Report Title: Rapid Screening and Confirmation of Organic GSR using Electrospray Mass Spectrometry

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Abstract

The primary goal of this project was to develop a rapid separation and detection method for identifying common smokeless powder additives and their decomposition products using ultra performance liquid chromatography/ mass spectrometry. Smokeless powders can be used as the explosive component in pipe bombs as well as the propellant in modern ammunition. When used in ammunition, the powder is ignited by the primer and burns rapidly to produce a large volume of gas at high pressures and temperatures. It is this buildup of pressure that forces the projectile out of the firearm. In addition to the bullet, there is a release of primer chemicals and unburned/partially burned powder that may get deposited onto the shooter's skin, hair, clothing, and in the nearby environment. These particulates are generally described as gunshot residue (GSR). To date, the major focus of GSR detection techniques has been on the recovery of primer residue, primarily barium, antimony, and lead. However, the removal of heavy metals from firearm ammunition, due to health and safety concerns, makes traditional techniques less effective for detecting GSR. An alternative approach focuses on the detection of the organic compounds present in the residue from the propellant. Because each manufacturer changes the composition of the propellant powder so that it performs in a specific manner, it is also possible to use variances in composition to differentiate between brands and potentially lots of the same powder.

To test this theory, Ultra Performance Liquid Chromatography (UPLC) with Tandem Mass Spectrometry (MS/MS) was used for the analysis of the smokeless powders and organic gunshot residue. UPLC was chosen because it is able to accommodate higher backpressures and smaller particle columns than HPLC and also permits users to perform more efficient and rapid separations. In order to detect the wide array of compounds found in smokeless powder, tandem mass spectrometry (MS/MS) was utilized along with ESCi®. The addition of ESCi® allows for simultaneous monitoring of ions in positive and negative electrospray ionization (ESI) as well as negative atmospheric pressure chemical ionization (APCI). Confirmation was achieved by monitoring different product-to-precursor transitions for each compound in multiple reaction monitoring (MRM) mode. Standards of common smokeless powder additives and their decomposition products were used to develop the UPLC/MS/MS method. Some of these included diphenylamine (DPA), ethyl centralite (EC), dibutyl phthalate (DBP), nitroglycerin (NG), and 2,6-dinitrotoluene (2,6-DNT). The limits of detection that were achieved with the UV method ranged from 0.08 to 2.6 ng injected and 0.4 to 64 ng injected for the MRM method. Once the UPLC/MS/MS method was optimized, a small-scale study was performed to examine compositional differences of various smokeless powders. By identifying these differences, it should be possible to link organic gunshot residue found on a shooter to ammunition and spent cartridges found at the crime scene. Results of a small-scale study showed that quantifiable differences are present in the additive profile of powders from different brands and lots of smokeless powder; however, a larger population of powders will need to be characterized to allow us to determine the probative nature of these differences. In the next portion of the study, gunshot residue samples were collected from the hands of a shooter and analyzed to test the method's applicability in firearm cases. Various collection devices and extraction methods were

first tested in order to determine the best technique for recovering the organic compounds present in the residue. It was found that cotton swabs moistened with an isopropanol:water mixture for GSR recovery and later extracted with acetonitrile provided the highest recoveries. Hand samples collected after shooting different ammunition types in the same weapon type were compared to look for differences in the additive profile. The results show characteristic differences in the UV and MRM profiles that depended on the type of ammunition fired. These results indicate the potential of the technique for distinguishing class differences based on ammunition type in addition to the standard determination of GSR on the shooter's hands.

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Executive Summary

The chemical speciation of gunshot residue primarily comprises the analysis of primer and smokeless powder composition. The primer cup is a mixture of different chemicals that combust when struck by the firing pin. These residue produced from this action includes particulates containing barium, antimony, and lead. Smokeless powder, on the other hand, contains a variety of additives that are utilized to adjust burn rates, improve stability, and achieve other objectives in the manufacturing process. When a weapon is fired, the firing pin hits the primer cup and ignites it. The hot particles from the primer then ignite the smokeless powder and cause it to combust. The combustion of the powder causes an increase in temperature and pressure inside of the weapon that forces the projectile out of the weapon. Vapors and particulates released during this process get deposited on the shooter's skin and clothing. It is also deposited on nearby surfaces. These particles are generally referred to as gunshot residue (GSR), which is composed of both primer and powder residue.

To date, the major focus of GSR analysis has been on the detection of barium, antimony, lead, and other metals from the primer; however, concerns over the health and safety of the shooter as well as environment impacts have led manufacturers to begin removing heavy metals from ammunition. The removal of these elements compromises the effectiveness of current GSR detection techniques. An alternative is to examine the composition of the smokeless powder present in the GSR. Individual brands of smokeless powders contain characteristic chemical additive packages that influence stability and burn rate. These additive packages can vary depending on the intended use of the powder. As a result, the components of the powder can provide a link from a shooter to a type of ammunition. By looking at the powder residue, it is

possible to compare other types of associated physical evidence that may be recovered from the crime scene. These include the weapon as well as spent cartridges.

The proposed method for organic GSR analysis utilizes ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC/MS/MS). Ultra performance chromatography uses smaller particles within its columns to improve the efficiency and resolution of the separation when compared to traditional HPLC. In addition, the system can accommodate higher backpressures, allowing increased flow rates and faster analysis times. The MS/MS provides the sensitivity and selectivity needed for confirmation of each component in the sample. Because of the wide array of potential compounds present in the powders, it was necessary to run ESCi® mode to ensure MS detection. In ESCi® mode, the instrument switches at high speeds between three modes: positive electrospray ionization (ESI+), negative electrospray ionization (ESI-), and negative atmospheric pressure chemical ionization (APCI-). This facilitates the detection of all components in the same run. For example, positive ESI ionization can detect diphenylamines and other stabilizers, negative ESI can detect energetic compounds such as NG, and negative ESCi® ionization permits the determination of nitrotoluenes. For confirmation, two precursor-to-product transitions were monitored for most of the compounds in multiple reaction monitoring (MRM) mode. In this mode, the molecular ion (or adduct) is selected in the first quadrupole, the ion is fragmented in the collision cell, and a product ion is selected in the third quadrupole.

In this project, twenty common smokeless powders additives and decomposition products were investigated. These included diphenylamine (DPA), N-nitrosodiphenylamine (N-NsDPA), 2-nitrodiphenylamine (2-NDPA), 4-nitrodiphenylamine (4-NDPA), 2,4'-dinitrodiphenylamine (2,4'-DNDPA), 4,4'-dinitrodiphenylamine (4,4'-DNDPA), 4-nitrosodiphenylamine (4-NsDPA),

methyl centralite (MC), ethyl centralite (EC), dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), nitroglycerin (NG), 2-nitrotoluene (2-NT), 3-nitrotoluene (3-NT), 4-nitrotoluene (4-NT), 2,3-nitrotoluene (2,3-DNT), 2,4-dinitrotoluene (2,4-NT), 2,6-dinitrotoluene (2,6-NT), 3,4-nitrotoluene (3,4-DNT), and 2-naphthol. Each standard was dissolved in organic solvent and diluted to the appropriate concentrations using a mixture of acetonitrile and water with ammonium salts added.

The research design included four major steps. The first step was to develop a UPLC/MS/MS method for the separation and detection of the smokeless powder additives. Some of the parameters that were investigated included the column type, column temperature, flow rate, separation gradient, and mobile phase composition. Different columns were tested for their ability to separate the different components; however, the 100 mm BEH C18 column with 1.7 μm particles from Waters Corporation provided the best separation. The final parameters that were selected for the UPLC separation were: flow rate of 0.5 mL/min, UV wavelength of 210 nm, column temperature of 40 °C, aqueous mobile phase consisting of 90:10 water:acetonitrile with 2mM ammonium acetate and 0.2mM ammonium chloride, and organic mobile phase consisting of 95:5 acetonitrile:methanol with 2mM ammonium acetate and 0.2mM ammonium chloride. Samples were prepared for injection in a mixture of 40:60 acetonitrile:water with 6mM ammonium acetate and 40 mM ammonium chloride. Increasing the amount of ammonium chloride in the sample helped to increase the detection of nitroglycerin. The ammonium chloride provides a source of chloride ions that nitroglycerin can use to form stable adducts. The final parameters that were selected for the MS/MS detection were: capillary voltage of 3.20 kV (ESCi+) and 4.30 kV (ESCi-), corona current of 20.0 μA, desolvation gas temperature of 500 °C, source temperature of 150 °C, desolvation gas flow of 850 L/hr, cone gas flow of 50 L/hr, and

collision cell pirani pressure of ~$3.6x10^{-3}$ mbar. The limits of detection for the UV data ranged from 0.08 to 2.6 ng injected, whereas the MS data limits of detection ranged from 0.4 to 64 ng injected. The UV limits of detection are better in some cases than the MS limits because there is some loss in sensitivity when switching between ionization mode using ESCi®. Additionally, there is some loss in sensitivity because of the salts added to enhance ionization of specific compounds. Nevertheless, it can be seen that this method is fast and sensitive for the separation and detection of common smokeless powder additives.

The second step was to apply the developed method to the analysis of actual smokeless powders. Five different brands – including differing lots of each powder - were extracted with methylene chloride for 6 hours in the absence of light, separated by UPLC, and confirmed by MS/MS. The percent composition was then calculated for each component in the sample. The compositions were compared using the t-test and ANOVA, depending on the number of lots. Based on the results, it can be seen that this method is applicable to the analysis of smokeless powders. Single and double base powders can be differentiated based on the presence of NG in double base powders. Furthermore, powders with similar additive profiles can be differentiated based on the percentages of each component in the sample. These results demonstrate the power of the technique; however, a larger population study would be needed to determine the method's full discrimination power.

The third step in this project involved the development of an extraction method for recovering the organic gunshot residue from the hands of shooters. Different collection devices were tested, including cotton swabs, masking tape, aluminum stubs with carbon tape, and alcohol swabs. For the cotton swabs, two devices were tested for the extraction: syringes with filter tips and centrifuge tubes with nylon filters. The centrifuge tubes proved to be easier and more

efficient at extracting the smokeless powder standards. The extraction procedure for the centrifuge tubes involved spiking the cotton tip with the standard GSR mixture, adding organic solvent, and centrifuging the tube. Following this step, the extract was collected, evaporated to dryness under a gentle stream of nitrogen gas, and reconstituted in sample dilutor. Masking tape was also used for sample collection. Small pieces of tape were rolled up, spiked with the GSR standard mixture, and placed in a clean vial. Methanol was then added to extract the organic compounds. After sonicating for 15 minutes, the tape was removed and the extract was evaporated until dry and reconstituted in sample dilutor. However, it was very difficult to collect hand samples with the tape alone, and awkward to remove the sticky tape. Aluminum stubs were also evaluated because they are currently being used for the collection of inorganic GSR. These stubs are mounted with double-sided carbon tape and dabbed against the hands or clothing of the shooter. For analysis, the stubs are placed under a scanning electron microscope and examined for GSR particles having the correct morphology. The Scientific Working Group for Gunshot Residue (SWGGSR) requires that the particles be spheroidal or that they exhibit signs of having been molten. Other particles around the suspect particle must also be considered, as fireworks and other products may show GSR-like particles. Once a particle has been identified, energy dispersive X-ray spectrometry is applied to the sample in order to determine the elemental composition. This takes several hours to complete and only identifies the metallic content of the sample. Aluminum stubs were evaluated for concomitant collection of organic GSR. Standards were spiked onto the stub and extracted with organic solvent. Results showed significant background interferences and low recoveries. The final device that was tested for organic GSR collection was an alcohol swab. The alcohol swabs were pre-moistened with 70:30 isopropanol:water. These swabs were spiked with the standard GSR mixture, placed in a

centrifuge tube, and extracted with organic solvent. After centrifuging the tubes, the extract was removed, evaporated until dry, and reconstituted in sample dilutor. Limited results were obtained and further studies are necessary to determine if recoveries can be improved.

The fourth step was to apply the developed extraction, separation, and detection methods to the analysis of live-fire residue samples. The live-fire samples were samples collected from the hands of shooters. These samples were collected with the assistance of the Firearms Division of the Miami Dade Police Department Crime Laboratory under the guidance of appropriate Institutional Review Board (IRB) protocols. Their indoor firing range and personnel were used for the sample collection. Both cotton and tape samples showed positive results for organic GSR and indicated the capability of the procedure to detect residue during the live fire exercises.

In conclusion, this project has demonstrated the applicability of UPLC/MS/MS with multimode ESCi® ionization. Unlike many previous studies on organic GSR, this procedure permits the determination of all applicable chemical components in the additive package of each powder. Positive ESI ionization can detect diphenylamines and other stabilizers, negative ESI can detect NG, and ESCi® ionization permits the determination of nitrotoluenes. The procedure is sensitive and specific, permitting the determination of lot-to-lot differences between similar bulk powders. Testing the procedure following live fire of ammunition demonstrates the successful use of the technique for detecting the presence of organic gunshot residue. Overall, this procedure should provide laboratories performing explosives residue detection and GSR analyses with an additional and powerful tool for the determination of GSR and smokeless powder based evidence.

I. Introduction

A. Statement of the problem

Gunshot residue generally refers to the vapors and particulates released upon firing a weapon. The analysis of gunshot residue can be classified into two categories: inorganic GSR analysis and organic GSR analysis. The inorganic components refer to those compounds released from the cartridge (primer, propellant, and bullet) following discharge of the firearm. The primer is a metal cup containing a mixture of different energetic materials that combust upon impact by the firing pin to produce a flame that ignites the propellant powder. Depending on the firearm it is designed for, the primer may be present in the rim of cartridge (rim-fire ammunition) or as a cup in the center of the cartridge case head (center-fire ammunition). The primer formulations may also vary based on the manufacturer but the general primer composition includes lead, barium, and antimony, which vaporize and then condense into droplets after being ignited. The organic compounds present in gunshot residue may originate from the lubricants, primer mix, smokeless powder, and other parts of the ammunition. However, a significant portion of the organic GSR is due to unburned or partially burned smokeless powder. The powder serves as the propellant in the ammunition and is ignited by the hot primer particles. The burning of the smokeless powder results in an increase in pressure and temperature inside of the cartridge; and as a result of this pressure buildup, the projectile is forced out of the firearm.

When a gun is fired, the organic and inorganic compounds get deposited on the shooter's skin, hair, and clothing and on nearby surfaces. As a result of safety and environmental concerns, many manufacturers have begun to produce "green" or "non-toxic" ammunition that are lead and heavy metal-free. In some instances, these metals are removed only from the primer and the bullet isn't lead-free. In other cases, the bullet is encased in nylon or copper instead of lead to

reduce airborne concentrations. However, when the bullet strikes the target and fragments, lead inside of the bullet still gets released into the air.

These modern formulations of ammunition that are lead-free pose a significant challenge to gunshot residue analysts. Inorganic GSR techniques focus on the detection of various elements in collected particles, including barium, antimony, and lead. As manufacturers move away from primers containing these compounds, current techniques become less effective at identifying the particles as GSR, producing a false negative. A false negative occurs when a test fails to recognize a result as positive or true. Another issue with scanning electron microscopic (SEM) analysis of the inorganics is that it can take between 2-6 hours to search one stub for GSR particles. These suspect particles must then be relocated and manually confirmed by the analyst, which can be time consuming. Following this process, the elemental composition of each particle is acquired using energy dispersive X-ray spectroscopy (EDX). We have proposed a solution to the identification of GSR that instead focuses on the identification of the organic components present in the smokeless powder. This method would be fast, sensitive, and selective. A review of the literature, method, results, and conclusions are presented in this report.

B. Literature citations and review

In 2010, an overview of the analysis of gunshot residue was published by Dalby et al. in the Journal of Forensic Sciences (1). The article discusses the formation of both organic and inorganic gunshot residue as well as sample collection, preparation, and analysis of the residues. The primary technique used for the analysis of inorganic GSR is scanning electron microscopy with energy dispersive X-ray spectrometry (SEM/EDX). Using SEM/EDX, GSR particles are identified in the sample and then the elemental composition is determined for each particle. The

analysis of propellant powder and residue has been performed using other analytical techniques, including gas chromatography (GC), micellar electrokinetic capillary electrophoresis (MEKC), and high performance liquid chromatography. GC has been employed for the separation and identification of different smokeless powder constituents (1,2,3). One major issue with gas chromatography is that the high temperatures in the injection port and column may cause decomposition of thermally labile compounds such as nitroglycerin. Limits of detection were reported by Zeichner (4) in the nanogram range for NG, 2,4-DNT, and 2,6-DNT using GC with thermal energy analysis (TEA) and ion mobility spectrometry (IMS), but NG was detected as two peaks due to thermal decomposition. It was also reported in various studies that high GC temperatures can cause denitrosation of nitrosodiphenylamines; and as a result, DPA and nitrated DPA may be detected instead (5,6,7).

Different liquid techniques have also been employed for the separation and characterization of smokeless powders, including MEKC and HPLC (8, 9,10,11, 12,13). Northrop (14) reported detection limits in the range of 0.9-3.8 picograms for standard solutions of 13 organic GSR compounds using MEKC. This technique produces high-resolution separations with very small amounts of sample and buffer for analysis but confirmation by MS is limited by the addition of surfactants in the buffer (15). Very little has been published on the application of ultra performance liquid chromatography to smokeless powders and organic GSR analysis. Mathis and McCord (10) reported an HPLC method with electrospray ionization mass spectrometry (ESIMS) for the analysis of different smokeless powders using a linear gradient of 50-95% methanol in 25 minutes. In 2007, Laza et al. (16) published a UPLC/MS/MS method for the analysis of common propellant stabilizers in gunshot residue, including akardite II, ethyl centralite, diphenylamine, methyl centralite, N-nitrosodiphenylamine, 2-nitrodiphenylamine, and

4-nitrodiphenylamine. Limits of detection for hand samples ranged from 5 to 115 μg injected for five powder stabilizers detected after the firing of a 9mm weapon. However, the published method focused on the stabilizers present in the propellant and only monitored one MS/MS transition for confirmation of each component. It is useful to monitor more than one transition in order to accurately identify each smokeless powder compound. Furthermore, it is important to detect all components in the residue, including nitrotoluenes, nitroglycerine, and stabilizers.

In this project, we are proposing a method that can be used to extract, separate, detect, and confirm 20 common smokeless powder components by monitoring two MRM transitions for each chemical. These include stabilizers, deterrents, energetics, flash suppressants and plasticizers. The project utilizes ultra performance liquid chromatography with tandem mass spectrometry in ESCi mode, permitting simultaneous use of positive/negative ionization electrospray and APCI detection modes. UPLC has been applied to the analysis of explosives (17); however, a wider scope of compounds was investigated in this project due to our ability to perform multimode mass spectrometry. An article by Gallagher et al. also provides history on the development of combined ESI-APCI sources and its application to LC-MS analyses (18). It is advantageous to utilize the combined source as it provides higher sample throughput and reduced analysis times.

C. Statement of hypothesis or rationale for the research

In this study, we propose a rapid separation and detection method for the analysis of 20 organic compounds present in single and double base smokeless powders (SP) using ultra performance liquid chromatography with tandem mass spectrometry. Smokeless powders are composed of a wide array of additives, including deterrents, dyes, energetics, flash suppressants,

opacifiers, plasticizers, and stabilizers (19). Using UPLC, we hypothesize that these additives can be separated with good efficiency by using smaller particle columns and higher flow rates. Previous results by Mathis showed that LC with ESIMS could be applied to powder analysis. By focusing on the organic compounds present in GSR, it is also possible to analyze powder recovered from unfired ammunition and spent cartridges by swabbing the inside of the casing. We also hypothesize that MS/MS will provide the sensitivity and specificity needed to confirm each component in the sample based on the results published by Laza et al. (16). To test the UPLC/MS/MS method's applicability in firearm cases, actual smokeless powders and live-fire residue samples were collected and analyzed. It is expected that some of the powders will have different formulations, as manufacturers alter the composition so that it performs in a specific manner and for a specific application. These differences will be examined in order to link organic GSR present on the hands of a shooter to a particular ammunition type. The results of this study should provide an alternative method for smokeless powder analysis and organic GSR detection.

II. Methods

A. Materials

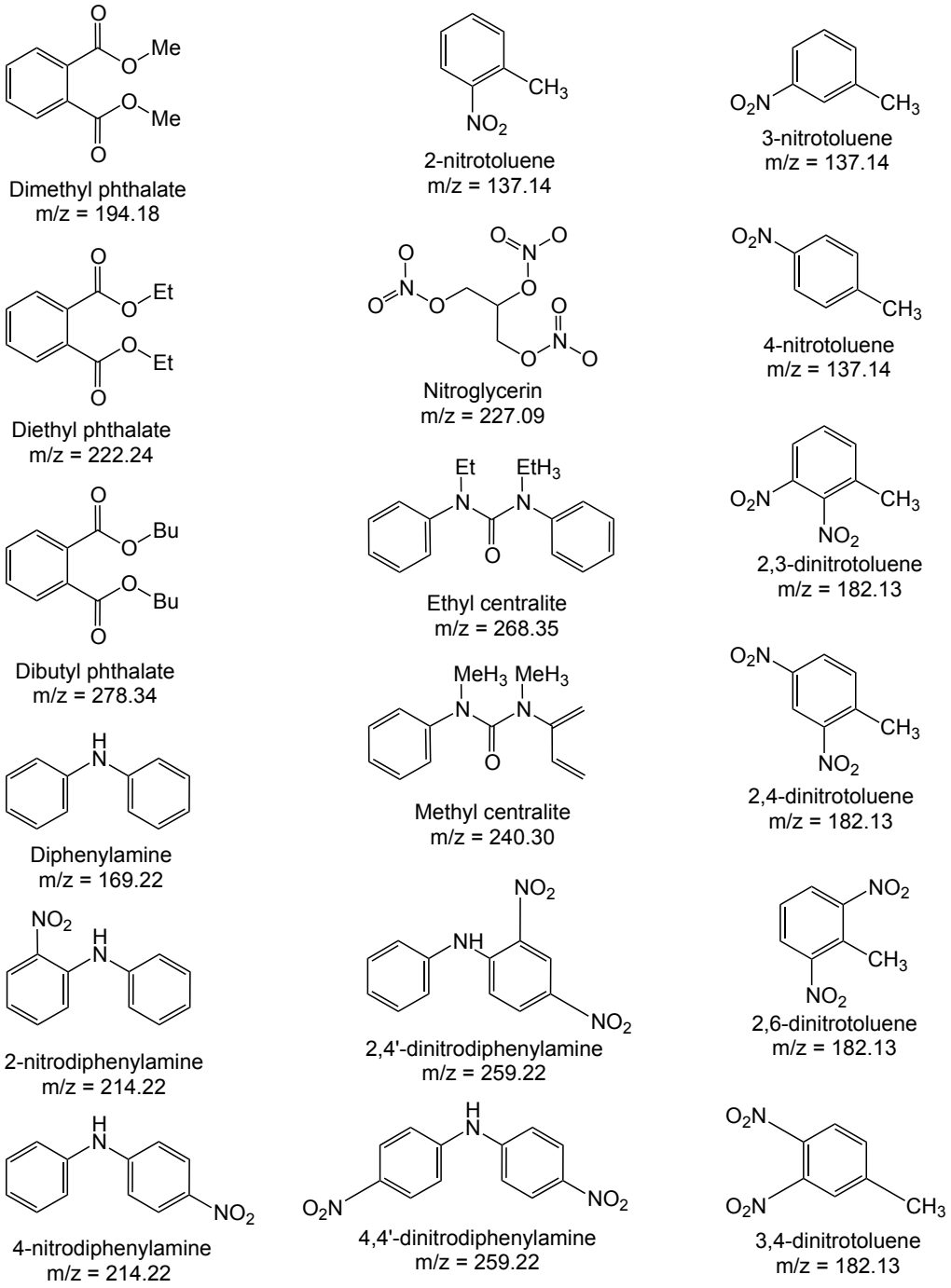
A wide array of compounds was used in this project. Highlighted in Table 2.1 is a list of common smokeless powder additives and decomposition products that may be found in gunshot residue samples (14,20). Standards of these compounds were obtained from different manufacturers, including Acros, Cerilliant, Fluka, Restek, and Sigma, and prepared as stock solutions in acetonitrile at 1mg/mL. Stock solutions were then diluted using a mixture of acetonitrile and water with ammonium acetate and ammonium chloride. Ammonium chloride, ammonium acetate, and LC/MS optima grade solvents (acetonitrile, methanol, and water) were also used to prepare the aqueous and organic mobile phases. 2-naphthol was used as an internal standard and also prepared in acetonitrile at 1 mg/mL. All of the stock solutions of standards were refrigerated.

Table 2.1. List of characteristic organic smokeless powder constituents and their usage in smokeless powders (14,20).

