Analysis of Forensic Laboratory Instrumentation Used for DUI/DUID Cases

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Thesis Title

Kara Sitton

Author's Name

July 2020

Date

Jackson College of Graduate Studies at the University of Central Oklahoma

A THESIS APPROVED FOR

The W. Roger Webb Forensic Science Institute

By

Committee Chairperson

Committee Member

Committee Member

Committee Member

Executive Summary

The widespread conversion of drunk drivers to drugged drivers means that forensic laboratories have had to drastically increase the scope of their DUI/DUID testing. This increased scope has caused an expansion of the types of laboratory instrumentation used. In an attempt to identify the instrument best suited for DUI/DUID casework, an in-depth analysis was performed that evaluated presently-applied techniques as well as emerging techniques. This was followed by an analysis of the analytical efficiency of each instrument, see Table 1 below.

Table 1.

			Robustness of the Entire Analytical Workflow				
Type of Test	**Type of Instrumentation**	**Type of Technique**	**Instrument Set-up**	**Sample Preparation**	**Instrument Results**	**Data Analysis**	**Total**
Alcohol Quantitation	Headspace GC/FID	Presently-Applied	5	5	4	5	19
	Headspace GC/FID/MS	Emerging	5	5	5	5	20
Presumptive Screening	Traditional Immunoassays	Presently-Applied	5	5	4	3	17
	Biochip Array Technology	Emerging	5	5	4	4	18
	LC/MS/MS	Emerging	3	4	5	4	16
Drug Confirmation and Quantitation	GC/MS	Presently-Applied	5	3	4	5	17
	LC/MS/MS	Emerging	4	5	4	4	17

This study showed that there is not a one-size-fits-all preferred instrument for the analysis of DUI/DUID case samples. The variations in laboratory needs, capabilities, and definitions of analytical efficiency are why there is not a single best instrument. Instead, there are several instruments available that complement one another in the analysis of DUI/DUID case samples when used in conjunction with each other. While some of the emerging techniques appear to be more analytically efficient than presently-applied techniques, this does not discount the overall utility and analytical efficiency of the presently-applied techniques.

Introduction

In August of 1997, the world was shocked by the sudden death of Diana, Princess of Wales. While there were many conspiracy theories surrounding the cause of her death, eventually, the automobile accident that claimed her life was determined to have been caused by her driver having been "driving under the influence". Henri Paul, the Ritz Security Officer and the driver of the vehicle, was found to have had both alcohol (three times the French legal limit) and drugs (antidepressants and antipsychotics) in his system. The alcohol is believed to have enhanced the effects of the antidepressants and antipsychotics in his system, thus increasing the effect of the impairment caused by the alcohol alone. (Stevens, 2006).

According to the U.S. Centers for Disease Control and Prevention, about one third of U.S. fatal car accidents are caused by alcohol-impaired driving, nearly 11,000 deaths annually (Bergen, Shults, & Rudd, 2011). According to the U.S. Substance Abuse and Mental Health Services Administration (SAMHSA), in 2014 the U.S. suffered approximately one fatality every 53 minutes that was caused by alcohol-impaired driving. SAMHSA also reported that in 2014 11.1% of drivers aged 16 or older drove under the influence of alcohol, and 4.1% of drivers aged 16 or older drove under the influence of illicit drugs. (Lipari, Hughes, & Bose, 2016). The U.S. Governors Highway Safety Association (GHSA) observed a slight *decrease* in alcohol-impaired driving fatalities, and a noticeable *increase* in drug-impaired driving fatalities from 2006-2016. In 2015 and 2016, the GHSA observed that more impaired-driving fatalities were caused by drugs than by alcohol. The GHSA has also observed that increasingly, impaired-drivers are under the influence of some combination of drugs and alcohol. (Hedlund, 2018). The U.S. National Highway Traffic Safety Administration (NHTSA) noted a 3.6% decrease in alcohol-impaired driving fatalities from 2017 to 2018, where alcohol-impairment was defined as a blood

alcohol concentration over 0.08 g/dL. In 2018, alcohol-impaired driving accounted for 29% of the total fatal motor vehicle crashes. Unfortunately, the NHTSA failed to identify or quantify any motor vehicle fatalities that may have been caused by drug-impaired driving. (National Highway Traffic Safety Administration, 2019).

These statistics cause one to wonder what DUI really means. Driving under the influence (DUI) is illegal in all 50 U.S. states. Originally, DUI's were associated exclusively with drunk driving, however as drug use and abuse issues accelerate, drugged driving has also come to be associated with the term DUI. States have now begun to differentiate between drunk driving and drugged driving by charging drugged drivers with the offense of driving under the influence of drugs (DUID). (National Institute on Drug Abuse, National Institutes of Health, & U.S. Department of Health and Human Services, 2019; Hedlund, 2018).

So, what is the difference between drunk driving and drugged driving? Drunk driving is driving under the influence of alcohol. 49 U.S. states consider a driver over the age of 21 to be alcohol-impaired if their blood alcohol content (BAC) is over 0.08 g/dL; Utah is the outlier, as it recently lowered its legal limit to 0.05 g/dL. Some states have a form of zero-tolerance law for alcohol-impaired driving under the age of 21, while many states have a limit of 0.02 g/dL for underage drivers. (Nolo, n.d.). Drugged driving is driving under the influence of drugs, with marijuana and opioids being the most prevalent. Drugged driving is particularly dangerous because impairment can be caused by both prescription and illicit drugs. A key issue with determining drugged driving is that the concentration of drug(s) required to cause impairment is not well understood; there is not a direct relationship between drug concentration and impairment. Individual drugs, combinations of multiple drugs, or drugs and alcohol together cause different effects of impairment, none of which are well understood. However, it is

generally accepted that alcohol in combination with drugs acts synergistically to increase impairment. Thus, there are no uniform concentrations levels used for the identification of a drug-impaired driver. Instead, many states have zero-tolerance drugged driving laws, where drivers found driving with any detectable drug, prescribed and/or illicit, are considered impaired, irrespective of actual impairment. Alternatively, some states have set "per se" limits on specific drugs, prescribed and/or illicit, which means that if a driver is found with drug concentrations above a certain limit, they are considered impaired, again, irrespective of actual impairment. Since the presence of drugs does not necessary imply impairment, many states are waiting for conclusive research over drug concentration and level of impairment prior to the development of "per se" laws. Reaching conclusive consensus is challenging; for example, in regard to THC concentrations, there are many conflicting studies correlating blood concentration and level of impairment. (National Institute on Drug Abuse, National Institutes of Health, & U.S. Department of Health and Human Services, 2019; Hedlund, 2018; Compton & Berning, 2015; Armentano, 2013).

Statement of the Problem

The widespread conversion of drunk drivers to drugged drivers means that forensic laboratories have had to drastically increase the scope of their DUI/DUID testing. This increased scope has caused an expansion of the types of laboratory instrumentation used.

Daubert is the legal precedent used to determine the admissibility of analytically-derived evidence; its five prongs are: validation, controlled conditions (repeatability), peer review, erroneous results, and general acceptance in the relevant scientific community. (Koon, 1994). Before an analytical instrument can be used for casework, it must be evaluated by all of the Daubert prongs. Methods are typically peer reviewed and generally accepted by the relevant

scientific community prior to the purchase of an analytical instrument. Upon installation, the method must undergo extensive validation, which is performed under controlled conditions, theoretically minimizing the potential for erroneous results.

This literature review evaluates presently-applied techniques which have already undergone peer review and are generally accepted in the forensic science community, and evaluates emerging techniques that are still undergoing the process of peer review and general acceptance in the forensic science community. The methods validated, the conditions used for testing, and the potential for erroneous results have been reviewed. This review includes an analysis of each instrument against a set of evaluation parameters, the definition of analytical efficiency, and the author's conclusions regarding which instrument provides the best analytical efficiency for DUI/DUID casework.

Background

Drunk driving has been a problem for more than 100 years, with the first recorded drunk driving incident occurring in London, England in 1897. A taxi driver named George Smith crashed his cab into a building, and upon pleading guilty, was fined 25 shillings. In 1906, New Jersey became the first state to enact a drunk driving law, followed by New York in 1910. Because these early laws were not based on a quantitative definition of alcohol-impairment, outward signs of intoxication were used to identify alcohol-impairment. The first instrumentation used to identify alcohol-impairment was the Drunkometer in 1936, followed by the Breathalyzer in 1953. The Breathalyzer was determined to be more manageable and more accurate than the Drunkometer, which is why variations of it are still in use. (History.com, 2009; Birchfield v. North Dakota, 2016).

Today, breathalyzers are still used for roadside testing of impaired drivers. Roadside tests include: breathalyzers, field sobriety tests, and the use of drug recognition experts (Hedlund, 2018). While roadside tests are outside the scope of the literature review, they are important because they provide the probable cause necessary for the collection of a biological sample, and the subsequent evidentiary DUI/DUID tests performed on that sample, all of which are the focus of the literature review.

Purpose of Study

The purpose of this literature review is to evaluate the various types of presently-applied and emerging laboratory instrumentation most commonly available for use in the identification of drugs and alcohol in impaired driving cases. This analysis involves the evaluation of these instruments against a specific set of parameters covering: instrument set-up, sample preparation, instrument results, and data analysis. Margin of error and cost will also be discussed, but in a more general sense, because information covering these topics is not readily available in the published literature. This literature review has attempted to identify the forensic laboratory instrumentation with the highest analytical efficiency for DUI/DUID casework. This analysis includes: a description of current forensic laboratory DUI/DUID workflow; a description of the substrates used for DUI/DUID cases; and, a description of target drugs/drug classes that are commonly identified in DUI/DUID samples. Because analytical efficiency has a subjective definition, this literature review also includes a review of the topic of analytical efficiency, and the objective parameters the author used for the evaluation of the instrumentation.

Scope of Study

The scope of this literature review was limited by the following four factors:

1. It did not involve a bench study comparing the different types of instrumentation that can be used for DUI/DUID cases. This research was a literature review that evaluated the different types of instrumentation on a theoretical basis. Interested parties may choose to transition some of these theoretical instrumental approaches to validation studies.
2. The drugs/drug classes involved in this evaluation were limited to those most commonly encountered in DUI/DUID cases; all are Tier I compounds as described by the National Safety Council's Alcohol, Drugs and Impairment Division (Logan, et al., 2017).
3. It did not include an evaluation of roadside tests, such as breathalyzers, field sobriety tests, or the use of drug recognition experts, though the results of these roadside tests are taken into consideration when performing DUI/DUID laboratory analyses. Instead, this study focused on the instrumental approaches used by forensic laboratories to perform evidentiary DUI/DUID tests on biological samples.
4. The instrumental approaches were evaluated against a specific set of parameters; however, these parameters are not all-inclusive. They were limited by the information that is available in published literature.

Significance to the Field

This study should assist forensic laboratories in identifying the laboratory instrumentation with the highest analytical efficiency for their specific DUI/DUID casework. The goal of forensic laboratories should be to produce accurate and reliable results as efficiently as possible. Ideally, this literature review will aid forensic laboratories in making purchasing decisions for new instrumentation, and help them learn how to better utilize their existing instrumentation.

Even though the focus of this literature review is DUI/DUID casework, this analysis of laboratory instrumentation can also be applicable to other forensic toxicology subsets, such as drug-assisted sexual assault and post-mortem drug analysis. Additional research may be required for these applications, as they often employ the use of matrices that are outside the scope of this literature review.

This study's theoretical descriptions of the presently-applied and emerging techniques used in forensic science laboratories, may also be used to aid the comprehension and understanding of court officials, lawyers, and law enforcement officers. This is significant, because it may help contribute to the general acceptance of these techniques in a court of law.

Methods

The research methodology used for this analysis included: a review of published literature, a review of textbooks, a review of the websites of instrument manufacturers, attempts to contact instrument manufacturers for more specific information, and interviews of relevant individuals. This literature review was followed by an evaluation of the data gathered against the previously-described evaluation parameters, followed by an analysis of the analytical efficiency of the presently-applied and emerging DUI/DUID analysis techniques.

The review of published literature included searches in the University of Central Oklahoma Max Chambers Library, using the "Central Search" function, as well as searches in the Science Direct database accessed through the library. Several articles were also requested and received through the Chambers Library Interlibrary Loan. This review also included general Internet searches using the Google search engine to identify additional resources that might be relevant. Internet searches facilitated the identification of regulatory guidance related to forensic science analyses, and patents related to the instrumentation being reviewed.

The review of textbooks included those owned by the author that were obtained in both undergraduate and graduate studies. The review also included textbooks that were available online through University of Central Oklahoma Max Chambers Library. Several textbooks pertaining to forensic science, chromatography, toxicology, pharmacology, and measurement uncertainty were identified and used.

The instrument manufacturer websites that were reviewed included: Thermo Fisher Scientific, Agilent Technologies, Sciex, Waters Corporation, Perkin Elmer, and Shimadzu Corporation. These instrument manufacturers were selected based on the American Chemical Society's (ACS) *Top Instrument Firms of 2018* ranking. The ACS ranked the top 20 instrument manufacturers worldwide based on 2018 instrument sales. This list included eight American companies; the websites for these companies were reviewed, and the five most relevant companies were selected: Thermo Fisher Scientific, Agilent Technologies, Danaher (the company that owns Sciex), Waters Corporation, and PerkinElmer. Shimadzu was a later addition to the instrument manufacturers reviewed, but it was also in the ACS top 20 ranking. (Reisch, 2019).

The purpose of the website reviews was twofold: identify the relevant instruments offered by the selected instrument manufacturers, and identify the resources manufacturers have available on their websites to aid in method development. This information was then put into a table to compare instrument manufacturers against one another for the specific instruments reviewed. The results of this analysis are in Appendix A – Instrument Manufacturer Analysis.

Because the information available in published literature and on instrument manufacturer websites does not include objective information related to cost, instrument sensitivity, time required for instrument set-up, instrument maintenance, and other various criteria, an attempt

was made to contact instrument manufacturers for more specifics on those criteria. The selected manufacturers were sent a cover letter explaining the purpose of the research, as well as a questionnaire to fill out (see Appendices B and C). These cover letters and questionnaires were sent to the customer service e-mail addresses on the instrument manufacturer websites for Agilent Technologies, Sciex, and Waters Corporation. Thermo Fisher Scientific and PerkinElmer did not list customer service e-mails, so the cover letter was sent through the "Contact Us" page of these websites. A copy of this cover letter and questionnaire were also sent to a sales representative at Shimadzu, who is a "friend of a friend", and later to an applications support scientist at Sciex who is a contemporary of the author. Because the survey did not involve the identification of particular individuals, or persons from disadvantaged/marginalized groups, Institutional Review Board approval was not required. (Appendix B – Instrument Manufacturer Cover Letter; Appendix C – Instrument Manufacturer Questionnaire).

Individuals interviewed for this review included the Technical Manager of the Toxicology Unit of the Oklahoma State Bureau of Investigation (OSBI), and two representatives from Sciex. The OSBI analyst was contacted in order for the author to gain a better understanding of the workflow of forensic laboratories, as well as examples of presently-applied and emerging analytical techniques that were recommended for review. The Sciex representatives were interviewed in relation to the instrument manufacturer survey they received; rather than fill out the survey, they preferred a conference call discussion.

Literature Review

Brief History of Toxicological Techniques

The study and practice of toxicology date back to pre-historic humans, and their use of plant poisons and animal venoms for hunting and war. The use of analytical techniques to identify poisons in human remains dates back to the nineteenth century, while the use of analytical instrumentation for drug analysis only dates back to the mid-twentieth century. The use of analytical instrumentation for drug analysis has included: the use of thin layer chromatography, starting in the 1950s; the use of ultraviolet spectrophotometry, starting in the 1950s and 1960s; the use of immunoassays, starting with radioimmunoassay (RIA) in the 1950s and 1960s; the introduction of enzyme-linked immunosorbent assay (ELISA) and enzyme-multiplied immunoassay technique (EMIT) in the 1970s, and a whole succession of various immunoassay techniques since then; the use of capillary electrophoresis, starting in the 1960s; the use of REMEDi-HPLC, popular in the 1990s and early 2000s; and finally, the classic mainstays of gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC), and hyphenated techniques utilizing mass spectrometry. While much of this instrumentation is outside the scope of the literature review, it is important to recognize how relatively new instrumental analytical techniques are, and to appreciate the development and refinement of these techniques in such a short period of time. (Klaassen, 2008; Langman & Kapur, 2006; Monroe, 1984).

Chromatography is the current cornerstone of toxicology and drug analysis, because it involves the separation of mixtures. It is a differential migration process that is used to separate mixtures of matrices/endogenous substances and drugs, mixtures of drugs and metabolites, and mixtures of multiple drugs and/or metabolites. The discovery of chromatography is typically

credited to Mikhail Semyonovich Tswett, a Russian chemist, who in 1903 separated leaf pigments using a chalk column. Tswett named this technique chromatography, or color writing, using the Greek words "croma" (color) and "grafeih" (writing). Since that time, there have been several different types of chromatography developed, which include: solid state gas chromatography, also known as gas-solid chromatography, in 1941; liquid-liquid chromatography (which became HPLC), in 1941; paper chromatography, in 1944; and gas-liquid chromatography (most commonly referred to as gas chromatography), also in the 1940s. (Langman & Kapur, 2006).

Mass spectrometry is considered by many to be the gold standard for toxicological drug confirmation. It is a strong-held belief in forensic science, that unless there is a spectral match, a drug has not been properly confirmed. The development of mass spectrometry is credited to Cambridge University professor Sir Joseph John Thompson, who in 1907 developed a device to positively charge ions, which were then forced through a magnetic field, then struck a screen and created unique patterns. It was noted at the time, that pure compounds always created the same pattern, and could be easily identified, but mixtures proved to be problematic. (Houck & Siegel, 2011; Jones, 2019).

Current Forensic Laboratory DUI/DUID Workflow

While the methods used to process DUI/DUID cases are lab specific, they generally include both presumptive and confirmatory testing of DUI/DUID samples. Presumptive tests, also referred to as preliminary tests, are used to screen samples for the presence of common drugs/drug classes. Presumptive tests are typically in the form of immunoassays; however, they can also include chromatographic methods. Major advantages of presumptive tests are that most of them require little or no sample prep, and can produce results fairly quickly. Presumptive

tests are used to direct the path of confirmation tests; this is important because different drugs/drug classes may require different extraction techniques, or the use of different types of instrumentation. Confirmation tests typically use chromatography, coupled with mass spectrometry, to confirm the specific drug(s) in a sample. Confirmation tests are different from presumptive tests, because presumptive tests may only be able to identify a drug class, whereas confirmation tests will be able to identify the specific drug(s) in the indicated drug class(s). Though differences may be subtle, every compound has a unique splitting pattern; this allows for spectral matches to conclusively identify individual drug(s), and as such, spectral confirmation is the industry standard for drug confirmation. Different methods, such as derivatization, can be employed to help differentiate among the subtleties of splitting patterns. (Houck & Siegel, 2011; Anderson, 2005; Jones, 2019; Lin, et.al., 2008).

Since impaired driving events have been traditionally caused by alcohol-impairment, alcohol is typically the first drug for which DUI/DUID samples are evaluated. In the Oklahoma State Bureau of Investigation (OSBI) Forensic Science Center in Edmond, if alcohol is identified and quantified above the legal limit, no other tests are performed. If alcohol is not identified, or is not quantified above the legal limit, then further testing is performed. This testing includes screening by ELISA, and confirmation of any presumptive positives, as well as a review of the drug recognition expert's (DRE) analysis for drugs/drug classes that cannot be screened for by ELISA, followed by confirmation testing for these substances. Since Oklahoma is a zero-tolerance drug state, as soon as a drug (therapeutic or illicit) is identified, no other tests are performed. States with "per se" limits on drugs may additionally require quantitation of confirmed drugs. This process can be limited by the quantity of biological sample received by the laboratory, which can dramatically vary in size. Presumptive testing is important, because it

directs the consumption of the sample down the most efficient path, toward the assays most likely to produce the required results. (Ross-Carr, 4/20/20).

