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Development Of Novel Analytical Methods For Detection And Determination Of Tamoxifen In Oral Fluid And Discrimination Between Regioisomeric Aromatase Inhibitors For Doping Control Purposes

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DEVELOPMENT OF NOVEL ANALYTICAL METHODS FOR DETECTION AND DETERMINATION OF TAMOXIFEN IN ORAL FLUID AND DISCRIMINATION BETWEEN REGIOISOMERIC AROMATASE INHIBITORS FOR DOPING CONTROL PURPOSES

BY

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ABSTRACT

The use of SERMs (Selective Estrogen Receptor Modulators) – most notably the drug Tamoxifen – has significantly increased among athletes over the past decade to enhance athletic performance and/or negate certain side effects of using anabolic steroids. This "doping" is always banned by WADA (World Anti-Doping Agency) for all athletes, but SERMs can naturally increase testosterone production so they are a tempting alternative to using steroids or androgenic supplements that can cause significant health problems (e.g. acne, breast development, frequent urge to urinate, low libido, etc.). Similarly, the use of AIs (Aromatase Inhibitors) has also increased among athletes for identical reasons, and they are also banned by WADA within the same drug classification. This project's overall goal was to develop and optimize a simple, sensitive and selective method for the detection and determination of Tamoxifen in oral fluid (OF) for drug testing purposes using Gas Chromatography/Mass Spectrometry (GC/MS) that could be utilized to supplement WADA's current testing protocol which uses blood and urine specimens. Because method development for analysis of oral fluid specimens containing highly protein bound analytes typically relies on the use of newer LC-MS/MS instrumentation (and the costs associated with acquiring and using this technology), a GC method remains advantageous. A calibration curve was created; the developed methods proved to be successful for the recovery and detection of Tamoxifen from artificial saliva samples with high sensitivity.

A second part of this project was to develop additional analytical methods for discrimination and chromatographic resolution of three regioisomeric aromatase

inhibitors (5-alpha-Androstan-17-one, 5-alpha-Androst-16-en-3α-ol and 17-beta-Hydroxy-5-alpha-androst-2-ene) per their representation of similar ingredients utilized in some black market dietary supplements that promise gain of muscle mass but also violate WADA's policies. Using Gas Chromatography/Mass Spectrometry (GC/MS) and Fourier Transform Infrared Spectroscopy (FT-IR), this project investigated detection and differentiation between these regioisomeric AIs which have the same molecular formulae, same nominal and exact masses and almost identical elution properties.

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ABBREVIATIONS

AAF.....Adverse Analytical Finding

AAS..... Anabolic Androgenic Steroid

ABP.....Athlete Biological Passport

ADAMS..... Anti-Doping Administration and Management System

AIs ..... Aromatase Inhibitors

ANOVA .....Analysis of Variance

APEDs.....Appearance and Performance Enhancing Drugs

ATF .....Atypical Finding

D/H Ratio ..... Deuterium/Hydrogen Ratio

DBS.....Dried Blood Spot

DHHS.....Department of Health and Human Services

DI Water.....De-ionized Water

EIMS..... Electron Impact Mass Spectrometry

Fcv..... F Critical Value

FSH ..... Follicle Stimulating Hormone

FT-IR .....Fourier-Transform Infrared Spectroscopy

FWHM..... Full Width at Half Max

GC .....Gas Chromatography

HETP.....Height Equivalent to a Theoretical Plate

HGH.....Human Growth Hormone

HSD ..... (Tukey) Honest Significant Difference Test

ICH ..... (International Council on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use)

IS ..... Internal Standard

LC ..... Liquid Chromatography

LH ..... Luteinizing Hormone

LOD ..... Limit of Detection

LOQ ..... Limit of Quantitation

MS ..... Mass Spectrometry

NIDA ..... National Institute on Drug Abuse

NMR ..... Nuclear Magnetic Resonance

OF ..... Oral Fluid

OFMG ..... Oral Fluid Mandatory Guidelines

PEDs ..... Performance Enhancing Drugs

SAMHSA ..... Substance Abuse and Mental Health Services Administration

SERMs ..... Selective Estrogen Receptor Modulators

SIM ..... Selected Ion Monitoring

SPE ..... Solid Phase Extraction

UFC ..... Ultimate Fighting Championship

US ..... United States

USADA ..... United States Anti-Doping Agency

WADA ..... World Anti-Doping Agency

1. INTRODUCTION

There is increasing interest among scientists to further research the use of oral fluid (OF) as an alternative biological matrix for drug testing. Currently, blood and urine remain the most predominantly collected matrices for determining or monitoring occurrence of drug use (e.g. in the workplace, criminal justice systems, medical facilities, and sports), but each has significant disadvantages and limitations (Drummer, 2006, p. 150). Provided samples can be tampered with or manipulated to conceal or obscure a person's true drug use behavior, and collection can be invasive or dependent on the availability of medical personnel or gender-dependent observers (Athlete Biological Passport, 2017). Issues of expense, invasiveness and spontaneity definitely complicate or even stifle drug testing policies and procedures – whether the test is administered for employment screening, monitoring of an athlete, or any other purpose. Because oral fluid collection is considered non-invasive and nearly tamper-proof, OF drug testing could supplement (not replace) blood and urine testing (Drummer, 2006, p. 150). Each biological specimen has its own advantages and disadvantages, but one cannot substitute for another. Each specimen has unique detection windows, and the concentration of parent compounds and/or drug metabolites often differ according to which matrix is tested.

Some analytical challenges for OF testing have been resolved or improved over the past two decades (e.g. low volume samples and separation techniques due to new technology), but qualitative and quantitative methods for OF testing have only recently reached the cusp of having broad scientific acceptance. Significant evidence

of this acceptance is the U.S. federal agency Substance Abuse and Mental Health Services Administration (SAMHSA) – part of the U.S. Department of Health and Human Services (DHHS) – now allowing oral fluid as a drug testing specimen as part of its drug testing programs for federal employees, implemented January 1, 2020 (Bradbury, 2019). SAMHSA guidelines for drug testing federal employees have been in place since 1988 to support federal drug-free workplace laws, but oral fluid drug testing had previously been deemed unreliable in comparison with urine analysis. Drug testing itself is a deterrent method aimed to prevent problems or accidents in any work situation but especially where transportation or security issues are involved. Workplace drug testing laws do not apply to most private businesses, but the laws still influence hiring practices and safety practices across the United States. SAMSHA's release of its Oral Fluid Mandatory Guidelines (OFMG) therefore initiated a major change not only for federal employees but also toxicology laboratories and analysts, manufacturers of OF drug test kits, manufacturers of analytical instruments, etc. (Bradbury, 2019).

Additional evidence of growing scientific acceptance of oral fluid drug testing is the U.S. Anti-Doping Agency (USADA) now piloting an OF testing program implemented in December 2019 to detect use of in-competition banned substances (New Testing Method: Oral Fluid Collections: USADA, 2019). Specifically, the USADA test pilot program involves collected OF samples from UFC athletes (Ultimate Fighting Championship – mixed martial arts) to focus on validating its sample collection process and analytical methods. The OF tests will supplement, not replace, blood and urine

testing of athletes for compliance with abstinence from performance-enhancing drugs and other prohibited substances. Again, this program will majorly influence athletics and anti-doping practices across the United States.

To continue improvement of both qualitative and quantitative analytical methods of oral fluid specimens utilized for drug testing and to earn support for OF testing from scientists world-wide, on-going research is warranted, specifically in regard to pharmacokinetics (drug absorption, distribution, metabolism, and excretion) of targeted analytes and drug concentrations in OF (Drummer, 2006, p. 150). With oral fluid, the rules of drug protein binding and membrane permeability correspond with ability to detect unbound drug analytes in saliva excretions. Drugs with negligible transport from blood to saliva – where drug concentration in saliva is very low – it is hard to measure or difficult to interpret them (Haeckel & Hanecke, 1996, p. 171). Little to no research has been performed on detection of drug analytes known to have very high protein binding and possibly negligible transport to saliva (e.g. Tamoxifen).

Part two of this project addresses the very important issue in forensic drug chemistry which is the differentiation of regioisomeric compounds. This issue is extreme for many drug categories (e.g. stimulants, opioids and synthetic cannabinoids) and must be continuously addressed, but it is important to be able to distinguish between regioisomers within all drug categories in order to be able to confirm a drug's identity. The differentiation, after all, identifies the drug as legal or illegal dependent upon how legislation of a particular country is worded (Awad, Deruiter, & Clark, 2008,

p. 672). Specifically, this project investigates the differentiation of the regioisomeric AIs 5-alpha-Androstan-17-one, 5-alpha-Androst-16-en-3α-ol, and 17-beta-Hydroxy-5-alpha-androst-2-ene which all have the same molecular formula, and the same nominal and exact masses. Developed methods using GC/MS and FT-IR to separate and uniquely identify these regioisomers could be useful for forensic drug chemists working in laboratories where instrumentation such as nuclear magnetic resonance (NMR) is not readily available. The cost of this instrument and the advanced skill needed for the analysis are prohibitive. The specific identification of each isomer, then, relies on a combination of mass spectral data and chromatographic resolution of the regioisomeric AIs studied.

1.1 Project Overview and Purpose

This thesis project contributes to needed research of oral fluid used as a biological specimen for the purpose of non-invasive drug testing and commands ability to apply knowledge and understanding of drug chemistry principles in developing and optimizing sensitive, accurate and precise analytical methods for detection and determination of the performance-enhancing drug (PED) Tamoxifen. This project researches WADA's S4 class of substances that are banned for use by athletes at all times: hormones and metabolic modulators. The S4 class includes selective estrogen receptor modulators (SERMs – e.g. Tamoxifen) and aromatase inhibitors which accounted for 17% of all detected banned substances in 2016 as tested by WADA (2016 Anti-Doping Testing Figures, 2016, p. 29) and for 9% of all detected banned substances in 2018 as tested by WADA – according to WADA's most current published

data (2018 Anti-Doping Testing Figures, 2018, p. 27). Of significance is that Tamoxifen excretion into oral fluid occurs but at very low concentrations. Tamoxifen analytes have low permeability and high protein binding (Lien, et al., 1989, p. 2175).

This thesis project further contributes to needed research for the differentiation of three reigioisomers aromatase inhibitors in that WADA's Prohibited List includes all "chemically closely related" banned substances whether or not they are identified by name, and it includes such substances even when sold within dietary supplements (Testing Specification, 2020). Research and method development are warranted; separating regioisomers by mass spectrometry due to shared mass, charge and molecular formula is very difficult, then FT-IR spectroscopy is necessary for differentiation of drugs that have the same MS spectrometric finger print (Abdel-Hay, Deruiter, & Clark, 2014, p. 135).

1.2 Project Objectives: Part One and Part Two

The aim of the first part of this experiment was to develop and optimize analytical methods for the detection of the PED Tamoxifen in low concentration levels in oral fluid. The objectives included spiking of artificial oral fluid with Tamoxifen and efficient extraction of the modulator (parent compound and/or metabolites) from oral fluid for subsequent analysis. The desired endpoint for all developed methods was to attain the highest level of sensitivity with the lowest limits of detection possible; no comparisons could be found for detection of Tamoxifen in oral fluid using GC-MS. The physicochemical properties of Tamoxifen were studied to estimate the likelihood of this drug transporting to saliva by means of active transport, passive diffusion, and/or

ultrafiltration. The properties of Tamoxifen (specifically being highly protein bound and largely ionized in blood) significantly limit drug concentration levels in saliva, and oral fluid collection procedures can negatively affect saliva pH levels that are favorable to excretion of Tamoxifen. Of significant interest was the study of Tamoxifen ionization and possible ion-trapping in saliva. Special consideration of oral fluid collection procedures was made due to their effect on pH, which effects drug concentration levels, and special consideration was given to saliva flow rate as it, too, effects saliva pH levels.

Method development was approached systematically: learning chemical and physical properties of both the drug Tamoxifen and the biological matrix of oral fluid, focusing on the goal of detecting the drug Tamoxifen in low concentration, utilizing available analytical instrumentation and supplies (and working with their parameters), recording results using original set parameters, and checking for overall performance of each performed technique (i.e. method validation). It began with the performance of a scouting gradient with varied oven temperatures to determine when analytes elute. For method optimization and detection of Tamoxifen at very low concentration levels, each test parameter value was individually examined as a means for determining which parameters provided best sensitivity and level of detection. This demonstrates the ability to interpret and evaluate the results of each method. Throughout development, adjustment of conditions was also performed to resolve resolution and poor peak shapes. This demonstrates ability to apply understanding of each analysis regarding the instruments used. This first part of this project

demonstrates gained and applied understanding of analytical principles necessary for improving and/or resolving challenging aspects of oral fluid drug testing: from sample collection to instrumental analysis to data analysis and interpretation.

Aims and objectives of this project's second part was to successfully discriminate and resolve regioisomeric aromatase inhibitors that are listed on the WADA's Prohibited List within its S4 category of Hormone and Metabolic Modulators. This is a challenging but very important task for forensic drug chemists because "in most cases, legal controls are placed on only one or two of the conceivable isomers" (Negishi, et al., p. 338). Substances can be synthesized in clandestine labs that vary their structural placement of functional groups just differently enough to be technically legal, then utilized in products such as dietary supplements. Athletes who ultimately use these products to enhance their performance may find no desired effect or they may experience the worst scenario: a very unsafe drug.

2. RESEARCH

2.1 Background Information

This project integrates increased scientific interest in continued research of oral fluid drug testing with the prospect of an organization such as the World Anti-Doping Agency (WADA) utilizing oral fluid as a third drug testing matrix to supplement its current use of blood and urine. WADA is an independent, international agency responsible for overseeing and enforcing doping-control policies and procedures that are implemented world-wide for the purpose of ensuring that sporting events like the Olympics maintain integrity and legitimacy (Who We Are, 2018). WADA's annually published "Prohibited List" for banned use of substances has also become the gold standard for anti-doping regulations in world-wide professional sports, amateur sports, college sports, etc. (PROHIBITED LIST, 2020).

The History of WADA begins with the "Festina Affair" (The Festina Affair, 2008). This involved doping at the 1998 Tour de France, the annual bicycle race held in France known to be prestigious but difficult. Willy Voet, a member of the Festina cycling team of Spain, was found with a variety of performance enhancing drugs in his car. This discovery led to police investigations, raiding of other teams' headquarters and athletes' hotel rooms, and increased blood testing for athletes. There was a suspension of two teams followed by several strikes organized by other riders who said the enhanced police tactics and measures were uncalled for and rather extreme. There were actually fewer than 100 riders in that year's Tour de France; emotions and tensions were high (it troubled a lot of athletes that the integrity of the very sporting

event that they had trained to do was being questioned, and directors of the sporting events were anxious about what changes would need to happen to prevent another scandal). For these reasons the International Olympic Committee held a World Conference on Doping in February 1999 in Lausanne, Switzerland. Here, the "Lausanne Declaration on Doping" was constructed (Fraser, 2004, p. 171). The Olympic Movement Association pledged to establish an independent, International anti-doping agency before the 2000 Sydney Olympics (Who We Are, 2018). This was achieved on November 10, 1999 with the formation of the World Anti-Doping Agency. WADA's core values were firmly established: integrity, accountability, and excellence. The agency encourages "respect for the game and the people that play it." WADA is a partnership between sports and governing agencies world-wide whose vision is a world where all athletes can compete in a doping-free sporting environment. Today, over a hundred governing and sports agencies (including the U.S.) have agreed to support WADA's implementation of its "Code" – the promotion of fighting against doping in sport. All organizations that implement WADA's code are called "Code Signatories," and they are responsible for applying code provisions through policy and regulation (Code Compliance, 2019). This code defines what doping is and describes the rights and responsibilities of the athletes, the anti-doping agencies, the governing bodies, and the government agencies involved. One of the most important things WADA does is publish its Prohibited Substance List (PROHIBITED LIST, 2020). The first prohibited list came out in late 2004 (published as 2005), and

WADA has published one annually ever since. In order to be placed on the prohibited list, a substance must meet two of the three qualifications:

1. Poses a potential risk to the athlete's health.
2. Has the ability to enhance performance.
3. Violates the spirit of sport, characterized by values such as fairness, honesty, respecting rules and other athletes, etc.

The Prohibited List is separated into three categories and then further separated into drug classifications grouped by pharmacological effect on the body. The first category details all substances banned from use at all times (in and out of competition). The second category details substances banned during competition, and the third category details substances banned for particular sports. The possession, trafficking, administration or even attempted administration of these prohibited substances (and/or a refusal to submit to drug testing or failing to report "where about" information) can and does lead to several, severe consequences for an athlete. It is very important, too, that athletes understand that banned substances include substances that are not listed on the Prohibitive List if they meet certain criteria (e.g. unregulated designer drugs). Banned substances within the second category that cannot be used during competition may or may not be illegal substances for some people in some places, but they are substances that could potentially enhance performance for some athletes. Banned substances within the third category that cannot be used by athletes in particular sports must also be honored. It is the first category of banned substances (banned in and out of competition) that includes several drug classifications which are not only performance-enhancing while taking or using them, but they may provide enhancement long after stopping use of the

substance. SERMs and AIs fall within the S4 classification within the first category: Hormone and Metabolic Modulators. All substances listed within the S4 classification have been banned since the original Prohibited List (created 2004, published 2005) for their potential abuse: boosting free testosterone levels and countering negative side-effects of anabolic androgenic steroid use and/or androgenic supplements (The 2005 Prohibited List International Standard, 2005).

Both random and scheduled drug testing of athletes is done as a deterrent for using PEDs and as a way to uphold WADA's motto to "play true" (Who We Are, 2018). It is important to prevent the use of PEDs not only for the sake of maintaining fairness among athletes but also for health reasons. Some PEDs can have dire physical and/or emotional consequences of using or abusing them. Despite this, a competitive drive (and possibly a lack of self-confidence) puts athletes at risk for succumbing to temptation, albeit a decision that could ruin or harm a career and reputation, to partake in PED use. The list is long; several famous athletes have been caught by drug testing protocol for using banned substances. Planned drug testing continues to occur for all competition athletes, but some athletes are targeted for additional monitoring and testing due to risk of using steroid PEDs or manipulating their strength by blood or gene doping. Since 2009, WADA has formally utilized its ADAMS system (Anti-Doping Administration and Management System) with its "Athlete Biological Passport" (ABP) program and "Where About" program (ADAMS Knowledge Base). The passport program targets detection of steroid use, blood doping, gene doping and Human Growth Hormone (HGH) use by monitoring an athlete's biological variables over time

that can reveal effects of the doping because it creates a record of test results for comparison purposes: the "Haematological Module" blood collection and the "Steroidal Module" urine collection (Athlete Biological Passport, 2017, pp. 5-6). These tests are not intended to immediately find proof of "cheating," but they can serve as evidence that "cheating" has occurred. Each athlete's own "normal" levels of various hormones can be compared over time after collecting samples over months or even years. Athletes are never given advance notice, but they are tested for the passport program at least once a year. WADA's "Where About" program (actually in place since 2004) requires the athletes to provide detailed information every three months as to where they will be and to assure they can be available for unannounced drug testing. WADA conducts random drug testing and has no limitations in how often they can test athletes, but all testing must follow very strict guidelines (e.g. a doping control officer of the same gender must unobstructedly observe urine leaving the body) (Doping Control Process, p. 2). If a drug test result is found to be positive for a banned substance, WADA classifies it as an "Adverse Analytical Finding" (AAF). If a drug test result needs additional analysis because the result is unclear, WADA classifies it as an "Atypical Finding" (ATF) (PROHIBITED LIST, 2020).

It is extremely impressive that WADA drug tests more than 300,000 athletes every year utilizing an average of 30 WADA approved laboratories world-wide (List of WADA Accredited Laboratories, 2020). It requires a top-notch, coordinated system (reliant on government and other organizations and agencies) of tracking athletes, testing athletes, analyzing drug tests, reporting results, and comparing prior test

results. WADA's standards definitely set the bar for all monitoring programs for athletes. Every effort is made to fairly monitor athletes for use of banned substances but to also stay a step ahead of new tactics they may use to falsify their drug test results. WADA's use of blood as a testing specimen has specific advantages (e.g. longitudinal profiling of drug and hormone biomarkers) while its use of urine as a testing specimen also has specific advantages (e.g. detecting a large number of substance metabolites from drug use over days or weeks) (Hadland & Levy, 2016, pp. 2-3). Doping methods caught by blood analysis can include misuse of steroids, SERMs, AIs, hormones, or other banned substances, and blood analysis can help detect doping methods like manipulation of blood (blood transfusions or artificially increasing oxygen delivery) or gene doping (ADAMS Knowledge Base).

It is the disadvantages associated with blood or urine drug testing that makes the use of a third matrix like oral fluid (as a supplement) almost necessary. Detection windows between blood and saliva are approximately the same based on broad estimates that do not specifically account for the type of substance, amount used, or other contributing factors (as seen in Figure 1), but blood collection is costly and requires medical staff. Blood collection is not suitable for spontaneous testing, and it does present a level of risk for infection. In general, athletes view the collection of their blood as extraordinarily invasive. Some athletes learn how to cheat blood testing by working with doctors to strategically dose substances in ways that show no proof, even when their own samples are compared over time.

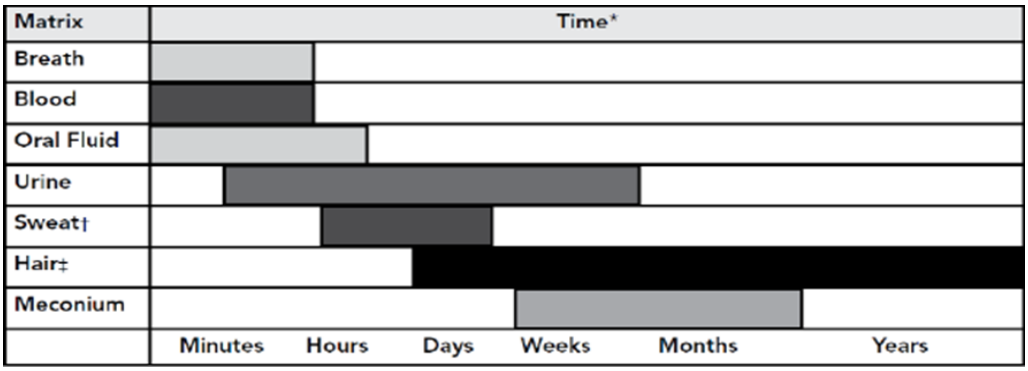


Figure 1 Detection Times for Drug Testing Different Biological Specimens.
Source:
Hadland, S. E., & Levy, S. (2016, July). Objective Testing - Urine and Other Drug Tests. *Child and Adolescent Psychiatric Clinics of North America, U.S. National Library of Medicine*. doi:doi:10.1016/j.chc.2016.02.005

Urine collection is suitable for spontaneous testing and provides a longer detection window than either blood or oral fluid, but most athletes feel it is also very invasive to have a collector directly observe their urination. The collector must be of the same gender as the athlete, and many steps must be taken by the collector to check for tampering. By far, the greatest disadvantage of urine drug tests is the possibility of tampering, with the internet teaching most athletes how to "beat" a test. The most common forms of tampering with urine samples or adulterating test results include substitution of urine with synthetic urine, using another person's urine, wearing a fake but anatomically correct device to hide use of a substituted sample, diluting urine (e.g. adding toilet water), "flushing" out the drugs by gulping water or other liquids to produce diluted samples, or adding an adulterant to the sample after it is produced (Dasgupta, 2015). Both the collectors and the lab analysts must remain diligent in looking for evidence of tampering and/or adulterants.

The key advantages to drug testing oral fluid include non-invasiveness of the collection process, less costly than testing blood, detects the parent compound of drug use (as does blood), detects recent drug use, may be tamper-proof, collection is not dependent on a gender-specific person, and oral fluid is not considered a hazardous waste (Huestis, et al., 2011, pp. 3-4).

WADA actively and constantly pursues ways to improve its monitoring methods and to implement procedures that cut down on costs and/or reduces inconvenience or discomfort for athletes. It very recently announced in October 2019 that a new method for collecting blood samples from athletes will soon be tried and evaluated for partial implementation for the 2020 Summer Olympic and Paralympic Games and full implementation for the 2022 Winter Olympic and Paralympic Games, called "dried-blood-spot" testing (DBS) (WADA Leads Exciting Collaboration on Dried-Blood-Spot Testing, 2019). This method allows for blood collection to be as simple as a finger prick.

> WADA Senior Executive Director, Sciences and International Partnerships, Dr. Olivier Rabin said: "The possible advantages of DBS are clear. It has the potential to add to the current global anti-doping program by complementing existing urine and blood testing to expand upon the program's testing coverage and capacity to better reveal doping practices. WADA is committed to making available new ways of protecting clean sport that reduce the inconvenience or discomfort for athletes and is easier, more effective and cheaper to carry out. In that way, it could be that DBS will be a major breakthrough in global anti-doping testing capacity (WADA Leads Exciting Collaboration on Dried-Blood-Spot Testing, 2019).

Like DBS testing, the use of oral fluid drug testing could supplement WADA's blood and urine testing program with very similar potential advantages: simplified sample collection with reduced invasiveness, reduced costs for collection and

transportation of samples, and less space needed for sample storage. Per WADA's announcement, "All these advantages could allow testing authorities to target more athletes and collect more samples, including in some geographically remote areas" (WADA Leads Exciting Collaboration on Dried-Blood-Spot Testing, 2019). Storage of samples is vital; they may need retested when there is a future discovery of how an athlete cheated a test.

WADA publishes annual reports to publicize its role in fighting against doping in sports. This provided information painstakingly details summarized data that includes not only numbers of collected and analyzed blood and urine samples, but also the adverse and atypical findings of tests according to the sports categories they represent and the drug classifications of all detected substances. Based on WADA's most recent published data that compiles 2018 statistics, the increase in detection of S4 class of substances that includes Tamoxifen and AIs went from its lowest finding of 70 in 2011 (Overview of Results, 2011, p. 9) to a total of 213 findings in 2018 (2018 Anti-Doping Testing Figures, 2018). Demonstrated in Figure 2, this increase clearly supports the importance of further researching oral fluid drug testing that could supplement WADA's existing methods and maximize its program efficiency.

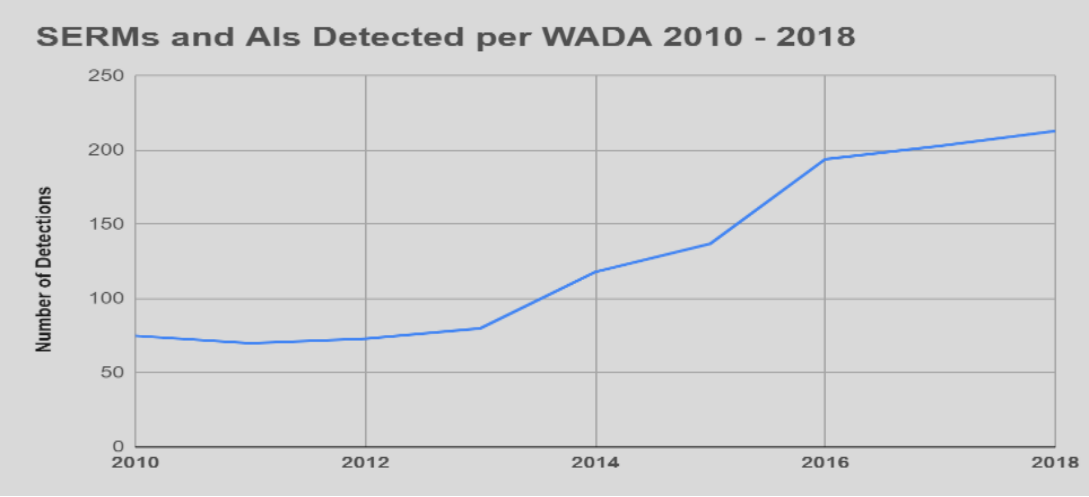


Figure 2 Detection of S4 Substances 2010-2018.
Includes Tamoxifen and AIs. Increased from 70 AAF and ATF findings in 2011 to a total of 213 AAF and ATF findings in 2018. Adapted from:
Overview of Results. (2011). Retrieved from World Anti-Doping Agency: https://www.wada-ama.org/sites/default/files/resources/files/WADA-2011-Laboratory-Testing-Figures.pdf
2018 Anti-Doping Testing Figures. (2018). Retrieved from Wada-Ama.org: www.wada-ama.org/en/resources/laboratories/anti-doping-testing-figures-report

2.2 Literature Review

WADA's biological passport testing protocol coupled with its "Where About" program and other strict drug testing policies more thoroughly and methodically address anti-doping than any other monitoring system it has previously employed. Every year, hundreds of thousands of blood and urine samples are collected from athletes around the world and analyzed for detection of prohibited substance use, and a significant number of samples are found to be "adverse" or "atypical." Unfortunately, because the internet is a treasure trove of information about how to beat these drug tests and because some doctors are willing to help athletes manipulate their test results by timing certain drug use or using chemicals that go undetected, there is more work to be done to close gaps which blood and

urine tests do present when it comes to issues like spontaneity of testing, the costs involved, the invasiveness of an athlete's privacy, and the most difficult issue of all: the likelihood of a specimen being adulterated. The use of oral fluid for drug testing could supplement WADA's use of blood and urine as test matrices. Much research has been done over the past two decades to improve and address any problems that have kept oral fluid from becoming just as routinely used as urine (e.g. low volume sample and low drug concentration levels), but most of the focus has been on developing analytical methods that detect and quantify substances most commonly abused in the general population: cocaine, heroin, marijuana, etc. (Drummer, 2006, p. 147). Many performance-enhancing drugs have only recently become known (usually after an athlete confesses to PED use and reveals the intricate information about how he or she got away with using the substance for years), so it has only been recently that laboratories have needed to steer their research toward detection of an extremely wide variety of these types of drugs. For almost ten years, one of the more prominently abused prohibited substances has been Tamoxifen along with other SERMs, as they provide a mask for athletes who abuse anabolic steroids or androgenic supplements but need or want to minimize the effects such as male breast enlargement. As well, SERMs – including AIs – provide a way to naturally increase levels of testosterone. While Tamoxifen has been thoroughly researched due to its use for decades to help prevent return of breast cancer, it is a perfect example of a PED that may be used intermittently and/or in low dosages to avoid detection in either blood or urine. If an athlete were to be more randomly and spontaneously drug tested

for SERMs utilizing oral fluid, a detection gap could possibly be closed. Development of a novel method for detection of Tamoxifen and other SERMs in oral fluid using GC/MS (commonly available with fewer costs) contributes to scientific research necessary for reaching a goal of supplementing WADA's testing procedures with OF testing.

Athletes' Use of Anabolic-Androgenic Steroids

Anabolic-androgenic steroids have been associated with "doping" by athletes since the 1980's when it became more commonly known they can help build muscle mass and enhance athletic performance. Most often just called "steroids," they are synthetic substances that mimic the male hormone testosterone (Steroids and Other Appearance and Performance Enhancing Drugs (APEDs), 2018, p. 4). When used as part of a weight training program, the increase of muscle mass also makes a person stronger and perform better than without anabolic steroid use. "They promote the growth of skeletal muscle (anabolic effects) and the development of male sexual characteristics (androgenic effects) in both males and females" (Steroids and Other Appearance and Performance Enhancing Drugs (APEDs), 2018, p. 4). It is mostly males who use steroids as PEDs, but not exclusively. Until 1990 in the United States when anabolic steroids were classified as controlled substances and outlawed, even many non-professional athletes were using them to improve personal appearance. To get around the 1990 federal law, dietary supplements with steroid precursors (e.g. androstenedione – aka "Andro") hit the shelves and could be legally purchased. If taken in very large quantities, they could also increase a person's testosterone

levels. Congress passed another law in 2004 to ban the sale, manufacturing and possession of these steroid precursors, too, but illegal sources have continued. For most steroid users, taking multiple types of supplements is common to either complement their increased strength gained from training with steroids (e.g. will also take stimulants or creatine) or for need to minimize unwanted side effects from increased testosterone levels (e.g. will take estrogen blockers to stop irreversible male breast enlargement or to reverse shrinking testicles) (Steroids and Other Appearance and Performance Enhancing Drugs (APEDs), 2018, pp. 10, 16). Such side effects occur because the enzyme aromatase converts androgen (increased testosterone) to estrogen (Hilborn, Stal, & Jansson, 2017, p. 3).

Usually, professional or Olympic athletes who use steroids "cycle," "stack" or even "pyramid" their doses and days to get benefits from the drugs. Sometimes they change up doses to try to avoid damaging their bodies (e.g. liver) or develop tolerance, but they focus on the results of their methods. Cycling steroids means taking different steroids in smaller doses but steadily; stacking steroids means taking very large amounts of steroids to gain fast effects; and pyramiding means taking steroids in low to high and back to low dosages. Again, concurrently, most athletes who use steroids also use anti-estrogens to combat steroid effects and will avoid steroid use altogether to allow for their bodies' hormonal systems to recuperate (Steroids and Other Appearance and Performance Enhancing Drugs (APEDs), 2018, p. 13). This goes together with trying to make sure their ability to "pass" drug tests can be achieved prior to events or other times when they suspect they will be tested – by blood tests or

by more easily adulterated urine tests. Likewise, the athletes who also take estrogen blockers will use them intermittently and/or in low doses to be more likely to "pass" drug tests. WADA chemists and others have been successful in discovering various steroid metabolites and this allows for detection of most steroid use even after discontinued use but trying to stay ahead of which new substances an athlete may be using is very consuming. New "designer drugs" may go undetected; an athlete's blood and urine results need to be compared to what is considered "normal" for testosterone levels of people in the general public, and the results need to continuously be compared to the athlete's own records as kept in their biological passport data. Comparison not only reveals questionable increases in testosterone, but the comparison can also reveal suppression of endogenous steroids which then serves as evidence of "doping" (Steroids and Other Appearance and Performance Enhancing Drugs (APEDs), 2018, pp. 24-25)

SERMs / Aromatase Inhibitors – "Indirect Androgen Doping"

Athletes who cheat rules of competition in order to be better at their sport are trying to improve their performance in one or more ways:

> Sports performance has 4 major components: skill, strength, endurance and recovery, with each sport employing a distinct combination of these elements. These performance characteristics also correspond to the most potent and effective forms of doping. Sports requiring explosive power are most susceptible to androgen doping through their effect on increasing muscle mass and strength whereas sports that require endurance are most enhanced by hemoglobin (blood) doping which increases oxygen delivering capacity to exercising tissues. Performance in contact sports and those involving intense physical activity or training may also be enhanced by growth hormone and its secretagogues through speeding of tissue recovery from injury. Hormones remain the most potent and widely detected doping agents being responsible for about 2/3 of anti-doping rule violations detected by increasingly

> sophisticated detection methods. At present, the vast majority of positives are still due to a wide variety of androgens, including marketed and illicit (nutraceutical, designer) synthetic androgens as well as exogenous natural androgens, while the peptide hormones (erythropoiesis stimulating agents, growth hormone and its secretagogues) and autologous blood transfusion remain difficult to detect (Handelsman, 2015, p. 1).

Their attempt to increase muscle mass for increased strength by "cheating" continues to rely mostly on direct doping – meaning they are using androgen products sold on the black market – but indirect doping is also frequently done.

Direct doping of androgen products involves the use of "exogenous" steroids. These steroids may be testosterone esters or synthetic anabolic steroids, but they are not naturally produced by the person taking them. Urine drug testing can be used to detect abnormal levels of testosterone (e.g. ratio of testosterone to converted 17α-epimer epitestosterone) and/or biomarkers (e.g. suppressed hormone production) of exogenous testosterone use – especially when comparing levels against a person's own urine samples over time (Handelsman, 2015, p. 10).

Indirect doping is a strategy used to increase "endogenous" testosterone. This means certain substances (e.g. growth hormone, estrogen receptor blockers, aromatase inhibitors, etc.) are used that cause a natural increase in testosterone production. The theory is that blocking a conversion of testosterone into estrogen allows for testosterone to naturally increase without having to use exogenous steroids (Ronde & Jong, 2011, pp. 2-3). This strategy is very attractive to athletes; they may be able to increase muscle mass and strength without failing a drug test if they carefully follow a plan of dosage (i.e. low dosage or intermittent use). Results from blocked estrogen conversion with daily use of prescribed aromatase inhibitors, for example,

have shown capability to increase testosterone levels in men by as much as 40% over 36 weeks (Ronde & Jong, 2011, p. 3). Results like these may not be typical but they are achievable. To detect the use of SERMs, anti-doping laboratories test for SERMs as well as luteinizing hormone (LH) suppression caused by the increased testosterone. At this time, detection of indirect doping is typically performed using MS-based methods (Handelsman, 2015, p. 11).