Compound	Abbreviation	Usage
Diphenylamine	DPA	Stabilizer
N-Nitrosodiphenylamine	N-NsDPA	Stabilizer reaction product
4-Nitrosodiphenylamine	4-NsDPA	Stabilizer reaction product
2-Nitrodiphenylamine	2-NDPA	Stabilizer reaction product
4-Nitrodiphenylamine	4-NDPA	Stabilizer reaction product
2,4'-Dinitrodiphenylamine	2,4'-DNDPA	Stabilizer reaction product
4,4'-Dinitrodiphenylamine	4,4'-DNDPA	Stabilizer reaction product
Dibutyl phthalate	DBP	Plasticizer
Diethyl phthalate	DEP	Plasticizer
Dimethyl phthalate	DMP	Plasticizer
Ethyl centralite	EC	Stabilizer
Methyl centralite	MC	Stabilizer
Nitroglycerin	NG	Propellant
2-Nitrotoluene	2-NT	Product
3-Nitrotoluene	3-NT	Product

4-Nitrotoluene	4-NT	Product
2,3-Dinitrotoluene	2,3-DNT	Flash inhibitor
2,4-Dinitrotoluene	2,4-DNT	Flash inhibitor
2,6-Dinitrotoluene	2,6-DNT	Flash inhibitor
3,4-Dinitrotoluene	3,4-DNT	Flash inhibitor

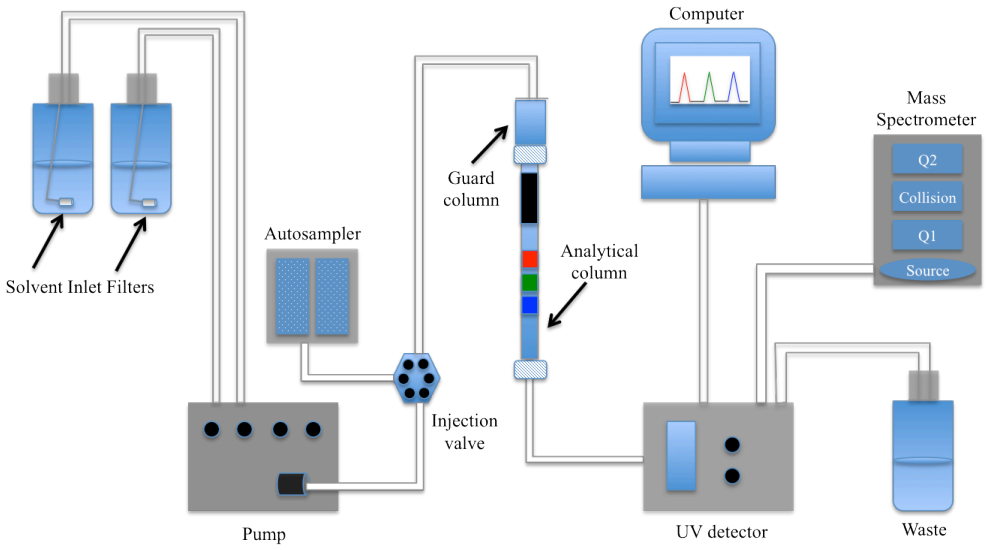
Figure 2.1 Chemical structures of common organic gunshot residue components.



B. Ultra performance Liquid Chromatography

Separations were carried out on an Acquity UPLC™ system (Waters) with a tunable UV (TUV) detector. A basic schematic of an LC system can be seen in Figure 2.2. Ultra performance liquid chromatography is an advancement over traditional HPLC systems, as users can achieve faster separations and increased resolution due to the use of smaller particle columns that help to minimize band spreading and the pumping system's ability to accommodate higher backpressures (21,22). The UPLC accommodates backpressures of up to 15,000 psi, allowing higher flow rates to be used for separations and ultimately, faster analysis times without sacrificing resolution. The system also accommodates smaller particle columns that contribute to improved resolution.

Figure 2.2. Basic schematic of a liquid chromatographic system.



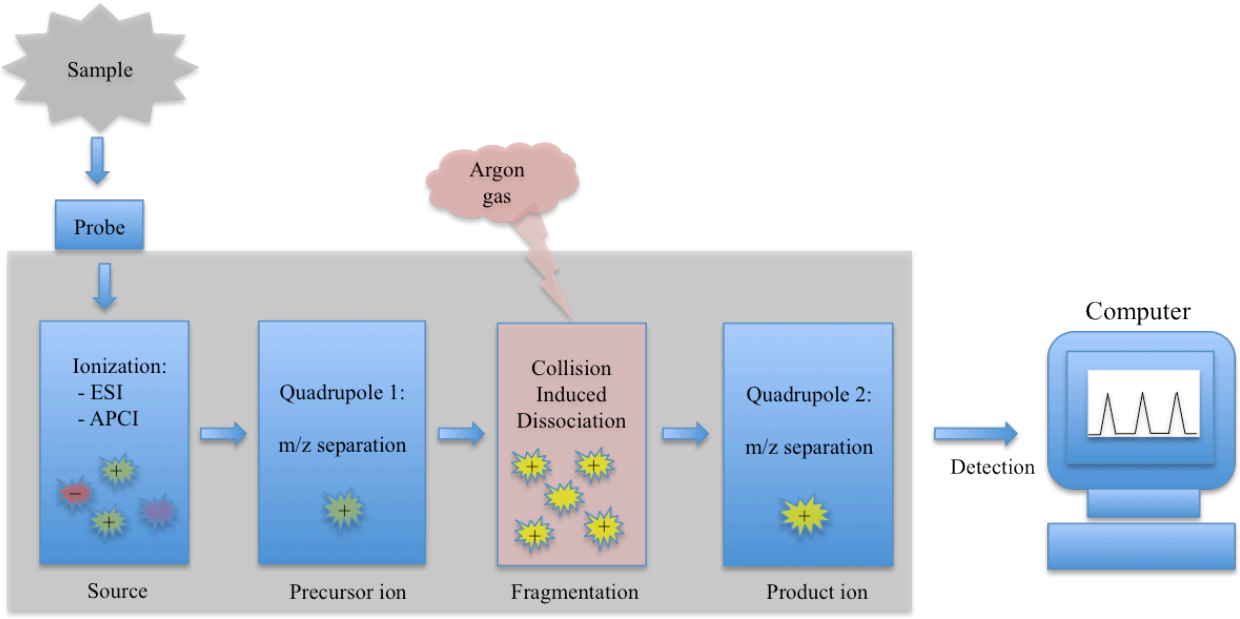
C. Mass Spectrometry

Following separation by UPLC, compounds were identified using a Waters Quattro Micro API™ tandem mass spectrometer (MS/MS) controlled by Mass Lynx software (v4.1). It

was advantageous to use MS/MS for detection because it offers great sensitivity, selectivity, and fast acquisition speeds. The tandem mass spectrometer included an ESCi® source for desolvation and ionization, a collision cell for fragmentation, and two quadrupoles for mass focusing. Figure 2.3 gives a general diagram of the MS/MS process. Analysis involved first ionizing the compounds in the source by electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). The ESCi® source allows for switching between the two modes at high speeds during the same run, saving both time and sample. A video highlighting the ESCi mode can be viewed at: http://www.waters.com/waters/promotionDetail.htm?id=10084787&locale=en_US. In addition, it permits the detection of a wide range of analytes – including the diphenylamines, nitrotoluenes, and nitrate esters present in smokeless powders – to be detected in a single analysis. After passing through the source, the ions were separated based on their mass-to-charge ratio (m/z).

To enhance detection, the instrument was set to run in multiple reaction monitoring (MRM) mode. In this mode, ions are separated in the first quadrupole based on their mass-to-charge ratio (m/z), a precursor ion is selected and fragmented in the collision cell, and these product ions are then filtered in the second quadrupole and detected. The instrument was set to monitor two precursor-to-product transitions for most compounds to ensure accurate identification of each one in a mixture. Fragmentation was achieved by introducing argon gas into the collision cell. A high-pressure liquid nitrogen tank provided the cone gas and desolvation gas to the MS source.

Figure 2.3. General schematic of compound fragmentation by MS/MS.



D. Experimental Design

The overall goal of this project was to develop a rapid separation and detection method for analyzing smokeless powder additives and organic gunshot residue. In order to achieve this goal, several objectives were established and are listed below.

1. Develop a UPLC/MS/MS method for analyzing common smokeless powder additives and their decomposition products
2. Apply the developed UPLC/MS/MS method to the analysis of commercially available smokeless powders in order to determine compositional profiles
3. Develop an extraction method for recovering organic GSR from the hands of shooters
4. Analyze live-fire residue samples collected from the hands of shooters

The first goal was to develop a UPLC/MS/MS method for analyzing common smokeless powder additives and their decomposition products. As mentioned previously, the compounds chosen were identified through various literature searches and are presented in Table 1.1 under the Methods section. Standard mixtures were prepared and separated into individual peaks by varying the mobile phase, stationary phase, column temperature, and flow rate. These conditions were optimized to minimize co-elution, which occurs when two or more compounds have similar affinities to the stationary and/or mobile phases and elute at the same time. Following separation, the compounds were ionized and detected by MS/MS in MRM mode. In order to determine the correct parameters for detection by MS/MS, standards of each compound were infused into the instrument. Using autotune, two product ions were identified for the molecular ion of most of the compounds. Isomers were challenging and only one product ion was selected for these compounds.

Once the UPLC/MS/MS method was optimized, it was applied to the analysis of commercially available smokeless powders. Different brands and lots of the same powder were extracted with methylene chloride and analyzed in order to determine compositional profiles for each powder. Using these profiles, it may be possible to link organic GSR found on a shooter's hands to ammunition containing a powder with the same profile. Statistical testing was applied to the data and included the ANOVA and t-tests. In this project, the t-test was used to compare two lots of the same powder to determine if the percent compositions were significantly different. For powder brands with 3 or more lots present in the lab, the ANOVA test was applied to their results in order to compare lots and each compound in the lot.

Extraction methods were developed and tested using GSR standards and then applied to live-fire residue samples. Different collection devices and methods were evaluated for recovery

of the organic compounds. An MS method was also developed for the rapid screening of GSR samples. Due to time constraints and instrument difficulties, a persistence and background interference study wasn't performed. However, a review of the literature highlights other studies that address persistence and interferences. Persistence refers to how long the organic compounds will remain on the shooter's hands after firing or handling a recently fired weapon. The length of time the compounds remain on the hand is affected by many variables, one of which includes whether or not they washed their hands. A person's job and/or environment may also influence the type of compounds present on their hands. As a result, it is important to question the individual about their daily routine to determine possible background interferences. These issues are further discussed in the conclusion section.

III. Results

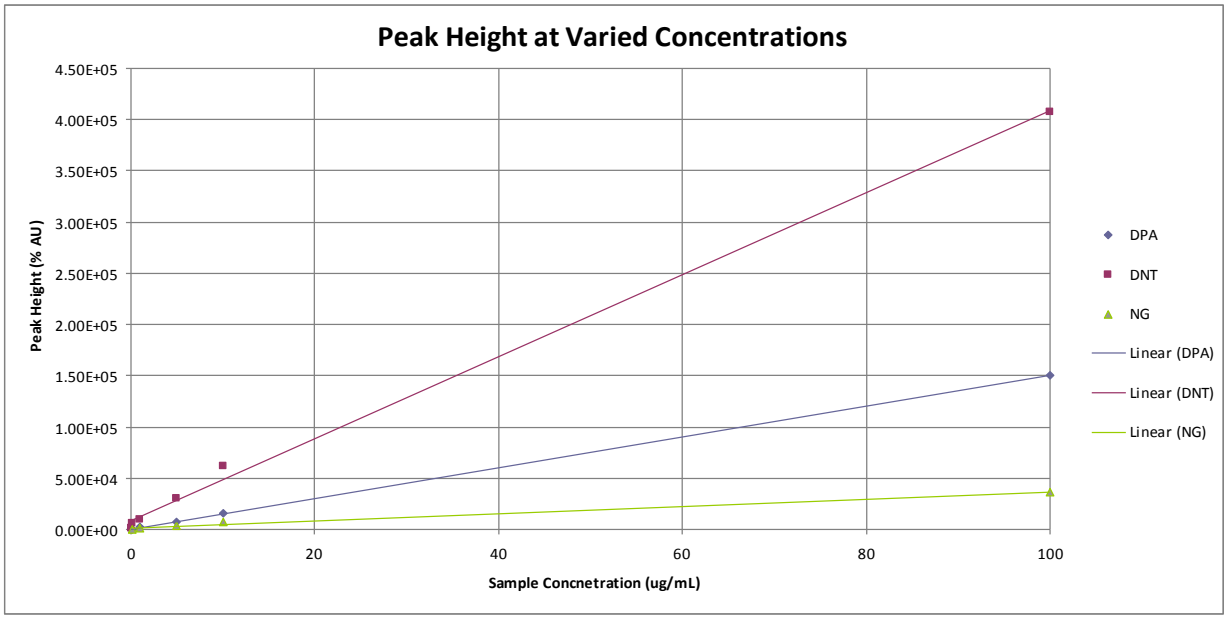
A. DEVELOPMENT OF THE SEPARATION AND DETECTION METHODS

1. UPLC Method Development

The first step in method development involved determining the best conditions for separating the smokeless powder additives by UPLC. Various UPLC parameters were evaluated, including the type of column, flow rate, gradient, and mobile phase used during the separation. The aqueous mobile phase (A) was 100% water with 2mM ammonium acetate (AA). For the organic mobile phase (B), two different solvents were investigated, acetonitrile (ACN) and methanol (MeOH). Both mobile phases contained 2mM ammonium acetate. However, it was found that the acetonitrile produced a more stabile baseline when compared to the methanol and it was chosen as the main component for the organic mobile phase. For the rest of this study, the column temperature and UV detection wavelength was fixed at 40°C and 210 nm, unless stated otherwise.

The smokeless powder standards were prepared at a concentration of 1 mg/mL in organic solvent (ACN or MeOH), combined to form the GSR mixture, and then diluted to various concentrations using a sample dilutor. The dilutor was a 50:50 mixture of acetonitrile and water with 2mM ammonium acetate. The acetate promotes efficient electrospray ionization. Various concentrations of the GSR mixture were then tested to determine initial limits of detection. These included standards ranging from 100 µg/mL to 1 ng/mL. UV and MS results yielded a linear calibration curve (Figure 3.1) with overall detection limits of approximately 5 ug/mL (UV) and below 1 ng/mL for the extracted MS ions. A more thorough study was done to determine individual limits of detection for all standards and is reported in Section 4.

Figure 3.1: Calibration curves for DPA, 2,4-DNT, and NG for estimating detection limits.



A C8 column and a C18 column were evaluated for their ability to separate 20 common smokeless powder components and decomposition products that may potentially be found in GSR. The properties of these two columns can be seen in Table 3.1. Both of the columns were Acquity UPLC BEH (Bridged Ethyl Hybrid) analytical columns. It was found that the C8 column – with the extra column length and increased polarity provided an overall improvement in separation of the GSR mixture. The final gradient procedure used is listed in Table 3.2 and an example chromatogram is shown in Figure 3.2. The mobile phases were 100% water and 100% acetonitrile and both contained 2mM ammonium acetate to promote ionization. A higher flow rate (0.500 mL/min) provided decreased analysis times and enhanced separation of the mixture, with 18 peaks visible in the chromatogram. Two of the peaks were due to co-eluting compounds and the separation was achieved in less than 10 minutes.

Table 3.1: UPLC columns examined this study.

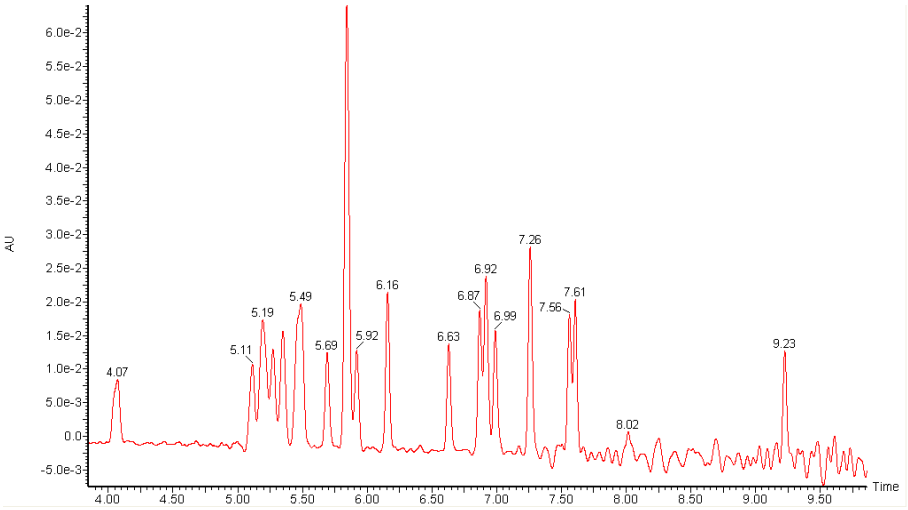
	Column 1	Column 2
Chemistry	C18	C8
Particle size	1.7 µm	1.7 µm
Length	50 mm	100 mm
Internal Diameter	2.1 mm	2.1 mm
Mode	Reversed-phase	Reversed-phase

Table 3.2: Gradient conditions for Figure 2. The curve profile signifies either a linear slope (6) or an immediate switch to a different parameter at a specific time during the run (1).

Time	Flow rate (mL/min)	% Aqueous	% Organic
0.00	0.500	90	10
12.00	0.500	20	80
13.50	0.500	5	95
13.60	0.500	80	20
15.00	0.500	80	20

A: Water + 2mM ammonium acetate
B: Acetonitrile + 2mM ammonium acetate

Figure 3.2: UPLC separation of a 20-component standard GSR mixture at an overall concentration of 10 µg/mL on a C8 column (100 mm, 2.1 mm i.d.). The gradient program was set to run from 10% - 80% organic in 12 minutes at 0.5 mL/min. 2mM ammonium acetate was added to both aqueous and organic mobile phases. The UV detection wavelength was 230 nm.



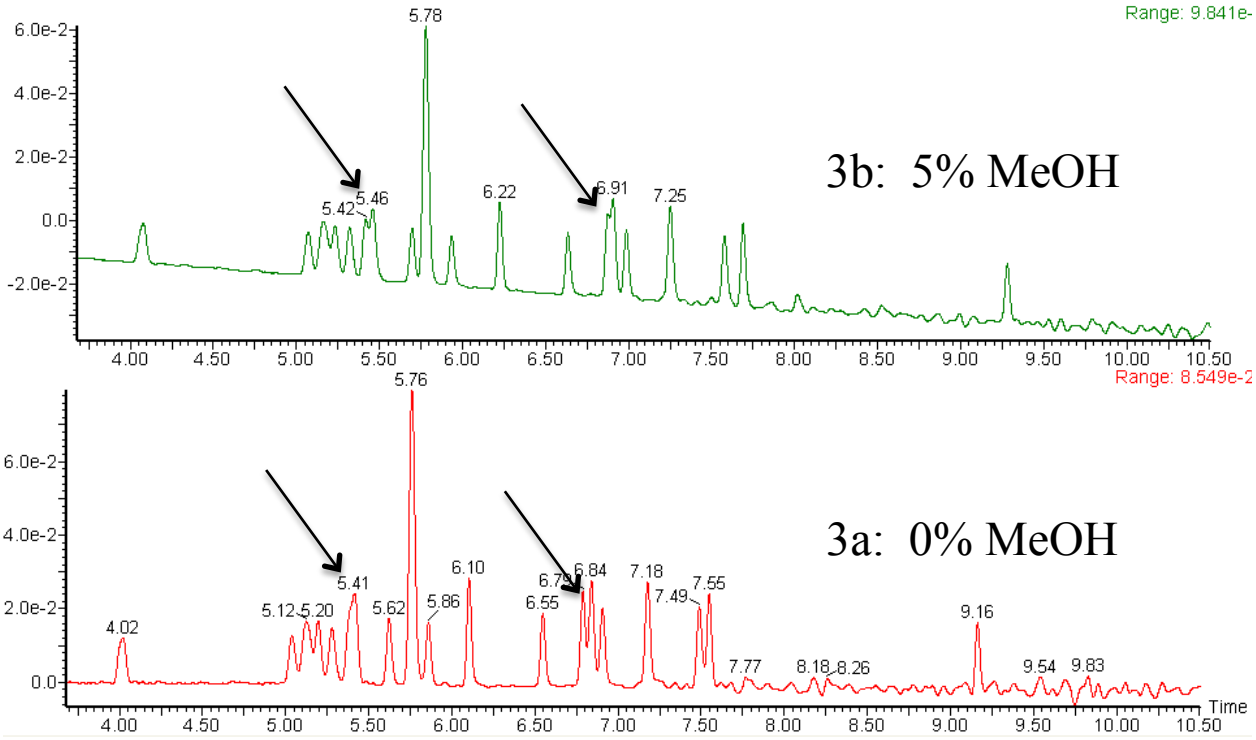
As the number of runs injected on column increased, it became necessary to modify the mobile phases in order to improve the stability of separation and detection of the smokeless powder standards as well as decrease the background baseline. Acetonitrile was added to the aqueous mobile phase to improve reproducibility and solubility and to limit bacterial growth. Table 3.3 gives the new gradient program that accounts for the addition of the acetonitrile. A study was then conducted to examine the effects of adding methanol (0-30%) to the organic mobile phase to improve separation and it was determined that the 5% solution worked the best. Higher percentages of methanol produced backpressures that exceeded the system limit of 15,000 psi. The differences in separation with and without methanol can be seen in Figure 3.3.

Table 3.3: New gradient program to account for the addition of acetonitrile in the aqueous mobile phase to prevent bacterial growth.

Time (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)	Curve
Initial	0.500	100.0	0.0	Initial
12.00	0.500	30.0	70.0	6
13.50	0.500	5.0	95.0	1
13.60	0.500	100.0	0.0	1
15.00	0.500	100.0	0.0	1

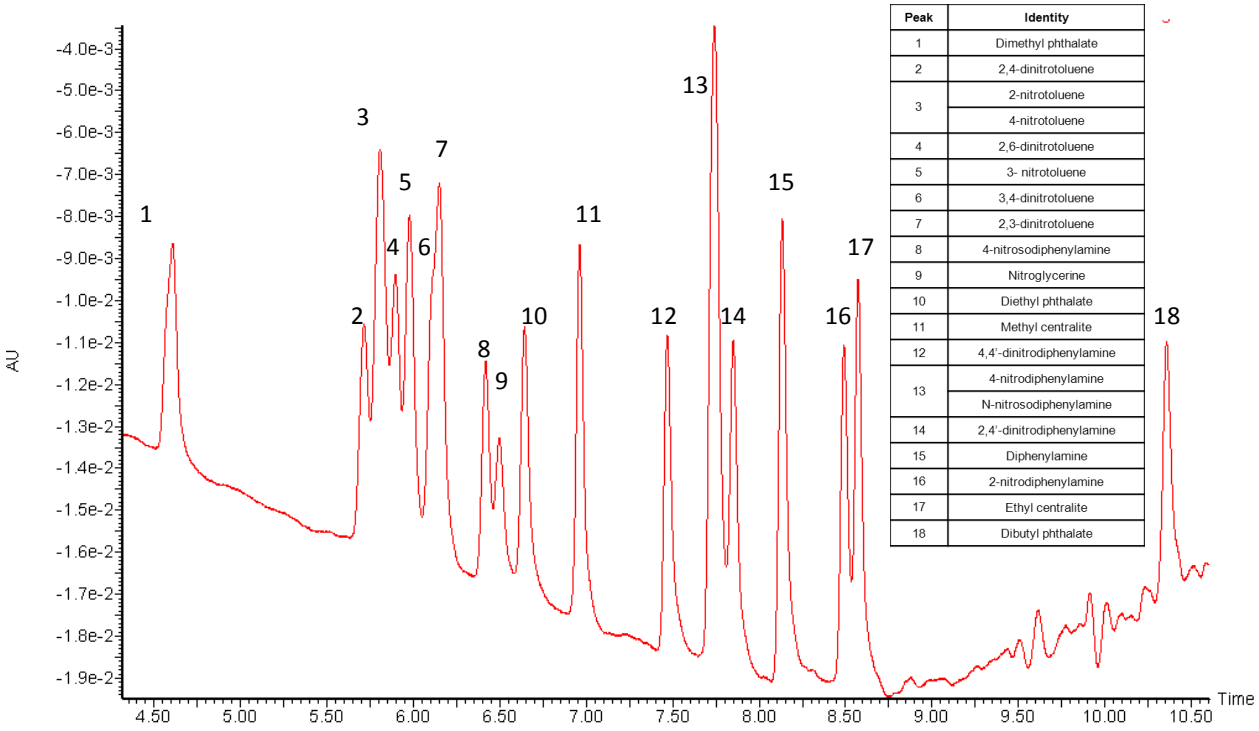
A: 90% Water + 10% Acetonitrile + 2mM ammonium acetate
B1: Acetonitrile + 2mM ammonium acetate
B2: 95% Acetonitrile + 5% Methanol + 2mM ammonium acetate

Figure 3.3: UPLC separations showing the effects of methanol when added to the organic mobile phase containing acetonitrile and 2mM ammonium acetate.



A guard column (BEH C8 Vanguard; 130 Å; 1.7 µm particles; 2.1 mm x 5 mm) was also inserted before the analytical column to protect it from strongly retained impurities. With the guard column, there was a slight increase in analysis times but more importantly, a decrease in resolution of certain peaks was seen in the chromatogram (Figure 3.4). The decrease in resolution may be due to the added column length and possible affinity to the guard column. The C8 guard column was chosen over the C18 one because it is less retentive.

