Study of Selectivity in Extraction, Separation, and Detection on Drugs Using Gas Chromatography

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STUDY OF SELECTIVITY IN EXTRACTION, SEPARATION, AND DETECTION ON DRUGS USING GAS CHROMATOGRAPHY

Submitted by

Anumeha P. Muthal

Dissertation

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

May 2017
We certify that we have read this dissertation and that in our opinion it is adequate to scientific scope and quality as a dissertation for the degree of Doctor of Philosophy.

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DEDICATION

Dedicated to my father Dr. Pradeep Muthal for his encouragement and support to pursue higher education and in loving memory of my mother late Mrs. Kumud Muthal.
ABSTRACT

The selectivity of a method is defined by its ability to determine a particular analyte without interference in a complex mixture. Selectivity in gas chromatography (GC) can be obtained at different points in the analysis including the sample preparation, sample introduction, separation on column and detection. In GC, the first dimension of selectivity can be obtained in sample preparation; the second dimension in chromatographic separation and the third dimension in detection. This research focuses on the relationships between selectivity in these different dimensions, studied using some non-steroidal anti-inflammatory drugs, glucocorticoids, fatty acid methyl esters and polycyclic aromatic hydrocarbons. Recent methodologies used for selective sample preparation such as supercritical fluid extraction, QuEChERs extraction and solid phase microextraction (SPME) are ‘greener’ approaches. Column selectivity is explained using comprehensive gas chromatography (GCxGC), using various column combinations. Detector selectivity is explained using new detection technologies, including multidimensional mass spectrometry (MS-MS) and vacuum ultraviolet detection (VUV), which show potential for better detection at low detection limits using MS-MS, and better separation of isomers using VUV. In method development, considering selectivity in all separation dimensions can provide the most efficient, and most sensitive method.

One goal of this work was to expand the multidimensional analysis of drugs using different extraction (SPME), separation (GCxGC-TOFMS) and detection (GC-MS-MS) technology in the gas chromatographic analysis as reviewed in Chapter 1. Multidimensional methods are less explored in the pharmaceutical applications as compared to applications in fuel and natural gas industry. The original research started with the separation of non-steroidal anti-inflammatory drugs (NSAIDs) in water without derivatization using SPME as extraction technique with a
comprehensive two-dimensional gas chromatography time of flight mass spectrometer (GCxGC-TOFMS) to separate a complex mixture and is detailed in Chapter 2. Comprehensive two-dimensional gas chromatography (GCxGC) (entire sample is separated on both columns) uses two columns in series with different polarity stationary phases to give an orthogonal separation. Extraction parameters such as pH, temperature, time of extraction, time of desorption were evaluated using gas chromatograph coupled with a triple quadrupole mass spectrometer (GC-MS-MS). Here, the same class of drugs, NSAIDs, were separated using two mass spectrometers in series.

A mixture of polycyclic aromatic hydrocarbons (PAHs) was separated to show the selectivity of extraction combined with detection. PAHs are carcinogens harmful to living populations which are exposed to them through the polluted environment from oil spills, vehicle exhaust, etc. This study was previously performed using SPME with GC-MS to separate PAHs in fish oil, extracted from real fish samples affected by the Deepwater Horizon oil spill (British Petroleum-operated) of 2010. Chapter 3 discusses the study of a same class of PAHs in fish oil but using a more selective technique with GC-MS-MS, where co-eluting PAHs can be separated and detected at trace levels of analysis in a complex matrix. Since, these PAHs degrade into their metabolites, this study was broadened to separate PAH metabolites in the fish oil samples from 2010. This experiment shows the separation of two isomers of PAH metabolites: 1-Naphthol and 2-Naphthol.

Finally, the original application involves a new detector technology, gas chromatography with vacuum ultraviolet detector (GC-VUV) for detection of glucocorticoids as discussed in Chapter 4. This study was also extended to observe the water content from sample preparation by SPME for NSAIDs. The VUV detector is sensitive to water, allowing easy water detection using GC which has been a major limitation.
Finally, Chapter 5 covers the application of GCxGC to study fatty acid methyl esters (FAMEs) on different column combinations and also the effect of modulation time and temperature, effect of the hot pulse. Modulation is the characteristic of the analysis using GCxGC-MS where analytes eluting from the first column are focused on to the second column in small fractions with a narrow bandwidth, which also increases the peak capacity. Hot pulse time determines how long the sample will be at the modulator to be released the secondary column which affects the sensitivity and resolution of the analysis. This was a short project for company Phenomenex to evaluate their test columns.

This research will demonstrate the use of multidimensional-gas chromatographic techniques to analyze NSAIDs without derivatizing. Study PAHs at low levels of detection and selectivity of FAMEs. SPME coupled to GC-MS and multidimensional separation can be used to separate NSAIDs residues in water as potential pollutants, PAHs and its metabolites as pollutants in fish oil, which can be applied in forensics and medicine.
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CHAPTER 1    INTRODUCTION

1.1    Abstract

This chapter summarizes selectivity in separation sciences focusing on three important dimensions of separation with instrumental analysis using gas chromatography coupled to mass spectrometric detectors (GC-MS). The first section focuses on the most common techniques used for the extraction of different analytes in complex matrices, with an emphasis on the extraction technique used predominantly during this research: solid phase microextraction (SPME). Later a background on separation using gas chromatography is discussed with a review of multidimensional instrumentation enhancing the scope of separations in two dimensions with respect to chromatography and detection.

1.2    Selectivity in chromatography

A selective method can be defined as a method in which a particular analyte can be determined without interference in a complex mixture [1]. Selectivity in gas chromatography can be obtained at different levels of analysis including sample preparation, analyte separation, and detection. Figure 1 shows a flow chart for different levels of selectivity in the gas chromatographic analysis. In GC, the first dimension of selectivity can be obtained in the sample preparation, where it can involve different techniques such as classical liquid-liquid extraction, solid-liquid extraction, solid phase extraction, soxhlet extraction, supercritical fluid extraction, QuEChERs extraction and solid phase microextraction for example. The second dimension of selectivity involves chromatographic separation achieved using two column in series with different polarity and the third dimension of selectivity involves the detection selectivity achieved using two quadrupole mass spectrometers in series.
Figure 1 Flow chart for different dimensions affecting separations in gas chromatography

(All images in this dissertation including Figures and Tables were created by Anumeha P. Muthal)
Selectivity in gas chromatography is studied by a separation factor ($\alpha$). $\alpha$ can be defined as a ratio of distribution constants for substance A and B which are measured under identical conditions, this factor is usually greater than 1 if components are separated. The separation factor is used to study how well the components in a mixture are separated from each other and is determined by a ratio of retention factors of any two peaks in the chromatogram. Figure 2 represents a relative retention showing selectivity/retention factor. This chromatogram shows an ideal separation of two components A and B with respective retention time $t_rA$ and $t_rB$ in mins. The $t_m$ represents the time for the non-retained component. These retention times can be used to determine the separation factor ($\alpha$) for separation by taking their ratios. For two peaks to be separated from each other a $\alpha$ greater than 1.2 is required. [2] [3]

In gas chromatography, components in a complex mixture are separated by interactions with a stationary phase which depend on physical and chemical properties of the chromatographic system. Chromatographic separation occurs by adsorption and desorption of components from the stationary phase. This behavior can be studied experimentally by evaluating thermodynamics at varied temperatures. The heat of adsorption is a measurable thermodynamic parameter in chromatographic separation which provides the information on quantification of interactions between adsorbate (component) and adsorbent (stationary phase). The thermodynamics of processes at the interface can be evaluated using the equation 1 for free energy ($\Delta G$) which can be determined by adsorbent parameter $V_g$ at different temperatures. This equation can also be used to determine enthalpy ($\Delta H$) and entropy ($\Delta S$) of adsorption. [2]

$$\Delta G = \Delta H - T\Delta S$$

Equation 1
Figure 2 Relative retention showing selectivity/retention factor

(Adapted from Dr. Yuri Kazakevich separation lecture slides on gas chromatography, 2013 [3])
As said by Dr. Colin Poole (Professor, Wayne state University) ‘All chromatographers need a working knowledge of selectivity to facilitate the development of separation methods with the desirable properties of adequate resolution in a reasonable time’. [4]

Resolution can be affected by selectivity. Equation 2 below shows how this can be determined.

\[
R_S = \left(\frac{\sqrt{N}}{4}\right) \left(\frac{k}{k+1}\right) \left(\frac{\alpha-1}{\alpha}\right)
\]

Equation 2

Where, \((\sqrt{N}/4)\) is based on the efficiency of the column that is defined as the number of theoretical plates, \((k/k+1)\) is the retention factor that is defined as the ratio of retention time of a component on the column to the retention time of a non- retained component and \((\alpha-1/\alpha)\) is the selectivity (separation factor) that can be defined as the ratio of distribution constants of two solutes. \(\alpha\) gives the information on the interaction of the solutes with the stationary phase and the magnitude of their differences. If \(\alpha > 1\) separation of two peaks is possible, higher the value of \(\alpha\) the easier the separation. Using the above equation, a capillary column with higher plate number is required to resolve the peaks with smaller values for \(\alpha\) (about 1.02) can be used to get a good separation, changes in these three parameters can improve the separation. [5] In isothermal conditions, \(N\) and \(k\) generally refer to the later eluted components; \(k\) and \(\alpha\) are constant for a given column, resolution can be dependent on the number of theoretical plates (\(N\)). With the decrease in temperature, the \(k\) term increases, with an increase in \(\alpha\) to less extent. To achieve the same separation at low temperatures, fewer theoretical plates/shorter column can be used. [2]
Fast GC

The gas chromatographic analysis is a relatively faster option as compared to other methods of separation. Fast GC involves changes in the column length, heating, faster flow rates, and modified inlet devices that can inject small sample volumes. To detect such fast eluting samples GC should be coupled to detectors that are capable of high-speed data collection such as FID, MS, and TOF detectors. Fast GC analysis separation time is about 1-10 minutes or less, unlike conventional GC. [6] To achieve such separation with desirable $\alpha$ values there is a need to optimize the speed, selectivity, and sensitivity of the techniques for optimization of chromatographic separations. Figure 3 shows the practical ways to a better separation of complex mixtures. Each corner of the triangle represents one pure attribute required to achieve separation. For example: if sensitivity is to be optimized at its maximum there is a need to analyze major components with the interfering components in a complex mixture, however, to achieve this resolution and time of analysis would be compromised giving long run times with low resolution. At the resolution corner, a lower speed and sensitivity will be achieved and at the speed corner, a low resolution and sensitivity will be achieved. So one variable can be optimized by compromising other, optimizing a separation method depends on the specific needs of the analysis and efficiency of the gas chromatograph and columns used. [2] Some limitations of conventional GC can be overcome by modifying the instrument and also using multidimensional GC or multidimensional MS which is are good alternatives for achieving a fast separation. Multidimensional techniques of separation are gaining popularity to shorten the analysis time with a good selectivity. There is a need to achieve the most prioritized feature between speed, sensitivity, and selectivity (as shown in Figure 3) which can be used to optimize GC-MS separations. There are also new instrumentation available, equipped with a high-resolution MS (HRMS) to achieve high sensitivity of the separation with a good resolving power.
Figure 3 Selectivity triangle optimization of chromatographic separation
1.3 First dimension of selectivity: Extraction

Extraction techniques are required to extract the analytes of interest from complex matrices, which also serves as the first dimension of selectivity. As gas chromatographic techniques require the analyte to be volatile in nature in order to be detected, using a specific extraction technique can serve as a clean-up step before injecting a sample into the gas chromatograph. Many extraction techniques require the use of organic solvents to extract the analytes from the matrix. These may not be safe if used in larger amounts and they produce larger volumes of laboratory waste. Modern extraction techniques also focus on a greener approach, using smaller amounts of organic solvents with the same analytical results. [7] There are various techniques in analytical separations such as classical: liquid-liquid extraction (LLE), solid phase extraction (SPE), soxhlet extraction, supercritical fluid extraction, etc. and modern extraction techniques: ionic liquid single drop microextraction (IL-SDME), stir-bar sorptive extraction, ultrasonication assisted extraction, QuEChERS extraction, solid phase microextraction (SPME). [8] [9] Figure 4 shows a comparison of SPME and other extraction techniques. This figure shows how SPME can be better over classical extraction techniques such as LLE, SPE etc. SPME uses a fiber assembly to extract and concentrate the analyte at the same time, which eliminates the use of harmful organic solvents to extract analytes, whereas classical methods use larger volumes of organic solvents with multiple step processes. Using SPME an extraction can be faster due to its automation and can be used to extract analytes at trace levels. This research used the solid phase microextraction (SPME) technique of separation to separate different class of analytes.
Figure 4 Comparison of SPME over different extraction techniques.

**SPME** (Solid Phase micro extraction)
- Solvent less technique
- Requires optimization of fibers
- Small sample required
- Sample preparation and extraction in one step
- Automated

**Classical Techniques** (LLE, SPE, etc.)
- Exposure to less safe solvents
- Multiple clean up steps
- Macro-scale sample required
- Error involved
- Limited extraction range
1.3.1.1: Classical extractions

Classical methods of extraction consist of liquid-liquid extraction, solid-liquid extraction, solid phase extraction (SPE), soxhlet extraction, etc. Most of the 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:

$$[A]_{\text{matrix}} \leftrightarrow [A]_{\text{medium}}$$  \hspace{1cm} \text{Equation 3}

Then the equilibrium constant can be determined by:

$$K_D = \frac{[A]_{\text{medium}}}{[A]_{\text{matrix}}}$$  \hspace{1cm} \text{Equation 4}

Where $K_D$ is the equilibrium constant or distribution constant. $K_D$ is used to study the amount of analyte distributed into two phases. If $K_D > 1$ majority of the analyte is extracted, if $K_D < 1$ most (but not all) of the analyte remains in the matrix and if $K_D \gg 1$ indicates that the analyte is extracted from the matrix by exhaustive extraction but there is always a finite amount in both phases. [10] [11] Figure 5 shows a typical separation of an analyte using liquid-liquid extraction, where aqueous phase is mixed with the organic phase to separate an aqueous analyte (green color) from the organic phase. After phase, separation into aqueous and organic layer the analyte is partitioned into both the layer. [12] The major factors affecting extractions are pH of the matrix and pKa of the analyte to be extracted where pH of the matrix needs to be adjusted to at least two units away from the pKa of the analyte in order to extract unionized analytes, which is applicable to ionizable compounds. Next is the extraction temperature, which affects the free energy of extraction for all the analytes (discussed for NSAIDs in Chapter 2). The amount of agitation and extraction medium also greatly affects recovery of the analytes.
Figure 5 Schematic of liquid-liquid extraction (LLE)
1.3.1.2: Solid phase microextraction (SPME)

Sample preparation is important for extraction of the analytes of interest from complex matrices. SPME was invented by Pawliszyn in 1990. This is a solvent-free technique for sample preparation where a fused silica fiber coated with a stationary phase is exposed to the liquid matrix containing the analyte of interest. [13] SPME combines analyte extraction and pre-concentration into a single step due to the equilibrium established between the analyte in the sample and on the fiber by the aid of agitation. Desorption of analyte from the fiber takes place at a high temperature at the GC inlet. The amount of analyte extracted depends on the partition coefficient between the sample and the sorbent layer (fiber) and their respective volumes. SPME is a technique, which can be applied to a wide range of volatile analytes in complex matrices such as food, biological fluids, and environmental samples. SPME can be used for the analysis of volatile and semi-volatile substances using specific coatings on fibers. [14] [15] The choice of the fiber depends on the polarity and molecular weight of the analyte, in order to get maximum extraction, the fiber should have similar polarity to the analyte. Figure 6 and Figure 7 explains the different choice of fibers depending on their polarity and molecular weight of the analyte respectively. The basic types of extraction performed using SPME are direct immersion and headspace extraction. [16] Figure 8 show the sample preparation and injection using SPME. Direct immersion extraction shortens the life of fiber as it is exposed to the solvent matrix which needs to be considered while selection of fiber, this method was used in the analysis of NSAIDs in water due to the semipolar nature of the analytes with the aid of a semi-polar fiber (PDMS/DVB/carboxen) was used. Headspace extraction using a nonpolar fiber (Polydimethylsiloxane, PDMS) was used to extract PAHs from fish oil where fiber was exposed to the headspace of the vial as PAHs are volatile and then injected into GC inlet.
Figure 6 Fiber selection with fiber polarity.

Reprinted with permission from dissertations: QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) Extraction - Gas Chromatography for the Analysis of Drugs, Schmidt, M., 2014. [17]
Figure 7 Fiber selection with analyte molecular weight.
Figure 8  Extraction steps using solid phase microextraction (SPME)
SPME is an equilibrium technique which is attained by optimization of parameters including fiber selection, extraction time and temperature, agitation time and temperature, desorption time at the inlet and effect of pH. After optimization of these parameters, the fiber is placed onto the GC inlet. The analytes adsorbed/absorbed on the fiber that are thermally desorbed due to the heated inlet and enter directly onto the column for separation.

Fiber selection

Fiber selection is the most important in SPME. This depends on the type of analyte to be extracted. According to the rule of thumb ‘like dissolves like’ applies, polar fiber is required to extract polar analytes and non-polar fibers for non-polar analytes. Fibers are commercially available with a different polymer coating, thickness, and absorption or adsorption type. The fiber coating determines the distribution constant at the equilibrium. Thicker the fiber coating more analytes will be extracted. For example, volatile analytes will be absorbed and retained in the fiber until they are transferred to GC inlet. SPME is an equilibrium process and some analytes with higher boiling point tend to remain on the fiber; hence, the fiber bake is necessary to make sure there is not any carry over from the previous run. [15] [18]

Sample agitation

Sample agitation is a kinetic effect on the sample where analyte extraction is increased with a decrease in extraction time. Agitation helps attain equilibrium in a shorter time due to increase in the rate of mass transfer from sample matrix to the fiber. There are various methods of agitation such as magnetic stirring, sonication, vortex stirring. In this research, an automated SPME autosampler was used on GC, which was equipped with a heated agitation chamber. [15]
Extraction temperature

As mentioned previously, extraction techniques are affected by the change in temperature. This affects the extraction thermodynamic parameters such as enthalpy ($\Delta H$) and entropy ($\Delta S$). As the temperature of the system increases, kinetic energy is increased which allows more heat to be absorbed, making it an endothermic process. The kinetic energy and enthalpy of the analyte at higher temperatures, increases resulting in a more favorable endothermic reaction thereby making extraction more favorable for SPME. However, the temperature and speed need only be high enough to agitate the analytes to be extracted without allowing them to escape the sample matrix and to maintain repeatability of the SPME method. Entropy is defined as a disorder of the system, which is inversely proportional to temperature. During extraction onto the fiber as the disorder of system increases, the analyte is extracted from matrix due to agitation. While concentration process, disorder decreases as the analyte is absorbed/adsorbed on the fiber. [13] [19] This is explained in Chapter 2 with NSAIDs.