Sample Matrices and Extraction Methods

There are a number of matrices that can be evaluated to determine drug use. Some, like blood and oral fluid, give the live concentration of an analyte. Others, such as urine and hair, are able to indicate historical drug use, but do not provide information on the drugs affecting an individual at the time of sample collection. This section will review whole blood and oral fluid as matrices that can be used for the analysis of DUI/DUID cases, as well as the common sample preparation methods used for these matrices. These matrices were chosen based on the results of the toxicology survey that was part of the National Safety Council's Alcohol, Drugs and Impairment Division's *Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities – 2017 Update*. (Logan, et.al., 2017). Of the laboratories surveyed, 90% reported that they test whole blood samples for DUI/DUID cases, 68% reported that they test urine samples for DUI/DUID cases, and 1% reported that they test oral fluid for DUI/DUID cases. Since the report also referenced a 2013 recommendation discouraging the use of urine as a specimen for DUI/DUID cases, because urine results provide historical data rather than the live concentration, urine will not be discussed. Because whole blood is the matrix most commonly analyzed in DUI/DUID cases, it will be the focus of the remainder of the literature review.

Whole Blood

Whole blood (referred to simply as blood going forward) is the matrix most commonly collected for analysis in DUI/DUID cases, because blood provides the live concentration of drugs at the time of sample collection. The presence of free, unbound drugs in blood indicates

recent use; because unbound drugs are pharmacologically active, they are a likely cause of impairment. Protein-bound drugs are not typically pharmacologically active, thus not the cause of impairment; this is why most DUI/DUID detection methods do not include a hydrolysis step to free protein-bound drugs. (Langman & Kapur, 2006; Raes, Verstraete, & Wennig, 2008).

Broadly speaking, blood is composed of two components: plasma and blood cells. The composition of plasma includes: proteins (including antibodies, transport molecules, and enzymes), minerals, and aqueous glucose. Plasma is the fraction of blood in which most drugs concentrate; however, some drugs also concentrate in red blood cells, the primary component of the blood cell fraction. The unequal distribution of drugs between blood fractions is why whole blood is a more appropriate matrix than plasma for DUI/DUID testing. (Koen & Bowers, 2017; Langman & Kapur, 2006).

The unequal distribution of drugs between blood fractions, in combination with the varying chemical properties of different drugs, is why there is not a one-size-fits-all preferred extraction process for DUI/DUID testing. (The chemical properties of specific drugs/drug classes will be discussed in more detail later, with the description of the drugs/drug classes.) The most common extraction techniques used for blood samples include: liquid-liquid extraction, solid-phase extraction, and protein precipitation. It is important to note that the type of extraction technique used can affect the quality of the chromatographic separation and subsequent detection of target analytes. (Leung & Fong, 2014; Jemal, 2000).

Liquid-Liquid Extraction

Liquid-liquid extractions (LLE) involve the partitioning of target analytes from an aqueous matrix into an organic solvent which is then evaporated. Evaporation is followed by a reconstitution step, which concentrates the extracted analytes. Generally, drugs fall into three

classes, basic, neutral, and acidic, with a majority of relevant drugs being basic or neutral. Because of the varying chemical properties of the drugs involved, LLE are often broken down into two types, basic extraction and acidic extraction, with neutral drugs being extracted by both methods. Basic extractions involve the basification of the matrix with a buffer, and the subsequent application of an extraction solvent. Acidic extractions involve the acidification of the matrix with a buffer, and the subsequent application of an extraction solvent. Extraction solvents need to be optimized in order to fully extract target analytes, while also preventing/minimizing the coextraction of interfering endogenous substances. LLE can be automated, and when performed properly, can give very clean extracts. (Kostakis Harpas, & Stockham, 2013; Leung & Fong, 2014; Jemal, 2000; Thermo Fisher Scientific, n.d.).

Solid-Phase Extraction

Solid-phase extractions (SPE) involve the use of a sorbent, typically in a cartridge or a 96-well plate, that has an affinity for the target analytes in the matrix. The matrix is passed through the sorbent, and the sorbent retains the target analytes by non-bonding interactions. An elution solvent, with a higher affinity for the target analytes than the sorbent, is then applied to the sorbent, thus releasing the target analytes. This solvent may then be injected into the chromatographic column, or evaporated and reconstituted to concentrate the target analytes prior to analysis. There are many different sorbents that may be used for SPE; they have varying separation mechanisms, and may be used with a variety of elution solvents. Some sorbents may include ion-exchange moieties, which allow for the separate elution of basic and acidic target analytes. SPE can be automated, and when performed properly, can give clean extracts. (Kostakis Harpas, & Stockham, 2013; Leung & Fong, 2014; Jemal, 2000; Thermo Fisher Scientific, n.d.).

Solid-Phase Micro-Extraction

Solid-Phase Micro-Extraction (SPME) is an extraction technique that has been gaining popularity for volatile and thermally-stable drugs; it is hailed as a "green" technique, because it is a solventless extraction. Similar to SPE, the matrix is passed through a fiber-containing cartridge and the target analytes are adsorbed onto the fiber. Unlike with SPE, the target analytes are not eluted from the fiber using an elution solvent; instead, the fiber is placed in the injector port of a gas chromatograph, and the target analytes are desorbed into the instrument. This technique can also be used with headspace gas chromatography. SPME offers much potential because: it uses small volumes of sample matrix; can be automated; and, by reducing sample manipulation, it reduces both the time required for sample preparation and the potential for analytical errors. Also, this technique is known for producing clean extracts. (Langman & Kapur, 2006; DeGiovanni & Fucci, 2008; Bogusz, 2008).

Protein Precipitation

Protein precipitation (PPT) is a simple clean-up procedure that is regarded for its speed and simplicity. Its advantages are somewhat offset by the output of an extract with more endogenous contaminants, because the primary endogenous substances removed are the proteins. PPT is achieved by exploiting the solubility of proteins, and is most commonly performed by the application of an organic solvent with a small dielectric constant, or the application of a salt that outcompetes the protein for water. PPT is considered to be universally applicable, because it can be used to extract almost any analyte, independent of chemical properties. Because the extract produced often contains matrix contaminants, PPT is sometimes performed as a pretreatment step for LLE or SPE. PPT can also be automated. (Leung & Fong, 2014; Jemal, 2000; Thermo Fisher Scientific, n.d.).

Chemical Derivatization

Depending upon the chromatography technique used, and the drugs being analyzed, samples may require chemical derivatization. Derivatization is generally performed on samples that are going to be analyzed by gas chromatography mass spectrometry (GC/MS), though studies have been performed to determine if there are potential benefits in derivatizing samples for liquid chromatography mass spectrometry or tandem mass spectrometry (LC/MS and LC/MS/MS) analysis. Chemical derivatization of analytes for LC/MS and LC/MS/MS is generally directed toward reactive carbonyl metabolites that exhibit chemical instability, such as acetoacetate and oxaloacetate, which, as endogenous compounds, are outside the scope of this study. Of the articles analyzed for this literature review, almost all derivatized drugs in preparation for GC/MS analysis, but the derivatization of drugs in preparation for LC/MS/MS analysis was not mentioned. Therefore, when discussing derivatization in this literature review, it will exclusively refer to GC/MS analysis. (Lin, et.al., 2008; Lin, et.al., 2016).

Typically, drugs are derivatized prior to GC/MS analysis for one or more reasons: 1) to improve analyte compatibility with the chromatographic environment; 2) to improve separation efficiency or achieve required analyte separation; or 3) to improve analyte detection and structural characterization. Since GC/MS is not as compatible with analytes that are thermally instable, highly polar, or semi-/non-volatile, improving analyte compatibility is the primary reason chemical derivatization is performed. Derivatization can improve analyte volatility, thermal stability, polarity, and recovery; it can also improve chromatographic properties, such as peak shape. Derivatizing agents may create more ideal analytical conditions for separation efficiency by bringing the retention times of analytes into a more desirable range. Achievement of required analyte separation may be used in the application of enantiomeric determination,

whereby enantiomers may be separated by the use of chiral derivatizing agents instead of, or in combination with, chiral stational phases. Chemical derivatization can improve analyte detection, with the ability to lower the limit of detection for certain analytes when analyzed in negative chemical ionization mode versus positive chemical ionization mode. Structural characterization can be improved by the production of better-defined mass spectra, thereby allowing for easier recognition of the molecular weight of analytes. It is important to note that the use of columns with different stationary phases, or the use of temperature programing (versus isothermal operation), may change the derivatization requirements of certain analytes from mandatory to optional. Though optional in some instances, derivatization may still be favored for the improvements it provides on GC/MS performance characteristics. (Lin, et.al., 2008; Lin, et.al., 2016; Kabir & Furton, 2012).

Derivatization can occur pre-column, as part of the sample preparation method, or on-column as part of the instrumental analysis. The most common derivatization reactions used for drug analysis are silylation, acylation, and alkylation. The sites most commonly derivatized are labile protons on heteroatoms which contain the following functional groups: -COOH, -OH, -NH, and -HN_2; derivatization at carbon sites has also been reported. Analyte-specific derivatization will be reviewed with the description of drugs/drug classes. (Lin, et.al., 2008; Lin, et.al., 2016).

Oral Fluid

Oral fluid is a matrix that is slowly gaining popularity as an alternative matrix for DUI/DUID cases; its primary advantage is that the collection process is non-invasive, compared to the invasive collection of blood. Because of oral fluid’s increasing use and because oral fluid

can be tested on the same instrumentation used for blood samples, a short review of oral fluid has been included.

Oral fluid, like blood, provides the live concentration of drugs at the time of sample collection, because it contains free drugs; it also has a tendency to contain parent drugs rather than metabolites. Oral fluid has a detection window similar to that of blood, and drug concentrations in oral fluid have a good correlation with those found in blood. Oral fluid is more than 97% water; it also contains electrolytes, immunoglobins, enzymes and some proteins. This simple composition reduces the potential for interference caused by endogenous substances. The extent of protein binding in oral fluid is minimal compared to blood, which forces drugs to convert into a water-soluble form to be retained in the oral fluid. For most drugs, water-solubility is attained via ionization, thus the concentration of drugs is dependent upon oral fluid pH. The pH of oral fluid is normally in the range of 5.5-7.9; this wide range is a contrast to the fairly constant pH value of blood. The pH of oral fluid can be affected by factors such as stimulation, flow rate, and smoking; interestingly, pH is not affected by alcohol consumption. Because oral fluid pH can be quite fluid, and analyte concentration can be dependent upon pH, collection devices typically include a collection buffer to stabilize pH, thus stabilizing analyte concentrations. (DeGiovanni & Fucci, 2008; Rodrigues, et.al., 2013; Raes, Verstraete, & Wennig, 2008).

Oral fluid's biggest downfall appears to be the variable influence of the collection method employed. Studies comparing different collection devices have shown wide variability in drug concentrations between the different devices. The reason for this variability is likely the collection buffers used, since each collection device uses a proprietary buffer; however, the variability can also be influenced by the method used to collect the sample, i.e. stimulated or not

stimulated collection. This lack of uniformity of sample collection means that oral fluid methods are dependent on the specific collection device used; they do not usually have the ability to be applied to a wide variety of collection devices. Additionally, until recently, there were not forensic guidelines for oral fluid cutoff concentrations. The *Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities – 2017 Update* referenced earlier provided some of the initial guidance on oral fluid testing in the context of DUI/DUID testing. It should be noted that the cutoff concentrations used for oral fluid testing are generally lower than those used for whole blood analysis, because the concentration of drugs in oral fluid is lower than the concentration of drugs in whole blood. (DeGiovanni & Fucci, 2008; Rodrigues, et.al., 2013; Raes, Verstraete, & Wennig, 2008; Langman & Kapur, 2006; Logan, et.al., 2017).

Dilute-and-shoot

There are several extraction methods that can be used with oral fluid samples, many of which are variations of those used for the extraction of blood; they include: SPE, SPME, LLE, PPT, and dilute-and-shoot. Because oral fluid has fewer endogenous components than blood, PPT produces a cleaner extract when extracting oral fluid samples than it does with blood. Dilute-and-shoot is a sample preparation method whereby the oral fluid sample is diluted by the extraction buffer, then an aliquot of the dilution is mixed with an internal standard (IS). This mixture is centrifuged in some instances, but the centrifugation step is not performed in other instances. The resulting dilution-IS solution is then injected into the instrument without any further sample processing. Because oral fluid is a rather clean matrix to begin with, dilute-and-shoot is a popular extraction technique. (DeGiovanni & Fucci, 2008; Rodrigues, et.al., 2013; Raes, Verstraete, & Wennig, 2008).

Description of Drugs/Drug Classes

The drugs/drug classes involved in this evaluation were limited to those most commonly encountered in DUI/DUID cases, and will be the focus of the remainder of the literature review. This approach was an effort to keep this literature review concise, relevant, and focused, as the number of potential drugs that could be encountered in DUI/DUID cases is seemingly limitless because of the growing number of novel, synthetic, and designer drugs. All of the drugs/drug classes reviewed were Tier I compounds as described by the National Safety Council's Alcohol, Drugs and Impairment Division, and were chosen by a review of the results of the toxicology survey that was part of the *Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities – 2017 Update* (Logan, et al., 2017).

Alcohol

As discussed previously, early impaired-driving laws specifically applied to alcohol intoxication, and the early instrumentation that was used in DUI cases was alcohol specific. In the United States, according to the Treasury Department, "The term *alcohol* means that substance known as ethyl alcohol [ethanol]". Alcohol is a central nervous system (CNS) depressant, specifically a sedative-hypnotic that depresses the CNS in a dose-dependent fashion. Alcohol is the only drug to have a known correlation between concentration and level of impairment. Alcohol-impairment affects many behaviors that are necessary for driving including balance, coordination, reaction time, attention, decision-making, risk taking, and judgement. There are decades of research correlating increased accident risk with increased alcohol concentration; this research is the basis for the legal limits set by each state. (Beale & Block, 2011; Hedlund, 2018; Berning, Compton, & Wochinger, 2015).

Benzodiazepines

Benzodiazepines are CNS depressants, specifically sedative-hypnotics; they cause sedation, hypnosis, blurred vision, confusion, slowed reflexes, slurred speech, and hypotension. Benzodiazepines have been used for the medical treatment of anxiety, insomnia, seizures, and pain management. Benzodiazepines are also prone to illicit abuse. Structurally, benzodiazepines can be divided into several categories: 1,4-benzodiazepines, nitro-benzodiazepines, imidazo-benzodiazepines, triazolo-benzodiazepines, and miscellaneous benzodiazepines. Pharmacologically, benzodiazepines are divided into two categories low dose and high dose. 1,4-Benzodiazepines are high-dose benzodiazepines that have oxazepam as a common metabolite; common 1,4-benzodiazepines include diazepam, nordiazepam, temazepam, and oxazepam. Nitro-benzodiazepines are low-dose benzodiazepines that that commonly produce 7-amino metabolites; common nitro-benzodiazepines include clonazepam and flunitrazepam. Imidazo-benzodiazepines are low-dose benzodiazepines that do not appear to have a common metabolite; midazolam is a common imidazo-benzodiazepine. Triazolo-benzodiazepines are low-dose benzodiazepines that have alpha-hydroxy-alprazolam as a common metabolite; common triazolo-benzodiazepines include alprazolam and triazolam. Benzodiazepines are alkaline in nature, so they are best extracted in basic conditions. Benzodiazepines are thermally unstable and generally require derivatization by silylation or acylation for compatibility with GC/MS. Derivatization improves the thermal stability of benzodiazepines, improves the chromatographic resolution, and produces better-defined mass spectra. (Beale & Block, 2011; Logan, et al., 2017; Hutchings & Widdop, 2013; Bertol & Vaiano, 2016; Drummer, 1998; Corey, Czakó, & Kürti, 2007).

Amphetamine/Methamphetamine

Amphetamines are CNS stimulants, specifically central sympathomimetic agents, also known as psychomotor stimulants; they cause elevated mood, increased alertness, self-confidence, and concentration. Their prolonged use can lead to weight loss, hallucinations, and paranoid psychosis. Amphetamines have been used for the medical treatment of obesity, appetite suppression, attention-deficit disorders, narcolepsy, and Parkinson disease. Amphetamines are also prone to illicit abuse; designer amphetamines, such as methylenedioxyamphetamine (MDMA), do not have an accepted medicinal use. Common amphetamines include amphetamine, methamphetamine, phentermine, and MDMA; amphetamine is their common metabolite. Amphetamines are alkaline in nature, so they are best extracted in basic conditions. In order to achieve the requisite chromatographic separation, and to generate better-defined mass spectra, derivatization by acylation is generally required for compatibility with GC/MS. However, caution should be taken when using heptafluorobutyric anhydride or 4-carboxyhexaflurobutyl chloride, because there is evidence that high injection-port temperatures can cause the artificial conversion of derivatized ephedrine to methamphetamine when ephedrine is present in high concentrations. (Beale & Block, 2011; Hutchings & Widdop, 2013; Valentine & Middleton, 2000; Lin, et.al., 2008).

Cocaine/Benzoylecgonine

Cocaine is a CNS stimulant, specifically a euphoriant-stimulant; it is also a local anesthetic. Cocaine causes feelings of well-being, decreased fatigue, increased alertness, compulsion, vasoconstriction, increased blood pressure, increased heart rate, and increased body temperature; its prolonged use can lead to psychosis. Cocaine has traditionally been used as a local anesthetic in surgery, but is more commonly abused illicitly. Cocaine rapidly metabolizes

in the body and is more commonly identified by its primary metabolite, benzoylecgonine. When cocaine and alcohol are taken simultaneously, the production of cocaethylene results, this metabolite is unique to the combination of cocaine and alcohol. Benzoylecgonine and cocaine are alkaline in nature, so they are best extracted in basic conditions. To improve chromatographic resolution, derivatization by acylation is generally required for compatibility with GC/MS. (Beale & Block, 2011; Hutchings & Widdop, 2013; Lin, et.al., 2016)

Opiates/Opioids

Opiates and opioids are narcotic analgesics; they cause analgesia, euphoria, narcosis, pinpoint pupils, and respiratory depression. Opiates and opioids have been used for the medical treatment of acute pain, the management of chronic pain, and sometimes as antitussives. Opiates and opioids are highly addictive, and are prone to illicit abuse. Regular use of opiates and opioids leads to tolerance, which is why in 2017 a U.S. national public health emergency was declared in regard to the opioid crisis. Opiates are drugs that are found in the unripe seed pod of the opium poppy; they include morphine and codeine. Morphine was first isolated in 1805 by F.W.A. Sertürner, a German pharmacy assistant; he named it after Morpheus, the Greek god of dreams. Opioids are semisynthetic or fully synthetic derivatives of opiates. Semisynthetic 6-keto-opioids include hydrocodone, hydromorphone, oxycodone, and oxymorphone. Synthetic opioids include fentanyl, methadone, and buprenorphine. Opiates and opioids are alkaline in nature, so they are best extracted in basic conditions. To achieve the requisite chromatographic separation, and to generate better-defined mass spectra, derivatization by acylation or silylation is generally required for compatibility with GC/MS. However, caution should be taken when using silylation, because the 6-keto-opioids have the potential to produce multiple derivatives as

a result of their keto- or enol forms. (Beale & Block, 2011; Hutchings & Widdop, 2013; Corey, Czakó, & Kürti, 2007; Bogusz, 2008; Hedlund, 2018).