Figure 3.4: UPLC separation with the addition of the C8 guard column. The gradient program was 15 min going from 0%-70% organic at a flow rate of 0.5 mL/min. The aqueous mobile phase was 90:10 water:ACN and the organic mobile phase was 95:5 ACN:methanol, both with 2mM ammonium acetate.



Peak	Identity
1	Dimethyl phthalate
2	2,4-dinitrotoluene
3	2-nitrotoluene
	4-nitrotoluene
4	2,6-dinitrotoluene
5	3- nitrotoluene
6	3,4-dinitrotoluene
7	2,3-dinitrotoluene
8	4-nitrosodiphenylamine
9	Nitroglycerine
10	Diethyl phthalate
11	Methyl centralite
12	4,4'-dinitrodiphenylamine
13	4-nitrodiphenylamine
	N-nitrosodiphenylamine
14	2,4'-dinitrodiphenylamine
15	Diphenylamine
16	2-nitrodiphenylamine
17	Ethyl centralite
18	Dibutyl phthalate

Ultimately, a new column was evaluated for its ability to enhance the separation of the GSR standard mixture. As previously mentioned, a 100mm BEH C8 column with a Vanguard C8 guard column was used to separate the mixtures prior to MS detection. The separation achieved with this column can be seen again in Figure 3.4 and the corresponding gradient is in Table 3.3. The aqueous mobile phase was 90:10 water:acetonitrile and the organic mobile phase was 95:5 acetonitrile:methanol, both containing 2mM ammonium acetate. The new column that was investigated was a BEH C18 column provided by Waters Corporation (100 mm length, 2.1 mm internal diameter, 1.7 µm particles). It was believed that the extra length (100 mm vs 50 mm

tested previously) would help to improve the UPLC separation. The guard column, mobile phases, and sample dilutor solution were the same ones used in previous analyses on the C8 analytical column. The gradient described in Table 3.3 didn't separate the compounds on the new C18 column very well; therefore, other gradients were tested.

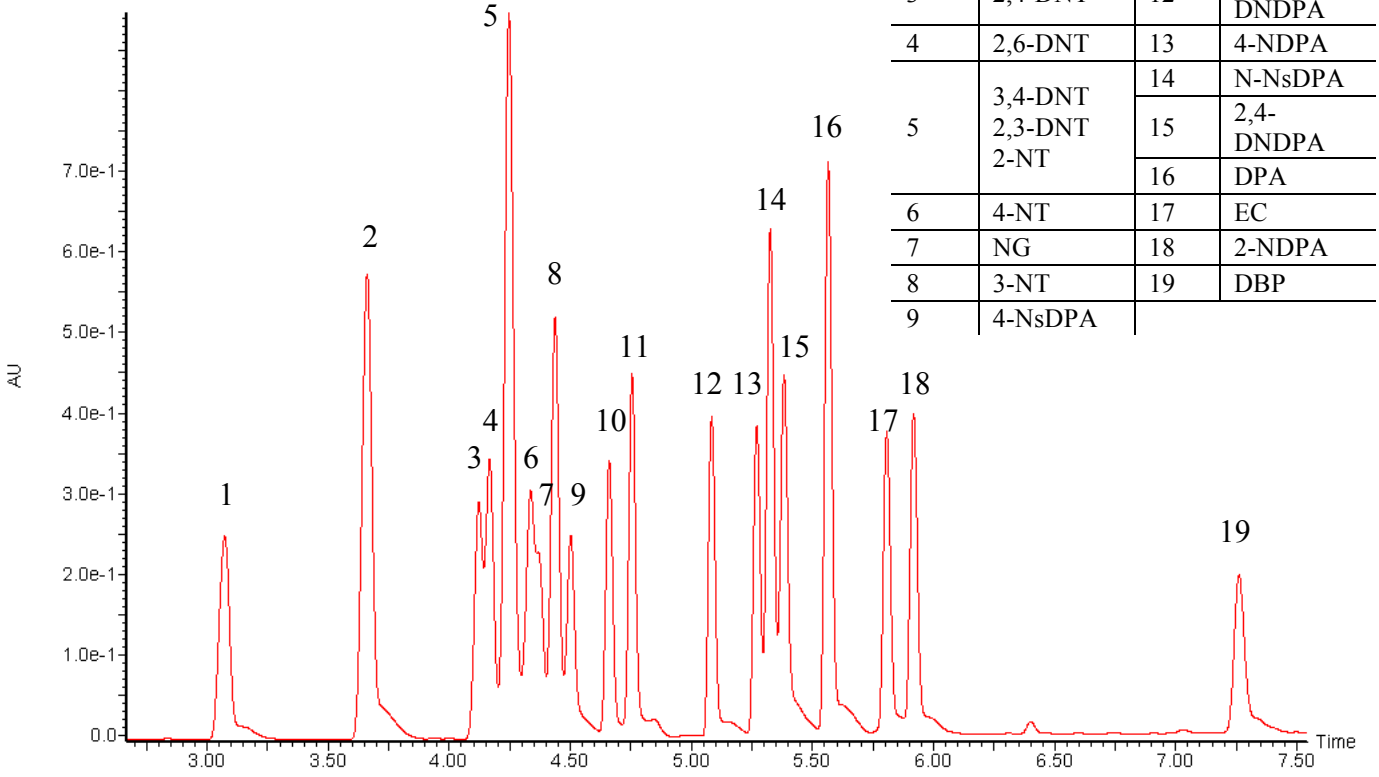
Using the gradient described in Table 3.4, we were able to separate the 21 standard mixture, with naphthol added as an internal standard, in less than 8 minutes with some co-elution. The UV chromatogram of this separation is given in Figure 3.5 along with numbers identifying each peak. Isomers such as the four DNT's are difficult to separate because of their structural similarities. Nevertheless, for other major smokeless powder components like ethyl centralite and diphenylamine, we are able to achieve very good separations.

Table 3.4. Gradient method used for the separation of a 21-component standard mixture using the C18 column with a guard column.

Time (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)	Curve
Initial	0.500	100	0.0	Initial
0.50	0.500	85	15	6
0.60	0.500	84	16	6
1.50	0.500	82	18	6
1.75	0.500	75	25	6
3.00	0.500	70	30	6
4.50	0.500	50	50	6
5.50	0.500	40	60	6
8.00	0.500	37	63	6
10.10	0.500	5	95	1

A: 90% Water + 10% Acetonitrile + 2mM ammonium acetate
B: 95% Acetonitrile + 5% Methanol + 2mM ammonium acetate

Figure 3.5: C18 separation of a 200 µg/mL standard mixture containing 21 compounds. The individual concentrations and amount injected of each compound were approximately 10 µg/mL and 100 ng, respectively. Separation conditions are listed in Table 3.4.



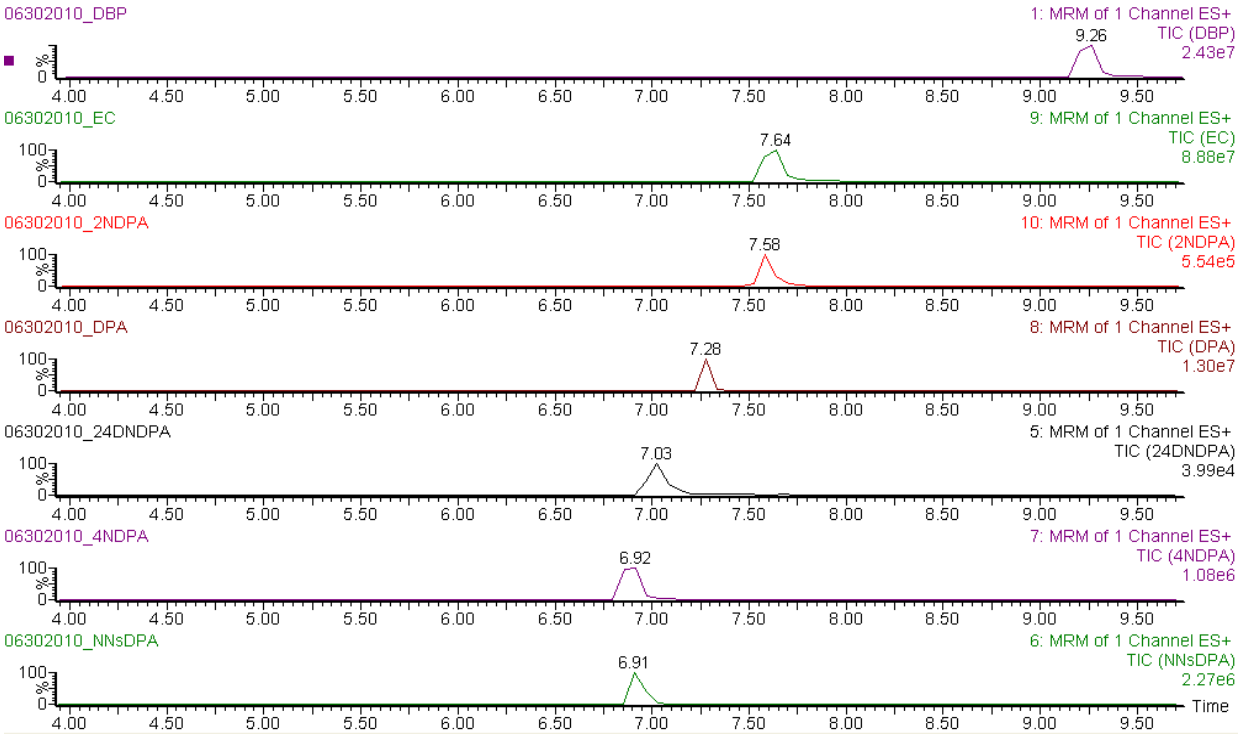
Peak	Compound	Peak	Compound
1	DMP	10	DEP
2	2-naphthol	11	MC
3	2,4-DNT	12	4,4'-DNDPA
4	2,6-DNT	13	4-NDPA
5	3,4-DNT 2,3-DNT 2-NT	14	N-NsDPA
		15	2,4-DNDPA
		16	DPA
6	4-NT	17	EC
7	NG	18	2-NDPA
8	3-NT	19	DBP
9	4-NsDPA		

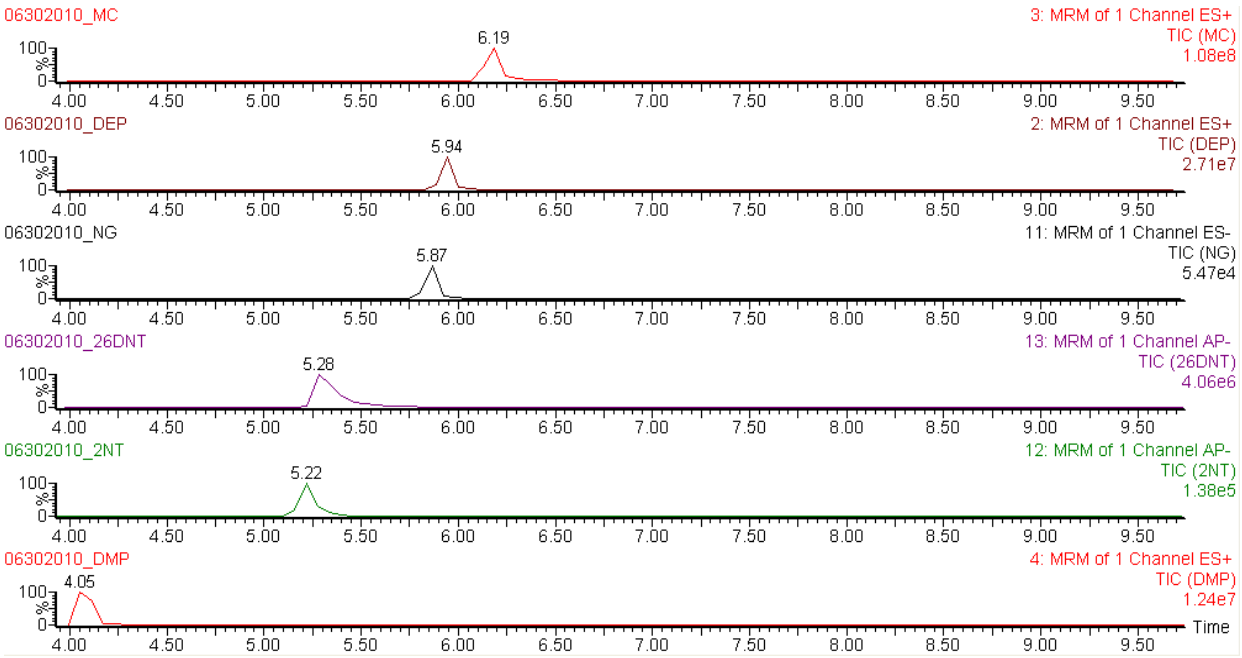
2. MS/MS Method Development

While optimizing the gradient separation, work was also conducted to determine characteristic ion transitions for each compound using the multiple reaction monitoring (MRM) feature of the tandem mass spectrometer. The goal was to determine the best conditions for detecting the smokeless powder additives and decomposition products. Figure 3.6 demonstrates a set of MRM ions produced for the separation displayed in Figure 3.2. The first UPLC separation

was performed on a C8 column (100 mm, 2.1 mm i.d.) prior to the 20-component standard GSR mixture being injected into the MS. The gradient program was set to run from 10%-80% organic in 12 minutes at a flow rate of 0.5 mL/min. The aqueous mobile was a 90:10 solution of water and acetonitrile, whereas the organic mobile phase was a 95:5 solution of acetonitrile and methanol. Both mobile phases contained 2mM ammonium acetate. The UV detection wavelength was 210 nm.

Figure 3.6: Stacked MRM chromatogram of the individual standards (10µg/mL) using a gradient of 10-80% organic in 12 min at 0.5 mL/min and a Waters BEH C8 analytical column.





The corresponding UV and MRM detection times as well as the MS/MS conditions are summarized in Table 3.5. Only one precursor-to-product transition was monitored for each compound, as seen by the line "MRM of 1 Channel" in Figure 3.6. For confirmation and accurate identification of the smokeless powder additives, it would be beneficial to monitor two transitions for each compound. Therefore, a study was conducted to identify other transitions and is mentioned later on in this section.

Table 3.5. UV and MRM data collected for the individual components in the GSR mixture using the gradient procedure listed in Table 3.2.

	Time (min)		MRM method					
Chemical	UV	MRM	Molecular weight	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)	Mode
DMP	4.06	4.05	198.14	195.07	163.09	15	10	ESI+
2,4-DNT	5.10	-	182.13	-	-	-	-	-
2-NT	5.17	5.22	137.14	137.00	45.70	12	12	APCI-
4-NT	5.20	-	137.14	-	-	-	-	-
2,6-DNT	5.26	5.28	182.13	182.00	152.00	15	10	APCI-
3-NT	5.34	-	137.14	-	-	-	-	-
3,4-DNT	5.44	-	182.13	-	-	-	-	-
2,3-DNT	5.48	-	182.13	-	-	-	-	-
4-NsDPA	5.68	-	198.22	-	-	-	-	-
NG	5.83	5.87	227.09	285.97	61.85	10	10	ESI-
DEP	5.90	5.94	222.24	223.10	148.80	15	16	ESI+
MC	6.14	6.19	240.30	241.10	133.70	15	16	ESI+
4,4'-DNDPA	6.62	-	259.22	-	-	-	-	-
4-NDPA	6.86	6.92	214.22	215.09	198.09	30	14	ESI+
N-NsDPA	6.91	6.91	198.22	199.08	169.11	20	12	ESI+
2,4'-DNDPA	6.99	7.03	259.22	260.10	243.30	30	16	ESI+
DPA	7.25	7.28	169.22	170.11	92.84	35	24	ESI+
2-NDPA	7.56	7.58	214.22	215.00	180.00	20	20	ESI+
EC	7.59	7.64	268.35	269.21	119.96	15	22	ESI+
DBP	9.21	9.26	278.34	279.20	148.80	20	12	ESI+

In order to determine the best conditions for detecting the different compounds by MS/MS, concentrated samples were directly infused into the instrument. The goal was to identify missing transitions for some of the compounds (e.g. DNT). Individual standards were dissolved in organic solvent and then diluted to various concentrations for infusion work and UPLC/MS/MS analysis. The sample dilutor was a mixture of acetonitrile and water (50:50) along with 2mM ammonium acetate.

The investigated parameters were: the cone voltage, collision energy, and ionization mode. The cone voltages and collision energies affect the fragmentation of the molecule and were adjusted manually to improve detection of the compounds. A lower cone voltage facilitates

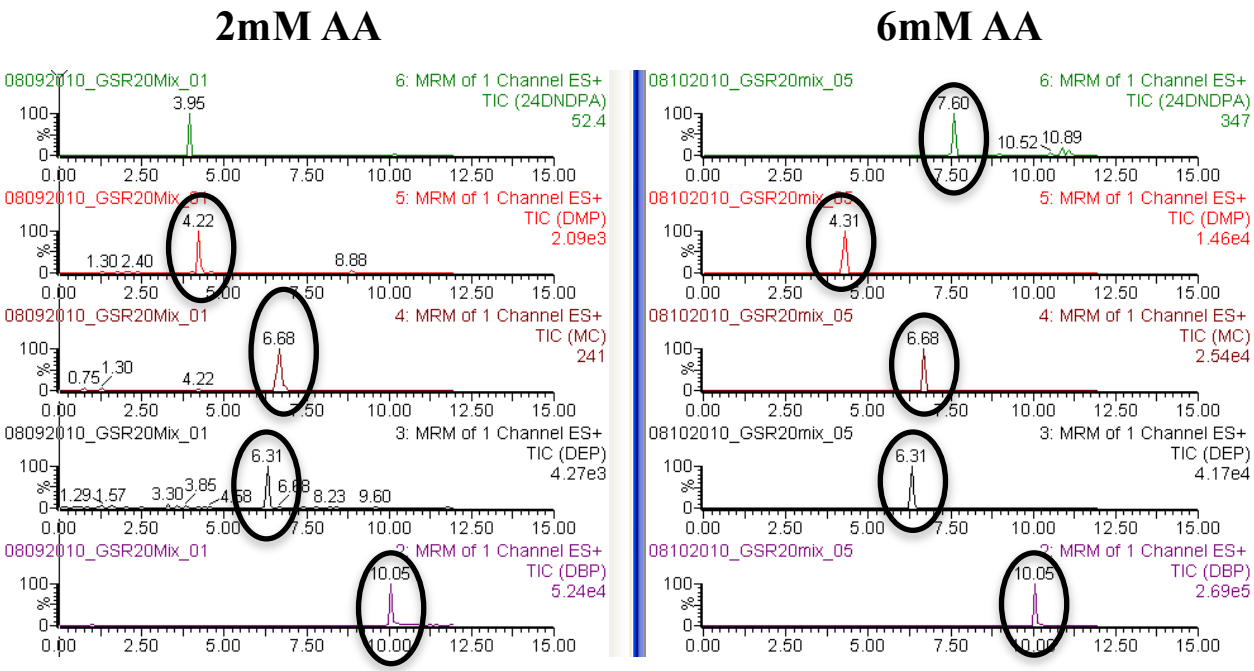
the passage of an intact parent molecule into the first quadrupole. The collision energy is applied in the collision cell to fragment a specific precursor ion into various product ions. These ions are then separated in the second quadrupole and detected by the mass spectrometer. The dwell time is the time focused on a specific mass during the analysis. The ionization mode was selected based on the structure of the compound. The [M+H] ion was monitored for most compounds in positive ESI mode. An adduct was chosen as the precursor ion in negative ESI mode for nitroglycerin because the molecular ion wasn't visible in the spectrum. The nitrotoluenes were all detected in negative APCI mode using a corona discharge pin. The conditions determined thus far for detecting each compound are listed in Table 3.6. The parameters were determined via infusion of concentrated samples directly into the MS and optimized using the Autotune software provided by Waters. The dwell time was set to 0.070 seconds.

Table 3.6. MRM method used for detection of samples by MS/MS. These values were

Compound	Ionization mode	Molecular weight	Precursor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision energy (eV)
Diphenylamine	ES+	169.22	170.11	92.84	35	24
N-Nitrosodiphenylamine	ES+	198.22	199.08	169.11	20	12
4-Nitrosodiphenylamine	ES+	198.22	199.09	181.20	35	26
2-Nitrodiphenylamine	ES+	214.22	215.00	180.00	20	20
4-Nitrodiphenylamine	ES+	214.22	215.09	198.09	30	14
2,4-Dinitrodiphenylamine	ES+	259.22	260.10	243.30	30	16
4,4'-Dinitrodiphenylamine	ES+	259.22	260.07	167.08	25	30
Dibutyl phthalate	ES+	278.34	279.20	148.80	20	12
Diethyl phthalate	ES+	222.24	223.10	148.80	15	16
Dimethyl phthalate	ES+	198.14	195.07	163.09	15	10
Ethyl centralite	ES+	268.35	269.21	119.96	15	22
Methyl centralite	ES+	240.30	241.10	133.70	15	16
Nitroglycerin	ES-	227.09	285.97	61.85	10	10
2-Nitrotoluene	API-	137.14	137.00	45.70	30	20
3-Nitrotoluene	API-	137.14	137.00	107.01	15	20
4-Nitrotoluene	API-	137.14	137.00	45.78	15	30
2,3-Dinitrotoluene	API-	182.13	182.00	151.95	15	10
2,4-Dinitrotoluene	API-	182.13	182.00	165.00	15	10
2,6-Dinitrotoluene	API-	182.13	182.00	152.00	15	10
3,4-Dinitrotoluene	API-	182.13	182.00	45.79	15	20

A study was also performed to determine if the MS signal could be improved by adding salts for use as potential adduct ions to the sample dilutor. Both ammonium acetate and ammonium chloride were evaluated for their effects on MS sensitivity. Previously reported analyses used 2mM ammonium acetate in a dilutor solution that was 50:50 water:acetonitrile. By comparing the signal intensity, it can be seen in Figure 3.7 that the 6mM improves analyte detection in many cases by as much as an order of magnitude.

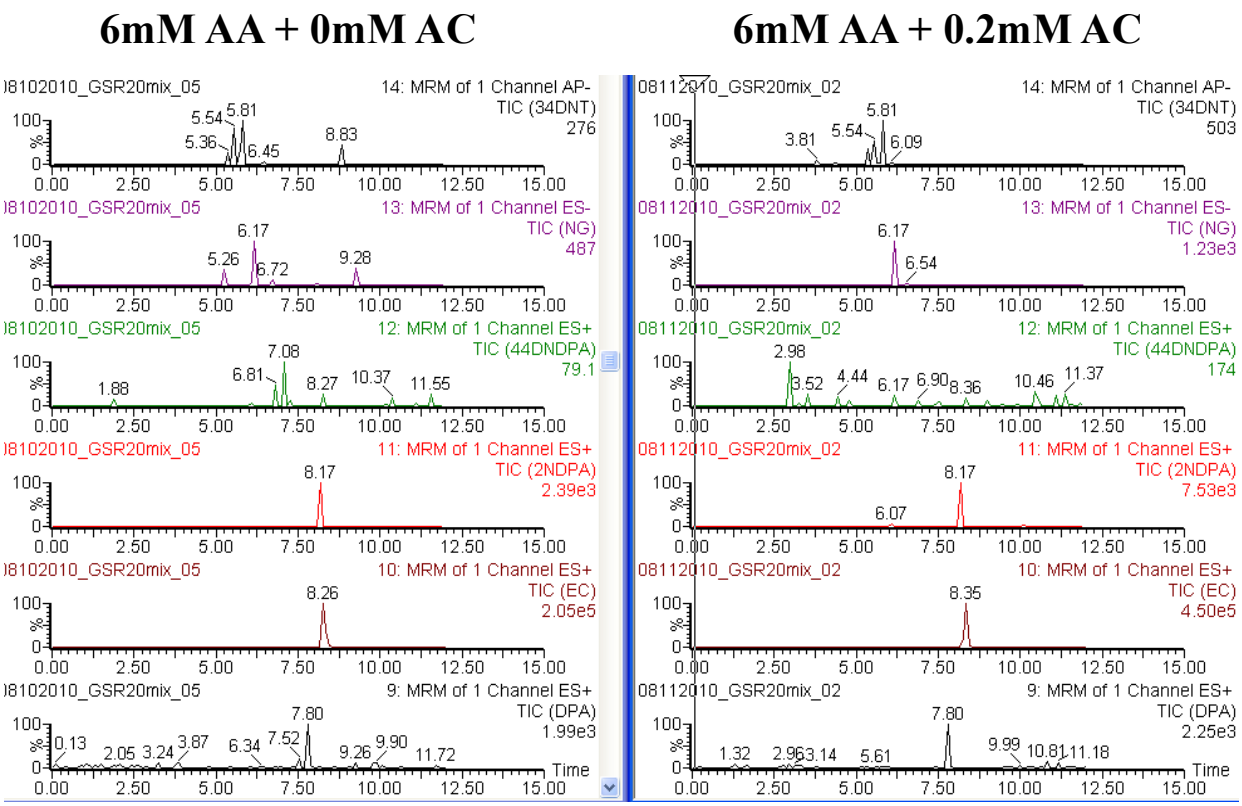
Figure 3.7. MS comparisons for diluted samples with 2mM and 6mM ammonium acetate (AA) demonstrating improvement in signal with additional ammonium acetate. Signal intensities are listed on the right hand side of each panel. Relevant peaks are circled for each compound.