Adulteration and/or Manipulation of Drug Testing Specimens

Using illicit drugs continues to be a major public health problem in the United States. Just five years ago, it was estimated that 9.4% of the U.S. population over the age of 12 was abusing at least one drug – most commonly marijuana, cocaine, heroin and hallucinogens (Dasgupta, 2015). Because people are often required to drug test for employment or for monitoring purposes (e.g. athletics, criminal justice system), an attempt to "cheat" is common.

Mostly urine is collected for drug testing purposes and sent to laboratories for analysis. If specimens have been adulterated, it is essential for laboratories to be able to identify that an adulterant has been added to the urine or to be able to show that the sample is synthetic. Examples of common additives in products sold as adulterants include strong oxidizing agents, potassium nitrite, a combination product of peroxidase powder followed by liquid hydrogen peroxide, glutaraldehyde products, and zinc sulfate (Dasgupta, 2015).

The three ways people usually try to cheat urine testing is to use synthetic urine or some other person's "clean" urine; they try to drink products that promise to

"flush" out the drugs; or they try to add an adulterant to their own urine (Dasgupta, 2015). Various ways that people may try to alter oral fluid drug test results were thoroughly analyzed (e.g. the commercial adulterant "Fizzy Flush Spit" and the mouthwash "Listerine"), but nothing was found that contaminated the results after a wait of 15-30 minutes to allow for the rapid turnover of oral fluid (Drummer, 2006, pp. 147-148).

The internet provides a very large assortment of promising products, but the one method that does tend to work even when a collected sample is closely observed, is a person drinking large quantities of water (e.g. 2 quarts) for approximately two hours before a test in order to naturally dilute the specimen. This method does not tend to cause problems that adulterants can cause such as being too hot/cool or by having unnatural readings for specific gravity, pH, or creatinine (Dasgupta, 2015).

Oral Fluid Collection

Saliva is produced by multiple glands: three pairs of major glands which contribute to 90% of all saliva secretions (the parotid, submandibular and the sublingual salivary glands) as well as minor glands that contribute to the remaining saliva secretions (Iorgulescu, 2009, p. 303). "Whole" saliva is the term used to describe the sum of all that is produced. In all, saliva is 99% water 1% protein and salts (Iorgulescu, 2009, p. 303).

The unstimulated flow rate of whole saliva is approximately 0.3-0.4 ml/minute and increases up to 1.0-5.0 ml/minute during eating, chewing or other stimulating activities (Iorgulescu, 2009, p. 303). Mechanically stimulated saliva contains more

buffering agents (e.g. bicarbonate) than unstimulated saliva and causes salivary pH to increase (Cunha-Cruz, et al., 2013, p. 2). The buffering agents help maintain a neutral pH range of 6.0-7.5 within the mouth (Iorgulescu, 2009, p. 304).

Researchers have found that non-stimulated spitting "was the most effective technique" for oral fluid collection because flow rate of saliva also affects drug concentration (i.e. allowing saliva to drip from the mouth is best) (Crouch, Day, Baudys, & Fatah, 2005, p. 11). This research details the finding that "stimulated" saliva collection presents potential problems: materials used for collection may absorb some drugs, salivary stimulants (e.g. lemon drop) may change saliva pH and consequently alter drug concentrations, or mechanical stimulation of saliva using chewing gum (or something similar) may change the salivary composition which also affects saliva-drug concentration (Crouch, Day, Baudys, & Fatah, 2005, p. 11).

When drug concentrations are present, it is the parent drugs and not the drug metabolites that are most often found. This, as Crouch stated, is because parent drugs are more lipid soluble and pass more easily through capillary and acinar membranes from blood into saliva. Because it is mostly the parent drugs present in oral fluid, conformational analysis of a substance may not require derivatization (e.g. parent drugs that are less polar and more easily extracted) (Crouch, Day, Baudys, & Fatah, 2005, p. 8).

2.3 Drugs Studied

Tamoxifen

Chemical and Physical Properties

Tamoxifen is an "antineoplastic nonsteroidal" selective estrogen receptor modulator (SERM). Its molecular formula is $C_{26}H_{29}NO$, and its molecular weight is 371.5 g/mol (Tamoxifen). Its pK_a is approximately 8.85, it has a melting point of 96-98°C, and Tamoxifen is practically insoluble in water (solubility is <0.01%, 20°C) (Sigma Product Information, p. 1). Tamoxifen's structure can be seen in Figure 3.

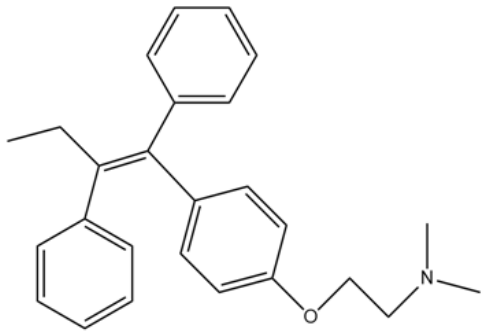


Figure 3 Chemical Structure of Tamoxifen.

Pharmacology

Tamoxifen inhibits the binding of estradiol (an estrogenic steroid produced in the ovaries) to estrogen receptors, and this results in a reduction in cellular response to estrogen, mostly in mammary tissue – which reduces risk for tumor growth in the breast. Tamoxifen is most often prescribed to women enduring breast cancer treatment, but it is sometimes prescribed to men for infertility treatments. The Tamoxifen increases a male's level of luteinizing hormone (LH) and follicle-stimulating

hormone (FSH), so it naturally increases a male's level of testosterone as well (Tamoxifen).

Metabolism

Tamoxifen is slowly absorbed (oral dosage) from the intestines and extensively metabolized with peak serum concentrations of 17 ng/mL occurring between 3 to 6 hours after a single 10-mg dose (Tamoxifen). When dosage is increased to twice daily and followed for three months, the average steady-state plasma concentrations of Tamoxifen range from 67 ng/mL to 183 ng/mL while its major metabolite N-desmethyltamoxifen generally ranges 1-2 times greater – from 148 ng/mL to 654 ng/mL (Tamoxifen). Research regarding concentration of Tamoxifen in oral fluid is scant; there has been no finding of strict correlation between concentration levels of Tamoxifen in saliva and the levels in serum (Lien, et al., 1989, p. 2180). Tamoxifen's half-life is typically 4 to 7 days, and its route of elimination is mainly feces, as much as 75% or higher. "Negligible" collection of Tamoxifen has been recovered from urine; only 1 human study has shown 26.7% recovery of Tamoxifen in urine (Tamoxifen).

Literature Review: Methods of Analysis

Biological samples containing Tamoxifen and its metabolites were studied for the purpose of understanding the rate of elimination. The samples were from thirty-six breast cancer patients chronically taking Tamoxifen and included: serum, pleural, pericardial and peritoneal effusions, cerebrospinal fluid, saliva, bile, feces, and urine (Lien, et al., 1989, p. 2175). It was determined that the protein binding of tamoxifen and its major metabolites was 98% or higher as albumin was the predominant carrier

for tamoxifen in human plasma (Lien, et al., 1989, p. 2175). Concentration levels of Tamoxifen and its metabolites were measured in each biological specimen. In saliva, Tamoxifen and one of its metabolites (*N*-desmethyltamoxifen) referred to as "X," were detected. Because these detected amounts exceeded the amounts of free drug in serum – a disparity in distribution – the researchers suggested the lipid soluble drug readily crossed the gland epithelium and the compounds were actively transported or trapped in the salivary gland "through interaction with salivary proteins including albumin" and then trapped per "pH-dependent change in drug ionization" (Lien, et al., 1989, pp. 2175, 2182). They further concluded that Tamoxifen is extensively metabolized and that most of the drug is excreted via bile into the feces with less than 20% of the drug eliminated in urine. Regarding plasma concentration, they found that patients given a single dose of Tamoxifen experienced peak plasma concentration after 4-7 hours with the terminal half-life being longer than 7 days (Lien, et al., 1989, p. 2175). The researchers noted that despite wide use of Tamoxifen (at the time of this research in 1989), "knowledge on its fate in humans is sparse."

Analysis of saliva from 11 patients showed that Tamoxifen and metabolite "X" were present in moderate quantities while the other metabolites were essentially absent (Lien, et al., 1989, pp. 2179-2180). These quantities ranged from 0.6 ng/mL to 14.6 ng/mL. Images of chromatograms were included and showed the researchers' identification of Tamoxifen and its metabolites in each of the tested biological fluids and extracts. As seen in Figure 4, an analyzed extract from saliva reveals a slight

response for LC-SIM traces of the M^+ ion for tamoxifen (374 *m/z*) and metabolite BX (372 *m/z*) (Lien, et al., 1989, p. 2177).

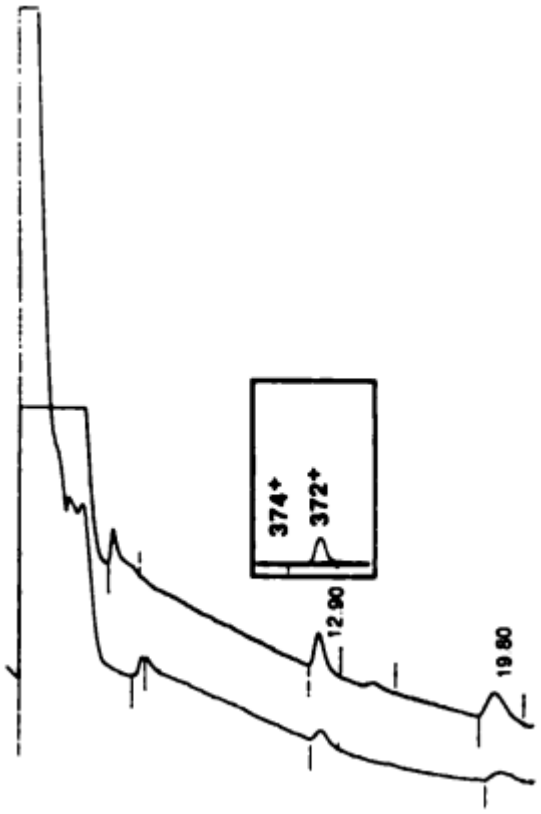


Figure 4 Chromatography of Extract from Saliva.
Source:
Lien, E. A., Solheim, E., Lea, O. A., Lundgrer, S., Kvinnslar, S., & Ueland, M. (1989, April 15). Distribution of 4-Hydroxy-N-Desmethyltamoxifen and Other Tamoxifen Metabolites in Human Biological Fluids during Tamoxifen Treatment. *American Association for Cancer Research*. Retrieved from cancerres.aacrjournals.org/content/49/8/2175

Authors Sebastien Anizan and Marilyn Huestis – both chemists conducting research for the National Institute on Drug Abuse (NIDA) in Baltimore, Maryland – outlined present day knowledge, advantages, disadvantages and limitations of using oral fluid (OF) drug testing as a viable alternative to WADA's current specimen

matrices (blood and urine). They pinpointed need to consider how different drugs are transferred into oral fluid from blood. A drug's physicochemical properties effect transfer. A drug's molecular weight, pK_a, protein binding and lipid solubility are examples of properties that affect whether or not it enters oral fluid by passive diffusion (Anizan & Huestis, 2014, p. 3). Most drugs transfer from blood to oral fluid by passive diffusion (moving from an area of higher concentration to lower concentration) because their molecules are small and uncharged, but molecules with high molecular weight may require binding to a transmembrane protein for active transport into oral fluid. Another way for transport into oral fluid is ultrafiltration – when the molecules are small enough to go through pores in the cell membrane (Anizan & Huestis, 2014, p. 3). Molecules with high weight and high pK_a value that are also highly protein-binding and already ionized in blood, transfer very poorly between blood and saliva (Cone & Huestis, 2007, p. 3). It is necessary for saliva to be at its lowest pH for this type of molecule to transfer and then become trapped ("ion trapping") as it becomes ionized (Anizan & Huestis, 2014, p. 3). This increases drug concentration in saliva but if the drug molecule is a weak base and stimulation is used for oral fluid collection – which raises the saliva pH significantly – ion-trapping may cause a false-negative drug test result (Allen, 2011, p. 533). Oral fluid collection method and stimulation techniques for collection (e.g. acidic candy) are extremely important challenges to research prior to oral fluid drug testing becoming a testing matrix for WADA (Anizan & Huestis, 2014, p. 3).

Regarding use of oral fluid to test for WADA Class S4 substances (hormones and metabolic modulators), the scientists highlighted the trend of Tamoxifen taken by males to "induce androgenic steroid production" but noted it is currently detected only in urine (Anizan & Huestis, 2014, p. 12). They further advised that based on amine group presence in Tamoxifen, "it should be detectable in oral fluid." The scientists stated need for determining Tamoxifen's detectability in OF and to determine its detection windows.

5-alpha-Androstan-17-one

Chemical and Physical Properties

The chemical structure of the regioisomer aromatase inhibitor 5-alpha-Androstan-17-one, a ketone, can be seen in Figure 5 below. Its molecular formula is $C_{19}H_{30}O$, and its molecular weight is 274.4 g/mol (U.S. National Library of Medicine, 2020).

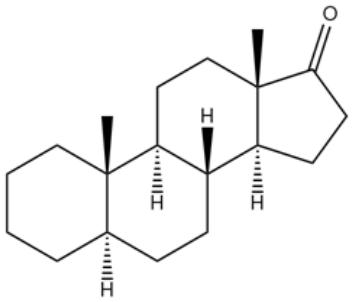


Figure 5 Chemical Structure of 5-alpha-Androstan-17-one.

Pharmacology

This regioisomer AI is a steroidal pheromone that is androgen-derived and released primarily in sweat. It is most concentrated in the sweat and urine of males (50 times higher in males than females) and may contribute to signaling for sexual

response (Sergeant, 2010, p. 25). It is described as an unpleasant odor by most people but for humans, there are some studies that show some women (i.e. ovulating) are attracted to it (Araneda & Firestein, 2004, p. 1). Some on-line bloggers discussing use of steroidal pheromones in dietary supplements believe a pheromone is a cognitive performance enhancer in that it may promote feelings of confidence and lead to success through dominating behaviors.

Metabolism

This regioisomer AI type is found in the urine and sweat in humans as a byproduct of the hormone testosterone (Araneda & Firestein, 2004, p. 1). This regioisomer AI type was initially included in WADA's Prohibited List within the S1 Classification of substances banned at all times because of its chemical similarity to anabolic steroids, but it was moved to WADA's S4 Classification in 2019 because this "better reflects its biological activity" (Summary of Major Modifications and Explanatory Notes, 2019). WADA's S4 Class of Prohibited Substances is the Hormone and Metabolic Modulators.

Literature Review: Methods of Analysis

To the best of our knowledge, no literature pertaining to the analytical methods for determining this drug has been reported. This makes this proposed analytical study novel and new.

17-beta-Hydroxy-5-alpha-androst-2-ene

Chemical and Physical Properties

The chemical structure of the aromatase inhibitor 17-beta-Hydroxy-5-alpha-androst-2-ene can be seen in Figure 6 below. Its molecular formula is $C_{19}H_{30}O$, and its molecular weight is 274.4 g/mol (U.S. National Library of Medicine, 2020). It is classified as an adrostanoid; it is "a steroid based on an androstane skeleton and its derivatives" (NCBO BioPortal, 2020).

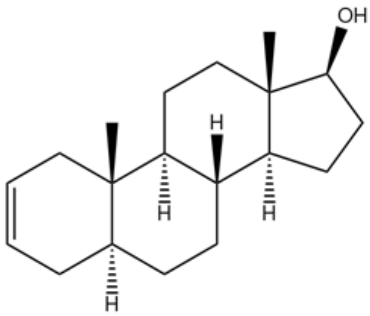


Figure 6 Chemical Structure of 17-beta-Hydroxy-5-alpha-androst-2-ene.

Pharmacology

To the best of our knowledge, no literature pertaining to medical use of this drug has been reported.

Metabolism

This regioisomer AI type is chemically related to anabolic steroids – a metabolite of testosterone and prohibited by WADA at all times for use in and out of competition (classified as an anabolic agent within WADA's S1 Classification of prohibited substances). Metabolites of testosterone such as this are prohibited by WADA because they are considered an anabolic androgenic steroid (AAS) administered

exogenously: a substance which is not ordinarily produced by the body naturally (PROHIBITED LIST, 2020, pp. 2-3). This regioisomer AI type was initially included within the S1 Classification because of its chemical similarity to anabolic steroids, but it was moved to WADA's S4 Classification in 2019 because this "better reflects its biological activity" (Summary of Major Modifications and Explanatory Notes, 2019). WADA's S4 Class of Prohibited Substances is the Hormone and Metabolic Modulators.

Literature Review: Methods of Analysis

To the best of our knowledge, no literature pertaining to the analytical methods for determining this drug has been reported. This makes this proposed analytical study novel and new.

5-alpha-Androst-16-en-3α-ol

Chemical and Physical Properties

The chemical structure of the regioisomer aromatase inhibitor 5-alpha-Androst-16-en-3α-ol, an alcohol, can be seen in Figure 7 below. Its molecular formula is $C_{19}H_{30}O$, and its molecular weight is 274.4 g/mol (U.S. National Library of Medicine, 2020).

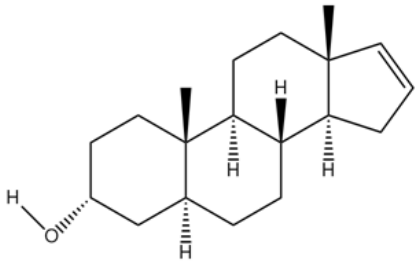


Figure 7 Chemical Structure of 5-alpha-Androst-16-en-3α-ol.

It is classified with organic compounds known as androgens and androgen derivatives (3-hydroxylated C19 steroid hormones) that favor the development of masculine characteristics, particularly affecting scalp and body hair in humans (Showing Metabocard for Androstenol, 2020).

Pharmacology

This regioisomeric AI is an androgen believed to act as a pheromone. 5-alpha-Androst-16-en-3α-ol (also labeled 3α-androstenol) belongs to a group of 16-androstenes, first isolated from boar testes and later found in humans (Kaminski, Marini, Ortinski, Vicini, & Rogawski, 2006, p. 694). It has been shown to have neurosteroid-like activity as a $GABA_A$ receptor modulator which means it may affect mood and act as an anxiolytic, anti-aggressive, sedative/anesthetic, and anti-epileptic agent in both animals and humans (Wang, 2011, p. 1). Musky smelling, it is a steroid that humans produce (i.e. in sweat) that may signal another person to react.

5-alpha-Androst-16-en-3α-ol is biosynthesized in the testes of boars and humans, and it can be found in human urine, blood plasma, saliva and sweat (Kaminski, Marini, Ortinski, Vicini, & Rogawski, 2006, p. 694). Possibly, this steroid or pheromone is one of several compounds released by humans that causes cerebral activation, most notably triggered mating behavior (Savic & Berglund, 2010, p. 1). Additionally, it may influence the secretion of the luteinizing hormone (LH) in human females which, in turn, may explain synchronization of menstrual cycles when women live together or work closely together (Savic & Berglund, 2010, p. 1).

Metabolism

This regioisomeric aromatase inhibitor 5-alpha-Androst-16-en-3α-ol is excreted in urine; its biological roles include being a hormone (regulates the activity of certain organs or control processes) and signaling molecule (participates in signal transduction processes) (Showing Metabocard for Androstenol, 2020).

This regioisomer type – an androstanol – was also initially included in WADA's Prohibited List within the S1 Classification of substances banned at all times because of its chemical similarity to anabolic steroids, but it was moved to WADA's S4 Classification in 2019 because this "better reflects its biological activity" (Summary of Major Modifications and Explanatory Notes, 2019). WADA's S4 Class of Prohibited Substances is the Hormone and Metabolic Modulators.

Literature Review: Methods of Analysis

For doping control purposes, this steroid has been used to develop a GC/thermal conversion/isotope ratio mass spectrometry method for the determination of the deuterium/hydrogen (D/H) ratio of endogenous urinary steroids (Sigma-Aldrich, 2020). A study conducted in 2008 in Germany included 5-alpha-Androst-16-en-3α-ol as one of 10 steroids measured from a urine sample to determine naturally occurring D/H ratios. From this, the researchers were able to "calculate preliminary reference limits for relevant Δ values among the steroids and to test the method for its possible application for doping control purposes" (Piper, Flenker, & Schänzer, 2008, p. 181). The method used a combined gas chromatography/thermal combustion/isotope ratio mass spectrometry for measurement; high performance

liquid chromatography (HPLC) was used with sample preparation; and 18 male volunteers provided urine samples. Results showed clear potential for D/H determinations detecting misuse of steroids by athletes, but the researchers noted that further research was needed (Piper, Flenker, & Schänzer, 2008, p. 189).

3. EXPERIMENTAL DESIGN

3.1 Project One

Detection and Determination of Tamoxifen in Oral Fluid

This project's experimental design utilized available gas chromatography (GC) instrumentation to perform separation and detection of molecules in artificial oral fluid (e.g. internal standard Propranolol and analyte of interest Tamoxifen) followed by utilization of mass spectrometry to perform identification of molecules and a measurement of the abundance of ions with high sensitivity and specificity. Emphasis was placed on determining how to lower detection limits of Tamoxifen within a low volume sample by optimizing OF collection and optimizing use of GC instrumentation (e.g. adjusting inlet temperature, changing from split to splitless injection, and temperature programming). Oral fluid and its collection procedures were carefully studied to determine how to make sure only the highest levels of Tamoxifen concentration would remain in collected OF (upon application of this project's method) and ensure it can be extracted from and detectable in samples. Following GC-MS, an extraction method (Tamoxifen from oral fluid) was developed and optimized in order to successfully extract the available Tamoxifen.

3.2 Project Two

Discrimination Between Regioisomeric Aromatase Inhibitors

Following chromatographic separation of three regioisomeric aromatase inhibitors, this project's experimental design utilized mass spectrometry and FT-IR instrumentation for discrimination and differentiation of each AI. The spectra and chromatographs were utilized to compare their structures, gaining valuable information about their functional groups for identification purposes.

4. MATERIALS AND INSTRUMENTATION

Materials, supplies, instrumentation and all equipment utilized for this project were provided by the Eastern Kentucky University Department of Chemistry.

Purchased supplies included:

- Artificial Saliva (for Pharmaceutical Research and Laboratory Use); 200 mL produced by Pickering Laboratories.
- Tamoxifen (2x 25 mg); produced by Cayman Chemical Company
- Propranolol (hydrochloride); 1 g produced by Sigma Aldrich
- 5-alpha-Androstan-17-one; 100 mg produced by Toronto Research Chemicals
- 5-alpha-Androst-16-en-3α-ol; 100 mg produced by Sigma Aldrich
- 17-beta-Hydroxy-5-alpha-androst-2-ene; produced by Alfa Chemistry.

General materials and supplies included:
(Previously purchased from Fisher Scientific, Sigma-Aldrich and VWR Chemicals)

HPLC grade methanol, benzene, cyclohexane, hexane, methyl ethyl ketone, ethyl acetate, chloroform, ammonium hydroxide, isopropanol, potassium phosphate monobasic, potassium phosphate dibasic, sodium acetate, glacial acetic acid and pH strips (Insta-Chek 0-13 Jumbo) by Micro Essential Laboratory.

Instrumentation and equipment included:

- GC-MS (by Agilent Technologies) 7890B gas chromatograph and a 7683B auto injector coupled with a 5977B MSD Agilent mass selective detector (Figure 8)
- Agilent J&W HP-5 GC Column (5%-phenyl)-methylpolysiloxane nonpolar column (30m x 0.250mm and 0.25 Micron ID)
- Vacuum Manifold
- Vortex Mixer by Fisher Scientific
- Perkin Elmer Spectrum 100 FTIR with Diamond ATR (Figure 9)

Figure 8 Agilent 7890B GC (and Auto Injector with MS Detector).

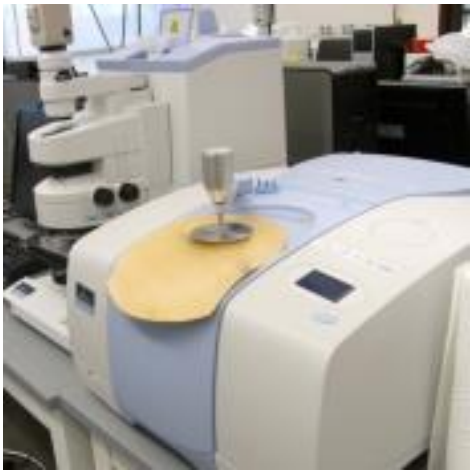


Figure 9 Perkin Elmer Spectrum 100 FT-IR (with Diamond ATR).

5. THEORY

5.1 Gas Chromatography (GC)

The eluted solution containing Tamoxifen analytes goes through an evaporation process (hot water bath) before the analytes are reconstituted with methanol and an internal standard (Propranolol) is added. An internal standard is added in order to account for instrument variation. The specimen is then ready for GC analysis. GC instrumentation is used for separating compounds that have different properties such as different boiling points and molecular weights. The specimen sample is injected into the heated inlet by way of an autosampler. After injection, the sample is vaporized into a gas. A nonreactive carrier gas such as helium, nitrogen, or hydrogen is utilized at a selected flow rate to push the vaporized sample through a column that is located in the instrument's oven. The column is a long, coiled tube where the separation of compounds occurs. The separation happens due to analytes having different affinities for the stationary phase (liquid coating of the column) as they move through the column. The stronger the analyte is attracted to the stationary phase the more time the analyte will spend bonded to it, thus determining the analyte's specific retention time. Each analyte elutes from the column at different moments in time, leading to separation of compounds. Based on physical and chemical properties, individual components of a sample will elute from the column one-by-one to then be detected and quantified. Detection happens as an individual component is leaving the column and passes an electronic detector (in this case, a mass spectrometer). (See Figure 10 for a basic GC/MS diagram.)

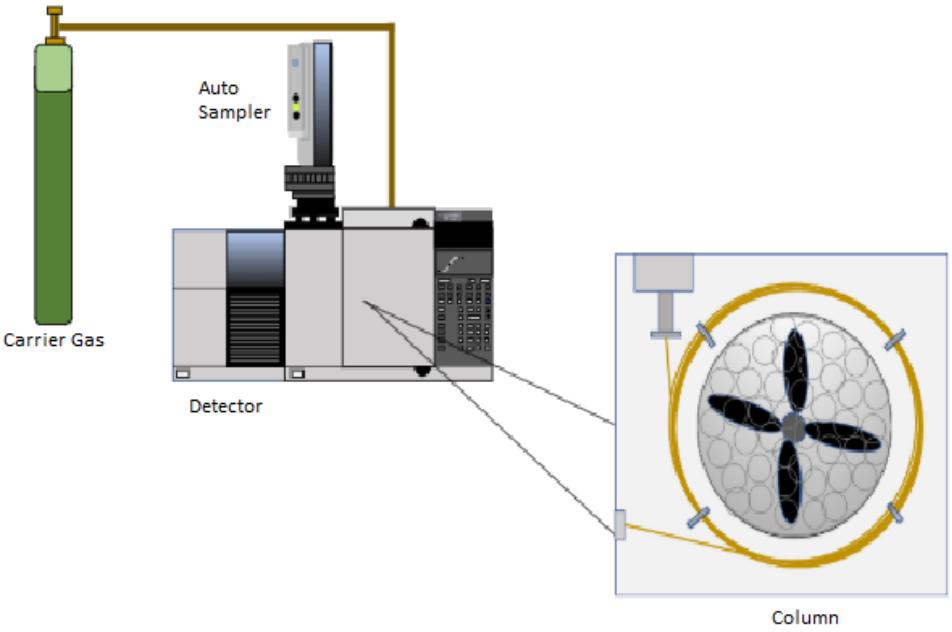


Figure 10 GC/MS Diagram.

With regard to GC there is one piece of data; it is a chromatogram with peaks. Retention time is interpreted by looking at when a peak occurs. When GC parameters are kept constant, retention times can be used for analyte identification. The area under each peak can be utilized in determining the amount of a component that is present. A calibration curve must be created using a range of analyte concentrations. As concentration increases, so does the area under the peak. A calibration curve is also created when an internal standard is utilized. A constant amount of internal standard is added to each sample of increasing analyte concentration. The peak area of the analyte is compared to the peak area of the internal standard. Each analyte concentration corresponds to a specific ratio of the peak areas. Desired peak shape is a factor that makes a chromatogram good quality: narrow, tall, symmetrical peaks.

Peak shape is also important so the analyte may be accurately quantified. The quality of the chromatograph can change as each parameter is changed and optimized.

Measurement of peak width can be done to assess separation results. For optimization of a method using GC, this is critical. Calculating asymmetry factors and tailing factors determines which peaks are as close to 1 as possible – which means both sides of a peak have the exact same width. This information helps determine which parameter values tested for method development are optimal. Peak shape is related to peak height which is related to sensitivity; thus, when sensitivity is an important factor for a method, scrutinizing minute differences is important. Tailing occurs when the analyte spends more time in the mobile phase than the stationary phase. To measure asymmetric peaks, either (or both) the asymmetric factor or the tailing factor is used. For nonpharmaceutical laboratories, the asymmetry factor is used and calculated by measuring the back half-width of the peak at 10% of the peak height and dividing it by the front half-width – with tailing factor acceptability being less than 1.5 and not over 2.0 (Dolan, 2012). See Figure 11 for illustration and explanation for measuring peaks.

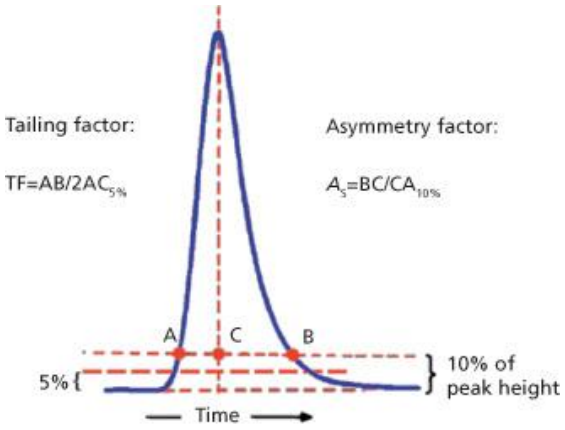


Figure 11 Measurement of Asymmetric Factor and Tailing Factor.

Source:

Dolan, J. W. (2012, July 1). Troubleshooting Basics, Part 4: Peak Shape Problems. *LCGC Europe: Solutions for Separation Scientists, 25*(7), 370-374. Retrieved from Cromatography Online: http://www.chromatographyonline.com/troubleshooting-basics-part-4-peak-shape-problems

The Van Deemter Equation

A theoretical plate is a layer within the GC column; it is not truly a physical layer, just a hypothetical layer that is used to describe increments within the column where an analyte moves through it. The analyte does actually move through the column and gets distributed between phases in the column: stationary phase (where it is dissolved) and mobile phase (immiscible with the stationary phase where it is further dissolved). The analyte moves by being distributed between the phases, in equilibrium. And the analyte must go through separate equilibrations as it gets distributed between phases. "Where" the equilibrations occur is in each theoretical (layer) plate. So, the more layers in the column where equilibrations are occurring means the number of theoretical plates is higher. "The more plates the better." The

higher the number of plates, the column is deemed as efficient. The equation utilized for calculating the number of theoretical plates can be seen in Equation 1:

$$N = 5.545(t\mathrm{R}\ /w\mathrm{h})^2$$

Equation 1 Theoretical Plate Number Equation.

The number of theoretical plates is represented by "N;" the time the analyte stays in the stationary phase is represented by "t_r" while the peak width measured at half height is represented by "w_h." This calculation of the theoretical number gives an indicator of column performance (its separation capacity).

The equation utilized for calculating the theoretical plate height (which is inversely proportional to plate number and calculated to determine column efficiency) can be seen in Equation 2:

$$H = L/N$$

Equation 2 Theoretical Plate Height Equation.

The "L" represents column length and the "N" is the calculated theoretical plate number. This calculation of plate height is interchangeably written as HETP (Height Equivalent to a Theoretical Plate).

Where the HETP is lowest, gas flow is at its optimal mobile phase flow rate. The mobile phase flow rate is typically one of the most important factors that leads to broadening of peaks because if the target analyte moves through the column too slowly, it takes more time to diffuse. As well, if the flow rate is overly increased there is greater spread due to resistance to mass transfer. When flow rate is optimal,

the broadening of peaks is lessened. Narrow peaks – not broad peaks – appear when a sample moves perfectly through a column with perfect conditions. The plotted effect of flow rate on column efficiency utilizes a Van Deemter Equation (Determination of the Optimum Flow Rate Agilent, n.d.). The Van Deemter Equation (as seen in Equation 3) mathematically predicts the optimal flow rate utilizing three factors that influence the theoretical plate height.

$$HETP = A + B/u + Cu$$

Equation 3 The Van Deemter Equation.

Getting the highest number of theoretical plates and the smallest plate height as possible is the ideal. The higher the number of theoretical plates, the better the separation. The three factors utilized in the equation that influence plate height are:

A – Eddy diffusion (refers to the multiple paths and matters when using a packed column)
B/u –Longitudinal diffusion
C – Resistance to mass transfer
u – Average mobile phase flow rate (linear velocity) (Engewald & Dettmer-Wilde, 2014).

This project did not use a packed column, so an Eddy diffusion factor was irrelevant. Longitudinal diffusion refers to analytes moving through the column with some analytes diffusing from higher to lower concentration – which is inversely proportional to the flow rate. This factor is influenced by flow rate in that a faster flow rate means less time for the "band" (analytes) to spend in the column and less likelihood of peak broadening due to this diffusion. Mass transfer refers to a "finite equilibrium time," meaning some solute is in the mobile phase and continues to move

while some solute is in the stationary phase and has stopped moving. This difference is directly proportional to the flow rate affecting band spread. Each of these factors is important and each changes according to mobile phase velocity or flow rate, except factor "A" because it is an independent factor. As seen in Figure 12 below, a Van Deemter graph illustrates where minimum plate height and a moderate flow rate can predict optimal column efficiency.

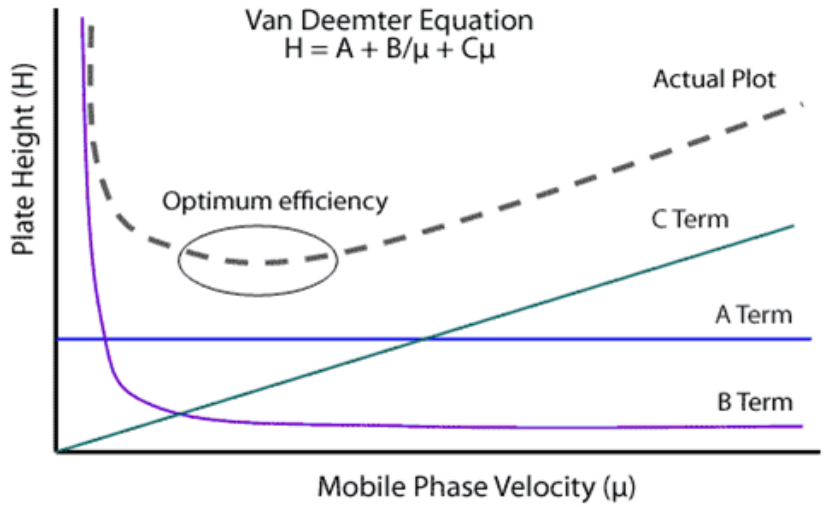


Figure 12 Illustration: The Van Deemter Equation.
Describes the Relationship between column flow rate and peak efficiency.
Source:
Lake, R. (2020). *How Do Small Particle Size Columns Increase Sample Throughput?* Retrieved from RESTEK: https://www.restek.com/Technical-Resources/Technical-Library/Pharmaceutical/pharm_A016

5.2 Mass Spectrometry (MS)

From the GC column and through a transfer line, the vaporized compounds (Tamoxifen and Propranolol) individually reach an Electron Impact (EI) ionization source within the MS, an instrument used for accurately measuring the molecular mass of a specimen sample. It generates a "mass spectrum" by ionizing a sample and measuring the mass-to-charge ratio of its ions, then recording their abundance. Electron Impact ionization entails an electric current (70V) heating a filament inside an ionization chamber that emits an electron beam that causes neutral molecules to emit one of their electrons from their highest occupied orbital when they pass through the electron beam. This leads to the formation of radical cations. This technique is a hard ionization technique, meaning the energy that causes the analyte molecules to remove their outer shell electrons is very high. Only very rarely will the positive molecular ions stay whole and not be further broken down into charged and neutral fragments. Produced ion fragments are electrically pushed out of the ion source by the positive voltage on the repeller electrode. These fragments are important because they help identify analytes; the mass-to-charge-ratio of the parent fragment tells the molecular weight of the substance, and the intensity of both the parent and fragments ions is what gives each analyte a unique molecular identification.