Effect of pH

pH of sample matrix determines the amount of analytes to be extracted with respect to their pKa. The pH of the matrix is adjusted when weak acids and weak bases are extracted. Some neutral compounds does not possess any readily ionizable groups in their structure, thus there is no effect on extraction due to pH of the matrix. According to the rule, pH of the matrix is adjusted to be at least two pH units away from the pKa of the analyte in order to prevent ionization [2]. pH is typically adjusted to be lower than the pKa for acidic compounds and higher for basic compounds. This is discussed in Chapter 2. Equation below shows ionization of weak acids and bases:

\[ HA + H_2O \leftrightarrow H_3O^+ + A^- \quad \text{(For weak acid)} \]  \hspace{1cm} \text{Equation 5}

\[ B + H_2O \leftrightarrow BH^+ + OH^- \quad \text{(For weak base)} \]  \hspace{1cm} \text{Equation 6}
1.4 Second dimension selectivity: Chromatography

1.4.1 Basics of gas chromatography-mass spectrometry (GC-MS)

1.4.1.1 Separation by gas chromatography

Michael Tswett is the ‘father of chromatography’ who was the first scientist to explain chromatography. Since then, different techniques have evolved and Ramsey studied chromatography with the separation of gases. [20] [21] Gas chromatography is a separation technique, which separates the analytes in a mixture by their vapor pressure. This occurs due to the partitioning of the analyte in the stationary phase and the mobile phase: which is an inert gas. During the beginnings of gas chromatography, in 1941, A. Martin and R. Synge demonstrated the first model for column efficiency and developed liquid-liquid chromatography. [22] Later in 1952, A. James and A. Martin introduced to gas-liquid chromatography. [23] Figure 9 shows a diagram of a gas chromatograph coupled to the mass spectrometer. [24] A typical gas chromatograph consists of carrier gas; which is an inert gas, an inlet; where the sample is introduced, a capillary column; where separation occurs and a detector. In the gas chromatographic analysis, a sample is vaporized at the heated inlet which then enters onto the column with the carrier gas which then partitions in the stationary phase (in the coating) and each analyte elutes out based on their relative vapor pressure and finally to the detector. Gas chromatography being a fast technique it has some advantages and limitations. Table 1 shows a list of advantages and limitations for a sample analysis using gas chromatographic techniques. [5] This comparison shows that GC is a faster technique, which can be used to separate a complex mixture by less than one µL of the sample with high efficiency, which can be expressed using the plate number of a column, which is typically several hundred thousand. This is a non-destructive type of separation, which can be coupled to different detectors making it sensitive to lower limits of detection. GC have some towards non-volatile samples, as they need to be derivatized to be GC compatible.
Figure 9 Diagram of a gas chromatograph coupled to the mass spectrometer.
Table 1 Advantages and disadvantages of gas chromatography [25]

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
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<tbody>
<tr>
<td>Fast analysis, usually in minutes</td>
<td>Only for volatile samples (analytes with lower vapor pressure)</td>
</tr>
<tr>
<td>Separation efficiency with a good resolution (greater than 1.5)</td>
<td>Only for thermally stable compounds (Needs derivatization for non-volatile samples)</td>
</tr>
<tr>
<td>Sensitivity in detecting at ppb and ppt levels</td>
<td>Difficult for larger volume of samples (Ex. 1 mL injections will overload the column)</td>
</tr>
<tr>
<td>Requires small sample sizes</td>
<td>Requires a detection technique to confirm the identity of a molecule such as spectroscopy</td>
</tr>
<tr>
<td>(1 µL injections or less)</td>
<td></td>
</tr>
</tbody>
</table>
In addition, a larger volume of injection may overload the column. To summarize, GC is a method of choice to separate volatile components due to its speed, high resolving power, ease of use. Recent techniques have to be used to overcome such challenges in gas chromatographic analyses by modifying sample introduction techniques, modification in columns and new detectors with selective and sensitive identification. This research will discuss some challenges and how they were evaluated with GC still being a faster technique. Gas chromatographic separations occur in the capillary column, which is coated with different stationary phases depending on the type of analytes to be separated. Stationary phases for capillary columns are typically a liquid phase cross-linked or chemically bonded to the fused silica surface. Figure 10 shows the inner view of a column separation where the solute with a weaker interactions with stationary phase (green squares) spends more time in the mobile phase and moves faster through the column, eluting first. The compound with stronger interaction with the stationary phase (blue triangles) spends less time in the gas phase and moves slower through the column. [26] The coating determines the polarity of the column, which is required in the selection of column type for effective separation. The separation occurs due to the interaction of compounds with stationary phases by intermolecular forces such as van der Waals forces (dispersive, induced dipole or dipole-dipole interactions) and hydrogen bonds. [27] [28] The rule of thumb ‘like dissolves like’ states that a nonpolar stationary phase is required to separate nonpolar compounds and polar phase for polar compounds. [5] There are hundreds of stationary phases available, which can be evaluated on basis of analyte polarity, volatility etc. to separate different class of analytes. Considering these factors there are columns of various diameters, length and thickness. [29] [30] The most commonly used stationary phase is a 5% diphenyl: 95% dimethyl polysiloxane phase.
Figure 10 Inner view of a capillary column.
1.4.1.2 Sample introduction in gas chromatography

The injection port of a gas chromatograph is heated for the analytes to be vaporized. It is important for the analytes to be vaporized in order to enter into the capillary column, which are introduced through carrier gas. There are different ways to introduce a sample into GC such as a split, splitless, direct, on-column and cold on-column injections. Figure 11 shows the most common inlets which are split and splitless. Split injection is gas injection where the amount of sample fraction entering the column can be controlled, the rest of vaporized sample and excess carrier gas passes out through the purge valve. This technique is simple and can be operated by opening and closing of the split/ purge valve by setting a split ratio. However, this has a limitation in performing trace levels of analysis since only a fraction of the sample enters the column. Split injection, typically uses a flow of carrier gas through the glass liner (50–100 mL/min). The injected sample is vaporized and mixed with the carrier gas, which is then released to the column at the end of inlet liner or purged at the purge vent. The ratio of the volumetric flow rate out of the purge vent to the volumetric flow rate in the capillary column (1 mL/min) is termed the split ratio that gives the actual volume of sample entering the column.

On the other hand, splitless injections are best for the trace levels of analysis. In this type, the purge valve is closed while the sample is introduced and then opened to remove any residual solvents. This improves the sensitivity of the method. The backpressure regulator controls the inlet flow entering into the column. When the purge valve is off, after sample introduction, there is no place to go from the glass sleeve, but into the column. The splitless inlet is heated to ensure sample, which is also prepared in volatile solvent, vaporization and mixing with the carrier gas. The split valve is opened to release any residual vapors from the inlet. Initially, only the volatile solvent is
vaporized and carried through the column. While this is process is going on, the samples are refocused into a narrow band. After sometimes, the analytes are vaporized at the hot column and separated. Thus high resolution of the high boiling analytes is observed. The splitless mode is 20 to 50 folds more sensitive than a split mode. [5] [31] [32]

A typical sample volume for gas chromatographic analysis is about 1 μL for split liquid injections creating a very narrow and fast injection profile. Such injections can result in solvent effects. [5] [17] Figure 12 shows the solvent effects while the separation occurs which needs to be considered during development of a method which occurs for higher-volatility analytes. These occur in two stages, firstly, the solvent vapor enters the column and recondenses at a temperature below its boiling point which results in reduced volume and trapping the analyte. Secondly, carrier gas flows through the column continuously which flows through a zone where the analyte is trapped, it evaporates and concentrates the analyte. These injections can cause the band broadening which arises due to the time required for injected components to release from the inlet and to focus on the column. Another reason for band broadening in space occurs from the spreading of dissolved analyte in the solvent, as it condenses inside the initial length of the capillary column. Then, a cold trapping occurs for low-volatility analytes. If the initial column temperature is low enough, lower volatility analytes will be trapped in a narrow band on the column. [3]
Figure 11 Schematic of a split and a splitless inlet in gas chromatograph

(Adapted from Restek technical resources: Grob, K.; Why 5cm syringe needles for capillary GC?, Advantage; 2, 1995. [31])
Figure 12 Solvent focusing: a) Sample spreads after injection, b) A solvent plug forms and then elutes from the column

(Adapted from Dr. Yuri Kazakevich separation lecture slides on Gas chromatography, 2013 [3])
1.4.2 GCxGC-TOF-MS Instrumentation

Traditional 1-dimensional gas chromatography coupled with mass spectrometry (GC-MS) has been used to analyze mixtures of specific components such as volatiles, which frequently co-elute. [33] [34] GCxGC can provide a greater peak capacity for complex samples with two columns, where the first column is a conventional column and is typically nonpolar and the second column is typically a short (0.5-1.5m) polar column with a cryogenic modulator as the interface. The modulator focuses the first column eluent into the second column. [35] [36] [37] [38] This combination of nonpolar-polar columns is considered as an orthogonal configuration that increases the resolving power and enhances sensitivity for the trace level analysis of components from complex matrices. For detection in GCxGC, the detector must be fast and sensitive; hence, GCxGC is often coupled with a time of flight mass spectrometer, TOF-MS or FID.

Figure 13 shows a diagram of GCxGC-TOF-MS from Leco.com; this is the instrumental setup in our lab. The diagram shows the arrangement of the two-dimensional gas chromatograph coupled to Time of flight mass spectrometer, which is the Pegasus, 4D from Leco and represents all the conditions required for the mass spectrometer. After the sample is injected into the first dimension column in gas chromatograph for separation, the analyte separates and is focused with the aid of modulator on the second column where the analyte peaks are sliced. These slices then enter through a heated transfer line into the TOFMS consisting of ion source (for fragmentation of analytes by electron ionization), flight tube (1m travel path for fragments to reach to the detector) and a detector for further detection.
To achieve an optimum detection and maintain the inert environment for the fragments coming in from the ion source, flight tube is maintained under heated conditions and the high vacuum created by turbo-molecular pumps.

Figure 14 shows the setup for a comprehensive two-dimensional gas chromatograph. Here the first long column is coupled to the short second column with a press fit. A modulator is attached to focus analytes entering from the first column (non-polar) to get on second column (polar) by alternating hot and cold jets (cooled by liquid nitrogen). Each column has their own oven to maintain the temperature program of separation and Figure 15 shows a schematic representation of the modulation process (steps A-E) in a comprehensive two-dimensional gas chromatograph. [39] [40] Step A represents the analyte mixture (red, yellow and green analytes) separated from the first column waiting to enter into the second column through the modulator for separation in the second column. Step B shows a hot jet started to release first set of analytes. Step C shows a cold jet started to hold this analyte to slice them one by one. Step D shows a cold jet started to release the analytes to the second column with different polarity for further separation. Step E shows all the analytes separated into their respective groups by the slices entering one at a time into the second column. These analytes then enter the mass spectrometer to be detected and the data system combines the elution of analytes from first and second dimension columns to produce a chromatogram. [41] [42] [43]
Figure 13  Diagram of GCxGC-TOF-MS.

Material(s) used with permission from LECO Corporation [44]
Figure 14 Schematic of GCxGC.
Figure 15 Modulation operation (shapes represent different analyte)
Peak modulation is characteristic of the analysis using GCxGC where analytes eluting from the first column are focused on to the second column in small fractions with a narrow bandwidth. The modulator traps the peaks from the first column, which are the slices of each fraction, where each slice represents one component of a mixture. This increases the peak capacity for each separation. There are different types of modulators available, in this study; a quad-jet cryotrap modulator was used where there are alternately hot and cold nitrogen gas jets. The hot jets keep the fraction entering the modulator moving through the modulator and nitrogen gas is cooled using liquid nitrogen, which traps the fraction of the elute in the modulator. This is a continuous process, which keeps fractions from any interferences. In this type of modulator, the hot pulse is shorter than the cold pulse to preserve the bandwidth and get a good orthogonal separation. Therefore, these conditions for modulation time and the hot pulse time needs to be optimized to get a good separation. [35] [45] [41]

*Two-dimensional separation*

Peak capacity is the number of peaks that can fit in the space of chromatogram. For the complex separations, GCxGC can provide a higher peak capacity as compared to a one-dimensional GC. For example, comparing this column combination if a peak capacity for a single dimension column is 142 but when a second column is connected in series the peak capacity increases to 5822 suggesting that the peak resolving power is higher when a two dimension separation occurs. [41]

Following equations can be used to calculate the peak capacities. Equation 7 can be used to calculate the peak capacities for a single column and Equation 8 can be used to determine the peak capacity for a two-dimensional column combination.
\[ n = \frac{\sqrt{N}}{2R} \ln \frac{t_2}{t_1} + 1 \]  

Equation 7

\[ n_{\text{GCxGC}} = n_{\text{column1}} \times n_{\text{column2}} \]  

Equation 8

where \( n \) is the total number of peaks resolved, \( N \) is the column efficiency, \( R \) is the resolution between two peaks, \( t_2 \) and \( t_1 \) are the end time and start time of the run respectively. \(^{[35]} \) \(^{[46]} \) \(^{[38]} \)

After completion of the GCxGC separation, a data system is required to process and visualize the output. In this research, LECO’s Pegasus 4D was used for comprehensive two-dimensional analysis (GCxGC-TOFMS) this is equipped with ChromaTOF software; which transforms the raw data into a three-dimensional chromatogram. How it stacks the second dimension separations side by side with respect to the modulation time to match the retention times of the first dimension for each peak which is explained schematically in Figure 16, showing the step by step process of the formation of two-dimensional chromatogram of a real separation \(^{[47]} \) \(^{[48]} \).
Figure 16 GCxGC data analysis showing data processing from modulation to visualization.

(Printed with permission from Purcaro, Giorgia; Comprehensive Two-Dimensional Gas Chromatography (GC x GC) for Lipid Analysis; AÖCS Lipid Library (accessed March 19, 2017). [47]
1.5 Third dimension selectivity: Detection

1.5.1 Detectors in gas chromatography

Detectors in gas chromatography operate on the incoming flow from the separated analytes from the column. In gas chromatographic separation, the sample is volatilized and arrives at the detector, hence, these analyses require fast detection systems with a high sensitivity and a good dynamic range, for precise and accurate quantitation. There are various detectors that can be coupled to GC, which are chosen according to the requirements of the method. Some detectors are more selective towards specific compounds when analyzed in a complex matrix. [2]

Detectors for GC include flame ionization detector (FID), thermal conductivity detector (TCD), electron capture detector (ECD), Nitrogen phosphorus detector (NPD), infrared (IR), vacuum ultraviolet (VUV), mass spectrometer (MS), etc. Mass spectrometry is the most efficient detector, which can provide information on mass to charge ratio (m/z) used to identify the compounds. Table 2 summarizes commonly used gas chromatographic detectors with their selectivity and limits of detection. TCD and ECD are the concentration-sensitive types of detectors while as MS and FID are mass-sensitive detectors. Comparing the detectors, VUV and IR are a non-destructive where the analytes are not altered chemically. Non-destructive type of detectors gives an option to couple it to another detector in series for more accurate analysis. [49]
Table 2 Comparison of some common GC detectors

<table>
<thead>
<tr>
<th>Detector</th>
<th>Selectivity</th>
<th>Detection limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>FID</td>
<td>Selective for C-H bonds</td>
<td>0.1-10 pg</td>
</tr>
<tr>
<td>TCD</td>
<td>Universal</td>
<td>1-20 ng</td>
</tr>
<tr>
<td>ECD</td>
<td>Selective for halogens</td>
<td>0.1-1000 pg</td>
</tr>
<tr>
<td>NPD</td>
<td>Selective for nitrogen and phosphorus</td>
<td>1-10 pg</td>
</tr>
<tr>
<td>IR</td>
<td>Universal or selective</td>
<td>&lt; 200 pg</td>
</tr>
<tr>
<td>MSD</td>
<td>Universal or selective</td>
<td>1-10 pg</td>
</tr>
<tr>
<td>VUV</td>
<td>Universal or selective</td>
<td>20-200 pg</td>
</tr>
</tbody>
</table>
Mass spectrometry is a universal detector, which combines both selectivity and sensitivity for a separation of components with a high resolution. Analytes need to be thermally stable and volatile to be analyzed using MS, which is maintained under high, vacuum (range from $10^{-5}$-10$^{-7}$ torr), this minimizes the ion-molecule collision to achieve its sensitivity.

This research used two types of mass spectrometers: quadrupole and time of flight. MS provides information on the qualitative and quantitative identification of unknown compounds by their structure, elemental and molecular composition. A typical mass spectrometer has three main components: ionization source, a mass analyzer, and a detector. [50] These three components are discussed in the following section.

1.5.1.1 Ionization source

In this research, the two MS were equipped with an electron impact (EI) ionization source. In EI, the analytes eluting from the GC are ionized in order to be attracted by electric fields. Figure 17 shows a schematic of electron impact ionization source with the blue electrons produced from the filament attached to the ion source. [51] The ion source is heated and maintained under high vacuum so that most of the analytes are vaporized and ionized in an inert atmosphere. Ionization occurs by an electron beam at -70 eV from a filament. Due to this high-energy impact, the analytes are ionized and further fragmented. This produces positive ions with a charge on the fragments. The equation below shows the charge formation on the fragments. [5] [41]

$$M^+ + e^- \rightarrow M^+ + 2e^- \quad \text{Equation 9}$$
Figure 17 Electron impact ionization source
1.5.1.2 Mass analyzers

After the ionization at the ion source, the charged particles need to be analyzed. Here, the charged ions are repelled by charged lenses and then into the mass analyzers. These are separated by mass to charge ratio (m/z) using electric fields. There are different types of mass analyzers such as quadrupole, ion trap and time of flight mass analyzers. In this research, a multidimensional quadrupole (MS-MS) and a time of flight (TOF) mass analyzers were used.

Single quadrupole mass spectrometer (MS)

A single quadrupole is made of four hyperbolic rods at right angles with a DC voltage with opposite charges as shown in Figure 18. These charges are rapidly switched due to which analytes are moved rapidly. These ions are then filtered by a combination of radio frequency and DC voltages to reach the detector. The ions with different m/z ratios are a strike out of the quadrupole or enter into the vacuum. The mass filters can be set to filter or scan all m/z ratio entering the quadrupole for example 60-600. [5] [52] [53] During this research, a triple quadrupole was used where three quadrupoles are in a series and mass analyze the analytes in multiple reaction-monitoring modes (this is explained in Chapter 2 and 4).