Heroin/6-MAM

Heroin is a narcotic analgesic that causes the same effects as opiates and opioids, but with a much stronger addiction potential. Heroin is a semisynthetic opioid with no accepted medical use; it is extremely prone to illicit abuse. Heroin was first synthesized in 1898 by Bayer as a (hopefully) non-addictive alternative to morphine; it was named heroin because it made the test subjects feel heroic. Heroin rapidly metabolizes in the body and is identified by its primary metabolite, 6-monoacetylmorphine (6-MAM). 6-MAM is alkaline in nature, so it is best extracted in basic conditions. Like opiates and opioids, 6-MAM generally requires derivatization by acylation or silylation for compatibility with GC/MS in order to achieve the requisite chromatographic separation and to generate better-defined mass spectra. (Beale & Block, 2011; Hutchings & Widdop, 2013; Bogusz, 2008).

THC/THC-COOH

Delta-9-tetrahydrocannabinol (THC) is the primary psychoactive component of marijuana (cannabis), and is a depressant-intoxicant; it causes vigilance, drowsiness, delayed reaction time, divided attention, poor coordination and balance, bloodshot eyes, increased heart rate, and increased appetite. Prolonged use of THC can lead to dysphoria, hallucinations, and paranoia. The medico-legal status of THC is complicated. Federally, it does not have an accepted medical use and is illegal; at the state level, it depends on the state. Some states have implemented the acceptance of “Medical Marijuana”, whereby individuals may use marijuana for the treatment of various ailments, including cancer chemotherapy (as an antinauseant), asthma, multiple sclerosis, and glaucoma. Some states have decriminalized marijuana

altogether, while some states still consider it to be illegal, per federal guidelines. Amid this everchanging medico-legal landscape, THC is prone to abuse, whether it be by medical use, by decriminalized recreational use, or by illicit use. THC is generally identified by both THC (parent drug) and its primary metabolite 11-nor-9-carboxy-THC (THC-COOH), though it can also be identified by several other metabolites. THC and THC-COOH are acidic in nature, so they are best extracted in acidic conditions. THC-COOH contains a carboxylic acid group, which means it has the potential to form strong hydrogen bonds with components in the chromatographic system, causing peak loss or tailing. Therefore, samples generally require derivatization by silylation or acylation for compatibility with GC/MS. Derivatization by acylation has been shown to improve the limit of detection for THC-COOH in negative chemical ionization GC/MS applications. (Beale & Block, 2011; Hutchings & Widdop, 2013; Lin, et.al., 2008; Lin, et.al., 2016).

Alcohol Quantitation

In forensic laboratories, the first step of a DUI/DUID workflow typically involves the quantitation of blood alcohol concentration. The instrumentation that is primarily used for the quantitation of blood alcohol concentrations is headspace gas chromatography flame-ionization detection (HS-GC/FID). Though this method is the industry standard, it does not provide mass spectral confirmation, thereby requiring some forensic labs to run additional confirmation tests. By contrast, headspace gas chromatography flame-ionization detection mass spectrometry (HS-GC/FID/MS) has all of the capabilities of HS-GC/FID, with the added benefit of spectral confirmation, thus eliminating the need for additional confirmation tests. This section will include a description of HS-GC/FID and HS-GC/FID/MS, as well as an assessment of each instrument against the following parameters: instrument set-up, sample preparation, instrument

results, and data analysis. Instrument performance in relation to these parameters will be evaluated as part of the analytical efficiency discussion. (Sithersingh & Snow, 2012; Restek, 2000; Agilent, n.d.).

Headspace Gas Chromatography Flame-Ionization Detection – Presently-Applied Technique

Headspace Gas Chromatography Flame-Ionization Detection is the industry standard for blood alcohol quantitation. HS-GC/FID can be executed one of two ways: static or dynamic. Static HS-GC/FID is the method most commonly employed for forensic analyses, whereby the instrument samples the vapor phase of an equilibrated sample, and then injects the vapor directly into the chromatographic column(s) for separation. The flame ionization detector measures the response of the analytes, and this response is then used for quantification of blood alcohol concentration. (Thermo Fisher Scientific, n.d.; Sithersingh & Snow, 2012; Agilent Technologies, n.d.).

What makes this technique unique, and ideal for the analysis of blood alcohol quantitation, is the headspace sampling, also referred to as headspace extraction. Headspace extractions rely on the volatility of target analytes, such as ethanol, which has a low boiling point (78 ºC). The other drugs/drug classes reviewed are not typically analyzed in this fashion because they do not meet the volatility requirements. In headspace extractions, sealed sample vials are heated at a specified temperature for a specified time; this is to move analytes from the liquid sample into the vapor phase. Once sample/vapor phase equilibrium is achieved, the concentration of the volatile analyte is constant across both the liquid and vapor phases. This equilibrium allows for the extraction of the vapor phase, and the direct correlation of the concentration in the vapor phase to the liquid sample. (Sithersingh & Snow, 2012; Restek Corporation, 2000; Beale & Block, 2011).

Instrument Set-up: Instrument set-up can be divided into two categories method development and method validation.

Method development involves the selection and programing of instrument parameters for HS-GC/FID instruments; parameters include equilibration time and temperature, injection method, column selection, temperature gradient, and optimization of sample preparation. Some instrument manufacturers sell HS-GC/FID instruments that have been pre-configured for the sole purpose of blood alcohol quantitation. This pre-configuration, in combination with the resources available on the websites of most instrument manufacturers, minimizes the time and effort required for method development. (Sithersingh & Snow, 2012; Appendix A – Instrument Manufacturer Analysis).

Equilibration time and temperature are important factors to optimize during method development, as extended exposure to equilibration temperatures may cause analyte degradation. Additionally, if sample/vapor phase equilibrium is not achieved, vapor concentration cannot be accurately correlated back to sample concentration. (Tiscione et.al., 2011; Sithersingh & Snow, 2012).

An important aspect of instrument set-up is the selection of the injection method used to inject the sample into the chromatographic column; this is critical to the repeatability and quality of the resulting chromatogram. Traditionally, a gas tight syringe (GTS) was used for this transfer step. GTSs are the simplest, easiest, and most inexpensive method used for retrofitting standard GCs into headspace-GCs. This method is no longer favored because it does not produce acceptable repeatability; this is likely due to sample loss caused by samples recondensing in syringes that are not equilibrated to the same temperature as the sample vials. Transfer-line-based systems, also called balanced-pressure systems, are used for sample injection

with a high degree of repeatability. However, this method does not inject a quantifiable sample volume into the system, which makes it less than ideal for forensic analysis. The injection method used by most headspace samplers intended for forensic use is the sample-loop system, also called the pressure-loop system. This method employs the use of a pressurized loop system to inject a known volume of sample; reproducibility is improved by the use of the fixed volume loop. (Sithersingh & Snow, 2012; Restek Corporation, 2000).

Typically, HS-GC/FID uses capillary columns, which are generally 30-meters. The internal diameters and widths of the stationary phase vary depending on the column. Columns affect analyte separation and retention time. Another factor that affects analyte separation and retention time is the oven temperature. Runs can be isothermal, or can be run on a temperature gradient. When developing methods, labs need to select an appropriate column, and then optimize the temperature gradient to be used in order to effect quality separation. (Sithersingh & Snow, 2012; Restek Corporation, 2000; Kaya, et.al., 2011; Cooper, Riccardino, & Cojocariu, 2019).

After instrument methods have been developed, they must be validated prior to use for casework. According to the *Standard Practices for Method Validation in Forensic Toxicology*, written by the Academy Standards Board (ASB), "Validation is the process of performing a set of experiments to establish objective evidence that a method is fit-for-purpose and to identify the method's limitations under normal operating conditions." Validation parameters for quantitative forensic analyses at minimum must include: bias, calibration model, carryover, interference studies, ionization suppression/enhancement (for applicable techniques, such as LC/MS), limit of detection (LOD), limit of quantitation (LOQ), precision, dilution integrity (if applicable), and processed sample stability (if applicable). (Academy Standards Board, 2019).

Carryover is the potential for a sample with a high concentration to contaminate the sample(s) that come after it. If carryover is not properly assessed during validation, it has the potential to cause false positives (erroneous results). Carryover is evaluated by running blank samples after samples with high concentrations, typically the highest calibrator. If the blank samples don't show a response above the method's LOD, then the carryover assay is acceptable. If carryover is observed, then modifications need to be made to the method to remove or reduce carryover. Carryover can be minimized by purging the sample loop, by increasing the injection-port temperature, or by baking-out the oven. (Academy Standards Board, 2019; Restek Corporation, 2000).

Interference is the potential for the sample matrix, the stable-isotope internal standards, or other commonly-encountered analytes, such as over-the-counter medications, to hinder the effectiveness of an analytical method. Alcohol quantitation does not typically involve the use of stable-isotope internal standards. Matrix interference can be reduced by the sample preparation method used. Interference from other analytes can be tested in a variety of ways; for alcohol quantitation, a mixed alcohol calibration working standard is typically used. This standard includes typical volatile organic compounds that have the potential to be partitioned into the headspace and interfere with the analysis. Analytes may include methanol, ethanol, acetone, isopropanol, acetonitrile, ethyl acetate, and 1-propanol. Depending on the column and the chromatographic method used, these analytes have different, known retention times and can be effectively differentiated from one another. (Academy Standards Board, 2019; Cooper, Riccardino, & Cojocariu, 2019).

According to the ASB, labs have the option of either making their lowest non-zero calibrator, or using the decision point to define both their LOD and LOQ. This appears to be a

common practice for blood alcohol quantitation, with 0.02 g/dL being the decision point. This use of a non-zero calibrator, or decision point LOQ, could explain why many states have a limit of 0.02 g/dL for DUI drivers under 21; the LOQ of their tests is likely 0.02 g/dL. (Academy Standards Board, 2019; Cooper, Riccardino, & Cojocariu, 2019; Nolo, n.d).

Sample Preparation: HS-GC/FID sample preparation is relatively simple for a chromatographic technique, because the only sample preparation that is required is the addition of a known concentration of internal standard (IS), to a known volume of matrix in a headspace vial. The vial is then crimped closed to trap the vapor phase in the vial, and is ready for instrumental analysis. Alcohol quantitation does not typically require derivatization. The volume of biological sample used may vary from lab to lab, but it can be less than 1 mL. The IS used, and the concentration of the IS varies from lab to lab, but it is typically a volatile organic compound (VOC), similar to those described in the interference study. Standards, QCs, and blanks are included in the instrument run in addition to the case sample(s). (Cooper, Riccardino, & Cojocariu, 2019; Kaya, et.al., 2011).

Instrument Results: HS-GC/FID instruments can be constructed with a single-column or dual-column configuration. Some forensic labs use dual-column systems with two columns of different chemistries and selectivities to change the elution order and/or retention times of analytes. This technique enables the second column to be used for the qualitative confirmation of alcohol. (Cooper, Riccardino, & Cojocariu, 2019; Restek Corporation, 2000).

When calculating the time required to run a sample, it is important to remember that headspace analyses require an incubation period prior to GC analysis. Incubation time, and the time required for the chromatographic run can vary from lab to lab and instrument to instrument. Cooper, Riccardino, & Cojocariu incubated for 15 minutes and had a run time of 5 minutes.

Kaya, et.al. incubated for 10 minutes and had a run time of 6 minutes. Ghorbani, et.al. incubated for 15 minutes and had a run time of 12 minutes. Restek Corporation incubated for 15 minutes and had a run time of 3 minutes. Shimadzu incubated for 13 minutes and had a run time of 5.5 minutes. These studies used different incubation temperatures, different columns, and different temperature gradients. All were able to reliably quantitate and identify ethanol in less than 30 minutes. (Cooper, Riccardino, & Cojocariu, 2019; Kaya, et.al., 2011; Ghorbani, et.al., 2018; Restek Corporation, 2000; Shimadzu Corporation, 2019).

Data Analysis: The type of data obtained in a HS-GC/FID analysis is a chromatogram. Data is generally processed using integration software that comes with the instrument. Software is generally specific to instrument brands; for example, Agilent instruments use ChemStation, and Thermo Fisher Scientific instruments use Chromeleon. Though software may be used to aid data analysis, manual review and verification of results are essential. Labianca describes a case where the results produced by integration software were not manually verified; this caused the case to be acquitted. In this instance, the calibration curve produced by the software did not have an acceptable linearity, which caused an inaccurate calculation of the blood alcohol concentration of the case sample. (Cooper, Riccardino, & Cojocariu, 2019; Ghorbani, et.al., 2018; Labianca, 2015).

The ethanol peak is identified by retention time; when using a dual-column system, ethanol should have different retention times on the two columns, and can be qualitatively confirmed if both retention times match the reference. After qualitative analyte identification comes quantitation. The standards are used to build a calibration curve, and the QCs are used to verify the accuracy of the curve. Typically, a run will include at least one positive and one negative QC. Once QCs are determined to be acceptable, the case samples are then compared to

the curve, and samples above cutoff are quantitated. (Cooper, Riccardino, & Cojocariu, 2019; Ghorbani, et.al., 2018; Labianca, 2015).

Headspace Gas Chromatography Flame-Ionization Detection Mass Spectrometry – Emerging Technique

Headspace Gas Chromatography Flame-Ionization Detection Mass Spectrometry is the progeny of HS-GC/FID and GC/MS. HS-GC/FID/MS is a hybrid instrument that allows for a single injection to provide simultaneous quantification (FID) and spectral confirmation (MS). This hybridization is achieved by the installation of a two-way splitter, similar to a Dean's Switch, to the terminal end of the chromatographic column. The ends of the splitter are connected to the FID and MS in a 1:1 split ratio using deactivated columns. (Agilent Technologies, n.d.; Tiscione et.al., 2011; Tiscione et.al., 2013).

Instrument Set-up: Instrument set-up for HS-GC/FID/MS is very similar to instrument set-up for HS-GC/FID, and can also be divided into two categories, method development and method validation.

Method development for HS-GC/FID/MS also involves the selection and programing of instrument parameters, and includes equilibration time and temperature, injection method, column selection, temperature gradient, optimization of mass spectrometer parameters, and optimization of sample preparation. For mass spectrometers analyzing in selected ion monitoring (SIM) mode, method development may also include the establishment and optimization of parameters for SIM analysis. Some instrument manufacturers are now selling HS-GC/FID/MS instruments that have been pre-configured for blood alcohol quantitation. This pre-configuration, in combination with the resources available on the websites of many instrument manufacturers, minimizes the time and effort required for method development.

(Sithersingh & Snow, 2012; Shimadzu Corporation, n.d.; Appendix A – Instrument Manufacturer Analysis).

The validation requirements for HS-GC/FID/MS are the same as those for HS-GC/FID, and at minimum must include bias, calibration model, carryover, interference studies, ionization suppression/enhancement (for applicable techniques, such as LC/MS), LOD, LOQ, precision, dilution integrity (if applicable), and processed sample stability (if applicable). (Academy Standards Board, 2019).

Sample Preparation: Sample preparation for HS-GC/FID/MS is the same as that for HS-GC/FID, including the use of a VOC IS. HS-GC/FID/MS also includes standards, QCs, and blanks in the instrument run. (Tiscione, et.al., 2011).

Instrument Results: The MS analysis of HS-GC/FID/MS methods can be performed in one of two analysis modes, full-scan (scan) or SIM. Scan mode is used for both qualitative and quantitative analysis, and is a full ion scan. SIM mode is typically used for quantitative analysis; it requires prior knowledge of the specific masses to be measured; this gives it much better sensitivity than scan mode. Since HS-GC/FID/MS analysis uses the MS for the qualitative identification of ethanol, scan mode is generally used. (Shimadzu Corporation, n.d.; Tiscione, et.al., 2011).

Since HS-GC/FID/MS is an extension of HS-GC/FID, the time required for sample equilibration and run time is approximately the same. Tiscione, et.al. incubated for 20 minutes and had a run time of 8.5 minutes, thus enabling them to quantify ethanol concentration and qualify ethanol via spectral confirmation in less than 30 minutes. (Tiscione, et.al., 2011).

Data Analysis: Data analysis of the FID data is the same as that for HS-GC/FID. Like the FID data, the MS data is also processed using integration software; the same software is

typically used to process both types of data. Scan mode produces a total ion chromatogram (TIC); the peaks on the TIC can be selected, and the mass spectrum is viewed. Retention time and mass spectral confirmation are used for qualitative confirmation of ethanol. Spectral confirmation occurs when the ratios of diagnostic ions, those that reveal distinctive information about the target analyte, either match those of a reference standard *or* they match a library spectrum at a defined match factor. Manual verification of results produced by software is required. (Shimadzu Corporation, n.d.; Tiscione, et.al., 2011; Academy Standards Board, n.d., Standard for Identification Criteria in Forensic Toxicology; Academy Standards Board, n.d., Standard for Mass Spectral Data Acceptance in Forensic Toxicology).

Immunoassay Screening

In forensic laboratories, if alcohol is identified and quantified above the legal limit, often, no other tests are performed. If alcohol is not identified, or is not quantified above the legal limit, then further testing is performed. This further testing includes immunoassay screening, which is typically the enzyme-linked immunosorbent assay (ELISA), but may be a different type of immunoassay, such as the enzyme multiplied immunoassay technique (EMIT). This section will include a description of traditional immunoassays and Biochip Array Technology (BAT), as well as an assessment of each analytical system against the following parameters: instrument set-up, sample preparation, instrument results, and data analysis. System performance in relation to these parameters will be evaluated as part of the analytical efficiency discussion. (Logan, et.al., 2017; Monroe, 1984; Randox Laboratories, n.d.).

Traditional Immunoassays – Presently-Applied Techniques

Immunoassays are screening tools that utilize analyte-specific antibodies to presumptively identify the drugs/drug classes present in a sample. Whenever xenobiotics are

consumed, the body registers those invasive substances as antigens, and then deploys antibodies to counteract the effects of the antigens. Some antibodies are antigen specific for those comprised of certain molecular configurations. When an antibody attacks an antigen, an immune complex bond is formed; the formation of this bond is what is used to identify drugs/drug classes in unknown samples. In enzyme immunoassays, the antibodies or antigens that are used for these reactions may be enzyme labeled; this enzyme labeling is used to colorimetrically measure the concentration of the target analyte in the sample. (Datta, 2019; Monroe, 1984; Vashist & Luong, 2018; Immunalysis Corporation, 2016-2018).

Immunoassays used for drug screening are typically direct competitive assays. They utilize the competitive binding of the antibody by both the enzyme labeled antigens (in the reagents) and unlabeled antigens (in the sample matrix). Theoretically, competitive assays can be developed for any analyte. These assays are typically either homogenous or heterogenous. In homogenous assays, such as EMIT, the separation of excess enzyme-labeled antigens is not necessary. Concentration in homogenous assays is directly proportional to the measured enzyme activity. In heterogenous assays, such as ELISA, the separation of excess enzyme-labeled antigens is necessary, and requires the inclusion of a wash step(s) prior to signal measurement. Concentration in heterogenous assays is inversely proportional to the measured enzyme activity. (Datta, 2019; Monroe, 1984; Vashist & Luong, 2018; Immunalysis Corporation, 2016-2018; Cox & Baum, 1998; Sundström, Pelander, & Ojanperä, 2014; Engvall, 1980).