5.3 Quadrupole Mass Analyzer

This is the type of mass analyzer used in the mass spectrometer. A quadrupole mass analyzer consists of four cylindrical metal rods. Two of the metal rods are placed across from one another and are positively charged while the other two adjacent

metal rods are negatively charged. These charged rods inevitably work in tandem to affect ion movement. (See Figure 13 for a basic quadrupole mass analyzer schematic.)

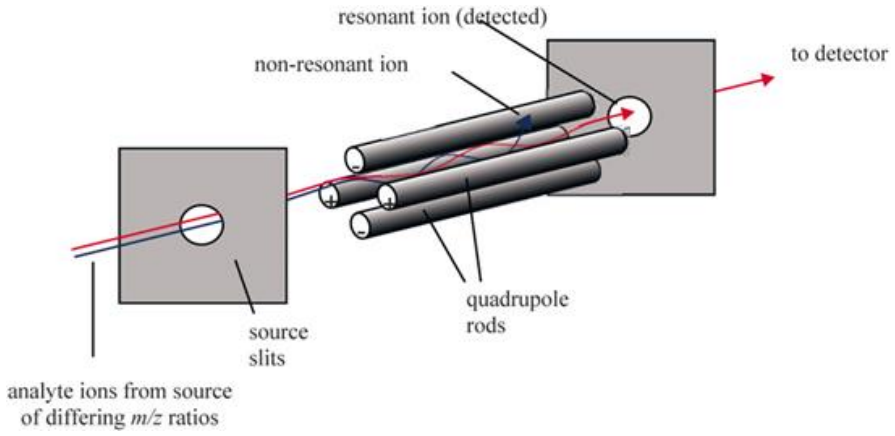


Figure 13 Quadrupole Mass Analyzer Schematic.

Source:

Homer, N. Z. M. (2011, June 1). *Clinical Mass Spectrometry: A Short Review*. Retrieved from Spectroscopy Europe Asia: https://www.spectroscopyeurope.com/article/clinical-mass-spectrometry-short-review

A quadrupole allows for all mass to charge ratios to be scanned and results to be viewed by the analyst. It accomplishes this by alternating voltages on the charged rods so that only one mass charge makes it through the quadrupole. At its core, a quadrupole works due to the concept that like charges repel and opposite charges attract. Both the ion charge and size are critical in how an ion maneuvers through the quadrupole. The positive poles have a positive direct current that is varied as the mass analyzer scans each mass to charge ratio. Also attached to the poles is an alternating current that is able to switch from a negative to positive value. This alternating current is essential in making the quadrupole an effective mass analyzer. If only the positive DC voltage was present, all positive ions would be repelled by the rods and

make it through the quadrupole. As the AC voltage becomes negative and becomes a bigger value than the positive direct current, the rods momentarily become negative, thus attracting the positively charged ions. Smaller ions quickly move towards the rods and crash (being neutralized) while ions with larger masses do not move as rapidly. By the time the larger ions make it close to the rods, the AC current has been switched back to being positive and the ions can continue moving through the quadrupole. Essentially, the positive rods are the high mass filter. The two negative rods have a negative DC voltage attached. Again, there is also an alternating current that can go from a positive voltage to a negative voltage. The two negative rods become the low mass filter and work very similarly to the positive poles. As the alternating current on the negative rods becomes positive and a larger value than the negative direct current, the rods momentarily become positive, thus repelling the positively charged ions. Smaller ions make it through the quadrupole while larger ions keep their general trajectory towards the rods. They crash and become neutralized. Only the ions that have a stable trajectory (do not collide with the rods) and a selected mass-to-charge ratio (or range) will reach the detector. The detector is an electron multiplier which is used to detect the filtered ions and to detect an ion with a mass to charge ratio of interest. This can be done by way of a discreet dynode electron multiplier or a continuous dynode electron multiplier. (See Figure 14 for a discreet dynode system schematic and see Figure 15 for a continuous dynode system schematic.)

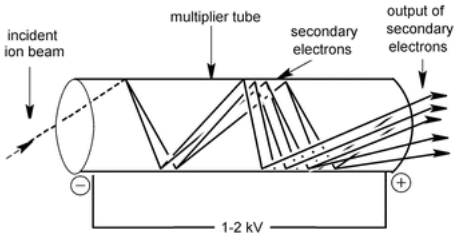


Figure 14 Discreet Dynode System.

Source:
Gross, J. H. (2017). Instrumentation. *In: Mass Spectrometry* (pp. 151-292). Springer. doi:https://doi.org/10.1007/978-3-319-54398-7_4

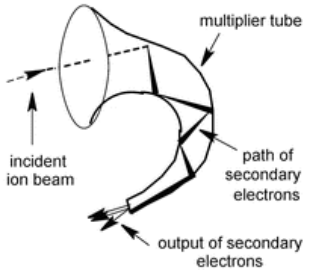


Figure 15 Continuous Dynode System.

Source:
Gross, J. H. (2017). Instrumentation. *In: Mass Spectrometry* (pp. 151-292). Springer. doi:https://doi.org/10.1007/978-3-319-54398-7_4

The discreet dynode system converts the ion(s) to a measurable signal by first making it an electron; it is converted to a current by way of impacting a cathode and causing liberation of electrons that multiply through the dynodes. As the electrons multiply and move toward the anode, the dynodes get progressively more positive. When they reach the anode, the electrons generate a current that is then converted to a voltage which can be measured. The measurement of ions is based on the generated current. For continuous multiplication – using the continuous dynode system – the ion(s) is converted to electrons with the use of a conversion dynode. Instead of

electrons held at discreet dynodes at positive values that attract negative electrons, the electrons are multiplied when they hit the sides of the horn-shaped funnel that has lead-doped glass and they go deeper into the funnel toward the more positive voltage (Gross, 2017).

5.4 Fourier Transform Infrared Spectroscopy (FT-IR)

This instrument uses infrared light to scan samples so they will absorb radiation. Bonds between atoms in the samples absorb this light energy at different frequencies, so their levels of absorbed radiation are completely unique. Infrared light is not the same as visible light; infrared light has a longer wavelength and a lower frequency than visible light. The absorbed radiation converts to vibrational energy within each sample, and the act of the bonds between atoms absorbing energy produces a range of signals (peaks): a spectrum of energy. Each sample's absorption is unique in its pattern and ultimately results in a recorded molecular fingerprint. A sample's range of signals is measured and graphed: the sample's absorbance or infrared radiation on a vertical axis with its measured frequency (recorded as "wavenumber") on a horizontal axis. The graphing of the absorption shows a molecule's (or functional group) absorption bands that serve to identify it. In summary, a sample absorbs energy from infrared light at different wavelengths dependent on that sample's unique molecular makeup. (See Figure 16 for a basic FT-IR diagram.)

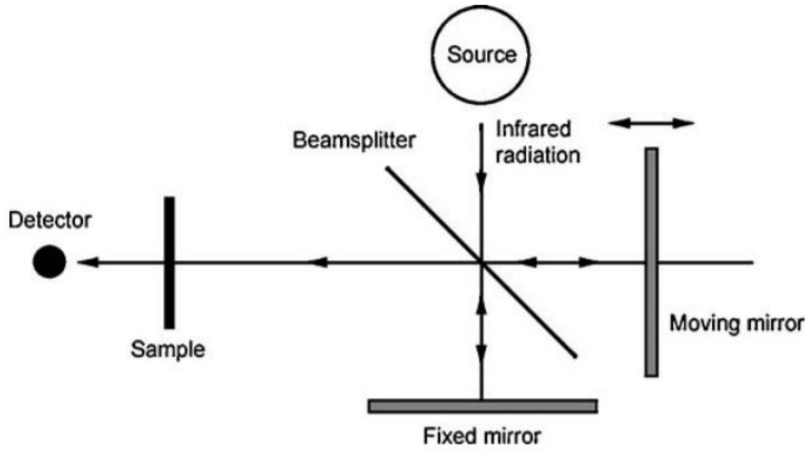


Figure 16 Basic FT-IR Diagram.

Source:

FTIR: Fourier-Transform Infrared Spectroscopy Principles and Applications. (2019, March 27). Retrieved from FindLight Blog: www.findlight.net/blog/2019/03/27/ftir-principles-applications/

Absorption is dependent on factors such as temperature, but bonds of each molecule (and molecules within function groups) behaves differently (e.g. stretching, bending, wagging, etc.) and therefore absorbs light only when the frequency of their movements match the frequency of the light beam. When no movement matches the light frequency, the light gets transmitted and detected. An interferometer device is used to determine a sample's identity by repeatedly changing the light beam's frequencies for detection: using a stationary mirror, a motor, a moving mirror and a beam splitter. The light first hits a beam splitter. Half the light is transmitted to a moveable mirror and half the light is reflected to a stationary mirror. Once the light hits both mirrors, it is reflected again, back to the beam splitter. Light coming from the stationary mirror can either be transmitted to the sample or it is reflected back to the light source. Light coming from the moving mirror can either get reflected to the

sample or transmitted back to the light source. An interference pattern happens when the beams of light are recombined (from both mirrors). This interference is seen as constructive when light waves are "in phase" and seen as deconstructive when light waves are "out of phase" by ½ λ. "In phase" refers to the light waves traveling the same distance before they are detected and "out of phase" refers to the light waves canceling each other out – which means very little energy gets to the detector. As the moving mirror changes or travels a certain distance, therefore, the light waves that make up the infrared radiation and go in or out of phase produce an interferogram (a plot of the detector response and the intensity of the absorbed radiation) (Experiment 7: IR Spectroscopy, 2017). The sample's signal (obtained from the detector) is measured and decoded by computer using a mathematical technique known as Fourier transformation that transforms a function of time to a function of frequency to reveal its spectrum, to be searched against reference libraries for identification.

5.5 Method Validation Parameters

Specificity

Specificity of an analytical method is the ability to identify an analyte of interest. This is an assurance that a "specific" analyte can be identified regardless of other compounds being present in a sample. It is the method's capability to discriminate between analytes. Specificity of a method is achieved by analyzing a sample containing the analyte of interest mixed with a structurally similar analyte(s) with specific identification of the target analyte, or by analyzing a sample containing structurally similar analytes but not the analyte of interest and not getting specific

identification of the target analyte. When achieved, it is shown by clear resolution of different peaks. This parameter is an assurance that potentially interfering substances or cross-reactions do not hinder the method's ability to identify only the specific target analyte. ("Selectivity" parameters are utilized for method validation when detecting multiple analytes.)

Linearity

Linearity of an analytical method is the parameter that demonstrates the method's quality. It is an important assessment of the method's ability to perform; it is achieved when the method's results (ranging from a measure of low concentration to high concentration) are directly proportional to the concentration of an analyte in a test sample (Reportable Range, Linearity & Calibration Verificatio, 2013). Determining linearity involves performing the developed method on at least five samples containing five different concentrations of an analyte of interest (e.g. Tamoxifen) and graphing all test results: the concentration of each sample as a known value on the x-axis vs. the peak area response of each tested sample as a dependent variable on the y-axis. From this graphing, evaluation is done by a statistical method known as "least squares regression" or "method of least squares." With the linear equation $y = mx+b$ (b is the y-intercept and m is the slope), a regression line is calculated, and a correlation coefficient "r" is determined. This "r" is a statistic, using a scatterplot of paired data to look for a trend in its overall distribution (Taylor, 2020). If paired data fall along a straight-line pattern, this shows a linear relationship. The value of "r" is always between -1 and +1; and it measures the strength and direction of the

relationship between plotted results. When "r" is close to +1, this shows a positive relationship, but when "r" is close to -1, this shows a negative relationship. The variance of the concentration causes a variation of the peak area (i.e. if x increases, then y increases). The coefficient of determination is "r squared" and shows the proportion of variance fluctuation of y that is predictable from x. It "denotes the strength of the linear association between x and y" (must be between 0 and 1) (Correlation Coefficient, 2020). Using the software program Excel (which provides a trend line after graphing each result), the r-squared value is calculated with 0.99 representing a straight line, a very strong linear relationship. In addition to the correlation coefficient, standard error of estimate "$S_{y/x}$" is another important parameter that indicates the extent of variation of an observation made around the computed regression line. The smaller the value of a standard error of estimate the closer are the dots to the regression line and better is the estimate based on the equation of the line (Miller & Miller). The "$S_{y/x}$" can be used to calculate both the standard deviation of intercept "S_a" and the standard deviation of slope "S_b." The smaller the value of the $S_{y/x}$, the smaller the values of both S_a and S_b (Miller & Miller).

Precision

Precision of an analytical method is the extent to which it can be repeated with reproducible results. Multiple test runs need to be made utilizing test samples made from the same batch but with varied concentrations of the analyte of interest (i.e. nine samples in total with three different concentration levels and replicated runs three

times each). Each of the three trials are compared, and the closer they are in agreement, the better the precision.

Limit of Detection (LOD)

Limit of detection is the parameter of an analytical method that establishes its lowest concentration of a target analyte that can be reliably detected (not quantitated). To estimate the method's detection limit, the signal-to-noise ratio should be 3:1 or 2:1, determined by comparing measured signals from samples with known low concentrations of analyte with those of blank samples. Another method may also be used and was applied to this project; the LOD is calculated based on a created calibration curve. From the calibration curve, the slope as well as the standard error of estimate is determined. The LOD is calculated using Equation 4 below:

$$LOD = \frac{3.3s}{m}$$

Equation 4 Limit of Detection Equation.

Where:
s = Standard Deviation
m = Slope of the Calibration Curve

Limit of Quantitation (LOQ)

Limit of quantitation is the parameter of an analytical method that establishes its lowest concentration of a target analyte that is not only detectable, but it can be quantitated or measured with accuracy. A target analyte must be sufficient enough in concentration to produce a signal distinguishable from "noise." To estimate the

method's quantitation limit, the signal-to-noise ratio should be 10:1. For this project, the LOQ was calculated by multiplying the LOD by 3.

5.6 Solvent Partitioning

This technique depends on solubility and is sometimes just called "extraction." Literally, this technique entails using one solvent to draw out a target analyte from another solvent (the two are immiscible) by creating a choice for the analyte based on solubility. The target analyte partitions into the solvent it is attracted to the most. The degree of partitioning is determined by a distribution coefficient. The larger the distribution coefficient, the more likely the analyte is to be found in the organic layer. This technique to extract a drug analyte allows for high level of recovery and purification.

6. METHODS

Project One: Detection and Determination of Tamoxifen in Oral Fluid

With little to no research previously done regarding detection of Tamoxifen or similar SERMs in oral fluid (with some exception of detecting Tamoxifen utilizing liquid chromatography instrumentation), a detection method for use of GC instrumentation was developed. The performance of an scouting gradient utilizing Tamoxifen and the internal standard Propranolol dissolved in methanol was done to reveal which oven temperatures would improve analyte separation and peak shapes (Optimising GC Temperature Programming, n.d.). This technique began with an oven temperature of 30°C then increased to 325°C at a rate of 10°C/min. with a hold time of ten minutes. It was revealed that Propranolol analytes eluted from the column at approximately 250°C (before the Tamoxifen), and the Tamoxifen analytes eluted at approximately 270°C. This scouting run proved to be very long, almost an hour, but it did provide a basic method to be optimized. Based on thorough research, the collection method of oral fluid requires optimization in that different collection methods and utilized devices affect the pH of oral fluid samples which, in turn, affects drug concentration levels and ability to detect Tamoxifen in a low volume sample. GC-MS method optimization was performed to reduce run time, improve peak shapes, gain the highest sensitivity level as possible, and to achieve the lowest limit of detection as possible. Asymmetry factors and tailing factors as well as the number of theoretical plates were calculated in order to assess peak shape and separation efficiency.

6.1 Oral Fluid Collection Optimization

Different collection methods of oral fluid have different effects on drug concentrations. No literature can be found regarding research done comparing Tamoxifen drug concentration levels in oral fluid based on different collection methods, but a limited amount of research has been done for other drugs. A consistent finding is that as the flow rate of saliva increases (e.g. by stimulated OF collection), the pH level of saliva also increases because of an influx of bicarbonate ions (O'Neal, Crouch, Rollins, & Fatah, 2000, pp. 536-537). This increase in bicarbonate ions in the saliva actually neutralizes the saliva just as it does when a person eats certain foods, and the saliva helps to eliminate sugar that feeds bacteria growth. For oral fluid collection, the neutralization of saliva decreases or prohibits ionization of basic drugs such as Tamoxifen (pK_a of approx. 8.85) and therefore disrupts the possibility of "ion trapping" to occur (Sigma Product Information, p. 1). Ion-trapping is when a basic drug does cross membranes from blood to saliva, and the free uncharged basic drug becomes ionized (i.e. protonated) because the saliva has a lower pH and is acidic; this ionization then traps the drug and keeps it from crossing back into the blood (Allen, 2011, p. 533). The result is increased drug concentration in saliva. What is unique about Tamoxifen aside from it being extremely protein bound, is that it is mostly already ionized in the blood and not free to cross membranes into saliva. This is supported by the application of the Henderson-Hasselbalch equation; Tamoxifen is mostly protonated in blood (Chen, Schindler, & Simon, 1999, p. 18366). A lower pH of

saliva is necessary for any uncharged Tamoxifen to cross membranes and to then be ionized and trapped if any amount of Tamoxifen is to be detectable in saliva.

Understanding the application of the Henderson-Hasselbalch equation allows for determining Tamoxifen's potential for crossing semipermeable membranes into saliva. Since it is already ionized in blood, it cannot easily cross. Only unionized forms of a drug are absorbable. The crossing of any available unionized Tamoxifen is dependent on its pK_a (its degree of ionization) and the pH of saliva. If the saliva is acidic – not neutralized from stimulating saliva collection – available unionized Tamoxifen will likely endure absorption, cross through the membranes and concentrate in the saliva. The pH of saliva needs to be as low as naturally possible to get Tamoxifen to ionize into the saliva for greatest concentration. Resting, unstimulated saliva typically has a pH range between 5.7 and 6.2 while the pH of stimulated saliva can reach 8 (Kubala, et al., 2018, p. 2). Equation 5, the Henderson-Hasselbalch equation, estimates the degree of ionization of a weak basic drug:

$$pH = pKa + \log\{[unionized\ form]/[ionized\ form]\}.$$

Equation 5 Henderson-Hasselbalch Equation (Weak Basic Drug).

Oral fluid collection for drug testing is typically done in one of several ways: the draining method (freely dripping or drooling saliva from the mouth into a container), expectoration (spitting), or a mechanically stimulated saliva method followed by spitting, suctioning, absorbing or swabbing (e.g. after eating a lemon candy or chewing gum) (Crouch, Day, Baudys, & Fatah, 2005, p. 11). Rarely acknowledged in literature is

that subtle variables in how oral fluid is collected can have remarkable effect on the levels of drug concentration in oral fluid samples (Crouch D. , 2005, p. 167). Stimulated collection can significantly reduce drug concentrations by interrupting ion-trapping (thereby lowering Tamoxifen drug concentration).

Researched methods of collecting oral fluid and the effects each method has on pH consistently show that any stimulation (chewing gum, candy, spitting) causes the collected saliva to be more neutral and that collection performed with swabs additionally creates problems with some analytes remaining on the absorption material rather than being available for analysis, leading to false-negative results. Such collection devices also present issues because they contain a buffer. This must be compensated for and effects sample volume and drug concentration (i.e. buffers improve measurement but dilute concentrations) (Bosker & Huestis, 2009, p. 4). Though subtle, the act of trying to produce saliva is stimulation in and of itself (increased flow rate), and drug concentration is lowered:

> OF drug concentration also is dependent on OF excretion stimulation, which may occur even to a small extent by placement of a collector in the mouth. Thus, it is impossible to prevent an increase in OF excretion during collection, except by employing passive drool. Older methods of increasing OF excretion included chewing on paraffin, and newer approaches embed citric acid or other chemicals onto collection devices. Early studies, including those in our laboratory, used devices that stimulated OF excretion; however, we learned that stimulation ultimately lowered rather than increased drug concentrations and complicated interpretation of results (Bosker & Huestis, 2009, p. 4).

For optimization, this project would require that human oral fluid samples be collected using the (passive drool) draining method. The person providing a sample would be asked to rinse their mouth with provided water, wait 15 minutes under

observation, and then gently tilt their head forward to allow saliva to drip into a graduated funnel-like tube until 2 mL - 12 mL was collected. This method is not preferred by people who are providing oral fluid samples, but people almost unanimously agree that it is still more comfortable to provide oral fluid (e.g. with a special technique device) than to have another person closely watch a collection of urine (Casolin, 2016, p. 483). Tamoxifen is too highly protein bound (and already largely ionized in blood) to risk collection of oral fluid by any other method, risking inability to detect ample drug concentration of Tamoxifen.

6.2 GC/MS Method Optimization

Column Selection

The column utilized was an HP-5MS UI column manufactured by Agilent Technologies, Inc. This column is relatively non-polar, and its stationary phase is a polysiloxane polymer with 5% Phenyl and 95% Methyl that can undergo dispersive interactions because of the methyl and pi-pi interactions because of the phenyl. Different analytes undergo differing amounts of these types of interactions dependent upon their chemical or physical properties. The dispersive interactions caused by an induced dipole are weak and will not last for a long time; but the less volatile an analyte is (e.g. Tamoxifen has low volatility per its size), the longer the retention time. Phenyl ring compounds (e.g. Tamoxifen has 3 benzene rings) would have increased retention time on this column because of the pi-pi interactions. Equally important as to which interactions will occur are the relative amounts of each. This stationary phase will undergo mostly dispersive interactions because it is 95% methyl. This

column is described by Agilent as ideal for GC-MS; that being so non-polar, it is rugged and can withstand high temperatures. However, with Tamoxifen being a very polar analyte, a more polar column would have been ideal. This column was chosen because of its availability and wide use in most labs. With this column having a 0.25mm inner diameter, resolution is good but separation times can be slow. Resolution is a calculation of how well peaks can be differentiated in a chromatographic separation, considering retention times of the analytes and the widths of the peaks. When different peaks that are narrow, tall and symmetrical can be seen on a chromatograph, resolution is said to be good. When peaks on a chromatograph are broad, overlapping, and/or asymmetrical, resolution is said to be poor. The smaller the inner diameter of the column, the better the resolution; this is because smaller inner diameters – compared to larger inner diameters – generate more theoretical plates and therefore have better separation. With a smaller inner diameter, the flow rate has to decrease and that is why separation times can be slow. This column's film thickness of 0.25µm is relatively thin and recommended for use with high boiling point analytes like Tamoxifen, and its film thickness provides good resolution, sharper peaks and decreased retention. With the thin film, there is rapid "equilibration" of analytes in the mobile and stationary phases. Finally, this column is of intermediate length: 30 meters. This is good in that it provides good resolution although a shorter column would allow shorter run times with less inlet pressure, and a longer column would improve resolution (meaning, a higher number of theoretical plates).

Injection Method

Both split and splitless injection techniques required consideration. With Tamoxifen as the analyte of interest and Tamoxifen having a very low concentration in oral fluid, effort had to be made to make sure most of the analytes make it to the column. With split injection, a portion of the analyte flows along with the carrier gas into the split line, avoiding flow into the column. With Tamoxifen as a targeted analyte in a low volume sample, analyte concentration levels would ultimately be too low for detection if any amount of analyte was not directed into the column. The term "split ratio" can be defined as the split vent flow rate over the column flow rate; it is not a proportion of injected sample being vented away from the column vs an amount of sample reaching the column. The amount of sample that does reach the column is calculated by dividing the column flow rate by the sum of the column flow rate and split vent flow rate. Split ratios typically range from <5:1 to >400:1 depending on column application (Injection Techniques for Capillary GC, 2014). Split injection works very well with high concentration samples; it is a way to do internal dilution that avoids overwhelming the detector. Also, the choice of split injection makes sense when a sample contains a large drug concentration and benefits from splitting as a lower concentration gives sharper peaks. With splitless injection, the split valve is initially closed and a sample with low analyte concentration (e.g. Tamoxifen in oral fluid) is almost fully directed into the column. The valve only opens and closes very quickly to "flush" the liner of any remaining vaporized solvent. This sometimes happens as soon as ten seconds after injection, but this period of time can be custom

programmed, a technique known as “splitless hold time.” By default, the use of splitless injection was found to be necessary in order to capture enough drug concentration to successfully detect Tamoxifen. Choosing to use splitless injection because of low Tamoxifen concentration allowed for exploring the effects of different hold times and enabled more analyte to reach the column before the valve would open.

Splitless Time

With splitless injection, the split valve is closed at first to make sure all of the sample reaches the column. The valve must open to clear out the injection port liner of remaining solvent vapors, but the timing of when it opens affects the trapping of sample vapors at the head of the GC column. The sample vapors need to be as fully transferred as possible from the inlet before the split valve opens, assured by using a low initial oven temperature. The amount of time that passes before opening the split valve is very important. Once the valve is opened, if there are sample vapors that have not made it to the head of the column, these vapors will immediately be cleared out of the injection port liner through the split vent that had been closed off. Typically, splitless hold time is between 60 and 90 seconds, but the time depends on the sample composition, column length and ID, carrier gas flow rate, injection port liner configuration, sample solvent, and sample size (Operating Hints for Using Split / Splitless Injectors, 2002, p. 7). The optimal splitless hold time is determined by experimenting with different time lengths (shorter to longer), but holding too long can

produce tailing peaks and broad peaks (Operating Hints for Using Split / Splitless Injectors, 2002, p. 7).

Sample Volume and Inlet Temperature

The problem of "backflash" can occur in the GC injection port if the sample's solvent vapors expand to be larger than the injection port liner volume as seen in Figure 17. The expanded vapors overfill the injector liner and injection port, and there is sample loss along with chromatographic problems (e.g. ghost peaks, split peaks, tailing peaks) (Backflash and Its Impact on GC Analyses, n.d., p. 2). The overfill causes vapors to contaminate the gas lines that come into and out of the inlet, too.

Determining the inlet temperature for the splitless injection was also extremely important for method optimization. Temperature must be high enough to quickly and completely vaporize the sample's analytes but not too high because this causes sample degradation; the compounds break down. With the sample being in the injection port longer (splitless injection vs. split injection time), the needed temperature for vaporization must be adjusted. If the temperature is set too low, then sensitivity will be low. The compounds either fail to vaporize or they take an extra-long time to vaporize and enter the column. This temperature problem causes tailing peaks (Backflash and Its Impact on GC Analyses, n.d., p. 2).

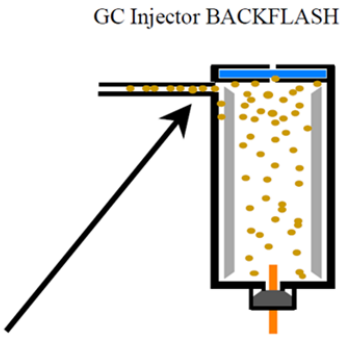


Figure 17 GC Injector Backflash.
Sample expands and overfills injector liner and injection port.
Source:
Backflash and Its Impact on GC Analyses. (n.d.). Retrieved fromAgilent: www.agilent.com/cs/library/support/documents/a16183.pdf.

Temperature Programming

Temperature programming plays an incredibly important role in optimizing a GC-MS method. Temperature programming can affect critical aspects such as band width, resolution, retention time of compounds, and total sample run time. For this project, temperature programming introduced a battle between achieving the lowest limit of detection and the highest sensitivity of the method. Results of this project's scouting gradient served as the basis for developing and following a generic temperature program for optimization. Table 1 lists the variables utilized for the scouting gradient. It is important to make clear that the results of the scouting gradient prompted need to make changes before developing a generic temperature program to use as a starting point for GC optimization. The scouting gradient run time was extraordinarily long and undesirable; therefore, two ramps were added and hold time was lessened.

Table 1 Generic Temperature Program.

	Rate (℃/ min)	Value (℃)	Hold time (Minutes)
Initial		**30**	**3**
Ramp 1	**50**	**150**	**3**
Ramp 2	**5**	**250**	**5**
Ramp 3	**15**	**300**	**1**

General knowledge about temperature programming was also utilized. This project's compounds have very high boiling points, so oven temperature initially set low could not be kept low during a sample run. The run would need to involve increasing oven temperature plus closely controlling the rates and hold times before getting good peak resolution and identifying the peaks. Resolution of the late eluting peaks can be improved by increasing temperature while rate change and hold times can also be adjusted to obtain good separation. To work toward achieving the best separation with shortest run times, the different parameters of each factor (rate, temperature and hold times) were further investigated and analyzed. If temperature is increased to improve separation and decrease retention time, the ramp rate may need to be faster or the hold time may need to be shorter.

Calibration and Linearity

Linearity of the proposed instrumental method of analysis is essential for its validity. It establishes relationships between peaks for known amounts of analyte in standards by way of "calibration curves" that can then be used for estimating amounts of that same analyte in samples of unknown concentration. An instrument response

needs to be consistently proportional to the concentration of the analyte, meaning the detection falls within a calibration range for that analyte. Protocol for instrument calibration involves creating solutions of known concentrations using standards and observing the response of the instrument to each analyte amount. Once a GC method is optimized, an ideal calibration technique needs to be chosen: the external standard method of calibration, the method of standard addition, or the internal standard method of calibration.

The external standard method is a very widely used calibration method by analytical scientists. It consists of comparing instrument responses to a sample with an unknown analyte to documented instrument responses to known analytes called "standards." The compared responses are peak heights (or peak areas), indicating analyte concentration. This method involves creating a series of standards (samples) that contain known concentrations of a known analyte, with an increased concentration of the analyte in each and plotting the instrument's responses to produce a "calibration curve." The plotted calibration curve can then be used to figure the concentration of the unknown analyte.

For this project, the internal standard method was chosen as an ideal calibration technique which is commonly used for GC in analytical chemistry to improve the precision of quantitative analysis. This method involves addition of an analyte that is different from an analyte of interest to a sample; both the IS (internal standard Propranolol) and the analyte of interest (Tamoxifen) are in the same analyzed sample(s) so that comparison of measurements can be made at the same

time. Instrument signals for both analytes are used to calculate a ratio (based on peak area) which is proportional to their concentration in the sample (Venton, 2020). Because the IS is in the same sample(s) as the analyte of interest, any variations or irregularities with the instrument ultimately affect the measurement of both of them in the same way; the ratio of their signals does not change. Also, when there are differences in sample volume (i.e. from multi-step sample preparation), having an IS helps correct any potential errors because the ratio of IS to the analyte of interest does not change. Reproducibility is not affected. It is very important to select an IS analyte that gives a signal that is almost identical to the signal of the analyte of interest (similar properties such as volatility, molecular weight, functional groups, polarity, etc.) but different enough the signals can be distinguishable with no overlap and, most definitely, not already be present in the sample or reactive with utilized solvent. If using MS, the use of a deuterated analog of the target analyte is common; if using a non-MS detector, the target analyte may coelute with the deuterated analog and be problematic for quantitation (Kelly, 2020). Because the deuterated analog is usually costly, analysts sometimes keep records of internal standards they have used with good outcome. The signals do help an analyst see if peaks are eluting within normal variation since the IS will elute near the analyte of interest. The similarity between the IS and the analyte of interest prevents other factors from causing the instrument response to be too dissimilar.

6.3 Sample Preparation Optimization

Solvent Partitioning

Solvent partitioning was necessary to first draw Tamoxifen out of fortified artificial oral fluid samples. To optimize the solvent partitioning, three trials using buffers made with five different pH levels were performed (to determine the optimal pH for Tamoxifen to transfer to a solvent), and three trials using a buffer with a constant pH and six different solvents with different polarity were performed (to determine the optimal solvent for recovery of Tamoxifen). Sample preparation began with this separation technique because saliva is 99% water and not miscible with a solvent needed for dissolving Tamoxifen. Tamoxifen is practically insoluble in a solvent like water; but Tamoxifen is readily soluble in an organic solvent. Solvent partitioning requires two solvents that are not miscible because the compound to be extracted will need to naturally transfer to the solvent it is most readily soluble in.

Tamoxifen is a weak base. It is able to act as a Bronsted-Lowry base when the lone pair of electrons on the Nitrogen accepts a proton. As shown in Figure 18, Tamoxifen accepts a proton, resulting in ionized Tamoxifen and hydroxide ions. The un-ionized form of Tamoxifen is more attracted to an organic solvent while the ionized molecules prefer to stay in the aqueous layer (Robinson & Cha, 1985, p. 18).

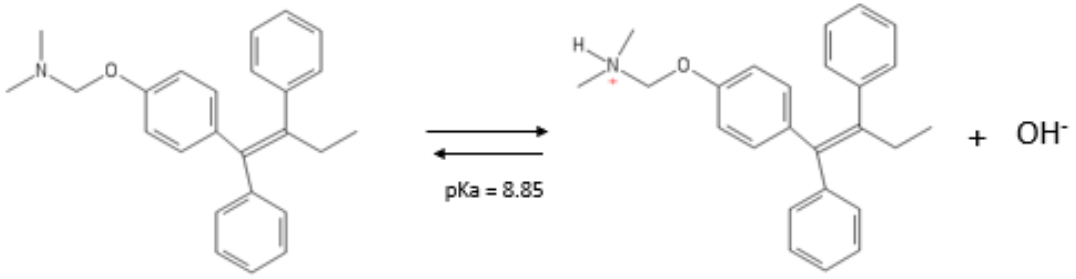


Figure 18 Ionization of Tamoxifen.

To look at this technique numerically, the distribution coefficient can be calculated. The distribution coefficient is the ratio of the analyte concentration in the extract to the analyte concentration remaining in the aqueous layer when extraction is complete. For this project, this means the ratio of Tamoxifen's concentration in both the oral fluid and the organic solvent is equal to the ratio of the Tamoxifen's solubility within each. The equation represents the distribution coefficient (also known as the distribution ratio and the partition coefficient) being equal to the molar concentration of the analyte of interest in the organic layer divided by the same analyte's molar concentration in the aqueous layer. To examine how different organic solvents and different pH buffers affected Tamoxifen's distribution coefficient, a calibration curve was created (see Figure 19).

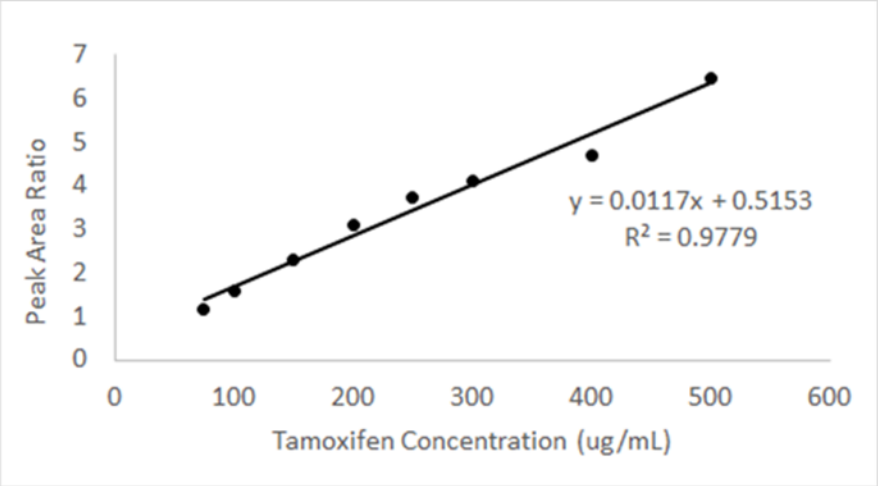


Figure 19 Calibration Curve for Extraction Optimization.