Time of Flight Mass spectrometer (TOF-MS)

TOF-MS is a more sensitive mass analyzer where the analytes are mass analyzed by their kinetic energy (KE) to travel a fixed distance. All the ions coming from ion source have potential energy, which is kicked by the repeller voltage at the same time with same kinetic energy. Since all ions get same kinetic energy, the smaller ions tend to travel faster than the larger ions. A typical flight tube is 1.0 meter in length. The following equations show that kinetic energy is proportional to the mass. [5] [54]
Kinetic energy (KE) = ½ mv² \hspace{1cm} \text{Equation 10}

Potential energy = qV \hspace{1cm} \text{Equation 11}

Where m is the mass of ion and v is the velocity of the ion. q is ion charge, V is accelerating potential. After combining these equations and rearranging yields an equation to determine the time spent by the ion in flight tube.

\[ qV = ½ mv^2 \quad \text{where} \quad v = \sqrt{2Vq/m} \quad \text{Equation 12} \]

Velocity is distance divided by time. Hence,

\[ t = L \sqrt{m/(2Vq)} \quad \text{Equation 13} \]

Where t is the time spent by the ion in flight tube and L is the length of the tube. The time of flight for each ion corresponds to their respective masses. The flight tubes are made shorter where there is a reflectron design that folds the tube and with a reflecting mirror, the ions are reflected back to the tube. [41] This doubles the path traveled by the ions. TOF mass analyzers are faster and sensitive. Figure 19 shows a typical time of flight mass analyzer. In this research, LECO’s Pegasus 4D was used.

1.5.1.3 Detector

After separation by a mass analyzer the ions produced enters the detector. A typical detector is a continuous dynode version of electron multiplier tube as in Figure 18. This counts ion and generates a spectrum. Ions are accelerated by the potential difference at the semi-conductive surface with a release of electrons. This is repeated until the signal is amplified and collected by the data system. [5] [55]
Figure 18 Diagram of quadrupole mass spectrometer with the electron multiplier tube
(Reproduced with permission from www.chem.libretexts.org [56])
Figure 19 Schematic of time of flight mass spectrometer (TOF-MS).

Reprinted with permission from www.chem.libretexts.org [57]
1.5.2 GC-MS-MS Instrumentation

In this instrument, the gas chromatograph is coupled to a triple quadrupole mass spectrometer, also termed a tandem mass spectrometer, MS-MS or MS\(^2\). Figure 20 shows a schematic of a GC-MS-MS. MS-MS involves the selection of a specific m/z ion (precursor ion) in the first mass analyzer (Q1) followed by, collision induced dissociation (CID) in the collision cell (Q2) which is filled with a neutral gas such as argon or nitrogen. The fragment ions are then sorted according to their mass to charge ratio in the second mass analyzer (Q3), and recorded by the detector. The triple quadrupole mass spectrometer provides fast ion transport with rapid ion removal that enables trace analysis of analytes in the sample at ppt levels. Multiple reaction monitoring (MRM) separates the masses in two stages, which makes the system more selective and particularly effective for the trace level analysis of analytes in complex matrices. MRM can reduce chemical noise from the signal and provide very high sensitivity and selectivity.

Figure 21 shows a schematic of MRM. In this research, with SPME extraction coupled to GC-MS-MS, NSAIDs will be identified and quantified in water and other matrices. Similar work has been performed for the separation of steroids and other drugs in water using SPME-GC-MS-MS as discussed in detail in Chapter 2 and 4. [15] [60] [61]
Figure 20 Diagram of gas chromatograph coupled to triple quadrupole (GC-MS-MS)
Figure 21 Schematic of triple quadrupole mass spectrometer: multiple reaction monitoring (MRM)
1.6 Conclusions

Multidimensional techniques of analysis can be used to study the selectivity in extraction using Solid phase micro-extraction (SPME), separation using comprehensive two-dimensional gas chromatography (GCxGC) and detection using triple quadrupole (GC-MS-MS). These techniques introduce another dimension of separation to analyze a specific class of analytes in complex matrices. This research focuses on the study of selectivity using gas chromatographic techniques that increase the GC separation in a broader range with overcoming some challenges to analyze semi-volatile analytes using SPME coupled to multidimensional gas chromatography.

Chapter 2 introduces the applications of all the three dimensions of analysis where the first dimension of selectivity: SPME would be explored to extract semi-volatile analytes (NSAIDs) without derivatization. These would be further separated using the chromatographic selectivity (second dimension) to separate these class of drugs underivatized. A detector selectivity (third dimension) will be used to separate and detect these drugs using two detectors (MS-MS). These studies will demonstrate the selectivity in all three dimensions.

Further, in Chapter 3 demonstrates the study of selectivity using first dimension: extraction (SPME) and second dimension: detection (GC-MS-MS) to separate polycyclic aromatic hydrocarbons (PAHs) and its metabolites from a complex matrix such as fish oil. Here, the detector selectivity was used to separate all the PAHs without the matrix interference. Chapter 4 demonstrates a new method of detection in gas chromatography GC-VUV, which shows a unique selectivity for drugs and trace levels of water using a vacuum-ultraviolet detector (third dimension
of selectivity). Chapter 5 studies the selectivity using the second dimension (GCxGC). The selectivity of the two chromatographic columns was evaluated using fatty acid methyl esters (FAMES) when used in different combinations. This study also demonstrated the effect on modulation parameters using comprehensive two-dimensional separation, which greatly affects the selectivity in separation.

All these techniques when combined together can provide an enormous amount of information on extraction, separation, and detection of a different class of analytes.
CHAPTER 2   MULTIDIMENSIONAL ANALYSIS OF NSAIDS IN AQUEOUS SAMPLES USING GCXGC-TOFMS AND GC-MS-MS

2.1  Abstract

Recently, the residues of non-steroidal anti-inflammatory drugs (NSAIDs) are studied as emerging pollutants in water, which enter the environment while they are manufactured, during improper disposal of expired or unused drugs and through human and animal excretion. These are difficult to detect using gas chromatography (GC) due to their acidic, highly polar and hydrophilic nature and cause adverse effects to the aquatic life and are a potential risk to human health at low concentration (ng/L). Mostly the analysis of NSAIDs using GC is done by incorporating derivatization techniques such as methylation and other methods to make them volatile and heat resistant with the limit of detection of 2-6 ng/L. In this work, Solid phase microextraction (SPME) coupled to two multidimensional techniques: GCxGC-TOF-MS and GC-MS-MS were used to study the NSAIDs using the chromatographic and detection selectivity of the two techniques respectively without derivatization. Upon analysis, this method can also be applied to determine the NSAIDs in complex matrices such as urine, blood for clinical toxicology for trace level analysis.

This study demonstrates the unique selectivity in extraction using Solid phase micro-extraction (SPME), separation using comprehensive two-dimensional gas chromatography (GCxGC) and detection using triple quadrupole (GC-MS-MS).
2.2 Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are drugs, which have analgesic, anti-inflammatory, and antipyretic effects. NSAIDs are easily available as over-the-counter (OTC) drugs that include ibuprofen, naproxen, ketoprofen, etc. NSAIDs can be misused for suicidal overdose due to easy availability. The residues of drugs are also emerging pollutants in water that enter the environment while they are manufactured, during improper disposal of drugs and through human and animal excretion. These are difficult to detect due to their acidic, highly polar and hydrophilic nature and cause adverse effects to the aquatic life and are a potential risk to human health at low concentration (ng/L) but these studies have not determined long-term toxicological effects. Table 3 shows the structures of the nine NSAIDs used in this study, including the most common of all: aspirin, along with other common NSAIDs. A variety of functionalities, including the characteristic aromatic and acid groups, ketones, nitrogen, and chlorine.

Trace analysis of NSAIDs is mostly done using high-performance liquid chromatography (HPLC) due to their low volatility and acidic nature with a limit of detection at 1-10 ng/L; it is challenging to perform the trace analysis of these drugs using gas chromatography (GC). Mostly the analysis of NSAIDs using GC is done by incorporating derivatization techniques such as methylation and other methods to make them volatile and heat resistant with the limit of detection at 2-6 ng/L. For the extraction of NSAIDs from water, techniques such as solid phase extraction (SPE) and solid phase microextraction based on coatings with sol-gel and carbon nanotubes have been employed. However, these methods can be time-consuming, and utilize large amounts of organic solvents, are not automated or are not readily or commercially available.
Solid phase microextraction (SPME) is a solvent-free extraction technique that has been in common use for drug analysis for about two decades. SPME combines analyte extraction and pre-concentration into a single step due to the equilibrium established between the analyte in the sample and on the fiber by the aid of agitation. Desorption of analyte from the fiber takes place at a high temperature in the GC inlet. The amount of analyte extracted depends on the partition coefficient between the sample and the sorbent layer (fiber) and their respective volumes. SPME is a technique, which can be applied to a wide range of volatile analytes in complex matrices such as food, biological fluids, and environmental samples. SPME combined with GC and GC-MS can be used for the analysis of a large variety of volatile and semi-volatile substances. [13] [59] [69] [70] [71]

Comprehensive two-dimensional gas chromatography (GCxGC) is multidimensional separation technique in which two columns are in a series that are coated with different stationary phases. Commonly, the first dimension column is a non-polar and second dimension is a polar column. This combination of nonpolar-polar columns is considered as an orthogonal configuration, which increases the resolving power and enhances sensitivity for the trace level analysis of co-eluting components from complex matrices. For detection in GCxGC, the detector must be fast and sensitive due to the rapid eluting from the second dimension; hence, GCxGC is most often coupled with a flame ionization detector or a time of flight mass spectrometer (TOF-MS). The high sensitivity and selectivity of TOFMS make it an ideal detector for GCxGC. GCxGC has been used for the analysis of drugs, especially drugs of abuse in clinical settings almost since its inception. [34] [33] [35] [46] [72] [38] [73] [74] In this study, non-steroidal anti-inflammatory drugs (NSAIDs) are extracted from water using SPME and separated using GCxGC-TOFMS, without derivatization. GCxGC-TOF-MS provides a different approach to multidimensional separations.
Upon analysis of these parameters, this method can also be applied to determine the NSAIDs in complex matrices such as urine, blood for clinical toxicology and the determination of NSAIDs concentration in drug formulations with easy sample preparation technique.

In GC-MS-MS, the gas chromatograph is coupled to a triple quadrupole mass spectrometer, also termed tandem mass spectrometry, MS-MS or $\text{MS}^2$. MS-MS involves the selection of a specific m/z ion (precursor ion) in the first mass analyzer (Q1) followed by, collision induced dissociation (CID) in the collision cell (Q2) which is filled with a neutral gas such as argon or nitrogen. The fragment ions are then sorted according to their mass to charge ratio in the second mass analyzer (Q3) and recorded by the detector. The triple quadrupole mass spectrometer provides fast ion transport with rapid ion removal that enables trace analysis of analytes in the sample at ppt levels. Multiple reaction monitoring (MRM) separates the masses in two stages, which makes the system more selective and particularly effective for the trace level analysis of analytes in complex matrices. MRM can reduce chemical noise from the signal and provide very high sensitivity and selectivity. [15] [74] In this research, with SPME extraction coupled to GC-MS-MS, NSAIDs will be identified and quantified in water and other matrices.

Figure 22 shows the challenges in NSAIDs analysis using gas chromatography. This flow chart shows the direction of research where the underivatized NSAIDs will be analyzed using GC to overcome its semi-volatility, heat labile, acidic nature of NSAIDs using SPME sample introduction. NSAIDs will be extracted in water due to their semi-volatile nature a direct immersion technique will be used to adsorb the analytes on the fiber and then injected directly on the column to minimize the degradation of NSAIDs in the injector port where the heat of injector will be used to desorb the analytes from the fiber. In this study, a PDMS/DVB/Carboxen fiber was used due to its semi-polar coating, which extracted most of the NSAIDs in a mixture.
Figure 22 Challenges in NSAID analysis by gas chromatography (left) and direction of research (right)

- **Semi volatile**
  - Extracted using PDMS/DVB/Car SPME fiber without any modification
  - Comparison using GC-MS-MS and GCxGC-TOFMS

- **Acidic**
  - Analyzed without derivatization
  - Separation on DB-5 column

- **Heat**
  - Injection degrades the analytes, SPME will be used
  - Column oven initial temperatures will be low

- **Study of selectivity**
  - GC-MS-MS: Trace level analysis of NSAIDs residues
  - GCxGC-TOF-MS: Analysis in complex matrix
2.3 Materials and methods

The NSAIDs used in this study ibuprofen, naproxen, and ketoprofen were obtained from Sigma-Aldrich (St. Louis, MO): and diclofenac, mefenamic acid, oxaprozin, tolfenamic acid, aceclofenac were obtained from VWR (Randor, PA). A Milli-Q Plus purification system, (Millipore, Milford, MA) was used to obtain Ultra-pure water in the laboratory. Table 3 shows the NSAIDs used in this study.

A Pegasus 4D comprehensive GCxGC-TOF-MS (LECO, St. Joseph, MI) equipped with an auto-sampler with SPME capability (Gerstel, Columbia, MD) was used in this work. SPME fibers: PA – polyacrylate, PDMS – polydimethylsiloxane, PDMS/DVB - polydimethylsiloxane/divinylbenzene, PDMS/CAR/DVB – polydimethylsiloxane/carboxen/divinylbenzene were obtained from Sigma-Aldrich (Supelco, Bellefonte, PA).

NSAIDs standards at 1000 ppm were prepared individually in methanol. Then a mixture of NSAIDs was prepared by spiking 20 µL of the standard to 20 mL ultra-pure water, which was pH adjusted using hydrochloric acid. Specific pH conditions are described in the discussion with the final pH selected to be 3.2. SPME experiments in this study were performed in direct immersion mode. A 20 mL screw cap vial was pre-incubated in the agitator for 10 min, followed by extraction for 30 min and desorption into a splitless inlet for 3 min with a post extraction bake in the inlet under split conditions for 10 min. Extraction temperature was optimized as discussed below with the final temperature selected at 70°C. In this study, weak acids were extracted from water as a matrix. Since weak acids and bases tend to ionize in water, pH of the water was adjusted to two units below the pKa of analytes, which ranged approximately in the range of 3-5 to prevent ionization to ensure that all analytes are present in non-ionized form to get maximum extraction; hence, these nine NSAIDs were selected for further studies. [41] [16]
<table>
<thead>
<tr>
<th>Name</th>
<th>Synonym</th>
<th>pKa</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>Acetylsalicylic acid</td>
<td>3.48</td>
<td><img src="image" alt="Aspirin Structure" /></td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>Benzeneacetic acid, α-methyl-4-(2-methylpropyl)-</td>
<td>4.41</td>
<td><img src="image" alt="Ibuprofen Structure" /></td>
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<tr>
<td>Naproxen</td>
<td>2-Naphthaleneacetic acid, 6-methoxy-α-methyl</td>
<td>4.84</td>
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<tr>
<td>Ketoprofen</td>
<td>Benzeneacetic acid, 3-benzoyl-α-methyl-</td>
<td>4.45</td>
<td><img src="image" alt="Ketoprofen Structure" /></td>
</tr>
<tr>
<td>Tolfenamic Acid</td>
<td>Benzoic acid, 2-[(3-chloro-2-methylphenyl)amino]-</td>
<td>3.69</td>
<td><img src="image" alt="Tolfenamic Acid Structure" /></td>
</tr>
<tr>
<td>Mefenamic Acid</td>
<td>Benzoic acid, 2-[(2,3-dimethylphenyl)amino]-</td>
<td>3.73</td>
<td><img src="image" alt="Mefenamic Acid Structure" /></td>
</tr>
<tr>
<td>Diclofenac</td>
<td>Benzeneacetic acid, 2-[(2,6-dichlorophenyl)amino]-</td>
<td>4.18</td>
<td><img src="image" alt="Diclofenac Structure" /></td>
</tr>
<tr>
<td>Aceclofenac</td>
<td>2-[2-[2-[(2,6-dichlorophenyl)amino]phenyl]acetyl]oxyacetic acid</td>
<td>3.44</td>
<td><img src="image" alt="Aceclofenac Structure" /></td>
</tr>
<tr>
<td>Oxaprozin</td>
<td>3-(4,5-Diphenyloxazol-2-yl)propanoic acid</td>
<td>4.28</td>
<td><img src="image" alt="Oxaprozin Structure" /></td>
</tr>
</tbody>
</table>
**GCxGC-TOFMS conditions**

GCxGC with a primary RTX-5MS, 5% phenyl polydimethylsiloxane, (15 m x 0.1 mm x 0.08 µm) and a secondary RTX-200, trifluoro propyl/methyl polysiloxane (1.5 m x 0.1 mm x 0.1 µm) column, (Restek, Bellefonte, PA) was performed with a constant flow of 1 mL/min. GC inlet was maintained at 230°C. The oven was programmed at temperature 100°C for 1 min, 10°C/min to 180 °C for 2 min, 4°C/min to 200°C for 10 min with the secondary oven maintained 5°C higher than the primary. The second dimension time was 5 sec. The transfer line was maintained at 280°C and ion source at 250°C. Modulator parameters were maintained at hot pulse for 0.60 sec and cold pulse for 1.90 sec with a temperature offset at 20°C. The acquisition delay was 120 sec. Masses were scanned from 40-400 amu at an acquisition rate of 100 spectra per second.

**GC-MS-MS and SPME conditions**

SPME experiments in this study were performed in direct immersion mode. A 10 mL screw cap vial was pre-incubated in the agitator for 10 min, followed by extraction for 30 min and desorption into a splitless inlet for 3 min with a post extraction bake in the inlet under split conditions for 10 min. Extraction temperature was optimized as discussed below with the final temperature selected at 70°C. On Shimadzu’s GC-TQ8030 with an  RTX-5MS, 5% phenyl polydimethylsiloxane, (15 m x 0.25 mm x 0.25 µm) (Restek, Bellefonte, PA) was performed with a constant flow of 1 mL/min. GC inlet was maintained at 250°C, the oven was programmed at temperature 80°C for 3 min, 20°C/min to 230°C for 2 min and transfer line was maintained at 280°C.
2.4 Results and discussion

2.4.1 Solid phase microextraction (SPME)

The SPME conditions were optimized using three representative NSAIDs for simplicity: ibuprofen, naproxen, and ketoprofen. When the full mixture was later used, extraction performance was satisfactory for all nine NSAIDs. Fiber phase, extraction pH and temperature were all optimized for a 30 min direct immersion extraction. The extraction time was later confirmed using the optimized conditions. While selectivity in GCxGC-TOFMS methods is most often discussed in terms of column selection and detection, it is clear that the choice of extraction phases (or solvents in a classical extraction) can also greatly influence selectivity.

2.4.1.1 Fiber Selection

In an SPME method, the greatest impact on selectivity is generated by selection of the appropriate extraction fiber. To date, most SPME methods employ the classical non-polar PDMS fiber or polar PA fiber. Figure 23 shows the extraction results for ibuprofen, naproxen and ketoprofen on three fiber phases: polydimethylsiloxane/carboxen/divinylbenzene (PDMS/CAR/DVB), polydimethylsiloxane/divinylbenzene (PDMS/DVB) and polyacrylate (PA). For ibuprofen, which is the smallest of the three analytes, with the least hydrocarbon backbone, the polar PA fiber provided the highest response. For naproxen, which includes two fused aromatic rings, indicating a large potential for pi-pi interactions, the mixed phase PDMS-DVB provided the highest response, indicating stronger interactions between the rings on the analyte and on the stationary phase. Finally, for ketoprofen, which has the two aromatic rings separated by a ketone, the mixed PDMS/CAR/DVB phase provided the highest response. This is likely due to the presence of the ketone group interfering with pi-pi interactions between the aromatic rings on ketoprofen and the DVB rings in the fiber. Overall, the best performance was obtained using the mixed-phase fiber:
Figure 23 Effect of fiber selection on the extraction of naproxen, ibuprofen, and ketoprofen
PDMS/CAR/DVB. This is a moderately polar phase that can be effectively used for direct immersion SPME of drugs and other moderately polar, semi-volatile compounds [40].

