Document Title:	**A Paradigm Shift in Forensic Toxicology Screening: The Development and Validation of Two Automated Sample Preparation Techniques for the Comprehensive Screening of Biological Matrices Using High Resolution Mass Spectrometry with Comparison to Conventional Screening Techniques**
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Project Title:	A Paradigm Shift in Forensic Toxicology Screening: The Development and Validation of Two Automated Sample Preparation Techniques for the Comprehensive Screening of Biological Matrices Using High Resolution Mass Spectrometry with Comparison to Conventional Screening Techniques

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Award Recipient Organization: Virginia Department of Forensic Science

Project Period: January 1, 2019 – December 31, 2022

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SUMMARY OF PROJECT

Major Goals and Objectives

The goal of this research project was to develop and validate two fully automated sample preparation techniques for the qualitative analysis of whole blood and additional biological matrices in accordance with the guidelines promulgated previously by SWGTOX.

The objectives for this project were: 1) develop, validate, and compare tubular and 96-well plate fully automated solid phase extraction sample preparation techniques to an existing manual sample preparation technique for comprehensive screening of antemortem and postmortem biological samples using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-qTOF); 2) compare LC-qTOF screening results with current qualitative screening protocols including blood alcohol stop testing limits, enzyme-linked immunosorbent assay (ELISA) results, and gas chromatography-mass spectrometry (GC-MS) screening results.

Research Questions

The main research question to be answered with this research project was the applicability of implementing a comprehensive high resolution mass spectrometry screen for biological matrices in both antemortem and postmortem specimens. Does the method provide a more efficient screening protocol? Does the method provide more specificity regarding compounds present within a sample when compared to traditional screening techniques? Does the method provide a more cost-effective approach to comprehensive screening in toxicology?

Research Design, Methods, Analytical and Data Analysis Techniques

The project design was intended to be a twofold process consisting of method development and validation prior to the comparison of current methods to the comprehensive screening results. The method development and validation components of the project consisted of using an Agilent Technologies 1290 liquid chromatograph (LC) system coupled to an Agilent Technologies 6550 dual jet stream electrospray ionization quadrupole time-of-flight mass spectrometer (qTOF) with iFunnel technology for comprehensive screening of biological matrices. The comprehensive targeted screen included over 250 target compounds encompassing nearly all compounds qualitatively identified by the Virginia Department of Forensic Science (DFS). In addition to the development of analytical instrument parameters, a sample preparation method was developed to efficiently extract the target compounds from biological matrices. The optimal sample preparation technique was determined to be a solid phase extraction (SPE) which was subsequently automated using a Hamilton Microlab STAR liquid handling system.

For experimental ease, the developed method including sample preparation and instrumental analysis was validated using the manual extraction process. Subsequent verification of the Hamilton Microlab STAR liquid handling system was performed. The final component of the project design was to be a comparison of the newly validated comprehensive high resolution mass spectrometry screening method with the conventional analytical workflow including ELISA and GC-MS screening.

Expected Applicability of the Research

The expectations within this research project were to develop a comprehensive high resolution mass spectrometry screen for biological matrices within forensic toxicology. The implementation of a method of this caliber would significantly impact the forensic science community enabling a more efficient, comprehensive, streamlined process for screening specimens. The nature of high-resolution mass spectrometry in comparison to traditional ELISA screening technology enables direct identification of a compound within a specimen as opposed to broad drug class information. Further, automation of the sample preparation using a Hamilton Microlab STAR liquid handling system enables a hands-free approach to sample preparation increasing efficiency within the laboratory.