After each extraction procedure, the ratio of Tamoxifen's concentration in the organic layer (determined by using the analyte: internal standard peak area ratios obtained from the calibration curve) was divided by the concentration of Tamoxifen remaining in the oral fluid. For each extraction completed, the Tamoxifen concentration of the fortified OF sample created was 500 ug/mL. The amount of Tamoxifen remaining in the aqueous layer was determined by subtracting the organic layer's Tamoxifen concentration from 500 ug/mL.

Three trials using buffers made with five different pH levels were performed. 50 mL of buffer solutions were first prepared in beakers at pH levels 4, 5, 6, 7 and 8. Utilizing the Henderson-Hasselbalch equation, the amount of the acid and the amount of the conjugate base needed was calculated for each of the buffers in order to be able to use pH levels of 4-8. See Table 2 for buffer compositions.

Table 2 Buffer Composition.

Buffer Composition		
pH	**NaOAc (g)**	**Glacial Acetic Acid (uL)**
4	.0607	237
5	.2603	101
	K_2HP0_4 (g)	KH_2PO_4 (g)
6	.0581	.6409
7	.332	.406
8	.474	.0949

After making buffers, available pH test strips were utilized to make certain of the pH levels being tested. See Figure 20 which illustrates accuracy in making the buffers range as desired: pH 4 - pH 8.

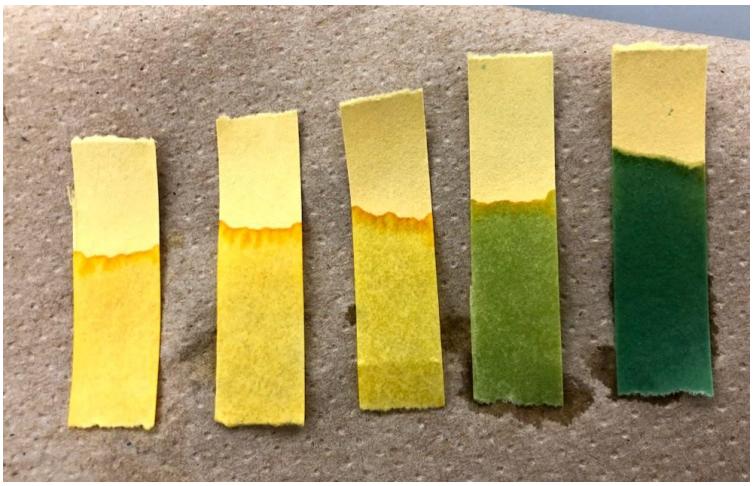


Figure 20 pH Test Strips.

One mL of artificial oral fluid ("saliva") was placed into a clean test tube, and the saliva was fortified (or "spiked") with the desired concentration of Tamoxifen – 500 µg. Added to the test tube was 1 mL of buffer and 2 mL of solvent. After each

time the buffer was added, the sample was vortexed, and each time solvent was added, the sample was again vortexed. After both the buffer and solvent had been added and vortexed for all samples, each sample was set aside to allow the solvents to again separate. During this time, the Tamoxifen naturally transferred (per its distribution coefficient) to the organic solvent layer. As seen in Figure 21, the Tamoxifen transferred to the top organic solvent layer and could be pipetted.

Figure 21 Solvent Partitioning.
Extraction of Tamoxifen from fortified oral fluid sample.

The solvent was then evaporated off with a hot water bath. The Tamoxifen on the sides of all test tubes was reconstituted with 800 µL of methanol per test tube and vortexed again. The 800 µL of extract was collected and placed in a GC vial. Next, 200 µg of the internal standard was added to the vial to make a total volume of 1 mL. GC/MS analysis was completed to determine which pH allowed for the most Tamoxifen recovery. For solvent selection, a total of 6 different solvents (benzene, cyclohexane, hexane, methyl-ethyl ketone, ethyl acetate and chloroform) at 2 mL each, ranging in polarity from 0.1 to 4.9 (ranked on the polarity index) were ran three times. This provided evidence of which solvent was a best choice for optimizing the

developed method. Table 3 below illustrates the procedures performed to examine buffer pH and solvent efficiency.

Table 3 Procedures for Buffer and Solvent Efficiency.

Procedure (3 Trials each)	Buffer pH	Organic Solvent
1	4	Ethyl Acetate
2	5	
3	6	
4	7	
5	8	
6	No Buffer Added	Benzene
7		Cyclohexane
8		Hexane
9		Methyl Ethyl Ketone
10		Ethyl Acetate
11		Chloroform

6.4 Statistical Analysis

t-Test

A t-Test was done in order to compare extractions performed on fortified artificial oral fluid samples and extractions performed on samples that did not contain OF. At each concentration level, a t-Value was calculated and compared to the value on a t-Table. If a calculated value is greater than the t-Table value, the null hypothesis can be rejected.

The purpose of doing a t-Test was to evaluate whether or not there was a statistically different instrument response when OF was present in the extraction sample and when it was not. This is a step beyond simply calculating the means of the samples at the different concentrations (three trials done at high, medium and low concentration) because it can account for varied distribution between the samples. Application of the t-Test is illustrated below in Equation 6 and Equation 7. It follows

three steps: defining the null hypothesis (an assumption there is no difference between instrument response when OF is used and not used); calculating the $t\text{-}_{calc}$ value from the data obtained; then comparing the $t\text{-}_{calc}$ value to the $t\text{-}_{table}$ value (Stone, 2019).

1. Null Hypothesis

   H_o: $\mu_A = \mu_B$ (the mean of OF samples = mean of non-OF samples)

2. Calculate t_{calc} value:

$$S_{pooled} = \sqrt{\frac{(N_1 - 1){S_1}^2 + (N_2 - 1){S_2}^2}{N_1 + N_2 - 2}}$$

Equation 6 S_{pooled} Equation.

$$t_{calc} = \frac{\overline{X_1} - \overline{X_2}}{S_{pooled}\sqrt{\frac{N_1 + N_2}{N_1 N_2}}}$$

Equation 7 t_{calc} Equation.

3. Compare the $t\text{-}_{calc}$ value to the $t\text{-}_{table}$ value.

ANOVA

Because optimization for this first project's sample preparation included a variety of solvents and buffers with different pH levels, an ANOVA (Analysis of Variance) test was done for each variable (One-Way ANOVA, 2020). This determined whether or not there were statistically significant differences of the percent recoveries between the solvents and the pH levels. This test is done when there are three or more groups to compare, and it tests the null hypothesis. For this optimization step,

six solvents were varied while the Tamoxifen concentration, volume of OF, volume of solvent, and the number of extractions remained constant. Next, buffers were varied using 5 different created pH levels while the Tamoxifen concentration, volume of OF, volume of solvent used, buffer volume and the number of extractions remained constant.

Application of the one-way ANOVA test is illustrated below. See Table 4 for utilized equations. The "one way" test for this project was applicable because one variable was tested at a time for multiple samples. It follows several steps (One Way ANOVA by Hand, 2020):

1. Null Hypothesis
   H_o: $\mu_1 = \mu_2 = \mu_3 = \mu_4 = \ldots = \mu_{\#\text{ of groups}}$
2. Find Degrees of Freedom
   *First, between groups (# of groups minus 1) *Second, within groups (total # of observations minus # of groups)
3. Obtain Fcv (critical value) from F-Table
4. Calculate Sum of Squares
   *the total sum of squares, the sum of squares within groups, and the sum of squares between groups
5. Calculate the Variances
   *between groups and within groups
6. Calculate the F Statistic
7. Compare the F Statistic to the Fcv (Utilize F Table)
8. If the F Statistic is greater than the Fcv, the Null Hypothesis is rejected (if not, there is no statistical difference)

Table 4 One Way ANOVA Formulas.
Source:
How to Perform Analysis of Variance (ANOVA) - Step By Step Procedure. (2019, March 29). Retrieved from The Genius Blog: kindsonthegenius.com/blog/2018/03/how-to-perform-analysis-of-variance-anova-step-by-step-procedure.html

ANOVA				
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares (MS)	F
Within	$SS_w = \sum_{j=1}^{k}\sum_{j=1}^{l}(X - \overline{X_j})^2$	$df_w = k - 1$	$MS_w = \frac{SS_w}{df_w}$	$F = \frac{MS_b}{MS_w}$
Between	$SS_b = \sum_{j=1}^{k}(\overline{X_j} - \overline{X})^2$	$df_b = n - k$	$MS_b = \frac{SS_b}{df_b}$	
Total	$SS_t = \sum_{j=1}^{n}(\overline{X_j} - \overline{X})^2$	$df_t = n - 1$		

Tukey Honest Significant Difference Test

It is a common acceptance that a procedure like the Tukey Honest Significant Difference Test (HSD) is completed when an ANOVA is done and the null hypothesis is rejected; however, the Tukey test can be done whether or not an ANOVA resulted in a statistical difference. The Tukey HSD can provide additional information about the differences among means of each group.

The Tukey HSD compares all possible pairs of means and is based on the studentized range distribution (Tukey Test / Tukey Procedure / Honest Significant Difference, 2017). It is intended to show which specific group(s) means are different when comparing to the other groups tested. This project ran six solvents, testing the difference between all six pairs. Application of the Tukey HSD test is illustrated below utilizing the q_{Tukey} equation (see Equation 8):

$$q_{Tukey} = \frac{M_i - M_j}{\sqrt{\frac{MS_w}{n}}}$$

Equation 8 q_{Tukey} Equation.

Where: M_k-M_j is the difference between the pair of means, MS_w is the Mean Square Within, and n is the number of groups tested.

1. Perform the ANOVA test
2. Choose two means from the ANOVA results (means, Mean Square Within, number of group(s) and Degrees of Freedom Within
3. Calculate q_{Tukey} value
4. Find the value in the Q Table (Use Critical Value Table)
5. Compare the calculated q_{Tukey} with the score found in the Q Table. If the q_{Tukey} value is larger, the two means are significantly different.

Project Two: Discrimination Between Regioisomeric Aromatase Inhibitors

This project incorporated an initial study of separation and determination of three regioisomeric aromatase inhibitors (AIs): 5-alpha-Androstan-17-one, 5-alpha-Androst-16-en-3α-ol and 17-beta-Hydroxy-5-alpha-androst-2-ene. The term "Regioisomeric" refers to these substances as being positional isomers – meaning they have the same carbon skeleton and the same functional groups but differ from each other in their location in the carbon chain (Gunawardena, 2019). AIs are increasingly utilized as PEDs by athletes who want to increase their testosterone levels and/or minimize negative effects of anabolic steroid use, and regioisomeric AI's, particularly, may be attractive if they claim ability to help a person gain strength without the negative effects of steroids or likelihood of drug detection that comes with steroid use. This project compared the differentiation of three regioisomeric AIs utilizing both GC/MS and FT-IR to determine which analytical technique would serve as the best tool

for identification purposes. 5-alpha-Androstan-17-one (the ketone) is the indirect regioisomer of the two androstenols (i.e. it has the same molecular formula, same nominal and exact masses but different arrangement of the atoms in the molecular structure). 17-beta-Hydroxy-5-alpha-androst-2-ene and 5-alpha-Androst-16-en-3α-ol (the two alcohols) are directly regioisomeric to each other. This means that both have the same molecular formula, same nominal and exact masses but different position of the substituents. Chemical structures of each regioisomeric AI can be seen in Figure 22.

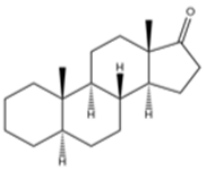
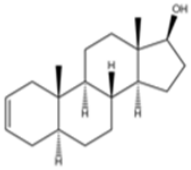
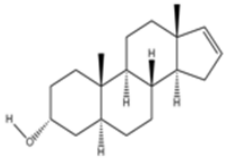
Regioisomeric Aromatase Inhibitors		
5-alpha-Androstan-17-one	**17-beta-Hydroxy-5-alpha-androst-2-ene**	**5-alpha-Androst-16-en-3α-ol**
		
Nominal Mass: 274.3 g/mol **Exact Mass:** 274.3 g/mol	**Nominal Mass:** 274.3 g/mol **Exact Mass:** 274.3 g/mol	**Nominal Mass:** 274.3 g/mol **Exact Mass:** 274.3 g/mol

Figure 22 Regioisomeric Aromatase Inhibitors.

Each of the three AIs was run separately using GC/MS. Each was first dissolved in methanol, then ran on the GC using a scouting gradient (30℃ to 300℃ at 20℃ per minute). This was done to gain a general idea of how difficult separation of the three compounds would be and to determine when each compound would elute. Next, a generic temperature program was developed to use as a starting point for obtaining separation. To reduce run time, two ramps were chosen: 30℃ to 225℃ and 225℃ to

300℃, both at 20℃ per minute. All three compounds were combined and mixed in one solution of methanol to run through each ramp time. The Ramp 1 Value and Ramp 1 Rate were optimized to get the best resolution between compounds. To calculate resolution, Equation 9 below was utilized (About Resolution, 2020). Concurrently with GC method optimization, FT-IR spectra were obtained for each AI in order to analyze spectral differences.

$$\boldsymbol{R = 1.18(\frac{t_{R2} - t_{R1}}{w_{h_1} + w_{h_2}})}$$

Equation 9 Resolution Equation.

Where:
t_{R1} = retention time of first peak
t_{R2} = retention time of second peak
w_{h1} = peak width at half height, first peak (in units of time)
w_{h2}= peak width at half height, second peak (in units of time)

For further determination of method development strategy, additional factors regarding resolution were explored. This was to help reach the best separation. Calculating the Purnell Equation is a modification of the Resolution Equation that can help improve chromatographic resolution by combining three factors: retention, selectivity and efficiency. Equation 10 for the Purnell Equation can be seen below (Samanidou, 2015, p. 31).

$$\boldsymbol{R_s = (\frac{\sqrt{N}}{4})(\frac{a - 1}{a})(\frac{k_2}{k_2 + 1})}$$

Equation 10 Purnell Equation.

Where:
N = the number of theoretical plates

α = the selectivity
k_2 = the retention factor for peak 2

To calculate the retention factor, see the Equation 11 below:

$$\boldsymbol{k} = \frac{(\boldsymbol{t_R} - \boldsymbol{t_0})}{\boldsymbol{t_0}}$$

Equation 11 Retention Factor Equation.

Where:
t_R = analyte's retention time
t_0 = column dead time

To calculate selectivity (separation factor), see the Equation 12 below:

$$\boldsymbol{a} = \frac{(\boldsymbol{t_{R2}} - \boldsymbol{t_0})}{(\boldsymbol{t_{R1}} - \boldsymbol{t_0})}$$

Equation 12 Selectivity Equation.

Where:
t_{R2} = analyte 2 retention time
t_{R1} = analyte 1 retention time

7. RESULTS AND DISCUSSION: Project One

Detection and Determination of Tamoxifen in Oral Fluid

7.1 GC/MS Method Parameters

Mass Spectrometry

Figure 23 and Figure 24 illustrate the mass spectra of Tamoxifen and this project's internal standard Propranolol.

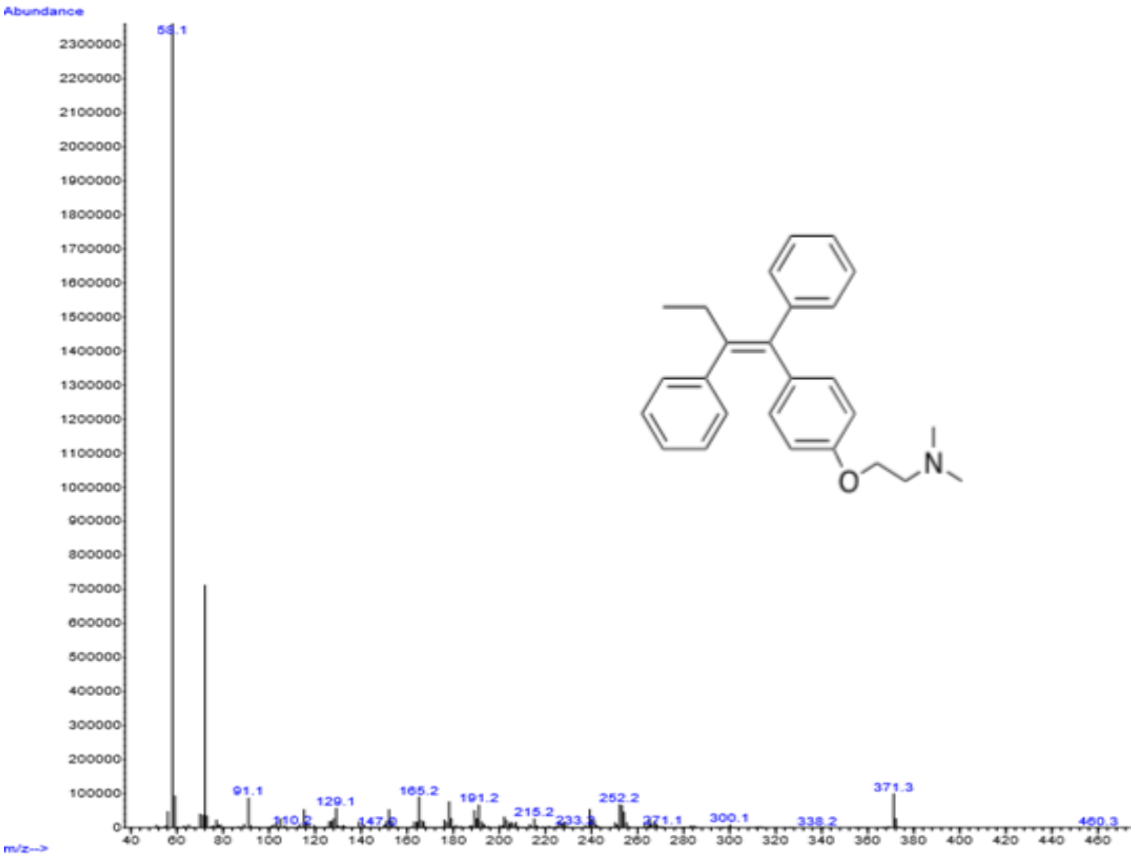


Figure 23 Mass Spectrum of Tamoxifen.

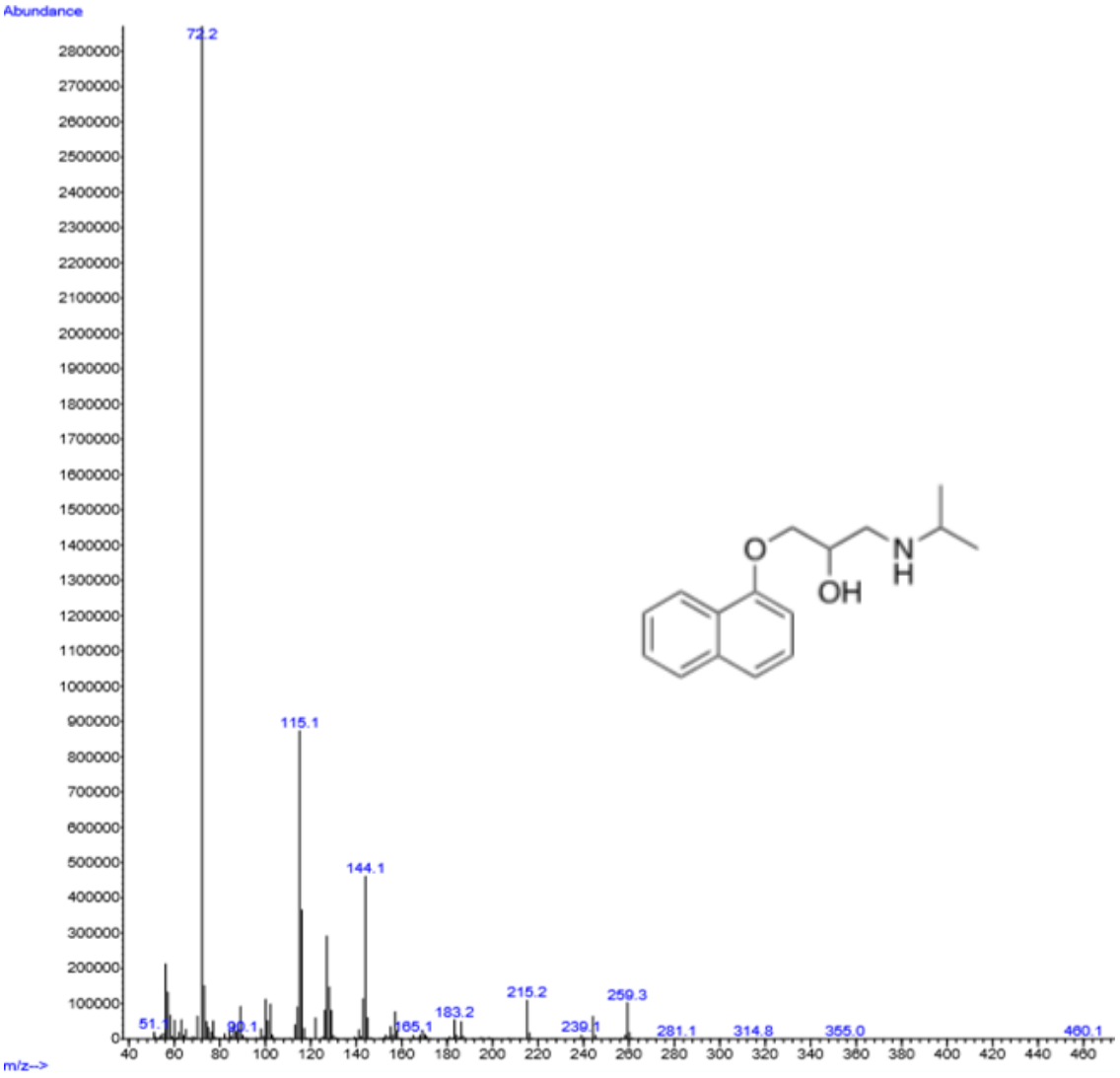


Figure 24 Mass Spectrum of Propranolol.

In EI mass spectrometry, fragmentation occurs via homolytic cleavage or heterolytic cleavage. The difference in the two pathways revolves around whether there is movement of a pair of electrons or movement of single electrons. Functional groups of the compound play key roles in determining fragmentation patterns. Tamoxifen consists of a tertiary amine group. With amine groups, the most abundant mass to charge ratio (also known as the base peak) is a result of cleavage of the β

bond. Tamoxifen is no exception. The cation has a radical on a heteroatom or an unsaturated functional group. The driving force of fragmentation is the strong tendency of the radical ion for electron pairing. Cleavage occurs when the radical and an odd electron from the bonds adjacent to the radical migrate to form a bond between the alpha carbon and either the heteroatom or the unsaturated functional group (Dass, p. 220). Figure 25 illustrates the electron ionization fragmentation of Tamoxifen.

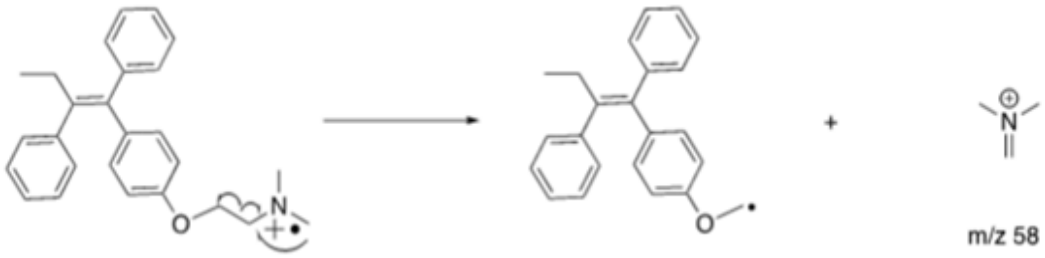


Figure 25 EIMS Fragmentation of Tamoxifen.

Full scan monitoring provides a qualitative picture of the composition of the sample, and it involves scanning the mass range beginning at the smallest mass of fragment ions to the highest mass expected for the fragments. In the selected ion monitoring mode (referred to as "SIM"), data is collected on only the selected masses of interest rather than over a wide range of masses. This improves the specificity of the instrument resulting in an increase in detection limits. This feature helps eliminate interferences arising due to complex sample matrix such as oral fluid. Based on this information, SIM mode was used (not full scan), and ions were chosen to be monitored (Scan Mode and SIM Mode, 2020).

When looking at the fragmentation patterns of both the Tamoxifen analyte and the Propranolol internal standard, it was determined that both produced positive ions with a mass to charge ratio of 58 and 72 (as seen in Figure 26 and Figure 27). Because of this, the ions chosen for monitoring were: 58, 72 and 259 – the molecular weight of Propranolol – and 371, the molecular weight of Tamoxifen.

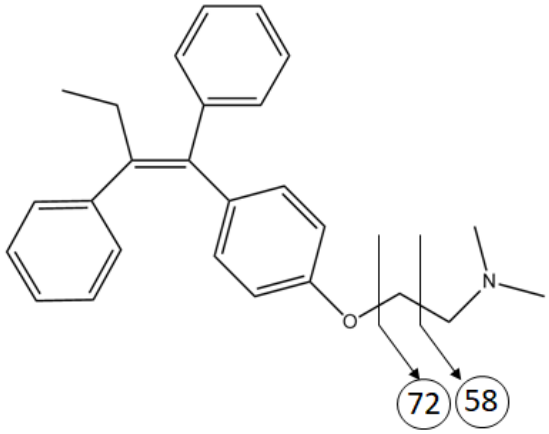


Figure 26 Tamoxifen (m/z 58 and 72).

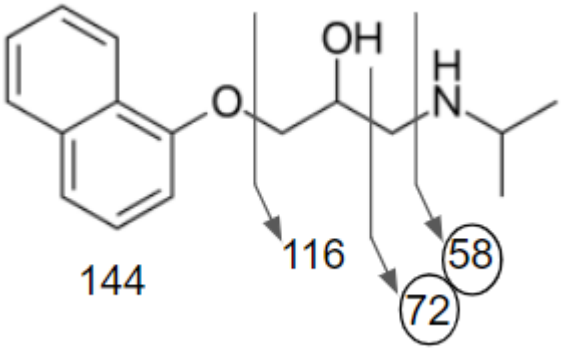


Figure 27 Propranolol (m/z 58, 72, 116 and 144).

Temperature Programming

Ramp 1 Value

Assessment of Ramp 1 Value variables (100°C - 210°C) revealed important information regarding retention times and peak separation. As the temperature increased, the retention time of both the Propranolol and the Tamoxifen decreased. This demonstrated that as temperature increases, the Propranolol and Tamoxifen molecules have fewer interactions with the stationary phase. Fewer interactions were expected, however, because an increase in temperature would decrease the stationary phase partition coefficient (aka: the equilibrium constant K_s) and therefore decrease retention factor. Also, peak separation decreased as the temperature increased. This can be explained by the Van Deemter Equation, meaning that as the temperature increases, the resistance to mass transfer also increases (Engewald & Dettmer-Wilde, 2014, pp. 37-40). Because of this, the theoretical plate height increases and therefore lowers the number of theoretical plates. The lower number of theoretical plates means peak separation decreases. See Figure 28 for illustration of effect on the molecules' retention time and peak separation.

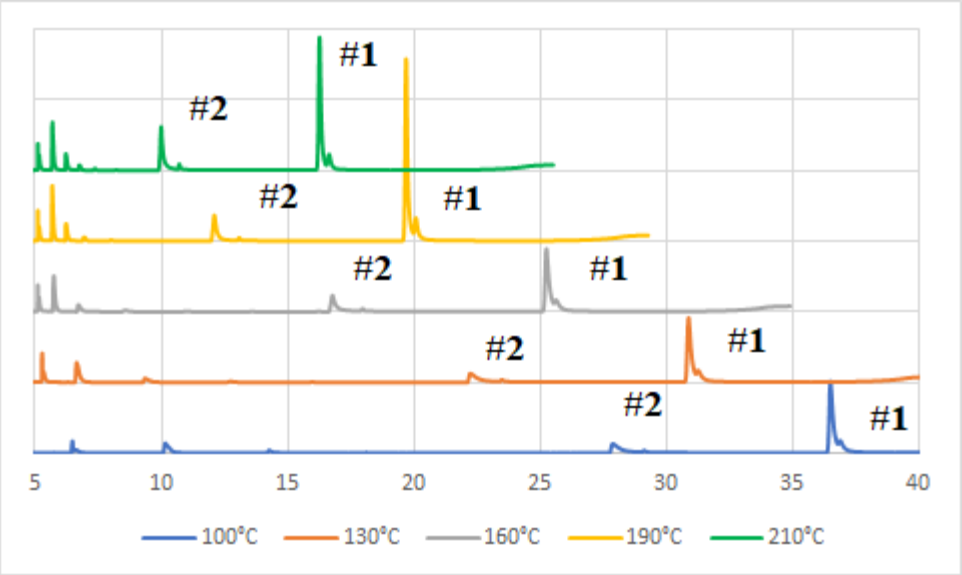


Figure 28 Increase of Ramp 1 Value: GC Chromatograph Overlay of Tamoxifen (#1) at 75 ug/mL and Propranolol (#2) at 200 ug/mL.

Data in Table 5 lists the calculated results of peak measurement (half peaks a and b), the number of theoretical plates (N), the asymmetrical factor (A_s) and the tailing factor (T_f) for each of the five Ramp 1 Values at Tamoxifen concentration levels ranging from 75 ug/mL to 300 ug/mL. Observable trends within the data include the number of theoretical plates decreasing as the Ramp 1 Value increases; the retention time decreasing as the Ramp 1 Value increases; the changes of peak shape are more significant with lower concentrations; and consistently, the lower Ramp 1 Value produced the most asymmetric peaks – most significantly, at the lowest concentration level of Tamoxifen.

Table 5 Ramp 1 Value Data

Ramp 1 Value						
Ramp 1 Value (°C)	**Tamoxifen Retention Time (t_r)**	**a (Min)**	**b (Min)**	**N**	**A_s**	**T_f**
75 ug/mL						
100	36.487	0.048	0.104	317727.1	2.181	1.591
130	30.882	0.068	0.088	217342.1	1.298	1.149
160	25.239	0.049	0.105	149667.1	2.157	1.579
190	19.696	0.044	0.060	197593.6	1.349	1.174
210	16.26	0.041	0.071	116508.9	1.711	1.356
100 ug/mL						
100	36.474	0.043	0.081	478008.7	1.868	1.434
130	30.856	0.047	0.081	326239.1	1.731	1.366
160	25.226	0.044	0.077	237498.9	1.742	1.371
190	19.684	0.035	0.057	253687.7	1.656	1.328
210	16.248	0.037	0.052	187624.3	1.404	1.202
150 ug/mL						
100	36.462	0.040	0.068	640418.9	1.704	1.352
130	30.844	0.045	0.063	456975.9	1.412	1.206
160	25.214	0.038	0.061	360440.8	1.622	1.311
190	19.684	0.040	0.045	295096.7	1.108	1.054
210	16.235	0.030	0.054	206915.3	1.816	1.408
200 ug/mL						
100	36.449	0.033	0.065	764013	1.990	1.495
130	30.831	0.037	0.059	576715.4	1.581	1.291
160	25.201	0.032	0.062	399688.5	1.954	1.477
190	19.671	0.029	0.049	347407.9	1.666	1.333
210	16.235	0.032	0.050	220243.9	1.565	1.282
250 ug/mL						
100	36.449	0.034	0.057	886744.5	1.681	1.341
130	30.831	0.040	0.049	659749.8	1.214	1.107
160	25.201	0.033	0.057	435082	1.740	1.370
190	19.684	0.045	0.036	327985.9	0.815	0.907
210	16.248	0.044	0.037	224697.8	0.837	0.919
300 ug/mL						
100	36.449	0.034	0.054	953818.8	1.613	1.306
130	30.832	0.044	0.044	675054.7	0.988	0.994
160	25.214	0.044	0.043	464900.7	0.977	0.989
190	19.684	0.045	0.036	327090.6	0.815	0.907
210	16.248	0.043	0.033	254193.4	0.755	0.878

In reference to Table 6, the Ramp 1 Values of 130°C and 160°C had the lowest limit of detection. Additionally, Values 130°C and 160°C both had an r^2 value of 0.999 for the calibration curves – although each of the values had r-squared values greater than 0.99.

Table 6 Ramp 1 Value Regression Analysis.

Regression Analysis: Ramp 1 Value					
Ramp 1 Value(°C)	**r^2**	**Sy**	**m**	**LOD (ug/mL)**	**LOQ (ug/mL)**
100	0.994	0.789	0.105	24.766	74.299
130	0.999	0.263	0.101	8.613	25.840
160	0.999	0.238	0.089	8.776	26.328
190	0.996	0.437	0.071	20.374	61.121
210	0.997	0.306	0.055	18.314	54.942

As the Ramp 1 Value increased, the peaks for Tamoxifen and Propranolol became more narrow and taller. This can be explained by the analytes having faster exchanges between the mobile and stationary phases with increased temperature. This observation is true for both Tamoxifen as seen in Figure 29 and for Propranolol as seen in Figure 30.

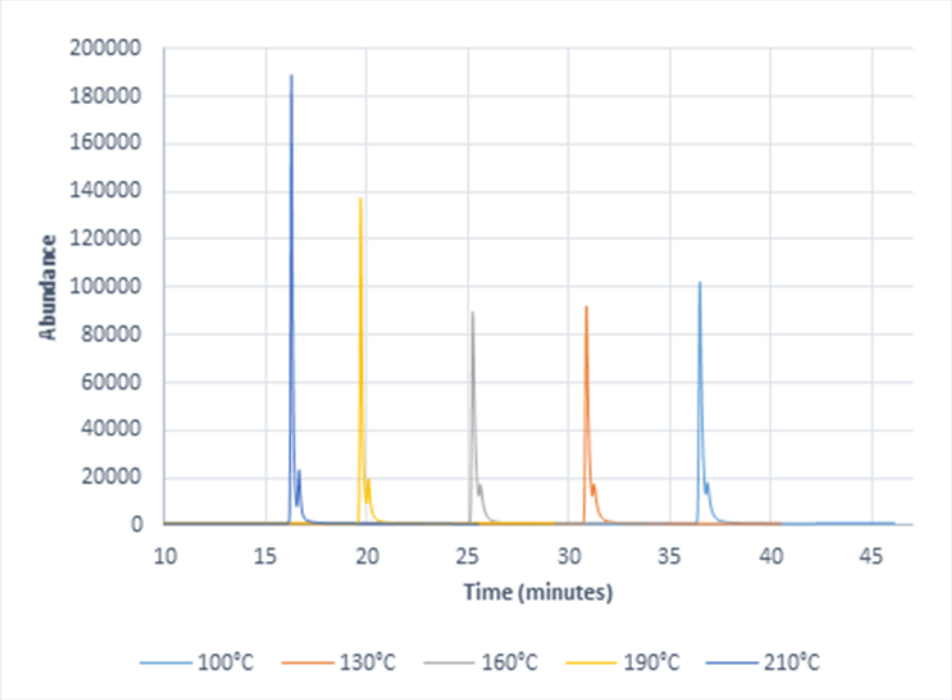


Figure 29 Ramp 1 Value: Tamoxifen at 75 ug/mL .

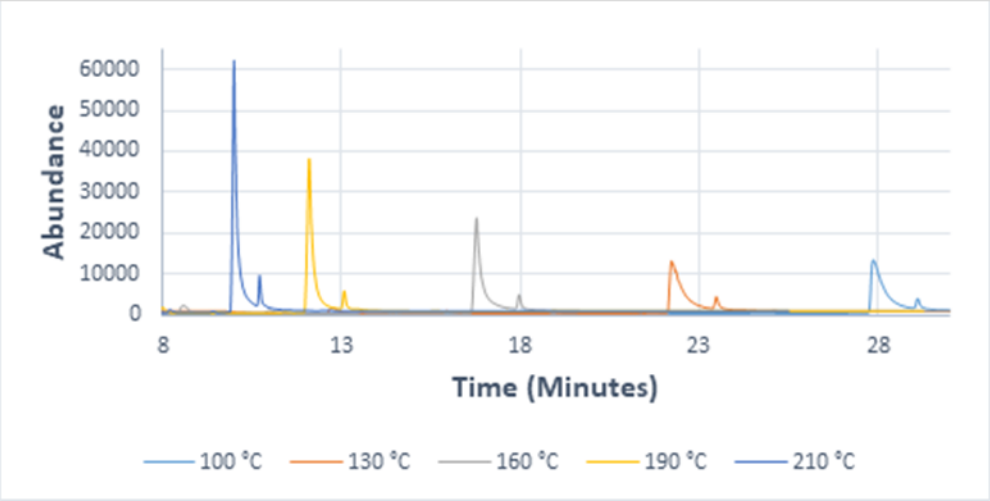


Figure 30 Ramp 1 Value: Propranolol 200 ug/mL .