2.4.1.2 Effect of pH

NSAIDs all have a characteristic carboxylic acid group at one end of the molecule; therefore, all nine analytes in this study are weak acids. The extraction pH was studied across a wide pH range to both determine the optimum pH for the extraction and to determine the pH range for which the extraction can be done and an instrument response still is seen. This can provide insight to the utility of SPME to extract NSAIDs from samples in which no sample preparation is possible, such as in vivo blood sampling. The pKa’s of the NSAIDS are between 4-5, indicating that a lower pH of about 3 or lower should maximize the proportion of the ionizable NSAID that is in the neutral form and therefore available for extraction. [71].

Figure 24 shows the effect of pH on the extraction of the three representative NSAIDs from water at 70°C using the PDMS/DVB/CAR fiber. As expected, the highest response for all three NSAIDs is seen at pH 3.2. At pH 2, there may be some fiber degradation or fouling due to the higher ionic strength of the solution. As the pH increases, as expected, the response drops. However, finite responses were seen at pH as high as 7.7, indicating that, with sensitive instrumentation, NSAIDs could be extracted directly from solutions at physiological pH without further sample preparation. For ionizable compounds, the combination of fiber selection and pH are the major drivers of extraction selectivity. If the extraction goal is to maximize the instrument response, both should be optimized to provide the maximum response, where amount extracted is expected to inversely track the percent ionization. If simplified or no additional preparation is required, then selectivity can be used to predict whether the extraction is possible at the given non-optimum conditions.
Figure 24 Effect of pH on extraction of naproxen, ibuprofen, and ketoprofen
2.4.1.3 Effect of extraction temperature

Extraction from an aqueous solution into an organic extraction phase that does not contain any analyte is a spontaneous process. If the partition coefficient for the process is greater than one, the standard Gibbs’ free energy change involved is negative and the extraction process is most likely exothermic. In the case of SPME, with $K > 1$, extraction must be exothermic. Analytes are concentrated into the fiber, therefore, the entropy change $\Delta S$ must be negative. $\Delta H$ must therefore also be negative and larger than $T\Delta S$ for $\Delta G$ to remain negative, indicating a spontaneous process. Therefore, all other variables being equal, an increase in extraction temperature will decrease the amount of analyte extracted, at equilibrium. However, for weak acids, such as NSAIDs, the acid dissociation equilibrium will also be affected by temperature. Generally, weak acid dissociation is endothermic, meaning that increased temperature will drive that process in the direction of ionization, reducing the amount of the neutral molecule in solution available to extract. Temperature also affects kinetics; increased temperature reduces the time required to reach equilibrium. Figure 25 shows the effect of extraction temperature on the responses for the three representative analytes using the PDMS/DVB/CAR fiber at pH 3.2 and a 30-minute extraction. It is seen that 70°C provided the highest response for all three, so this was chosen for all further work. Responses are seen to increase until 70°C, likely a kinetic effect, indicating that equilibrium has not been fully reached, with a decrease above 70°C, likely a thermodynamic effect as exothermic extraction pushes that process back toward the aqueous phase and endothermic acid dissociation pushes that process toward the ionic form. [13] [19]

The final optimized extraction conditions were PDMS/CAR/DVB fiber, pH 3.2, 30 min direct immersion extraction at 70°C.
Figure 25 Effect of extraction temperature on extraction of ibuprofen, naproxen, and ketoprofen
2.4.2 GCxGC-TOF-MS

Figure 26 shows a total ion chromatogram of a standard containing the three representative NSAIDs (ibuprofen, naproxen, and ketoprofen) extracted using SPME and analyzed using GCxGC-TOF-MS under the optimized conditions. In the first dimension, a 5% phenyl polydimethylsiloxane column was used. This stationary phase separates mainly based on a combination of dispersive interactions on the PDMS backbone with limited pi-pi interactions generated by the phenyl groups. Broadly, it is considered a non-polar stationary phase. These three analytes are well separated, as expected, due to the addition of the second fused aromatic ring on naproxen and the second ring with ketone on ketoprofen. The second dimension column was 100% trifluoro propyl polysiloxane, a moderately polar phase. This stationary phase separates based on a combination of dispersive interactions and interactions between the lone pair electrons on fluorine with lone pair electrons on the analytes. It is especially selective for compounds containing nitrogen, oxygen, and halogens. [75] As the selectivity of the two columns are quite different, pi-pi versus lone pair interactions, this column set may be considered orthogonal. Orthogonality is related to the peak capacity. In separation, an angle of 90º represents theoretical maximum of peak capacity. [76] In the second dimension, for the three representative NSAIDS, it is seen that ketoprofen is more strongly retained than ibuprofen and naproxen, mainly due to the presence of the ketone.

Figure 27 shows a total ion chromatogram of all nine NSAIDs used in this study, extracted from water under the optimized SPME conditions. This chromatogram demonstrates both the benefits and challenges of adding selectivity to a chromatographic system by using the second dimension column. It is clear that all nine compounds are separated from each other in the first dimension alone, however, they may not be fully separated from matrix interferences (there are a number of
additional, unidentified peaks in this chromatogram). In addition, drugs often have low volatility, resulting in longer retention times and higher elution temperatures; there may be column or septum bleed, which would also interfere with a single-dimension separation. The additional selectivity afforded by the second dimension column will also reduce interferences due to additional endogenous compounds that may be extracted from a true biological sample.

Examining some peak pairs in Figure 27, both the benefits and difficulties of adding the second dimension column are seen. For peaks 1 and 2, which represent aspirin and ibuprofen respectively, are well-separated in the first dimension and not so well separated in the second. This is not surprising as the main structural difference between the two molecules is the presence of additional hydrocarbons on ibuprofen. There is, however, some separation from the unidentified matrix components. Peaks 3 and 4, naproxen and ketoprofen were discussed above; the presence of the ketone in ketoprofen, with lone electron pairs interacting with the fluorine moieties in the Rtx-200 stationary phase increases its second dimension retention significantly. Peaks 6 and 7, mefenamic acid and tolfenamic acid differ primarily by the presence of chlorine on tolfenamic acid. Stronger retention on Rtx-200 would be expected for tolfenamic acid but this is not observed on the chromatogram. However, tolfenamic acid is wrapped around, as is peak 8, diclofenac. While wrap around is generally avoided, in this case, as the two peaks appear within the useful separation space, they do not co-elute with any of the other analytes or interferences, so this does not present a problem and actually simplifies the separation, allowing a shorter second dimension time.
Figure 26 Total ion chromatogram of the three representative NSAIDs
Figure 27 Total ion chromatogram of 9 NSAIDs extracted from water

<table>
<thead>
<tr>
<th>NSAIDs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
</tbody>
</table>
Finally, the extraction and separation of these nine NSAIDs were performed without derivatization, which is most commonly used for many of them. This greatly simplifies sample preparation. The added selectivity and sensitivity provided by GCxGC-TOFMS coupled with optimized SPME increases the range of compounds on which GC can be performed without derivatization, which often significantly complicates the sample preparation. Although the underivatized NSAIDs are generally polar compounds, they exhibit satisfactory resolution and peak shapes. While there may be some degradation or adsorption of the NSAIDs in the inlet of the gas chromatograph, there was sufficient recovery of the analytes onto the column to allow separation and detection.

2.4.3 GC-MS-MS

2.4.3.1 MS-MS Conditions

The ion source was maintained at 280ºC. The standard mixture was run on the full scan at Q3 where it will scan all the ions fragmenting at the ion source. The parent mass or precursor ion was selected and ran at different collision energies ranging from 5-35 eV. Three most stable transitions were chosen, where first is a quantitation ion and other two are confirmation ions, from the product ion scan which ran on MRM mode where NSAIDs were confirmed with their retention times and quantitation ions. Figure 28 shows the step-by-step flow chart for the method development for MRM, which shows a summary of all the steps from MRM. Here, a full scan for a particular mixture needs to be optimized to study the retention times then a product scan is optimized by selecting a precursor and product ion, then ran at different collision energies. Finally, after optimizing product scan a MRM method is used for each individual analyte. This is explained in details in next section.
2.4.3.2 NSAIDs analysis using GC-MS-MS

The standard mix of NSAIDs with concentration ranging from (200-20 µg/mL) was studied in Q3 (full scan mode) to determine the retention times for each standard.

Figure 29 shows the full scan for three representative NSAIDs (ibuprofen, naproxen, ketoprofen) in GC-MS mode where all fragments from the ion source are mass analyzed at the detector. This chromatogram shows separation of all three NSAIDs but with an overlap with matrix interferences, which can be observed by the other interfering peaks as compared to the previous analysis using GCxGC. For example ketoprofen tends to retain more in the second dimension but in GC-MS mode with a single column shows less selectivity (according to their peak heights) as compared to ibuprofen and naproxen. This study was extended to more NSAIDs where a mixture of 7 NSAIDs was analyzed.

Figure 30 shows the overlay of 7 NSAIDs where each was ran individually to show and confirmation of the retention times and peak identities. NSAIDs showed a good sensitivity for the method but when these were ran as a 7 component mixture all the analytes except (ibuprofen and naproxen) were co-eluting in the single dimension separation. Figure 31 shows the full scan of the standard NSAIDs mix spiked in water. This study analyzed only detect 7 NSAIDs as compared to GCxGC analysis where 9 NSAIDs were detected, due to the presence of a second dimension column with different polarity.
The product scan was ran at different collision energies at 5 eV to 35 eV to determine the product ions and the method was optimized by choosing the fragments individually for each component (Refer MRM method summary for NSAIDs below for step by step explanation on the optimization of MRM).

By comparing these the collision energies were optimized for further MRM analysis. After optimizing all the standards by MRM method to build up a standard method of analysis, these standards were spiked in the water to study the selectivity using SPME fiber to extract these NSAIDs with all the interfering peaks from the mixture and then separate selectively using MRM method without any interfering peaks from water as a matrix. Figure 36 shows how MRM selectively separated only 7 NSAIDs from complex matrices. Since some of the NSAIDs such as Diclofenac were not stable after a certain amount exposure to water and light which interfered the results for other NSAIDs as a mixture. So, after the method development mixture of three NSAIDs were validated to determine the figures of merit for the method.
Figure 28 Step-by-step flow chart for optimization MRM method for NSAIDs

- Optimization of full scan with NSAIDs
- Selection of precursor ion m/z
- Optimization of collision energy during product scan
- Selection of product ion m/z
- Multiple reaction monitoring (MRM)
Figure 29 Full scan of NSAIDs on GC-MS
Figure 30 Overlay of 7 individual NSAIDs
Figure 31 Full scan of 7 NSAIDs mixture
**MRM method summary for NSAIDs**

Chapter 1 discussed the details of instrumentation for GC-MS-MS. This section will discuss the scanning of ions in triple quadrupole mode (MS-MS). Figure 20 shows a schematic of a triple quadrupole mass spectrometer. When the analytes are separated in the GC column, they undergo ionization at the ion source, and then they are mass analyzed in the triple quadrupole mass analyzer that has three quadrupoles: Q1, Q2, and Q3. In Q1 all, the analytes are mass analyzed according to their mass to charge ratio. Then they enter Q2 where they are further fragmented by argon gas, which is a soft ionization, this quadrupole acts as a Collision-induced cell where the dissociation of fragments occur (CID). These fragments then enter Q3 where they are further mass analyzed and detected at the detector. In triple quadrupole MS, different scanning modes can be set selectively separate the ions for specific analytes and separate the co-eluting peaks. Figure 32 shows different scan modes in MS-MS: full scan mode, product ion scan and multiple reaction monitoring (MRM).

In full scan mode, as seen in the schematic (Figure 32: step 1), all fragments coming out from the ion source are scanned and are detected at the detector. Full scan determines the retention time of the analytes and mass spectrum helps to determine the precursor ion, which is the most abundant. A representative ibuprofen peak in chromatogram and mass spectrum of NSAID mix is shown in Figure 33, which is a full scan of all the fragments that were detected at the detector. From the mass spectrum, it can be seen that 161 m/z was the most abundant and chosen as the precursor ion for further analysis.
Figure 32 GC-MS-MS scan modes
Figure 33 Step 1 in optimization of MRM for ibuprofen
In product ion scan mode, as seen in the schematic (Figure 32: step 2), the fragmentation occurs in CID (Q2) where the precursor ion chosen in step 1 gets further fragmented by an argon gas to break down by collision induced dissociation. These fragments collide at different collision energies (ranging from 5-35 eV) these are then compared for choosing the product ions, which can have varied % abundance at different collision energies. These collision energies are then optimized for specific fragmentation pattern for the analyte of interest, here, ibuprofen. Figure 34 shows the fragmentation of ibuprofen at collision energy 10 eV. In this example, 119, 105, and 57 were chosen at the stable transitions for precursor ion, which was 161. However, these collision energies can be different for different transitions. In Multiple reaction monitoring (MRM) scan mode, as seen in the schematic (Figure 32: step 3), after the selection of stable transitions one transition is a quantitative ion and other two transitions are the confirmatory ions which can be more than two if required by the method. In MRM mode, all the optimized parameters are used for further analysis. Here Q1 and Q2 are fixed for the optimized mass analyzing and collision energies respectively.

Figure 35 shows the optimized MRM scan for ibuprofen. In this spectrum, all the transitions are represented: each color represents one transition and the mass spectrum only scans the product ions that were fixed in the Q1 and Q2 here, only 119, 105 and 57 were mass analyzed in Q3. Table 4 shows all the optimized conditions with their precursor ions and collision energies for each NSAID. Figure 36 shows all the 7 NSAIDs were separated by their transitions in MRM mode (all the colors represent an individual transition for a specific analyte at specific retention time) with the interference of few degrading compounds having similar fragmentation pattern, which made it difficult to monitor the same transition each time. So only three NSAIDs were validated in a mixture.
Figure 34 Step 2 in optimization of MRM for ibuprofen
Figure 35 Step 3 in optimization of MRM for ibuprofen
Table 4 Optimized MRM conditions used for GC-MS-MS analysis of NSAIDs

<table>
<thead>
<tr>
<th>ID#</th>
<th>Name</th>
<th>tr (min)</th>
<th>Precursor Ion</th>
<th>Product Ions</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ibuprofen</td>
<td>8.067</td>
<td>161</td>
<td>119,105,57</td>
<td>10,10,10</td>
</tr>
<tr>
<td>2</td>
<td>Naproxen</td>
<td>10.498</td>
<td>185</td>
<td>170,153,141</td>
<td>10,20,25</td>
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<tr>
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<td>Mefenamic acid</td>
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<td>208,194,180</td>
<td>20,20,20</td>
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<tr>
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<td>Aceclofenac</td>
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<td>10,10,30</td>
</tr>
<tr>
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<td>Ketoprofen</td>
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<td>81,69,63</td>
<td>10,10,30</td>
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<tr>
<td>6</td>
<td>Tolfenamic acid</td>
<td>11.169</td>
<td>208</td>
<td>180,179,152</td>
<td>15,20,20</td>
</tr>
<tr>
<td>7</td>
<td>Diclofenac</td>
<td>11.47</td>
<td>214</td>
<td>179,178,151</td>
<td>5,5,30</td>
</tr>
</tbody>
</table>
Figure 36 Multiple reaction monitoring on NSAIDs
Analytical figures of merit

Linear range

The Linear fit plot for each of the standards can be evaluated by the appearance of the trend line with $R^2$ for each standard line. A linear regression program from Microsoft Excel was used to determine the slope of each calibration curves.

% Recovery:

% Recovery from each sample was calculated using following equation:

\[
\% \text{ Recovery} = \left( \frac{\text{Concentration extracted}}{\text{Concentration spiked}} \right) \times 100
\]

Equation 14

The extracted concentration was calculated using the equation for calibration curves determined using linear regression algorithm in Microsoft Excel.

The extracted concentration was calculated using the equation for calibration curves determined using linear regression algorithm in Microsoft Excel. % Recoveries were obtained in the range from 74-92%. Samples were spiked with reference standards before extraction.

Linear range, Limit of detection (LOD) and Limit of Quantitation (LOQ):

LOD/LOQ was determined using the IUPAC method. Below is the equation used to calculate these limits.

\[
LOD = \frac{kS_b}{m}
\]

Equation 15
Where \( k \) is the S/N threshold that is equal to 3 for LOD and equal to 10 for LOQ. The standard deviation \( S_b \) of the blank, which was determined by taking the standard deviation of the noise readings from 10 data points adjacent to the peak at S/N between 2 and 3. [77]

The results for NSAIDs standards showed linearity in the range from 2-100 ng/mL using an external standard method with \( R^2 \) ranging from 0.99-0.92 as shown in Figure 37. Linearity was also affected due to the poor peak shapes as the NSAIDs were extracted underivatized.

The reported values for LOD ranged from 2-8 ng/mL and LOQ ranged from 6-26 ng/mL.

The validation parameters for NSAIDs are summarized in Table 5.

Real samples of water were obtained from some local places in New Jersey and tested for the presence of NSAIDs. However, none of them detected the presence of NSAIDs. This method using MRM can be used to determine the presence of NSAIDs at trace levels in different matrices. Table 6 shows a summary of real sample analysis.
Figure 37 Calibration curves for ibuprofen, naproxen, and ketoprofen
Table 5 Summary of the linearity study of NSAIDs using GC-MS-MS

<table>
<thead>
<tr>
<th>NSAIDs</th>
<th>$R^2$</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibuprofen</td>
<td>0.9252</td>
<td>2.0</td>
<td>6.7</td>
<td>76</td>
</tr>
<tr>
<td>Naproxen</td>
<td>0.9945</td>
<td>2.0</td>
<td>6.7</td>
<td>92</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>0.9597</td>
<td>8.0</td>
<td>26.7</td>
<td>74</td>
</tr>
</tbody>
</table>

Table 6 Summary of real sample analysis for NSAIDs

<table>
<thead>
<tr>
<th>NSAIDs</th>
<th>Ibuprofen</th>
<th>Naproxen</th>
<th>Ketoprofen</th>
</tr>
</thead>
<tbody>
<tr>
<td>River water</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Drinking water 1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Drinking water 2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = not detected
**Chromatographic figures of merit**

Chromatographic parameters are used to evaluate the performance of an analytical separation. These include retention factor, selectivity, efficiency and resolution, which are used to evaluate and compare a method, which is in use to a new method of separation. [78] In GC, the separation can occur using a constant temperature or temperature changed at a constant rate. An isothermal separation occurs when the temperature remains constant for the entire run, which can be used to separate samples containing a single component or a mixture of few components. It can be used to determine chromatographic figures of merit such as the void time of the column, $t_0$; the retention factor, $k$; the selectivity, $\alpha$; and the column efficiency, $N$. A temperature-programmed separation occurs when the temperature is increased at a constant rate throughout a single run, which can be useful in separating multiple analytes in a single run in shorter time. [2] [5] This section studies and compare the one-dimensional (1D) (a 15m column) and a two-dimensional (2D) separation (15m in the first dimension and a 1.5m in the second dimension) which were used to evaluate selectivity of NSAIDs in a temperature programmed separation.