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name:	Rebecca Wagner, PhD
Project Role:	Principal Investigator
Contribution to Project:	As the principal investigator, Dr. Wagner was responsible for the coordination of the project including collaboration with vendors to mitigate analytical findings with the instrumentation and programming of the automated liquid handling system. Furthermore, Dr. Wagner coordinated the method development and validation efforts of the Research Specialist.
Funding Support:	Virginia Department of Forensic Science
Foreign Country Collaboration:	No

Name:	Richard Barron, B.S.
Project Role:	Research Specialist
Contribution to Project:	As the Research Specialist, Richard Barron was responsible for performing the experiments required for method development and validation. Additionally, Richard Barron was responsible for preparing and maintaining standards, supplies, and requests for orders.
Funding Support:	National Institute of Justice/Virginia Department of Forensic Science
Foreign Country Collaboration:	No

CHANGES IN APPROACH FROM ORIGINAL DESIGN AND REASON FOR CHANGE

There were no significant changes made to the original project design rather the completion of the project was hindered by the findings of the method validation data. The original validation of the method indicated problems attaining appropriate mass accuracy results with target compounds. This included poor mass accuracies and the inability for the software to integrate and calculate the mass accuracy. The software algorithms were changed to employ SureMass for liquid chromatography instrumentation to

rectify the issues. This resulted in the release of a new software revision from Agilent Technologies for the high resolution mass spectrometry platform.

With this change, the data collection mode was required to be in profile mode as opposed to the original centroid mode. The initial validation was performed in centroid mode and therefore revalidation was required. With the change from centroid mode collection to profile mode collection, data file size became a significant issue requiring the computer to be upgraded to a larger hard drive prior to revalidation.

Upon revalidation, additional shortcomings were noted that hindered the ability for the method to be validated. These shortcomings are delineated in subsequent sections. From these setbacks, a fully validated method was not achieved and therefore the validation of a method was not complete. The project period consisted of troubleshooting and determining the applicability of a high-resolution mass spectrometry method for screening biological matrices using the aforementioned instrumentation.

OUTCOMES

Activities/Accomplishments

Instrumental Method

An instrumental method was originally developed to create a comprehensive qualitative screening method using high resolution mass spectrometry. The instrumental method was developed on an Agilent Technologies 1290 liquid chromatograph coupled to an Agilent Technologies 6550 iFunnel qTOF with jet stream electrospray ionization. Two data acquisition methods were developed to enable the evaluation of acidic and basic compounds. Both data acquisition methods were performed using MS mode on the instrument with a mass range of 50-1000 *m/z* for positive ionization mode and 35-1000 *m/z* for negative ionization mode. All data was initially collected and stored in centroid data storage mode. Table 1 describes the positive ionization mode instrumental parameters.

Table 1 Positive ionization mode instrumental method

LC Instrumental Parameters			
Parameter	Setting		
Column	Agilent Technologies Poroshell 120 SB-C18, 2.1 x 100 mm, 2.7 µm		
Guard Column	Agilent Technologies Zorbax SB-C18, 2.1 x 5 mm, 1.8 µm		
Injection Volume	5 µL		
Needle Wash	3 seconds		
Flow Rate	0.5 mL/min		
Mobile Phase A	Water with 0.01% formic acid and 5 mM ammonium formate		
Mobile Phase B	Methanol with 0.01% formic acid		
Gradient	Time (min)	% A	% B
	0.00	95	5
	0.10	95	5
	3.00	65	35
	8.50	40	60
	9.25	5	95
	11.25	5	95
Post Time	1.75 minutes		
Column Temperature	55°C		

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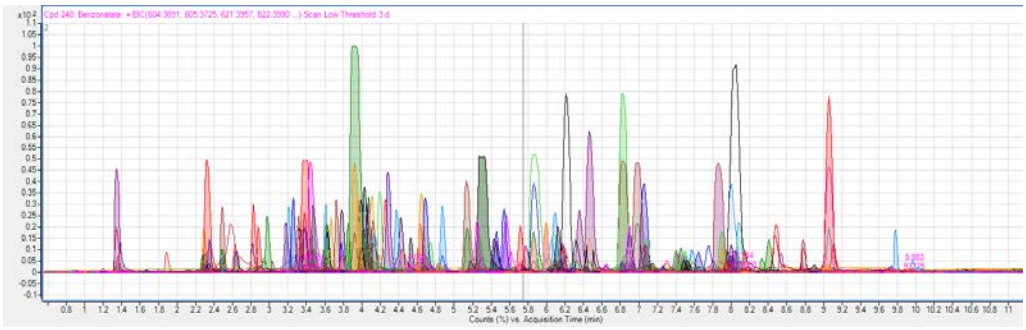
The positive mode instrumental parameters had a total of three time segments for analysis. Time segment one was from 0.0 minutes to 0.5 minutes. Time segment two was from 0.5 minutes to 11.2 minutes. Time segment three was from 11.2 minutes to the end of the analytical run. The first and third time segments were waste segments while the second time segment had the diverter valve set to the mass spectrometer. All other parameters including source conditions and iFunnel settings were the same for all time segments. The qTOF instrumental parameters are delineated in Table 2.