Figure 31 illustrates that the slope of the calibration curve decreases as the Ramp 1 Value is increased. This information is important because it shows that when the Ramp 1 value is lowest, there is the largest change in instrument signal per smallest change in analyte concentration – also known as sensitivity. Figure 31 also demonstrates that as Ramp 1 Value is increased, the sensitivity decreases; however, the limit of detection increases. This information supported a choice for a higher temperature value because the lower temperatures did not produce the lower limit of detection. The limit of detection is so crucial for detecting Tamoxifen in oral fluid that choosing the Ramp 1 Value of 160°C would be optimal because it struck a balance with sensitivity.

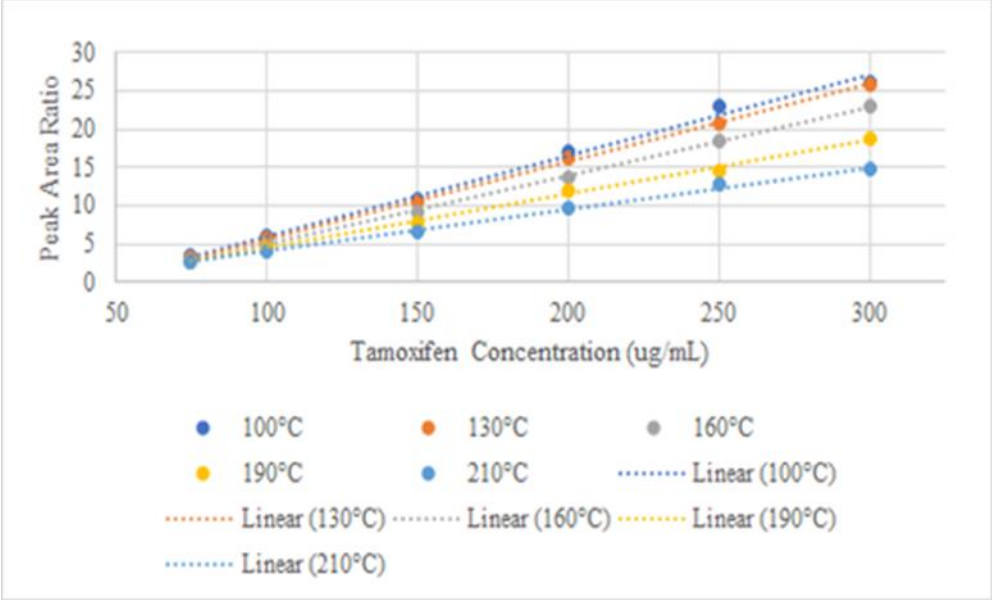


Figure 31 Ramp 1 Value: Peak Area Ratio vs. Concentration.

Ramp 1 Rate

Increased rates for each run resulted in no significant changes or differences for sensitivity or LOD. The calibration curves demonstrate the very minor differences. The lowest three Ramp 1 Rates of 20℃/min, 40℃/min and 60℃/min did show the largest changes in retention times. For both the IS and Tamoxifen, an average of 3 minutes was eliminated upon each of the first three rate changes. See Figure 32 for illustration of reduced retention times. With no significant changes in retention times past the 80℃/min rate, however, it was determined the optimal Ramp 1 Rate would be 80℃/min.

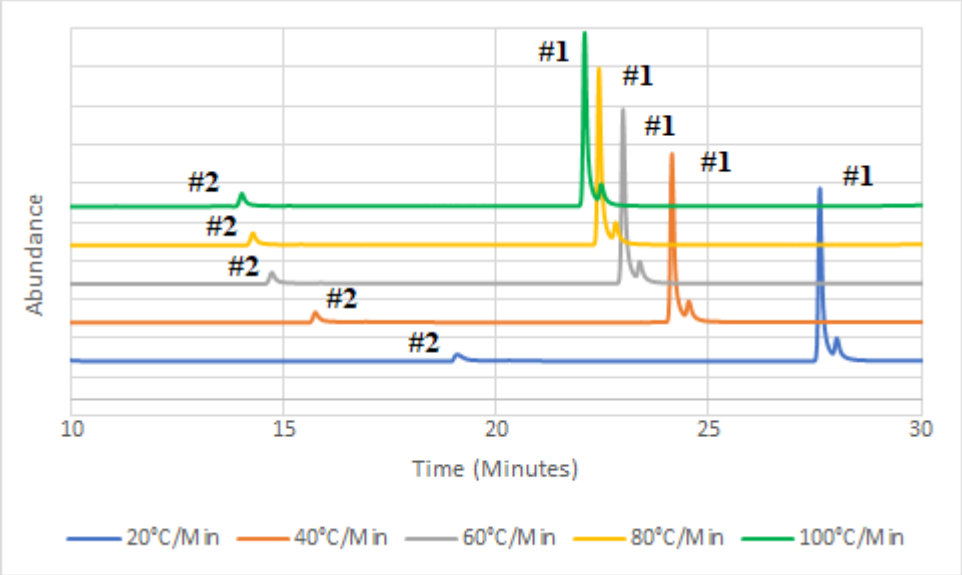


Figure 32 Increase of Ramp 1 Rate: GC Chromatograph Overlay of Tamoxifen (#1) at 75 ug/mL and Propranolol (#2) at 200 ug/mL.

Observable trends within the data listed in Table 7 include observation that Tamoxifen peaks become more asymmetrical as the Ramp 1 Rate increases. As the Rate also increases, the number of theoretical plates decrease. This mirrors the trend observed in Ramp 1 Value increases.

Table 7 Ramp 1 Rate Data.

Ramp 1 Rate						
Ramp 1 Rate (°C/Min)	**Tamoxifen Retention Time (t_r)**	**a (Min)**	**b (Min)**	**N**	**A_s**	**T_f**
75 ug/mL						
20	27.634	0.048	0.067	318370.494	1.404	1.202
40	24.148	0.043	0.073	241235.5387	1.687	1.343
60	22.994	0.041	0.079	203908.4063	1.959	1.479
80	22.43	0.042	0.083	178651.1774	1.960	1.480
100	22.091	0.041	0.081	179287.9069	1.968	1.484
100 ug/mL						
20	27.621	0.041	0.060	411961.0005	1.444	1.222
40	24.135	0.034	0.066	318665.9799	1.920	1.460
60	22.994	0.043	0.057	292875.6852	1.303	1.151
80	22.469	0.085	0.015	278558.2198	0.183	0.591
100	22.091	0.044	0.059	257229.5536	1.348	1.174
150 ug/mL						
20	27.621	0.044	0.049	486873.1894	1.132	1.066
40	24.135	0.039	0.052	388435.1876	1.317	1.158
60	22.982	0.035	0.055	360203.8894	1.547	1.273
80	22.417	0.038	0.053	339054.9728	1.394	1.197
100	22.079	0.038	0.054	318064.3021	1.433	1.217
200 ug/mL						
20	27.609	0.031	0.057	543790.7083	1.800	1.400
40	24.123	0.031	0.057	415116.5968	1.806	1.403
60	22.982	0.040	0.046	392749.4383	1.162	1.081
80	22.417	0.039	0.046	380020.2337	1.173	1.087
100	22.079	0.039	0.048	350463.9668	1.224	1.112
250 ug/mL						
20	27.621	0.044	0.040	608848.76318	0.910	0.955
40	24.135	0.045	0.044	409516.07380	0.989	0.995
60	22.982	0.041	0.045	397475.55527	1.083	1.042
80	22.417	0.041	0.044	385847.32054	1.070	1.035
100	22.079	0.039	0.048	350780.05676	1.230	1.115
300 ug/mL						
20	27.621	0.044	0.042	582031.4801	0.954	0.977
40	24.135	0.044	0.042	436624.593	0.958	0.979
60	22.982	0.042	0.043	396687.7074	1.024	1.012
80	22.417	0.041	0.043	392538.1968	1.045	1.022
100	22.079	0.042	0.043	369563.7296	1.012	1.006

In reference to Table 8, the Ramp 1 Rates 40°C/min and 80°C/min had the lowest limit of detection: 12.564 ug/mL and 11.790 ug/mL, respectively. Additionally, rate 80°C/min had an r^2 value of 0.999 – although each of the values had excellent r-squared values.

Table 8 Ramp 1 Rate Regression Analysis.

Regression Analysis: Ramp 1 Rate					
Ramp 1 Rate (°C/min)	**r^2**	**Sy**	**m**	**LOD (ug/mL)**	**LOQ (ug/mL)**
20	0.997	0.567	0.103	18.194	54.583
40	0.998	0.354	0.093	12.564	37.691
60	0.994	0.710	0.092	25.485	76.456
80	0.999	0.309	0.086	11.790	35.370
100	0.996	0.545	0.083	21.558	64.673

Figure 33 shows minor differences in the slope of the calibration curves. This indicates there should not be significant differences in the LOD regarding which rate is chosen. The slope decreases only slightly (0.1 to 0.08); but there is a trend. As the Ramp 1 Rate increases, the limit of detection also increases albeit slightly.

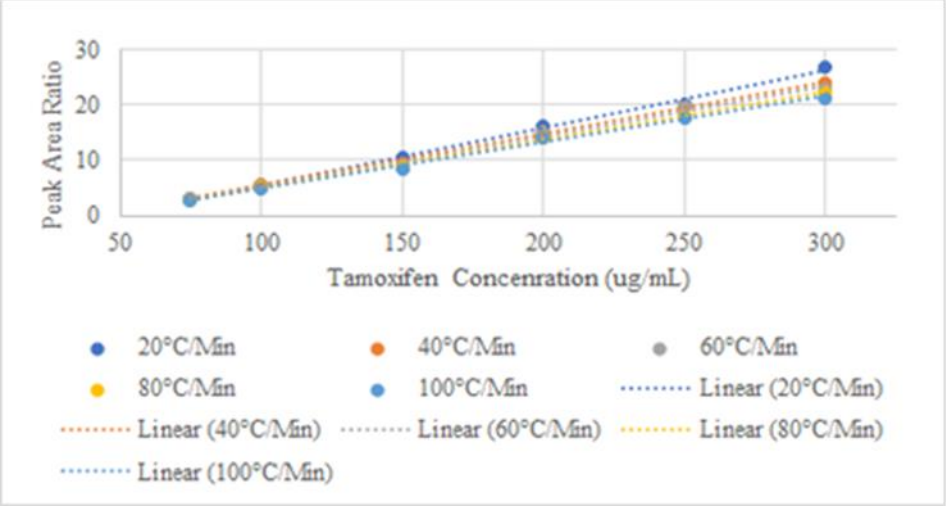


Figure 33 Ramp 1 Rate: Peak Area Ratio vs. Concentration.

Ramp 2 Value

The one observed change was a decreased retention time for Tamoxifen of approximately 1 minute when the Ramp 2 Value increased from 230℃ to 245℃. The retention time for the internal standard Propranolol, however, remained constant.

This can be seen very clearly in Figure 34. The decreased retention time for Tamoxifen was expected because raising the column temperature generally reduces retention time due to a decreased number of interactions with the stationary phase. Possibly, the Propranolol eluted from the column prior to the temperature increase.

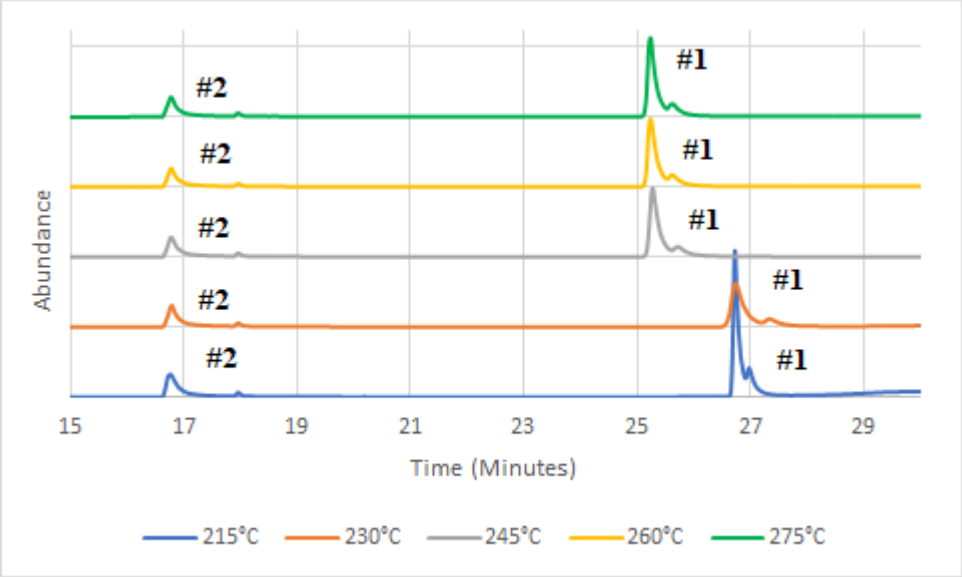


Figure 34 Increase of Ramp 2 Value: GC Chromatograph Overlay of Tamoxifen (#1) at 75 ug/mL and Propranolol (#2) at 200 ug/mL.

Observable trends within the data listed in Table 9 include the retention time decreases as the Ramp 2 Value increases. The number of theoretical plates also decreases as the temperature is increased. This once more parallels the decreased number of theoretical plates when temperature is increased.

Table 9 Ramp 2 Value Data.

Ramp 2 Value						
Ramp 2 Value (°C)	**Tamoxifen Retention Time (t_r)**	**a (Min)**	**b (Min)**	**N**	**A_s**	**T_f**
75 ug/mL						
215	26.731	0.032	0.057	508780.6	1.791	1.396
230	26.744	0.087	0.138	77984.1	1.577	1.288
245	25.276	0.056	0.099	147035.3	1.782	1.391
260	25.239	0.056	0.094	156566.3	1.667	1.333
275	25.239	0.057	0.082	184112.5	1.443	1.221
100 ug/mL						
215	26.718	0.027	0.051	648167.5	1.875	1.438
230	26.706	0.067	0.118	115109.1	1.755	1.378
245	25.264	0.056	0.070	221990.8	1.238	1.119
260	25.226	0.047	0.067	274337.2	1.424	1.212
275	25.213	0.036	0.070	315208.7	1.953	1.477
150 ug/mL						
215	26.718	0.031	0.036	869002.4	1.176	1.088
230	26.693	0.067	0.101	140231.8	1.519	1.259
245	25.251	0.044	0.057	349765.3	1.287	1.143
260	25.214	0.044	0.056	351890.7	1.276	1.138
275	25.214	0.044	0.056	349696	1.255	1.128
200 ug/mL						
215	26.706	0.019	0.044	1009232	2.376	1.688
230	26.693	0.069	0.082	173730.4	1.195	1.098
245	25.239	0.045	0.056	349594.8	1.254	1.127
260	25.214	0.041	0.058	355290.4	1.424	1.212
275	25.201	0.031	0.056	454839.2	1.793	1.396
250 ug/mL						
215	26.706	0.019	0.043	1013657	2.290	1.645
230	26.693	0.068	0.082	173769.4	1.201	1.100
245	25.239	0.044	0.056	352318.1	1.275	1.138
260	25.201	0.044	0.057	349679.1	1.297	1.149
275	25.201	0.032	0.044	617551.4	1.391	1.195
300 ug/mL						
215	26.718	0.031	0.032	1008980	1.019	1.009
230	26.693	0.081	0.069	176191.4	0.859	0.929
245	25.239	0.044	0.056	351982.9	1.270	1.135
260	25.201	0.032	0.044	615446.8	1.398	1.199
275	25.201	0.031	0.044	628107.2	1.418	1.209

In reference to Table 10, the Ramp 2 Value of 260°C had the lowest limit of detection (4.534). This value was significantly different than the other Ramp 2 Values. Additionally, the Ramp 2 Value of 260°C had an r^2 value of 1.000 – although each of the values had excellent r-squared values.

Table 10 Ramp 2 Value Regression Analysis.

Regression Analysis: Ramp 2 Value					
Ramp 2 Value(°C)	**r^2**	**Sy**	**m**	**LOD (ug/mL)**	**LOQ (ug/mL)**
215	0.998	0.323	0.073	14.577	43.731
230	0.999	0.276	0.074	12.286	36.857
245	0.998	0.334	0.078	14.083	42.249
260	1.000	0.108	0.079	4.534	13.602
275	0.998	0.365	0.080	14.978	44.935

Again, as seen in Figure 35, calibration curves demonstrate very minor differences for Ramp 2 Value. The slope of the calibration curves only slightly increase (the 0.073 to 0.08) as the Ramp 2 Value increases. It was determined that the optimal Ramp 2 Value would be 260℃ because the retention times of both the IS and Tamoxifen compounds were satisfactory and 260℃ avoids an additional, increased run time of two minutes when compared with the Ramp 2 Value of 275℃.

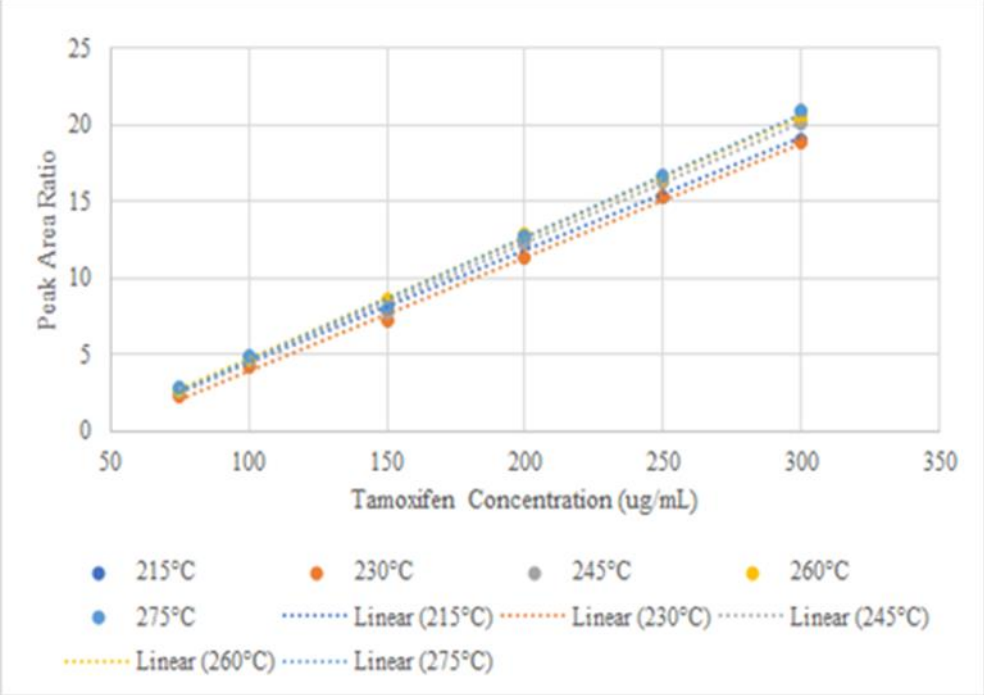


Figure 35 Ramp 2 Value: Peak Area Ratio vs. Concentration.

Ramp 2 Rate

Each run resulted in significant changes for both sensitivity and limit of detection. The overall result, however, was that the highest Ramp 2 Rate had the lowest sensitivity and the lowest limit of detection. Conversely, the lowest Ramp 2 Rate had the highest sensitivity and the "next to highest" limit of detection. This is a dilemma because neither of these ramp rates could be optimal (i.e. having both high sensitivity and low detection limit). The overall trend within this parameter was that ramp rate increase resulted in decreased peak separation and decreased retention time for both Tamoxifen and Propranolol. This observed trend is illustrated in Figure 36.

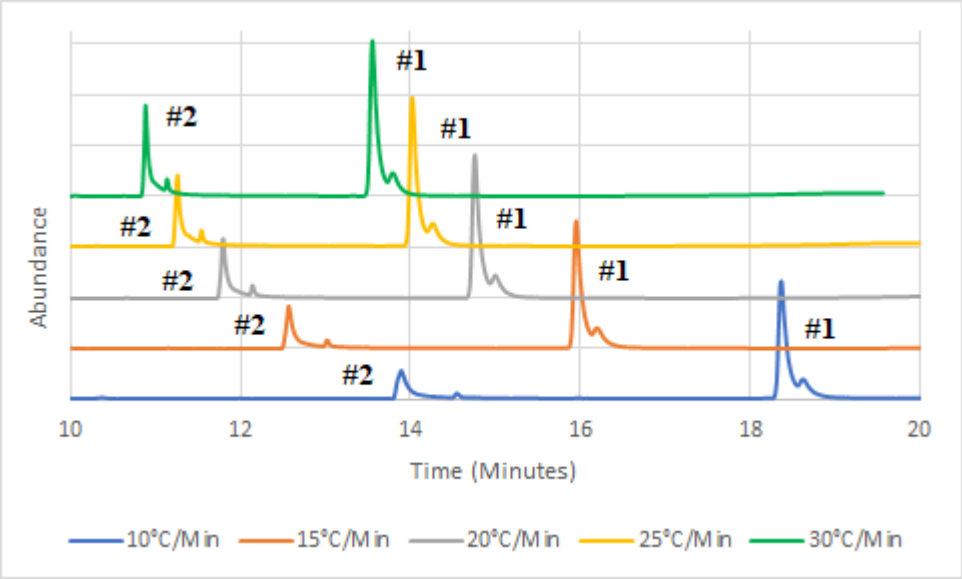


Figure 36 Increase of Ramp 2 Rate: GC Chromatograph Overlay of Tamoxifen (#1) at 75 ug/mL and Propranolol (#2) at 200 ug/mL.

Analysis of data presented in both Table 11 and Table 12 reveals that peak width decreases as the Ramp 2 Rate increases. As well, for all but the lowest concentration level of Tamoxifen (75 ug/mL), the number of theoretical plates decreased as the Ramp 2 Rate increased due to the decrease in peak width and peak separation. Narrowing of the peak widths caused lower limits of detection.

Table 11 Ramp 2 Rate Data.

Ramp 2 Rate						
Ramp 2 Rate (°C/Min)	**Tamoxifen Retention Time (t_r)**	**a (Min)**	**b (Min)**	**N**	**A_s**	**T_f**
75 ug/mL						
10	18.367	0.044	0.056	185750.1567	1.268	1.134
15	15.959	0.031	0.054	195259.5787	1.730	1.365
20	14.756	0.032	0.043	211455.4015	1.350	1.175
25	14.028	0.031	0.057	142004.8923	1.827	1.414
30	13.552	0.032	0.044	179965.5798	1.386	1.193
100 ug/mL						
10	18.354	0.031	0.049	294553.3469	1.564	1.282
15	15.947	0.031	0.044	249849.41	1.389	1.195
20	14.743	0.031	0.043	214602.5847	1.379	1.189
25	14.016	0.032	0.045	185000.6339	1.422	1.211
30	13.539	0.032	0.044	177137.3954	1.396	1.198
150 ug/mL						
10	18.342	0.019	0.045	453419.2678	2.379	1.689
15	15.934	0.018	0.044	360073.0359	2.403	1.702
20	14.73	0.019	0.044	307152.6508	2.374	1.687
25	14.016	0.032	0.031	277659.9651	0.981	0.990
30	13.539	0.032	0.038	213259.6686	1.190	1.095
200 ug/mL						
10	18.342	0.019	0.044	477616.6498	2.303	1.651
15	15.934	0.019	0.044	353655.3634	2.300	1.650
20	14.73	0.028	0.041	256078.037	1.446	1.223
25	14.016	0.032	0.031	277817.1185	0.981	0.991
30	13.539	0.032	0.032	255859.6741	1.000	1.000
250 ug/mL						
10	18.342	0.032	0.038	390791.2564	1.188	1.094
15	15.934	0.031	0.032	360091.5874	1.016	1.008
20	14.73	0.032	0.032	302854.5896	1.000	1.000
25	14.015	0.031	0.032	277527.4256	1.012	1.006
30	13.539	0.031	0.032	259253.5212	1.013	1.007
300 ug/mL						
10	18.342	0.032	0.044	330176.8756	1.380	1.190
15	15.934	0.031	0.032	359011.6567	1.013	1.007
20	14.73	0.031	0.032	301389.3047	1.028	1.014
25	14.015	0.031	0.032	278427.5556	1.016	1.008
30	13.539	0.031	0.032	259696.2406	1.015	1.008

Table 12 Ramp 2 Rate Regression Analysis.

Regression Analysis: Ramp 2 Rate					
Ramp 2 Rate (°C/min)	**r^2**	**Sy**	**m**	**LOD (ug/mL)**	**LOQ (ug/mL)**
10	0.989	0.701	0.067	34.448	103.344
15	0.989	0.619	0.059	34.700	104.099
20	0.990	0.517	0.053	32.135	96.404
25	0.991	0.428	0.045	31.171	93.514
30	0.993	0.348	0.043	26.550	79.649

The slope m decreases as the Ramp 2 Rate increases. This can be seen in Figure 37. The r^2 value gradually increases and the Sy term gradually decreases, as supported by calculated data also seen in Table 12. It was determined that the optimal Ramp 2 Rate would be 25℃/min because it struck a balance with sensitivity and had the "next best" limit of detection at 31.171.

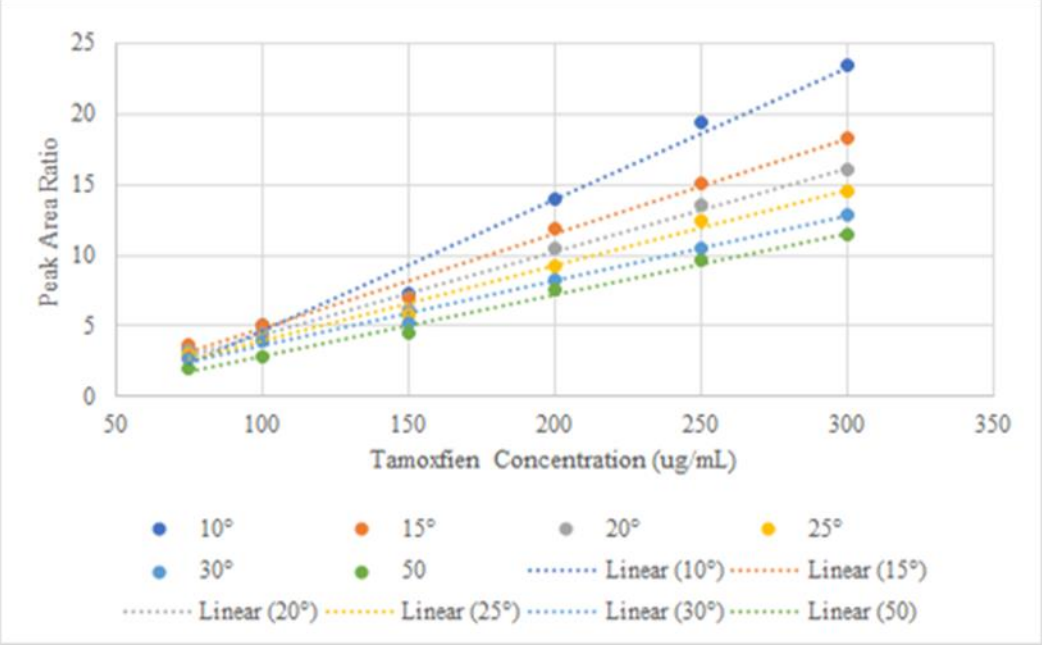


Figure 37 Ramp 2 Rate: Peak Area Ratio vs. Concentration.

Ramp 3 Value

Increased temperature changes for the Ramp 3 Value resulted in no significant changes or differences regarding sensitivity or LOD. The separation is taking place after the Tamoxifen and Propranolol have eluted. Retention times did not change, as illustrated in Figure 38.

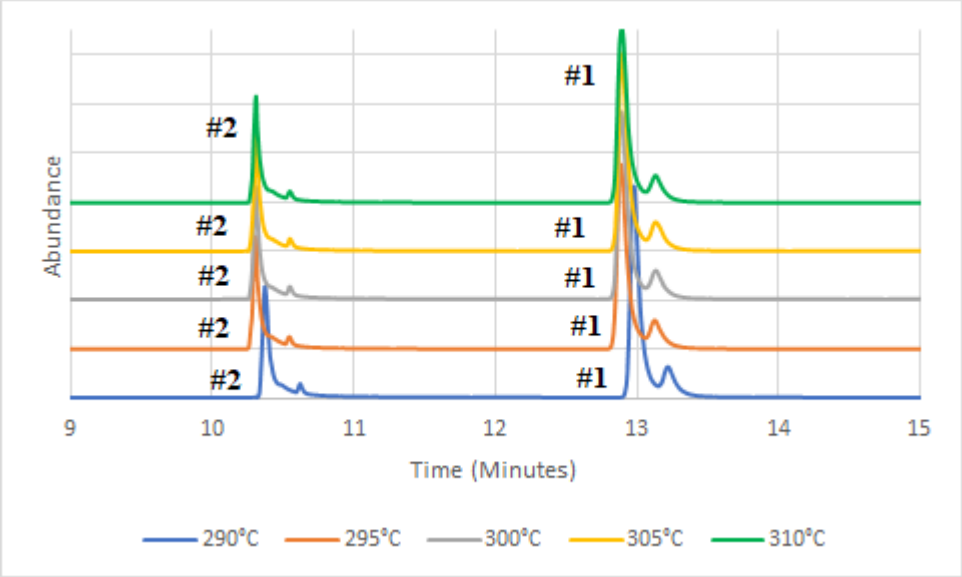


Figure 38 Increase of Ramp 3 Value: GC Chromatograph Overlay of Tamoxifen (#1) at 75 ug/mL and Propranolol (#2) at 200 ug/mL.

Observable trends within the data listed in Table 13 include the number of theoretical plates remaining fairly consistent. This is true for retention time, too and only slight variation with peak width regardless of concentration levels of Tamoxifen.

Table 13 Ramp 3 Value Data.

Ramp 3 Value						
Ramp 3 Value (°C)	**Tamoxifen Retention Time (t_r)**	**a (Min)**	**b (Min)**	**N**	**A_s**	**T_f**
75 ug/mL						
290	12.975	0.032	0.038	195818.2	1.190	1.095
295	12.887	0.031	0.044	163398.1	1.424	1.212
300	12.887	0.032	0.044	161093.5	1.396	1.198
305	12.887	0.019	0.044	229449.5	2.302	1.651
310	12.887	0.031	0.044	164324.9	1.421	1.210
100 ug/mL						
290	12.887	0.019	0.044	230654.7	2.305	1.652
295	12.875	0.032	0.044	160381.7	1.365	1.182
300	12.875	0.031	0.032	235133.3	1.016	1.008
305	12.875	0.019	0.043	231684.4	2.230	1.615
310	12.875	0.031	0.031	235337.9	0.985	0.993
150 ug/mL						
290	12.887	0.032	0.032	231810.1	1.000	1.000
295	12.875	0.032	0.032	231378.6	1.000	1.000
300	12.887	0.019	0.044	232147.3	2.253	1.627
305	12.887	0.032	0.032	231810.1	1.000	1.000
310	12.887	0.038	0.038	163565.2	1.000	1.000
200 ug/mL						
290	12.875	0.031	0.032	230753.1	1.032	1.016
295	12.875	0.032	0.031	231474.9	0.965	0.983
300	12.875	0.031	0.032	231117.7	1.029	1.015
305	12.875	0.020	0.031	359102.6	1.588	1.294
310	12.875	0.032	0.031	230988.6	0.970	0.985
250 ug/mL						
290	12.874	0.031	0.032	230046.4	1.036	1.018
295	12.874	0.019	0.032	357955.2	1.732	1.366
300	12.874	0.031	0.032	234981.8	1.016	1.008
305	12.874	0.031	0.032	235299.7	0.501	1.009
310	12.874	0.031	0.031	235066.6	0.984	0.992
300 ug/mL						
290	12.874	0.031	0.032	230798.9	1.035	1.017
295	12.874	0.019	0.031	367400.7	1.702	1.351
300	12.874	0.031	0.032	231257.2	1.031	1.016
305	12.874	0.032	0.019	359018	0.603	0.801
310	12.874	0.032	0.031	234903.6	0.983	0.992

Again, calibration curves demonstrate very minor differences (Figure 39). Without differences, it was determined the optimal Ramp 3 Value would be 290°C to avoid getting too close to the maximum oven temperature of 325°C for the column. Maximum temperature could degrade the column and lead to column bleed.

Avoiding the maximum temperature helped avoid background signal. An increase in background signal can lead to decrease in sensitivity.

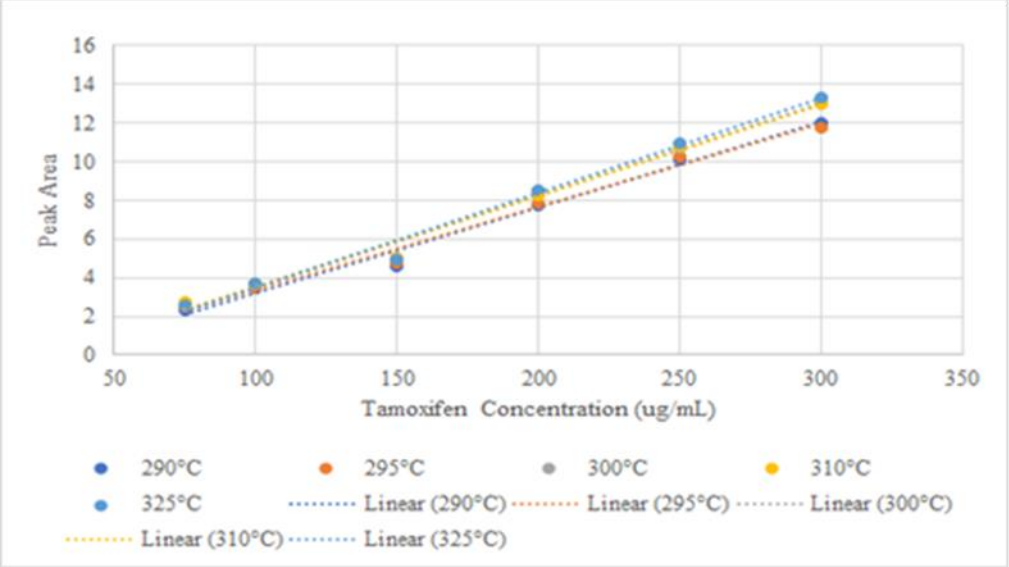


Figure 39 Ramp 3 Value: Peak Area Ratio vs. Concentration.

Ramp 3 Rate

Increased run rates for the Ramp 3 Rate variable resulted in no significant changes or differences regarding sensitivity or LOD. The separation is taking place after the Tamoxifen and Propranolol have eluted. Again, calibration curves demonstrate these very minor differences (Figure 40). It was determined the optimal Ramp 3 Rate would be $10℃/\mathrm{min}$.

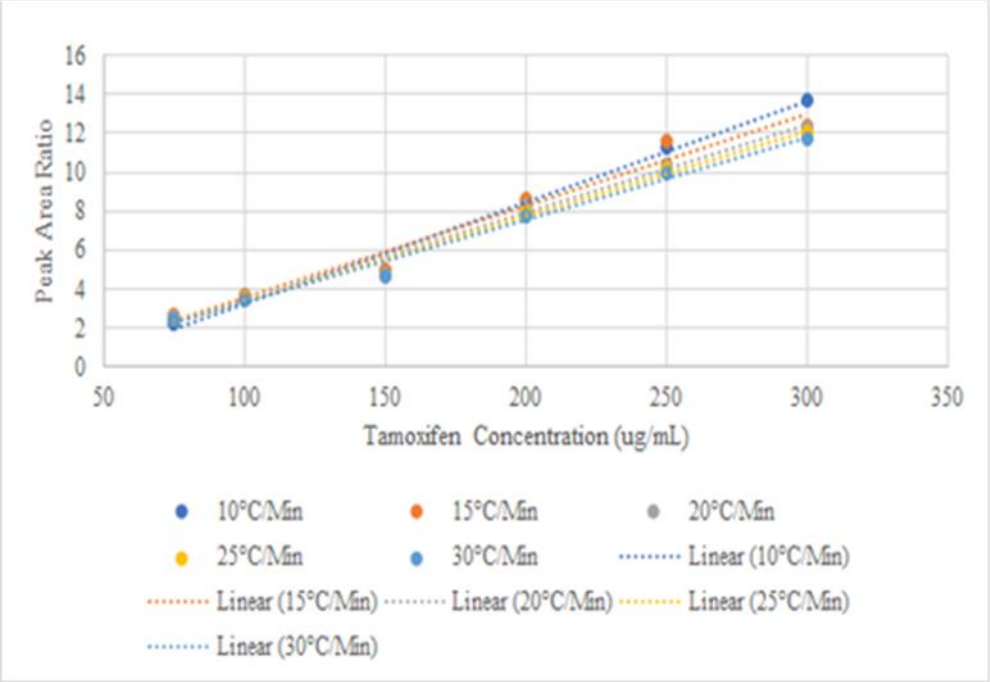


Figure 40 Ramp 3 Rate: Peak Area Ratio vs. Concentration.