**Retention factor**

Retention factor or capacity factor ($k$) evaluates the retention of the analyte and gives information of the time of interaction with the stationary phase. A high value of $k$ shows that it is highly retained. Retention factor was determined using the equation below:

$$k = (t_r - t_0)/ t_0 \quad \text{Equation 16}$$
where $t_r$ is the retention time of analyte and $t_0$ is hold up time. In this example, 2D retention factor shows increased retention as compared to 1D retention factor values. This is due to the change in polarity, which is also based on the polarity of the molecule. For an analyte with a good retention on a GC column have a value for retention factor in the range of 2-10. [2] As seen in Table 7 the retention factor for ibuprofen and naproxen with lower values which shows poor retention on the secondary column but the value for ketoprofen shows a good retention in the second dimension which is higher than 2 if compared to the 1 D-separation.

**Selectivity**

Selectivity is the ratio of retention factors between two peaks adjacent to each other and gives the information on separation of peaks from each other. The value of $\alpha$ should be higher than 1.0 in order to separate the peaks. Selectivity also discusses the interaction between solutes and stationary phase for each of the analytes in a mixture and can be used as a measure to study relative intermolecular forces. The values obtained for NSAIDs separation were higher than 1.0, which shows that the peaks are separated from each other even though the retention was poor. [5]
Efficiency

The efficiency (N) is a measure of dispersion of analyte in the column as it travels through. This can be calculated by:

\[ N = 16 \left( \frac{t_r}{w_b}\right)^2 \quad \text{Equation 17} \]

Where \( t_r \) is the retention time of the analyte and \( w_b \) is the width of the peak. Efficiency is also affected by the column flow rate and pressure of carrier gas. In this study, the second dimension separation shows lower efficiency as compared to the 1D, this is due to the shorter length of the 2D column, which gives narrow peak widths. This variation of peak widths is an effect of analyte interaction with the stationary phase at a higher velocity in the shorter second column.

Tailing factor and peak shape

During the chromatographic separation, analytes aggregate at the same retention time after sorption and desorption from the stationary phase. This result into a peak with a normal or a Gaussian shapes on a chromatogram. Some peaks show asymmetry representing that there was an undesirable interaction between solute and stationary phase. For example, some peaks show broadening (occurs due slow kinetics of mass transfer), tailing or fronting. Tailing or fronting I the peak can be determined by evaluating the location of the peak asymmetry by measuring the peak height at 10% of the peak height. If the tailing factor is greater than one then there is tailing in the peak, if tailing factor is less than one there is fronting.
Table 7 Comparison of chromatographic parameters for NSAIDs with 1 dimensional and 2-dimensional separation.

<table>
<thead>
<tr>
<th>NSAID</th>
<th>GCxGC-MS</th>
<th></th>
<th>GC-MS-MS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average 2D tr (sec)</td>
<td>k</td>
<td>N</td>
<td>Average 1D tr (min)</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>1.65</td>
<td>0.41</td>
<td>4.36E+01</td>
<td>8.01</td>
</tr>
<tr>
<td>Naproxen</td>
<td>2.7</td>
<td>1.31</td>
<td>1.17E+02</td>
<td>10.46</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>3.9</td>
<td>2.33</td>
<td>2.43E+02</td>
<td>11.09</td>
</tr>
</tbody>
</table>
Comparison of two techniques

NSAIDs used in this study were separated using two different techniques GCXGC-TOFMS and GC-MS-MS. One being GCxGC, which used a two-column combination with different polarity making it orthogonal type of separation to separate analytes from a complex matrix with different polarity, which increases peak capacity. The other one being GC-MS-MS which separates analytes using one dimension of separation with the use of single column which cannot separate the coeluting analytes and analytes with different polarity but this instrument can resolve the coeluting peaks with the use of two MS to show detector selectivity. Table 8 summarizes the comparison of two techniques.

This study evaluated the separation ability of each instrument for NSAIDs mixture, which was injected without derivatization using SPME technique. Both the techniques demonstrated a good separation efficiency with different optimization parameters for each instrument. In GCXGC-TOFMS, the modulator optimization plays an important role in the separation using two columns. In GC-MS-MS, selection of specific ion transition for particular analytes to use in multiple reaction monitoring mode (MRM) which was a challenge to optimize and separate NSAIDs mixture due to some degradation on analytes, which was also evaluated by interference study. Each instrument gives a good separation depending on the type of analytes to be separated so none is superior over other, but when combined to study the similar class of analytes provides important information on how to approach a problem in separation science. A comparison of the two multidimensional techniques as shown in Table 8 showing each has their own advantages. It can be decided on the type of sample matrix used to separate and analytes of interest.
Table 8 Summary for comparison of two techniques

<table>
<thead>
<tr>
<th>Instrument:</th>
<th>GC-MS-MS</th>
<th>GCxGC-TOF-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column:</td>
<td>One column</td>
<td>Two columns</td>
</tr>
<tr>
<td>Detector type:</td>
<td>MS-MS</td>
<td>TOF-MS</td>
</tr>
<tr>
<td>Modulator:</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Collision cell:</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Separation:</td>
<td>Standard</td>
<td>Orthogonal (increased peak capacity)</td>
</tr>
<tr>
<td>Detection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mass spectrometer</td>
<td>- Selective</td>
<td>- Universal</td>
</tr>
<tr>
<td></td>
<td>(Operate in FS, SIM and MRM mode)</td>
<td>(Only FS and SIM mode)</td>
</tr>
<tr>
<td></td>
<td>- High sensitivity</td>
<td>- Higher sensitivity</td>
</tr>
<tr>
<td></td>
<td>- Standard acquisition rate</td>
<td>- Fast acquisition rate</td>
</tr>
</tbody>
</table>
2.5 Conclusions

Solid phase microextraction coupled to comprehensive two-dimensional gas chromatography-time of flight mass spectrometry (SPME-GCxGC-TOFMS) and solid phase microextraction coupled to gas chromatography triple quadrupole mass spectrometry (SPME-GC-MS-MS) was used to extract nine and seven non-steroidal anti-inflammatory drugs from water and separate them without derivatization respectively. This analysis provides insight into the many variables that can affect selectivity in multi-dimensional chromatographic methods. In the extraction, selectivity was affected by fiber phase selection, extraction temperature, and pH. The added selectivity provided by adding the moderately polar second dimension column in GCxGC and multiple reaction monitoring for detection in GC-MS-MS both reduces the possibility of problems with matrix interferences and provides added separation space for additional analytes or interferences. The next chapter studies a selectivity using first dimension (extraction selectivity) and the third dimension (Detection selectivity) with a different class of analytes in a complex matrix.
CHAPTER 3 ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS AND ITS METABOLITES IN FISH OIL USING SPME-GC-MS-MS

3.1 Abstract

Polycyclic aromatic hydrocarbons (PAHs) are carcinogens that are released to the environment due to human activities and from disasters such as oil spills. This study aimed at determining the trace levels of PAHs and metabolites, which are generally mono-hydroxy derivatives present in fish oil and food using solid phase microextraction coupled to gas chromatography with two-dimensional mass spectrometric detection (SPME-GC-MS-MS) using the first and third dimension of selectivity. The combination of the concentrating power and ease of automation of SPME with the sensitive and selective detection afforded by MS-MS provides very low detection limits with a straightforward analysis.

PAHs were detected in aquatic life after the Deepwater Horizon oil spill of 2010. In previous work using headspace SPME with single quadrupole GC-MS, the limits of detection and quantitation varied from 0.1 to 50 ppb. This work extends detection to MS-MS using a triple quadrupole mass spectrometer to increase the selectivity for the PAHs and to test simultaneously for the mono-hydroxy metabolites of PAHs that are produced by fish as the PAHs are injected. The PAHs were extracted from samples of fish oil capsules spiked with the standard EPA 610 mixture and standard PAH metabolites, extracted using a polydimethylsiloxane fiber, and further analyzed using GC-MS-MS with multiple reaction monitoring.
3.2 Introduction

Polycyclic aromatic hydrocarbons (PAH) are a class of analytes that are often studied because of their carcinogenic and mutagenic properties. Contamination of PAHs has been a major issue in regards to the environment because they present a health hazard to the living population. PAHs can enter the environment from sources such as the burning of crude oils, smoke, smoked food, automobiles, etc. Different separation techniques are available for the separation and quantification of PAHs, however, the separation of some PAH isomers is found to be challenging. These isomers may have different effects on a person’s health, which is why it is important to study them individually through effective separation techniques.

The British Petroleum (BP) oil spill in the Gulf of Mexico (April 20, 2010) lasted for 87 days, harmfully affecting both the aquatic and mainland life of animals. During the oil spill, researchers conducted a study on the PAHs that contaminated the fish during the spill. The tested fish were taken locally from the Atlantic Ocean off the coast of New Jersey and from the Gulf of Mexico off the Louisiana coast where the oil spill had occurred. [15]

Polycyclic aromatic hydrocarbons are a ubiquitous group of several hundred chemically related, environmentally persistent organic compounds of various structures and varied toxicity. [79] It is important to test for PAHs because of the toxic and harmful effects they have on organisms. The menhaden fish collected, which are not consumed by humans, are a diet for fish humans do consume, such as swordfish, cod, and tuna. Thus, it was necessary to test for high levels of PAHs caused by the oil so researchers could know how food sources such as fish can affect humans. PAHs are produced naturally, during forest fires and volcano eruptions, and are manufactured, in cigarette smoke, vehicle exhaust, and wood or trash burning. PAHs can cause both short-term and long-term health problems, depending on the length and route of exposure.
Short-term effects include nausea, eye and skin irritation, diarrhea, and confusion. Long-term effects include immune dysfunction, cataracts, kidney and liver damage, cancer, genetic mutations, and birth defects [79]. There are many ways humans can be exposed to PAHs/metabolites, from drinking water to driving cars, and the surrounding air.

The extraction technique for the PAHs used was solid phase microextraction (SPME). SPME is a simple, efficient, solventless sample preparation method that is ideally GC coupled with mass spectrometry (MS) [69]. There are various types of SPME fibers, such as polydimethylsiloxane (PDMS), polydimethylsiloxane/divinylbenzene (PDMS/DVB), polydimethylsiloxane divinylbenzene carboxen (PDMS/DVB/Carboxen), which can be used for extraction. Each fiber differs in polarity, coating, and thickness, for this study PDMS fiber was selected due to its non-polar coating and a 100µm thickness, which could extract the non-polar PAHs from the headspace of the SPME vial and absorb all the volatile analytes onto the fiber until they reach the injection port.

The instrumentation used is a gas chromatograph- mass spectrometer (GC-MS-MS). Gas chromatography is a form of chromatography in which a gas is a mobile phase. [5]. MS-MS is a triple-quadrupole technique rather than a single-quadrupole (MS). Triple-quadrupole has two stages of mass analysis, which makes it more sensitive and highly selective that was discussed in details previously.
3.3 Materials and methods

A Shimadzu gas chromatograph coupled to a triple quadrupole mass spectrometer (GC-MS-TQ8030) equipped with fully automated PAL autosampler was used for the experiment. For separation of the compounds, a RTX-5 MS column (15m X 0.25 mm i.d. X 0.25 µm df) (Restek, Bellefonte, PA) was used. Column flow rate for the Helium was 1.0 mL/min with a splitless injection. The temperature of transfer line was maintained at 250°C and ion source at 230°C. For GC headspace injections sample mixture was injected into the heated GC inlet at 280°C. The temperature program 1. For PAHs study, which ramped from 50°C for 1 min, ramped to 325°C at a rate of 20°C/min for 2 min. 2. For PAH metabolite study which ramped from 80°C for 3 min, ramped to 230°C at a rate of 20°C/min for 2 min. Data was analyzed using Shimadzu’s Lab Solutions software version 4.2.

Chemicals for the experiment: a 16 component EPA 610 PAH mix and PAH metabolites study included 1-Naphthol, 2-Naphthol, 9-phenanthrol, 9-Fluorenol, and 1-Pyrenol.

Table 9 and Table 10 show the compounds, with their structures, used in this study that was obtained from Sigma-Aldrich (St. Louis, MO, USA). The fibers used for SPME: a 100 µm PDMS were purchased from Supelco (Bellefonte, PA). Solvent methanol and dichloromethane used in the study were obtained from Macron Fine chemicals (Center Valley, PA). 10 mL vials with magnetic screw caps were purchased from Sigma-Aldrich (St. Louis, MO).
Table 9 List of EPA 610 PAH mixture with their concentrations and mass monitored

<table>
<thead>
<tr>
<th>ID#</th>
<th>Compound</th>
<th>Concentration (µg/mL)</th>
<th>Major Masses (Precursor ion &gt; product ion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naphthalene</td>
<td>101.62</td>
<td>128&gt;127</td>
</tr>
<tr>
<td>2</td>
<td>Acenaphthylene</td>
<td>203.78</td>
<td>152&gt;151</td>
</tr>
<tr>
<td>3</td>
<td>Acenaphthene</td>
<td>101.97</td>
<td>152&gt;151</td>
</tr>
<tr>
<td>4</td>
<td>Fluorene</td>
<td>20.34</td>
<td>165&gt;164</td>
</tr>
<tr>
<td>5</td>
<td>Phenanthrene</td>
<td>10.39</td>
<td>178&gt;152</td>
</tr>
<tr>
<td>6</td>
<td>Anthracene</td>
<td>10.12</td>
<td>178&gt;177</td>
</tr>
<tr>
<td>7</td>
<td>Fluoranthene</td>
<td>20.33</td>
<td>202&gt;201</td>
</tr>
<tr>
<td>8</td>
<td>Pyrene</td>
<td>10.23</td>
<td>202&gt;201</td>
</tr>
<tr>
<td>9</td>
<td>Benzo(a)anthracene</td>
<td>10.22</td>
<td>228&gt;227</td>
</tr>
<tr>
<td>10</td>
<td>Chrysene</td>
<td>9.79</td>
<td>228&gt;227</td>
</tr>
<tr>
<td>11</td>
<td>Benzo(b)fluoranthene</td>
<td>20.36</td>
<td>252&gt;244</td>
</tr>
<tr>
<td>12</td>
<td>Benzo(k)fluoranthene</td>
<td>10.15</td>
<td>252&gt;242</td>
</tr>
<tr>
<td>13</td>
<td>Benzo(a)pyrene</td>
<td>10.91</td>
<td>252&gt;242</td>
</tr>
<tr>
<td>14</td>
<td>Indeno(1,2,3-cd)pyrene</td>
<td>20.17</td>
<td>276&gt;256</td>
</tr>
<tr>
<td>15</td>
<td>Dibenz(a,h)anthracene</td>
<td>20.89</td>
<td>276&gt;267</td>
</tr>
<tr>
<td>16</td>
<td>Benzo(g,h,i)perylene</td>
<td>10.23</td>
<td>276&gt;260</td>
</tr>
</tbody>
</table>
Table 10 Summary of PAH metabolites used in the study

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular weight</th>
<th>Mass monitored</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Naphthol</td>
<td>144.17</td>
<td>115&gt;89</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>2-Naphthol</td>
<td>144.17</td>
<td>115&gt;89</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>9-Fluorenol</td>
<td>182.22</td>
<td>181&gt;152</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>1-Pyrenol</td>
<td>218.25</td>
<td>189&gt;187</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
</tbody>
</table>
Sample preparation

Stock solutions for standardization were made in methanol and dichloromethane in 1:1 ratio and stored at refrigerated temperatures. The 610 EPA PAH mix had the concentrations of all PAHs in the range from 100-2000 ng/mL, which was further diluted in a 10 mL mixture of solvent and then used for further studies, concentrations for working standards were as shown in Table 9. For the analysis using SPME, 1 g of fish oil capsule (Nature’s Bounty, fish oil capsules, lot number: 372863-01) was placed in a 10 mL of SPME screw cap vial and desired concentration was attained by spiking standard EPA mixture stock solution to obtain desired PAH concentrations. Similarly, PAH metabolites samples were prepared by spiking with standard stock solutions (100 ng/mL) prepared in methanol and diluting to obtain desired concentration. Real fish oil samples for PAH metabolite analysis were used from the previous study on PAHs [15]

SPME conditions

In this study, the analytes were extracted in the headspace of a vial where analytes in the gaseous phase are extracted from that sample matrix at higher temperatures, which are equilibrated with the sample concentration. Headspace extraction is efficient for analytes that are volatile and the matrix interference can be avoided at the same time. These type of extractions requires less time of equilibration. A compound with a lower partition coefficient evaporates easily into headspace from the matrix with a larger response and low detection limits. Figure 38 shows the phases in the headspace of the vial, where the volatile analytes are partitioned between the headspace and sample matrix until the equilibrium is attained due to the presence of heat and agitation.

SPME conditions were used from the previous studies [15], where headspace solid phase microextraction was used (HS-SPME) with a non-polar polydimethylsiloxane (PDMS) fiber with a 100 µm thickness was used for volatile analytes here PAHs. The fibers were conditioned
according to the manufacturer’s instructions (Sigma-Aldrich-Supelco, Bellefonte, PA). HS-SPME was performed on an automated autosampler AOC-5000. PAHs and metabolites spiked in fish oil capsule were incubated in the agitator at 150°C for 15 min with agitator speed of 500 rpm, followed by headspace extraction for 30 minutes. The fiber was desorbed into the inlet of a gas chromatograph for 15 minutes. After the extraction was performed, the fiber was baked again at 360°C for 18 minutes to prevent carry over. This was confirmed by running blanks between each sample run.

**MS-MS Conditions**

The ion source was maintained at 230°C. The standard mixture was run on the full scan at Q3 where all the ions fragmenting at the ion source were scanned. The selected parent mass or precursor ion was selected and run at different collision energies ranging from 5-35 eV. The three most stable transitions were chosen, where the first is a quantitation ion and the other two are confirmation ions from the product ion scan, which ran in MRM mode. PAHs and metabolites were confirmed with retention times and quantitation ions. Figure 39 shows a step-by-step flow chart for MRM method development. First, a full scan of the standards is measured to determine the precursor ion, which is the most abundant ion for a specific analyte. Second, a product ion scan is measured; by sample runs at different collision energies in the collision cell. After selecting precursor and product ions and optimizing collision energies; a multiple reaction monitoring is programmed where three or more sets of reference ions (one quantitation ion and two confirmation ions) which program the MS to identify only specific ions at a specific retention time, which is unique for each analyte.
Figure 38 Phases in the headspace of a vial.