This review will focus primarily on ELISA immunoassays, as they are the ones predominantly used by forensic labs. This predominance is likely because ELISA assays are what the National Safety Council’s Alcohol, Drugs and Impairment Division recommends for screening blood. The results of the previously-mentioned toxicology survey that was part of the

Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities – 2017 Update, listed ELISA as the primary screening method. Of the laboratories surveyed, 74% reported the use of ELISA as their screening method for drugs in blood, while 11% reported the use of EMIT for screening blood for drugs; other labs also reported the use of various chromatographic methods for screening. (Logan, et.al., 2017).

Instrument Set-up: Because most immunoassays come in a semi-automated or fully-automated analytical system, there is not usually a method development step. Tests typically come in the form of a kit that provides the reagents, calibrators, and QCs necessary to perform a specific assay. However, if labs choose to employ the use of an automated liquid handling system, such as a TECAN Genesis liquid handling workstation, additional programing and regular calibration may be required. (Immunalysis Corporation, 2016-2018; Xie, et.al., 2004).

The validation requirements for immunoassay screening are quite simple as they only include LOD, precision (at the decision point), and processed sample stability (if applicable). Often, labs may choose to use the manufacturer recommended cutoff as both the LOD and decision point for immunoassays. Since most immunoassays used for forensic purposes use a zero calibrator and a cutoff calibrator, the concentration of the cutoff calibrator is the manufacturer recommended cutoff, and is used for both the decision point and the LOD. In this instance, labs are required to validate the LOD of all cross-reactive analytes in the same drug class that have cross-reactivity lesser than the target drug. For example, if an opiates assay uses morphine as the target analyte, but the lab wants to claim the potential for detecting hydromorphone (cross-reactivity of 50% in blood) with this assay, the lab must validate its ability to detect hydromorphone at double the cutoff of the opiates assay. However, this is not required for drugs that have cross-reactivities higher than the target drug. Continuing with the

example of the opiates assay, this means that codeine (cross-reactivity of 200% in blood), does not require additional validation because it is detected below the cutoff of the opiates assay. If labs choose to use a concentration other than the manufacturer recommendation as their decision point, additional LOD validation is required. (Academy Standards Board, 2019; Immunalysis Corporation, 2016-2018).

Sample Preparation: Sample preparation for immunoassays generally involves the use of an unaltered sample matrix, or the dilution of a sample matrix with buffer. After this step, samples are ready for analysis on fully-automated instruments. Semi-automated instruments may require additional steps involving the addition of reagents, incubation, and wash steps. However, it is not uncommon for labs to utilize automated liquid handling systems to turn semi-automated assays into fully-automated assays. The volume of sample required for immunoassays is generally assay specific, but is usually quite low (10-75 µL). Immunoassays also require calibration, usually a single calibrator at the decision point, as well as positive and negative QCs. (Tiscione & Wegner, 2017; Immunalysis Corporation, 2016-2018; KPL, 2013; Xie, et.al., 2004).

Instrument Results: Typically, immunoassays are designed to target a specific drug or drug class, which means that a sample is usually tested with multiple immunoassays. Even then, a single immunoassay may not cover an entire drug class. For example, the opiates/opioids drug class includes several different sub-classes of opiates/opioids. There is not an all-inclusive immunoassay that can be used to test for opiates, semi-synthetic opioids, and fully synthetic opioids. Typically, an opiates assay is required for opiates and some of the semi-synthetic opioids; it uses morphine as a target analyte, and the others have known cross-reactivities. An oxycodone assay is required for oxycodone (target analyte), and oxymorphone (cross-reactive

species), and other semi-synthetic opioids. Each of the fully synthetic opioids (fentanyl, methadone, and buprenorphine) require their own assays. Amphetamine/methamphetamine is another drug class that may require the use of multiple assays to cover most of the target analytes. The necessity for multiple assays to cover a single drug class is something that labs need to take into account when they are building immunoassay test panels. (Immunalysis Corporation, 2016-2018).

Regarding the time required to perform immunoassays, the limiting step seems to be the required incubation period. The incubation required for immunoassays is generally assay specific, but can range from 20-60 minutes. Generally, fully-automated assays will produce results faster than semi-automated assays, because semi-automated systems may require manual addition of reagents and wash steps. These are steps that can be performed faster and more accurately when automated. (Immunalysis Corporation, 2016-2018; Xie, et.al., 2004).

Data Analysis: Generally, immunoassay results come in the form of classifying analytes as detected or not detected in a sample. Typical forensic laboratory DUI/DUID workflow will include a confirmation of the assays that are detected in the screen. However, there is a chance that the screen results and confirmation results may not match up. This is because immunoassays have a proclivity for erroneous results caused by interferences. Interferences may be caused: by cross-reactivity of structurally similar compounds; by endogenous substances, such as binding proteins or autoantibodies; or by exogenous interactions, such as sample turbidity and incomplete washing during sample preparation. False negative results can also be caused by the High-dose Hook Effect, which is a phenomenon whereby an extremely high concentration of an analyte alters antibody binding, thus causing an assay to produce a false negative result. (Krasowski et.al., 2009; Tate & Ward, 2004; Academy Standards Board, 2018).

What follows is a description of the potential for erroneous results, primarily interference caused by cross-reactivity, in the six drug/drug classes that were described previously. This information is intended as a guide, and may not be true for all immunoassays because different manufacturers may use different antibodies to target antigens; thus, same analyte interference may vary for assays from different manufacturers. (Tate & Ward, 2004; Krasowski et.al., 2009).

Benzodiazepine immunoassays have some potential for erroneous results. Not all assays are built to target the low dose benzodiazepines; this can result in false negative results. Additionally, oxaprozin, nefopam, and sertraline are known to cross-react with benzodiazepine assays and cause false positive results. (Dasgupta, 2019; Krasowski, et.al., 2009).

Amphetamine/methamphetamine immunoassays are rife with erroneous results. Drugs derived from phenethylamine are the most common causes of false positives; these include several over-the-counter drugs such as ephedrine and pseudoephedrine. The optical isomers of amphetamine and methamphetamine also have the potential to cause false positives; for example, the use of a Vicks Inhaler has been identified in several studies. Dietary weight loss products are also potential sources of false positive results, because many of the active drug ingredients metabolize to amphetamine. Additionally, ranitidine, bupropion, and trazodone are known to cross-react with amphetamine/methamphetamine assays and cause false positive results. Interestingly, amphetamine/methamphetamine immunoassays may not be able to detect bath salts and other designer drugs structurally similar to amphetamine. (Dasgupta, 2019; Hutchings & Widdop, 2013; Maharjan & Johnson-Davis, 2019).

Cocaine/benzoylecgonine immunoassays are fairly robust. The only erroneous results noted in studies are from cross-reactivity related to the consumption of herbal teas from Latin America. These teas are sometimes made with coca leaves, which contain trace amounts of

cocaine, which is then metabolized to benzoylecgonine. (Maharjan & Johnson-Davis, 2019; Dasgupta, 2019; Krasowski, et.al., 2009).

Opiates/opioid immunoassays have some potential for erroneous results. Opiate assays tend to have lesser cross-reactivity with semisynthetic 6-keto-opioids and practically no cross-reactivity with synthetic opioids. This lack of cross-reactivity with synthetic opioids is why most of them have drug specific immunoassays, which exhibit very little cross-reactivity with potentially interfering substances. The poor cross-reactivity with some semisynthetic 6-keto-opioids is why many labs have an oxycodone assay that is intended to cross-react with oxymorphone; an unintended cross-reactive substance is naloxone. False positive opiate results have been linked to the consumption of poppy seed-containing foods, since poppy seeds contain morphine and codeine. Additionally, rifampicin, naloxone, pholcodine, and certain fluoroquinolone antibiotics are known to cross react with opiate assays and cause false positive results. (Maharjan & Johnson-Davis, 2019; Dasgupta, 2019; Krasowski, et.al., 2009).

Heroin/6-MAM immunoassays are fairly robust. As mentioned previously, semisynthetic opioids have a lesser cross-reactivity for opiate immunoassays. Additionally, since 6-MAM assays are drug specific, they exhibit very little cross-reactivity with potentially interfering substances, and practically no cross-reactivity with other opiates/opioids. (Hutchings & Widdop, 2013).

THC/THC-COOH immunoassays have some potential for erroneous results. Marinol, synthetic marijuana, is known to cause positive marijuana results. Additionally, efavirenz, niflumic acid, and nonsteroidal drugs, such as naproxen and ibuprofen, are known to cross-react with cannabis assays and cause false positive results. (Maharjan & Johnson-Davis, 2019; Dasgupta, 2019).

Biochip Array Technology – Emerging Technique

In 2002, Randox Laboratories developed Biochip Array Technology (BAT), which utilizes the principles of ELISA on a microscale. Instead of using a 96 well plate to perform a battery of immunoassays, a single biochip (9x9mm) acts as the reaction vessel for a variety of immunoassays. Biochips are composed of up to 49 discrete test regions (DTRs), including four internal quality control DTRs, one visual reference DTR, and 44 DTRs which are used for simultaneous multi-analyte testing. Each DTR can contain analyte-specific antibodies for a drug/drug class immunoassay, thereby allowing up to 44 different screening tests to be performed on a single biochip concurrently. (Randox Laboratories, n.d.; Randox Laboratories, 2001; Randox Laboratories, 2014; Randox Laboratories, 2018).

Instrument Set-up: Randox offers both semi-automated and fully-automated instrumentation for BAT; these assays include detection via a chemiluminescent light source, and a charge coupled device (CCD) camera and imaging system. For their toxicology assays, Randox allows for user-defined (custom) cutoffs, in addition to their recommended cutoffs. (Randox Laboratories, n.d.; Randox Laboratories, 2018; Randox Laboratories, 2015).

The validation requirements for BAT are the same as those for immunoassay screening; they include LOD, precision (at the decision point), and processed sample stability (if applicable). Alvarez, et.al. and Castaneto, et.al. determined that BAT assay performance could be optimized for improved detection when the manufacturer recommended cutoffs were increased. Labs using custom cutoffs are required to perform additional LOD validation for analytes with cross-reactivities at or below the target analyte. Continuing with the previous example of the opiates assay, this means that codeine (cross-reactivity of 200% in blood) would

require validation of the ability to detect codeine at half the cutoff of the opiates assay. (Academy Standards Board, 2019; Alvarez, et.al., 2012; Castaneto, et.al., 2015).

Sample Preparation: Sample preparation for BAT generally involves the use of an unaltered sample matrix, or the dilution of a sample matrix with buffer. Following this step, samples are ready for analysis on the fully-automated instruments. The semi-automated instrument requires additional steps involving the addition of reagents, incubation, and wash steps. The volume of sample required for BAT is generally assay (biochip) specific, but is usually quite low, requiring as little as 7-25 µL per biochip. BAT assays also require calibration as well as positive and negative QCs. (McLaughlin et.al., 2012; McLaughlin et.al., 2019; Randox Laboratories, 2015; Randox Laboratories, 2018).

Instrument Results: Randox offers a variety of multiplexed biochips which allow for the simultaneous analysis of up to 22 drugs/drug classes in a single biochip. Randox offers biochips with several different DOA (drugs of abuse) panels, and also offers custom arrays. With custom arrays, labs are able to select up to 22 drugs/drugs classes they want included in their screening panel. Since specific drugs may be abused somewhat regionally, the use of a custom array allows labs to test for the drugs/drug classes most often encountered in their region. (Randox Laboratories, n.d.; Logan, et.al., 2017).

In theory, the results for a biochip can be obtained within 60 minutes of the commencement of sample processing when using the semi-automated system; this time-frame includes a 30-minute incubation. When using the fully-automated systems, results may be obtained within 55 minutes of the inception of testing, including a 30-minute incubation. (McLaughlin et.al., 2012; McLaughlin et.al., 2019; Randox Laboratories, 2015).

Data Analysis: BAT results typically come in the form of classifying analytes either as detected or not detected in a sample, for each assay reported. Randox instruments allow users to decide which tests to report, which means there may be tests that were performed on a biochip that were not reported. Randox instruments store both reported and unreported data, which allows for the retrieval of previously unreported results; this data-mining capability allows for samples to be reassessed without requiring reanalysis on the instrument. (Randox Laboratories, 2015; Randox Laboratories, 2018; FitzGerald, et.al., 2005; Academy Standards Board, 2018).

To reduce potential interference caused by cross-reactivity of structurally similar compounds and endogenous substances, biochips contain a scavenger antibody that has a high affinity for interfering substances, and a low affinity for target analytes. In two studies of postmortem blood samples, McLaughlin et.al. found that less than 1% of the samples screened using BAT and confirmed via chromatographic methods gave false positive results. False positive screen results were only seen in amphetamine assays, and were attributed to the decompositional state of the postmortem samples, exogenous interactions of putrefactive amines. There were also a few instances of false negative results, less than 1% of samples screened using BAT and confirmed via chromatographic methods. These false negatives were attributed to the cutoffs of the BAT screen being above the cutoffs of the chromatographic confirmation methods. Alvarez, et.al. and Castaneto, et.al. found that false positives could be reduced by increasing the cutoffs of the assays. Both studies increased the manufacturer recommended cutoffs anywhere from 5- to 20-fold, essentially moving the cutoffs into therapeutic ranges, versus the sub-therapeutic ranges recommended by Randox. (Randox Laboratories, 2018; McLaughlin et.al., 2012; McLaughlin et.al., 2019; Alvarez, et.al., 2012; Castaneto, et.al., 2015).

Drug Confirmation and Quantitation

Typical forensic laboratory DUI/DUID workflow includes: a confirmation of the drugs/drug classes that screen as detected; a review of the DRE analysis for drugs/drug classes that can't be screened for; and confirmation testing for these substances. Confirmation testing may be divided into two categories, systematic toxicological analysis, or targeted analyte analysis. In systematic toxicological analysis, samples are prepared and analyzed in a fashion that is favorable for the qualitative analysis of a broad spectrum of substances, for example acidic drugs. Depending on the laws of the locality where the testing is being performed, analytes that are qualified may additionally require quantitation. In targeted analyte analysis, samples are prepared and analyzed in a fashion that is favorable for the qualitative and/or quantitative analysis of a single drug or drug class, (for example, lorazepam and/or benzodiazepines). Depending on the technique used, qualification and quantitation may be performed simultaneously or independently. This section will include a description of gas chromatography mass spectrometry (GC/MS) and liquid chromatography tandem mass spectrometry (LC/MS/MS), as well as an assessment of each instrument against the following parameters: instrument set-up, sample preparation, instrument results, and data analysis. Instrument performance in relation to these parameters will be evaluated as part of the analytical efficiency discussion. (Kabir & Furton, 2012; Kostakis, Harpas, & Stockham, 2013; Sciex, 2010).

Gas Chromatography Mass Spectrometry – Presently-Applied Technique

Gas Chromatography Mass Spectrometry (GC/MS) is considered by many to be the gold standard for drug confirmation, because every analyte creates a unique spectrum. GC/MS was developed in the 1950s, when two Dow Chemical Company researchers combined the separation

power of gas chromatography with the spectral identification capabilities of mass spectrometry. GC/MS was also simultaneously developed by two Philip Morris Incorporated researchers. The Dow Chemical Company researchers used a fast mass spectrometer, while the Philip Morris Incorporated researchers used a slower mass spectrometer. Importantly, none of these researchers patented GC/MS, thus allowing others to quickly adapt the method and make necessary improvements to allow the technology to go mainstream. (Jones, 2019).

Gas chromatography is a separation technique where analytes in a sample interact with the chromatographic column while it is simultaneously being heated. The analyte interactions with the column, in combination with the different boiling points of the analytes, cause separation. Separation is observed by the elution of analytes at retention times. After elution from the column, analytes enter the mass spectrometer where they produce a spectrum displaying ion peaks versus their relative concentrations. Each analyte has a unique splitting pattern, creating a unique spectrum, which allows for the utilization of reference libraries to match unknown spectra with known standards. (Thermo Fisher Scientific, n.d; Finkle, Taylor, & Bonelli, 1972; Kabir & Furton, 2012; Silverstein, Webster, & Kiemle, 2005).

Instrument Set-up: Instrument set-up can be divided into two categories, method development and method validation.

Method development involves the selection and programing of instrument parameters for GC/MS instruments; parameters include column selection, temperature gradient, ionization method, and optimization of sample preparation. For mass spectrometers analyzing in SIM mode, method development may also include the establishment and optimization of parameters for SIM analysis. Some instrument manufacturers sell GC/MS instruments that have been pre-configured for forensic toxicology analysis. This pre-configuration, in combination with the

resources available on the websites of most instrument manufacturers, minimizes the time and effort required for method development. (Kabir & Furton, 2012; Shimadzu Corporation, n.d.; Appendix A – Instrument Manufacturer Analysis).

Typically, GC/MS uses capillary columns; common lengths for columns are 10-15 meters. The internal diameters and widths of the stationary phase vary depending on the column. Packed columns have also been used for GC/MS analysis. Columns affect analyte separation and retention time. Column selection may also impact whether analyte derivatization is necessary or optional. Runs can be isothermal or utilize a temperature gradient. When developing methods, labs need to select an appropriate column, and then optimize the temperature gradient to be used in order to effect quality separation. (Thermo Fisher Scientific, n.d.; Agilent Technologies, n.d.; Kabir & Furton, 2012; Schaaf, Gerhards, & Dobbeleer, 2018; Gujar, 2018; Lambing, Phillips, & Robarge, 2010; Finkle, Taylor, & Bonelli, 1972; Finkle, Foltz, & Taylor, 1974).

MS ionization typically utilizes one of three gas-phase ionization methods, positive chemical ionization (PCI), negative chemical ionization (NCI), or electron impact ionization (EI). EI is considered to be “hard ionization”, as it often causes substantial fragmentation. PCI and NCI are considered to be “soft ionization”, as they tend to produce spectra with less fragmentation. Traditionally, EI was the primary ionization method used, in-part due to the fact that it was the first ionization method available. PCI is also frequently used for drug analysis because it allows for the easy identification of drugs and their metabolites. NCI is gaining popularity in drug analysis, particularly for electrophilic molecules, such as THC-COOH, because it has been shown to lower the LOD. Labs will generally choose the ionization method that produces the best results; this may involve a comparison among the different ionization

methods, or the selection of an ionization method for its compatibility with a certain library or database. (Silverstein, Webster, & Kiemle, 2005; Gujar, 2018; Kabir & Furton, 2012; Quimby, 2008; Schaaf, Gerhards, & Dobbeleer, 2018; Finkle, Foltz, & Taylor, 1974; Lin, et.al., 2008; Lin, et.al., 2016).

After instrument methods have been developed, they must be validated prior to use for casework. Validation parameters for qualitative forensic analysis at a minimum must include carryover, interference studies, ionization suppression/enhancement (for applicable techniques, such as LC/MS), LOD, and processed sample stability (if applicable). Validation parameters for quantitative forensic analyses require the previously listed qualitative parameters, and include the additional parameters of bias, calibration model, LOQ, precision, and dilution integrity (if applicable). Though it is not a validation requirement, labs additionally need to develop acceptance criteria for the analysis of MS data. These criteria must be used for validation, and for all case samples analyzed using the validated method. (Academy Standards Board, 2019; Academy Standards Board, n.d., Standard for Identification Criteria in Forensic Toxicology; Academy Standards Board, n.d., Standard for Mass Spectral Data Acceptance in Forensic Toxicology, n.d.).