Table 2 qTOF positive ionization mode instrumental parameters

qTOF Instrumental Parameters	
Parameter	Setting
Min Range	50 *m/z*
Max Range	1000 *m/z*
Scan Rate	2.50 spectra/second
Gas Temp	200°C
Gas Flow	17 L/min
Nebulizer Pressure	20 psig
Sheath Gas Temp	375°C
Sheath Gas Flow	12 L/min
Capillary	3000 V
Nozzle	0 V
Fragmentor	380 V
Skimmer 1	0 V
Octopole RF Peak	750
Funnel Exit DC	50 V
Funnel Delta V1	150 V
Funnel Delta V2	100 V
Fennel RF HP	150 V
Funnel RF LP	90 V
Reference Mass	121.0509 *m/z*
Reference Mass	922.0098 *m/z*

An example extracted ion chromatogram of the positive ionization mode method is shown in Figure 1.

Figure 1 Positive ionization mode extracted ion chromatogram example



The chromatography for the negative ionization mode method utilized a different analytical column as well as a different gradient and mobile phase composition. Given the ionization suppression in negative ionization mode with the utilization of formic acid, acetic acid was used as an additive in the aqueous and organic mobile phases. The liquid chromatography negative ionization mode instrumental parameters are delineated in Table 3.

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Table 3 Negative ionization mode instrumental method

LC Instrumental Parameters			
Parameter	Setting		
Column	Agilent Technologies Poroshell 120 Phenyl Hexyl, 2.1 x 100 mm, 2.7 µm		
Guard Column	Agilent Technologies Poroshell 120 Phenyl Hexyl, 2.1 x 5 mm, 2.7 µm		
Injection Volume	0.1 µL		
Needle Wash	3 seconds		
Flow Rate	0.5 mL/min		
Mobile Phase A	Water with 0.01% acetic acid		
Mobile Phase B	Acetonitrile with 0.01% acetic acid		
Gradient	Time (min)	% A	% B
	0.00	95	5
	4.00	5	95
Post Time	1.00 minutes		
Column Temperature	55°C		

The qTOF negative ionization mode instrumental parameters had a total of four time segments for analysis. The time segments are delineated in Table 4. The first time segment had the diverter valve set to the mass spectrometer due to the early elution time of GHB. The fourth time segment switched the diverter valve to waste.

Table 4 qTOF negative ionization mode time segments

qTOF MS Time Segments	
Time (min)	Diverter Valve Position
0.00	Mass spectrometer
1.95	Mass spectrometer
2.60	Mass spectrometer
4.00	Waste

Unlike the positive ionization mode method, in addition to the diverter valve switching, the negative ionization mode time segments had different capillary voltages, nozzle voltages, and acquisition rates. The qTOF instrumental parameters for the negative ionization mode method are delineated in Table 5.

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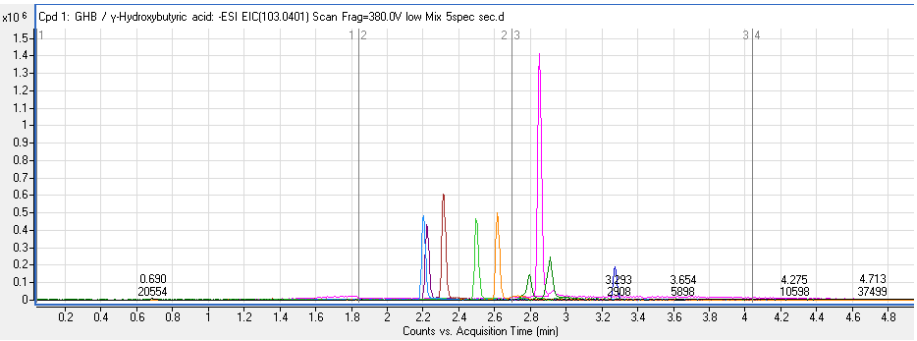
Table 5 qTOF negative ionization mode instrumental parameters