Approval for use of this image was given by Teledyne Tekmar. www.teledynetekmar.com [80].
Measure the standard sample (Q3 Analysis)

Determine the precursor ion

Measure the standard sample (Product Ion Scan Analysis)

Determine the product ion and collision energy

Create the MRM Analysis Method (Optimize collision energy in detail)

Set the reference ion ratio

Figure 39 Flow chart for MRM optimization for PAH analysis
3.4 Results and discussion

PAH standard mixture

The standard mix of PAHs with concentrations ranging from (200-20 µg/mL) was studied in Q3 (full scan mode) to determine the retention times for each standard. Figure 40 shows the full scan of the standard PAH mix representing all sixteen PAHs separated from each other to determine the retention times for each peak for further analysis. All the peaks were identified and confirmed by the MS library is listed in Table 9 that represent the numbers as labeled in the chromatogram. The product scan ran at different collision energies (CE) at 5 eV to 35 eV to determine the product ions and the method was optimized by choosing the fragments individually for each component. For instance, naphthalene at retention time 4.57 min shows maximum abundance at m/z 128, which was selected as a precursor ion. After the product ion scan, one quantitation ion (127 at CE 10) with next highest abundance was chosen, two confirmation ions with a next highest abundance (102 at CE 10 and 78 at CE 10) was selected for MRM analysis. Table 11 summarizes the above optimized parameters for PAH analysis.

Figure 41 shows a representative product scan at different collision energies foracenaphthene at retention time 6.67 minutes. To choose and compare the abundance of product ions different collision energies were compared. Figure 42 shows the comparison of CE 5 eV, 10 eV, 15 eV and 30 eV, where a specific fragment can be compared at different collision energies and can be used for further analysis by MRM. By comparing these, the collision energies were optimized for further MRM analysis. After optimizing all the standards by MRM method to build up a standard method of analysis these standards were spiked in the fish oil. This can be used to study
Figure 40 Full scan of standard PAH mixture

(Refer Table 9 peak retention times with peak numbers)
Table 11 Optimized MRM conditions used for GC-MS-MS analysis of PAH

<table>
<thead>
<tr>
<th>ID#</th>
<th>Name</th>
<th>tr (min)</th>
<th>Precursor Ion</th>
<th>Product Ions</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Naphthalene</td>
<td>4.57</td>
<td>128</td>
<td>127,102,78</td>
<td>10,10,10</td>
</tr>
<tr>
<td>2</td>
<td>Acenaphthylene</td>
<td>6.437</td>
<td>152</td>
<td>151,126,102</td>
<td>25,25,25</td>
</tr>
<tr>
<td>3</td>
<td>Acenaphthene</td>
<td>6.67</td>
<td>152</td>
<td>151,126,102</td>
<td>10,25,25</td>
</tr>
<tr>
<td>4</td>
<td>Fluorene</td>
<td>7.253</td>
<td>165</td>
<td>164,139,115</td>
<td>10,25,25</td>
</tr>
<tr>
<td>5</td>
<td>Phenanthrene</td>
<td>8.35</td>
<td>178</td>
<td>152,151,128</td>
<td>5,30,5</td>
</tr>
<tr>
<td>6</td>
<td>Anthracene</td>
<td>8.423</td>
<td>178</td>
<td>177,152,138</td>
<td>15,15,15</td>
</tr>
<tr>
<td>7</td>
<td>Fluoranthene</td>
<td>9.8</td>
<td>202</td>
<td>201,189,152</td>
<td>5,10,10</td>
</tr>
<tr>
<td>8</td>
<td>Pyrene</td>
<td>10.047</td>
<td>202</td>
<td>201,143,138</td>
<td>10,5,15</td>
</tr>
<tr>
<td>9</td>
<td>Benzo(a)anthracene</td>
<td>11.497</td>
<td>228</td>
<td>227,223,200</td>
<td>5,5,5</td>
</tr>
<tr>
<td>10</td>
<td>Chrysene</td>
<td>11.543</td>
<td>228</td>
<td>227,214,202</td>
<td>5,5,15</td>
</tr>
</tbody>
</table>
Figure 41 Representative product scan of PAH mixture at collision energy 5 eV.
Figure 42 Optimization of product scan at different collision energies

(Picture from Lab solutions software)
the selectivity using the SPME fiber to extract these PAHs in fish oil with all the interfering peaks from esters present in the fish oil and then to separate all PAHs selectively using the MRM method without any interfering peaks from oil matrices.

Figure 43 shows the full scan with the PAH standard mix spiked in Fish oil which, showing all the interfering peaks, which were identified as the esters from the fish oil that were also separated using a SPME fiber. To separate all the PAHs from fish oil, using the detector, a MRM program was developed. Figure 44 shows how MRM selectively separated with all the fragments selected in the MRM method, with different colors for 10 PAHs. Figure 43 and Figure 44, when compared, show the selectivity of the detector which increases in the signal for each PAHs without any other peaks from the sample matrix.

The method was then validated to determine the figures of merit. This study was further extended to extract PAH metabolites in fish oil, which were present in the fish oil samples present in the lab. This method can also be used to identify the presence of PAHs in different sample matrices such as fuels, soil samples where PAHs might be present and needs to quantify with all other interferences using SPME as a sample preparation technique to separate analytes from a matrix and GC-MS-MS to quantify at trace levels.
Figure 43 Full scan of PAHs mixture in fish oil showing the selectivity of SPME to extract analytes from a complex matrix.

(Refer Table 11 peak retention times with peak numbers)
Figure 44 MRM scan of PAHs mixture in fish oil showing detector selectivity to separate only analytes of interest from the sample matrix.

(Refer Table 11 peak retention times with peak numbers)
Validation

Linear range, Limit of detection (LOD) and Limit of Quantitation (LOQ), % Recovery:

The results for PAH standards showed linearity in the range from 0.008-400 ng/mL using the external standard method with R² ranging from 0.98-0.99 as shown in Figure 45 to Figure 54 show a good linearity for the 10 PAHs. The reported values for LOD ranged from 0.002-20 pg/mL and LOQ ranged from 0.01-67 pg/mL.

% Recoveries were obtained in the range from 80-105%. Samples were spiked with reference standards before extraction.

The validation parameters for PAHs are summarized in Table 12.

Real samples were tested for the presence of PAHs. Since these fish oil samples were extracted and stored a few years back from previous studies on GC-MS, no presence of PAHs was found. So this study was extended to determine PAH metabolites, which expected to form in the fish oil over the time. This PAHs method using MRM can still be applied to determine the presence of PAHs at trace levels in different matrices such as soil and other oil samples.

Next section shows the method development using MRM for PAH metabolites.
Figure 45 Calibration curve for naphthalene from the optimized GC-MS-MS analysis

\[ y = 1006.4x - 4154.6 \]

\[ R^2 = 0.9903 \]
Figure 46 Calibration curve for acenaphthylene from the optimized GC-MS-MS analysis
Figure 47 Calibration curve for acenaphthene from the optimized GC-MS-MS analysis

\[ y = 1768.1x - 9959.1 \]

\[ R^2 = 0.9941 \]
Figure 48 Calibration curve for fluorene from the optimized GC-MS-MS analysis

Fluorene

\[ y = 656.13x + 1318.2 \]
\[ R^2 = 0.9941 \]
Figure 49 Calibration curve for phenanthrene from the optimized GC-MS-MS analysis

\[ y = 662.1x + 1222.7 \]

\[ R^2 = 0.9952 \]
Figure 50 Calibration curve for anthracene from the optimized GC-MS-MS analysis
Figure 51 Calibration curve for fluoranthene from the optimized GC-MS-MS analysis

\[ y = 368.45x + 1170.2 \]
\[ R^2 = 0.9927 \]
Figure 52 Calibration curve for pyrene from the optimized GC-MS-MS analysis
Figure 53 Calibration curve for benzo(a)anthracene from the optimized GC-MS-MS analysis

\[ y = 121.03x + 892.79 \]

\[ R^2 = 0.9861 \]
Figure 54 Calibration curve for chrysene from the optimized GC-MS-MS analysis
Table 12 Summary of the linearity study of PAHs using GC-MS-MS

<table>
<thead>
<tr>
<th>PAH</th>
<th>$R^2$</th>
<th>LOD (pg/mL)</th>
<th>LOQ (pg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>0.9903</td>
<td>0.020</td>
<td>0.10</td>
<td>104</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>0.9929</td>
<td>8.0</td>
<td>27</td>
<td>105</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>0.9941</td>
<td>0.040</td>
<td>0.13</td>
<td>91</td>
</tr>
<tr>
<td>Fluorene</td>
<td>0.9941</td>
<td>0.0080</td>
<td>0.030</td>
<td>91</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>0.9952</td>
<td>0.0020</td>
<td>0.010</td>
<td>99</td>
</tr>
<tr>
<td>Anthracene</td>
<td>0.9973</td>
<td>20</td>
<td>67</td>
<td>107</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>0.9927</td>
<td>0.080</td>
<td>0.30</td>
<td>86</td>
</tr>
<tr>
<td>Pyrene</td>
<td>0.9934</td>
<td>0.040</td>
<td>0.13</td>
<td>86</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>0.9861</td>
<td>0.0040</td>
<td>0.010</td>
<td>97</td>
</tr>
<tr>
<td>Chrysene</td>
<td>0.9859</td>
<td>4.0</td>
<td>13</td>
<td>81</td>
</tr>
</tbody>
</table>
This section focuses on the analysis of polycyclic aromatic hydrocarbon metabolites, which are a ubiquitous group of several hundred chemically related, environmentally persistent organic compounds of various structures and varied toxicity. These are produced due to the degradation of the PAHs into their metabolites over a period. It is important to test these metabolites because of potentially toxic and harmful effects they have on organisms. These were studied the same way as the PAHs standard mixture to determine the full scan for the method as seen in Figure 55 and Table 13 show the optimized parameters for MRM scan. Figure 56 shows the MRM scan for the PAH metabolites in fish oil which also shows the isomeric separation of 1-naphtol and 2-naphtol using MRM. Figure 57 shows the fiber blank where the fiber was baked at a higher temperature of the inlet to confirm any carry over from the previous sample. This method was applied to the real fish oil samples obtained from the Day brook fish samples which were stored from the previous studies to determine the potential metabolites formation over the time. Figure 59 shows a sample analysis of real fish oil sample 1 which confirmed the presence of 1-pyrenol and fish oil sample 2 confirm the presence of 9-flurenol and 1-pyrenol. The amount of metabolites was determined using the validation studies.

To test the linearity a calibration curve was plotted with a standard mixture of PAH metabolites spiked in fish oil at 0.0001-100 µg/mL. The method showed linearity in this range from 0.91 to 0.99 for each metabolite as shown in Figure 58. The equation of line obtained from the calibration curve was used to calculate the concentration of real samples.
Table 13  Optimized MRM conditions used for GC-MS-MS analysis of PAH metabolites

<table>
<thead>
<tr>
<th>ID#</th>
<th>Name</th>
<th>tr (min)</th>
<th>Precursor Ion</th>
<th>Product Ions</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-Naphthol</td>
<td>8.353</td>
<td>115</td>
<td>89, 65, 63</td>
<td>15, 15, 25</td>
</tr>
<tr>
<td>2</td>
<td>2-Naphthol</td>
<td>8.400</td>
<td>115</td>
<td>89, 65, 63</td>
<td>15, 15, 25</td>
</tr>
<tr>
<td>3</td>
<td>9-Fluorenol</td>
<td>9.720</td>
<td>181</td>
<td>152, 127, 77</td>
<td>15, 25, 30</td>
</tr>
<tr>
<td>4</td>
<td>1-Pyrenol</td>
<td>13.217</td>
<td>189</td>
<td>187, 163, 139</td>
<td>25, 25, 25</td>
</tr>
</tbody>
</table>
Figure 55 Full scan of PAH metabolites mixture in fish oil

Shows the separation of the PAH metabolites through SPME in the full scan with the interfering peaks from the components of fish oil.
Figure 56 MRM scan of PAH metabolites in fish oil

Shows the PAH metabolites which were extracted using SPME and then selectively separated using MRM. Here the standards showed linearity with the detection limits ranging from 200-0.002 ng/mL with the $R^2$ ranging from 0.91-0.99.
Figure 57  Fiber blank of PAH metabolites in fish oil

Shows that there was no carry over from the fiber after the fiber bake for 40 mins and two intermediate blank runs.
Validation

Linear range, Limit of detection (LOD) and Limit of Quantitation (LOQ), % Recovery:

The results for PAH metabolite standards showed linearity in the range from 0.002-200 ng/mL using the external standard method with $R^2$ ranging from 0.99-0.91 as shown in Figure 58, representing the linearity for 1-naphthol, 2-naphthol, 9-fluorenaol and 1-pyrenol without the use of any internal standard. The parent compounds showed better linearity. Since in this study, a PDMS fiber was use to extract both PAHs and metabolites, the poorer linearity shows less selectivity at lower concentrations. To improve this a different fiber with mixed polarity can be tested, which might have better selectivity for the hydroxy-PAH metabolites, to improve the extraction at lower concentrations.

The reported values for LOD ranged from 2-20 pg/mL and LOQ ranged from 1.8-66 pg/mL.

% Recoveries were obtained in the range from 92-104%. Samples were spiked with reference standards before extraction.

The validation parameters for PAH metabolites are summarized in Table 14.

Real samples of fish oil from Menhaden fish captured in NJ and LA were tested for the presence of PAH metabolites. Since these fish oil samples were extracted and stored a few years back from previous studies on GC-MS, to study the presence of PAHs metabolites GC-MS-MS was used. This PAH metabolites method using MRM can be used to determine the presence of PAH metabolites at trace levels in different matrices. These samples only showed presence of 1-Pyrenol as seen in Figure 59 and Table 15.
Figure 58 Calibration curves for PAH metabolites
Table 14 Summary of the linearity study of PAH metabolites using GC-MS-MS

<table>
<thead>
<tr>
<th>PAH</th>
<th>$R^2$</th>
<th>LOD (pg/mL)</th>
<th>LOQ (pg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Naphthol</td>
<td>0.9747</td>
<td>2.0</td>
<td>6.8</td>
<td>103</td>
</tr>
<tr>
<td>2-Naphthol</td>
<td>0.9107</td>
<td>2.0</td>
<td>6.7</td>
<td>92</td>
</tr>
<tr>
<td>9-Fluoreno</td>
<td>0.9875</td>
<td>20</td>
<td>66</td>
<td>104</td>
</tr>
<tr>
<td>1-Pyrenol</td>
<td>0.9833</td>
<td>2.0</td>
<td>6.7</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 59 MRM scan of PAH metabolites in real sample

Shows the presence of only 1-Pyrenol present in the real fish oil, which was separated and detected at low levels (9.98 ng/mL) using SPME-GC-MS-MS and rest of the peaks were interference from the fish oil.
Table 15 Summary of real sample analysis

<table>
<thead>
<tr>
<th>PAH metabolites</th>
<th>1-Naphthol</th>
<th>2-Naphthol</th>
<th>9-Fluorenol</th>
<th>1-Pyrenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Real fish oil sample 1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>9.98 ng/mL</td>
</tr>
<tr>
<td>Real fish oil sample 2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
3.5 Conclusions

In this study, focused on the extraction and detector selectivity. The effects of the BP oil spill on the aquatic life were studied using the sensitive and selective MS-MS technique, so trace levels of PAHs/metabolites can be detected with a lower limit of detection and quantitation. If the levels are too high, it will become dangerous for both the aquatic life and the humans who are exposed to contaminated fish. HS-SPME is an effective technique for the extraction of PAHs from complex matrices, such as fish oil. GC-MS-MS selectively separates complex mixtures and provides low detection limits. HS-SPME, when coupled to GC-MS-MS, is an effective technique for separation and detection at trace levels of contamination.

The next chapter demonstrates a unique selectivity using a new detection technology VUV that is a complementary technique to MS.
CHAPTER 4 ANALYSIS OF GLUCOCORTICOIDS USING GAS CHROMATOGRAPHY-VACUUM ULTRAVIOLET SPECTROSCOPY

4.1 Abstract

This study focuses on the performance of a new vacuum ultraviolet detector (VUV) for analyzing glucocorticoids in the third dimension of selectivity. These steroidal hormones are commonly used for allergic or inflammatory conditions; however, they are frequently used in the adulteration of herbal medicinal products to enhance the healing process. This study used a vacuum ultraviolet detector (VGA-100) coupled to a gas chromatograph (GC) which can test gas phase absorption in the VUV range from 125-240 nm. Glucocorticoids were detected using VUV detector as this class of drugs showed absorbance with the limit of detection of 20-60 ng mass on column of the standard glucocorticoid mix. GC-VUV shows a high potential for gas chromatographic analysis of drugs, complementary to mass spectrometry. This technique can also be extended for the applications in various samples such as identification of water by SPME to overcome the shortcomings of the other traditional GC detectors, which will be discussed in this chapter.

4.2 Introduction

4.2.1 GC-VUV background

Gas chromatography (GC) is a highly researched sensitive and selective technique for the separation of complex mixtures. This is researched with various modifications for sample introduction and varied detectors such as flame ionization (FID), thermal conductivity detector (TCD), electron capture detector (ECD), Mass spectrometer (MS), etc. MS detector is the universal detector used for GC. There are limited universal detectors coupled to GC such as MS; which provides accurate information on mass to charge ratio (m/z) for a particular analyte with the use
of a most common type of ionization technique used for MS is electron ionization and confirms the fragmentation pattern using standard MS libraries.

The vacuum ultraviolet spectrophotometer was developed in 2014 and provided a new detector technology complimentary to other GC detectors such as MS, FID, ECD, etc. The VUV detector has been used and explored in various fields of applications, such as analysis of pesticides, fatty acid methyl esters, diesel fuel, isomeric separations, etc. since its launch. The literature published in scientific journals is growing rapidly from 2014 – 2016 which is also available on the VUV Analytics website. [81]

Many detectors have been used through years with GC which vary in their response with respect to the mode of operation, selectivity, sensitivity and linearity. Over the years, there is a massive literature available on gas chromatographic separations coupled to different detectors. Between 1950 - 2016: the most commonly used detector was MS; which shows 89.31% of the published articles and other detectors together was 10.69% including FID (6.51%), TCD (0.32%), ECD (1.57%), etc. GC-VUV is a new, universal and a mass selective detector which is easy to operate with the ability to deconvolute co-eluting peaks at 20-200 pg levels of detection. [49]
4.2.2 GC-VUV Instrumentation

The electromagnetic spectrum comprises a large range of wavelengths and frequencies. These are divided into different spectral regions depending on the various kinds of radiation.