The effects of adding ammonium chloride to the sample dilutor were also investigated. An improvement in MS detection was seen when 0.2 mM ammonium chloride was added to the sample dilutor already containing 6mM ammonium acetate. This was particularly important for

the detection of thermally labile or difficult compounds like nitroglycerin, as the signal for this compound increased 2.5 times with the addition of ammonium chloride. The MRM chromatograms are shown in Figure 3.8.

Figure 3.8. MS comparisons for diluted samples with 0mM and 0.2mM ammonium chloride (AC). The samples also contained 6mM ammonium acetate (AA). The results show a dramatic improvement in signal of NG with the addition of ammonium chloride. Signal intensities are listed on the right hand side of each panel. Relevant peaks are circled for each compound.



To improve the detection of NG and nitrotoluenes, temperatures and gas flows in the source were lowered during direct infusion to stabilize peak signals at low flow rates. The sample was also diluted with higher amounts of water. This this tends to produce more stable

signals in the ESCi® mode as it minimizes evaporative effects. Table 3.7 shows the effects of changing the percentage of water in the sample dilutor on the signal intensities of the nitrotoluenes, the dinitrotoluenes, and nitroglycerin. MS scans were collected for each compound for 0.5 min and the peak intensities were recorded for the molecular ion ([M+H] or [M-H]) or an adduct.

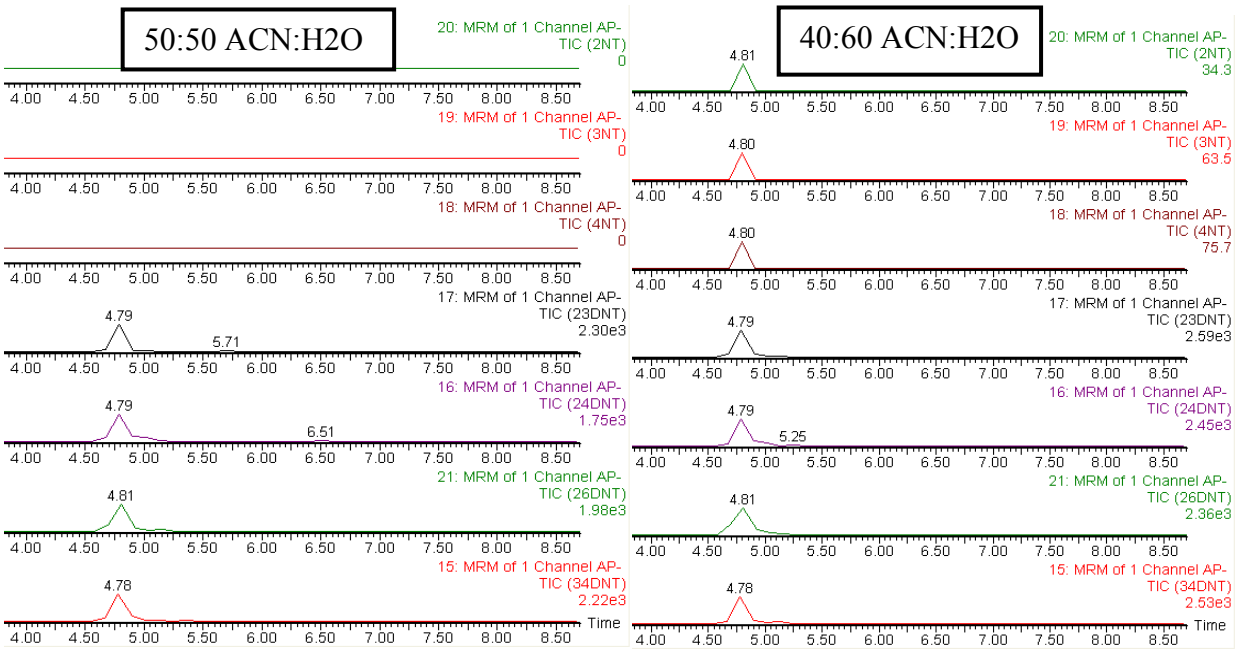
Table 3.7. Comparison of signal strengths for nitroglycerin (m/z 262), the nitrotoluenes (m/z 137), and the dinitrotoluenes (m/z 182) in different diluting solutions. 6 mM ammonium acetate (AA) and 0.2 mM ammonium chloride (AC) were added to increase ionization of compounds. Negative APCI mode was used for detecting nitrotoluenes, whereas negative ESI mode was used for NG detection.

Compound	**MS Signal Strength**		
	40:60 ACN:H2O	**50:50 ACN:H2O (AA+AC)**	**40:60 ACN:H2O (AA+AC)**
2-NT	2.01E3	1.69E3	3.13E3
3-NT	1.98E3	1.39E3	1.68E3
4-NT	2.39E3	1.78E3	2.45E3
2,3-DNT	4.77E3	2.88E3	5.01E3
2,4-DNT	2.24E3	2.67E3	4.02E3
2,6-DNT	5.56E3	1.01E4	1.24E4
3,4-DNT	2.65E3	3.85E3	6.26E3
NG (chloride)	1.42E5	5.40E5	1.18E4

After comparing the peak intensities for each compound's precursor ion, it was determined that a 40:60 mixture with ammonium acetate and ammonium chloride would be the best diluent. Overall, it provided the highest intensities for the nitrotoluenes and dinitrotoluenes, which is important when trying to perform MRM scans. It is easier to find and lock onto a precursor ion in the 1st quadrupole if the initial peak is intense. A mixture was tested to ensure that all peaks could actually be detected when first separated on column. It can be seen in Figure

3.9 that the 40:60 mixture with AA and AC provided better results than the 50:50 mixture. However, the signal intensities of nitroglycerin and some of the other compounds detected in ESI+ mode were reduced slightly with the additional water.

Figure 3.9. Comparison of MRM signals for NT and DNT in different diluting solutions. The results confirm that the use of higher amounts of water enhances the detection of the nitrotoluenes.



Following this, each compound was diluted in the 40:60 mixture containing ammonium acetate and ammonium chloride and infused directly into the mass spectrometer to search for new precursor-to-product transitions. The ESI capillary voltage and APCI current were adjusted manually to improve detection of the precursor ion and then the cone voltages and collision energies were optimized for isolation of the product ions. The new MRM method is outlined below in Table 3.8 for each compound. It was difficult to identify a second MRM transition for

the 2-, 3-, and 4-nitrotoluenes. This may be due to a combination of reasons: concentration too low to detect multiple product ions, the MS switching between ESI and APCI during the run, or high source temperatures.

Table 3.8. MRM method used for detection of samples by MS/MS. These values were determined via infusion of concentrated samples directly into the MS and optimized using the Autotune software provided by Waters. Two daughter ions were selected for most compounds.

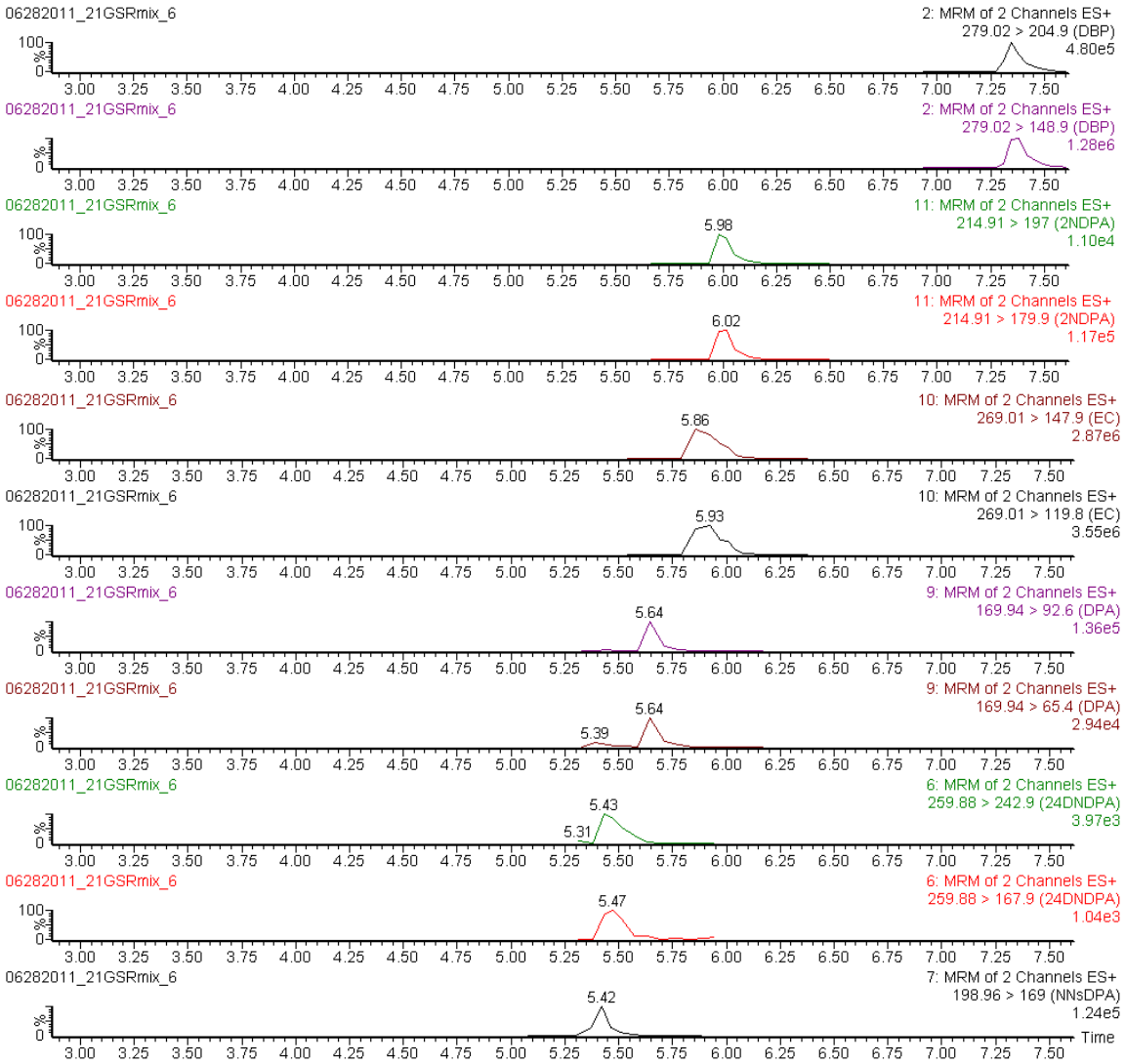
Compound	Ionization mode	Precursor ion (m/z)	Product ion 1 (m/z)	CV, CE (Voltage)	Product ion 2 (m/z)	CV, CE (Voltage)
Diphenylamine	ES+	169.94	65.4	34, 40	92.6	34, 22
N-Nitrosodiphenylamine	ES+	198.96	65.7	18, 26	169.0	18, 10
4-Nitrosodiphenylamine	ES+	198.96	127.8	32, 38	181.1	32, 22
2-Nitrodiphenylamine	ES+	214.91	179.9	20, 18	197.0	20, 8
4-Nitrodiphenylamine	ES+	214.91	167.0	28, 34	197.9	28, 12
2,4-Dinitrodiphenylamine	ES+	259.88	167.9	30, 24	242.9	30, 14
4,4'-Dinitrodiphenylamine	ES+	259.94	168.8	22, 36	243.0	22, 12
Dibutyl phthalate	ES+	279.02	148.9	16, 14	204.9	16, 6
Diethyl phthalate	ES+	222.98	64.8	14, 48	148.9	14, 18
Dimethyl phthalate	ES+	194.96	76.7	14, 32	162.9	14, 10
Ethyl centralite	ES+	269.01	119.8	20, 22	147.9	20, 12
Methyl centralite	ES+	240.99	105.8	20, 26	133.9	20, 14
Nitroglycerin	ES-	262.00	45.6	8, 6	61.7	8, 4
2-Nitrotoluene	API-	136.79	46.0	8, 10	---	---
3-Nitrotoluene	API-	136.79	46.0	8, 10	---	---
4-Nitrotoluene	API-	136.79	46.0	8, 10	---	---
2,3-Dinitrotoluene	API-	181.82	46.0	18, 12	152.0	18,12
2,4-Dinitrotoluene	API-	181.82	46.0	18, 12	164.9	20,10
2,6-Dinitrotoluene	API-	181.82	46.0	18, 12	152.2	20,10
3,4-Dinitrotoluene	API-	181.82	46.0	18, 12	152.0	18,12
2-naphthol	ES-	142.85	64.6	44, 24	114.8	44, 24

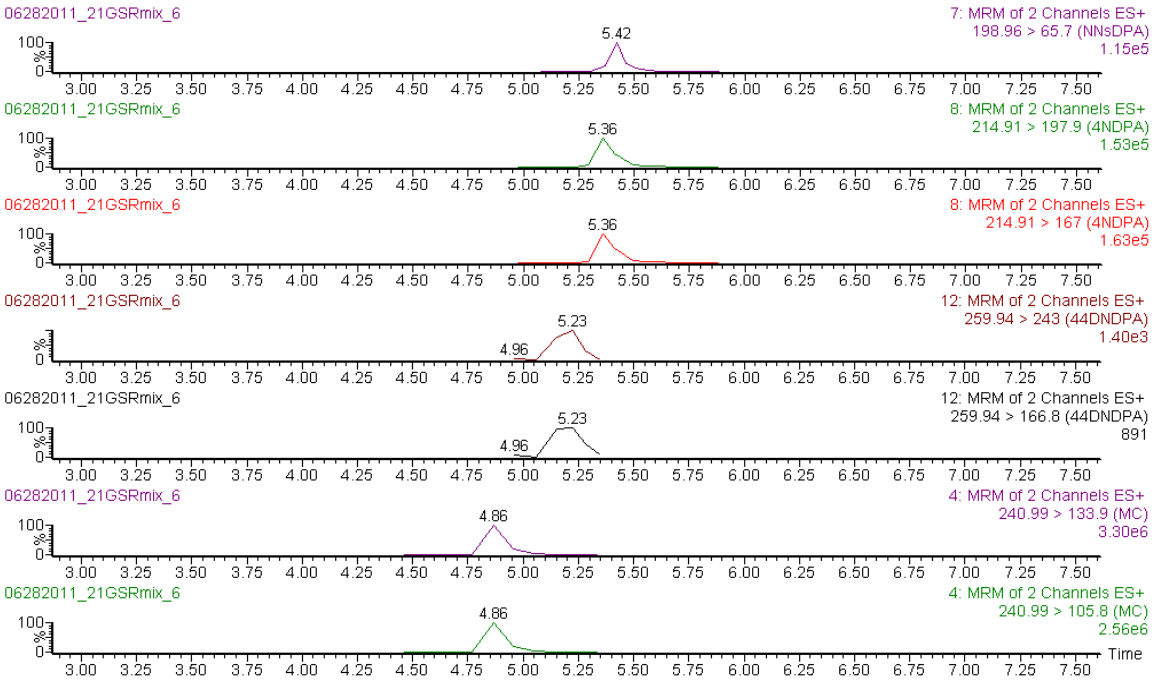
*ESCi+ capillary voltage = 3.20 kV; ESCi- capillary voltage = 4.30 kV, ESCi- current = 20.00 A

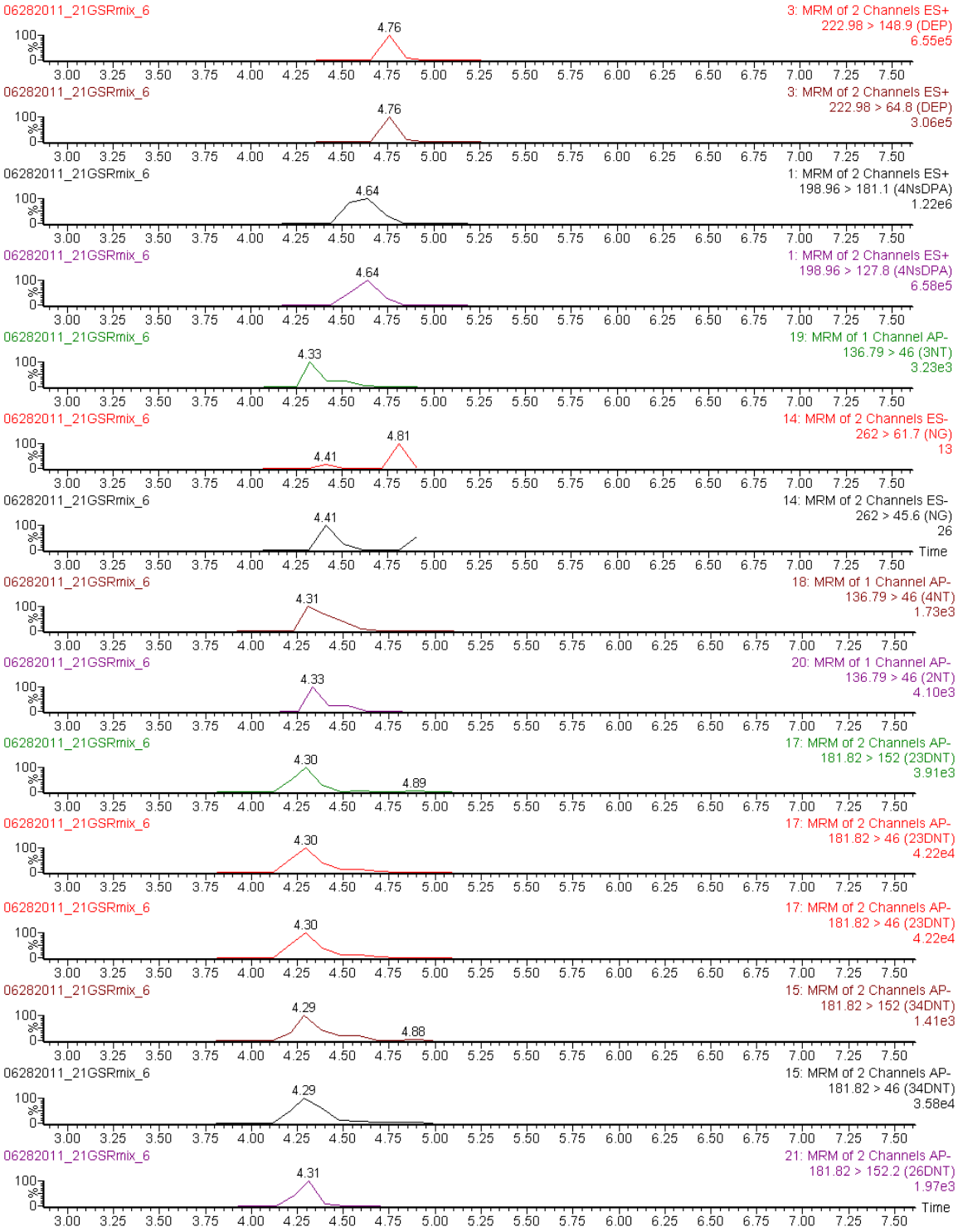
The new MRM method was applied to samples following UPLC separation. The signals detected for each compound can be seen in Figure 3.10. There are two chromatograms shown for all of the compounds except the nitrotoluenes. The channel represents one precursor-to-product

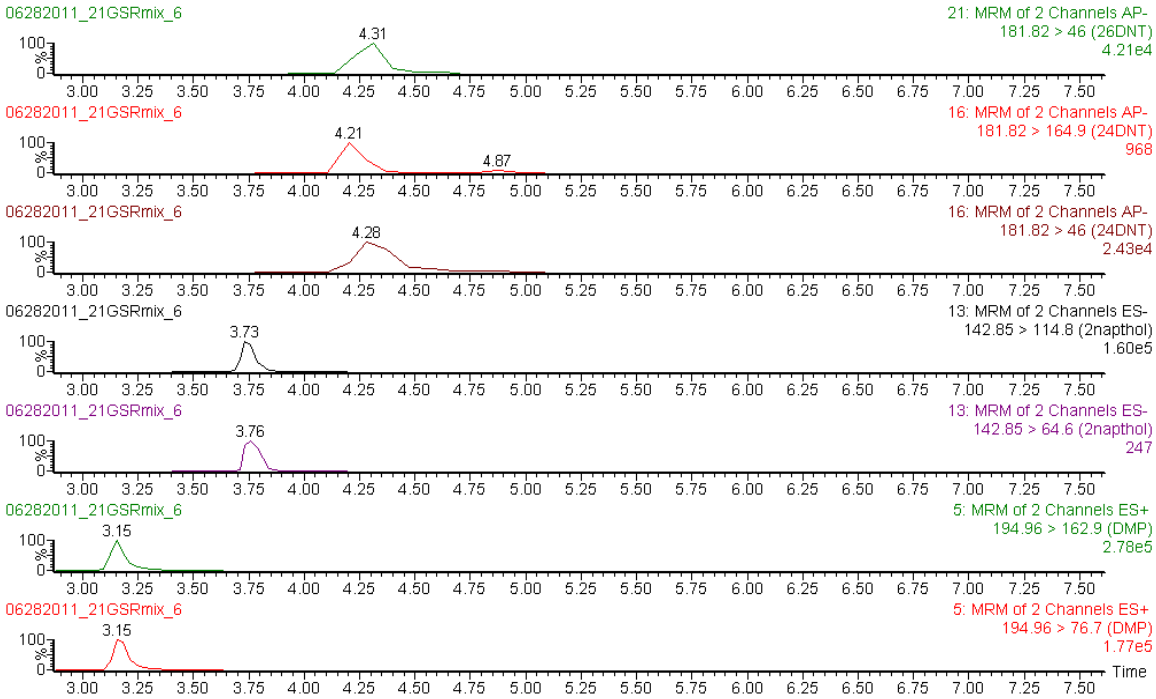
transition. Signal intensities and compound names are on the right hand side of the chromatogram, whereas the migration times are above the detected peaks.

Figure 3.10. MRM signals obtained following the separation of a 200 ug/mL standard mixture on a C18 column – see Table 3.4 and Figure 3.5 for UPLC separation and conditions. The individual concentrations and amount injected of each compound were approximately 10 µg/mL and 100 ng, respectively. Two channels representing two precursor-to-product transitions are given for most compounds with signal intensities and migration times.









With this new UPLC/MS/MS method, all 21 compounds we are able to be separated and detected by their UV retention times, MRM times, and MRM transitions. This allows for accurate identification of each chemical present in the mixture. For the nitrotoluenes, it was difficult to identify two transitions that could be used to identify each compound. This may be due to an insufficient signal for the precursor and/or product ion, which is also affected by the multi-ionization mode being employed. For the dinitrotoluenes, two transitions were identified for each isomer. However, it was also difficult to select two distinct transitions for each isomer because of their similarities in fragmentation. Nevertheless, most of the compounds can be distinguished based on one or more characteristic parameters.

Before ending this section on method development, it is important to note that a study was done to improve the MS signal for nitroglycerin. The MRM signal of nitroglycerin was very low when it came to analyzing mixtures and results were inconsistent over runs. The detection of nitroglycerin is important in the analyses because it is a primary component in double base

smokeless powders. Different amounts of ammonium chloride were added to the mobile phases in an attempt to improve detection of NG. Ammonium chloride was chosen as the additive because the chloride adduct of NG was monitored in MRM mode as the precursor ion.

Previous analyses utilized only 2mM ammonium acetate in the mobile phase. Two new sets of mobile phases were prepared and tested for their ability to improve the ionization of NG. To both mobile phases, either 0.2mM or 1mM ammonium chloride were added, in addition to the ammonium acetate already present. By comparing the signal intensities of NG, it can be seen that there was an improvement in NG detection with the addition of chloride (Table 3.9). However, there was also a reduction in signal intensities for some of the other compounds. 0.2mM ammonium chloride was chosen for future work because NG can be detected without significant reductions in other signal intensities. In addition, it was easier to dissolve lower amounts of AC in both the aqueous and organic mobile phases. An improvement in the signal for NG was also seen with higher concentrations of the standard mixture; however, this lead to more co-elution of certain compounds during the UPLC separation. Nitroglycerin was tested alone and the MRM signal was also stronger, signifying that the ionization is also a concentration issue.

Table 3.9 Comparison of MRM signal intensities of all 21 standards obtained after analyzing the GSR mixture with varying amounts of ammonium chloride in the mobile phase.