	qTOF Instrumental Parameters	
Parameter	Setting	
Min Range	35 *m/z*	
Max Range	1000 *m/z*	
Scan Rate	Time Segment (min)	Scan Rate (spctra/s)
	0	3
	1.95	5
	2.60	3
	4.00	3
Gas Temp	125°C	
Gas Flow	17 L/min	
Nebulizer Pressure	20 psig	
Sheath Gas Temp	375°C	
Sheath Gas Flow	12 L/min	
Capillary	Time Segment (min)	Capillary (V)
	0	5000
	1.95	1500
	2.60	5000
	4.00	5000
Nozzle	Time Segment (min)	Nozzle (V)
	0	2000
	1.95	0
	2.60	2000
	4.00	2000
Fragmentor	380 V	
Skimmer 1	0 V	
Octopole RF Peak	750	
Funnel Exit DC	-50 V	
Funnel Delta V1	-150 V	
Funnel Delta V2	-100 V	
Fennel RF HP	120 V	
Funnel RF LP	60 V	
Reference Mass	112.985587 *m/z*	
Reference Mass	980.016375 *m/z*	

The time segments were designed to assist in desensitization of the instrument for barbiturates. Barbiturates easily ionize and elute in the second time segment window. To decrease the amount of saturation, the capillary voltage and nozzle voltage were adjusted to decrease the overall instrumental response. The scan rate was also adjusted to collect more spectra per second creating more points across a peak and subsequently decreasing the overall abundance of the spectra. An example extracted ion chromatogram for the negative ionization mode method is shown in Figure 2.

Figure 2 Negative ionization mode extracted ion chromatogram example



During instrumental method development, the utilization of qualifier ratios was investigated. Given the linear range of the instrumentation and the dynamic ranges desired within the method, the qualifier ratios varied significantly from the low threshold concentration to the high threshold concentration. The utilization of fragmentation also impacts the sensitivity of the instrument. The limit of detection was higher when collision energies were applied for fragmentation due to the cycling of multiple collision energies. Therefore, qualifier ratios are unable to be used in the identification of a compound.

Sample Preparation Method

A solid phase extraction sample preparation method was developed for the extraction of over 250 compounds in biological specimens. The compounds were grouped into thirteen different groups based on the concentration ranges of drug classes to include subtherapeutic to toxic concentrations. Table 6 describes the drug class groupings and associated threshold concentrations evaluated.

Table 6 Drug classes and associated threshold concentrations

Group	Drug Classes	Low Threshold Concentration (mg/L)	High Threshold Concentration (mg/L)
1	Antihypertensive, Hallucinogen	0.0001	0.01
2	Fentanyl Derivatives	0.0005	0.05
3	THC	0.001/0.01	0.1/1
4	Antipsychotic, Fentanyl Derivatives, Muscle Relaxant, Opioids	0.001	0.1
5	Antihistamine, CNS Stimulant, Cocaine	0.01	0.5
6	Anesthetic, Antiarrhythmic, Anticholinergic, Antidepressant, Antidiarrheal, Antiemetic, Antihistamine, Antihypertensive, Antiphrastic, Antiparkinsonian, Antipsychotic, Anxiolytic, Benzodiazepine, CNS Depressant, Dissociative Anesthetic, Muscle Relaxant, Narcotic, Opioids, Poison, Amphetamines, Novel Psychoactive Substances	0.01	1
7	Amphetamines, Cocaine	0.01	2
8	Antidepressant, Antipsychotic, Antitussive, CNS Stimulant	0.1	1
9	Anesthetic, Opioids, Antipsychotic, Antitussive	0.1	2
10	CNS Stimulant, Anti-Epileptic, Benzodiazepine	1	20
11	Anti-Epileptic, Barbiturates, Muscle Relaxant	1	50
12	NSAIDs	10	100
13	GHB	30	100

The sample preparation method utilized a UCT Clean Screen (ZSDAU) copolymerized sorbent column with a 200 mg bed mass and 10 mL total volume capacity. Prior to extraction, 0.5 mL of biological matrix was diluted with 4.0 mL of 100 mM phosphate buffer. The samples were vortexed prior to centrifugation and subsequent solid phase extraction. Traditional solid phase extraction steps were employed with conditioning the columns with hexane, water, and buffer prior to the addition of diluted biological specimen. After the addition of the biological matrix, columns were washed with 3.0 mL of water followed by 2.0 mL of 100 mM acetic acid. The method contains three elution steps to collect the target compounds. Figure 3 describes the solid phase extraction workflow with the collection of the three eluate fractions.