As mentioned previously, carryover is the potential for a sample with a high concentration to contaminate the sample(s) that come after it. If carryover is not properly assessed during validation, it has the potential to cause false positives (erroneous results). Carryover can be minimized by baking-out the oven or by column backflushing. (Academy Standards Board, 2019; Quimby, 2008).

As mentioned previously, interference is the potential for the sample matrix, for stable-isotope internal standards, or for other commonly encountered analytes, such as over-the-counter

medications, to hinder the effectiveness of an analytical method. Matrix interference can be reduced by the sample preparation method, used in combination with the optimization of GC parameters. Interference from stable-isotope ISs can come from impurities in the standard of non-labeled compounds. This is assessed by spiking a blank sample with IS, and then analyzing it for non-labeled compounds above the LOD. Interference from other analytes can be tested in a variety of ways. For drug qualification and quantification, this may involve an evaluation of matrix samples containing compounds that may interfere the assay, including structurally-similar compounds, common drugs of abuse and their metabolites, and over-the-counter drugs. (Academy Standards Board, 2019; Quimby, 2008).

As mentioned previously, the ASB gives labs the option of either making their lowest non-zero calibrator, or using the decision point to define both their LOD and LOQ. The National Safety Council's Alcohol, Drugs and Impairment Division has recommended cutoffs for Tier I analytes, which can be used as the decision point to define both LOD and LOQ. The survey accompanying these recommendations found that only 17% of surveyed labs were in compliance with these cutoffs, and an additional 52% were working toward compliance. ASB also gives labs the option to estimate LOD using background noise, which may be achieved by the use of reference materials, statistical analysis of background, or a linear calibration curve. Labs that are not testing at the recommended cutoffs for Tier I analytes may use this alternative approach to LOD estimation. (Academy Standards Board, 2019; Logan, et.al., 2017).

Sample Preparation: GC/MS requires labor-intensive sample preparation, which directly impacts the integrity and quality of chromatographic data. Common extraction methods for blood were described previously; these included LLE, SPE, SPME, PPT, and a discussion on derivatization. Because GC/MS does not work as well for analytes that are thermally instable,

highly polar, or semi-/non-volatile, analytes exhibiting one or more of these issues can sometimes be derivatized to coerce compatibility with GC/MS. However, it should be noted that not all analytes are compatible with GC/MS. During the description of drugs/drug classes, it was further communicated that derivatization is required or recommended for the specific drug/drug classes reviewed for a variety of reasons, including thermal instability of target analyte, achievement of requisite chromatographic separation, and generation of better-defined mass spectra. The downside of derivatization is that it increases the complexity of the sample preparation process, as well as the length of time required for sample prep. (Kabir & Furton, 2012; Kostakis, Harpas, & Stockham, 2013; Lin, et.al., 2008; Lin, et.al., 2016).

Sample size can range from a few hundred microliters to a couple milliliters, and appears to be dependent on the extraction method being used. Large sample volume requirements result when a concentration step is required. The time required for sample preparation is also dependent on the extraction method and the simultaneous derivatization method that are being used; because GC/MS requires a clean sample matrix, this can be a long process, with sample prep taking several hours. As discussed earlier, confirmation testing may take the form of systematic toxicological analysis or targeted analyte analysis; these different analyses may also affect the required sample size and the time required for sample preparation. Standards, QCs, and blanks are included in the instrument run in addition to the case sample(s). Qualitative analyses may only include a cutoff calibrator, while quantitative analyses will include a calibration curve for each analyte being quantified. (Lambing, Phillips, & Robarge, 2010; Kostakis, Harpas, & Stockham, 2013; Raes, Verstraete, & Wennig, 2008; Jemal, 2000; Sciex, 2010; Waters Corporation, n.d.).

Instrument Results: The MS analysis of GC/MS methods can be performed in one of three analysis modes scan, SIM, or fast automated scan/SIM mode (FASST). Scan mode is used for both qualitative and quantitative analysis, and it is a full ion scan. SIM mode is typically used for quantitative analysis; it requires prior knowledge of the specific masses to be measured, which gives it much better sensitivity than scan mode. FASST mode acquires scan and SIM data simultaneously by rapidly switching back and forth between the modes. FASST mode works very well for multi-analyte qualitative methods, because the SIM mode identifies analytes that may have low sensitivity and may be missed in scan mode, while scan mode identifies compounds that may be overlooked in SIM mode. The type of assay being performed determines the type of analysis mode used. (Shimadzu Corporation, n.d.; Quimby, 2008).

GC/MS typically requires a relatively long analysis time of 10 – 60 minutes per sample; however, attempts are being made to shorten analysis time with the innovation of fast GC. Fast GC attempts to shorten run time by manipulation of the chromatographic parameters, without sacrificing chromatographic separation. Quimby reported the use of a fast GC run with an MS that was simultaneously collecting full scan and SIM data; this method screened for 725 target compounds in 9.75 minutes. (Kabir & Furton, 2012; Quimby, 2008).

Data Analysis: GC/MS data is generally processed using integration software that comes with the instrument. Software is generally specific to instrument brands; for example, Agilent instruments use ChemStation, and Thermo Fisher Scientific instruments use Chromeleon. Deconvolution software may also be used to aid data analysis of full scan data; Automated Mass Spectral Deconvolution & Identification System (AMDIS) is a deconvolution program developed by the National Institute of Standards and Technology (NIST). AMDIS uses computational techniques to produce "cleaned spectra" by the extraction of target spectra from

overlapping interference peaks; this produces spectra that are more compatible with library searching. (Cooper, Riccardino, & Cojocariu, 2019; Ghorbani, et.al., 2018; Quimby, 2008).

Analyte identification is a combination of retention time and mass spectral confirmation. Scan mode produces a total ion chromatogram (TIC); the peaks on the TIC can be selected, and the mass spectrum is viewed and run through a library. Spectral confirmation occurs when the ratios of diagnostic ions, those that reveal distinctive information about the target analyte, either match those of a reference standard *or* match a library spectrum at a defined match factor. Instruments that analyze in scan mode may have data mining capabilities. SIM mode evaluates samples for pre-established target and qualifier ions for each target analyte; each analyte will typically have at least three selected ions. Spectral confirmation occurs when observed diagnostic ion ratios agree with calculated ion ratios from a reference standard that is concurrently analyzed. Manual verification of results produced by software is required. (Shimadzu Corporation, n.d.; Quimby, 2008; Academy Standards Board, n.d., Standard for Identification Criteria in Forensic Toxicology; Academy Standards Board, n.d., Standard for Mass Spectral Data Acceptance in Forensic Toxicology; Lambing, Phillips, & Robarge, 2010).

Once qualitative analyte identification is complete, quantitation follows, when applicable. The standards are used to build a calibration curve, and the QCs are used to verify the accuracy of the curve. Typically, a run will include at least one positive and one negative QC. Once QCs are determined to be acceptable, the case samples are then compared to the curve, and samples above cutoff are quantitated. (Lambing, Phillips, & Robarge, 2010; Quimby, 2008).

Liquid Chromatography Tandem Mass Spectrometry – Emerging Techniques

The use of Liquid Chromatography Tandem Mass Spectrometry in clinical laboratories has increased substantially the last 20-25 years. Presently, LC/MS/MS is successfully

penetrating forensic science laboratories because it addresses the need for spectral confirmation of analytes that are traditionally detected via HPLC. (Grebe & Singh, 2011).

Liquid chromatography is a separation technique that employs the use of a column that is packed with a stationary phase, and typically two liquid mobile phases. One liquid mobile phase is aqueous, and one is organic; these mobile phases propel samples through the column. The analytes in the sample interact with both the stationary and mobile phases, thus causing separation. Separation is observed by the elution of analytes at retention times. After elution from the column, analytes enter the tandem mass spectrometer (MS/MS). There are a variety of options available for MS/MS analysis of DUI/DUID samples; they include triple quadrupole (QQQ), quadrupole linear ion trap (QLIT or QTRAP®), and quadrupole time of flight (QTOF). (Thermo Fisher Scientific, n.d.; Kostakis, Harpas, & Stockham, 2013).

It is important to note that while there are numerous options for detection methods, the same liquid chromatography techniques can be used with each of the different detection methods. It is also important to note that QLIT and QTOF have the ability to analyze in both targeted and untargeted scan modes, with the scan mode used determining whether the results obtained are considered to be presumptive or confirmatory. For forensic purposes, targeted scans are typically considered confirmatory and can be quantitated, while untargeted scans are typically considered presumptive, thus requiring additional confirmation. The advantage of an untargeted screen is the ability to presumptively identify novel, synthetic, or designer drugs that would be missed in targeted screens. The disadvantage of targeted screens is that one only finds what they are looking for. (Academy Standards Board, n.d., Standard for Mass Spectral Data Acceptance in Forensic Toxicology; Marquet, 2002; Kostakis, Harpas, & Stockham, 2013; Fleming, et.al., 2016).

Instrument Set-up: Instrument set-up can be divided into two categories, method development and method validation.

Method development for LC/MS/MS is typically more labor intensive than for GC/MS, because instruments used in multiple reaction monitoring (MRM) mode require optimization for every precursor and product ion being targeted. This is in addition to the selection and programing of other instrument parameters, such as column selection, mobile phase composition, mobile phase gradient, needle rinse, ionization method, and optimization of sample preparation. MS/MSs that are used in full scan or enhanced production ion (EPI) scan modes don't generally require extensive method development; instead, they require optimization of scan criteria and information dependent acquisition (IDA) parameters. Due to the more complex nature of LC/MS/MS, specifically the scheduling of MRMs, most LC/MS/MS instrument manufacturers do not offer preconfigured instruments. Some manufacturers have developed methods or created MRM libraries that can be used as a starting point for LC/MS/MS method development; these tools may require an additional purchase. Many manufacturers also offer resources on their websites that are intended to aid the method development process; these resources can include recommended sample preparation procedures, recommended LC conditions, and recommended MS/MS conditions. Even with these resources, LC/MS/MS method development can be a tedious, time consuming process. There are consultants who specialize in method development and validation of LC/MS/MS systems; they are an option, but add additional upfront cost. (Kostakis, Harpas, & Stockham, 2013; Lynch, et.al., 2010; Appendix A – Instrument Manufacturer Analysis).

LC columns come in a variety of stationary phases and particle sizes; labs will select the column that is most compatible with the separation they are trying to perform. The column most

commonly used for drug analysis is a reversed phase C18 column, though there are many different column options available for drug analysis, including biphenyl and phenyl-hexyl. Many LC systems also employ the use of a guard column to extend the life of the analytical column. Guard columns precede analytical columns, and are typically composed of the same stationary phase and the same particle size as the analytical column. Columns affect analyte separation and retention time. Mobile phase selection is just as important to analyte separation as column selection. LC systems employ the use of both aqueous and organic mobile phases that have been buffered; buffering mobile phases helps ensure retention time reproducibility. Mobile phases are typically buffered with either formic acid or ammonium formate, though other agents may be used. The organic mobile phase is typically methanol or acetonitrile. Jemal found that runs using buffered water/methanol mobile phases gave a significantly higher response than runs using buffered water/acetonitrile mobile phases. Runs can be either isocratic or utilize a gradient that changes the ratio of the aqueous and organic mobile phases during the run. Gradient elution is used more commonly than isocratic elution, because gradient elution maximizes the separation of analytes across a wide polarity range. When developing methods, labs need to select the appropriate column and mobile phases, and then optimize the mobile phase gradient to be used in order to effect quality separation. (Kostakis, Harpas, & Stockham, 2013; Grebe & Singh, 2011; Jemal, 2000; Maurer, 2005; Eeckhaut, et.al., 2009; Narayanasamy, et.al., 2019; Sosienski, 2019; Kintz, et.al., 2005; Li, Wang, & Jenkins, 2016; Cabrices, et.al., 2019; Xu, et.al., 2007).

MS/MS ionization typically utilizes one of three evaporative ionization methods positive electrospray ionization (ESI+), negative electrospray ionization (ESI-), or atmospheric pressure chemical ionization (APCI). ESI+/- and APCI are both considered to be “soft ionization”, as they tend to produce protonated (M+) or deprotonated (M-) molecular ions. ESI+/- is commonly

used to analyze a wide range of compounds, such as those in forensic analyses. APCI is generally less sensitive than ESI+/- for forensically relevant compounds, though it has been shown that APCI is less susceptible to matrix effects. ESI+ is the ionization method used most commonly, followed by APCI. ESI- is best suited for the analysis of THC-COOH and barbiturates because these analytes ionize preferentially in ESI-. Traditionally, ESI instruments were only able to be used in either positive *or* negative mode, which meant that if labs wanted to identify negative mode analytes, they needed to run samples twice. Since running samples twice was not ideal, some labs choose to only analyze samples in positive mode. More recently, ESI instruments have fast polarity switching capabilities, which allow for the analysis of samples in both ESI+ and ESI- almost simultaneously. Labs will generally choose the ionization method that produces the best results, but the selection of an ionization method may also be based on its compatibility with a certain library or database. (Silverstein, Webster, & Kiemle, 2005; Kostakis, Harpas, & Stockham, 2013; Maurer, 2005; Eeckhaut, et.al., 2009; Narayanasamy, et.al., 2019; Sosienski, 2019; Cabrices, et.al., 2019; Tran, et.al., 2017; He, et.al., 2015).

Instruments that are being used in MRM mode require optimization for the precursor and product ions of each analyte tested in the panel. First, the precursor ion of each target analyte must be identified. Then the product ions for each precursor ion must be identified, and the two product ions that are unique to a precursor ion are selected and optimized. Parameters optimized may include scan time, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP). DP, CE, and CXP are fragmentor voltages that are used for collision induced dissociation (CID) fragmentation. Many LC/MS/MS libraries are highly dependent upon CID fragmentation; as such, labs wishing to use those libraries will need to use fragmentation conditions that are similar to those used for the library. It has been noted that LC/MS/MS

libraries are manufacturer specific because of the inter-instrument differences in the relative intensities of product ion spectra, even when similar CID conditions are used. (Maurer, 2005; Jansen, Lachatre, & Marquet, 2005; He, et.al., 2015; Grebe & Singh, 2011).

After instrument methods have been developed, they must be validated prior to use for casework. Validation parameters for qualitative forensic analysis at a minimum must include carryover, interference studies, ionization suppression/enhancement (*for applicable techniques, such as LC/MS*), LOD, and processed sample stability (if applicable). Validation parameters for quantitative forensic analyses require the previously-listed qualitative parameters, and also include the additional parameters of bias, calibration model, LOQ, precision, and dilution integrity (if applicable). Validation parameters for non-immunoassay presumptive screening analyses, include interference studies, LOD, and ionization suppression/enhancement (*for applicable techniques, such as LC/MS*). Though it is not a validation requirement, labs additionally need to develop acceptance criteria and data analysis parameters for the analysis of MS/MS data. These criteria must be used for validation, and for all case samples analyzed using the validated method. Data analysis parameters are particularly important for untargeted scans because analyte identification is more dependent on data analysis rather than data collection. (Academy Standards Board, 2019; Academy Standards Board, n.d., Standard for Identification Criteria in Forensic Toxicology; Academy Standards Board, n.d., Standard for Mass Spectral Data Acceptance in Forensic Toxicology, n.d.; Colby, Thoren, & Lynch, 2018).

As mentioned previously, carryover is the potential for a sample with a high concentration to contaminate the subsequent sample(s) tested. If carryover is not properly assessed during validation, it has the potential to cause false positives (erroneous results). In LC techniques, carryover is primarily controlled by the needle rinse; key variables are the solution

used for the rinse and the rinse sequence. The needle is typically rinsed before and after injection, using a specified volume of needle rinse solution for a specified time, such as 1mL for 5 seconds. Needle rinse solution is typically composed of organic solvents, and may include acetonitrile, isopropanol, and methanol; solvent ratios may vary depending on the lab. (Academy Standards Board, 2019; Narayanasamy, et.al., 2019; Tran, et.al., 2017; He, et.al., 2015; Verplaeste, et.al., 2013; Lynch, et.al., 2010).

As mentioned previously, interference is the potential for the sample matrix, the stable-isotope internal standards, or other commonly-encountered analytes, such as over-the-counter medications, to hinder the effectiveness of an analytical method. Matrix interference will be discussed later in the ionization suppression/enhancement section. Interference from stable-isotope ISs can come from impurities in the standard of non-labeled compounds. This is assessed by spiking a blank sample with IS, and then analyzing it for non-labeled compounds above the LOD. Interference from other analytes can be tested in a variety of ways. For drug qualification and quantification, this can involve an evaluation of matrix samples containing compounds that may interfere with the assay, such as structurally-similar compounds, common drugs of abuse and their metabolites, and over-the-counter drugs. (Academy Standards Board, 2019; Fleming, et.al., 2016).

As mentioned previously, the ASB gives labs the option of either making their lowest non-zero calibrator, or using the decision point to define both their LOD and LOQ. Also mentioned previously were The National Safety Council's Alcohol, Drugs and Impairment Division's recommended cutoffs for Tier I analytes, which can be used as the decision point to define both LOD and LOQ. ASB also gives labs the option to estimate LOD using background noise, which may be achieved by the use of reference materials, statistical analysis of

background, or a linear calibration curve. Labs that are not testing at the recommended cutoffs for Tier I analytes may use this alternative approach to LOD estimation. (Academy Standards Board, 2019; Logan, et.al., 2017).

Ionization suppression/enhancement, which is also referred to as matrix effects, is the phenomenon caused by co-eluting endogenous substances that enhance or suppress the co-eluting target analyte. Labs need to assess matrix effects, and demonstrate that they do not have an impact on other validation parameters, such as LOD and LOQ. Matrix effects can be assessed by either post-column infusion or post-extraction addition. It is important to note that the evaluation of matrix effects includes multiple different sources of blank matrix to account for common interferences. Matrix effects can be reduced through several means, including modification of sample preparation, optimization of LC parameters, or the use of stable-isotope IS. Matrix effects are, in part, dependent upon the type of biological fluid being analyzed, because endogenous substances found in the sample matrix are the cause of interference. Because the offending compounds are endogenous, matrix effects can sometimes be reduced by modification of sample preparation techniques. Biological fluid specific matrix effects can also be ameliorated by the use of matrix matched standards and QCs. Additionally, matrix effects can be reduced by optimizing chromatography; this includes improving analyte separation, and adjustment of the mobile phase gradient so that interfering substances elute either before or after all target analytes. Stable-isotope IS can be used to compensate for matrix effects affecting the named analyte. The stable-isotope IS and the named analyte need to be at least three mass units apart for this technique to be effective. Limitations from this technique arise from the complication that not all compounds have stable-isotope IS. (Academy Standards Board, 2019;

Grebe & Singh, 2011; Jemal, 2000; Maurer, 2005; Eeckhaut, et.al., 2009; Li, Wang, & Jenkins, 2016; Casey et.al., 2020).

Sample Preparation: LC/MS/MS requires adequate sample preparation, because it directly impacts the integrity and quality of chromatographic data; however, it is not as sensitive to sample preparation as GC/MS. Common extraction methods for blood were described previously; these included LLE, SPE, SPME, PPT, and a discussion on derivatization. One of the hallmarks of LC/MS/MS is its compatibility with analytes that are not compliant with GC/MS because of their thermal instability, high polarity, or semi-/non-volatility. This LC/MS/MS compatibility comes with the added benefit of not requiring derivatization, which reduces both the time required for sample preparation, as well as the potential for errors to be made during the preparation process. (Kostakis, Harpas, & Stockham, 2013; Li, Wang, & Jenkins, 2016; Sciex, 2010; Cabrices, et.al., 2019; Maurer, 2005; Eeckhaut, et.al., 2009; Leung & Fong, 2014).