Optimal Values and Rates

A summary of each optimal variable and parameter that was tested for method optimization regarding temperature programming can be seen in Table 14.

Table 14 Temperature Programming Utilized for Optimization.

	Value	Rate	Hold Time
Initial	30℃		3 Minutes
Ramp 1	160℃	80℃/Min	3 Minutes
Ramp 2	260℃	25 ℃/Min	5 Minutes
Ramp 3	290℃	10 ℃/Min	1 Minute

Inlet Temperature

Inlet temperature was an important parameter to adjust to avoid degradation of the Tamoxifen and the internal standard Propranolol. The goal was to be able to set a temperature that was hot enough to make sure the analytes evaporated in their entirety but without causing them to undergo thermal degradation or backflash. Five inlet temperatures were explored using increments of 25°C (400°C down to 300°C), finding that 300°C still caused slight degradation. A wide, almost flat peak can be seen at 14.25 minutes for Tamoxifen when the inlet temperature was set at the lowest increment. To totally avoid degradation, the chosen inlet temperature for optimization was 225°C. This choice was made after trial and error, and this choice did avoid degradation and improved peak shape. Going to a lower temperature than 225°C would cause band broadening and risk complete evaporation of the analytes. See Figure 41 and Figure 42 that show how lowering inlet temperature during exploration of increments reduced thermal degradation of both Tamoxifen and Propranolol but support an even lower temperature for optimization.

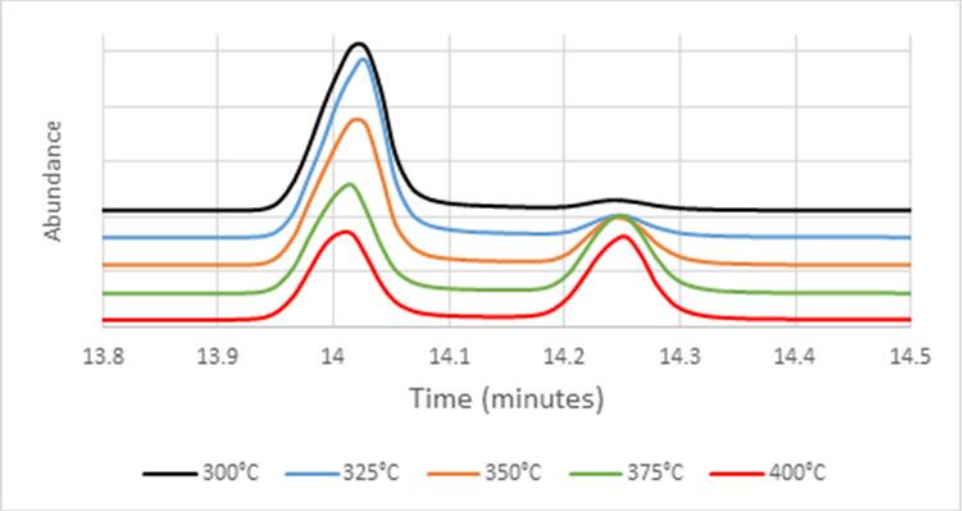


Figure 41 Inlet Temperature Optimization for Tamoxifen.

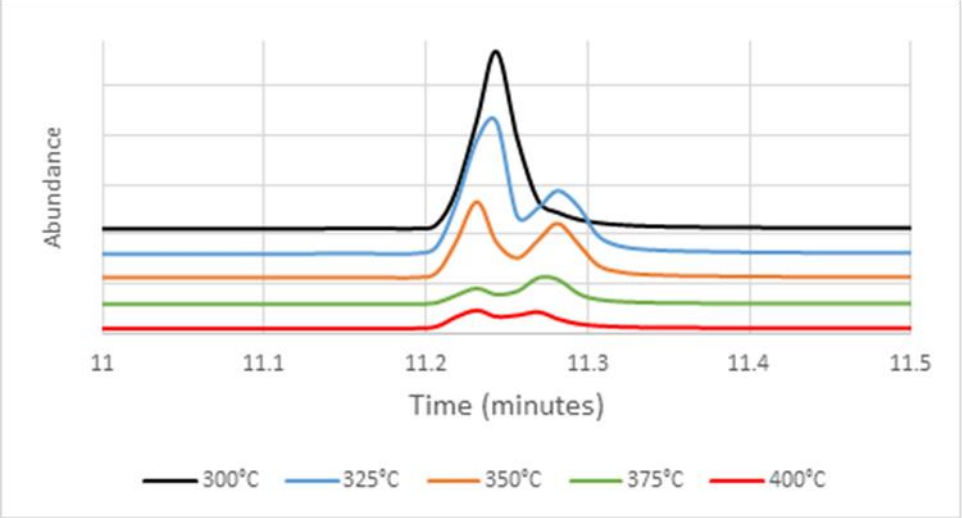


Figure 42 Inlet Temperature Optimization for Propranolol.

Column Flow

One factor regarding this parameter was the retention time of Tamoxifen at varied flow rates. The rates ranged from 1.2 mL/min – 4.0 mL/min with increments of 0.2 mL/min studied. It was observed from data that the most pronounced changes in retention times occurred at flow rates of 1.2 mL/min – 2.4 mL/min as can be seen in

Figure 43. The same was observed to be true for the IS Propranolol at the same varied flow rates and studied increments. This can be seen in Figure 44.

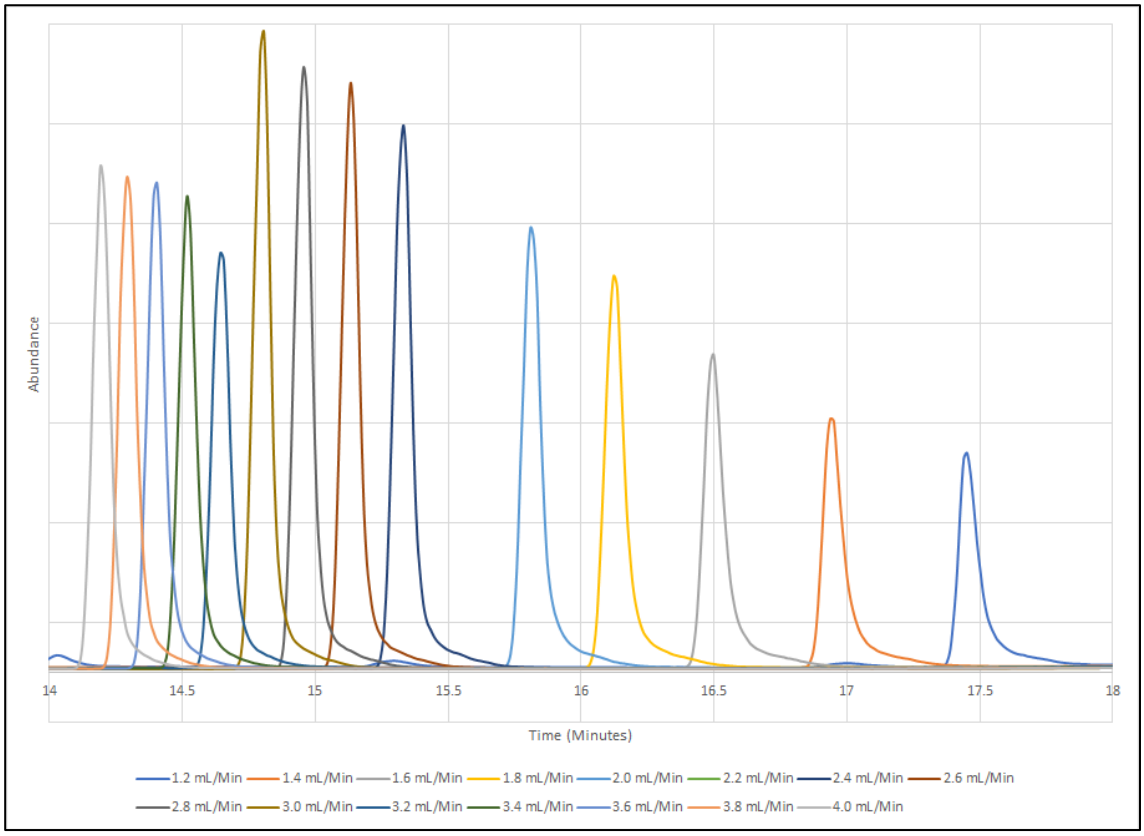


Figure 43 Retention Time of Tamoxifen at Varied Flow Rates.

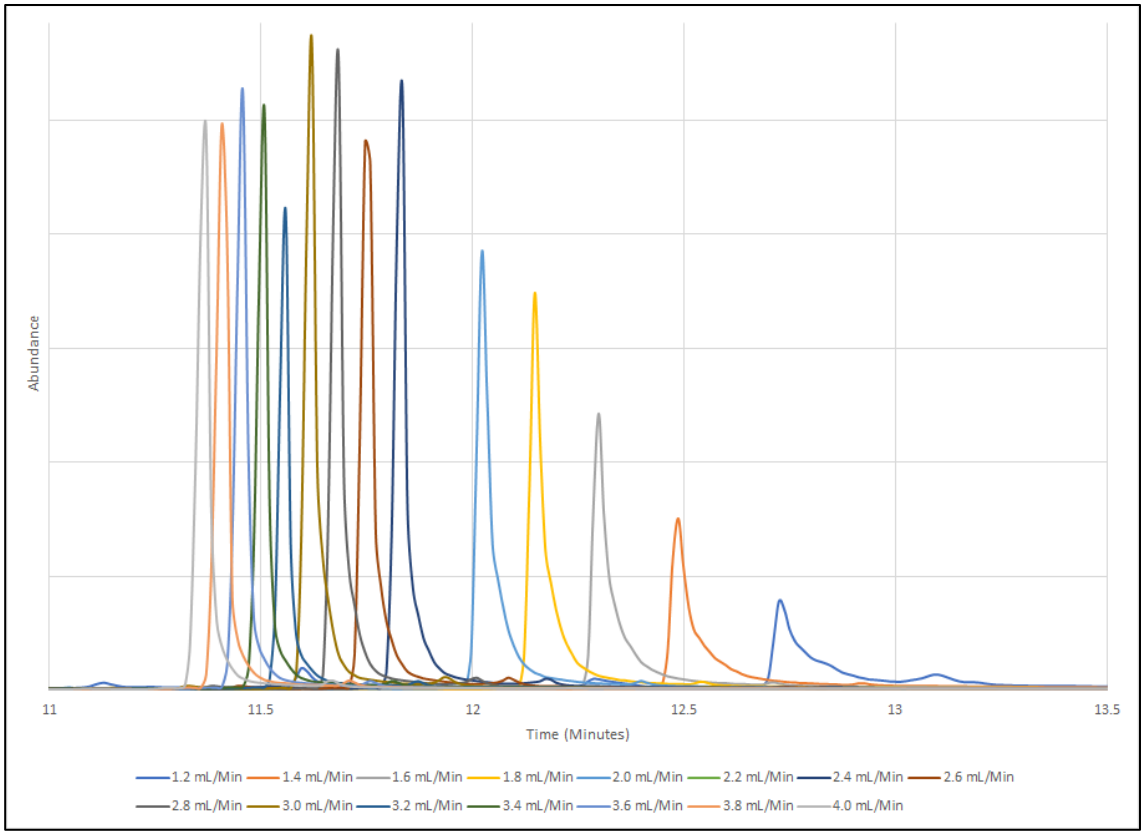


Figure 44 Retention Time of Propranolol at Varied Flow Rates.

A second factor for this parameter was to calculate the number of theoretical plates and the theoretical plate height in order to study the effectiveness of the GC separation (the column efficiency). By plotting HETP vs. flow rate, the effect of the flow rate on column efficiency can be seen. Flow rate of the gas through the column affects separation in that speeding up gas flow causes the analyte to move through the column more quickly. Faster flow rates push the analytes through the column faster and thus, there is less time for interaction with the stationary phase. Van Deemter curves were produced for both Tamoxifen and Propranolol. See Figure 45 and Figure 46.

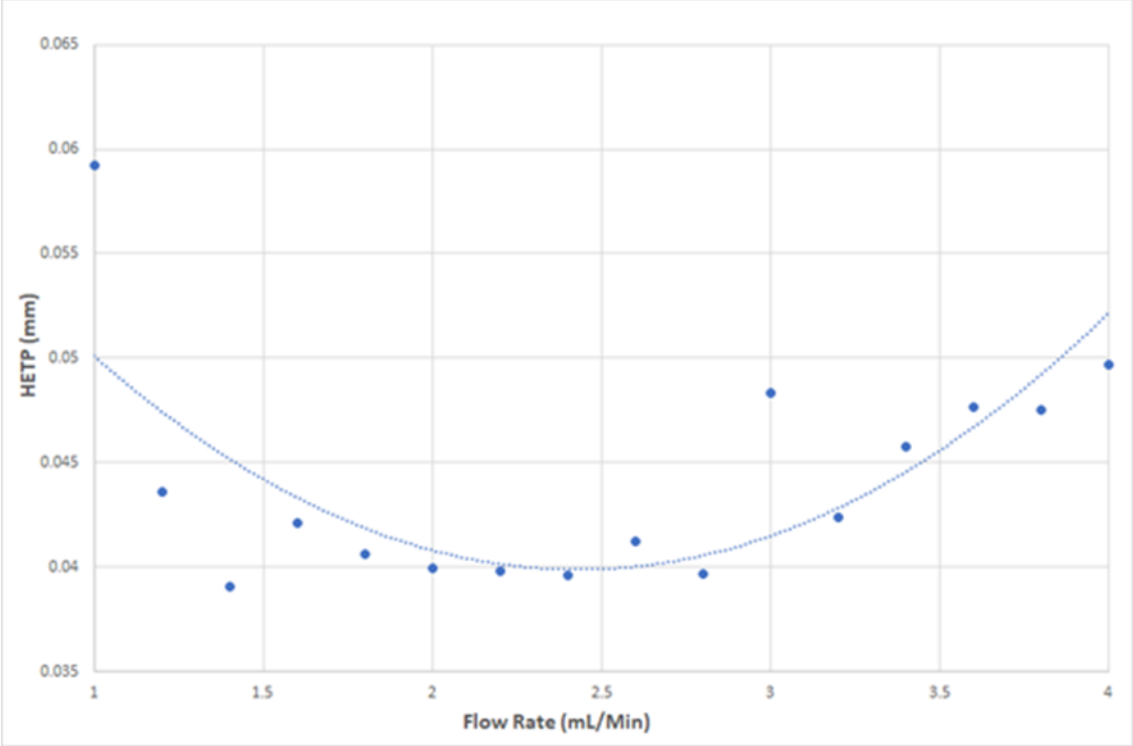


Figure 45 Theoretical Plate Height vs. Flow Rate: Tamoxifen.

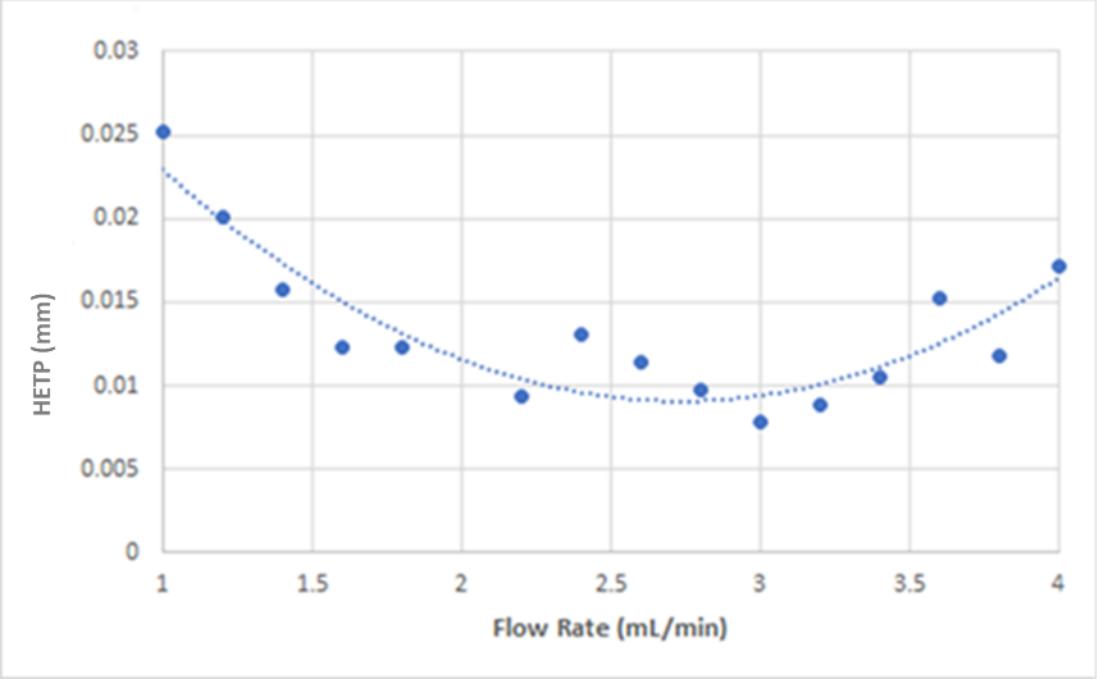


Figure 46 Theoretical Plate Height vs. Flow Rate: Propranolol.

All ran samples were observed for the effect of varied flow rates on peak shape. At the lowest rate, the IS peak was poorly shaped and in low abundance. As the flow rate increased, however, the retention times of both the IS and target analyte – as well as the peak area of both compounds and the peak shape of both compounds – improved. An issue of tailing was continuous throughout this project, however, and studied in order to resolve it. Common troubleshooting techniques for tailing peaks (bake out of the column, solvent rinse of the column and checking for leaks) were performed. Tailing is often caused by the analyte of interest interacting with some portions of the stationary phase more than others.

Based on these runs and personal observations (and practicality regarding analysis time), the chosen flow rate for optimization of this project's method was 4 mL/min. A lower flow rate would have been chosen if this method required more efficient separation of compounds but achieving a detection limit as low as possible was forefront.

7.2 Method Validation

To demonstrate validity of a GC/MS method to show its repeatability, reproducibility and reliability, parameters are assessed: specificity, linearity, precision, accuracy, limit of detection and limit of quantitation. This project utilized guidelines set forth by ICH (International Council on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) for validation purposes. (Quality Guidelines: Analytical Validation, 2020)

Specificity

The proposed method proved to be specific for identification of Tamoxifen in presence of solvent or saliva components (refer to Figure 47 which shows the chromatogram of Tamoxifen and Propranolol).

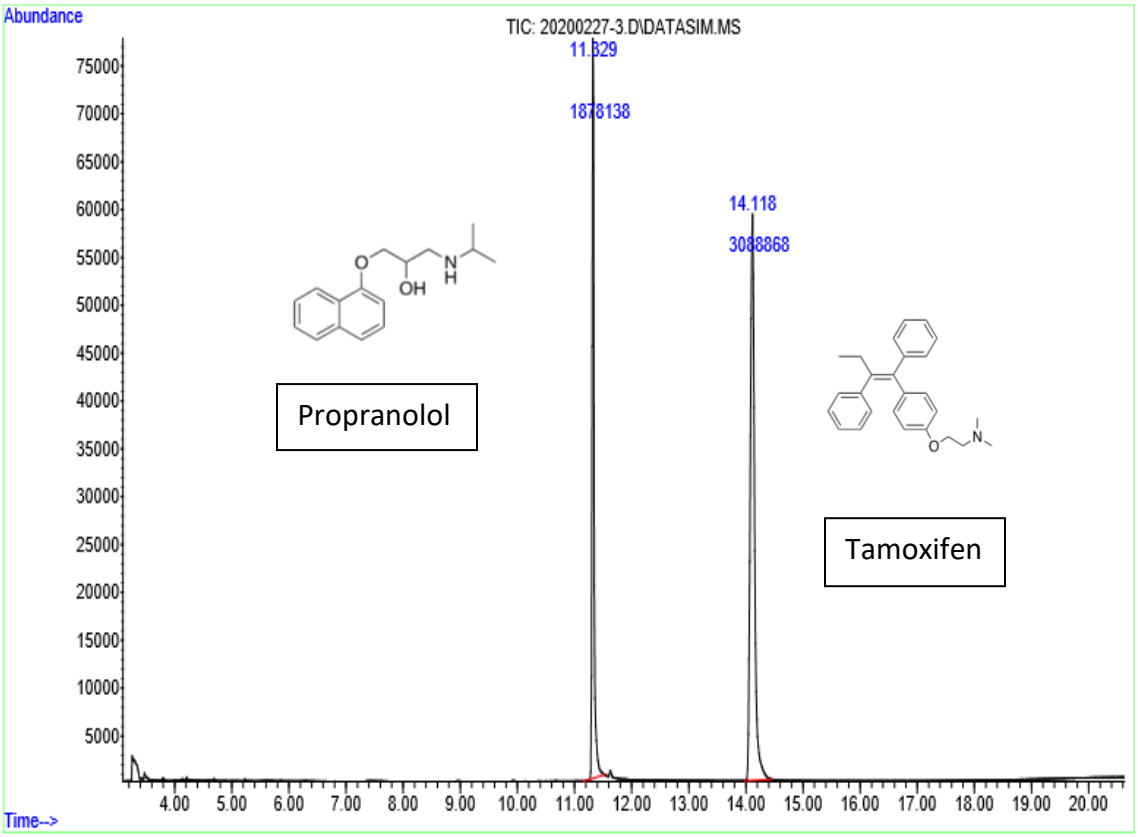


Figure 47 Chromatogram of Tamoxifen and Propranolol.

Linearity

When every variable and parameter was tested for method optimization, the Tamoxifen concentration was reduced to investigate how low the limit of detection could be. The calibration curve illustrated in Figure 48 shows the lowest LOD achieved was 1.22 µg/mL, making the lowest LOQ 3.66 µg/mL.

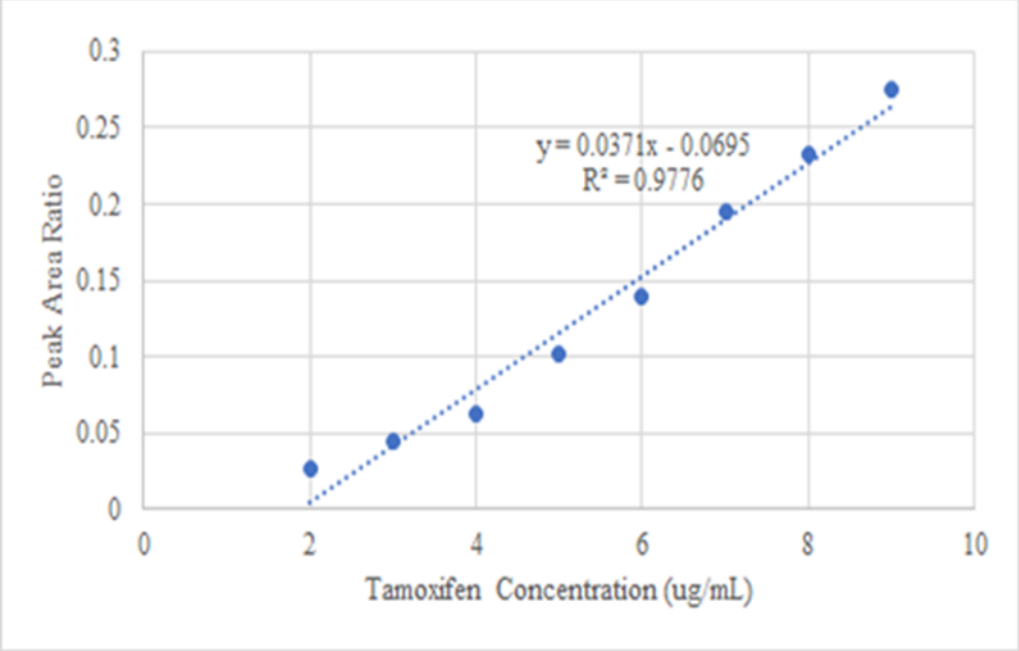


Figure 48 Calibration Curve: Peak Area Concentration.

After creating a calibration curve, a study was done of the regression of statistical parameters of the linearity of this project's developed method. The standard error of the estimate was determined to be a very good value: .014873. It was determined by taking the actual values, making a regression line, and then calculating estimated values. The result is the distance between the estimated and the actual values, where the distances are the errors. Much effort was given to minimize the errors of distance between the actual values and the estimated values. Of note, this formula is similar to the formula for standard. Next, there was determination of the limit of detection (LOD) and the limit of quantitation (LOQ). For calculating the LOD, the error of the estimate was divided by the slope and multiplied by 3.3. The LOQ is calculated by multiplying the LOD by three. Again, for this project, the

determined LOD was 1.22 µg/mL. Below, Table 15 shows the linearity and linear regression parameters of the proposed method results.

Table 15 Regression and Statistical Parameters of the Linearity of the Proposed Method.

a (Y Intercept)	**-0.0695**
b (Slope)	**.037129**
R^2	**.97759**
S_{yx} (Standard Error of the Estimate)	**.014873**
S_y (Standard Deviation of the Intercept)	**.013674**
S_b (Standard Deviation of the Slope)	**.002295**
LOD (Limit of Detection)	**1.22 ug/mL**
LOQ (Limit of Quantitation)	**3.66 ug/mL**

Precision

Table 16 shows the results of the repeatability and reproducibility of this project's proposed method. Two different aspects of this method were analyzed for precision. For intra-day precision, a calibration curve was run using Tamoxifen concentrations ranging from 75 µg/mL to 500 µg/mL – for a total of 8 concentration levels. This process was performed another two times within the same day. This provided a total of 24 Tamoxifen samples (3 different batches) which were then tested the same day using all the same supplies, chemicals and instrumentation. For inter-day precision, a total of three calibration curves were made, each using the same 8

Tamoxifen concentration levels but done over a three consecutive day period. Again, the same supplies, chemicals and instrumentation was used but the three "batches" of Tamoxifen samples were made and used on different days. The standard deviation is calculated; the smaller the standard deviation, the higher the degree of precision. Also, close agreement needs to occur when the developed method is repeatedly applied, even by different analysts but under the same conditions (e.g. comparable equipment).

Table 16 Precision of the Proposed Method.

Precision of the Proposed Method		
Concentration (µg/mL)	**Intra-day Precision (%RSD)**	**Inter-day Precision (%RSD)**
75	7.927	0.827
100	1.407	2.577
150	1.986	3.151
200	1.156	3.251
250	0.792	0.316
300	1.510	2.659
400	1.058	7.596
500	1.296	5.602
Mean	1.446	1.677

Accuracy

Accuracy of an analytical method is different than precision. Precision is a parameter involving repeatability of tests and getting replicated results. Accuracy is a parameter which measures the degree of closeness of the test results to the true values. The new method is performed on multiple samples containing known amounts of an added analyte (i.e. a minimum of nine samples in total with three different

concentrations with three run times each). The percentage of analyte recovered from each sample is then calculated and recorded, and the difference between the mean value of the recovery percentages and the accepted true value is then divided by the known value and multiplied by 100% to be reported as "percent error." Table 17 shows the results of the accuracy testing of the proposed method.

Table 17 Accuracy of the Proposed Method.

Accuracy of the Proposed Method	
Concentration (µg/mL)	**Percent Error (%)**
75	0.888
	3.635
	0.221
100	13.422
	4.669
	3.360
300	23.392
	12.809
	22.844

7.3 Solvent Partitioning

Testing for Matrix Effects

Investigation of matrix effect for this project was warranted because recovery of Tamoxifen exceeded 100%. Researching possible explanations for this, it was common to find that recovery limits are very important, but that reproducibility of the method is a more important piece of analytical criteria. It was common to read that 100% is an ideal recovery with acceptability typically ranging between 80% and 120%. Repeatedly, on-line sources pointed to "industry" guidelines issued by the U.S. Food and Drug Administration that supports:

> "[optimizing] the recovery of the analyte to ensure that the extraction is efficient and reproducible. Recovery need not be 100 percent, but the extent of the recovery of an analyte and the ISs should be consistent and reproducible" (Bioanalytical Method Validation Guidance for Industry, 2018).

Matrix effect on an analytical method occurs when the matrix (in this project, artificial oral fluid) – not the target analyte – has an influence on the quality of results. It can explain why an analyte responds differently when analyzed in different solutions or in different biological matrices (Øiestad, Johansen, & Christophersen, 2007). The matrix may contain components that interfere with (and possibly enhance) an analyte's signal. In this project, matrix effect is the difference between response for Tamoxifen in standard solution and its response at the same concentration in artificial oral fluid. Matrix effect can negatively affect method validation (i.e. limit of detection, accuracy, etc.); it is important to look at whether matrix effect has occurred. To find evidence of any matrix effect for this project, two experiments that tested extraction of Tamoxifen in artificial oral fluid as well as Tamoxifen in standard solution were performed. Within each experiment, three trials were run with three different concentrations (high, medium and low) of Tamoxifen. Visually, as seen in Figure 49 and Figure 50 there appears to be minimal difference in signals for all Tamoxifen concentrations between both the artificial OF and standard solution.

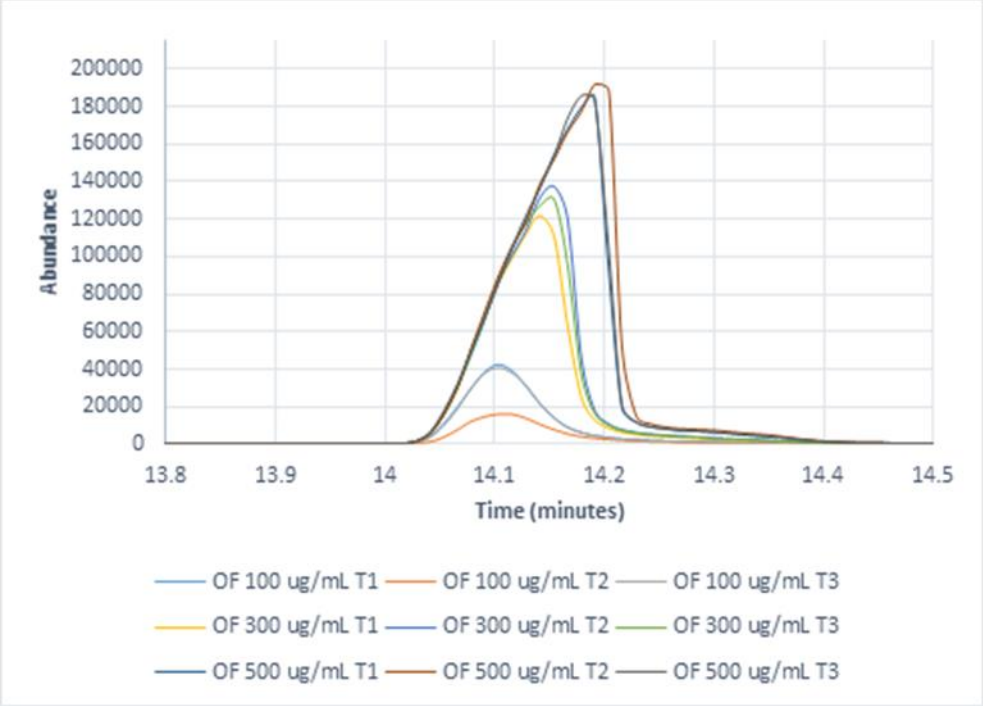


Figure 49 Extraction with Oral Fluid.

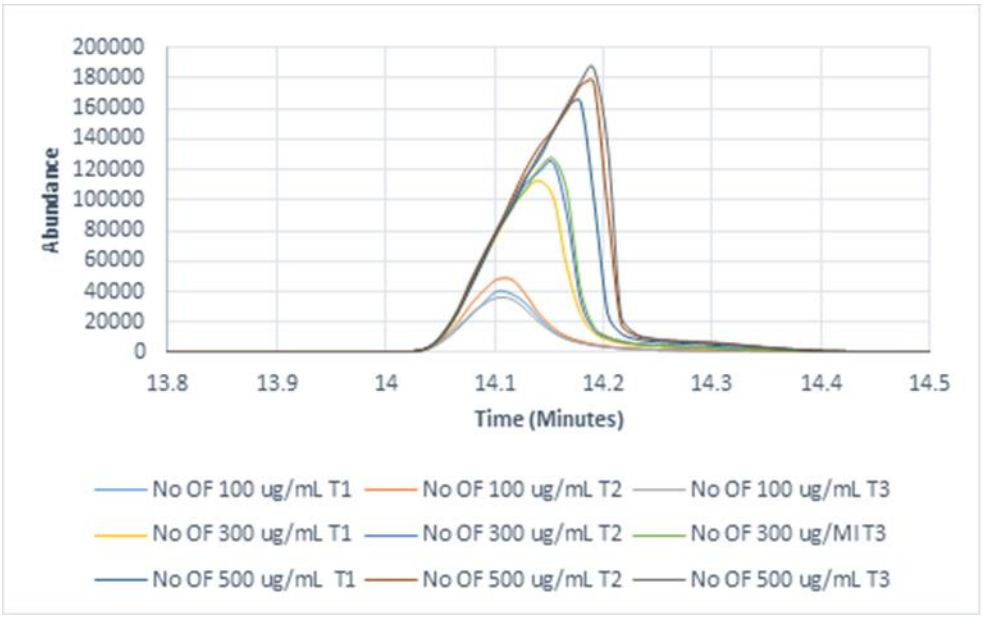


Figure 50 Extraction without Oral Fluid.

However, by comparing the analyte signals by peak area ratios in the samples made with OF and made without OF, there was significant difference. Normally, the explanation for ion enhancement is to look at co-eluting analytes or contamination. Figure 51 clearly shows evidence of ion enhancement, but there were no additional analytes run. While it remains unclear the cause of the ion enhancement when using oral fluid, this could be further investigated in future work.

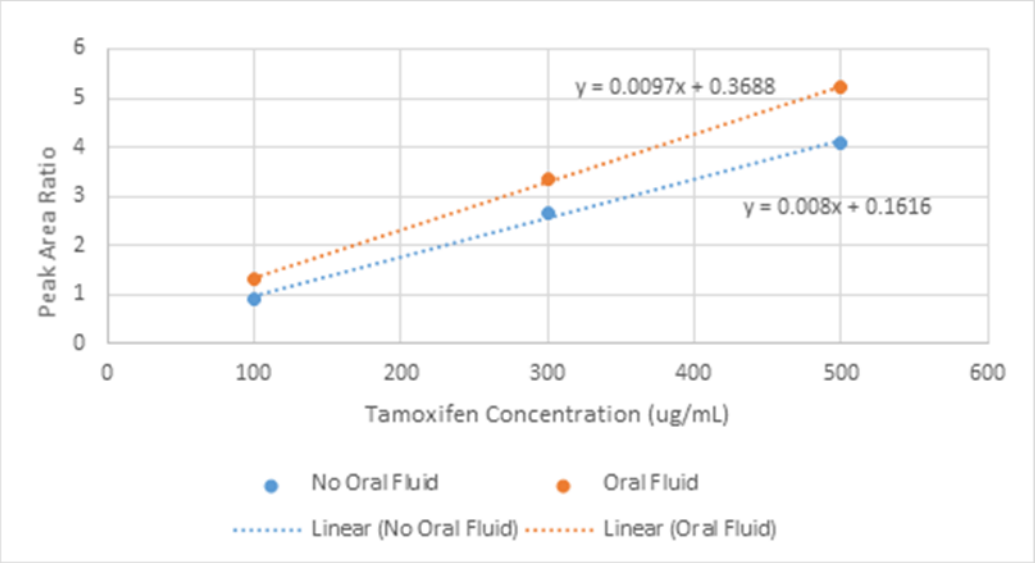


Figure 51 Matrix Effects Calibration Curve.