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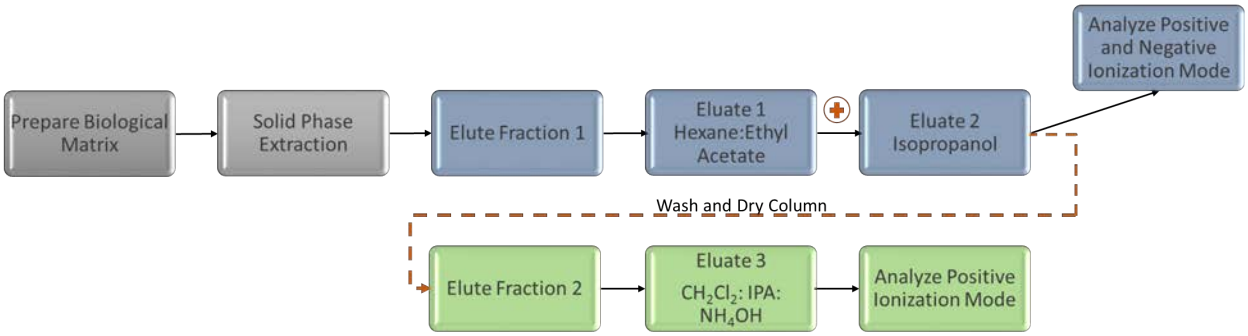
Figure 3 Solid phase extraction workflow



The first two eluates were collected together while the third eluate was collected separately. Prior to elution of Fraction 1, the columns were dried under full flow. Eluate Fraction 1 contained a 3.0 mL hexane:ethyl acetate (9:1) collection and a 3.0 mL isopropanol collection. After the collection of Fraction 1, a methanol wash was performed using 3.0 mL of methanol followed by drying the columns at full flow. Eluate Fraction 2 contained 3.0 mL methylene chloride:isopropanol:ammonium hydroxide (78:20:2). Both eluates were evaporated to dryness at <40°C under nitrogen. Prior to evaporation of Fraction 2 samples, 50 µL of 0.1 N HCl in isopropanol was added to each sample collection tube. Samples were subsequently reconstituted in 100 µL of 95:5 water:methanol.

An extensive evaluation was performed on the elution of compounds with the optimized solid phase extraction method. During development, fraction analysis was completed to determine in which fraction each compound eluted. This also enabled the optimization of the eluate fraction collection. To determine the optimal fraction collection combination, samples were extracted, and the eluates were collected for analysis. Table 7 describes the elution fraction collection experimental design.

Table 7 Fraction collection analysis

Sample	Fraction Collection
Sample 1	Fraction 1, 2, and 3 collected together
Sample 2	Fraction 1, 2, and 3 collected individually
Sample 3	Fraction 1 and 2 collected together and Fraction 3 individually
Sample 4	Fraction 1 collected individually and Fraction 2 and 3 collected together
Sample 5	Fraction 1 and 3 collected together and Fraction 2 individually
Sample 6	Methanol wash collected and evaluated

It was determined that the most efficient approach that does not compromise compound identification was to elute and collect fraction one and fraction two together and analyze the combined fraction with both the positive and negative ionization mode instrumental methods and collect fraction three individually and analyze the fraction with the positive ionization mode method. A total of three injections are subsequently required for the comprehensive analysis with a total analysis time of 32 minutes per sample.