Another sample preparation option that is unique to LC/MS/MS is fast-flow on-line extraction, also referred to as on-line SPE. This allows for the direct injection of whole samples that have been spiked with IS pre-column. This method process utilizes a specialized type of LC extraction column that acts as an SPE cartridge; this extraction column is in series with the analytical column. The mobile phases act as the wash and elution solvents for the SPE process. A six-port switching valve allows for the materials removed in the wash step to be sent to waste instead of to the analytical column, and then sends the subsequent elution of target analytes onto the analytical column. Like traditional SPE, there are many different sorbents that may be used, each with varying separation mechanisms, and a variety of solvent options. The type of SPE cartridge or column used also determines if each sample injected requires a new cartridge, or if

the same column can be used for an entire analytical run. The use of disposable cartridges can help reduce carryover. There are also systems that include the on-column addition of IS, which allows for the direct injection of whole samples. (Jemal, 2000; Xu, et.al., 2007).

Sample size can range from 50 µL to a few hundred microliters, and appears to be dependent on the extraction method that is being used. Smaller sample volumes can be used because LC/MS/MS has better sensitivity than GC/MS and does not require a concentration step. The time required for sample preparation is also dependent on the extraction method. LC/MS/MS sample preparation is generally faster than GC/MS sample prep because of the elimination of the derivatization step. In the instance of on-line SPE, sample preparation time is negligible. As discussed earlier, confirmation testing may take the form of systematic toxicological analysis or targeted analyte analysis; these different analyses may also affect the sample size and the time required for sample preparation. Standards, QCs, and blanks are included in the instrument run in addition to the case sample(s). Qualitative analyses may only include a cutoff calibrator, while quantitative analyses will include a calibration curve for each analyte being quantified. (Xu, et.al., 2007; Li, Wang, & Jenkins, 2016; Sciex, 2010; Cabrices, et.al., 2019; Tran, et.al., 2017; Narayanasamy, et.al., 2019; Sosienski, 2019).

QTOF instruments additionally require the use of a reference solution to calibrate the MS/MS so it can perform mass accuracy corrections. The frequency of these mass calibrations can vary dramatically, for example, from every 40 seconds, to one for every sample, to every 5 injections; it appears to be user determined. Lockspray solution is a QTOF calibration solution sold by Waters; it uses leucine enkephalin as the calibrant for both positive and negative ionization modes. Purine and hexakis(1H, 1H, 3H-tetrafluoropropoxy)-phosphazine are also common compounds used for QTOF calibration of positive and negative ionization modes.

(Bidny, et.al., 2016; Grapp, et.al., 2018; Sciex, n.d.; Kronstrand, et.al., 2014; Tsai, et.al., 2013; Broecker, et.al., 2011; Thoren, et.al., 2016).

Instrument Results: QQQ utilizes a targeted analysis process known as multiple reaction monitoring (MRM). In the first quadrupole (Q1), precursor ions are filtered by their mass to charge (m/z) ratios. Q2 is a collision cell in which product ions are created by collision induced dissociation (CID). In Q3, product ions are filtered by their m/z ratios. Each target analyte in an assay will have a specific product ion that is targeted in the MRM; this is referred to as a transition, and typically there are two transitions (product ions) monitored per precursor ion. MRM is a targeted analysis, and can only be used to detect analytes that are included in the method; it can be used for both qualification and quantitation. (Sciex, 2012; Waters Corporation, n.d.; Marquet, 2002).

QLIT has the ability to analyze samples in MRM mode, but can also utilize enhanced production ion (EPI) mode, an untargeted analysis. EPI mode is an untargeted screen which can be used to scan for analytes that are not included in the method; it can be used for presumptive qualification. In EPI mode, Q1 is used to filter precursor ions, and Q2 is used to create product ions by CID. Q3 is used as a linear ion trap to perform a full scan of all product ions produced in Q2. The resulting product ion scan can then be searched against a library to identify the analyte. Another common way QLIT is being used is in MRM mode, in conjunction with information dependent acquisition (IDA) and EPI mode; this is sometimes referred to as MRM-EPI, and is a targeted analysis. Targeted precursor ions are analyzed in MRM mode, then IDA identifies ions that meet user-determined conditions, and then automatically follows the MRM scan with an EPI scan. The resulting product ion scan can then be searched against a library to identify the analyte. (Sciex, 2012; Sciex, 2014; Lynch, et.al., 2010; Thoren, et.al., 2016; Verplaeste, et.al.,

2013; Sciex, 2020; Dresen, et.al., 2010; Herrin, McCurdy, & Wall, 2005; Jansen, Lachatre, & Marquet, 2005).

QTOF typically analyzes samples in auto MS/MS mode, which is an untargeted analysis. Auto MS/MS mode first performs a full scan in MS mode, then IDA identifies ions that meet user-determined conditions, and then automatically follows the full scan with an MS/MS scan. The resulting auto MS/MS scan can then be searched against a library to presumptively identify the analyte. Waters Corporation has developed a proprietary data independent QTOF acquisition mode called MS^E, which is also an untargeted scan. In MS^E mode, precursor and product ions are produced and collected almost simultaneously. Software is then used to deconvolute the data and organize it by retention time so it can undergo analysis. In addition to auto MS/MS mode, some QTOF instruments also have the ability to analyze samples in MRM mode. (Thoren, et.al., 2016; Colby, Thoren, & Lynch, 2018; Broecker, et.al., 2011; Broker, Herre, & Pragst, 2012; Kronstrand, et.al., 2014; Waters Corporation, 2011; Grapp, et.al., 2018; Bidny, et.al., 2016; Sciex, n.d.).

LC/MS/MS typically requires a shorter analysis time than GC/MS, typically less than 15 minutes per sample. Sosienski separated a 26-drug panel in 3.5 minutes. He et.al. separated a 93-drug panel in 6.5 minutes. Sciex performed a study where it compared several GC/MS panels against LC/MS/MS panels, and found that LC/MS/MS run times could be as much as 65% faster than GC/MS run times. The Sciex study was also able to combine opiate and 6-MAM panels into a single run, versus the two runs that would be required on GC/MS. It was observed that articles written between 2000 and 2010 cited longer run times than articles written between 2010 and 2020. One reason for this could be improvements made in regard to HPLC and UHPLC

techniques. (Kostakis, Harpas, & Stockham, 2013; Sosienski, 2019; He et.al., 2015; Sciex, 2010).

Data Analysis: LC/MS/MS data is generally processed using integration software that comes with the instrument. Software is generally specific to instrument brands. Sciex uses SCIEX OS; Waters uses UNFI; Agilent uses MassHunter; and Thermo uses Xcalibur. Due to the nature of the data gathered, LC/MS/MS data analysis is more time consuming than GC/MS data analysis. This is because analyte identification is more dependent on data analysis rather than data collection for untargeted scans. It is essential for labs to establish data analysis criteria. Data analysis can be aided by the optimization of library search criteria, however, all data must be manually reviewed and verified. (Lynch, et.al., 2010; Cabrices, et.al., 2019; Kronstrand, et.al., 2014; Grapp, et.al., 2018; Alvarez, et.al., 2012).

Analyte identification for targeted analyses depends on the analysis method used. MRM mode requires analytes to have matching retention times and the presence of target product ions in at least two transitions. Spectral confirmation occurs when observed diagnostic product ion ratios agree with calculated ion ratios from a reference standard that is concurrently analyzed. In MRM-EPI mode, analyte identification is a combination of retention time, the presence of a single transition, and mass spectral confirmation. MRM-EPI mode produces a product ion spectrum which can be viewed and run through a library. Spectral confirmation occurs when the ratios of diagnostic product ions, either match those of a reference standard *or* match a library spectrum at a defined match factor. (Academy Standards Board, n.d., Standard for Identification Criteria in Forensic Toxicology; Academy Standards Board, n.d., Standard for Mass Spectral Data Acceptance in Forensic Toxicology; Marquet, 2005; Thoren, et.al., 2016).

Once qualitative analyte identification is complete, quantitation follows, when applicable. The standards are used to build a calibration curve, and the QCs are used to verify the accuracy of the curve. Typically, a run will include at least one positive and one negative QC. Once QCs are determined to be acceptable, the case samples are then compared to the curve, and samples above cutoff are quantitated. (Tran, et.al., 2017; Narayanasamy, et.al., 2019; Sciex, 2020).

Presumptive analyte identification of untargeted analyses also depends on the analysis method used. EPI mode requires analytes to have retention times and product ion spectra that match those of a database at an acceptable level. Auto MS/MS mode and MS^E mode require analytes to have retention times, precursor masses, isotope peak patterns, and product ion spectra that match those of a database at an acceptable level. QTOF methods calculate the precursor mass and the isotope peak pattern, which, in combination with the right software, allows for the theoretical identification of <u>all</u> fragment spectra. Agilent's MassHunter software has an "Identify Metabolites" tool which uses an algorithm to find parent drugs or metabolites that may match the product ion spectra that did not have library matches. Untargeted ion acquisition modes allow for retrospective data analysis, typically referred to as data mining; this is important because it allows for samples to be reassessed without requiring reanalysis on the instrument. (Broecker, et.al., 2011; Broecker, et.al., 2010; Bidny, et.al., 2016; Thoren, et.al., 2016; Colby, Thoren, & Lynch, 2018; Grapp, et.al., 2018).

Margin of Error

The textbook description of margin of error, which is also referred to as measurement uncertainty or uncertainty, is a range in which the "true value" of a quantitative measurement is expected to fall. It is not possible to measure the "true value" of an article; instead a method is developed that yields a measurement which is believed to be close to the "true value". Statistical

uncertainty is the level of confidence associated with that measurement. The scientific purpose of reporting margin of error is to make the interpretation of results more meaningful by highlighting the confidence level of the reported result. In the context of DUI/DUID cases, ethanol and drug concentrations are the articles being measured; the entire process used to make measurements contributes to the uncertainty of the values reported. This process does not only include instrumental analysis, it also includes the human components of sample preparation and data analysis. Interestingly, the human components of measurement processes contribute considerably more to the margin of error than the instrument(s) used as part of those measurement processes. (Bell, 2016).

Estimation of uncertainty is linked to the method development and method validation processes. The process used for the estimation of uncertainty involves four key steps: 1) identification of potential uncertainty contributors, which often involves the use of a cause-and-effect diagram and a review of the measurement procedure; 2) minimization of dispersion, which involves an evaluation of uncertainty contributors, and then attempts to reduce their uncertainty through optimization of the method; 3) quantification of uncertainty contributors, which involves the assignment of numerical values to contributors; and 4) estimation of uncertainty, which involves the use of an uncertainty budget. Margin of error can be presented in the form of a percentage of the measurement, but is more commonly presented in the form of a range around the measurement. (Bell, 2016).

Traditionally, margin of error was a calculation reserved for the reporting of quantitative measurements. More recently, margin of error is also being used to estimate the level of confidence for qualitative data. For example, in the context of DUI/DUID cases, this could be the probability that the methamphetamine identified in a biological sample is in fact

methamphetamine, and not another drug, such as phentermine. There are fewer available guidelines for qualitative uncertainty calculations, and the qualitative nature of the data reported increases the complexity of these calculations. Bayes' theorem of posterior odds is commonly being used for qualitative margin of error calculations, however, there is not a uniform method for the calculation of the prior odds and likelihood ratios that are used in this calculation. (Jackson, 2016; Marquis, et.al., 2017; Morrison, Ballentyne, & Geoghegan, 2018).

The event that highlighted the importance of uncertainty measurements into the spotlight was the 2009 National Academy of Science Report (NAS Report), *Strengthening Forensic Science in the United States: A Path Forward*, which was written by several National Research Council committees. The NAS Report recommended that, "Forensic reports, and any courtroom testimony stemming from them, must include clear characterizations of the limitations of the analyses, including measures of uncertainty in reported results and associated estimated probabilities where possible." The legal intent behind reporting margin of error is best summarized by Christensen, et.al., "if a method can be applied, error may exist and should be acknowledged." The denial of measurement uncertainty can lead to the misrepresentation of forensic results in a court of law. However, the misunderstanding of measurement uncertainty by the court can also lead to a misrepresentation of forensic results. This discussion is outside the scope of this review. (National Research Council, 2009; Christensen, et.al., 2014).

In the context of DUI/DUID analysis, the process used to perform a measurement is the largest contributor to uncertainty. Consequently, there is not a meaningful way to compare the margin of error associated with each instrument included in this review, because margin of error must be calculated for each assay performed on each instrument in each lab. And, while multiple labs may use the same instrumentation to perform a measurement, no unified process is used for

these measurements. It is meaningful, however, to point out that as the number of steps in a measurement process increase, such as those used for sample preparation, so too will the potential uncertainty associated with that process increase. (Bell, 2016).

Cost

Information of costs of the laboratory instrumentation discussed herein is not readily available in published literature or on equipment manufacturer websites. All instrument manufacturer websites have an option to "Request Quote" on each instrument page. To gain an understanding of instrument cost, an attempt was made to contact a number of prominent instrument manufacturers; the procedure used for selecting manufacturers and contacting them is described in the "Methods" section. Of the manufacturers contacted by customer service e-mail or website inquiry, only one response was received, and it was a redirect of the inquiry. The redirect did not yield a response. The two representatives who were contacted directly did respond to the inquiry, and their completed questionnaires can be found in Appendices D and E.

Shimadzu produces both gas chromatography and liquid chromatography instruments, with a variety of detection methods available. As can be seen on the questionnaire, cost increases as the complexity of detection method increases; cost is lower for gas chromatography than liquid chromatography. This is consistent with the review performed by Wu et.al., where GC/MS was described as being approximately ¼ the cost of LC/MS/MS. It is important to note that the Wu et.al. study was the only one the author was able to find that listed approximate instrument pricing. (Appendix D – Shimadzu Questionnaire Response; Wu et.al., 2012).

Sciex produces liquid chromatography instruments. It offers multiple detection methods, and multiple models of each instrument. Instead of filling out the questionnaire, Sciex representatives chose to have a conference call to discuss the complexity of the cost question.

Their questionnaire was filled out by the author using the information obtained during the conference call. According to Sciex, a general estimation of cost is not quantifiable because the instrument and model selected depends on the needs of the lab. The cost ranges associated with their instruments are quite wide, and generally overlap, thereby reducing the usefulness of inter-instrument comparison. Intra-instrument comparisons are possible, but again, depend on the needs of the lab. The conclusion of this conversation was that cost is lab specific, and cannot be easily generalized. (Appendix E – Sciex Questionnaire Response).

Analytical Efficiency – Definition

Analytical efficiency is a term associated with both instrumentation/software efficiency, and whole laboratory efficiency. Broadly speaking, analytical efficiency is the useful information obtained, weighed against the efforts required to obtain it; or more simply, analytical efficiency is the ratio of input to output. According to a multiyear project sponsored by Agilent Technologies and executed by the Analytical Scientist, every lab has its own definition of analytical efficiency. Additionally, there is not a one-size-fits-all definition for analytical efficiency; each lab must select the input and output parameters that are most important to it. (The Analytical Scientist, 2018, Your Efficiency Challenge – Part II; Agilent Technologies, 2020).

The multiyear project sponsored by Agilent Technologies and executed by the Analytical Scientist began with a laboratory efficiency survey that was sent to over 1,200 liquid chromatography laboratories worldwide. 19% of the total laboratories surveyed were in North America, and 10% of the total laboratories surveyed were government organizations. When asked about the “Importance of Various Topics with Regard to Liquid Phase Separations”, the top four answers were: 1) robustness of the entire analytical workflow, i.e. reliability of results;

2) low limits of detection; 3) quick turnaround time for our samples; 4) high sample throughput. It was noted that robustness of the workflow was considered to be especially important to government organizations, and that turnaround time was especially important to facilities in North America. When asked about "Additional Major Challenges Regarding Efficiency and Liquid Separation Methods", the top answers included: methods, applications; resolution, sensitivity; sample preparation; software, data analysis; and, speed. (The Analytical Scientist, 2018, Laboratory Efficiency and Liquid Separations Survey Report).

Analytical Efficiency – Evaluation Criteria

The criteria that were ultimately selected for the analytical efficiency evaluation focused on robustness of the entire analytical workflow, which includes instrument set-up, sample preparation, instrument results, and data analysis. The robustness evaluation included a review of the relevant Daubert Prongs as applied to each step of the analytical workflow, as well as a review of LOD and turnaround time. The objective parameters the author used for the evaluation of the analytical efficiency of the instrumentation included those identified to be most important by both the government organizations and the North American laboratories surveyed; the parameters selected address the efficiency challenges described by the laboratories surveyed.

Margin of error and cost were not included as criteria for the analytical efficiency evaluation, because specific information covering these topics is not readily available in the published literature. Margin of error, like analytical efficiency, is lab and process specific. Thus, it was determined that an attempt to quantify margin of error in regard to analytical efficiency would not be meaningful. However, non-instrument factors, such as sample preparation, that contribute to margin of error may be discussed. Cost is also lab specific, in that each lab has different testing needs and a different budget. Additionally, labs may already own

the instrumentation being discussed, but could be seeking ways to use that instrumentation more efficiently. Thus, it was determined that cost would not be meaningful in regard to analytical efficiency.

Analytical Efficiency – Instrument Evaluations

An evaluation of the analytical efficiency of the instrumentation that was described above follows. Each instrument was evaluated against the four specifications detailed (Robustness of Instrument Set-up, Robustness of Sample Preparation, Robustness of Instrument Results, and Robustness of Data Analysis). In an attempt to quantify the analytical efficiency of each instrument, each specification was given a numerical value ranging from 1-5. This value was derived from an evaluation of the applicable Daubert Prongs, the level of complexity and time required for the specification, as well as various specification specific criteria, see Table 1 below.

Table 1

Point Value	**General Assessment Criteria**
1	Doesn't give desired results
2	Extremely complex and time consuming
3	Relatively higher level of complexity and ample time required
4	Moderate level of complexity and moderate time required
5	Lower level of complexity and minimal time required
	Specification Specific Assessment Criteria
Robustness of Instrument Set-up	Pre-configured instrument available? Develop method from scratch or modify exiting method from another instrument?
Robustness of Sample Preparation	Number of steps in sample preparation; as steps increase, so does potential for error. Volume of sample required.
Robustness of Instrument Results	Multiple tests required?
Robustness of Data Analysis	Potential for erroneous results?

HS-GC/FID – Presently-Applied Technique

HS-GC/FID has been the instrumentation of choice for the analysis of blood alcohol concentration for over 40 years; this has paved the way for its peer review and general acceptance by the relevant scientific community. Blood alcohol testing is unique because it exploits the inherent chemical properties of the target analyte. The extreme volatility of alcohol allows for the application of heat to move the alcohol out of the biological matrix and into the headspace where it can be sampled and analyzed. The optimization of this process makes HS-GC/FID very effective and efficient for the quantitation of blood alcohol concentration.

LOD is not an issue for this method, because all states have predetermined limits for blood alcohol concentration, and the limits are fairly high when considered against the capability of most instruments. Kaya et.al. reported an ethanol LOD as 0.00448 g/dL, which is more than 4 times lower than 0.02 g/dL, the LOQ many states appear to use. Turnaround time is not a key issue for this method, because the ease of the sample preparation process and the data analysis process make up for the somewhat longer run time. (Kaya et.al., 2011; Nolo, n.d).

Robustness of Instrument Set-up: As described above, HS-GC/FID instruments can be purchased pre-configured, ready for the comprehensive validation procedure that is performed under controlled conditions to evaluate the test system for erroneous results. Even when instruments are purchased that are not pre-configured, the familiarity that labs have with this method typically allows for easy method development. The establishment of this method, and the ease of method development are why Robustness of Instrument Set-up is receiving a 5-point rating.