Compound	0 mM AC	0.2 mM AC	1 mM AC	Change in MRM signal (0 -> 0.2mM)	Change in MRM signal (0 -> 1 mM)
DPA	2.20E+05	1.74E+05	1.41E+05	Decrease	Decrease
2-NDPA	1.09E+05	1.34E+05	1.44E+05	Increase	Increase
4-NDPA	6.71E+04	3.02E+05	2.44E+05	Increase	Increase
4-NsDPA	3.10E+06	5.58E+06	1.65E+06	Increase	Decrease
N-NsDPA	4.97E+05	1.77E+05	3.71E+05	Decrease	Decrease
2,4-DNDPA	2.34E+03	3.02E+03	2.99E+03	Increase	Increase
4,4'-DNDPA	2.05E+03	3.03E+03	2.20 E+03	Increase	Increase

DMP	7.61E+05	2.26E+05	4.54E+05	Decrease	Decrease
DEP	4.23E+05	1.12E+06	1.11E+06	Increase	Increase
DBP	1.59E+06	2.22E+06	1.63E+06	Increase	Same
EC	1.70E+07	1.38E+07	1.18E+07	Decrease	Decrease
MC	1.34E+07	1.29E+07	6.94E+06	Decrease	Decrease
NG	0.00E+00	3.96E+02	5.67E+02	Increase	Increase
2-NT	2.13E+03	2.47E+03	2.39E+03	Increase	Increase
3-NT	2.37E+03	2.83E+03	1.42E+03	Increase	Decrease
4-NT	2.48E+03	2.50E+03	2.04E+03	Same	Decrease
2,3-DNT	6.27E+04	6.65E+04	7.09E+04	Increase	Increase
2,4-DNT	4.29E+04	4.55E+04	5.36E+04	Increase	Increase
2,6-DNT	6.31E+04	4.96E+04	4.95E+04	Decrease	Decrease
3,4-DNT	5.76E+04	5.91E+04	7.05E+04	Increase	Increase
2-naphthol	2.71E+04	1.88E+04	6.82E+04	Decrease	Increase

Summary: For this study, it was important to have a method that permits all 20 organic GSR components to be identified accurately in a short amount of time. The compounds are separated on a reversed-phase column and initially detected by UV. Once the analytes pass the UV lamp, they enter the MS source and are ionized and detected. With these two methods, it is possible to accurately identify the analyte. Because the mixture contains a wide array of compounds, it is important to have a method for determining each one. For some of the samples that have similar UPLC retention times, it is still possible to distinguish them by MS. In terms of MS detection, it is important to make two points:

(1) some of the GSR components which have the same MS parent ion have different daughter ions and can be distinguished by MS (e.g. 2,3-DNT and 2,4-DNT).

(2) some of the GSR components which have the same MS daughter ions can be distinguished based on their retention times (Figure 6, e.g. 2-NDPA and 4-NDPA).

3. Calibration

The UPLC/MS/MS method was validated by running a mixture of the standards in triplicate from 0-750 µg/mL. The individual concentrations of each standard in the mixture and amounts injected are given in Table 3.10. The calibration samples were placed in a random order for analysis to get a more accurate determination of the limits of detection (LOD). The precision of the method and system was also evaluated by running blanks - containing only the diluting solution - and mixtures at 200 µg/mL at the beginning, middle, and/or end of the day. For the UV and MS data, the variability of the time, peak area/intensity, and efficiency was calculated (Tables 3.11-3.12). In order to reduce the variability between days, it was important to make new mobile phases at the start of the day.

Table 3.10 Concentrations of sample mixtures to be analyzed for determining limits of detection.

Total Concentration of 21 standard mixture	Individual Concentration of each standard in mixture	Amount detected with 10uL injection (ng)
750 µg/mL	35.7 µg/mL	357
500 µg/mL	23.8 µg/mL	238
250 µg/mL	11.9 µg/mL	119
100 µg/mL	4.76 µg/mL	47.6
50 µg/mL	2.38 µg/mL	23.8
10 µg/mL	476 ng/mL	4.76
5 µg/ mL	238 ng/mL	2.38

Based on the results in Table 3.11, it can be seen that there is very little deviation within and between days with this method for the UV data. Other than nitroglycerin, the area RSD for all of the compounds is below 7% and the time RSD is even smaller, signifying that the data is consistent and reliable.

Table 3.11: Figures of merit (n≥3) for the UV data.

Compound	Retention time (min)	Time RSD (%)	Capacity factor	Area	Area RSD (%)	Efficiency
DMP	3.08	1.51E-14	5.84	12633	4.90	19086
2-naphthol	3.66	3.80E-14	7.13	29796	4.32	30318
2,4-DNT	4.12	2.25E-14	8.16	10166	2.32	53950
2,6-DNT	4.17	1.18E-01	8.26	11141	1.90	57637
3,4-DNT	4.25	1.16E-01	8.44	33548	2.83	58787
2,3-DNT	4.25	1.16E-01	8.44	33548	2.83	58787
2-NT	4.25	1.16E-01	8.44	33548	2.83	58787
4-NT	4.34	1.14E-01	8.64	8887	1.81	74090
NG	4.37	2.12E-14	8.71	4422	32.89	106962
3-NT	4.44	2.09E-14	8.87	16178	1.42	88269
4-NsDPA	4.51	2.06E-14	9.02	6784	1.21	92119
DEP	4.67	1.06E-01	9.37	10357	4.44	114224
MC	4.76	1.95E-14	9.58	13921	3.24	121251
4,4'-DNDPA	5.09	9.67E-02	10.32	12473	4.19	151993
4-NDPA	5.27	9.34E-02	10.72	9804	3.75	149743
N-NsDPA	5.34	9.23E-02	10.86	16004	3.44	183161
2,4-DNDPA	5.39	9.13E-02	10.99	11258	6.24	178755
DPA	5.57	8.83E-02	11.39	23386	3.46	177063
EC	5.82	0.00E+00	11.93	12381	2.88	202054
2-NDPA	5.92	1.57E-14	12.16	13534	3.08	191697
DBP	7.28	0.00E+00	15.18	9490	4.24	121418

For the MRM results, the deviation between and within days is slightly higher (Table 3.12-3.13). The time RSD % is low but the area RSD % ranges from about 7-28%. 4,4'-DNDPA gave a very high RSD, but this may be attributed to sample decomposition. The overall higher RSD values may also be due to the use of multiple reaction monitoring mode.

Table 3.12. Figures of merit (n≥3) for the MRM data.

Compound	Retention time (min)	Time RSD (%)	Signal intensity	Signal Intensity RSD (%)	Efficiency
DMP	4.64	0.00E+00	2.36E+06	16.86	2892
2-naphthol	7.39	3.91E-01	2.51E+06	8.29	34026
2,4-DNT	4.76	1.95E-14	1.43E+06	15.15	7439
2,6-DNT	4.86	0.00E+00	1.07E+07	15.11	7937
3,4-DNT	3.16	4.88E-01	4.72E+05	14.77	7015
2,3-DNT	5.47	0.00E+00	3.93E+03	24.51	15572
2-NT	5.42	1.71E-14	3.37E+05	7.35	33332
4-NT	5.36	0.00E+00	2.53E+05	9.65	15472
NG	5.64	0.00E+00	1.58E+05	27.62	16579
3-NT	5.93	0.00E+00	1.16E+07	19.49	22048
4-NsDPA	6.02	1.54E-14	1.21E+05	8.90	19565
DEP	5.18	7.61E-01	1.39E+03	19.05	3917
MC	3.76	3.70E-14	3.01E+05	12.76	9289
4,4'-DNDPA	4.49	9.01E-01	1.40E+02	85.28	6788
4-NDPA	4.31	9.44E-01	6.69E+04	13.55	1578
N-NsDPA	4.33	1.07E+00	4.26E+04	13.31	1544
2,4-DNDPA	4.32	8.12E-01	7.65E+04	20.68	1854
DPA	4.39	5.91E-01	2.72E+03	13.83	2148
EC	4.33	2.14E-14	5.02E+03	33.95	3826
2-NDPA	4.33	2.14E-14	6.00E+03	25.09	7136
DBP	4.31	2.15E-14	7.48E+04	25.00	2307

Table 3.13. Limit of detection (n=3).

Compound	R2		Slope		LOD (ng/mL)		LOD (ng)	
	UV	MRM	UV	MRM	UV	MRM	UV	MRM
DMP	0.9667	0.9814	874	34238	141	137	1.41	1.37
2-naphthol	0.9824	0.9848	2111	12737	65	472	0.65	4.72
2,4-DNT	0.9900	0.9951	1437	4023	163	57	1.63	0.57
2,6-DNT	0.8854	0.9941	1670	5345	137	82	1.37	0.82
3,4-DNT	0.9989	0.9911	1408	6904	151	56	1.51	0.56
2,3-DNT	1.0000	0.9599	1362	4764	142	64	1.42	0.64
2-NT	0.9987	0.9829	1140	357	235	21	2.35	0.21
4-NT	0.8994	0.9872	1237	272	201	33	2.01	0.33
NG	0.8880	0.8927	1268	17	413	53	4.13	0.53
3-NT	0.9815	0.9896	1834	335	107	16	1.07	0.16
4-NsDPA	0.9331	0.9949	646	213587	231	172	2.31	1.72
DEP	0.8455	0.9428	694	68406	155	227	1.55	2.27
MC	0.9133	0.9648	1046	416543	125	460	1.25	4.60
4,4'-DNDPA	0.9787	0.7629	1116	102	89	2930	0.89	29.30
4-NDPA	0.9983	0.8104	1105	15811	121	482	1.21	4.82
N-NsDPA	0.9861	0.9326	2117	13803	77	156	0.77	1.56
2,4-DNDPA	0.9910	0.9283	1344	274	105	604	1.05	6.04
DPA	0.9996	0.9990	2443	18643	63	240	0.63	2.40
EC	0.9997	0.9947	1283	689714	144	267	1.44	2.67
2-NDPA	0.9999	0.8325	1428	5668	112	225	1.12	2.25
DBP	0.9998	0.9975	1058	155585	170	304	1.70	3.04

Summary: This method permits the accurate identification and confirmation of common smokeless powder components in GSR samples. Trace amounts of each organic compound can also be detected with the developed methods. The LOD ranges for the amount of sample detected by UV and MS/MS are 0.77-4.13 ng and 0.16-29.30 ng, respectively.

4. Final results for UPLC/MS/MS

The information in this section was published in the Journal of Forensic Sciences in May 2013 and a summary is given below (20). For the UPLC separations, a bridged ethyl hybrid (BEH, Waters) analytical column having the following specifications was used: C18, 2.1x100mm, and 1.7-µm particle size. A BEH C8 guard column was also installed to filter samples and protect the analytical column from strongly retained species. The temperature of the system was kept constant at 40ºC and the flow rate was set to 0.5 mL/min. In order to separate the wide range of analytes present in the mixtures, a reverse phase gradient program was utilized in this study (Table 3.14). The separation is presented in Figure 3.11.

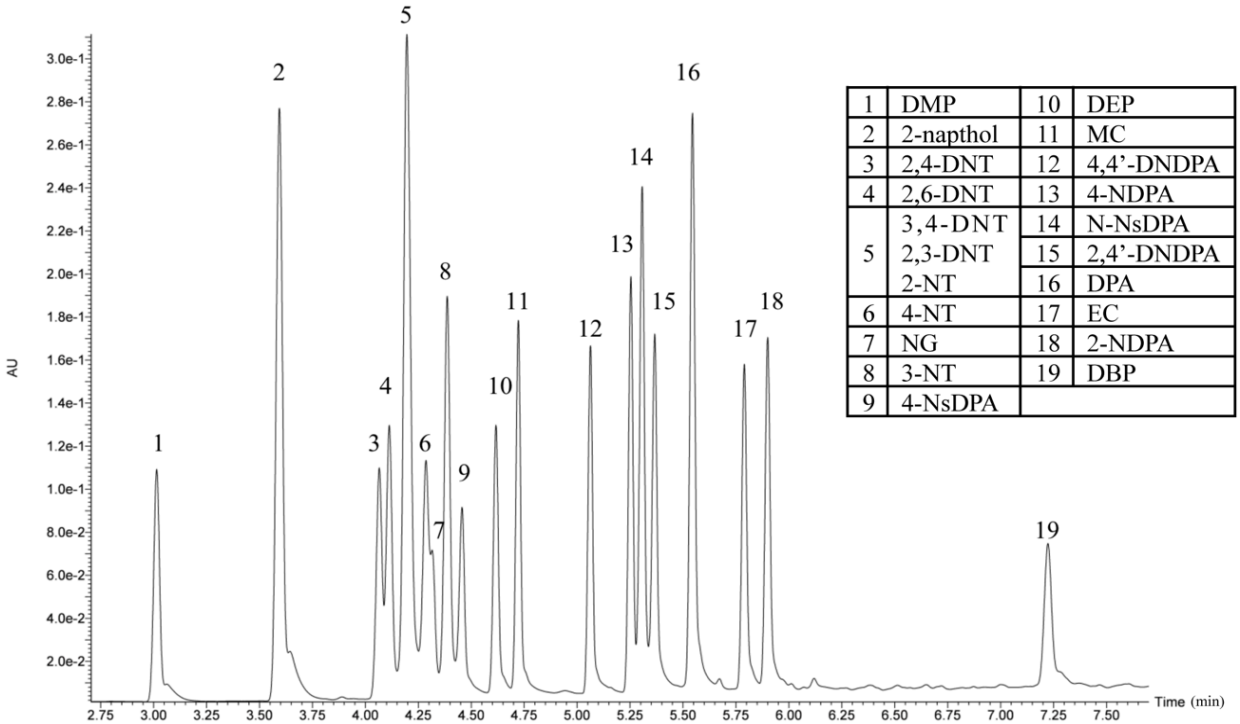
Table 3.14 UPLC gradient program for the separation of smokeless powder additives on a C18 column using an aqueous mobile phase of 90:10 water and acetonitrile and an organic mobile phase of 95:5 acetonitrile and methanol (20).

Time (min)	Solvent A (%)	Solvent B (%)
Initial	100	0
0.50	85	15
0.60	84	16
1.50	82	18
1.75	75	25
3.00	70	30
4.50	50	50
5.50	40	60
8.00	37	63
A: 90% Water + 10% Acetonitrile + 2mM ammonium acetate B: 95% Acetonitrile + 5% Methanol + 2mM ammonium acetate		

The final mixture chosen for the aqueous mobile phase was a 90:10 solution of water and acetonitrile, whereas the organic mobile phase was a 95:5 solution of acetonitrile and methanol. To promote MS ionization of the compounds, 2mM ammonium acetate and 0.2mM ammonium

chloride were added to both mobile phases (10,21). The salt was added to the two mobile phases in order to keep the salt content constant throughout the gradient. The acetate was added to enhance the detection of compounds in positive ion mode such as the diphenylamines. On the other hand, the chloride and NG form a stable adduct that makes it easier to detect NG in negative ESCi® mode. A low amount of chloride was chosen because it was easier to dissolve the salt in both mobile phases and had less of an impact on the ESI signal intensity when compared to higher amounts of salt. The final MS/MS conditions and detection parameters are given in Table 3.15 and Table 3.16, respectively.

Figure 3.11. C18 separation of a 100 µg/mL standard mixture containing 21 compounds (20). Approximately 50 ng of each compound was injected into the system and detected by UV at a wavelength of 210 nm.



1	DMP	10	DEP
2	2-napthol	11	MC
3	2,4-DNT	12	4,4'-DNDPA
4	2,6-DNT	13	4-NDPA
5	3,4-DNT	14	N-NsDPA
	2,3-DNT	15	2,4'-DNDPA
	2-NT	16	DPA
6	4-NT	17	EC
7	NG	18	2-NDPA
8	3-NT	19	DBP
9	4-NsDPA		

Table 3.15. MS/MS conditions for the detection of smokeless powder additives (20).

Condition	Value
Capillary voltage (ESCi+)	3.20 kV
Capillary voltage (ESCi-)	4.30 kV
API current (ESCi-)	20.0 μA
Source temperature	125 ºC
Desolvation temperature	400 ºC
Desolvation gas flow	600 L/hr
Cone gas flow	50 L/hr
Collision pressure on pirani gauge	~$3.6x10^{-3}$ mbar

Table 3.16. Multiple reaction monitoring method used for the detection of the smokeless powder additives by MS/MS (20). The cone voltages (CV) and collision energies (CE) were determined via infusion of each compound directly into the MS.

Compound	Ionization mode†	Precursor ion (m/z)	Product ion 1 (m/z)	CV, CE (Voltage)	Product ion 2 (m/z)	CV, CE (Voltage)
Diphenylamine	ES+	169.94	65.4	34, 40	92.6	34, 22
N-Nitrosodiphenylamine	ES+	198.96	65.7	18, 26	169.0	18, 10
4-Nitrosodiphenylamine	ES+	198.96	127.8	32, 38	181.1	32, 22
2-Nitrodiphenylamine	ES+	214.91	179.9	20, 18	197.0	20, 8
4-Nitrodiphenylamine	ES+	214.91	167.0	28, 34	197.9	28, 12
2,4-Dinitrodiphenylamine	ES+	259.88	167.9	30, 24	242.9	30, 14
4,4'-Dinitrodiphenylamine	ES+	259.94	168.8	22, 36	243.0	22, 12
Dibutyl phthalate	ES+	279.02	148.9	16, 14	204.9	16, 6
Diethyl phthalate	ES+	222.98	64.8	14, 48	148.9	14, 18
Dimethyl phthalate	ES+	194.96	76.7	14, 32	162.9	14, 10
Ethyl centralite	ES+	269.01	119.8	20, 22	147.9	20, 12
Methyl centralite	ES+	240.99	105.8	20, 26	133.9	20, 14
Nitroglycerin	ES-	262.00	45.6	8, 6	61.7	8, 4
2-Nitrotoluene	API-	136.79	46.0	8, 10	---	---
3-Nitrotoluene	API-	136.79	46.0	8, 10	---	---
4-Nitrotoluene	API-	136.79	46.0	8, 10	---	---
2,3-Dinitrotoluene	API-	181.82	46.0	18, 12	152.0	18,12
2,4-Dinitrotoluene	API-	181.82	46.0	18, 12	164.9	20,10
2,6-Dinitrotoluene	API-	181.82	46.0	18, 12	152.2	20,10

3,4-Dinitrotoluene	API-	181.82	46.0	18, 12	152.0	18,12
2-naphthol	ES-	142.85	64.6	44, 24	114.8	44, 24

†ESCi+ capillary voltage = 3.20 kV; ESCi- capillary voltage = 4.30 kV, ESCi- current = 20.00 A

Another validation test was performed to determine the linearity, repeatability, and sensitivity of the developed UPLC/MS/MS method using the conditions described previously in this section. Sample concentrations ranging from 0.2 to 5 ug/mL (2–50 ng injected) were analyzed and the results were used to make calibration curves. To determine the system's linearity, the coefficient of determination (R2), y-intercept, slope, and standard error of the slope were calculated with the data. The limit of detection (three times the standard deviation) and limit of quantitation (10 times the standard deviation) were calculated using the standard deviation of replicate samples at a low concentration and the slope of the calibration curve. UV detection and quantitation limits at 210 nm were as low as 0.08 to 0.5 ng injected, respectively (Table 3.17). MRM detection and quantitation limits were in the low nanogram range (Table 3.18). Low RSD values were obtained for the UV data; however, the MRM peak areas resulted in higher percentages. For example, NG and 4,4'-DNDPA showed RSD values of 43% and 61%, respectively. This may be attributed to thermal decomposition of the compounds in the hot source. The temperature was set high in order to minimize solvent accumulation on the corona pin and increase detection in negative APCI mode. The MRM results also suffer slightly because of the multi-mode ionization technique employed and the scan speed selected to detect all compounds. The MS switches between ESI and APCI modes in order to detect all analytes in a single analysis. This increases sample throughput; however, there may be some loss in reproducibility and sensitivity due to the switching.

Table 3.17 Figures of merit for the detection of smokeless powder additives by UV (n≥3) (20).

Compound	Capacity factor, k'	k' RSD (%)	Area RSD (%)	Linearity				Sensitivity	
				r2	y-intercept	Slope	Standard error of the slope	LOD (ng)	LOQ (ng)
DMP	5.7	0.053	0.78	0.9773	159	775	59	0.33	1.1
2-naphthol	7.0	0.040	0.92	0.9777	457	2154	163	0.39	1.3
2,4-DNT	8.0	0.023	1.7	0.9777	156	691	52	0.76	2.5
2,6-DNT	8.1	0.036	1.6	0.9781	177	778	58	0.70	2.3
2,3-DNT*	8.3	0.043	1.6	0.9768	511	2286	176	0.15	0.5
3,4-DNT*	8.3	0.043	1.6	0.9768	511	2286	176	2.6	8.7
2-NT*	8.3	0.043	1.6	0.9768	511	2286	176	0.08	0.3
4-NT	8.5	0.049	2.1	0.9795	132	617	45	0.89	3.0
NG	8.6	0.069	1.4	0.9762	57	249	19	0.61	2.0
3-NT	8.7	0.055	1.3	0.9769	242	1103	85	0.54	1.8
4-NsDPA	8.9	0.054	0.92	0.9777	89	433	33	0.39	1.3
DEP	9.3	0.048	1.3	0.9784	176	736	55	0.56	1.9
MC	9.5	0.040	3.1	0.9769	233	934	72	1.4	4.5
4,4'-DNDPA	10.2	0.065	1.8	0.9803	194	945	67	0.75	2.5
4-NDPA	10.7	0.039	1.2	0.9779	238	912	69	0.54	1.8
N-NsDPA	10.8	0.030	1.2	0.9783	237	1115	83	0.50	1.7
2,4-DNDPA	10.9	0.029	1.0	0.9787	169	794	59	0.44	1.5
DPA	11.3	0.026	2.3	0.9770	395	1615	124	1.0	3.4
EC	11.9	0.035	1.1	0.9765	213	875	68	0.46	1.5
2-NDPA	12.1	0.020	2.5	0.9767	201	1025	79	1.0	3.5
DBP	15.0	0.042	5.6	0.9537	248	623	69	2.6	8.8

*2,3-DNT, 3,4-DNT, and 2-NT were analyzed individually in order to determine sensitivity.

Table 3.18. Figures of merit for the detection of smokeless powder additives by MS/MS (n≥3) (20).

Compound	Average migration time (min)	Time RSD (%)	Area RSD (%)		Linearity			Sensitivity	
				r2	y-intercept	Slope	Standard error of the slope	LOD (ng)	LOQ (ng)
DMP	3.07	†	7.3	0.9549	699	7,309	790	2.9	10
2-naphthol	3.71	†	3.5	0.9806	750	2,724	190	1.4	4.7
2,4-DNT	4.27	†	11	0.9507	300	1,302	150	5.3	18
2,6-DNT	4.31	†	15	0.9744	198	1,035	84	6.9	23
2,3-DNT	4.28	†	12	0.9806	2,313	468	160	2.4	8.0
3,4-DNT	4.27	†	13	0.9864	2,494	465	150	0.4	1.3
2-NT	4.30	†	25	0.9827	74	-7	5	8.1	27
4-NT	4.29	†	19	0.9538	8	94	10	6.9	23
NG*	4.53	†	61	0.9712	-121	11	1.3	64	210
3-NT	4.30	†	28	0.9975	-6	85	2	9.1	30
4-NsDPA	4.60	†	5.8	0.9478	78,135	96,822	11,000	3.9	13
DEP	4.67	1.8	21	0.8096	384	1,364	330	9.6	32
MC	4.93	†	3.2	0.9843	6,703	24,295	150	1.5	5.1
4,4'-DNDPA*	5.57	†	43	0.8833	-154	42	6.8	17	57
4-NDPA	5.53	†	10	0.9834	163	937	61	4.8	16
N-NsDPA	5.36	4.2	11	0.9876	133	376	21	4.9	16
2,4'-DNDPA	5.45	2.1	22	0.9502	22	64	7	12	41
DPA	5.81	†	25	0.9871	33	140	8	13	44
EC	5.82	†	20	0.9431	8,457	37,285	4600	7.4	25
2-NDPA	6.10	†	16	0.9874	46	234	13	6.7	22
DBP	7.33	†	8.2	0.9801	40,095	5,3181	3800	5.2	17

*NG and 4,4'-DNDPA were analyzed at higher concentrations in order to determine sensitivity and linearity.

† The percent RSD is <0.01 for these compounds.

Summary: With the current UPLC/MS/MS method, a standard mixture of organic compounds commonly found in smokeless powder samples can be separated on a C18 column and detected in less than 8 minutes. Each compound is identified based on several parameters, including

capacity factor, MS migration time, and 1 or 2 MRM transitions. There is some co-elution of the nitrotoluenes in the UPLC chromatogram but these compounds aren't a major concern as they are manufacturing impurities.

B. APPLICATION OF DEVELOPED METHODS TO SMOKELESS POWDERS

1. Analysis of individual smokeless powders

In order to test the applicability of the developed UPLC/MS/MS method to actual smokeless powders, several powders were extracted and analyzed. The following smokeless powder samples were initially investigated: Red dot, IMR 485, Hodgdon H380, and Winchester 288. The powders were extracted with methylene chloride, evaporated, and reconstituted in sample dilutor. For the unburned samples, 250 μL was added to 5 mg of each powder and allowed to sit overnight in the absence of light prior to evaporation (10,13). The burned samples were first prepared by burning a small amount of the powder on a watch glass. Methylene chloride was added to the burned sample and then some of the solution was filtered, evaporated, and reconstituted in sample dilutor (40:60 acetonitrile:water with 6mM ammonium acetate and 0.2mM ammonium chloride). The results for each powder are given below (Figures 3.12-3.15). The top chromatogram is the burned sample, the middle chromatogram is the unburned sample, and the bottom chromatogram is the separated mixture of 20 GSR standards. The peaks are identified by abbreviations; however, there are other unknown peaks which appear in the UV chromatograms that are unlabeled. Further studies must be conducted to determine exactly what these compounds are in the sample. For the smokeless powder samples, compounds in the MS were deemed positive if the signal for that compound was above the one seen in the blank sample.