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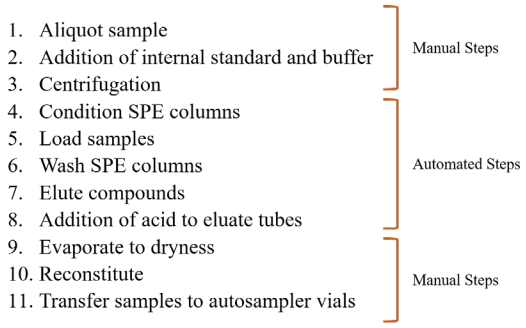
Automated Liquid Handling

The optimized solid phase extraction procedure was automated using a Hamilton Microlab STAR liquid handler. The original intent of the automation was to develop a fully automated process including sample evaporation and reconstitution. To accomplish this, the Microlab STAR was customized to be equipped with four vacuum manifolds in addition to MPE^2 evaporation modules. The evaporation module was capable of drying down samples in a 48-well or 96-well format. After extraction, samples were transferred from the collection tubes into a 48-well plate for dry down and reconstitution. During development, evaporative crosstalk was observed during sample dry down in the MPE^2. This evaporative crosstalk could not be mitigated and therefore the method was adjusted to have evaporation and reconstitution as manual extraction steps. The extraction process including manual and automated steps is shown in Figure 4.

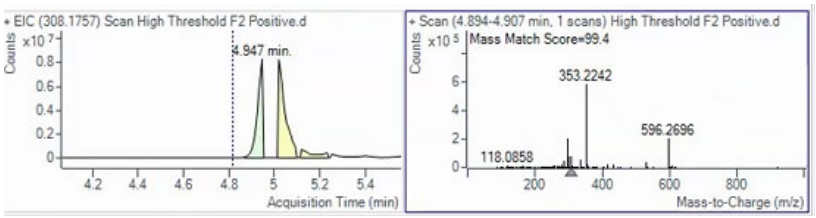
Figure 4 Description of manual and automated sample preparation steps



Results and Findings

An automated sample preparation technique for the comprehensive screening of biological specimens was successfully developed. During initial validation of the method, several compounds indicated issues with poor integration from missing data or poor mass accuracies. An investigation, in collaboration with Agilent Technologies, was performed to identify the source of the missing data and poor mass accuracies. Initial studies indicated peak saturation causing a shift in the mass accuracy of the peak. If the mass extraction window was set to a traditional value of ±20 ppm, data would be missing from the peak as shown in Figure 5.

Figure 5 Demonstration of missing data



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If the mass extraction window was set to a larger extraction window (±100 ppm), the data would return and show a chromatogram, but the mass accuracy would be unacceptable (>±10 ppm). To create an appropriate representation of the data collected, a change to the algorithms in the MassHunter Quantitative Analysis software was required. A new algorithm was developed using SureMass. Traditionally, SureMass was used for gas chromatography data. The concepts and algorithms were translated to liquid chromatography data and a new software version was created and deployed by Agilent Technologies.

During this process, several beta software versions were evaluated. To employ SureMass, data must be collected in profile mode. All previous validation data was collected in centroid mode to minimize file size. The final version of software was evaluated by assessing antemortem blood specimens using the optimized qualitative screen (including profile mode data storage) and confirming the positive opioid and cocaine results using previously validated liquid chromatography tandem mass spectrometry (LCMSMS) results. A total of 360 antemortem samples were evaluated with a total of 15 compounds for a total of 5400 evaluations. All results were compared to obtain a false positive and false negative rate for the screening method. The existing LCMSMS quantitative method was utilized as the ground truth result.

> False positive result: Screening result pending, LCMSMS result negative or less than the lower limit of quantitation.
>
> False negative result: Screening result below low threshold or negative, LCMSMS results produced quantitative value.

When evaluating the data, the false positive rate was calculated to be 0.11% while the false negative rate was calculated to be 0.31%. This was for a small subset of compounds and samples but did include compounds that performed poorly when evaluating the original software version.

Upon publication of the software version, the method was revalidated. Data was subsequently collected in centroid and profile mode to ensure that all data was captured. During revalidation, the ionization suppression and enhancement data suggested significant enhancement of several compounds (>150% enhancement). An investigation into the source of ionization enhancement was performed and the information was grouped into three problems. The issues included:

1. Missing peaks that are not integrated with “NO DATA” return.
2. Missing peaks and large mass error in matrix samples.
3. Significant ionization enhancement in neat and matrix samples.