Robustness of Sample Preparation: The sample preparation method used for this technique has also been well established, peer reviewed, and generally accepted. There are very

few steps in the sample preparation process; in addition to making this a swift process, it also reduces the potential for error. The simplicity of the sample preparation process is why Robustness of Sample Preparation is receiving a 5-point rating.

Robustness of Instrument Results: Including sample incubation and actual instrument run time, a single sample typically takes less than 30 minutes to run. Because these runs are for quantitative purposes, they require a full calibration curve and QCs in addition to case samples, which increases the total run time. Depending upon whether the instrument is single or dual columned, one or two chromatograms are produced by the instrument. The use of a second column allows for the qualitative identification of alcohol. However, because the practice of forensic science is moving toward spectral confirmation for qualitative identification, some labs are required to prepare and run additional samples. The need to run additional confirmation assays is why Robustness of Instrument Results is receiving a 4-point rating.

Robustness of Data Analysis: When performed properly, data analysis is a fairly simple process of building a calibration curve, verifying that curve with QCs, and then quantitating case samples. This process can be performed using manufacturer-provided software, though manual verification of results is required. The simplicity of this process is why Robustness of Data Analysis is receiving a 5-point rating.

HS-GC/FID/MS – Emerging Technique

HS-GC/FID/MS is a valuable method because it sustains the strengths of HS-GC/FID, while tackling its primary weakness, the lack of spectral confirmation. Peer review for this method comes in the form of articles detailing side by side comparisons that labs have executed as part of their validation of this method. Tiscione et.al. re-analyzed 81 case samples as part of its evaluation of HS-GC/FID/MS; its results showed exceptional correlation with their

established HS-GC/FID method. The primary advantage they cited for this method was the two-in-one quantitation and spectral confirmation. (Tiscione, et.al., 2011).

Robustness of Instrument Set-up: HS-GC/FID/MS is simply an upgraded version of a well-established, peer reviewed, generally accepted technique. The general acceptance of this technique is evidenced by the instrument manufacturers who are already offering pre-configured instruments specifically marketed for forensic labs. Even when instruments are purchased that are not pre-configured, the familiarity that labs have with both HS-GC/FID and GC/MS methods allows for easy method development. This ease of method development is why Robustness of Instrument Set-up is receiving a 5-point rating.

Robustness of Sample Preparation: The sample preparation method used for this technique is the same well established, peer reviewed, and generally accepted technique used for HS-GC/FID. There are very few steps in the sample preparation process, which in addition to making it a swift process, also reduces the potential for error. The simplicity of the sample preparation process is why Robustness of Sample Preparation is receiving a 5-point rating.

Robustness of Instrument Results: Including sample incubation and actual instrument run time, a single sample typically take less than 30 minutes to run. Because these runs are for quantitative purposes, they require a full calibration curve and QCs in addition to case samples, which increases the total run time. HS-GC/FID/MS allows for the simultaneous analysis of both blood alcohol quantitation and spectral confirmation, which makes this method highly efficient, as it eliminates the need for additional analyses. The dual functionality of quantitation and spectral confirmation is why Robustness of Instrument Results is receiving a 5-point rating.

Robustness of Data Analysis: When performed properly, data analysis is a fairly simple two-step process. The first step involves the construction of a calibration curve, the verification

of the curve with QCs, and then quantitation of case samples. The second step involves searching for ethanol in a spectral library. This process can be performed using manufacturer provided software, though manual verification of results is required. The straightforwardness of this process is why Robustness of Data Analysis is receiving a 5-point rating.

Traditional Immunoassays – Presently-Applied Techniques

Immunoassays have been peer reviewed and generally accepted for decades, but it is important to remember that immunoassays are merely a screening tool. Immunoassays are not generally analyte specific; instead they tend to target a drug class. Additionally, they are known to produce erroneous results, even when performed under controlled conditions and using validated methods. Immunoassay results cannot be the sole evidentiary test performed on a case sample because they do not satisfy all of the Daubert prongs. However, immunoassays are a good compass for confirmation testing.

Robustness of Instrument Set-up: Immunoassays typically come in the form of semi- or fully-automated systems that have reagent kits which can be purchased for each assay; as a result, instrument set-up is fairly easy. The lack of method development, paired with the ease of system set-up are why Robustness of Instrument Set-up is receiving a 5-point rating.

Robustness of Sample Preparation: Immunoassays typically use unprocessed samples, or require simple dilution prior to analysis. The lack of sample preparation required, is why Robustness of Sample Preparation is receiving a 5-point rating.

Robustness of Instrument Results: Immunoassays are advertised as being quick and easy tests. However, an issue that some labs encounter with traditional immunoassays, is the limited number of reagents that can be held in the reagent wheel of a semi-automated or fully-automated analytical system. For example, an automated analytical system for immunoassays can hold the

reagents for 12 assays, but forensic labs often screen for more than 12 assays, so labs must often run the analytical system twice for every sample, which slows turnaround time. Additionally, some immunoassays have long incubation periods, up to 60 minutes, which also slows turnaround time. The inefficiency of running a system twice, and the potentially long incubation times for "quick and easy tests" are why Robustness of Instrument Results is receiving a 4-point rating.

Robustness of Data Analysis: Immunoassay results come in the form of detected or not detected, however, the interpretation of these results is not as straightforward as it would seem because immunoassays are known to have erroneous results. There can be false negatives if cutoffs are above the cutoff of the corresponding confirmation assay. Or, false positives can be caused by cross-reacting substances. While false negatives are a problem, false positives are a greater problem, as they may mis-direct the course of confirmation testing. Not only does this hurt turnaround time, it also reduces the quantity of biological sample available for further testing. The propensity for erroneous results is why Robustness of Data Analysis is receiving a 3-point rating.

Biochip Array Technology – Emerging Technique

BAT is a method that has significant potential, because it uses the generally accepted technology of ELISA, and addresses some of its weaknesses. The multiplexing ability of these assays makes them very attractive to labs that are currently limited by the number of reagent positions on their analyzers. Perhaps the reason that BAT seems to be gaining general acceptance in forensic labs is because it is perceived as the next generation of immunoassays.

Robustness of Instrument Set-up: Like traditional immunoassays, BAT comes in the form of semi- or fully-automated systems; because of this, instrument set-up is fairly easy. The

lack of method development, paired with the ease of system set-up are why Robustness of Instrument Set-up is receiving a 5-point rating.

Robustness of Sample Preparation: BAT typically requires only simple dilution prior to analysis on fully-automated systems. The lack of sample preparation required, is why Robustness of Sample Preparation is receiving a 5-point rating.

Robustness of Instrument Results: BAT is advertised as being quick, easy, and accurate. As mentioned previously, each biochip contains internal quality control DTRs. If one or more of the internal quality control DTRs do not meet specifications, an error code is generated, and the sample will need to be reanalyzed. (Randox Laboratories, 2014). Additionally, some BAT assays have somewhat long incubation periods, up to 30 minutes, which slows the turnaround time. The associated incubation time for "quick and easy tests", and the potential to have to rerun samples due to faulty DTRs are why Robustness of Instrument Results is receiving a 4-point rating.

Robustness of Data Analysis: BAT biochips contain a scavenger antibody that seems to do a reasonably good job of preventing false positives. However, studies have also shown that when the cutoffs of the assays are at the manufacturer recommended levels (5 ng/mL), the number of false positives increases. The cutoff has to be raised to a therapeutically relevant level (25 ng/mL) to reduce false positives. (Castaneto, et.al., 2015). The possibility of erroneous results caused by manufacturer recommended test cutoffs being too low is why Robustness of Data Analysis is receiving a 4-point rating.

GC/MS – Presently-Applied Technique

GC/MS has been a lab staple for half a century; it was the first technique that coupled chromatography with mass spectrometry, and it has been generally accepted and peer reviewed

for decades. GC/MS is so engrained as the "gold standard" technique, that television shows and books even mention it by name when trying to make forensic science references. It is likely the most widely known forensic analytical instrument. Validated methods, performed under controlled conditions, have proven the lack of erroneous results. GC/MS has the ability to detect concentrations in the ng/mL range, but first, analytes need to be compatible with the testing technology. GC/MS is unique, in that many analytes must be forced into compatibility by derivatization, and even then, there are analytes that may not be compatible with GC/MS analysis.

Robustness of Instrument Set-up: As described above, GC/MS instruments can be purchased pre-configured, ready for the comprehensive validation procedure that is performed under controlled conditions and evaluates the test system for erroneous results. Even when instruments are purchased that are not pre-configured, the familiarity that labs have with this method allows for easy method development. The establishment of this method, and the ease of method development are why Robustness of Instrument Set-up is receiving a 5-point rating.

Robustness of Sample Preparation: Sample preparation for GC/MS is a long, tedious process; in addition to extraction, derivatization is often also required. Because GC/MS extracts need to be as clean as possible, sample preparation processes typically require multiple steps; subsequent derivatization can also be a multi-step process. All the steps in the sample preparation process are sites for potential error. If not performed properly, analytes may not derivatize properly, which will inhibit their detection. Also, GC/MS sample preparation procedures may require a considerable quantity of sample, because the sample prep process is intended to concentrate the target analytes. This reduces the quantity of sample available for other analyses. The complicated sample preparation process, in combination with the

requirement for derivatization and sample concentration are why Robustness of Sample Preparation is receiving a 3-point rating.

Robustness of Instrument Results: The long sample preparation procedure is usually followed by a long instrument run time (up to 60 minutes). An advantage that GC/MS has is the ability to simultaneously run SIM and scan analyses when using FASST mode. This ability to run simultaneous targeted and untargeted screens can prevent the need for future runs, however, not all instruments have this capability. The long analysis time is why Robustness of Instrument Results is receiving a 4-point rating.

Robustness of Data Analysis: The use of spectral libraries to identify unknown peaks dates back to the 1970s, when these libraries had to be accessed by telephone. It is interesting to note that even back in the 1970s, analysts recognized the importance of *both* retention time *and* spectral library matches to confirm analytes, as well as manual verification of library matches. (Finkle, Foltz, & Taylor, 1974; Finkle, Taylor, & Bonelli, 1972). The methods used for GC/MS analyte confirmation are generally accepted and peer reviewed. The use of manufacturer provided software, and additional software, like AMDIS help speed the data analysis process. The well-established methodology for analyte confirmation is why Robustness of Data Analysis is receiving a 5-point rating.

LC/MS/MS – Emerging Technique for Confirmatory Testing

LC/MS/MS is a technique that has been embraced wholeheartedly by clinical toxicologists since the late 1990s. According to Grebe & Singh, the reason for this acceptance is that "It [LC/MS/MS] seemed to offer all the advantages of GCMS without the disadvantages of compromised detection sensitivity and long chromatography run-times." (Grebe & Singh, 2011). According to Logan, et.al., LC/MS/MS is gaining traction in the forensic science community as

well. (Logan, et.al., 2017). However, LC/MS/MS has the stigma of being much more complex than GC/MS, which may discourage prospective users, and cause existing users to underutilize their instrumentation.

LC/MS/MS has been extensively peer reviewed; in spite of this, it has not yet received the same level of general acceptance as GC/MS. Many authors seem eager to point out the perceived weakness of LC/MS/MS in favor of the perceived strengths of GC/MS. Other authors eagerly describe LC/MS/MS as the perfect complement to GC/MS; this is because LC/MS/MS is compatible with analytes that don't work on GC/MS, or only work when forced. LC/MS/MS has the ability to detect concentrations in the ng/mL range, sub ng/mL range, and some instruments can even detect concentrations as low as the pg/mL range. (Sciex, n.d.) Validated methods, performed under controlled conditions have demonstrated the lack of erroneous results.

Before defining the analytical efficiency of LC/MS/MS, it is important to review the three common types that are being used. LC/QQQ uses a targeted MRM scan to identify analytes; the results obtained from this assay are considered confirmatory. LC/QLIT has the ability to analyze samples in MRM-EPI mode, which is a targeted scan used to identify analytes; it includes the use of a library search, the results of which are considered confirmatory. LC/QLIT also has the ability to perform a full scan of samples in EPI mode; this is an untargeted scan which is often considered to be a presumptive result. LC/QTRAP also has the ability to perform an untargeted full scan, in auto MS/MS mode; it is also often considered to be a presumptive result. The forensic science regulatory standards require that MS/MS assays have targeted diagnostic ions to be considered confirmatory. (Standard for Identification Criteria in Forensic Toxicology; Academy Standards Board, n.d., Standard for Mass Spectral Data

Acceptance in Forensic Toxicology, n.d.). For this reason, the analytical efficiency evaluation will only consider targeted analysis mode.

Robustness of Instrument Set-up: When LC/MS/MS is used in a targeted analysis mode, both the precursor and product ions must be known. Collision parameters for the product ions of each precursor ion must be optimized. There are resources available to aid this process, however, doing so requires more effort than any of the previous method development processes described. LC/MS/MS is very customizable, which goes hand in hand with the increased level of complexity required to bring an instrument online. The complexity and customizability of method development are why Robustness of Instrument Set-up is receiving a 4-point rating.

Robustness of Sample Preparation: The complexity of sample preparation for LC/MS/MS is much lower than the level of complexity of GC/MS sample prep. One of the reasons for this lower level of complexity is the absence of derivatization. The volume of sample required for LC/MS/MS extractions is generally smaller than the volume required for GC/MS extractions because LC/MS/MS does not typically require a concentration step. GC/MS also requires very clean samples; because of the increased sensitivity of LC/MS/MS, sample prep is required, but extracts do not have to be as clean as those necessary for GC/MS. A sample extraction method Sciex recommends for blood is PPT. This preparation method has the potential to cause matrix effects, but these can be ameliorated during the method development process by optimization of chromatography parameters and/or the utilization of stable-isotope ISs. As discussed, the fewer steps there are in the sample preparation process, the fewer places there are for error to occur. Additionally, LC/MS/MS has the unique ability to perform on-line sample extraction, which practically eliminates the sample preparation process, and drastically

reduces the potential for error. The ease of the sample preparation process is why Robustness of Sample Preparation is receiving a 5-point rating.

Robustness of Instrument Results: An advantage that LC/MS/MS has over GC/MS is its vastly reduced run time, typically less than 10 minutes. LC/MS/MS employs a targeted analysis, whereby analytes are only detected if they are included in the method. An advantage of targeted analysis is increased sensitivity for target analytes. While the targeted analysis approach employed by LC/MS/MS has advantages, a disadvantage is that the data received is limited to the pre-selected target analytes. The limitation of using a targeted analysis is why Robustness of Instrument Results is receiving a 4-point rating.

Robustness of Data Analysis: LC/MS/MS data is processed using manufacturer provided software, however, its analysis process is not as automated as the analysis process used for GC/MS. Peaks require manual verification, and may even require manual integration. The development of data analysis parameters is a very important step of method development and validation. The libraries available for use with LC/MS/MS are typically manufacturer specific; this is due to inter-instrument differences in CID fragmentation, which cause differences in the resulting product ion spectra. Generally, if a library is going to be used, the CID parameters need to be similar to those of the library. This limitation of the variability of libraries may be one reason why targeted analyses are required for confirmation. Once factor contributing to the popularity of MRM mode may be its targeted identification of multiple product ions, whereby it does not have to rely on the use of potentially variable libraries. The labor-intensive nature of data analysis is why Robustness of Data Analysis is receiving a 4-point rating.

LC/MS/MS – Emerging Technique for Presumptive Testing

As mentioned earlier, LC/MS/MSs used in untargeted modes only qualify as presumptive tests; this is a limitation when considering these instruments for confirmatory testing. However, its utility of untargeted scans for screening purposes is an advantage when comparing LC/MS/MS to immunoassay screening, as immunoassay screening is often limited to pre-established drugs/drug classes. When used for screening purposes, LC/MS/MS has the potential to presumptively identify novel, synthetic, and designer drugs that may not yet be widely known. These are drugs that would likely be missed by other methods for one of several reasons: they don't cross react well enough in immunoassays; due to lack of compatibility or lack of a library entry, they may be missed by general untargeted GC/MS screens; and, because cutting edge drugs may not be included in the panel, they would be missed by targeted LC/MS/MS analyses. LC/MS/MS screening is a technique that has undergone substantial peer review; it is also gaining traction as a generally accepted method because of its advantages over immunoassay screening. These advantages are why the analytical efficiency of LC/MS/MS as a screening tool was evaluated.

Robustness of Instrument Set-up: When LC/MS/MS is used in an untargeted analysis mode, it does not require as extensive method development as when it is used in targeted analyses. LC/MS/MS untargeted analyses require optimization of chromatography settings, optimization of scan criteria, and the selection of IDA parameters. The method development required for an LC/MS/MS screen is much more complex than the relative simplicity of purchasing a ready-to-use immunoassay system; this is why Robustness of Instrument Set-up is receiving a 3-point rating.

Robustness of Sample Preparation: Because LC/MS/MS is a chromatography technique, sample preparation is required. While the sample preparation required is generally *less* labor-intensive than GC/MS sample preparation, it is considerably *more* labor-intensive than the minimal sample preparation required for immunoassays. The requirement of sample preparation is why Robustness of Sample Preparation is receiving a 4-point rating.

Robustness of Instrument Results: LC/MS/MS has several advantages over immunoassays, including shorter run time, specific analyte presumptive identification (versus a drug class), increased sensitivity, and the ability to run an untargeted scan. An untargeted scan allows for presumptive identification of compounds that would be missed by immunoassays. It also allows for the presumptive identification of novel, synthetic, or designer drugs that may not yet have reference standards, or may not yet be in analyte libraries; these limitations would cause them to be missed by targeted LC/MS/MS analyses and untargeted GC/MS analyses. The presence of an analyte peak, even one that is unable to be identified, indicates the need for further analysis of the case sample, and could indicate a change in the drug habits of the geographic region. The data-mining capabilities of untargeted screens allow for future reanalysis of screen results, such as the development of a reference standard or the development of a library spectrum for novel, synthetic, or designer drugs. The advantage of employing an untargeted analysis is why Robustness of Instrument Results is receiving a 5-point rating.

Robustness of Data Analysis: Immunoassay systems automatically produce results upon completion of the system's analysis. LC/MS/MS data must be manually processed to produce a result. The process used for the analysis of untargeted data can be more labor-intensive than the one used for targeted data. Presumptive identification of untargeted analytes is more dependent on data analysis than on data collection, which is why the establishment of data analysis

parameters is so important. The requirement for data analysis, as well as the complex nature of data analysis, are why Robustness of Data Analysis is receiving a 4-point rating.

Conclusion

Each instrument was evaluated against the four analytical efficiency specifications detailed (Robustness of Instrument Set-up, Robustness of Sample Preparation, Robustness of Instrument Results, and Robustness of Data Analysis), and was assigned a numerical value ranging from 1-5, in an attempt to quantify their analytical efficiency. The values for each instrument were then totaled, see Table 2 below.

