-353.
96. Yalkowsky, S. H.; He, Y. Handbook of aqueous solubility data for organic compounds. *Aquasol database*, 2003.
97. Marta, Z. Bobaly, B.; Fekete, J.; Magda, B. Imre, T. Simultaneous determination of ten nonsteroidal anti-inflammatory drugs from drinking water, surface water and wastewater using micro UHPLC-MS/MS with on-line SPE system. *Journal of pharmaceutical and biomedical analysis,* 2018, 160, 99-108.
98. Rossini, D.; Ciofi, L.; Ancillotti, C.; Checchini, L.; Bruzzoniti, M. C.; Rivoira, L.; Fibbi, D.; Orlandini, S.; Del Bubba. M. Innovative combination of QuEChERS extraction with on-line solid-phase extract purification and pre-concentration, followed by liquid chromatography-tandem mass spectrometry for the determination of non-steroidal anti-inflammatory drug and their metabolites in sewage sludge. *Analytica chimica acta*, 2016, 935, 269-281.

99. Marchese, S.; Perret, D.; Curini, A. R.; Pastori, F. Determination of non-steroidal anti-inflammatory drugs in surface water and wastewater by liquid chromatography-tandem mass spectrometry. *Chromatographia*, 2003, 58, 263-269.
100. Muthal, A. Study of selectivity in extraction, separation, and detection on drugs using gas chromatography, chapter 2, dissertation, seton hall university, 2017, 48-91.
101. Jedziniak, P.; Pietruk, K.; Juszkiewicz, T. S.; Sledzinska, E.; Zmudzki, J. Determination of non-steroidal anti-inflammatory drugs and their metabolites in milk by liquid chromatography. *Analytical and bioanalytical chemistry*, 2012, 403, 2955-2963.
102. McNair, H. M.; Miller, J. M. Basic gas chromatography, 2nd ed., John Wiley and sons, inc., New York, 1998.
103. Dr. Deepak, Mass discrimination and analysis in mass spectrometry, lab-training.com, [online], https://lab-training.com/mass-discrimination-and-analysis-in-mass-spectroscopy/, accessed on March 6, 2022.
104. Benedikt, J.; Hecimovic, A.; Ellerweg, D.; Von Keudell, A. Quadrupole mass spectrometry of reactive plasmas. *Journal of Physics D: Applied Physics*, 2012, 45, 403001.