Figure 60 shows different regions of the electromagnetic spectrum. [82] The electromagnetic spectrum shows the various regions in the ultraviolet such as ultraviolet A, B, and C, vacuum ultraviolet, etc. as classified in Figure 61. Most of the ultraviolet regions have been researched for their use in detection of various analytes using different instrumentation such as liquid chromatography. Atmospheric oxygen, water and almost all chemical compounds absorb in the range 115nm-185nm. In the VUV region atomic species are probed due to the photons in $\pi \rightarrow \pi^*$ and $\sigma \rightarrow \sigma^*$ which cannot be probed in traditional ultraviolet-visible spectroscopy. [83]

GC has not been explored thoroughly with a VUV detector due to the limitations of wavelength ranges and limited applications, even though VUV spectroscopy has been used for many years. It has not been available commercially for gas chromatographic analysis. [84] [83] This technique uses a low energy VUV excitation, which is non-destructive and can scan a wavelength range from 125-240 nm. Photoabsorption of analytes in this VUV region produces spectra, which are complementary to the MS data produced by electron ionization, and this data can be compared to the libraries for identification of the analyte.
Figure 60 Electromagnetic spectrum to show VUV region.
**Classifications according to ISO 21348 – Definitions of Solar Irradiance Spectral Categories**

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Wavelength (nm)</th>
<th>Photon Energy (eV)</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultraviolet A</td>
<td>UVA</td>
<td>315 – 400</td>
<td>3.10 – 3.94</td>
<td>“Black” light, not absorbed by the ozone layer, vitamin D formation</td>
</tr>
<tr>
<td>Ultraviolet B</td>
<td>UVB</td>
<td>280 – 315</td>
<td>3.94 – 4.43</td>
<td>Mostly absorbed by the ozone layer, harmful to living organisms</td>
</tr>
<tr>
<td>Ultraviolet C</td>
<td>UVC</td>
<td>100 – 280</td>
<td>4.43 – 12.4</td>
<td>Completely absorbed by the atmosphere, germicidal applications</td>
</tr>
<tr>
<td>Hydrogen Lyman-Alpha</td>
<td>H Lyman-α</td>
<td>121 – 122</td>
<td>10.16 – 10.25</td>
<td>Spectral line at 121.6 nm, 10.20 eV</td>
</tr>
<tr>
<td>Extreme Ultraviolet</td>
<td>EUV</td>
<td>10 – 121</td>
<td>10.25 – 124</td>
<td>Ionizing radiation; extreme ultraviolet lithography</td>
</tr>
<tr>
<td>Vacuum Ultraviolet</td>
<td>VUV</td>
<td>10 – 200</td>
<td>6.20 – 124</td>
<td>Strongly absorbed by atmospheric oxygen and water, *UV cutoff at 105nm</td>
</tr>
<tr>
<td>Ultraviolet</td>
<td>UV</td>
<td>10 – 400</td>
<td>3.10 – 124</td>
<td></td>
</tr>
</tbody>
</table>

Figure 61  Classifications according to ISO 21348- For vacuum ultraviolet region.

Reprinted with permission from presentation on Harrison, D.; The Rise of Vacuum Ultraviolet Light, VUV analytics [85]
Figure 62 shows a schematic of the VUV detector. This benchtop detector is connected to a gas chromatograph by a heated transfer line. Figure 63 shows VGA-100 (VUV detector from VUV Analytics, Inc., Austin, Texas, USA) coupled to HP 5890 Gas Chromatograph. The VUV detector comprises a transfer line, a flow cell, source module and detector module. As in the schematic, a makeup flow of carrier gas is introduced into the transfer line; this helps to shorten sample residence time in detector cell. The analyte then enters a 10 cm pathlength flow cell where the analyte is exposed to VUV radiation in the 125-240 nm wavelength range by coated reflective optics and charged coupled device (CCD) light path monitor for the absorption coming from the analytes/peaks separating from GC column. These signals are then analyzed using the software to give the output as a chromatogram, with the separated peaks with the detailed absorption spectrum for respective peaks eluting from the column. VUV is sensitive to mass detector, which shows that response from the detector is directly proportional to the amount of compound present per unit time. [83]

This study demonstrates the use of VUV detector as a sensitive and universal detector for qualitative and quantitative analysis using GC. The introduction of new applications using VUV includes groups of compounds such as polycyclic aromatic hydrocarbons, fatty acid methyl esters, and drugs. This study also shows the separation and detection of water in a solvent mixture and in the solid phase microextraction (SPME) technique that uses water as a matrix using gas chromatograph. Also, overcomes the limitation of gas chromatographic analysis to quantitate water in sample mixtures. Previously studies were done using GC-MS/MS [17].
Figure 62 Schematic of GC-VUV
Figure 63 5890-GC coupled to VUV detector in our laboratory

(Image created by Anumeha P. Muthal)
4.3 Materials and methods

A VGA-100 vacuum ultraviolet detector (VUV Analytics, Inc., Austin, TX) coupled to an HP 5890 gas chromatograph was used with a Phenomenex ZB-5 MS Plus column (15m X 0.25 mm i.d. X 0.25 µm df) (Phenomenex, Inc., Torrance, CA). The column flow rate for the helium was 1.0 mL/min with a split ratio of 50:1. The temperature of the transfer line was maintained at 275°C. For injections, the sample mixture was injected manually into the heated GC inlet at 250°C. The temperature program for the drug study ramped from 150°C for 3 min, to 300°C at a rate of 15°C /min for 10 min. For the SPME study, it ramped from 80°C for 3 min ramped to 230°C at a rate of 20°C/min for 2 min. The VUV scan range was 125 nm – 240 nm wavelength.

Chemicals for the experiment: prednisone, prednisolone, methyl prednisolone, hydrocortisone and ibuprofen (for SPME study) were obtained from Sigma-Aldrich (St. Louis, MO, USA). (Table 16 shows compounds with their structure used in this study) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The fibers used for SPME: PDMS/DVB/Carboxen were purchased from Supelco (Bellefonte, PA) with a manual assembly. Solvent methanol and acetonitrile used in the study were obtained from Macron Fine chemicals (Center Valley, PA).
Table 16 Summary of compounds used in the study

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Molecular weight</th>
<th>Classifications</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisone</td>
<td>358.43</td>
<td>Synthetic corticosteroid</td>
<td><img src="image" alt="Prednisone structure" /></td>
</tr>
<tr>
<td>Prednisolone</td>
<td>360.44</td>
<td>Corticosteroid (active metabolite of prednisone)</td>
<td><img src="image" alt="Prednisolone structure" /></td>
</tr>
<tr>
<td>Methyl Prednisolone</td>
<td>374.47</td>
<td>Synthetic glucocorticoid</td>
<td><img src="image" alt="Methyl Prednisolone structure" /></td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>362.46</td>
<td>Glucocorticoid</td>
<td><img src="image" alt="Hydrocortisone structure" /></td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>206.29</td>
<td>Non-steroidal anti-inflammatory agent</td>
<td><img src="image" alt="Ibuprofen structure" /></td>
</tr>
</tbody>
</table>
4.4 Results and discussion

4.4.1 VUV separation of Glucocorticoids

Figure 64 shows the VUV separation of a 2000 ppm glucocorticoid standard mixture in acetonitrile injected under split conditions with an estimated mass-on-column ranging from 30-40 ng of each component. The respective spectrum for each glucocorticoid shows maximum absorption in the shorter wavelength range 125-160 nm (green color) this can occur due to carbon - carbon single bonds high-energy $\sigma \rightarrow \sigma^*$ transition.

Structures of drugs as shown in Table 16, this group of glucocorticoids has a similar base structure, which is a steroidal ring with different group substituted to it. The similar structure of glucocorticoids makes it challenging to separate and identify these compounds with traditional detectors such as FID. This group of glucocorticoids was separated using GC-VUV where the order of elution was prednisone, hydrocortisone, prednisolone and methyl prednisolone. Prednisolone was the one least retained which explains the structural difference on the C11 ketone group, with less hydrogen bonding with stationary phase as compared to other glucocorticoids this make it elute first. Hydrocortisone is the next due to the absence of unsaturation on C1 and C2 as compared prednisolone and methyl prednisolone and hence prednisolone and methyl prednisolone elute later.
Figure 65 shows the VUV spectrum for each glucocorticoid monitored at a 125-240 nm range (maximum range for the instrument) which shows the maximum absorption obtained for all glucocorticoids between 140-180 nm, and still the VUV identified them as individual components. These spectra were added to build up the VUV spectra library similar to the mass spectral libraries are used to confirm the identity of the analytes using the standard fragmentation pattern specific to the component. VUV spectral library can be used to confirm the identity of a component by comparing the $\lambda_{\text{max}}$ for an individual component, which makes it a complementary technique to MS where each component can be separated using gas chromatography and confirmed using VUV spectral libraries. Figure 66 shows an overlay of VUV spectra for four glucocorticoids to show a similar absorption spectrum. This separation of steroidal compounds with similar structures reflects the strength of GC-VUV.

Figure 67 and Figure 68 shows a linearity plots for Hydrocortisone and Prednisone. These were obtained in a triplicate with manual injections on GC-VUV, which shows a good linearity of 0.94 and 0.92 with a limit of detection of 20 ng for the mass-on-column. This demonstrates that VUV spectroscopy can be successfully used for the separation of drugs showing this technique is sensitive and selective to the molecular structure of the drugs. [86]
Figure 64 Chromatogram of separation of glucocorticoids by GC-VUV
Figure 65 VUV spectra of each glucocorticoid
Figure 66 Overlay of VUV spectra of all glucocorticoids
Figure 67 Linearity of hydrocortisone
Figure 68 Linearity of prednisone
4.4.2 VUV analysis of water in SPME

Solid phase microextraction (SPME) is a solventless technique used to extract a specific analyte in some complex matrices. This technique is selective for the class of compounds having similar characteristics and can be used to quantitate at parts per trillion levels. [15] Previous studies (Chapter 2) showed that NSAIDs a class of drugs were successfully separated using SPME-GC-MS without derivatization in water as a matrix. GC-MS being sensitive to moisture, water was not detected using MS. GC-VUV is a complimentary technique to GC-MS, a SPME run was carried out using manual SPME syringe with a PDMS/DVB/Carboxen fiber attached to it, VUV detector can quantify water present in the sample mixture. Due to the limitations of the silica column coating on the gas chromatographic column, only organic solvents were used for sample preparations. This study shows GC-VUV can be used to quantitate water not only in liquid injection but also detect residual water on the SPME fiber. An 80 µL of Ibuprofen stock sample solution (1000 ppm) was spiked in DI water at pH 3.3 to give a 4 ppm concentration which was extracted on the SPME fiber and injected manually into the GC inlet at 250°C.

Figure 69 shows a separation of a sharp water peak at 1 min and ibuprofen at 11 min. Ibuprofen shows a less absorption due to low sensitivity but water peak shows a good absorption about 0.6. Water can be quantitated using GC-VUV to determine the amount of water getting into GC column. Figure 70 and Figure 71 shows the VUV spectrum of water obtained on GC-VUV with $\lambda_{\text{max}}$ of the 165nm and VUV spectrum of Ibuprofen at $\lambda_{\text{max}}$ of 190nm.
Figure 69 Separation of water and ibuprofen by SPME injection
Figure 70 VUV spectrum of water
Figure 71 VUV spectrum of ibuprofen
4.5 Conclusions

Gas chromatography is a mature technique which can be used with varied types of detectors such as FID and MS. GC coupled to VUV detector adds up to GC toolbox to overcome some limitations in the analysis using different methods of detection as the third dimension of selectivity. GC-VUV proves to be a powerful technique to determine water content, separate isomers, separate co-eluting peaks, etc. using GC. This study demonstrated analysis of a different class of drugs detected at 20-60 ng mass on column with a split injection which shows the good sensitivity for the method using VUV detection, and SPME analysis showed a residual solvent (here water) in SPME sample preparation showing the efficiency of the detector to analyze water using gas chromatography. VUV detector shows distinct spectra for analytes at a different degree of absorption which makes it unique as a detection technique. The VUV detection technique is dependent on the molecular structure which shows that VUV is selective and a new powerful but non-destructive technique applied to a large group of compounds which haven’t been explored yet using GC-VUV. The next chapter shows an application of the second dimension of selectivity and factors affecting the separation using two columns.
CHAPTER 5       APPLICATION OF GCXGC-TOFMS TO ANALYZE FATTY ACID METHYL ESTERS (FAMEs)

5.1 Abstract

Traditional 1-dimensional gas chromatography coupled with mass spectrometry (GC-MS) have been used to analyze mixtures of specific components such as volatiles, which frequently co-elute. [34] [33] GCxGC can provide a greater peak capacity for complex samples with two columns, where the first column is a conventional column and is typically nonpolar and the second column is typically a short (0.5-1.5m) polar column with a cryogenic modulator as the interface. The modulator focuses the first column eluent into the second column. [35]- [38] This combination of nonpolar-polar columns is considered as an orthogonal configuration which increases the resolving power and enhances sensitivity for the trace level analysis of components from complex matrices. For detection in GCxGC, the detector must be fast and sensitive; hence, GCxGC coupled to a time of flight mass spectrometer (TOF-MS). In this experiment, the LECO® Pegasus 4D comprehensive GCxGC-TOF-MS was used (Details as described in Chapter2).

This application shows the second dimension of selectivity with the effect of different conditions on GCXGC with two different column combination: Traditional and reversed column combination using two different polarity columns (polar and a non-polar column).
5.2  PART I: Experimental (Instrumental and Results)

PART I study uses ZB-FAME as the Primary column and ZB-MS5 Plus as a secondary column.

**Sample preparation:** Supelco FAMEs mix (varied concentration 200-400 µg/mL) in dichloromethane.

**Instrumental conditions:**

5.2.1 Effect of modulator separation time

<table>
<thead>
<tr>
<th>Primary column and MS conditions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection</td>
<td>2 µL split 1:50, 250°C</td>
</tr>
<tr>
<td>Column</td>
<td>ZB-FAME: 30 m X 0.25 mm X 0.20 µm</td>
</tr>
<tr>
<td>Inlet temperature</td>
<td>250°C</td>
</tr>
<tr>
<td>Oven ramp</td>
<td>40°C hold for 2 min ramp 30°C /min to 160 °C, 2°C /min to 250°C hold for 1 min (solvent cut: 3 min)</td>
</tr>
<tr>
<td>Ion source temperature</td>
<td>220°C</td>
</tr>
<tr>
<td>Transfer line temperature</td>
<td>240°C</td>
</tr>
<tr>
<td>m/z range</td>
<td>45-650</td>
</tr>
<tr>
<td>Acquisition rate</td>
<td>100 spectra/second</td>
</tr>
<tr>
<td>Helium flow</td>
<td>1 mL/min</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary column and modulator conditions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>ZB-5 MS Plus: 1.5 m X 0.25 mm X 0.25 µm</td>
</tr>
<tr>
<td>Oven ramp</td>
<td>60°C hold for 2 min ramp 30°C /min to 180°C, 2°C /min to 270°C hold for 1 min</td>
</tr>
<tr>
<td>Modulator temperature offset</td>
<td>45°C</td>
</tr>
<tr>
<td><strong>Modulation time</strong></td>
<td>4, 6, 8 and 10 seconds</td>
</tr>
<tr>
<td>Hot pulse time (variable)</td>
<td>1.0 second</td>
</tr>
<tr>
<td>Total run time</td>
<td>56.00 min</td>
</tr>
</tbody>
</table>
5.2.1.1 Results and discussion:

Peak modulation is the characteristic of the analysis using GCxGC where analytes eluting from the first column are focused on to the second column in small fractions with a narrow bandwidth. Modulator traps the peaks from the first column, which are the slices of each fraction where each slice represents one component of a mixture. This increases the peak capacity for each separation. There are different types of modulators available, in this study; a quad-jet cryotrap modulator was used where there are alternately hot and cold nitrogen gas jets. The hot jets keep the fraction entering the modulator moving through the modulator and nitrogen gas is cooled using liquid nitrogen that traps the fraction of eluting in the modulator. This is a continuous process, which keeps fractions from any interferences. In this type of modulator, the hot pulse is shorter than the cold pulse to preserve the bandwidth and get a good orthogonal separation. Therefore, these conditions for modulation time and the hot pulse time needs to be optimized to get a good separation. [35] [45] [41]

In the first part of this experiment, the effect of modulation time was studied where the separation of FAMEs was subjected to 4s, 6s, 8s, and 10 seconds modulation time. In Figure 72 - Figure 75 the separation of C-18 FAMEs can be discussed where it shows these are separating at different retention times as the modulation time decreases with a wraparound of peaks. This can occur due to the interaction of eluent fraction interacting too strongly with the stationary phase in the second column, which prevents peaks from eluting at the same modulation time. So these peaks elute at a later modulation time, this wraparound can be corrected by changing the time of modulation which is typically between 4-10 seconds and the time of the hot pulse is typically between 0.6-1.2 seconds. In the second part, the hot pulse time can affect the separation in the second dimension.
This can also prevent any disruption from the cold jets and preserve the separation from the first column.

Figure 72 shows separation of C6-C17 series with a C18 wraparound at a later time due to 4 sec modulation. Since the second dimension retention is directly affected by modulation period, C18 series are scattered but separated in the second dimension due to shorter modulation time and other components are also separated in the space but do not show a good orthogonal separation. Hence, the same standard mixture was tested at different modulation times. Figure 73 shows separation of C6-C17 series with a C18 wraparound at a later time in a separation space due to 6 sec modulation. However, the C17 series do not show an orthogonal separation, instead, they are also wraparound due to the short modulation period. Figure 74 shows separation of C6-C17 series with a C18 wraparound at a later time and separation space due to 8 sec modulation with comprising resolution and still wraparound for one of the isomers for C17 cutting in the second dimension due to less modulation period.