In order to compensate for ion enhancement that appears to have been a matrix effect, percent recovery was calculated after an evaluation. Mentioned earlier, this evaluation consisted of spiking artificial saliva with Tamoxifen at different concentration levels (three trials using high, medium and low concentration), then completing the extraction process (utilizing 2 mL ethyl-acetate and vortexing). The same process was completed with stock solution containing Tamoxifen and methanol so that samples without OF could be run and used for comparison. For all samples,

stock solution with measured Tamoxifen levels (100, 300 and 500 µg/mL concentrations) was pipetted into test tubes. Twice, the ethyl-acetate was evaporated from each test tube but gently so that Tamoxifen would accumulate on the inner sides. Extraction of Tamoxifen was then completed for both OF samples and non-OF samples for comparison purposes. GC analysis was done for each extraction, and results were plotted on a graph. Very clearly, the graph shows ion enhancement (signals) for Tamoxifen extracted from saliva in comparison with detection of Tamoxifen in the stock solution. See Table 18 for matrix effects data and calculations. Equation 13 illustrates how to calculate matrix effect (Quantitative Estimation of Matrix Effect, Recovery and Process Efficiency, 2020):

$$\boldsymbol{ME = \frac{S_{OF} - S_{no\ OF}}{S_{no\ OF}} \times 100\%}$$

Equation 13 Calculating Matrix Effect by Signals.

Where:
S = peak area Signal
OF = Tamoxifen in Oral Fluid
No OF = Tamoxifen in solvent only

Table 18 Matrix Effects Calculations.

MATRIX EFFECTS			
	Tamoxifen Concentration (µg/mL)		
	100	**300**	**500**
No Oral Fluid Average Peak Area Ratio	0.911	2.640	4.092
No Oral Fluid Standard Deviation	0.065	0.221	0.212
Oral Fluid Average Peak Area Ratio	1.314	3.348	5.210
Oral Fluid Standard Deviation	0.230	0.128	0.145
Number of Trials (N)	3	3	3
Total Degrees of Freedom	4	4	4
S_{pooled}	0.169	0.181	0.182
t_{calc}	2.925	4.804	7.531
t_{value}	2.132	2.132	2.132
Matrix Effect (%)	44.237	26.826	27.330

Solvent Selection

The extraction recovery of Tamoxifen from saliva utilizing six different solvents was determined. For a total of 6 test tubes, 1 mL of artificial oral fluid fortified with Tamoxifen at a concentration of 500 µg/mL was added to each. Two mL of the utilized organic solvent (benzene, cyclohexane, hexane, methyl-ethyl-ketone, ethyl-acetate or

chloroform) was added to the 1 mL of fortified OF, and the test tube was vortexed for 10 seconds to allow for mixing of the aqueous OF and organic solvent. The test tubes were then set aside for 30 minutes as the target analyte partitioned between the two phases. After partitioning, the organic solvent layer of each test tube was pipetted off and placed in separate test tubes. The organic solvents were evaporated off by way of a hot water bath and the contents of each test tube was reconstituted with 800 µL of HPLC grade methanol. These 800 µL were transferred to a GC/MS autosampler vial. 200 µL of a 1 mg/mL stock solution of the internal standard Propranolol were added to the autosampler vial to obtain a total volume of 1 mL. Each extraction was analyzed using GC/MS (this project's optimized GC/MS method) to obtain the analyte to internal standard peak area ratio to then calculate the amounts of Tamoxifen recovered by way of a calibration curve. This entire process was completed a total of 3 times for each organic solvent. Figure 52 illustrates the amounts of Tamoxifen recovered per solvent with Ethyl Acetate having an approximate average Tamoxifen recovery of 435 µg and demonstrating the best solvent efficiency.

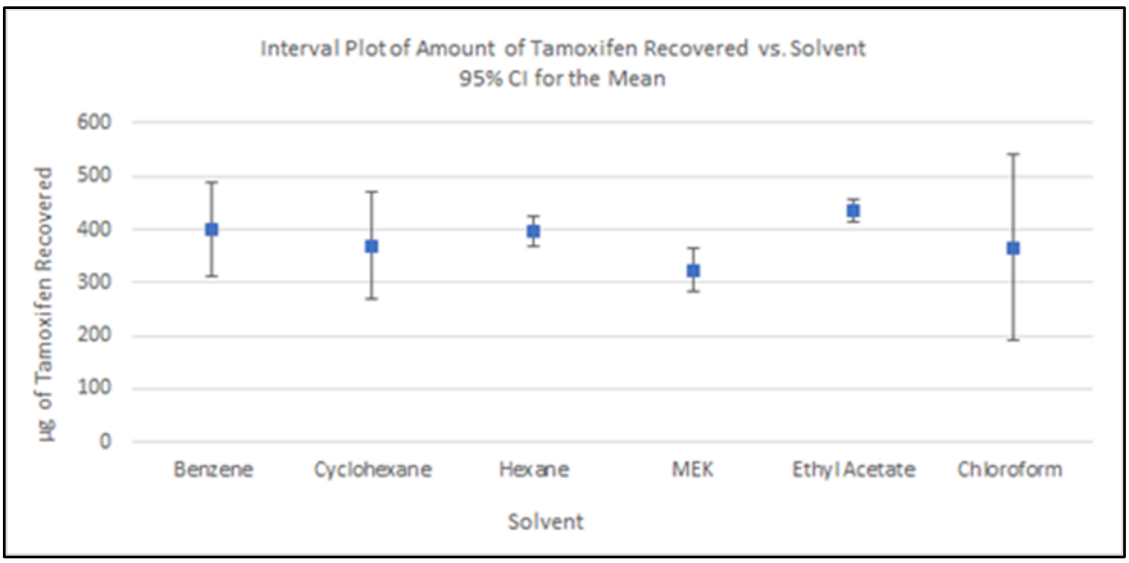


Figure 52 Extraction Efficiency: Solvents.

Statistical Analysis: Solvents

One-Way ANOVA Results

Table 19 shows the outcome of the performed One-Way ANOVA analysis regarding statistical difference between the group means (the extraction solvents). The significance value was 0.138 which is larger than the 0.05 significance level, resulting in no statistical difference among solvents. This supports not rejecting the Null Hypothesis and concluding that all means are coming from the same statistical population.

Table 19 One-Way ANOVA Results: Solvents.

Summary						
Solvent	Count	Average µg of Tamoxifen Recovered				
Benzene	3	400.2242188				
Cyclohexane	3	368.6781949				
Hexane	3	395.9653469				
Methyl Ethyl Ketone	3	323.2675058				
Ethyl Acetate	3	435.0244882				
Chloroform	3	365.3188681				
ANOVA						
	SS	df	MS	F	F crit	Significance
Between Groups	21725.76	5	4345.152	2.083976	3.105875	0.138024088
Within Groups	25020.36	12	2085.03			
Total	46746.11	17				

Tukey Honest Significant Difference Test

Although the ANOVA result showed no statistical difference among solvents, personal observation served as basis for completing the Tukey. Table 20 shows the absolute difference of means for all solvent comparisons which were used to calculate the q_{Tukey} value and compared to the $q_{critical}$ value located on a Q table. Because the $_{qTukey}$ value of the Methyl Ethyl Ketone and Ethyl Acetate comparison (5.995) was greater than the $q_{critical}$ value of 4.750, the null hypothesis is rejected and the means are considered to be significantly different.

Table 20 Tukey HSD Results: Solvents.

Tukey HSD				
Comparison	Absolute Difference	q_{Tukey}	$q_{critical}$	Means different?
Benzene/Cyclohexane	31.546	1.692	4.750	No
Benzene/Hexane	4.259	0.228	4.750	No
Benzene/MEK	76.957	4.128	4.750	No
Benzene/Ethyl Acetate	34.800	1.867	4.750	No
Benzene/Chloroform	34.905	1.872	4.750	No
Cyclohexane/Hexane	27.287	1.464	4.750	No
Cyclohexane/MEK	45.411	2.436	4.750	No
Cyclohexane/Ethyl Acetate	66.346	3.559	4.750	No
Cyclohexane/Chloroform	3.359	0.180	4.750	No
Hexane/MEK	72.698	3.900	4.750	No
Hexane/Ethyl Acetate	39.059	2.095	4.750	No
Hexane/Chloroform	30.646	1.644	4.750	No
MEK/Ethyl Acetate	111.757	5.995	4.750	Yes
MEK/Chloroform	42.051	2.256	4.750	No
Ethyl Acetate/Chloroform	69.706	3.739	4.750	No

Distribution Coefficient and Number of Extractions

By changing the solvent, it was investigated to see how the distribution coefficient is affected (the ratio of concentration of analyte in the solvent to the concentration of analyte in the artificial saliva). This is a measurement of which solvent proved to be the one to which Tamoxifen had the most affinity (the solvents ranged from 0.1 to 4.9 polarity). It was also explored using the distribution coefficient equation (Equation 14) to see if adding a buffer at a constant pH affected the distribution coefficient.

$$K = \frac{C_2}{C_1} = \frac{\left(\frac{mass_2}{volume_2}\right)}{\left(\frac{mass_1}{volume_1}\right)}$$

Equation 14 Distribution Coefficient Equation.

The solvent ethyl-acetate was selected because it led to the highest mass of Tamoxifen in the organic layer. Its polarity is 4.4, and it had the highest percent recovery. The calculated average distribution coefficient was 3.413.

Extraction Efficiency

To calculate the percentage of Tamoxifen that had partitioned into the chosen solvent Ethyl Acetate, the following extraction efficiency equation (Equation 15) was applied (Harvey, 2020):

$$(q_{aq})_1 = \frac{V_{aq}}{KV_{org} + V_{aq}}$$

Equation 15 Extraction Efficiency Equation.

Where: V_{aq} is volume of aqueous sample, K is the distribution coefficient, V_{org} is the volume of organic solvent utilized in the extraction, and q_{aq} is the fraction of analyte remaining in the aqueous sample.

For the fraction of Tamoxifen remaining in the aqueous sample, on average, q_{aq} was calculated to be 0.128. To then solve for the average extraction efficiency, q_{aq} was subtracted from 1.0 to get 0.872 (or 87.2% extraction efficiency). Although this is a fair extraction efficiency, some Tamoxifen still remains in the artificial oral fluid. To have successfully extracted 99.9% of the Tamoxifen, it was determined that approximately 293 mL of Ethyl Acetate would have been needed for just one extraction to be performed. To find the minimum number of extractions necessary for achieving 99.9% extraction efficiency using 2 mL of Ethyl Acetate, the extraction equation is modified to accommodate multiple extractions (Equation 16):

$$(q_{aq})_n = (\frac{V_{aq}}{KV_{org} + V_{aq}})^n$$

Equation 16 Extraction Efficiency Equation: Multiple Extractions.

Where: n is the number of performed extractions.

Solving for "n" required setting q_{aq} to 0.001 (meaning 99.9% is extracted). With 1 mL artificial oral fluid, the distribution coefficient of 3.413 and the 2 mL of Ethyl Acetate (for K and V_{org}), it was determined a minimum of 4 extractions (3.36) would be necessary for achieving 99.9% extraction efficiency.

Buffer Selection

Five buffers at pH levels ranging from 4 to 8 were tested to determine which pH level afforded the highest amount of Tamoxifen recovered. Again, 1 mL of artificial oral fluid fortified with Tamoxifen at a concentration of 500 µg/mL was utilized. One mL of a buffer at a specified pH (pH 4-8) was added to the OF sample, and vortexing was done for 10 seconds. Then, 2 mL of ethyl-acetate was added in order for solvent partitioning to occur. After the addition of the organic solvent, the sample was vortexed for 10 seconds for thorough mixing and set aside for 30 minutes to allow for Tamoxifen to partition between the two phases. After the allotted time had passed, the ethyl-acetate layer was transferred to a separate test tube. The ethyl-acetate was then evaporated off and the target analyte was reconstituted with 800 µL of HPLC grade methanol. The 800 µL were transferred to a GC/MS autosampler vial. 200 µL of a 1 mg/mL stock solution of the internal standard Propranolol were added to the autosampler vial to obtain a total volume of 1 mL. Each extraction was analyzed using

GC/MS (this project's optimized GC/MS method) to obtain the analyte to internal standard peak area ratio to then calculate the amounts of Tamoxifen recovered by way of a calibration curve. This entire process was completed a total of 3 times for each pH buffer. Of the five buffers, none were determined to benefit method optimization. The results can be seen for each buffer in Figure 53. In future work, buffers with pH levels above Tamoxifen's pK_a value of 8.85 will need to be explored. The higher pH values would leave Tamoxifen mostly in its neutral form, leading to improved transfer to the organic solvent layer. This would prove beneficial to the sample preparation procedure.

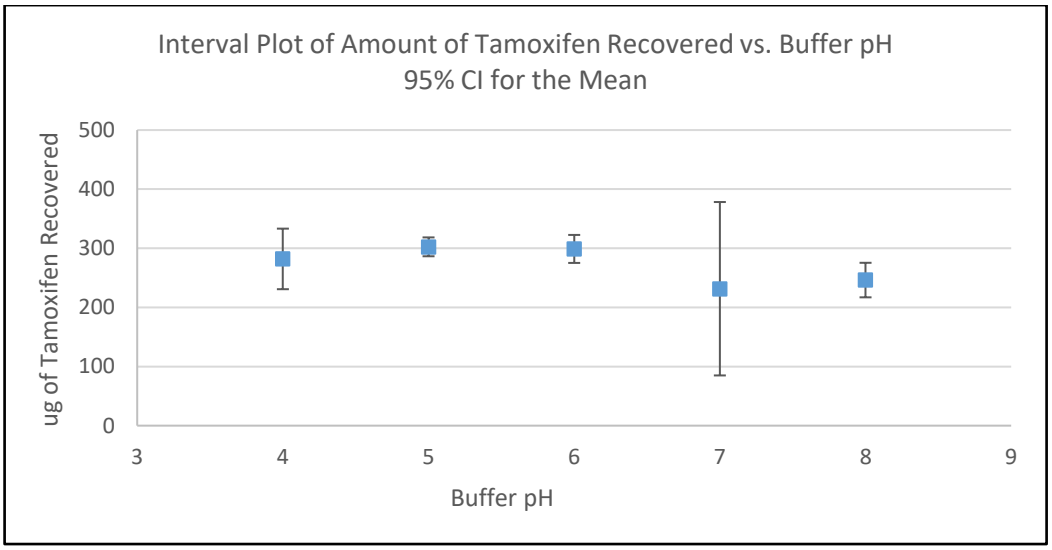


Figure 53 Extraction Efficiency: Buffer pH.

Statistical Analysis: Buffer pH

One Way ANOVA Results

Table 21 shows the outcome of the performed One-Way ANOVA analysis regarding statistical difference between the group means (the buffers). The

significance value was 0.117 which is larger than the 0.05 significance level, resulting in no statistical difference among buffers. This supports not rejecting the Null Hypothesis and concluding that all means are coming from the same statistical population.

Table 21 One Way ANOVA Results: Buffer pH.

Summary						
pH	**Count**				**Average ug of Tamoxifen Recovered**	
4	3				282.0572445048	
5	3				302.4965777823	
6	3				298.9464309899	
7	3				231.6144331698	
8	3				246.2893258642	
ANOVA						
	SS	**df**	**MS**	**F**	**F crit**	**Significance**
Between Groups	12146.81	4	3036.704	2.420739	3.47805	0.117109094
Within Groups	12544.53	10	1254.453			
Total	24691.34	14				

Tukey Honest Significant Difference Test

Although the ANOVA result showed no statistical difference regarding buffer pH, the Tukey was completed to see if it (like the solvents) would in fact reveal some level of significant differences. Table 22 shows the absolute difference of means for all buffer comparisons which were used to calculate the q_{Tukey} value and compared to the $q_{critical}$ value located on a Q table. Because the $_{qTukey}$ value of the buffer pH levels of 5 / 7 comparison (4.475) was greater than the $q_{critical}$ value of 4.410, the null hypothesis is rejected and the means are considered to be significantly different.

Table 22 Tukey HSD Results: Buffer pH.

Tukey HSD				
Comparison	Absolute Difference	q_{Tukey}	$q_{critical}$	Means Different?
pH 4 and 5	20.439	1.290	4.410	No
pH 4 and 6	16.889	1.066	4.410	No
pH 4 and 7	50.443	3.185	4.410	No
pH 4 and 8	35.768	2.258	4.410	No
pH 5 and 6	3.550	0.224	4.410	No
pH 5 and 7	70.882	4.475	4.410	Yes
pH 5 and 8	56.207	3.549	4.410	No
pH 6 and 7	67.332	4.251	4.410	No
pH 6 and 8	52.657	3.324	4.410	No
pH 7 and 8	14.675	0.926	4.410	No

8. RESULTS SUMMARY

Table 23 briefly summarizes optimization factors for each section of this project's method development for detection and determination of Tamoxifen in oral fluid. Overall, the results show that some parts of the process can be seen as "pros" (and either kept for use in the method or optimized further), and some parts of the process can be seen as "cons." The "cons" should not be ignored because trial and error sometimes reveal "what not to do" for optimization of a method. For this procedure, need for exploration of using buffers with higher pH levels was both revealed and confirmed (based on theory).

Table 23 Method Optimization Results.

Summary: Optimal Techniques, Values and Rates	
Oral Fluid Collection	Free drool method • Enables ion-trapping of Tamoxifen to achieve highest concentration levels
Sample Preparation	Solvent Partitioning • Avoids analyte loss / very simple and can result in a good extraction efficiency of a pure analyte Ethyl-acetate was extraction solvent of choice • Its calculated recovery of Tamoxifen from OF was highest No Selected Buffer • No effect on Efficiency
GC/MS Analysis	HP-5MS UI column • Readily available, rugged (can withstand high temperatures) Splitless Hold Time: 0.8 minutes • Splitless Injection Method avoids sample loss SIM Mode • Ions monitored: 52, 78, 259, 371 m/z • Increased Sensitivity **Ramp 1**: Value 160℃ and Rate 80 ℃/Min (Hold 3 min) **Ramp 2**: Value 260℃ and Rate 25℃/Min (Hold 5 min) **Ramp 3**: Value 290℃ and Rate 10℃/Min (Hold 1 min) • Improved retention time, peak shape, LOD, run time Inlet Temperature: 225℃ • Avoids thermal degradation Sample Volume: 1 µL • Calculated to avoid backflash Column Flow Rate: 4 mL /min • Limit of Detection was important

It should be said again that a main goal for this project was to utilize common laboratory instrumentation, equipment and supplies to develop a method that was intentionally simple but as highly sensitive as possible. It would definitely be very challenging: Tamoxifen is not a notable drug with a lot of method development research to use as resources. Most oral fluid drug testing research has been done for drugs such as cocaine and opioids – not a drug like Tamoxifen that some athletes sometimes use. That said, it turned out that this project contributed to OF research

that should prove very valuable for future researchers: one, that ion-trapping of Tamoxifen (and drugs with similar properties) require lower pH levels in saliva to avoid false negative results and to obtain sufficient drug concentration and two, that when sufficient concentration levels of Tamoxifen are in saliva a common solvent partitioning technique works very well for recovery and purity.

Very little research has been done regarding Tamoxifen detection in saliva. With Tamoxifen and other SERMs gaining popularity for use among athletes, it must be a matter of time before there will be specific interest in more oral fluid testing of athletes. There was no found "comparable" regarding limits of detection of Tamoxifen in oral fluid; only information regarding Tamoxifen amounts in urine of cancer patients could be found in multiple resources and only one research article could be found all together that showed Tamoxifen amounts in saliva. Even this one article was not a "comparable" because the saliva samples had been obtained from stimulated collection and the researchers did not explore sample pH. Analysis had also been performed after derivatization and using LC/MS – with detected amounts ranging from 0.6 ng/mL to 14.6 ng/mL (11 cancer patients) (Lien, et al., 1989).

This project's LOD was 1.22 μg/mL – not nearly low enough for the method to be applied to "real world" circumstances at this time, but definitely low enough to have excellent potential for additional optimization bringing the number down considerably. The "pros" for this method include good recovery, acceptable run time, and of course the use of GC/MS instead of more costly instrumentation. Ideas for this optimization would be to first select a slightly more polar column with a decreased film

thickness so that Tamoxifen (somewhat polar) analytes get sharper peaks and the signal to noise ratio is increased (meaning, a higher limit of detection). An internal standard choice would be to use deuterated Tamoxifen. This IS would behave more similarly to Tamoxifen than Propranolol (same functional groups and similar boiling point) and improve the accuracy and precision of the method. Higher pH levels for buffers would also be explored, anticipating that Tamoxifen would be mostly present in its un-ionized form and more readily transfer to the organic layer. Continued use of splitless injection would remain optimal and using a splitless GC inlet liner that tapers (or that is created with a complex flow path) and has wool would enhance vaporization of the larger molecules (i.e. Tamoxifen) due to larger surface area and the liner's high heat capacity. Hold times within the temperature programming aspect of this project's method would be optimized, and very definitely, use of derivatization prior to GC analysis would be done in order to increase the volatility of Tamoxifen (non-volatile) and get a lower limit of detection.

9. RESULTS AND DISCUSSION: Project Two

Discrimination Between Regioisomeric Aromatase Inhibitors

9.1 Mass Spectrometry

With the regioisomeric aromatase inhibitor 5-alpha-Androstan-17-one, a ketone, the base peak is the molecular ion (274.3 m/z) as seen in Figure 54.

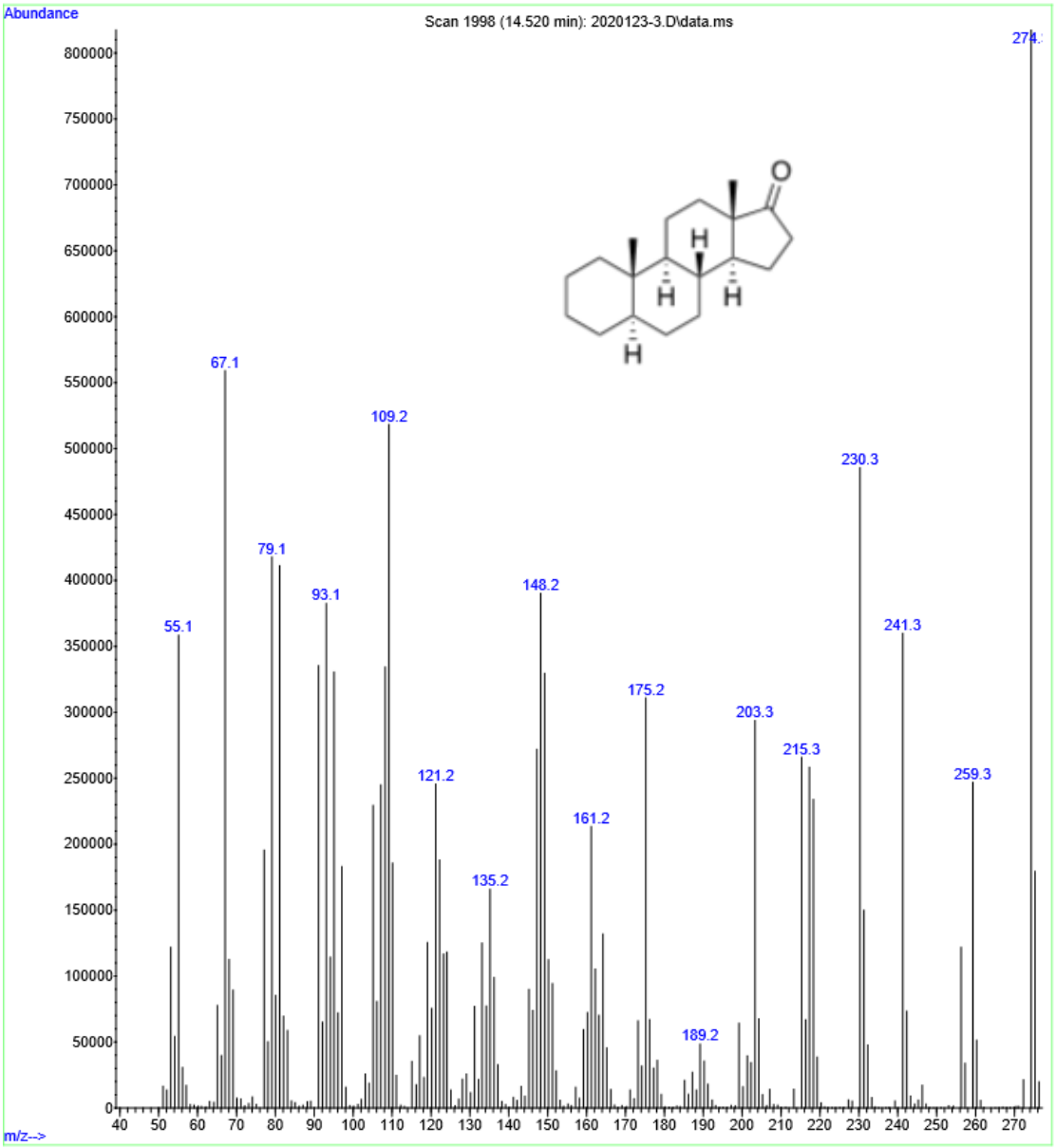


Figure 54 Mass Spectrum of 5-alpha-Androstan-17-one.

Its ring structure makes it a cyclic ketone, which means major cleavage is at the α bond. Unless another bond is broken, the cleavage gets detected as the molecular ion (Fragmentation and Interpretation of Spectra , 2017, p. 34). Figure 55 illustrates the ionized molecule's appearance after the α cleavage.

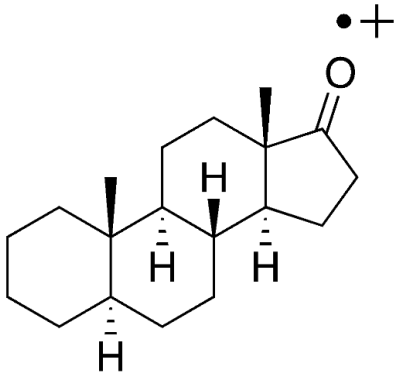


Figure 55 5-alpha-Androstan-17-one Molecular Ion.

After the homolytic α cleavage and the formation of the radical on the steroid ring, a heterolytic cleavage occurs and results in elimination of carbon monoxide (loss of 28 mass units) and formation of the distonic fragment cation at m/z 246 (Figure 56).

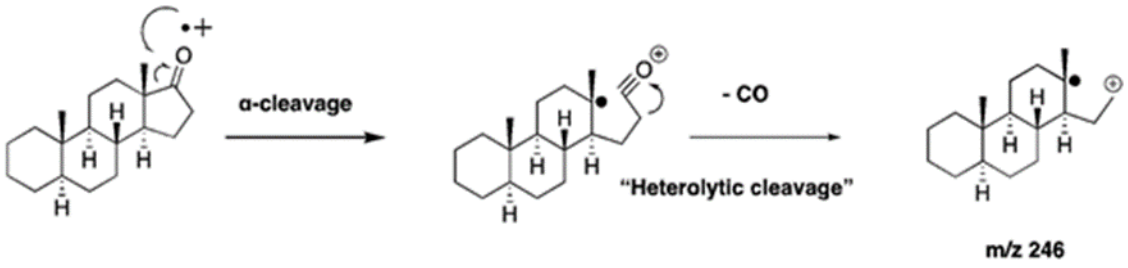


Figure 56 Mechanism of Formation of the Fragment at m/z 246.

With the regioisomeric aromatase inhibitor 5 alpha-Androst-16-en-3α-ol, an alcohol, the base peak is 148.2 m/z as seen in Figure 57.

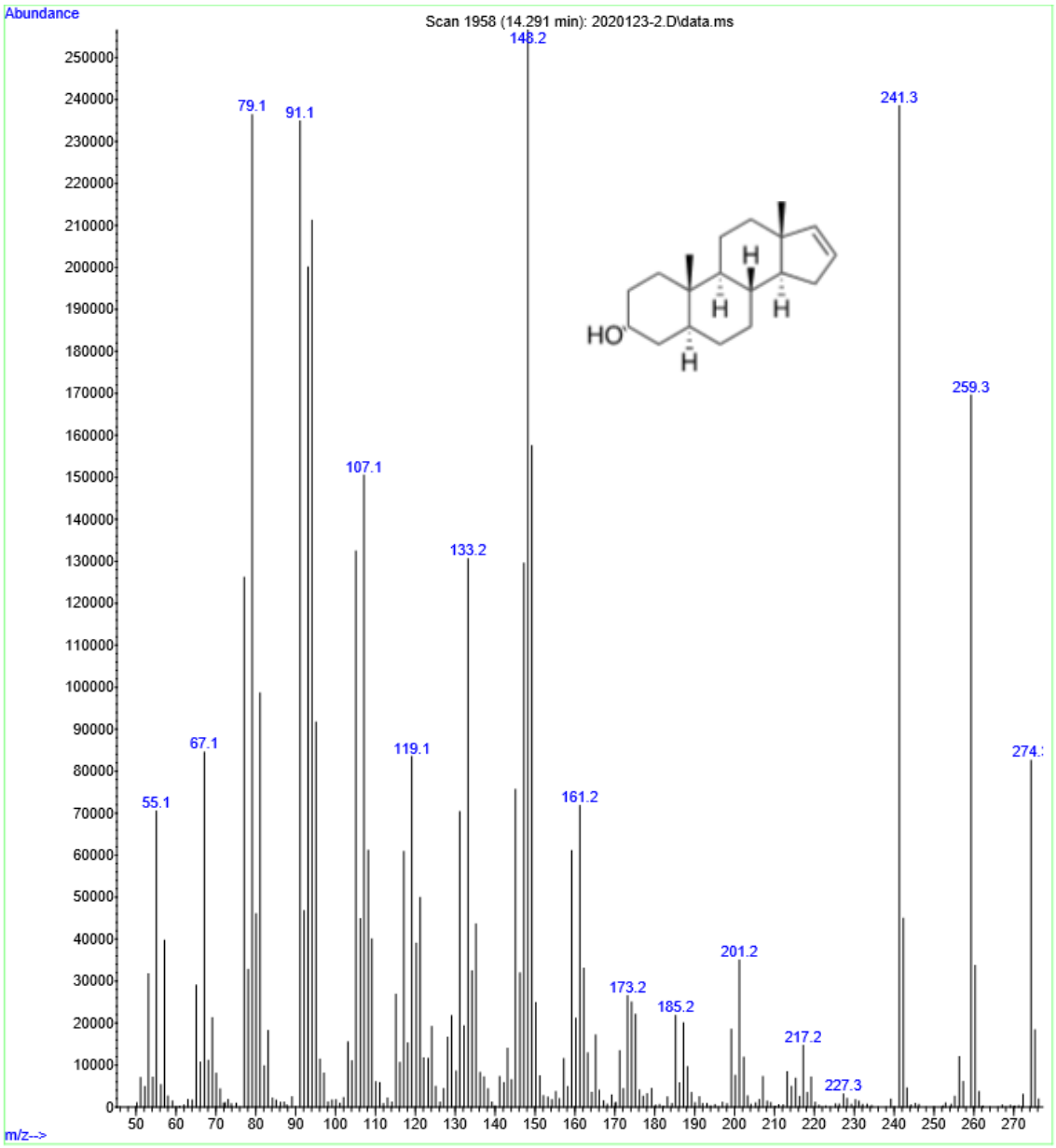


Figure 57 Mass Spectrum of 5 alpha-Androst-16-en-3α-ol.

With the regioisomeric aromatase inhibitor 17-beta-Hydroxy-5-alpha-androst-2-ene, which consists of an alcohol and an alkene, the base peak is the molecular ion (220.3 m/z) as seen in Figure 58.

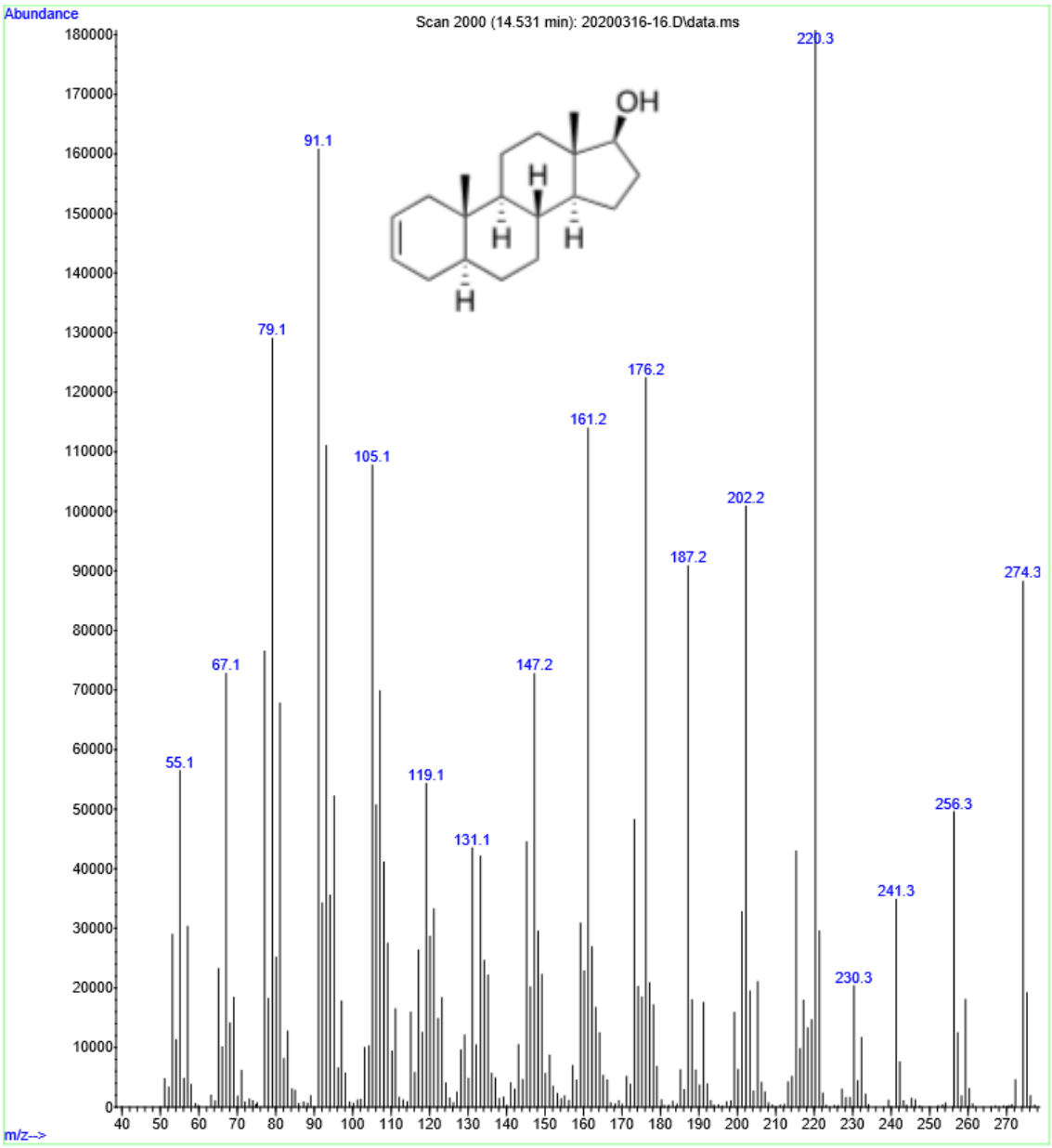


Figure 58 Mass Spectrum of 17-beta-Hydroxy-5-alpha-androst-2-ene.