Another collaboration ensued with Agilent Technologies to potentially rectify the aforementioned problems. Based on the collaboration, additional experimentation was performed resulting in the following conclusions.

1. Missing peaks that are not integrated with “NO DATA” return.

To evaluate this phenomenon, ten replicate injections were performed with the same neat standard containing a high concentration of the compounds of interest within the method. From that evaluation it was noted that several injections would return peaks that are not integrated or had no data associated with the compound. This was sporadic between the replicate injections. Additionally, this was not noted across all compounds, rather on a random conglomeration of compounds.

The root cause was determined to be a runtime mass calibration failure. The instrumentation employs an internal reference mass (IRM) that is analyzed in conjunction with the sample through a second nebulizer in the ionization source. The IRM serves as an in-run mass calibration. There are two reference masses, 121.0509 *m/z* and 922.0098 *m/z*, that will adjust the masses of the compounds identified based on the difference between the theoretical mass and mass obtained from the IRM during analysis. When evaluating the IRM over the course of the analytical run, spikes in the deviation between the theoretical and calculated masses were noted.

A typical mass deviation for the IRM is within 10 ppm. The deviation noted in a low concentration neat standard containing compounds of interest is reaching nearly 30 ppm when “spikes” were observed. This deviation would cause a shift in mass for the compounds of interest that elute during that time point in the analytical run. The current mass extraction window is 20 ppm. If the shift in IRM is greater than 20 ppm, the software is unable to identify the peak causing either no peak or a “NO DATA” return. A software issue was noted and is slated to be published in the next software revision.

2. Missing peaks and large mass errors in matrix samples.

To evaluate this phenomenon, both a neat standard and matrix sample were injected multiple times and evaluated. From this evaluation, similar results were noted as in the first issue. Several peaks were missing, and large mass errors were also noted. Although the results of missing peaks are similar between the two issues, the source of the problem is different. This problem is noted with matrix samples only. A matrix interference was noted with the 121.0509 *m/z* IRM causing the IRM to be overwhelmed. Given the response of the interfering compound, the wrong peak was selected for integration. This results in an inappropriate adjustment of the IRM causing a conversion bias from the large mass shift.

At least three interferences in matrix samples have been noted with the IRM. If the mass extraction window is expanded from 20 ppm to 50 ppm or even 100 ppm, the peaks are identified but the bias of the mass is so great that the mass accuracy is unacceptable (>10 ppm). It has been noted that if the IRM is not employed, the conversion bias does not occur allowing for peaks to be identified, integrated, and have an appropriate mass accuracy.

To resolve the issues, the IRM function may be turned off or a different IRM may be selected for analysis. By changing the IRM accuracy from ±100 ppm to ±30 ppm, an interferent with the IRM would no longer impact the mass correction of the target compounds. Removal of the IRM could significantly impact the instrumental mass drift over an analytical run. The change in reference mass may also have interferences and may not be a mass near the most common masses analyzed within the method.

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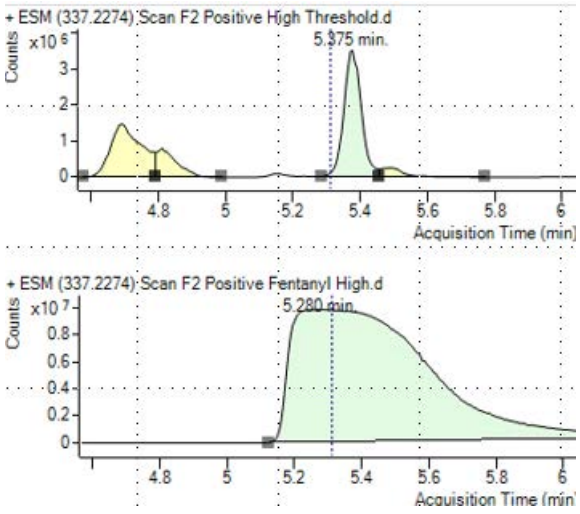
3. Significant ionization enhancement in neat and matrix samples.