Figure 3.12. Red dot powder. For the MS results, all five compounds detected in the unburned sample were also detected in the burned sample by the MS. The MS results were inconclusive for 4-NDPA, 4-NsDPA, and DNT.

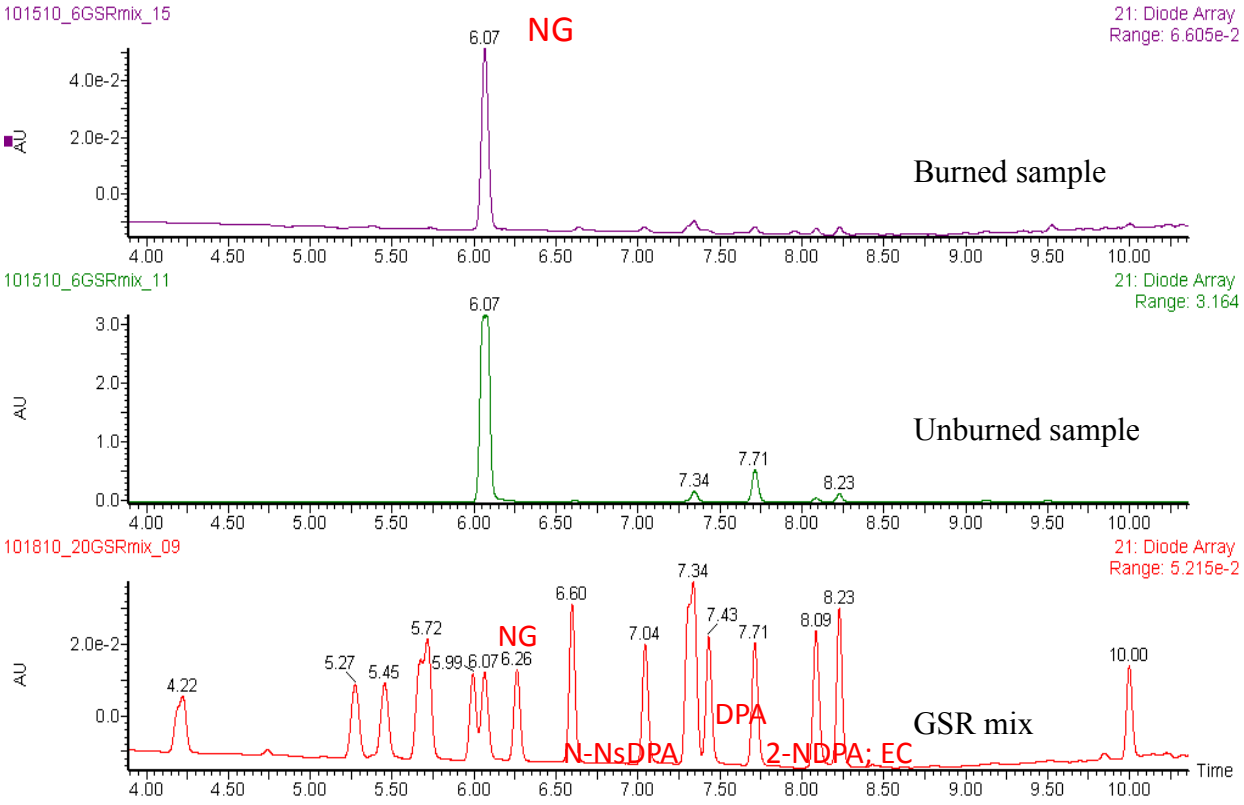


Figure 3.13. IMR 485 sample. For the burned samples, not all of the compounds present in the unburned samples were detected in the burned ones. The burned samples did seem to have 3-NT based on the MS results. Unlike Red dot, it appears that IMR has no NG or EC, allowing the two powders to be distinguished from each other.

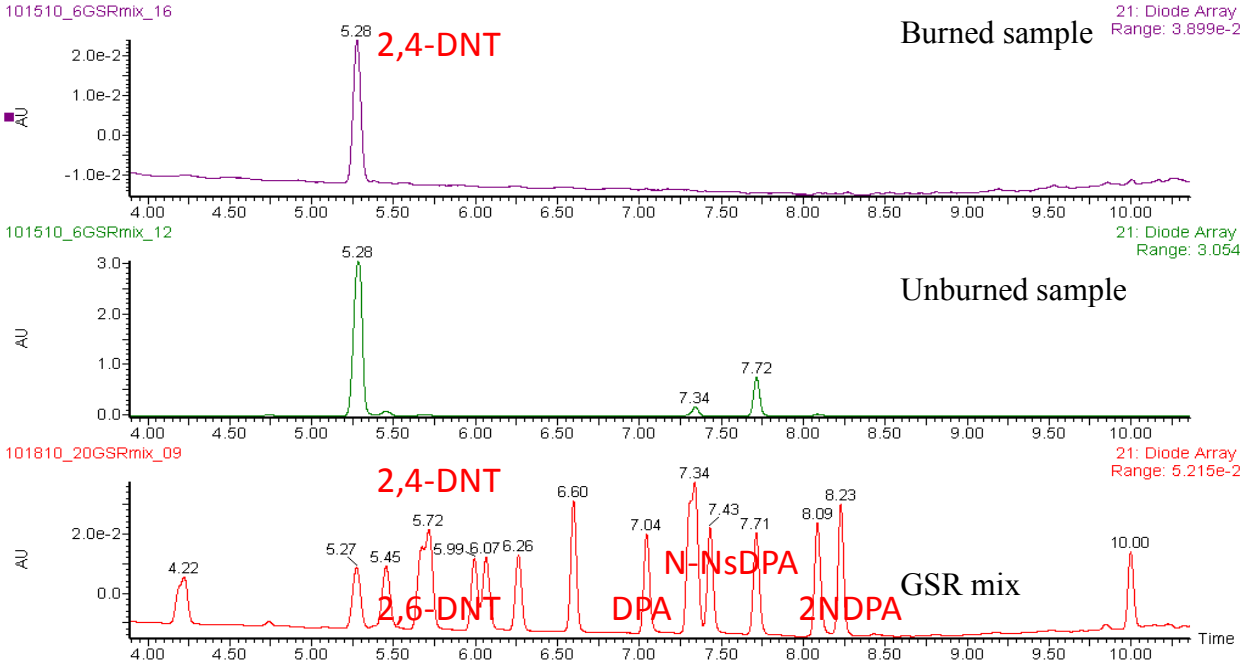


Figure 3.14. Hodgdon H380 sample. For the MS results, all six compounds detected in the unburned sample were also detected in the burned sample by the MS.

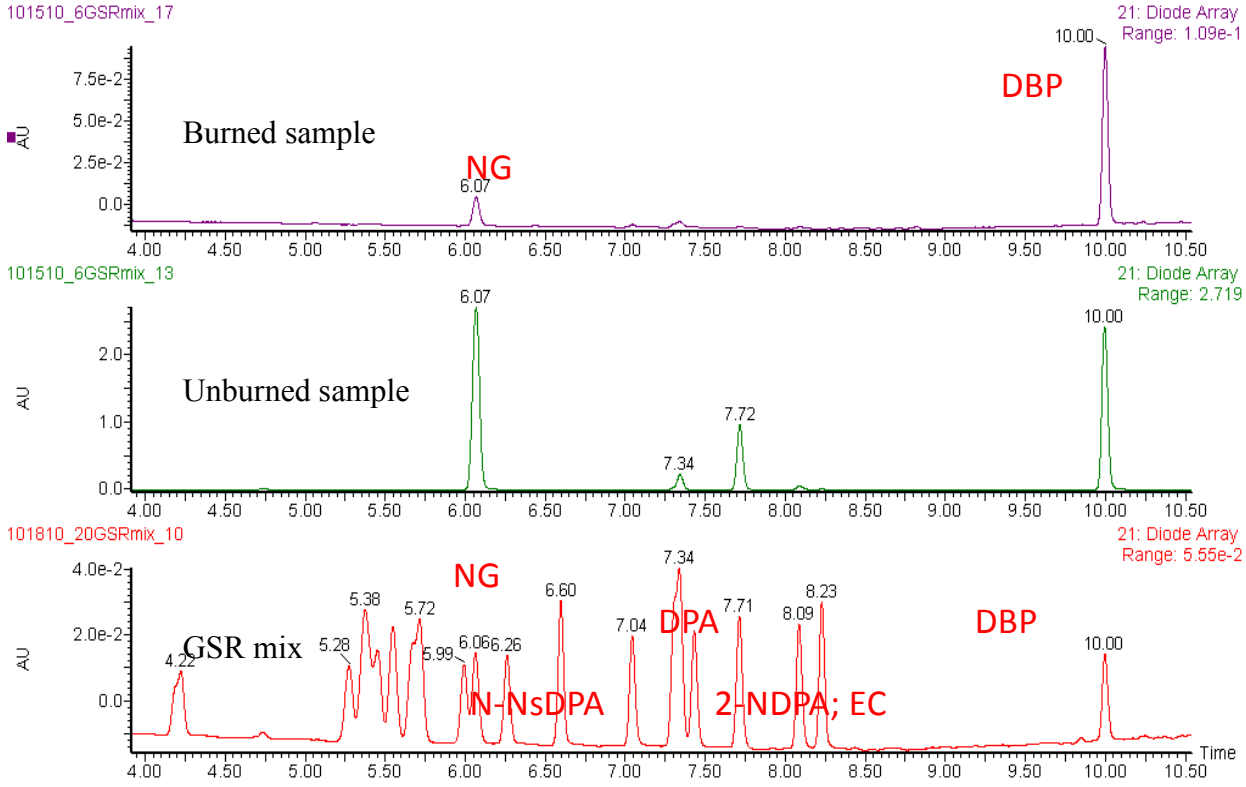
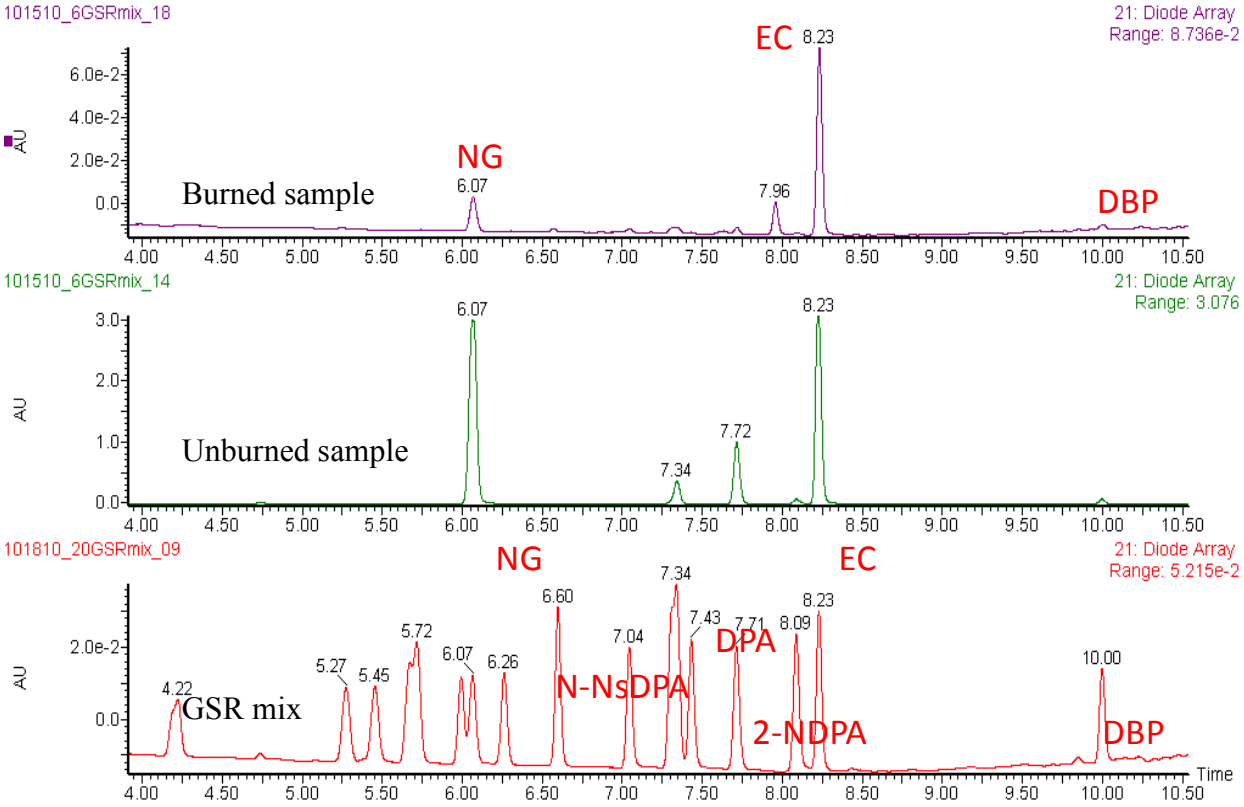


Figure 3.15. Winchester 288 sample. For the MS results, all six compounds detected in the unburned sample were also detected in the burned sample by the MS. The MS results were inconclusive for MC, 4-NDPA, 4-NDPA, and 4,4'-DNDPA.



2. Population Study

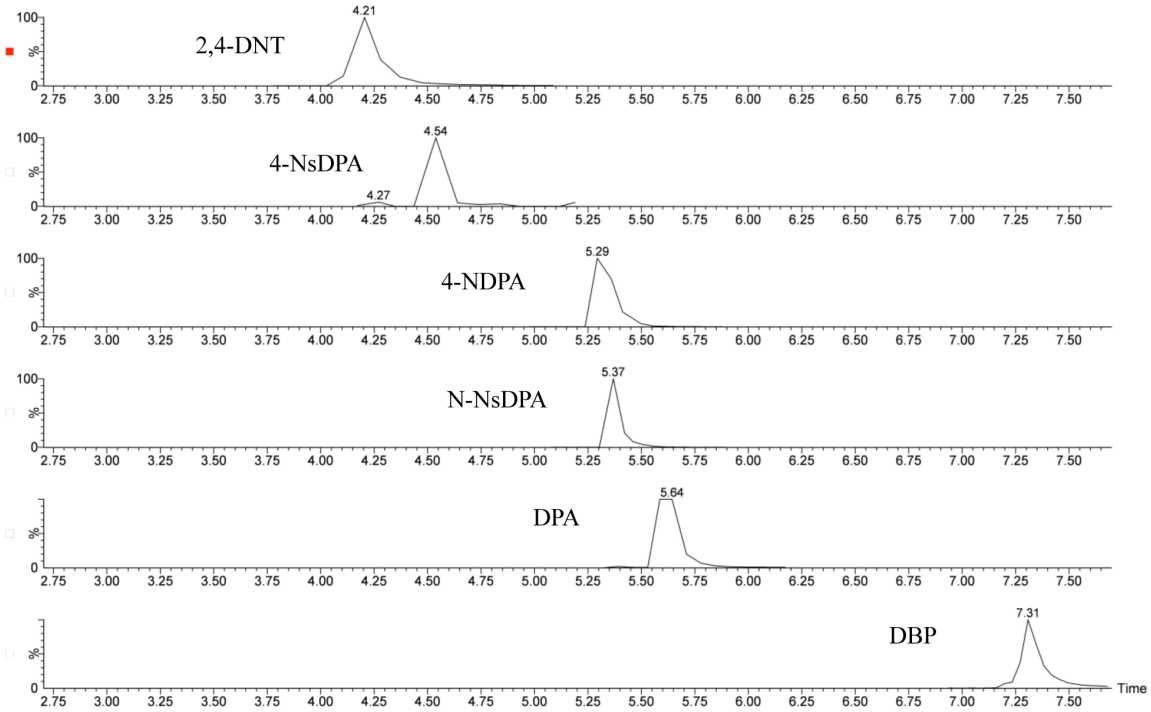
A population study was conducted to look at differences in the additive profile of various unburned smokeless powders. These powders were chosen from different manufacturers and obtained through collaborations with different law enforcement agencies. Both single and double base powders were selected with ages ranging from 0 - 14 years. This study (20) was published in the Journal of Forensic Sciences in May 2013 and the information is repeated below. Both single and double base powders were extracted and analyzed in order to determine the relative

composition of the each component in the powder. This was an important step because it evaluates the practical application of the method to powder analysis.

The extraction procedure is as follows. Five milligrams of the powder was extracted with 250 μL methylene chloride and allowed to sit in the absence of light for 6 hours. A 200 μL aliquot was transferred to a new vial, evaporated with nitrogen gas, and reconstituted in sample dilutor (40:60 acetonitrile:water with 0.6mM ammonium acetate and 0.02mM ammonium chloride). The percent composition was calculated for each smokeless powder sample using the detected UV signal and calibration curves; however, the MS data were used to confirm peak identity.

The MRM chromatograms for one of the smokeless powders – Brand 3 – are given in Figure 3.16. It shows the individual chromatograms for each component detected by the mass spectrometer and the time of detection. The MRM signals are a combination of two product-to-precursor transitions. By monitoring two channels, accurate identification of each component is possible. The last identified peak was dibutyl phthalate and it was detected in less than 8 minutes.

Figure 3.16. MRM chromatograms of smokeless powder additives identified in Brand 3 by UPLC/MS/MS. Each individual chromatogram is the result of two precursor-to-product transitions (Table 3.16).



Based on the presence or absence of specific additives in the powders, we were able to differentiate specific brands. Table 3.19 summarizes the results of the small population study. Numbers and letters designate the brands and lots, respectively. A clear difference is noticeable between Brand 3 and all other powders. Because Brand 3 is a single base powder, nitroglycerin is absent in the sample (Figure 3.16). The energetic in single base powders is only nitrocellulose. All of the other brands are double base powders and therefore, do contain both nitrocellulose and NG. Another major difference between Brand 3 and the other powders is the presence of 2,4-DNT, which is added as a plasticizer or burn rate modifier. After comparing Brands 1 and 5, similarities can be seen in their additive package; however, they can still be differentiated based

on the varying levels of each organic compound present in the powder. On average, Brand 1 has higher levels of 4-NDPA and NG, whereas Brand 5 has a higher concentration of EC and 2-NDPA.

Table 3.19. Percent composition of each organic compound present in different unburned smokeless powders. The powders were extracted with methylene chloride and analyzed by UPLC/MS/MS with a C18 BEH column.

Powder Compound	1a %	1b %	2a %	2b %	2c %	2d %	3a %	3b %	3c %	4a %	4b %
2,4-DNT							4.4*	4.1*	5.6*		
NG	14	12	16	16	16	16				10	9.2
3-NT	0.30†	0.60†									
MC								0.01†			
4-NDPA	0.14	0.12	0.01				0.02	0.02	0.02	0.13†	0.03
N-NsDPA	0.14*	0.03*					0.12	0.10	0.10	0.22	0.26
DPA	0.61*	0.19*	0.04*	0.04*	0.01*		0.56	0.53	0.60	0.74	0.62
EC	0.20*	0.86*	0.91*	1.08*	1.19*	1.20*				0.02	0.02
2-NDPA	0.08*	0.05*		0.30			0.04	0.03	0.04	0.09	0.07
DBP										5.3	4.6
RSD range	13-26	0.7-28	5.3-7.0	1.2-7.1	1.5-17	2.0-2.5	2.3-20	5.9-19	20-26	13-20	5.4-17

* On the basis of ANOVA (3+ lots) and *t*-test (2 lots) results of the means at the 95% confidence level, these lots showed significant differences in composition of specific compounds.

† These compounds have a relative standard deviation (RSD) value that fell in the range of 88-170%.

Significance testing was performed on the means of each lot at a 95% confidence level in order to further characterize the powders. Some variation ($p<0.05$) was seen between all of the lots except for powder 4. The most variation was identified in Powder 1, with significant

differences in percent composition of DPA, 2-NDPA, N-NsDPA, and EC between lots. The probability of having equal concentrations of DPA, EC, and N-NsDPA were 0.001, 0.0004, and 0.001 at the 1% level, respectively. The large difference in N-NsDPA between lots in Powder 1 can be attributed to the higher amounts of DPA present in lot A, as the nitroso product results from the degradation of DPA. For the range of smokeless powders tested, the calculated percentages were also consistent with the ones found in the manufacturer's material safety data sheets. This method is therefore applicable to the analysis of smokeless powder samples recovered in firearm cases and the data can be used to possibly link a shooter to a specific weapon or ammunition.

3. Extraction Method Testing

An extraction study was performed to compare recoveries of other extraction methods. Based on previous results, it was found that the modified Wissinger method (13) provided higher recoveries than the Northrop methanol extraction method (11) Therefore, the Wissinger method was used for the small population study. However, five other tests were performed on two different powders to compare each method again with additional criteria. Five milligrams of the powder was added to a clean vial and each extraction method was tested separately:

(i.) Extract for 6 hours in methylene chloride in the absence of light (13)

(ii.) Extract for 6 hours in methylene chloride in the absence of light with sonication

(iii.) Extract for 6 hours in methanol (MeOH) with sonication

(iv.) Extract for 15 minutes in methanol (MeOH) with sonication (11)

(v.) Extract for 15 minutes in methanol (MeOH) without sonication

The extracts were then transferred to a clean vial, evaporated to dryness using nitrogen gas, and then reconsituted in sample dilutor. The dilutor contained 40% acetonitrile, 60% water, ammonium acetate, and ammonium chloride. 2-napthol was added to all of the extracts as an internal standard. The different methods were compared to determine which one provided higher recoveries (Figures 3.17-3.19).

The results showed that the methylene chloride worked best for extracting the organic compounds over a 6 hour period when compared to methanol (see Figures B.3.1-3). The number on top of the peaks represent the migration time. Listed on the right side of each chromatogram is the following information from top to bottom: ionization mode, name of chemical, and peak itensity. The compounds with the highest intensities for each method are circled in black. The effects of the sonication fluctuated between samples and didn't give a significant improvement in sensitivity; therefore, this added step wasn't included in the extraction method.

Figure 3.17. MS comparison of ethyl centralite. The left side is Winchester powder, whereas the right side is Red dot. The 6hr extraction with methylene chloride improved the signal intensity of EC when compared to the methanol methods.

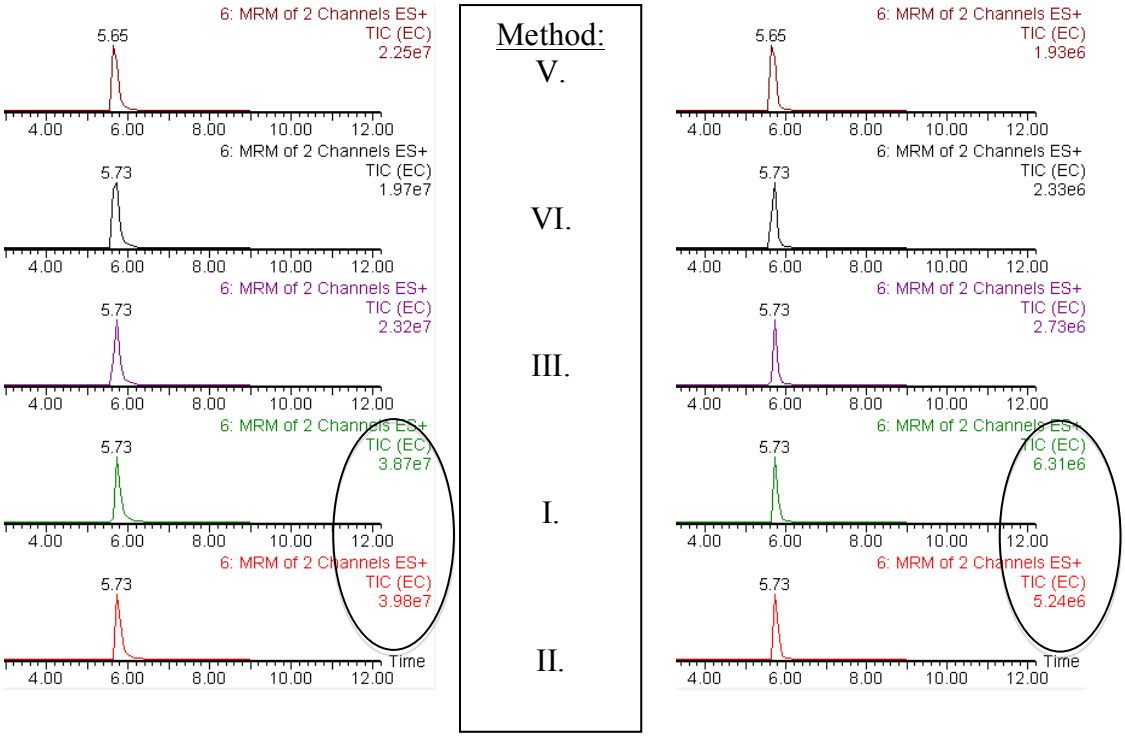


Figure 3.18. MS comparison of diphenylamine. The left side is Winchester powder and the right side is Red dot. The 6hr extraction with methylene chloride tripled the signal intensity of DPA for the Winchester powder.