Figure 75 shows optimized conditions for separation of C6-C17 series with no C18 wraparound due to 10 sec modulation with a good resolution. Here all the C6-C18 series of FAMEs shows a good orthogonal separation with utilizing the space in the second dimension with all the isomers for C18 separated from each other with a good resolution. Although, all components were separated in an orthogonal manner but some of the components were dependent on different variables for a good separation with better resolution. Hence, the next sections evaluate the effect of the hot pulse on the C6-C18 isomers and later showing the separation of all 34 components of FAMEs in the second dimension.
Figure 72  Zoomed to show separation of C18 in FAMEs mix with 4 sec modulation time
Figure 73 Zoomed to show separation of C18 in FAMEs mix with 6 sec modulation time
Figure 74 Zoomed to show separation of C18 in FAMEs mix with 8 sec modulation time
Figure 75 Zoomed to show separation of C18 in FAMEs mix with 10 sec modulation time
5.2.2 Effect of hot pulse time

**GCxGC-TOFMS (Leco’s Pegasus 4D)**

<table>
<thead>
<tr>
<th>Primary column and MS conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Injection</strong></td>
</tr>
<tr>
<td><strong>Column</strong></td>
</tr>
<tr>
<td><strong>Inlet temperature</strong></td>
</tr>
<tr>
<td><strong>Oven ramp</strong></td>
</tr>
<tr>
<td><strong>Ion source temperature</strong></td>
</tr>
<tr>
<td><strong>Transfer line temperature</strong></td>
</tr>
<tr>
<td><strong>m/z range</strong></td>
</tr>
<tr>
<td><strong>Acquisition rate</strong></td>
</tr>
<tr>
<td><strong>Solvent cut time</strong></td>
</tr>
<tr>
<td><strong>Helium flow</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary column and modulator conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
</tr>
<tr>
<td><strong>Oven ramp</strong></td>
</tr>
<tr>
<td><strong>Modulator temperature offset (above primary oven)</strong></td>
</tr>
<tr>
<td><strong>Modulation time</strong></td>
</tr>
<tr>
<td><strong>Hot pulse time (variable)</strong></td>
</tr>
<tr>
<td><strong>Total run time</strong></td>
</tr>
</tbody>
</table>
5.2.2.1 Results:

Figure 76- Figure 79 shows the effect of the hot pulse on the C-18 FAMEs, which shows the increased intensity of the peaks retained on the second dimension. Peak modulation is also dependent on the time of hot pulse and the cold pulse used to slice the analyte focusing on to the second column that is eluting from the first column. The hot pulse can be set in seconds with a maximum of 2.0 sec and the cold pulse sets by default by adjusting the difference for the hot pulse. This section evaluated the effect of the hot pulse that is also the main parameter to obtain a good orthogonal separation with higher intensity for the peaks with different polarity. Here, the FAMEs standard was tested at different hot pulse from 0.6 sec – 1.2 sec.

Figure 76 shows separation of C6-C17 series with C18 at optimized modulation time with 0.6 sec hot pulse time compromising sensitivity of FAMEs even though all peaks are separated in an orthogonal space but C18 isomers show less intensity which can be increased by increasing the time of the hot pulse. Figure 77 shows separation of C6-C17 series with C18 at optimized modulation time with 0.8 sec hot pulse time with a better sensitivity of FAMEs as compared to 0.6 sec hot pulse. It can be observed that, as the hot pulse time is increasing the intensity for C18 is increasing. Therefore, the same mixture was tested at 1.0 sec and 1.2 sec hot pulse.

Figure 78 shows separation of C6-C17 series with C18 at optimized modulation time with 1.0 sec hot pulse time with a better sensitivity of FAMEs as compared to 0.6 and 0.8 sec hot pulse.

Figure 79 shows separation of C6-C17 series with C18 at optimized modulation time with 1.2 sec hot pulse time with improved sensitivity of FAMEs, which was then optimized to separate the FAMEs mixture for 34 components that are discussed in the next section.
Figure 76 Zoomed in to show separation of C-18 series in FAMEs mix with 0.6 sec hot pulse
Figure 77 Zoomed in to show separation of C-18 series in FAMEs mix with 0.8 sec hot pulse
Figure 78 Zoomed in to show separation of C-18 series in FAMEs mix with 1.0 sec hot pulse
Figure 79 Zoomed in to show separation of C-18 series in FAMEs mix with 1.2 sec hot pulse
5.2.3 Optimized conditions

Instrumental conditions:

GCxGC-TOFMS (Leco’s Pegasus 4D)

<table>
<thead>
<tr>
<th>Primary column and MS conditions</th>
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<tr>
<td>Injection</td>
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<tr>
<td>Column</td>
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<td>Inlet temperature</td>
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<td>Oven ramp</td>
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<td>Ion source temperature</td>
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<tr>
<td>Transfer line temperature</td>
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<tr>
<td>m/z range</td>
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<tr>
<td>Acquisition rate</td>
</tr>
<tr>
<td>Solvent cut time</td>
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<tr>
<td>Helium flow</td>
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<table>
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<th>Secondary column and modulator conditions</th>
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</thead>
<tbody>
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<td>Column</td>
</tr>
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<tr>
<td>Modulator temperature offset (above primary oven)</td>
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<td>Modulation time</td>
</tr>
<tr>
<td>Hot pulse time</td>
</tr>
<tr>
<td>Total run time</td>
</tr>
</tbody>
</table>
5.2.3.1 Results

Figure 80 - Figure 82 and Table 17 shows all the FAMEs separated in different dimensional plots on the ChromaTOF software with the optimized conditions on ZB-FAME as primary column and ZB-MS5 Plus as a secondary column.

Figure 80 shows optimized two-dimensional chromatogram for FAMEs mix. This chromatogram shows a good separation of 34 FAMEs in the second dimension with utilizing second dimension separation space. Here, all the FAMEs showed a good retention using both the columns with a good retention selectivity and all components were separated from each other showing a good retention factor. Figure 81 shows a labeled chromatogram for separated C6-C18, which is also a zoomed in chromatogram from the separation of all 34 components. This shows separation after final conditions, which were optimized for a polar first dimension and a non-polar second dimension column combination with a list of separated FAMEs in Table 17. This table shows a list of 34 FAMEs which were separated using this column combination with each corresponding to the number labeled in the chromatogram with their retention times in both first dimension and second dimensions (in seconds).

Figure 82 represents a three-dimensional chromatogram, which shows a separation in the third dimension with a polar first dimension and a non-polar second dimension column combination for FAMEs separation. The colors represent the intensity of each peak red being the highest.

The next section studies the effect of reversing the column combination on the separation of FAMEs.
Figure 80 Optimized two-dimensional chromatogram for FAMEs mix

(Labelled peaks are shown in Table 17)
Figure 81 Zoomed in to show the separation for C6-C18 FAMEs

(Labelled peaks are shown in Table 17)
Figure 82 Optimized 3D chromatogram for FAMEs mix
Table 17 Peak table with library hits from the ChromaTOF software

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Name</th>
<th>1D tr (s)</th>
<th>2D tr (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexanoic acid, methyl ester</td>
<td>280</td>
<td>1.170</td>
</tr>
<tr>
<td>2</td>
<td>Octanoic acid, methyl ester</td>
<td>340</td>
<td>1.230</td>
</tr>
<tr>
<td>3</td>
<td>Decanoic acid, methyl ester</td>
<td>380</td>
<td>1.420</td>
</tr>
<tr>
<td>4</td>
<td>Undecanoic acid, methyl ester</td>
<td>410</td>
<td>1.600</td>
</tr>
<tr>
<td>5</td>
<td>Dodecanoic acid, methyl ester</td>
<td>430</td>
<td>1.920</td>
</tr>
<tr>
<td>6</td>
<td>Tridecanoic acid, methyl ester</td>
<td>450</td>
<td>2.370</td>
</tr>
<tr>
<td>7</td>
<td>Methyl tetradecanoate</td>
<td>480</td>
<td>3.020</td>
</tr>
<tr>
<td>8</td>
<td>10-Undecenoic acid, methyl ester</td>
<td>510</td>
<td>2.850</td>
</tr>
<tr>
<td>9</td>
<td>Pentadecanoic acid, methyl ester</td>
<td>520</td>
<td>3.870</td>
</tr>
<tr>
<td>10</td>
<td>7-Hexadecenoic acid, methyl ester, (Z)-</td>
<td>550</td>
<td>3.640</td>
</tr>
<tr>
<td>11</td>
<td>Pentadecanoic acid, 14-methyl-, methyl ester</td>
<td>560</td>
<td>5.080</td>
</tr>
<tr>
<td>12</td>
<td>9-Hexadecenoic acid, methyl ester, (Z)-</td>
<td>590</td>
<td>4.610</td>
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<tr>
<td>13</td>
<td>Hexadecanoic acid, 15-methyl-, methyl ester</td>
<td>620</td>
<td>6.560</td>
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<tr>
<td>14</td>
<td>Cyclopropaneoctanoic acid, 2-hexyl-, methyl ester</td>
<td>650</td>
<td>5.920</td>
</tr>
<tr>
<td>15</td>
<td>Octadecanoic acid, methyl ester</td>
<td>690</td>
<td>8.470</td>
</tr>
<tr>
<td>16</td>
<td>9-Octadecenoic acid (Z)-, methyl ester</td>
<td>710</td>
<td>7.550</td>
</tr>
<tr>
<td>17</td>
<td>9,12-Octadecadienoic acid, methyl ester, (E,E)-</td>
<td>740</td>
<td>7.300</td>
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<tr>
<td>18</td>
<td>11,14-Eicosadienoic acid, methyl ester</td>
<td>770</td>
<td>6.850</td>
</tr>
<tr>
<td>19</td>
<td>9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-</td>
<td>810</td>
<td>6.210</td>
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<td>20</td>
<td>11,14,17-Eicosatrienoic acid, methyl ester</td>
<td>840</td>
<td>6.440</td>
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<td>Eicosanoic acid, methyl ester</td>
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<td>3.390</td>
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<td>22</td>
<td>15-Tetracosenoic acid, methyl ester, (Z)-</td>
<td>900</td>
<td>1.640</td>
</tr>
<tr>
<td>23</td>
<td>11-Octadecynoic acid, methyl ester</td>
<td>970</td>
<td>0.410</td>
</tr>
<tr>
<td>24</td>
<td>Heneicosanoic acid, methyl ester</td>
<td>980</td>
<td>6.060</td>
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<td>25</td>
<td>11,14,17-Eicosatrienoic acid, methyl ester</td>
<td>1010</td>
<td>9.150</td>
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<tr>
<td>26</td>
<td>9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-</td>
<td>1050</td>
<td>9.500</td>
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<tr>
<td>27</td>
<td>Docosanoic acid, methyl ester</td>
<td>1100</td>
<td>8.980</td>
</tr>
<tr>
<td>28</td>
<td>15-Tetracosenoic acid, methyl ester, (Z)-</td>
<td>1130</td>
<td>6.460</td>
</tr>
<tr>
<td>29</td>
<td>4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-</td>
<td>1150</td>
<td>7.450</td>
</tr>
<tr>
<td>30</td>
<td>11,14-Eicosadienoic acid, methyl ester</td>
<td>1210</td>
<td>4.510</td>
</tr>
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<td>31</td>
<td>Heneicosanoic acid, methyl ester</td>
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<td>1.720</td>
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<td>32</td>
<td>Hexacosanoic acid, methyl ester</td>
<td>1380</td>
<td>4.490</td>
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<td>33</td>
<td>15-Tetracosenoic acid, methyl ester, (Z)-</td>
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<td>1.320</td>
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<tr>
<td>34</td>
<td>4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-</td>
<td>1450</td>
<td>9.020</td>
</tr>
</tbody>
</table>
5.3 PART II: Experimental (Instrumental and Results)

PART II study uses ZB-MS5 Plus as the primary column and ZB-FAME as a secondary column.

**Sample preparation:** Supelco FAMEs mix (varied concentration 200-400 µg/mL) in dichloromethane.

**Instrumental conditions:**

**GCxGC-TOFMS (Leco’s Pegasus 4D)**

<table>
<thead>
<tr>
<th>Primary column and MS conditions</th>
<th></th>
</tr>
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<tbody>
<tr>
<td><strong>Injection</strong></td>
<td>2 µL split 1:50, 250°C</td>
</tr>
<tr>
<td><strong>Column</strong></td>
<td>ZB-5 MS Plus: 15 m X 0.25 mm X 0.25 µm</td>
</tr>
<tr>
<td><strong>Inlet temperature</strong></td>
<td>250°C</td>
</tr>
<tr>
<td><strong>Oven ramp</strong></td>
<td>40°C hold for 3 min ramp 5°C/min to 250°C, hold for 1 min</td>
</tr>
<tr>
<td><strong>Ion source temperature</strong></td>
<td>220°C</td>
</tr>
<tr>
<td><strong>Transfer line temperature</strong></td>
<td>240°C</td>
</tr>
<tr>
<td><strong>m/z range</strong></td>
<td>45-650</td>
</tr>
<tr>
<td><strong>Acquisition rate</strong></td>
<td>100 spectra/second</td>
</tr>
<tr>
<td><strong>Helium flow</strong></td>
<td>1 mL/min</td>
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</tbody>
</table>

<table>
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<tr>
<th>Secondary column and modulator conditions</th>
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</thead>
<tbody>
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<td><strong>Column</strong></td>
<td>ZB-FAME:1.5 m X 0.25 mm X 0.20 µm</td>
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<td><strong>Oven ramp</strong></td>
<td>45°C hold for 3 min ramp 5°C/min to 255°C, hold for 1 min</td>
</tr>
<tr>
<td><strong>Modulator temperature offset (above secondary oven)</strong></td>
<td>15°C</td>
</tr>
<tr>
<td><strong>Modulation time</strong></td>
<td>5 seconds</td>
</tr>
<tr>
<td><strong>Hot pulse time</strong></td>
<td>0.6 second</td>
</tr>
<tr>
<td><strong>Total run time</strong></td>
<td>46.00 min</td>
</tr>
</tbody>
</table>
Results and discussion:

In part II of the experiment, column combination was reversed from the previous study. Here the ZB-5MS Plus as a primary column and ZB-FAME as a secondary column. Therefore, the selectivity of the analytes was also reversed. FAMEs showed a good separation of all C6-C24 in the ZB-5 MS Plus column and the isomers for C14, C15, C16, C17, C18, C20, C22 and C24 shows a good separation on the ZB-FAME, which is the secondary column. With this column combination, there was no wraparound of the peaks, and all peaks were separated from each other on the chromatograms above (Figure 83 - Figure 85) and Table 18 shows all the FAMEs in the reverse column combination.

Figure 83 shows separation of C6-C24 series without any wraparound. This combination separated C18 and C20 isomers with some co-eluting C18 isomers. The only difference using this column combination is it does not utilize the second dimension separation space. However, this column combination separated the isomers in the second dimension space.

Figure 84 represents a three-dimensional chromatogram, which shows a separation in the third dimension with a non-polar first dimension and a polar second dimension column combination for FAMEs separation. The colors represent the intensity of each peak red being the highest.

Figure 85 shows a separation of 34 FAMEs in a two-dimension model, which shows all 34 FAMEs numbered individually but C18 isomers tend to co-elute in the first dimension. Table 18 shows details of separated FAMEs with their names and first and second dimension retention times (in seconds) on a reverse column combination with their retention times demonstrating a good selectivity.
Figure 83 Two-dimensional chromatogram for FAMEs mix with reverse column combination
Figure 84 Chromatogram for FAMEs mix 3D view with reverse column combination
Figure 85 Separation for FAMEs with peak table with reverse column combination
Table 18 Peak table (for reverse column combination)

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Name (Library hits)</th>
<th>1D tr (s)</th>
<th>2D tr (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hexanoic acid, methyl ester</td>
<td>265</td>
<td>3.600</td>
</tr>
<tr>
<td>2</td>
<td>Octanoic acid, methyl ester</td>
<td>625</td>
<td>2.960</td>
</tr>
<tr>
<td>3</td>
<td>Decanoic acid, methyl ester</td>
<td>955</td>
<td>2.440</td>
</tr>
<tr>
<td>4</td>
<td>Undecanoic acid, methyl ester</td>
<td>1110</td>
<td>2.240</td>
</tr>
<tr>
<td>5</td>
<td>Dodecanoic acid, methyl ester</td>
<td>1255</td>
<td>2.100</td>
</tr>
<tr>
<td>6</td>
<td>Tridecanoic acid, methyl ester</td>
<td>1390</td>
<td>1.970</td>
</tr>
<tr>
<td>7</td>
<td>Methyl myristoleate</td>
<td>1500</td>
<td>2.170</td>
</tr>
<tr>
<td>8</td>
<td>Tridecanoic acid, 12-methyl-, methyl ester</td>
<td>1520</td>
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5.4 Conclusions

In this study, for the part I: the modulation time of 10s and a hot pulse time of 1.0s was optimized. Due to the smaller length of the second column, and to maintain the fast separation a limited modulation time was 10s. For the separation of the mixture of FAMEs in this column combination, a longer modulation time can be used to avoid the wraparound in the second dimension. This combination of the column shows a good orthogonal separation for the FAMEs.

In Part II of the study, the column combination was reversed which gave a good selectivity for all the FAMEs. This method was optimized with 5s modulation time and 0.6s hot pulse time. The comparison of two different column combinations shows the advantage of one over the other. While the traditional column combination separated all the FAMEs with utilizing the second dimension separation space but the reversed column combination also showed a good separation but losing its orthogonality with some co-eluting peaks in the second dimension with good selectivity. Different column combinations also affect the retention and selectivity of the analytes in the second dimension of selectivity. Thus, GCxGC can be used to achieve a best chromatographic separation with optimizing various parameters using different column combinations.

This work was performed in collaboration with Phenomenex (Torrance, CA) to evaluate the performance of their test columns on GCxGC.
OVERALL CONCLUSIONS AND FUTURE WORK

In recent years, multidimensional techniques have become an effective tool in petrochemical, criminal forensics, environmental monitoring, food and cosmetic industry, medicine and sports, etc.

This research demonstrated a novel approach to study NSAIDs without derivatization using gas chromatographic techniques. NSAIDs were analyzed using two multidimensional separation techniques: GC-MS-MS and GCxGC-TOF-MS, which are not yet, reported using solid phase microextraction (SPME) for NSAIDs without derivatization in river water, pharmaceutical wastewater, and other natural sources. GC-MS-MS and GCxGC-TOF-MS provide different approaches to multidimensional separations. This demonstrated the difference between chromatographic separation and mass spectrometric (MS) separation. To understand the relationships between selectivity generated from sample preparation, chromatographic separation, and detection for NSAIDs using single and multi-dimensional gas chromatographic analysis. This method can also be extended to determine the NSAIDs in complex matrices such as urine, blood for clinical toxicology and the determination of NSAIDs concentration in drug formulations with easy sample preparation technique. For future work, NSAIDs can be separated using ionic liquid columns in different dimensions using GC.

This research also validated trace levels of polycyclic aromatic hydrocarbons with their metabolites in fish oil using SPME-GC-MS-MS (multidimensional detection) to improve the figures of merit as compared to SPME-GC-MS (single dimensional detection) in Chapter 3.

Chapter 4 and 5 demonstrated some applications for gas chromatographic techniques. Chapter 4 evaluated a new detector Vacuum-ultraviolet (VUV) coupled to a GC. This is the first time where
GC-VUV was applied to analyze drugs with a same molecular structure and study the qualitative analysis of water in SPME. This is a recent technique, which can be extended to various fields of applications to overcome some GC limitations. Chapter 5 tested some fatty acid methyl esters on GCxGC-TOFMS and their selectivity using different research column combinations provided by Phenomenex, Torrance, CA.

This research demonstrated the use of multidimensional-GC techniques to analyze NSAIDs without derivatizing, PAHs at low levels of detection and selectivity of FAMEs. Using SPME coupled to GC-MS with lower detection limits and improved analytical figures of merit for NSAIDs residues in water as potential pollutants, PAHs, and its metabolites as pollutants in fish oil and demonstrated applications, which can be applied in forensics and medicine.

All these techniques when combined together can provide an enormous amount of information on extraction, separation, and detection on a different class of analytes as shown in Figure 86.
An approach to obtain selectivity in various dimensions of separation.

Figure 86 Demonstration of different dimensions in separation.
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