Among the regioisomer aromatase inhibitors, there are some common fragment ions. Of most significance is that all three share a molecular ion at 274.3 m/z plus fragment ions 55.1 m/z, 67.1 m/z, 79.1 m/z, 148.2 m/z (and 147.2 m/z), 161.2 m/z and 241.3 m/z. Other shared fragment ions between at least two of the AIs include 91.1 m/z, 175.2 m/z (and 176.2 m/z), 230.3 m/z and 259.3 m/z. Regarding ions unique

to each AI, the 5 alpha-Androst-16-en-3α-ol has a unique fragment ion at 107.1 m/z, 133.2 m/z, 173.2 m/z, 185.2 m/z, 201.2 m/z, 217.2 m/z and 227.3 m/z while the 17-beta-Hydroxy-5-alpha-androst-2-ene has a unique fragment ion at 105.1 m/z, 119.1 m/z, 131.1 m/z, 187.2 m/z, 202.2 m/z, 220.3 m/z and 256.3 m/z. The 5-alpha-Androstan-17-one has a unique fragment ion at 93.1 m/z, 109.2 m/z, 121.2 m/z, 135.2 m/z, 175.2 m/z, 189.2 m/z, 203.3 m/z and 215.3 m/z. Shared fragment ions likely share fragmentation pathways, making it extremely difficult to differentiate the regioisomers. However, the abundances of the common ions do have some significant differences, and this is important for differentiation. For 5-alpha-Androstan-17-one, the most abundant ions include 67.1 m/z, 109.2 m/z, 148.2 m/z, 230.3 m/z and 241.3 m/z. The 230.3 m/z represents an [M-44] ion, and the 256.3 m/z represents a loss of water. For 5 alpha-Androst-16-en-3α-ol, the most abundant ions include 79.1 m/z, 91.1 m/z, 148.2 m/z, 241.3 m/z and 259.3 m/z. The 259.3 m/z ion represents the loss of a methyl group; additionally, 241.3 m/z represents the loss of water. For 17-beta-Hydroxy-5-alpha-androst-2-ene, the most abundant ions include 79.1 m/z, 91.1 m/z, 105.1 m/z, 161.2 m/z, 176.2 m/z, 187.2 m/z, 202.2 m/z, 241.3 m/z and 256.3 m/z. The 256.3 m/z ion represents a loss of water; the 241.3 m/z ion represents the additional loss of a methyl group. See Table 24 for a summary of common and unique ions among the studied regioisomeric AIs.

Table 24 Recorded Ions of Regioisomeric AIs.

Common and Unique Ions: Regioisomeric AIs	
Common Ions	274.3, 55.1, 67.1, 79.1, 148.2 (and 147.2 m/z), 161.2 and 241.3 m/z
5-alpha-Androstan-17-one	93.1, 109.2, 121.2, 135.2, 175.2 , 189.2 , 203.3, 215.3 and 230.3 m/z
5 alpha-Androst-16-en-3α-ol	107.1 , 133.2 , 173.2 , 185.2, 201.2 , 217.2 and 227.3 m/z
17-beta-Hydroxy-5-alpha-androst-2-ene	105.1, 119.1, 131.1, 187.2 , 202.2 , 220.3 and 256.3 m/z

9.2 Fourier Transform-Infrared Spectroscopy

Within the scope of this project, interpretation of the FT-IR spectra was done. FT-IR allows for learning which functional groups are within which compounds so that regioisomeric compounds could be differentiated based on differences in their functional groups and substitution patterns. Infrared detection could provide compound specificity without the need for chemical modification of the drug molecule.

Figure 59 shows an overlay of the FT-IR spectra of the three AIs studied. Each compound shows an IR spectrum with absorption bands in the regions 700 - 1700 cm^{-1} and 2700 - 3100 cm^{-1}. In general, variations in the ring substitution pattern with no change in the main skeleton results in variations in the IR spectrum in the region 700 - 1700 cm^{-1} (Kempfert, 1988). Because the three AIs share the same steroid ring backbone, they share almost the same IR features in the region 2700 - 3100 cm^{-1}. However, they can be easily differentiated by the positions and intensities of several IR peaks in the region of 750 - 1750 cm^{-1}.

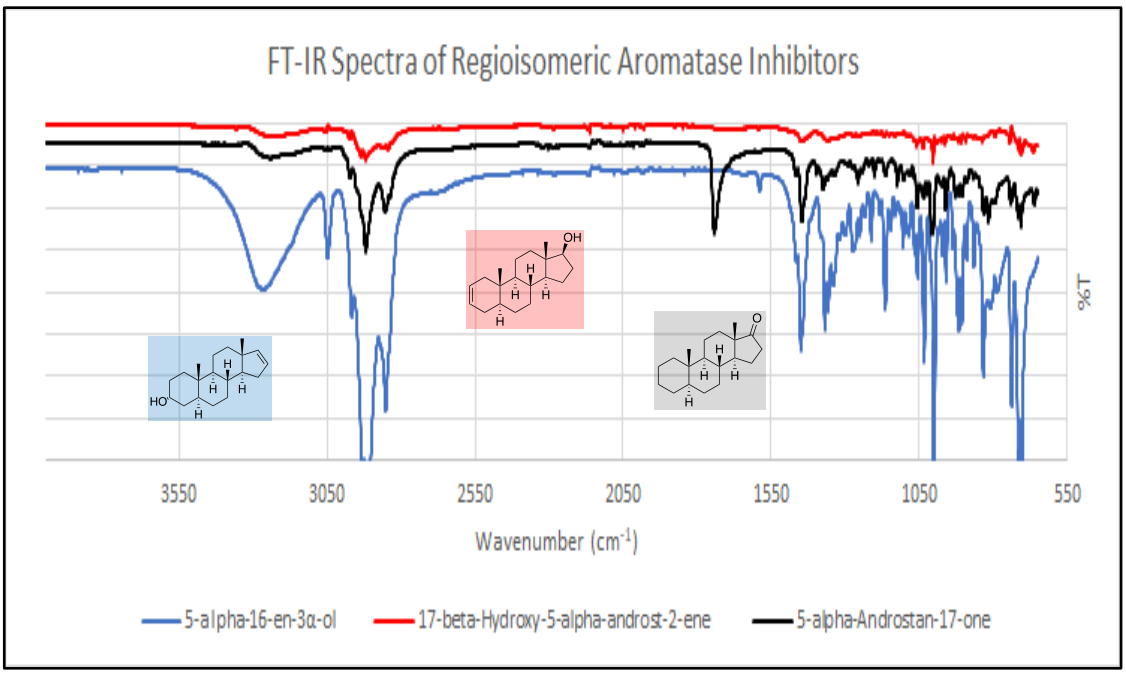


Figure 59 FT-IR Spectra Overlay of Regioisomeric AIs.

In order to analyze the data, the FT-IR spectrum has been divided into regions. The first region encompasses 4000 to 3000 cm^{-1}. O-H stretching is a unique characteristic of alcohols that appears between 3500 and 3200 cm-1 as a very broad and intense band. O-H stretching can be seen in the spectra of 17-beta-Hydroxy-5-alpha-androst-2-ene and 5-alpha-Androst-16-en-3α-ol while this band is absent in the spectrum of 5-alpha-Androstan-17-one. This observation is critical in the differentiation among the three regioisomers. Another significant difference between the spectra is the presence of peaks at slightly higher than 3000 cm^{-1} that are indicative of -C=C-H stretching vibrations. The spectrum of 5-alpha-Androstan-17-one does not possess this peak due to no sp^2 C-H stretching occurring within the molecule.

The second region encompasses 3000 to 2000 cm^{-1}. All three regioisomers are observed to have strong bands slightly under 3000 cm^{-1} which indicates sp^3 C-H

stretching due to the regioisomers' hydrocarbon skeleton. Absorptions due to C-H stretching come at lower frequency (lower wavenumber) than those of the -C=C-H bond in alkenes.

The third region encompasses 2000 to 1500 cm^{-1}. This region is of particular significance to chemists. Within this region appears the carbonyl stretching vibration band C=O at approximately 1700 cm^{-1}. These peaks are sharp with strong intensities. In the spectra of the three regioisomers in question, it can be observed that 5-alpha-Androstan-17-one possesses a strong peak at 1742.41 cm^{-1}, indicating C=O stretching specific to cyclopentanone. For ketones:

> The wave-number is characteristic of the structural type, and especially of the ring size. The most important distinction is between ketones in the five-membered ring D, which give C=O bands in the region 1,750 - 1,740 cm^{-1}, and those in any of the six-membered rings or in the side chain, which appear in the range 1,720 - 1,700 cm^{-1} (Makin & Gower, 2010, p. 58).

For an unsaturated steroid, C=C stretching bands occur between 1670 and 1625 cm^{-1}. Exactly where the band occurs is dependent upon the location of the C=C. Intensity of the C=C stretching band is increased with conjugation and the addition of polar substituents (Makin & Gower, 2010, pp. 54-55). The 5-alpha-Androst-16-en-3α-ol (belonging to the 16-ene group) has C-C stretching between 1630 and 1621 cm^{-1}, apparent in Figure 60. This band is weak because there is no conjugation. The other regioisomeric AI with an alkene group is 17-beta-Hydroxy-5-alpha-androst-2-ene. It belongs to the 2-ene group and has C=C stretching between 1657 and 1654 cm^{-1}, apparent in Figure 61.

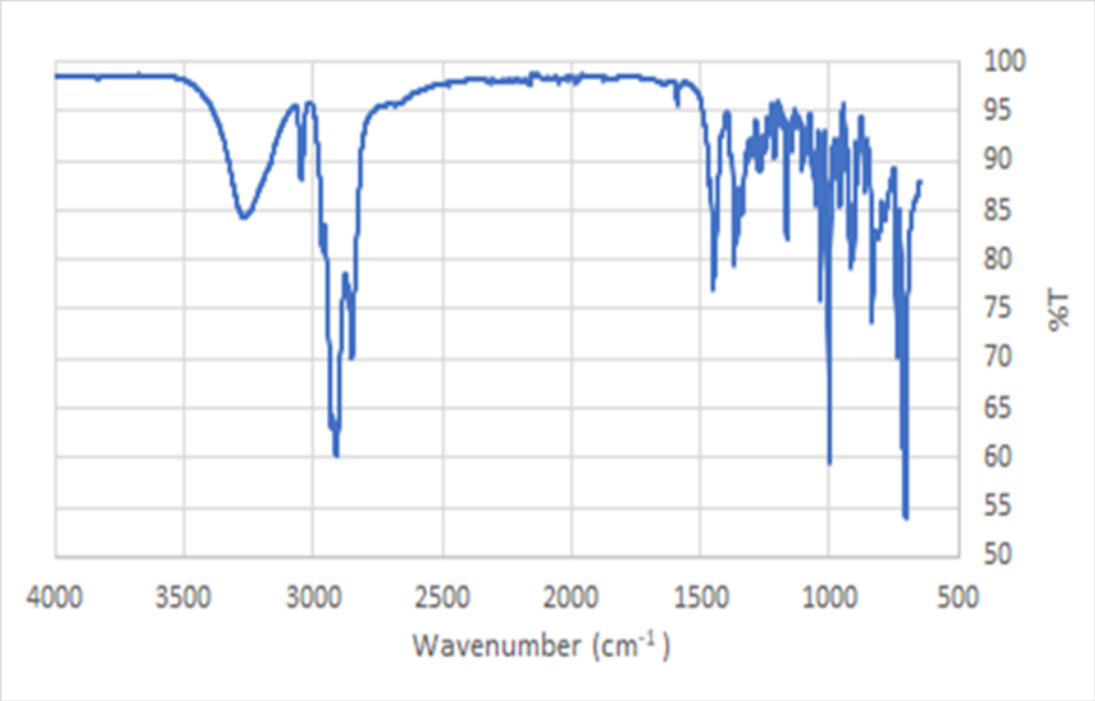


Figure 60 IR Spectrum 5-alpha-Androst-16-en-3α -ol.

Figure 61 IR Spectrum 17-beta-Hydroxy-5-alpha-androst-2-ene.

Region four encompasses 1500 to 1000 cm^{-1}. For all three regioisomers, there are recognizable peaks at approximately 1450 cm^{-1} and 1375 cm^{-1} which indicate the

presence of methyl groups. This is where hydrocarbons give broad and complex peaks due to scissoring or bending vibrations of the methyl groups. For the 5-alpha-Androstan-17-one, these bands occur at 1447.45 and 1375.65 cm^{-1} (apparent in Figure 62) and for 5-alpha-Androst-16-en-3α-ol, these bands occur at 1149.89 and 1368.97 cm^{-1}. Of additional significance is that 5-alpha-Androstan-17-one appears to have a shoulder peak at approximately 1408 cm^{-1} from CH_2-CO scissoring vibrations that identify a ring D ketone (Makin & Gower, 2010, p. 54). For the two regioisomers that possess alcohol groups, O-H bending bands can be seen between 1085 and 1050 cm^{-1}, and C-O stretching consistent with a secondary alicyclic five or six membered ring can be seen.

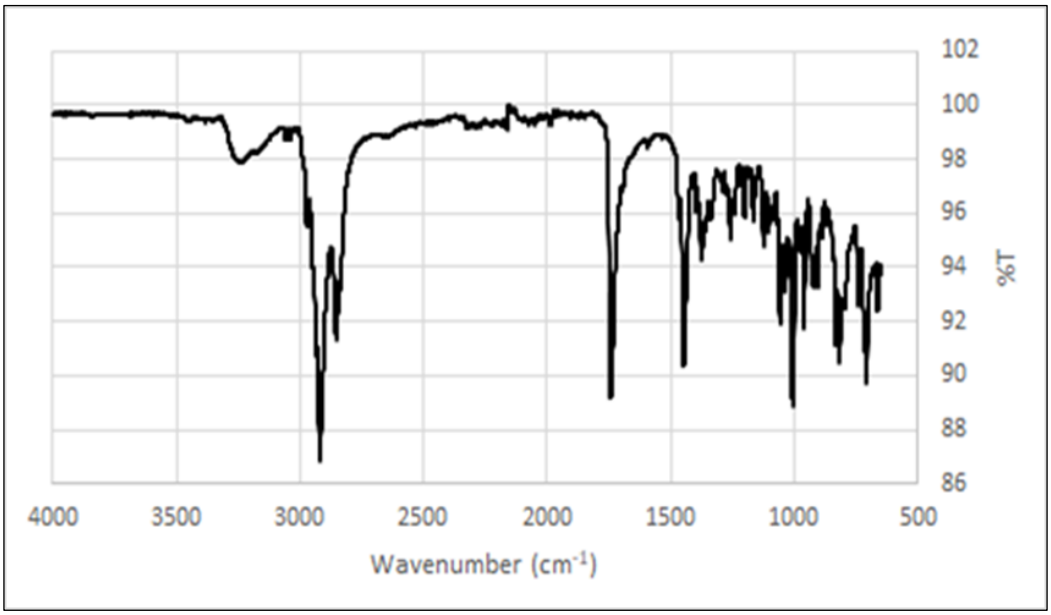


Figure 62 IR Spectrum 5-alpha-Androstan-17-one.

The final region encompasses 1000 to 650 cm^{-1}. The 5-alpha-Androst-16-en-3α-ol has a substantial peak at 714.58 cm^{-1} which is consistent with a 16-ene group for unsaturated steroids. For this group, C-H bending occurs between 715 and 710 cm^{-1}.

Also for this regioisomer, other significant peaks include 958.93 cm^{-1}, 915.81 cm^{-1}, 906.03 cm^{-1}, 833.76 cm^{-1} and 738.10 cm^{-1}. In comparison, the 5-alpha-Androstan-17-one has significant peaks at 960.81 cm^{-1}, 904.82 cm^{-1}, 831.20 cm^{-1} and 741.42 cm^{-1} while having unique peaks at 816.00 cm^{-1} and 706.82 cm^{-1}. Significant peaks for 17-beta-Hydroxy-5-alpha-androst-2-ene include 959.33 cm^{-1} and 830.23 cm^{-1}, while peaks at 849.82 cm^{-1} and 702.69 cm^{-1} are unique to the regioisomer.

9.3 Gas Chromatography

Scouting Gradient

See Figure 63, Figure 64, and Figure 65 – each of which are chromatographs showing the results of the scouting gradients.

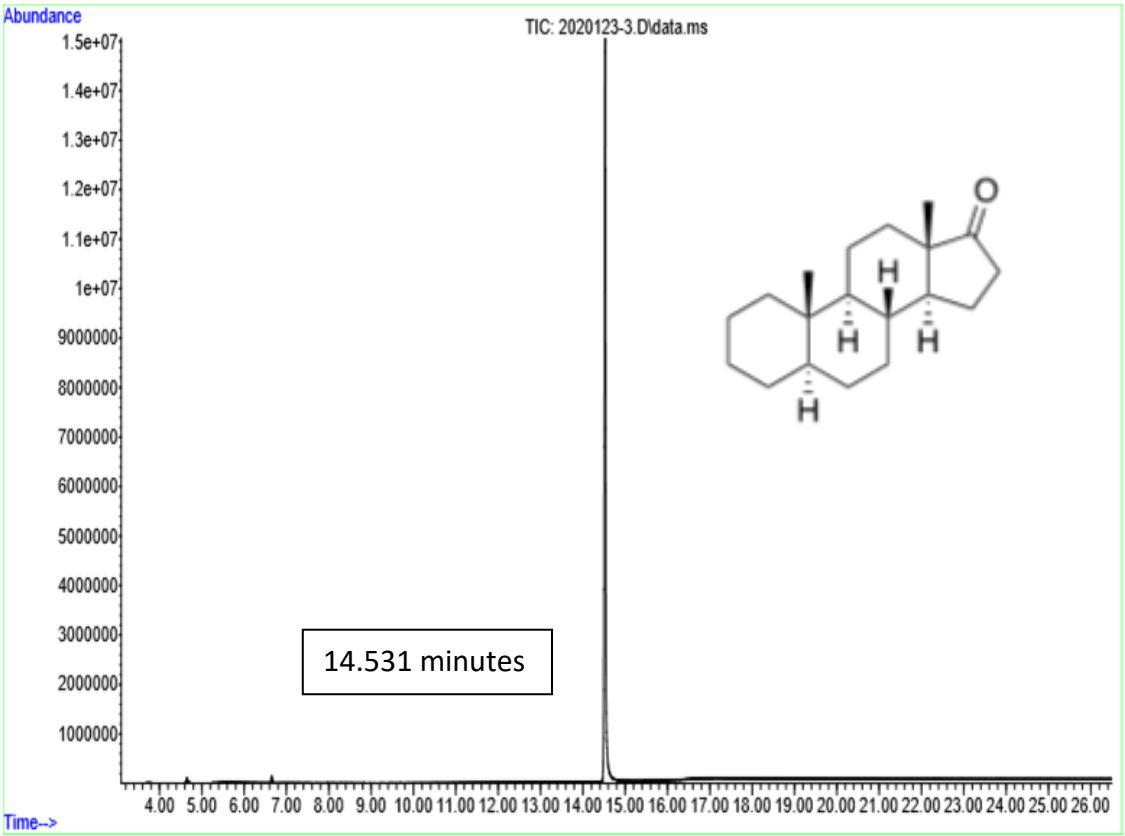


Figure 63 Scouting Gradient 5-alpha-Androstan-17-one.

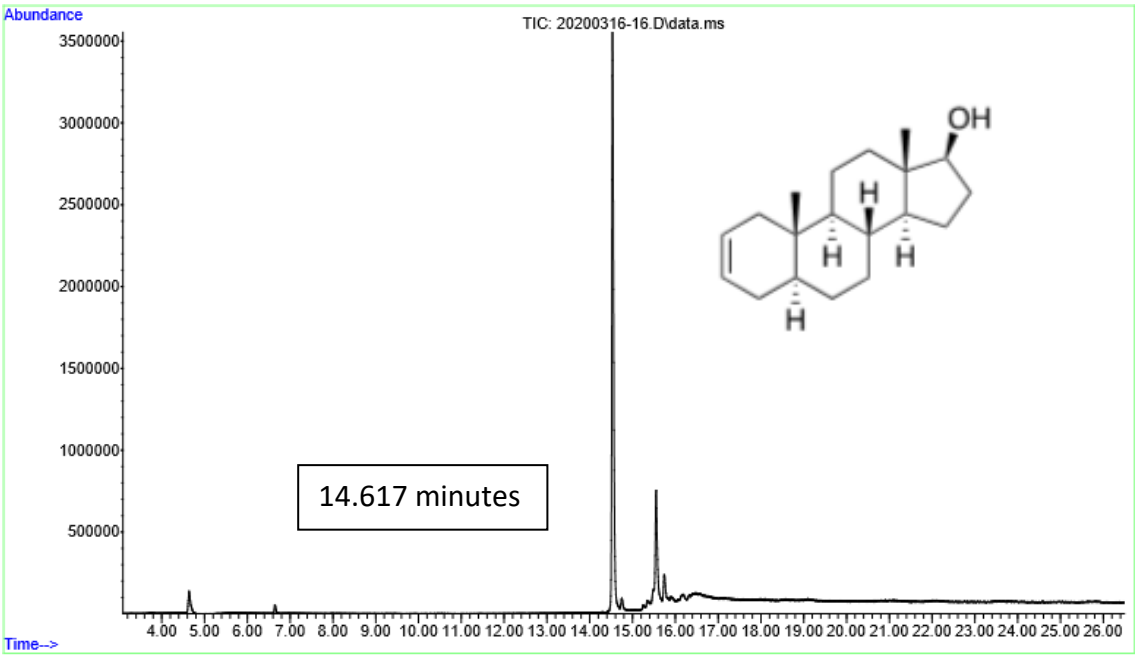


Figure 64 Scouting Gradient 17-beta-Hydroxy-5-alpha-androst-2-ene.

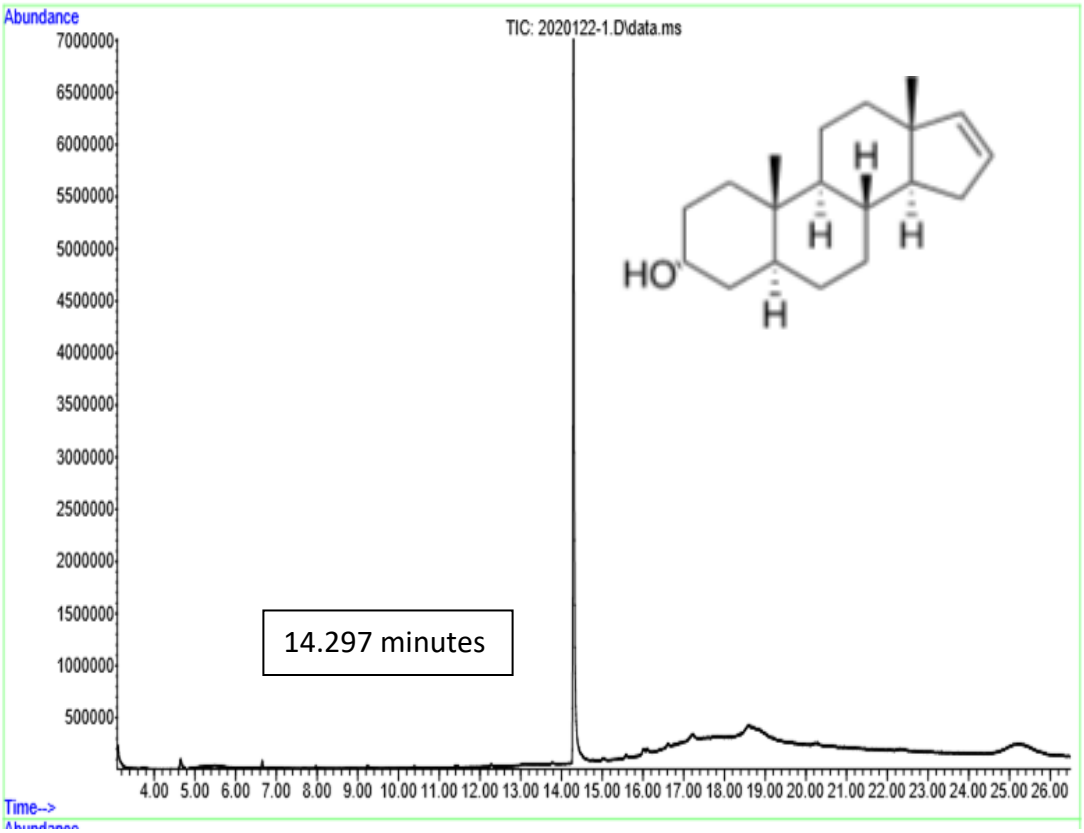


Figure 65 Scouting Gradient 5-alpha-Androst-16-en-3α-ol.

As predicted, the three regioisomers eluted almost simultaneously. It was found that 5-alpha-Androstan-17-one (a ketone) eluted at 14.531 minutes; the 17-beta-Hydroxy-5-alpha-androst-2-ene (an alkene and alcohol, too) eluted at 14.617 minutes; and the 5-alpha-Androst-16-en-3α-ol (an alcohol) eluted at 14.297 minutes. In this case, there is no correlation between the functional groups and the elution order. Further studies of the chromatographic properties and elution of the three AIs need to be done on stationary phases with different polarities.

Chromatographic Resolution

To get the best chromatographic resolution, column temperatures between 190°C and 250°C were explored. Determination of resolution involves accounting for both the amount of peak separation and peak widths while peak separation is simply the distance between two peaks (retention time), so resolution can change when either factor changes. The goal for resolution improvement is to have increased peak separation – more so than an increase in peak width. Figure 66 clearly shows a visual of peak separation occurring for each Ramp 1 Value. Two peaks are visible in each chromatogram with the 5-alpha-Androst-16-en-3α-ol eluting first succeeded by the 5-alpha-Androstan-17-one and the 17-beta-Hydroxy-5-alpha-androst-2-ene, the last two AIs eluting virtually simultaneously. More optimization will need to be completed to obtain baseline resolution between these two AIs. By appearance, 210°C has the best peak separation. For calculated resolution values, see Table 25 and Table 26.

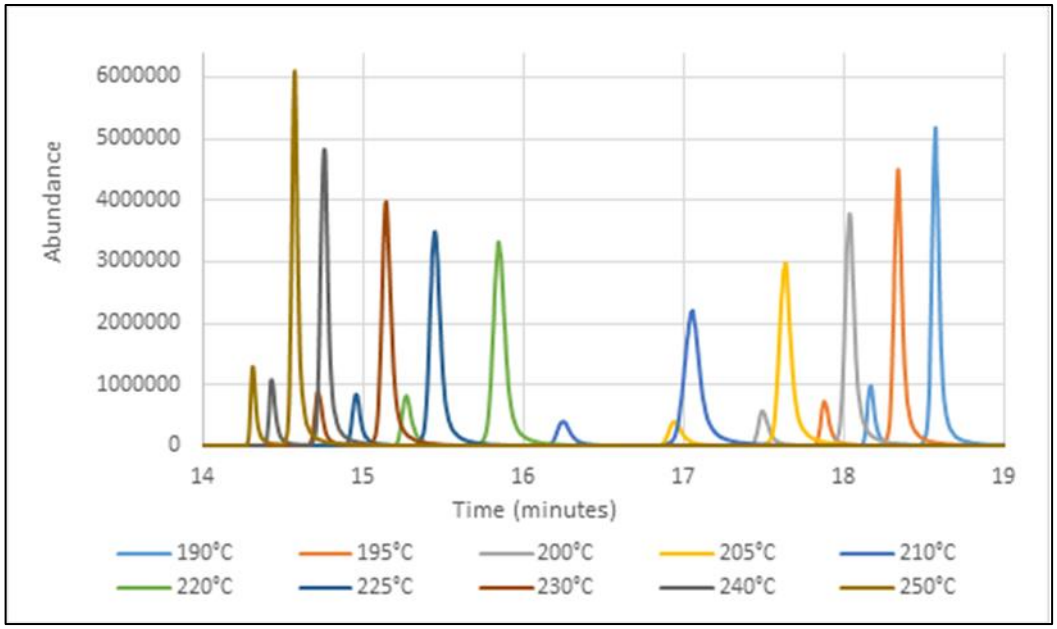


Figure 66 Ramp 1 Value Optimization: Regioisomers.

Table 25 Ramp 1 Value Optimization for Regioisomers.

Ramp 1 Value Optimization for Regioisomers					
Ramp 1 Value (°C)	t_{R1}	t_{R2}	Peak 1 width at half height	Peak 2 width at half height	Resolution
190	18.164	18.571	0.0499	0.0515	4.736
195	17.873	18.336	0.0571	0.0510	5.052
200	17.490	18.033	0.0744	0.0630	4.664
205	16.946	17.632	0.0909	0.0806	4.720
210	16.248	17.055	0.0916	0.1032	4.889
220	15.269	15.841	0.0630	0.0861	4.527
225	14.954	15.447	0.0534	0.0745	4.547
230	14.714	15.143	0.0514	0.0629	4.432
240	14.428	14.754	0.0461	0.0515	3.938
250	14.308	14.571	0.0340	0.0400	4.192

Table 26 Ramp 1 Value: Purnell Equation Calculations.

Resolution Calculations Using Purnell Equation: Ramp 1 Value Regioisomers							
Ramp 1 Value(°C)	**Compound A t_R**	**Compound B t_R**	**Compound B FWHM**	**Efficiency**	**Selectivity**	**Retention**	**Resolution**
190	18.164	18.571	0.0515	207.608	0.022	0.949	4.317
195	17.873	18.336	0.0510	206.263	0.025	0.948	4.939
200	17.490	18.033	0.0630	163.507	0.030	0.947	4.665
205	16.946	17.632	0.0806	123.821	0.039	0.946	4.559
210	16.248	17.055	0.1032	92.755	0.047	0.945	4.146
220	15.269	15.841	0.0861	104.466	0.036	0.941	3.548
225	14.954	15.447	0.0745	118.218	0.032	0.939	3.544
230	14.714	15.143	0.0629	137.873	0.028	0.938	3.664
240	14.428	14.754	0.0515	164.885	0.022	0.937	3.412
250	14.308	14.571	0.0400	210.548	0.018	0.936	3.556

Analyzing the data reveals virtually no change in the retention factor. However, applying the Purnell Equation allowed for further observation and evaluation based on which separation factors had the most effect on resolution according to Ramp 1 Value. For example, 195°C had a high efficiency while 210°C had the highest selectivity. For separating isomers, efficiency is thought to be most important for increasing resolution while selectivity is thought to be most important for separating analytes that are different in type or number of functional groups (Efficiency, Selectivity and Resolution, 2020). With this in mind, the optimal Ramp 1 Value would be 195°C because it is high in efficiency and has an acceptable selectivity value.

Figure 67 clearly shows the best peak separation occurring when the Ramp 1 Rate was 5°C / min. However, similar to comparisons within Ramp 1 Value Optimization there is a rate – which is 5°C / min – that is better regarding peak separation and another rate – which is 25°C / min – that is better regarding resolution due to decreased peak width. The 5°C / min rate does have a much longer run-time, so this creates what is known as the “general elution problem.” It provides good

resolution but an undesirable run time. Because efficiency is of most importance separating isomers, the 5°C / min was chosen as the best Ramp 1 Rate despite its run time. See Table 27 and Table 28 for all calculated resolution values.

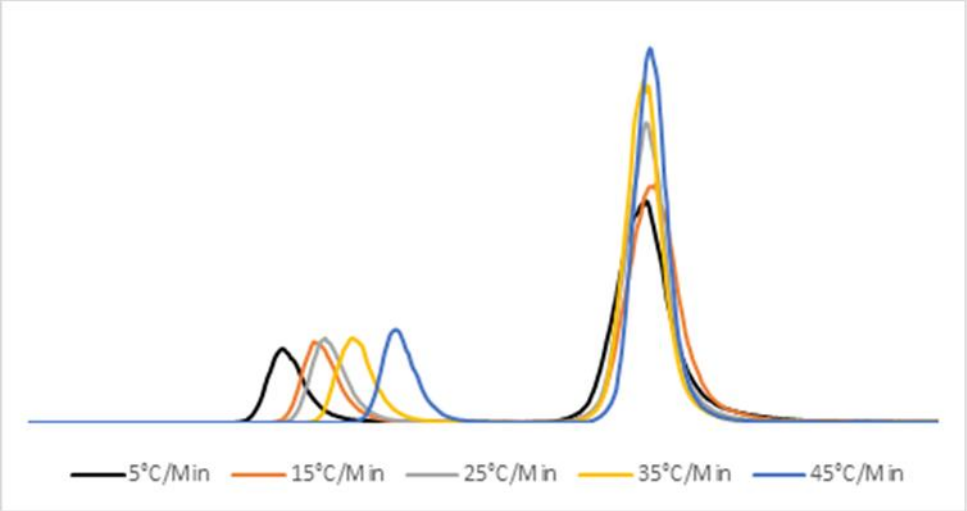


Figure 67 Ramp 1 Rate Optimization: Regioisomers.

Table 27 Ramp 1 Rate Optimization for Regioisomers.

Ramp 1 Rate Optimization for Regioisomers					
Ramp 1 Rate (°C/min)	t_{R1}	t_{R2}	Peak 1 width at half height	Peak 2 width at half height	Resolution
5	39.125	40.029	0.1032	0.1433	4.326
15	18.754	19.601	0.1030	0.1375	4.156
25	14.686	15.481	0.0972	0.1084	4.564
35	12.946	13.667	0.1031	0.0969	4.255
45	12.105	12.735	0.092	0.0915	4.047

Table 28 Ramp 1 Rate: Purnell Equation Calculations.

Resolution Calculations Using Purnell Equation: Ramp 1 Rate Regioisomers							
Ramp 1 Rate (°C/min)	**Compound A rt**	**Compound B rt**	**Compound B FWHM**	**Efficiency**	**Selectivity**	**Retention**	**Resolution**
5	39.125	40.029	0.1433	160.768	0.023	0.976	3.542
15	18.754	19.601	0.1375	80.330	0.043	0.951	3.303
25	14.686	15.481	0.1084	79.805	0.051	0.939	3.850
35	12.946	13.667	0.0969	78.723	0.053	0.932	3.870
45	12.105	12.735	0.0915	77.902	0.049	0.927	3.573

10. CONCLUSION

This project required insight, skill, and gained experience to develop a reliable method for the detection of Tamoxifen in artificial oral fluid – a practical method using GC/MS and other common laboratory equipment and supplies rather than using newer and more costly instrumentation such as LC/MS. As recommended by researchers who have been studying oral fluid and its potential to supplement blood and urine specimens, this project contributes to the needed study of drug concentration in saliva and its detectability in low sample volume. Specifically, this project utilized the drug Tamoxifen that is sometimes taken by athletes for performance enhancement but its detection in saliva is hindered because it is extremely protein bound, mostly ionized in the blood, and it has a high molecular weight. Its transfer to saliva from blood is not easy, so its concentration level in saliva can be negligible. It is hoped that WADA would consider further development of this method for use within its testing procedures. Each step of this developed method incorporated optimization techniques (e.g. oral fluid collection, GC/MS optimization and sample preparation); and careful data analysis was performed to choose results of the techniques to apply to the method to strike a balance between achieving a low limit of detection and high sensitivity.

The completed method for detection of Tamoxifen in artificial oral fluid was successful in achieving stated objectives. However, it is important to identify this method's limitations. Method validation requires the developed method to meet criteria for "specificity", but this project did not perform the analysis of additional

SERMs belonging to the same class of drugs as Tamoxifen. Analysis of Raloxifen or Toremifene, for example, would have been ideal to test the method's degree of specificity. It was decided to focus on detectability of Tamoxifen at this time, however, and incorporate an initial study of separating and differentiating regioisomeric aromatase inhibitors.

Stated objectives for this project's second part were also met. Method optimization was performed to separate the three regioisomeric aromatase inhibitors (5-alpha-Androstan-17-one, 5 alpha-Androst-16-en-3α-ol and 17-beta-Hydroxy-5-alpha-androst-2-ene), and both chromatographic and spectra analyses were performed for discrimination and differentiation between the AIs. Valuable information about each regioisomer's functional groups was revealed for identification purposes.

11. FUTURE DIRECTION

The first project lends itself to future research that builds on testing for SERMs and other PEDs in oral fluid. Oral fluid appears to be the most promising "new" biological matrix to be commonly utilized not only for drug testing purposes but also medication monitoring and diagnostics (although oral fluid research has actually been progressing for several years). OF drug testing for federal employees, for example, has just recently become more accepted by the scientific community in the United States as research and technology have been resolving the most troubling issues of low volume sample and low drug concentration. Building on this project's developed method could and should include analysis of SERMs within the same class as Tamoxifen (along with the metabolites of Tamoxifen) and use of this method should be further developed utilizing human saliva samples.

The second project initiates warranted research for differentiating substances that are newly discovered and becoming more prevalent in illicit use by athletes trying to enhance performance. The number of testosterone derivatives is constantly increasing; WADA statistics show that anabolic androgenic steroids and SERMs remain widely used. For this project's research, further studies of the chromatographic properties and elution of the three regioisomeric aromatase inhibitors need to be done on stationary phases with different polarities.

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