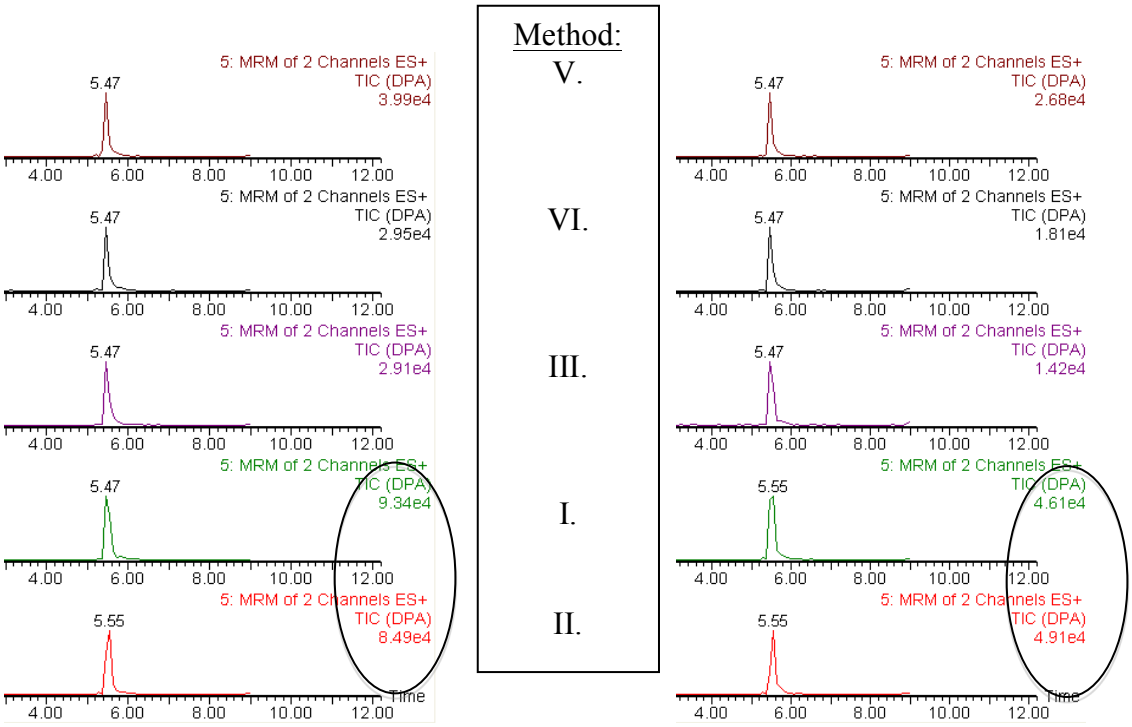
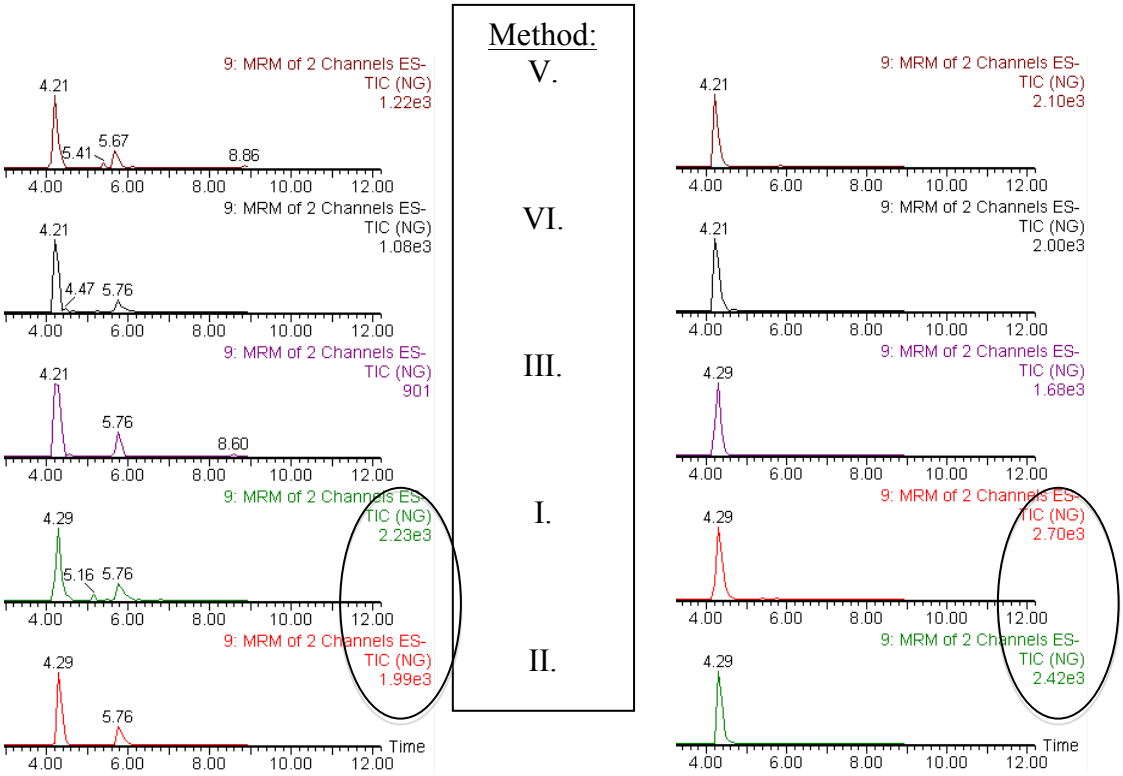


Figure 3.19. MS comparison of nitroglycerin. The left side is Winchester powder, whereas the right side is Red dot. The 6hr extraction with methylene chloride doubled the signal intensity of NG for the Winchester powder.



As previously mentioned, a larger population study of unburned smokeless powders would need to be analyzed in order to fully characterize the method's discriminatory power. The goal would be to create a library of results from various smokeless powders that could be used for comparing gunshot residue samples and defining the class and/or brand of powder used. That study is beyond the scope of this research, but similar data obtained via GC/MS has been published on the SWGFEX web site and has proven to be very useful. The UPLC results obtained in this study are less affected by pyrolysis in the GC and should be more quantitative.

C. DEVELOPMENT OF EXTRACTION PROCEDURES FOR ORGANIC GSR RECOVERY

1. Cotton extractions

The first extraction study was performed using cotton swabs attached to a wooden stick. Six standards were spiked onto the swab, including diphenylamine (DPA), nitroglycerin (NG), ethyl centralite (EC), dibutyl phthalate (DBP), 4-nitrodiphenylamine (4-NDPA), and 2,4-dinitrotoluene (2,4-DNT). The general extraction procedure for the cotton swabs is as follows. The tips of the swab were cut, spiked with different standards, and placed into a 2mL costar® Spin-X HPLC centrifuge tube containing a 0.2 μm Nylon filter. The standards were then extracted twice with acetone, each time for 5 minutes in the centrifuge. The centrifuge speed was 9 revolutions per minute (rpm). The liquids were then transferred to a small sample vial, evaporated to dryness under a stream of nitrogen gas, and then reconstituted in the sample dilutor.

The hand+spike samples – such as those seen in Figure 3.20 - were basically prepared using this same method; however, the hand was swabbed first with the cotton prior to spiking it with the standards. Studies were done using a syringe to filter the cotton samples but the extraction efficiency was much less when compared to the ones extracted with the centrifuge tubes. A study was also performed to test for interferences from the wood attached to the cotton swab and it was determined that it didn't produce any significant interference in the results.

Figure 3.20. Extraction of 6 different GSR standards using cotton swabs. The peaks in the spiked samples are identified in the box above the chromatograms. It can be seen that the hand matrix doesn't produce any major interferences in the UV chromatograms.

2,4-DNT	NG	4-NDPA	DPA	EC	DBP

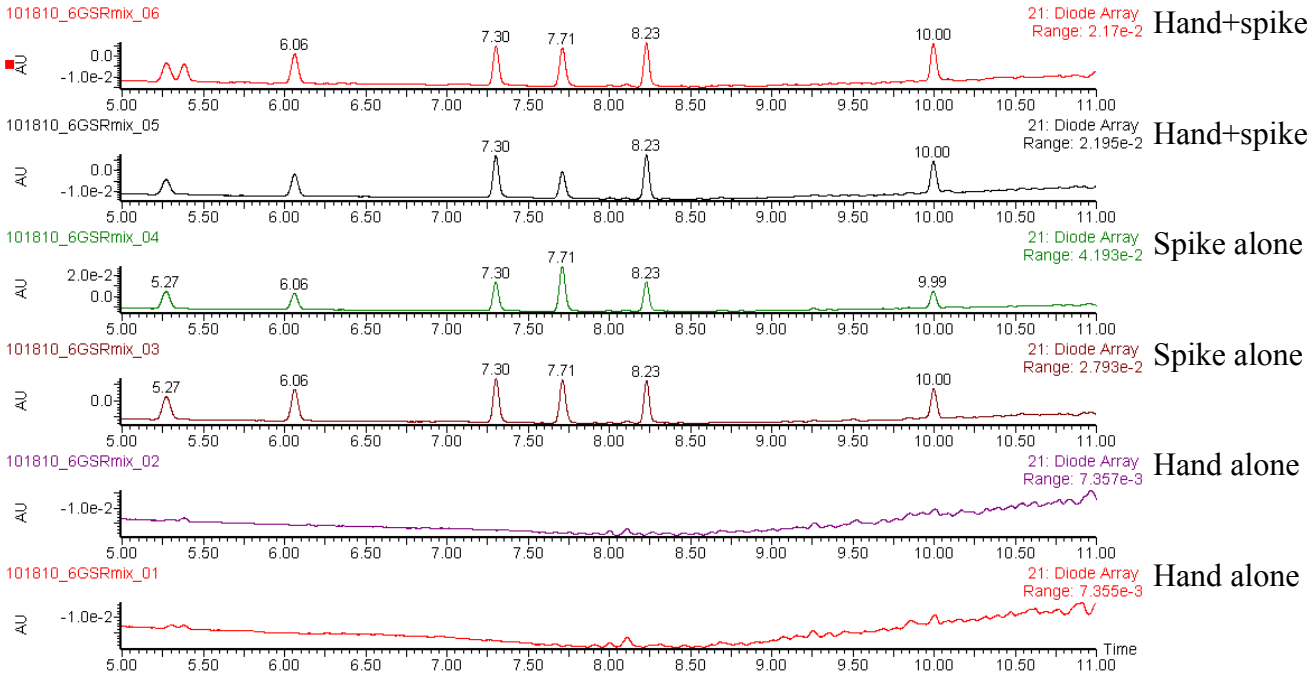
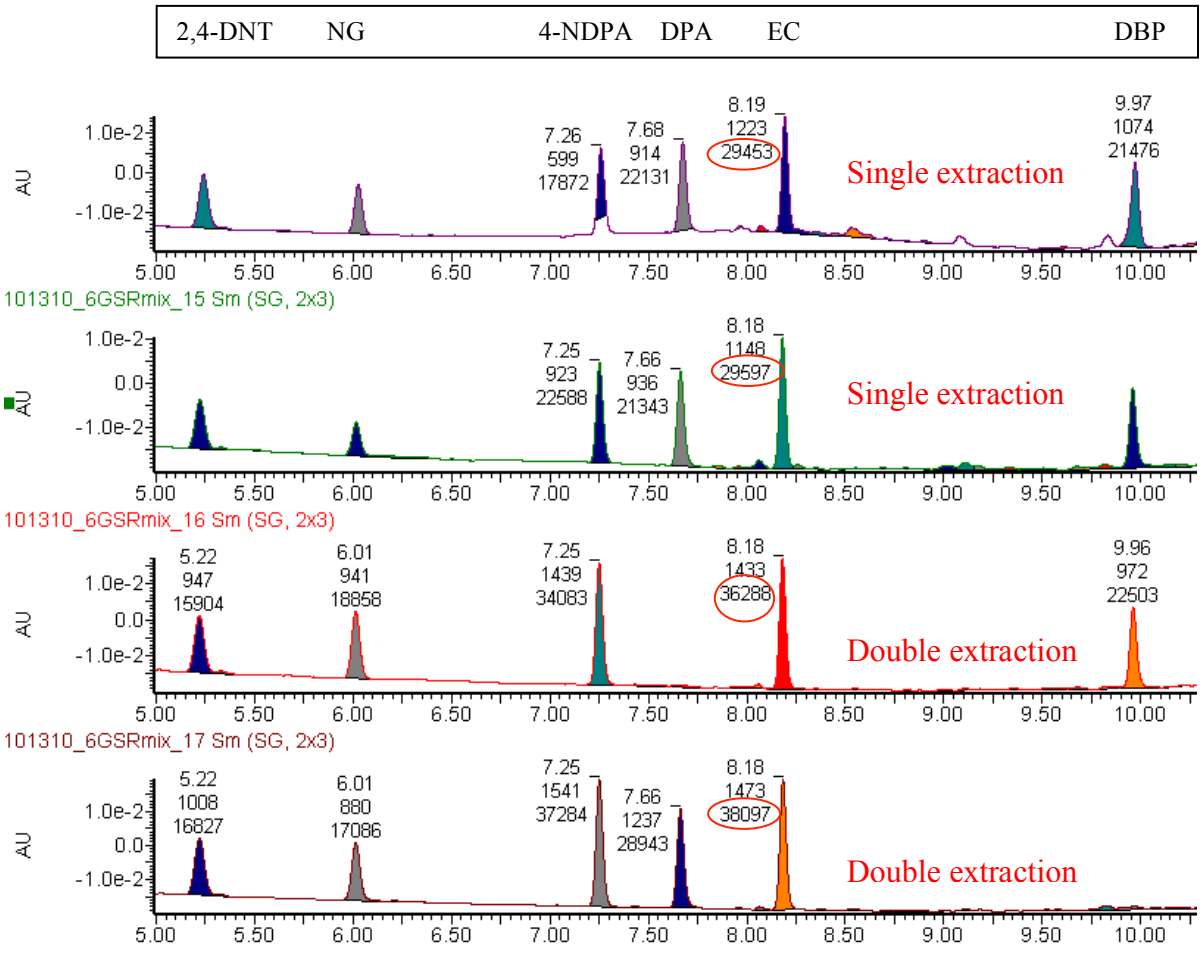


Figure 3.21. This shows the difference in peak area and height of samples extracted using the cotton swabs (see colored circles). The 2nd number near the peak is the peak area, while the third number is the peak height. There is an improvement in the peak area and height when the sample is extracted twice with acetone (double extraction). This was done by centrifuging the sample one time with 500uL of acetone and then adding another 500uL of acetone and centrifuging it again. There are some missing peaks due to errors by the technician preparing the samples; however, the results clearly show that recovery improves with a double extraction.



Another study that was conducted involved adjusting the amount of solvent and centrifuge time used for the extraction (Table 3.20). The general extraction procedure for the cotton swabs is as follows. The tips of the dry swab were cut, spiked with different standards, and placed in a centrifuge tube containing a 0.22 μm Nylon filter. The standards were then extracted with acetone and centrifuged. The liquid was transferred to a small sample vial, evaporated to dryness under a stream of nitrogen gas, and then reconstituted in the sample dilutor. Hand samples were prepared using this same method; however, the hand was swabbed first with the cotton or aluminum stub prior to spiking it with the standards. A full explanation of what the stubs are and how they are extracted are given in Part C, Section 2 of the extraction methods.

Table 3.20. Different extraction procedures tested for recovering organic GSR standards.

Collection device	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Cotton	500μL acetone	Centrifuge 5 min	----	-----
Cotton	500μL acetone	Centrifuge 5 min	500 μL acetone	Centrifuge 5 min
Cotton	500μL acetone	Centrifuge 2.5 min	500 μL acetone	Centrifuge 2.5 min
Cotton	1000μL acetone	Let sit in syringe	Filter	-----
Stub	5000μL acetone	Sonicate for 5 min	Filter with syringe	-----

Overall, the cotton swabs provided higher recoveries than the stub samples. The stubs showed a consistent recovery of only about 50%. Therefore, the cotton swabs were the preferred

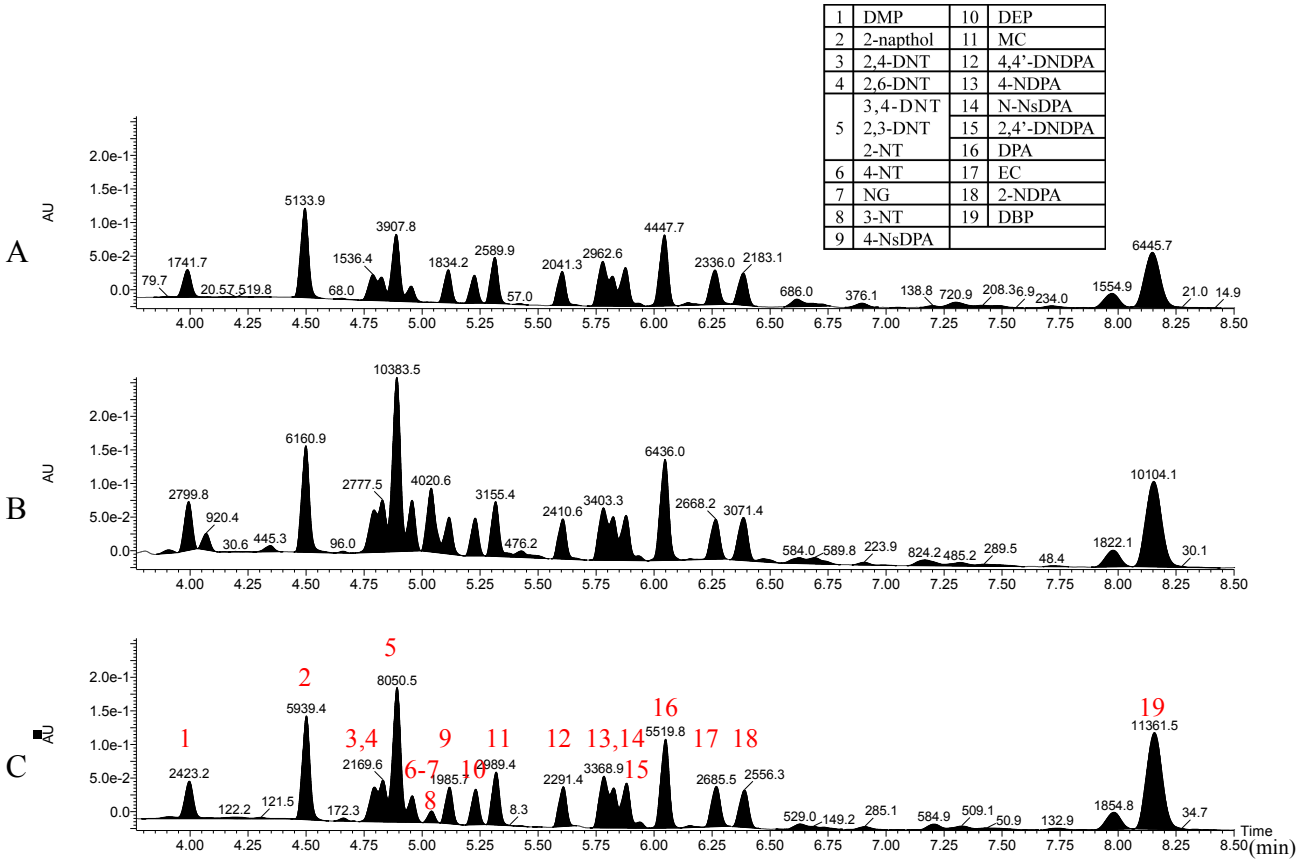
device for GSR collection. The swabs are also easier to work with when compared to the stubs. The syringe procedure for the cotton swabs was ruled out because it is tedious and there is more deviation when recovering the organic compounds. We chose to use the first method listed in Table C.1.1 as our general cotton extraction procedure because it provided similar results as the other swab methods and it was the easiest technique.

Due to an initial lack of recovery in live-fire residue samples, another test was performed to determine if recovery could be improved by adjusting the extraction solvent. Three different solvents were tested: acetone, acetonitrile, and a 75:25 mixture of isopropanol and water. A mixture of water and ethanol and acetonitrile were used by Detata et al. for extracting organic explosives including NT, DNT, and NG (22). Acetone was initially chosen for previous studies based on its popularity as an extraction solvent for explosive compounds. The general extraction procedure consisted of swabbing the hand with a cotton swab and then spiking the swab with 25uL of the standard mixture of organic GSR compounds. Following this, the cotton tips were cut and placed into a 2mL centrifuge tube. To each tube, 500uL of the extraction solvent was added and then centrifuged for 5 minutes. The extract was then transferred to a clean amber vial, dried under nitrogen gas, and reconstituted in a 60:40 mixture of water and acetonitrile with ammonium acetate and ammonium chloride. Higher recoveries were seen with acetonitrile for most of the compounds in the mixture (see Table 3.21 and Figure 3.22).

Table 3.21. Average UV peak areas and standard deviations for each extraction solvent (red highlights show highest average recovery for each compound.

Chemical	Acetone		Acetonitrile		Isopropanol:Water	
		stdev		stdev		stdev
DMP	2174	300	2778	19	1721	69
2-napthol	5686	351	6309	304	5175	131
2,4-DNT	1840	231	2466	11	1397	125
2,6-DNT	1983	281	2748	412	982	162
3,4-DNT;2,3-DNT;2-NT	6766	1727	10215	908	3691	205
4-NT	1245	185	2409	292	3062	3856
NG	686	675	2549	1385	7	4
3-NT	1946	45	2065	69	1881	57
4-NsDPA	1854	177	2143	100	1523	30
DEP	2857	215	3152	177	2628	83
MC	2233	79	2489	167	37	20
4,4-DNDPA	3212	140	3580	159	2058	51
4-NDPA	1724	236	2642	636	3008	69
N-NSDPA	2506	294	2847	231	1313	82
2,4-DNDPA	120	170	154	90	2467	26
DPA	5224	477	6388	292	4135	276
EC	2580	182	2762	182	2343	53
2-NDPA	2473	192	2876	189	2195	52
DBP	1793	92	1852	51	1574	39

Figure 3.22 UV chromatograms of three different samples: (A) isopropanol:water extraction, (B) acetonitrile extraction and (C) acetone extraction. Each swab was spiked with 25uL of a 1mg/mL standard mixture. The response areas are given above each peak.



2. Stub extractions

The aluminum stubs used in this study were purchased from Tri-Tech Forensics©. An example of the stub is given in Figure 3.23. It is covered with black double-sided adhesive carbon tape, which helps to grab the particles off of the hands of the shooter. Traditionally, these stubs are used for collecting inorganic GSR and are analyzed using scanning electron microscopy with energy dispersive X-ray spectroscopy. In this study, we are examining them to

be used for collecting organic GSR. It may be possible to collect residues and perform both inorganic and organic analyses on the sample in cases of false negatives due to lead-free primers.

Figure 3.23. SEM aluminum stub used for sample collection.

For the stub samples, a double extraction procedure was found in the literature for trace explosives (23). First, the stub was spiked with standards and extracted with a mixture of 80% water (with 0.1% azide) and 20% ethanol using sonication. Following this, a liquid/liquid extraction was performed using the solution from part one and methylene chloride. This however did not work when we attempted to extract the six standards. Figure 3.24 shows the results from this study. We suspected that the sample needed more organic solvent for the compounds to be extracted from the carbon tape on the stub. Because the acetone worked well for the cotton swabs, a similar procedure was examined for use with the aluminum stubs.

Because the stubs were too big to fit directly in the centrifuge tube, they were placed tape side down after being spiked in a beaker of 5 mL acetone. The beaker was then sonicated for 10 minutes and the liquid was filtered using a syringe and 0.2 µm Anotop filters. Similar to the

cotton swabs, the extracted sample was transferred to a small vial, evaporated to dryness, and then reconstituted in the sample dilutor. The results for the extraction studies are given below. Using this new technique, all six standards were extracted. The results can be seen in Figure 3.25. Further studies must be conducted to improve extraction efficiency (extract twice or use another solvent) and decrease the background noise (likely from the stub).

Figure 3.24 Unsuccessful extraction of six standards using the aluminum stubs and double extraction method.