Table 2

			Robustness of the Entire Analytical Workflow				
Type of Test	**Type of Instrumentation**	**Type of Technique**	**Instrument Set-up**	**Sample Preparation**	**Instrument Results**	**Data Analysis**	**Total**
Alcohol Quantitation	Headspace GC/FID	Presently-Applied	5	5	4	5	19
	Headspace GC/FID/MS	Emerging	5	5	5	5	20
Presumptive Screening	Traditional Immunoassays	Presently-Applied	5	5	4	3	17
	Biochip Array Technology	Emerging	5	5	4	4	18
	LC/MS/MS	Emerging	3	4	5	4	16
Drug Confirmation and Quantitation	GC/MS	Presently-Applied	5	3	4	5	17
	LC/MS/MS	Emerging	4	5	4	4	17

This study showed that there is not a one-size-fits-all preferred instrument for the analysis of DUI/DUID case samples. The variations in laboratory needs, capabilities, and definitions of analytical efficiency are why there is not a single best instrument. These variations are observed in the responses of the previously-mentioned surveys by Logan, et.al. and The Analytical Scientist. In the Logan, et.al. survey, some labs determined that the National Safety Council's Alcohol, Drugs and Impairment Division's recommendations were not relevant to their

laboratory, so they made no effort to be compliant with them. (Logan, et.al., 2017). In the Analytical Scientist's survey, some labs stated their resistance to change, and/or they questioned whether the benefits of adding new analyses outweighed the disadvantage of losing an analyst for an unspecified period of time in order to bring a new instrument online. (The Analytical Scientist, 2019, Your Efficiency Challenge – Part III).

The available instruments not only compete with one another, but can also complement one another when used together for the analysis of DUI/DUID case samples. While some of the emerging techniques appear to be more analytically efficient than presently-applied techniques, this does not discount the overall utility and analytical efficiency of the presently-applied techniques; the narrow spread of the analytical efficiency totals is evidence of this conclusion.

This study also showed that there is not a uniform definition of analytical efficiency; each lab must select the input and output parameters that are most important to them. When labs are looking to purchase instruments, they need to assess and identify their testing needs, their budget, and their capability to bring a new instrument online. Labs then need to partner with instrument manufacturers, who should help them select the right instrument for the task at hand. Instrument manufacturers may also be able to provide labs with resources to aid in method development. The ACS's *Top Instrument Firms of 2018* also included a top 10 manufacturer ranking based on R&D spending. Danaher, the parent company of Sciex, was ranked #1, followed by Thermo Fisher Scientific, Agilent Technologies, PerkinElmer, and Waters Corporation; Shimadzu Corporation did not make this list. (Reisch, 2019).

It was observed that when emerging technology is merely an enhancement or upgrade of existing technology, as is the case with HS-GC/FID/MS and BAT, it tends to gain general acceptance faster than emerging technology which employs an entirely new technique, such as

LC/MS/MS. Though LC/MS/MS is chromatography coupled to mass spectrometry, as is GC/MS, LC/MS/MS uses a different type of chromatography and a different type of mass spectrometry. The study by Agilent and the Analytical Scientist found that some labs are reluctant to move away from legacy methods, even if newer methods could improve analytical efficiency. The study also determined that labs view the time and effort necessary to improve current testing methods as a significant impediment. (The Analytical Scientist, 2019, Your Efficiency Challenge – Part III). The survey that was part of the *Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities – 2017 Update* noted that the most common rationales given by labs for their lack of compliance with recommended standards were lack of staff, lack of time, and budget restrictions. (Logan, et al., 2017). The lack of staff and lack of time issues can be compounded when a new instrument is introduced; because the primary function of forensic analysts is to perform casework, if they are required to develop and validate methods on a new instrument, then they are not doing casework, and a casework backlog can develop. Labs that are resistant to change often do not have the manpower necessary to learn how to develop methods on a new instrument, or the budget to hire a consultant to set up a new instrument. Instead, they may determine that purchasing a newer version of the instrument they need to replace is the most efficient option for their lab because of the ease with which they will be able to bring it online.

The previously-mentioned survey by Logan, et.al., listed instrument technology as a reason for lack of compliance with recommended guidelines, with 52% of labs working toward compliance with the standard. LC/MS/MS may be a good option for those labs looking to improve their compliance by upgrading their instrument technology if they are willing to either put in the time and effort required for method development, or if they have the budget to hire a

consultant. For labs looking to upgrade their instrument technology, but lack the time required for method development or the budget for a consultant, an option may be teaming up with local universities, farming method development out to graduate students. The previously-mentioned 2009 NAS Report states that educational programs should, "prepare the next generation of forensic practitioners" to better prepare forensic science students for casework. (National Research Council, 2009). Forensic labs farming out method development work to university students would benefit labs, as they would not have to sacrifice casework to bring a new instrument online. This approach would benefit university students, as it would expose them to the particulars of forensic labs, while training them over instrumentation they will be using should they become forensic analysts. This approach would subsequently aid forensic labs that hire these students; when the labs want to add additional tests to their instruments, their new hires will be familiar with the method development process, thereby enabling new tests to be added more efficiently.

While there does not appear to be a single best instrument for the analytical efficiency of all forensic labs, there are many instruments available from which to choose. Ultimately, it is up to each individual lab to select the instrument(s) that best suit their unique analytical efficiency needs. Hopefully, having a document that describes the similarities and differences among the capabilities of the available instruments will aid labs in their decision-making process. General considerations that labs may want to take into account during their decision-making process include:

- Labs currently using GC/MS that do not have analysts available to dedicate the time needed to develop methods on an LC/MS/MS, or the budget to hire a consultant, may be

best served by purchasing a newer version of GC/MS, because they will be able to bring it online more quickly.

- Labs currently using GC/MS that have analysts available to dedicate the time needed to develop methods on an LC/MS/MS, or have the budget to hire a consultant, may want to consider getting an LC/MS/MS to take advantage of the benefits it offers.
- Labs currently using HS-GC/FID, that are looking to boost the efficiency of their ethanol analyses, may want to consider a HS-GC/FID/MS, as it will allow them to combine two tests into one analysis.
- Labs that are limited by the reagent handling capabilities of their immunoassay systems may want to consider multiplexing with BAT analysis.
- Labs that are looking for screening systems with more sensitivity and increased analyte detection may want to consider LC/MS/MS untargeted screens.
- Labs using their LC/MS/MS instruments for targeted confirmation analyses may want to consider adding untargeted screening analyses to them.
- Labs using their LC/MS/MS instruments for untargeted screens may want to consider adding targeted confirmation analyses to them.
- Labs that are in regions where new drugs are constantly being introduced may want to consider LC/MS/MS untargeted screens as a way to keep up with these changes and spot new drugs as they hit the streets.
- Labs looking to lower their detection limits may want to consider LC/MS/MS.
- Labs with limited funds available for instrument purchases may be best served by pursuing GC/MS and traditional immunoassay options.

Limitations

One limitation was the subjective nature used to write peer-reviewed literature. Most of the available literature described each instrument reviewed as being well regarded for a series of traits including, but not limited to: sensitive, selective, having good resolution, accurate, precise, easy to use, and cost effective. Attempts to find quantitative reports of these qualities for the purpose of inter-instrument comparison produced very little. Even instrument manufacturers were not willing to share specifics on the capabilities of instrumentation. When conversing with Sciex representatives, the author was informed that much of the information requested in the questionnaire sent to them was confidential.

Another limitation was the lack of a uniform definition of both analytical efficiency and margin of error. It is quite difficult to perform an objective analysis when the topic being analyzed has a subjective and fluid definition. This was also complicated by the fact that every lab is different and has different needs/capabilities. The previously-mentioned survey by Logan, et.al., also listed lack of relevance as a reason for lack of compliance with recommended guidelines. Labs determined that the recommendations were not relevant because they may not encounter some of the analytes, or they encounter them at low rates. (Logan, et.al., 2017). This is an excellent example of the lack of uniformity of forensic analyses, which contributes to the complexity of making overarching recommendations.

The final limitation is that this review was performed from an outsider's perspective. Though the author has a background in clinical toxicology and laboratory accreditation as it pertains to medical laboratory testing, there are stark differences between clinical and forensic toxicology. This review could have been more relevant if it was written by a practicing forensic

toxicologist who better understands forensic laboratory testing needs and the corresponding regulatory requirements.

Future Research

One opportunity for future research includes in-lab comparisons of the different types of instrumentation described. Lynch, et.al. did a comparison of vendor-supplied methods for five different instruments, and used it as an inter-instrument comparison. Major limitations of this study were the lack of uniformity of sample preparation methods, and the analytes that each instrument was set up to detect. Another limitation was the use of patient samples for the inter-instrument comparison; it is unknown what drugs might have been in these samples, so reliability determinations are suspect, because perceived false positives could be drugs that were not tested for or identified by the original testing procedures. (Lynch, et.al., 2010). A more on-point inter-instrument study would involve more uniform sample preparation methods, a defined panel of target analytes that is fully validated on all instruments involved in the study, and the use of known samples. These known samples would be made using a blank matrix, and spiking it with known concentrations of the different drugs being analyzed; preferably, it would include multiple samples for target analytes at varying concentrations, to test across the linear ranges of the method on each instrument.

Appendices

Appendix A – Instrument Manufacturer Analysis

Instrument Manufacturers

Instrument Manufacturer	Pre-configured for Forensic Applications (y/n)	Comes with Analyte Library/Database	Resources Available on Website to Aid Method Development	Lists Forensic Applications in Instrument Description	Forensic Applications Information Page on Website	Polarity switching capabilities (LC Only)
Thermo Fisher Scientific: GC/MS, Headspace GC/FID, LC/MS/MS, LC/QLIT/MS Headspace GC/FID/MS						
Agilent Technologies: GC/MS, Headspace GC/FID, LC/MS/MS, LC/QTOF/MS, Headspace GC/FID/MS						
Sciex (Danaher): LC/MS/MS, LC/QLIT/MS, LC/QTOF/MS						
Waters Corporation: LC/MS/MS, LC/QTOF/MS						
Perkin Elmer: GC/MS, Headspace GC/FID, LC/MS/MS, LC/QTOF/MS, Headspace GC/FID/MS						
Shimadzu: GC/MS, Headspace GC/FID, LC/MS/MS, LC/QTOF/MS, Headspace GC/FID/MS						

Headspace GC/FID Manufacturers

Instrument Manufacturer	Pre-configured for Forensic Applications (y/n)	Comes with Analyte Library/Database	Resources Available on Website to Aid Method Development	Lists Forensic Applications in Instrument Description	Forensic Applications Information Page on Website
Thermo Fisher Scientific	does not mention	yes	yes	yes	yes
Agilent Technologies	yes	yes	yes	yes	yes
Perkin Elmer	does not mention	does not mention	no	yes	no
Shimadzu	yes	yes	yes	yes	yes

Headspace GC/FID/MS Manufacturers

Instrument Manufacturer	Pre-configured for Forensic Applications (y/n)	Comes with Analyte Library/Database	Resources Available on Website to Aid Method Development	Lists Forensic Applications in Instrument Description	Forensic Applications Information Page on Website
Thermo Fisher Scientific	does not mention	yes	yes	yes	yes
Agilent Technologies	yes	yes	yes	yes	yes
Perkin Elmer	does not mention	does not mention	no	no	no
Shimadzu	yes	yes	yes	yes	yes

GC/MS Manufacturers

Instrument Manufacturer	Pre-configured for Forensic Applications (y/n)	Comes with Analyte Library/Database	Resources Available on Website to Aid Method Development	Lists Forensic Applications in Instrument Description	Forensic Applications Information Page on Website
Thermo Fisher Scientific	does not mention	yes	yes	yes	yes
Agilent Technologies	yes	yes	yes	yes	yes
Perkin Elmer	does not mention	does not mention	no	yes	no
Shimadzu	yes	yes	yes	yes	yes

LC/QQQ Manufacturers

Instrument Manufacturer	Pre-configured for Forensic Applications (y/n)	Comes with Analyte Library/Database	Resources Available on Website to Aid Method Development	Lists Forensic Applications in Instrument Description	Forensic Applications Information Page on Website	Polarity switching capabilities (LC Only)
Thermo Fisher Scientific	does not mention	yes	yes	yes	yes	yes
Agilent Technologies	does not mention	available for purchase	yes	yes	yes	vaguely mentions it
Sciex (Danaher)	available for purchase	does not mention	yes	yes	yes	yes
Waters Corporation	does not mention	yes	yes	yes	yes	vaguely mentions it, but also mentions the use of 2 methods
Perkin Elmer	does not mention	does not mention	no	no	no	does not mention
Shimadzu	yes	yes	yes	yes	yes	yes

LC/QLIT Manufacturers

Instrument Manufacturer	Pre-configured for Forensic Applications (y/n)	Comes with Analyte Library/Database	Resources Available on Website to Aid Method Development	Lists Forensic Applications in Instrument Description	Forensic Applications Information Page on Website	Polarity switching capabilities (LC Only)
Thermo Fisher Scientific	yes	yes	yes	no	yes	yes
Sciex (Danaher)	available for purchase	available for purchase	yes	yes	yes	yes

LC/QTOF Manufacturers

Instrument Manufacturer	Pre-configured for Forensic Applications (y/n)	Comes with Analyte Library/Database	Resources Available on Website to Aid Method Development	Lists Forensic Applications in Instrument Description	Forensic Applications Information Page on Website	Polarity switching capabilities (LC Only)
Agilent Technologies	does not mention	available for purchase	yes	yes	yes	vaguely mentions it
Sciex (Danaher)	available for purchase	available for purchase	yes	yes	yes	yes
Waters Corporation	does not mention	yes	yes	yes	yes	vaguely mentions it, but also mentions the use of 2 methods
Perkin Elmer	does not mention	yes	yes	yes	no	does not mention
Shimadzu	yes	yes	yes	no	yes	yes

Appendix B – Instrument Manufacturer Cover Letter

Hello Sir or Madam,

I am a graduate student at the University of Central Oklahoma, pursuing a Master's Degree in Forensic Chemistry. My thesis project is a review covering the Analysis of Forensic Laboratory Instrumentation Used for DUI/DUID Cases. I am performing an evaluation of presently-applied analytical techniques, as well as emerging techniques, with a goal of identifying the technique(s) with the highest analytical efficiency.

Because part of my analysis includes an evaluation of instrumentation options, I am requesting that you have a member on your staff fill out the attached questionnaire with information regarding the capabilities and cost ranges of the instrument(s) your company offers for the forensic analysis of blood. The instruments I am interested in are: GC/MS, Headspace GC/FID, Headspace GC/FID/MS, LC/MS/MS, and LC/QTOF/MS.

I realize that the capabilities of an instrument have a somewhat direct relationship with its cost. I'm trying to understand the advantages or disadvantages of purchasing the least and most expensive instruments. I recognize that instrument costs are not published on your website for confidentiality reasons, which is why I am simply requesting that you list the instrumentation you offer in order by cost, from low to high. But if possible, can you give me common price ranges?

If you do not have time to fill out my questionnaire, I would appreciate it if you could send me a list of the instruments you have available for forensic toxicology applications, as well as any literature that may detail the differences between the instruments.

If possible, I would like to request this information by July 1st.

Thank you in advance for your assistance with my thesis research.

Best Regards,

Kara Sitton

Appendix C – Instrument Manufacturer Questionnaire

Instrument Manufacturer Questionnaire

Instrument Model(s) in order of cost, low to high	Forensic Specific Model	Pre-configured Assays	Comes with Analyte Library/Database	Resources Available to Aid Method Development	Compatible Software	Length of Time Required for Instrument Set-up	Sensitivity - Common LODs and LOQs	Specificity - Resolution	Complexity of Instrument Maintenance	Margin of Error - for Forensic Reporting	Polarity switching capabilities	Other Applicable Differences	Common Price Ranges

Information requested: Instrument Model(s) in order of cost, low to high; Forensic Specific Model; Pre-configured Assays; Comes with Analyte Library/Database; Resources Available to Aid Method Development; Compatible Software; Length of Time Required for Instrument Set-up; Sensitivity - Common LODs and LOQs; Specificity – Resolution; Complexity of Instrument Maintenance; Margin of Error - for Forensic Reporting; Polarity switching capabilities; Other Applicable Differences; Common Price Ranges

Appendix D – Shimadzu Questionnaire Response

Instrument Manufacturer Questionnaire

Shimadzu Scientific Instrumentation https://www.ssi.shimadzu.com/industry/forensics.html								
Instrument Model(s) in order of cost, low to high	**Picture**	**Forensic Specific Model**	**Pre-configured for Forensic Applications**	**Comes with Analyte Library/ Database**	**Length of Time Required for Instrument Set-up**	**Sensitivity - Common LODs and LOQs**	**Margin of Error**	**Polarity switching capabilities**
GC/FID/HS		Nexis GC-2030 / HS-20: BAC Dual column, dual FID	✓	✓	all installations typically take 1-3 days depending on applications and amount of training needed	application and compound dependent	depends on what you are measuring	N/A
GC/FID with AOC-6000 open bed headspace autosampler		Nexis GC-2030 / AOC-6000 HS: BAC Dual column, dual FID	✓	✓	all installations typically take 1-3 days depending on applications and amount of training needed	application and compound dependent	depends on what you are measuring	N/A
GC/MS single quad		GCMS-QP2020 NX	✓	✓	all installations typically take 1-3 days depending on applications and amount of training needed	application and compound dependent	depends on what you are measuring	EI and CI
GC/MS with AOC-6000 open bed headspace autosampler		GCMS-QP2020 NX / AOC-6000	✓	✓	all installations typically take 1-3 days depending on applications and amount of training needed	application and compound dependent	depends on what you are measuring	EI and CI
GC/FID/MS/HS single quad		GCMS-QP2020 NX /FID/HS-20	✓	✓	all installations typically take 1-3 days depending on applications and amount of training needed	application and compound dependent	depends on what you are measuring	EI and CI
GC/MS/MS triple quad		GCMS-TQ8050 NX	✓	✓	all installations typically take 1-3 days depending on applications and amount of training needed	application and compound dependent	depends on what you are measuring	EI and CI
LC/MS single quad		i-series/LCMS-2020	✓	✓	all installations typically take 1-3 days depending on applications and amount of training needed	application and compound dependent	depends on what you are measuring	EI/APCI/DUIS polarity switching
LC/MS/MS triple quad		Nexera HPLC with LCMS-8050	✓	✓	all installations typically take 1-3 days depending on applications and amount of training needed	application and compound dependent	depends on what you are measuring	EI/APCI/DUIS polarity switching
Q-TOF LCMS		Q-TOF LCMS-9030	✓	✓	all installations typically take 1-3 days depending on applications and amount of training needed	application and compound dependent	depends on what you are measuring	EI/APCI/DUIS polarity switching

Appendix E – Sciex Questionnaire Response

Instrument Manufacturer Questionnaire - Sciex

Instrument Model(s)	Forensic Specific Model	Pre-configured for Forensic Applications	Comes with Analyte Library/Database	Length of Time Required for Instrument Set-up	Sensitivity - Common LODs and LOQs	Margin of Error	Polarity switching capabilities
LC/MS/MS	Several models available	Offer packaged methods for the forensics market, additional purchase necessary	No, not necessary for MRM analysis	3-5 days depending on instrument, accessessories, and training	depends on instrument model and analyte being measured	Mass error ranges included in product specifications, labs need to account for other variables	depends on age of instrument, all newer models have polarity switching capabilities
LC/QTRAP	Several models available	Offer packaged methods for the forensics market, additional purchase necessary	Offer various analyte libraries, additional purchase necessary	3-5 days depending on instrument, accessessories, and training	depends on instrument model and analyte being measured	Mass error ranges included in product specifications, labs need to account for other variables	depends on age of instrument, all newer models have polarity switching capabilities
LC/QTOF	Several models available	Offer packaged methods for the forensics market, additional purchase necessary	Offer various analyte libraries, additional purchase necessary	3-5 days depending on instrument, accessessories, and training	depends on instrument model and analyte being measured	Mass error ranges included in product specifications, labs need to account for other variables	depends on age of instrument, all newer models have polarity switching capabilities

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