When evaluating the data collected from the ionization suppression/enhancement experiments, significant ionization enhancement was noted for the majority of the compounds. The extent of ionization enhancement was abnormal as it was greater than 200% in several cases. Subsequently, an investigation into the cause of the ionization enhancement was initiated. The first evaluation was with the total ion current of a sample and the internal reference mass. The total ion current abundance was noted to be as high as 2.75 $x10^8$. The total ion current is excessively high causing ionization suppression of the internal reference mass.

When the total ion current of the sample increases, the total ion current of the internal reference mass decreases causing an anti-correlation in the data. This data is suggesting that the ionization enhancement that was noted during validation was not enhancement but rather a suppression observed from exceeding the maximum ionization limit of the ionization source.

From this discovery, a comparison between a matrix high concentration control and neat standards of individual compounds at the same concentration was performed. In this evaluation, the instrumental response of the matrix high control was significantly less than the individual compounds. Figure 6 shows the chromatography for fentanyl in the high control (top) and the chromatography for fentanyl individuals in a neat standard (bottom).

Figure 6 Fentanyl chromatograms



The abundance of fentanyl in the high control mix was 12,819,924 peak area counts (top image) whereas the abundance at the same concentration in an individual standard was 281,596,315 peak area counts (bottom image). This significant difference in abundance further solidifies the concept of exceeding the maximum ionization limit of the instrumentation with the observed fentanyl response in a sample contained all compounds being significantly less than the fentanyl response when analyzed individually. Although the abundances are significantly different, the mass accuracy for the high concentration control and individual standard were -3.5357 ppm and -1.9903 ppm, respectively. Previous work to appropriately adjust the mass accuracies of saturated peaks produced the MassHunter Quantitative Analysis Version 11

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software that employed SureMass algorithms. The mass accuracies of the saturated fentanyl peaks in both the high concentration control mix and the individual control demonstrate that the SureMass software is functioning properly to account for peak saturation.

The original intent of this method was to evaluate a low and high threshold control containing all compounds of interest within every analytical batch. The low threshold was to be utilized as a decision point for further testing and confirmation analysis. The current functionality of the method would produce a significant number of false positive results due to the number of compounds in the control compared to an authentic sample. Authentic samples would produce a higher instrumental response for a compound than the threshold controls due to the differences in the number of compounds being ionized.

A correction factor may be applied to normalize the differences in instrumental response between a compound present individually in a sample and in combination. Preliminary investigations indicate that this relationship is not a linear relationship. Therefore, the relationship must be characterized prior to creating and applying an equation of the relationship for each compound. This experimental approach was beyond the scope of this project.

Limitations

Although a method was developed for the automated extraction of over 250 compounds in biological matrices, validation of the method in accordance with ANSI/ASB 036 Standard Practices for Method Validation in Forensic Toxicology proved to be challenging.

In the current state of the project, a method has not been validated to meet the requirements set forth in ANSI/ASB 036. A full characterization of the ionization relationship is required prior to revalidation. If the relationship between individual compounds and a mix of compounds cannot be experimentally determined, the controls could be separated into several different quality control samples at the low threshold and several at the high concentration to minimize the number of compounds competing for ionization in one specimen. This approach would increase the number of controls required to be extracted per batch subsequently reducing the number of unknown specimens that can be evaluated.

The advancements within this project have significantly impacted the field of forensic toxicology. The creation of a new software version for the MassHunter Quantitative Analysis software platform more accurately depicts the data collected by the high-resolution mass spectrometry instrumentation. This software has been employed in laboratories across the country.

Continued research into the relationship between compounds in conjunction with revalidation may produce a comprehensive automated method for the qualitative analysis of biological specimens using high resolution mass spectrometry.

ARTIFACTS

Wagner, R., Validation of Automated Sample Preparation Techniques for Comprehensive Screening of Biological Matrices using Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry, 2021 NIJ R&D Symposium, AAFS Virtual Meeting, Oral Presentation, February 2021

Barron, R and Wagner, R., Validation of Automated Sample Preparation Techniques for Comprehensive Screening of Biological Matrices using Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry, SOFT Conference, Nashville, TN, Poster, September 2021