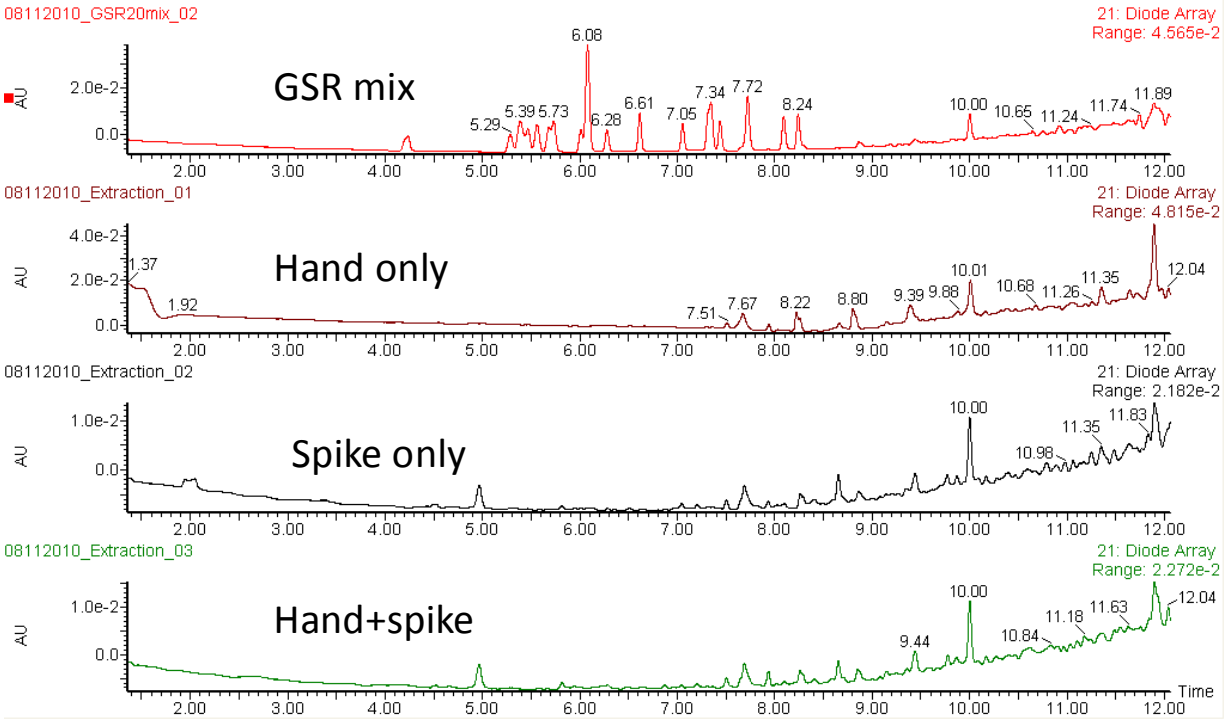
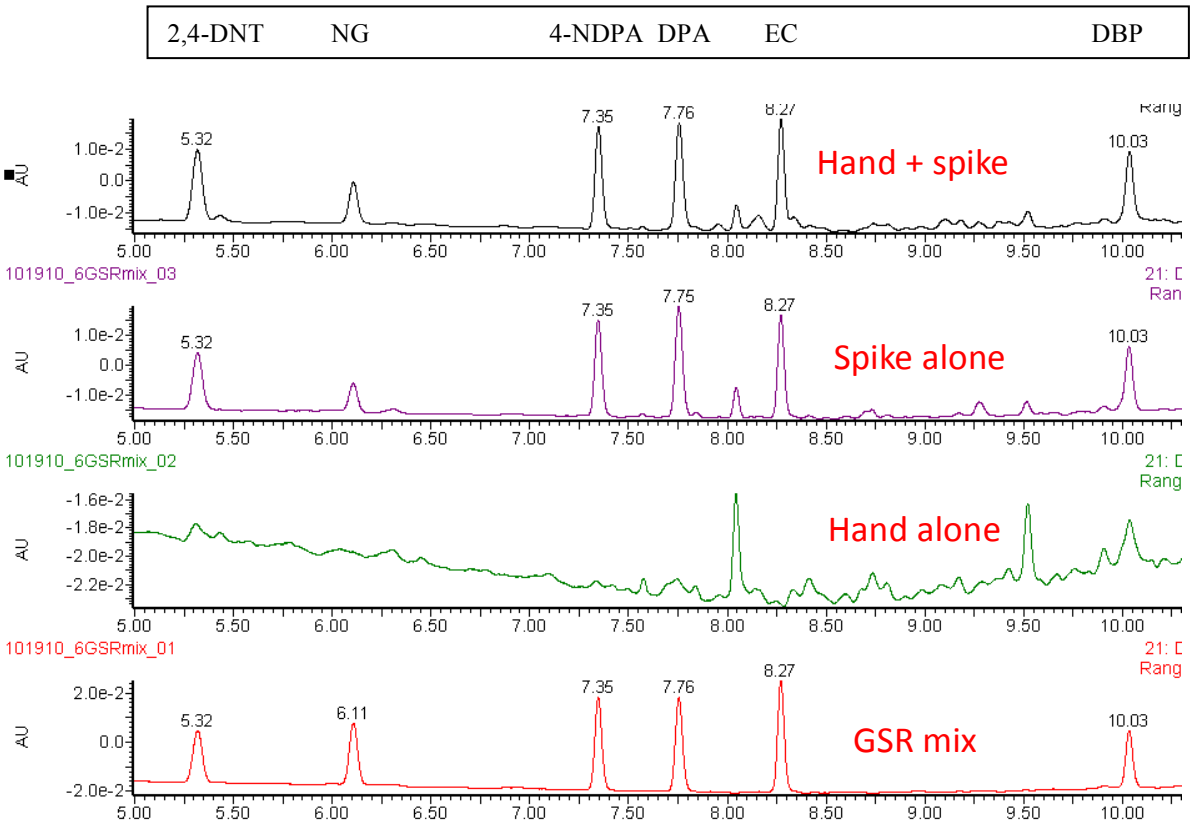


Figure 3.25 Successful extraction of six standards using the aluminum stubs and acetone. There are some background peaks that appear to be from the stub. It may be useful to use a different filter or extraction solvent to decrease matrix effects.



D. APPLICATION OF DEVELOPED METHODS TO GUNSHOT RESIDUE ANALYSIS

1. Sample Collection Overview

The collection of live-fire residue samples was made possible through collaboration with the Miami Dade Police Department crime laboratory, specifically the Firearms Division. They provided the indoor range, firearms, ammunition, and personnel used for the firing studies. Below is the general procedure used for sample collection:

1. The shooter will wash their hands and a blank hand sample will be collected
2. The shooter will fire a weapon and another hand sample will be collected
3. The shooter will wash their hands and a blank sample will be collected.
4. The shooter will wash their hands between different firing rounds and blanks will be taken prior to shooting and after each hand washing.
5. Spent casings and ammunition powder will also be collected

On December 1, 2010, a visit was taken to the crime laboratory at the Miami Dade Police Department. During this visit, a station was set up outside of the MDPD's firing range for sample collection and tested an initial set of swabs and stubs for collection of residue. Eighty samples were collected from 2 MDPD personnel that were certified to fire a weapon. Samples were collected from the shooter's hands, focusing on the following places: left palm, right palm, left back, right back, and fingers. These are some of the same areas that the MDPD swab when collecting inorganic GSR samples. A summary of the information obtained during this visit is given in Table 3.22. It highlights the type of weapons, ammunition, and collection devices that were used in this study. This visit to the crime lab allowed us to fine tune our sampling process

and collect preliminary samples for testing developed methods. Other proposed weapons and ammunition are highlighted in Tables 3.23 and 3.24.

Table 3.22. Types of weapons and ammunition used for organic GSR sample study.

Person	Weapon	Ammunition	Collection device	Samples collected	Total samples
A	Smith & Wesson	Remington 357	Cotton swabs	3 firing samples 4 blanks	28
	Smith & Wesson	Remington 357	Aluminum stubs	1 firing sample 2 blanks	12
B	Colt	Remington 357	Cotton swabs	3 firing samples 4 blanks	28
	Colt	Remington 357	Aluminum stubs	1 firing sample 2 blanks	12

Table 3.23. Types of weapons.

Type of Weapon	A	B	C
0.45 ACP	Glock (20)	Colt 1911 (20)	Smith&Wesson (20)
9mm	Glock (20)	Colt (20)	Smith&Wesson (20)
22mm	Colt pocket (10)	Beretta 21A (10)	Ruger Mark 1 (10)
22 revolver	ROHM	-	-
Rifle	AK 47 type (20)	M16 223 Remington (20)	-
Shotgun	Mossberg 12 gauge (10)	-	-

Table 3.24. Types of ammunition.

Ammunition	A	B
0.45	Federal	Winchester
9mm	Blazer	American Eagle
22mm	Stinger	-
Rifle	Federal	Wolf
Shotgun	Federal 12 gauge 2 buck shot	-

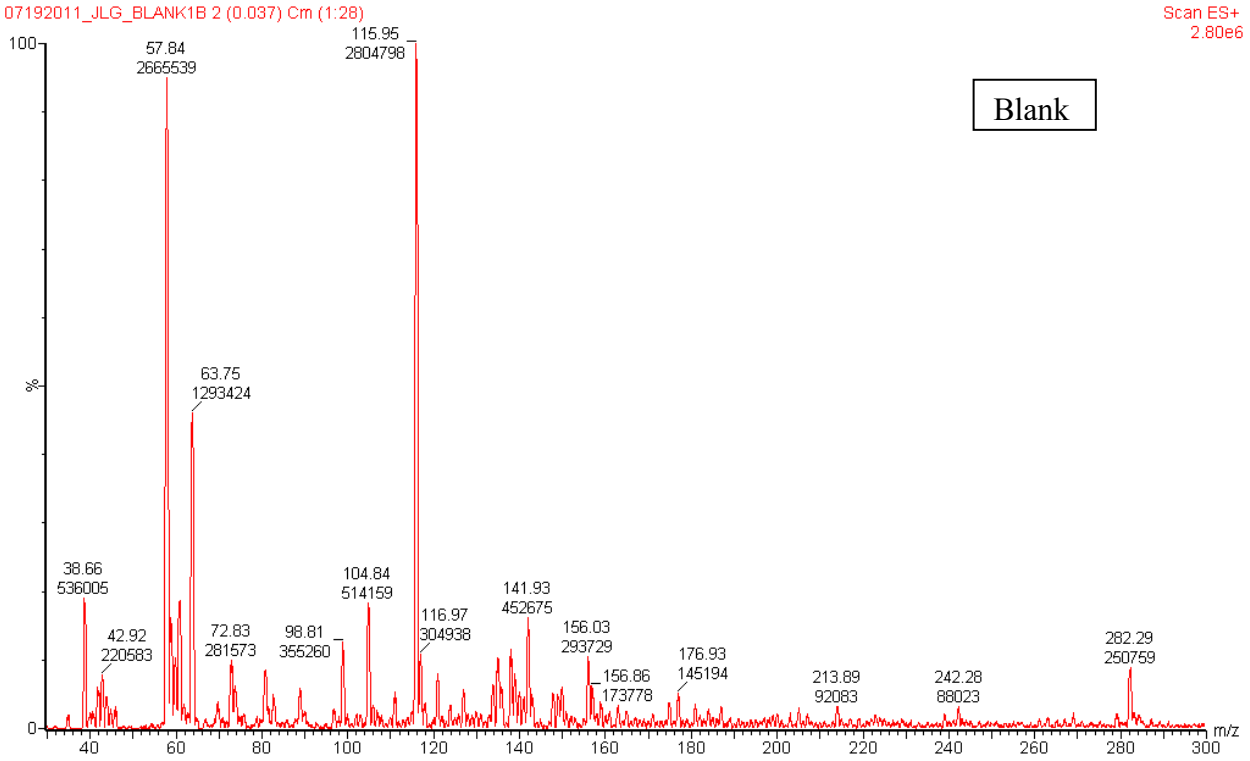
2. Infusion of Standard Mixtures to Test the System's Ability to be used as Screening Tool

It would be very useful to have a rapid method whereby GSR samples could be quickly screened for organic compounds. One method to do this would be to solubilize the extract and directly infuse it into the tandem mass spectrometer. While the sample is being infused into the MS, a spectrum can be collected for 0.5 min in each ionization mode: ESI+, ESI-, and APCI-. This must be done because the tandem mass spectrometer doesn't permit users to perform an MS scan in ESCi mode (all 3 ionization modes ran simultaneously) directly from the tune page. Nevertheless, it takes less than 10 minutes to acquire a spectrum of a sample in each ionization mode, extract the data, and search for the compounds of interest. This includes running a blank of sample dilutor before each run in all 3 ionization modes. The general MS conditions used for the infusion experiments are summarized in Table 3.25. The only difference between each ionization mode was the voltage applied on the capillary or corona discharge pin (APCI). The extracted spectrum for a standard GSR mixture analyzed in ESI+, ESI-, and APCI- modes are given in Figure 3.26, 3.27, and 3.28, respectively. The MS scans were collected over an m/z range of 30 to 300 amu.

Table 3.25 MS conditions for the infusion experiments.

Condition	Value	Condition	Value
ESI capillary voltage (+)	3.20	High mass resolution	15
ESI capillary voltage (-)	4.30	Ion energy	0.6
APCI current (-)	20.0	Entrance	50
Extractor	3 V	Collision	3
RF lens	0.1 V	Exit	50
Source temperature	125 ºC	LM resolution 2	14
Desolvation temperature	225 ºC	HM resolution 2	14
Desolvation gas flow	425 L/hr	Ion energy 2	0.6
Cone gas flow	50 L/hr	Multiplier	650
Low mass resolution	15	Collision pirani	~3.6x10^3

Figure 3.26. MS scan in positive ESI mode. The parent ions for 2,4- and 4,4'-dinitrodiphenylamine are not as pronounced as the other compounds. It is believed that this is a concentration issue, as a more defined peak is visible in the spectrum when each standard is run alone.



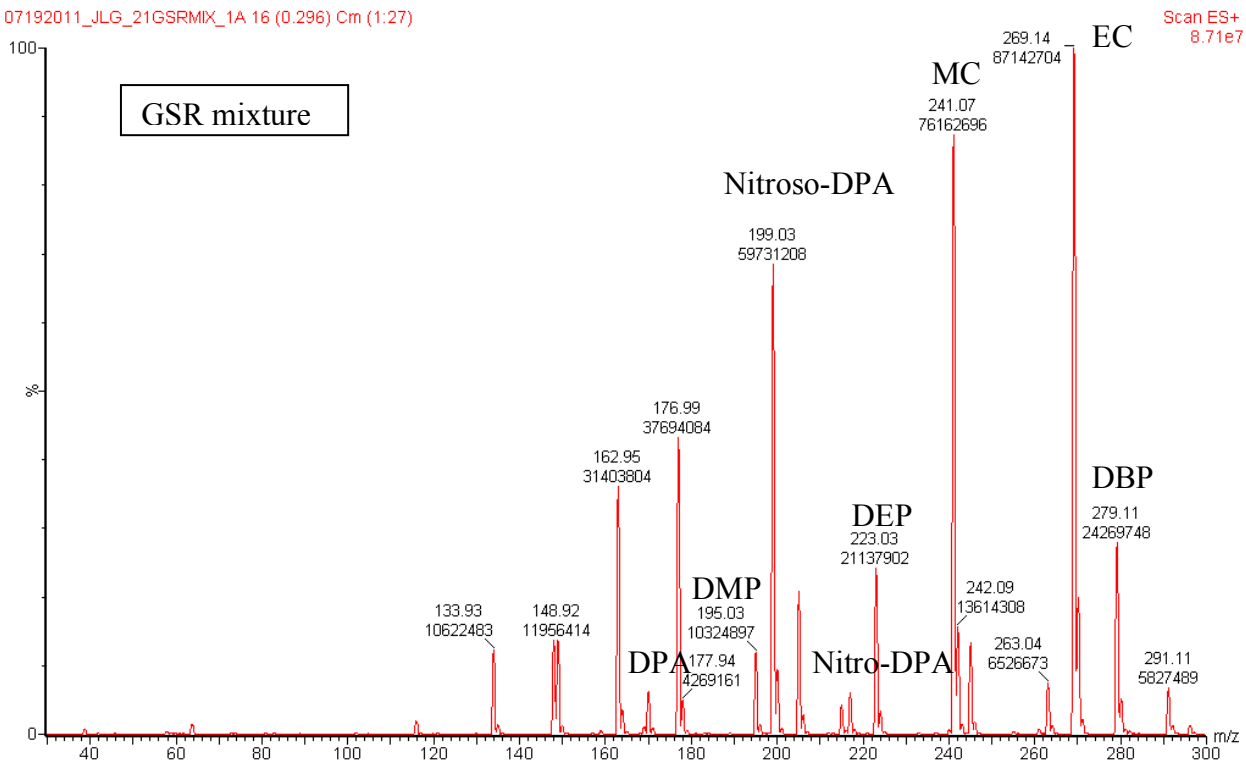
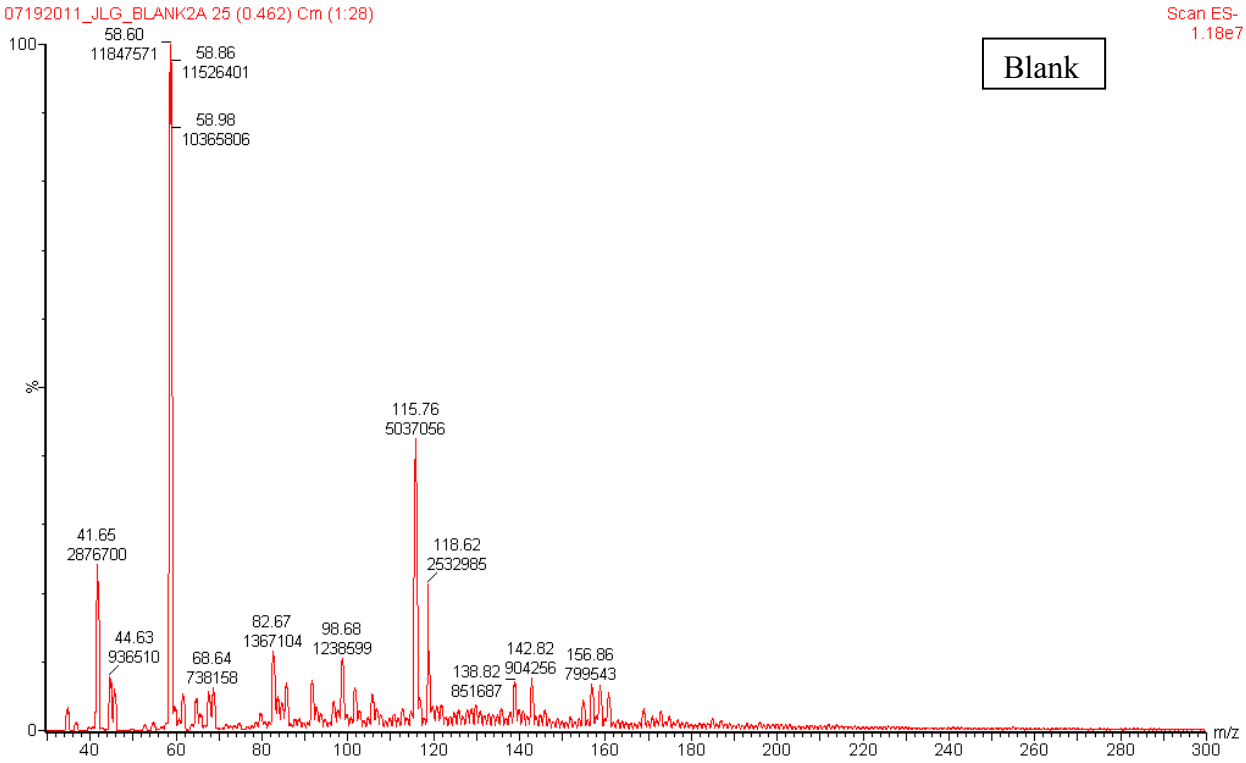


Figure 3.27. MS scan in negative ESI mode. For nitroglycerin, we can see the chloride adduct at m/z 262 and the two fragments used in the MRM method: m/z 46 peak (NO_2) and m/z 62 (NO_3). For 2-naphthol, we can see the parent ion at m/z 143 and one of its fragments at m/z 65.



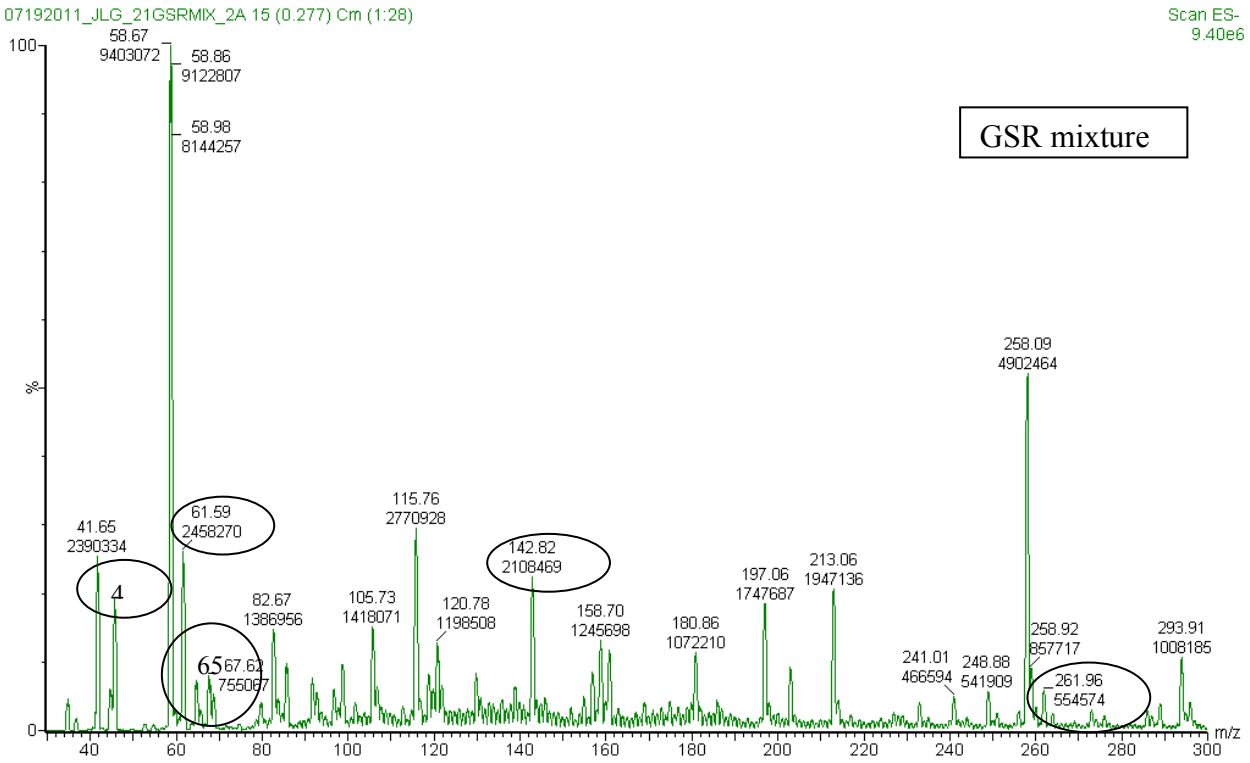
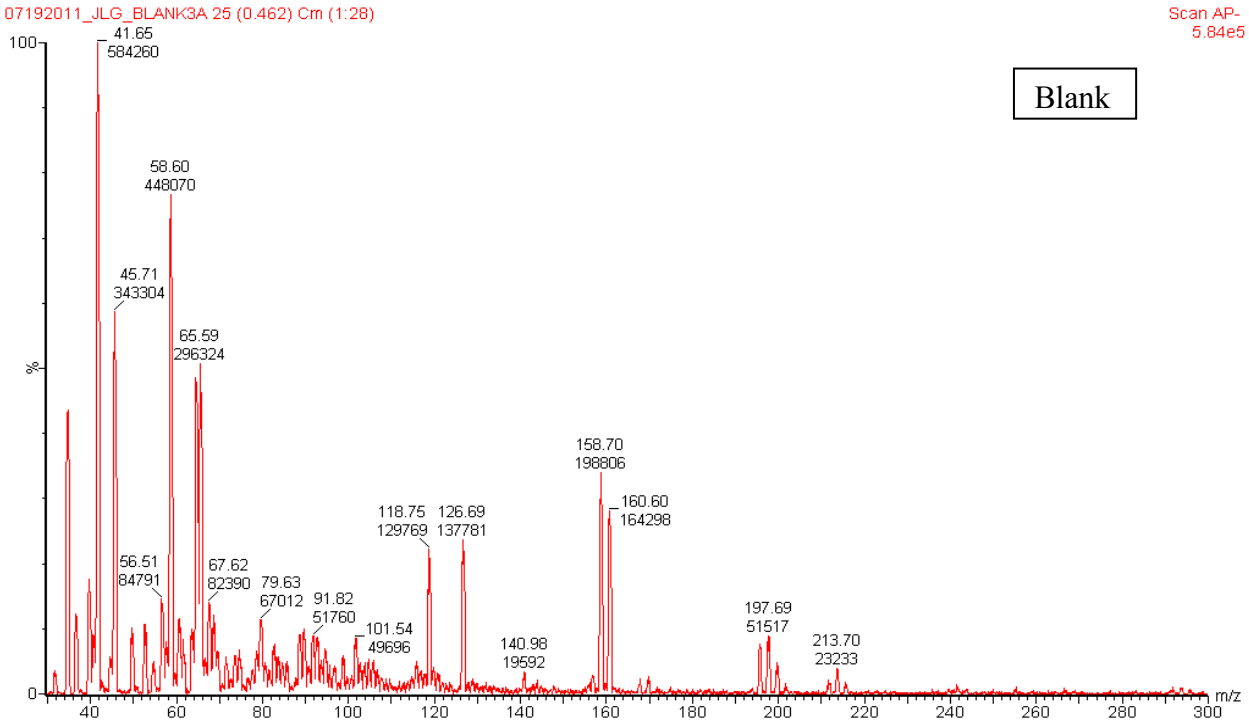
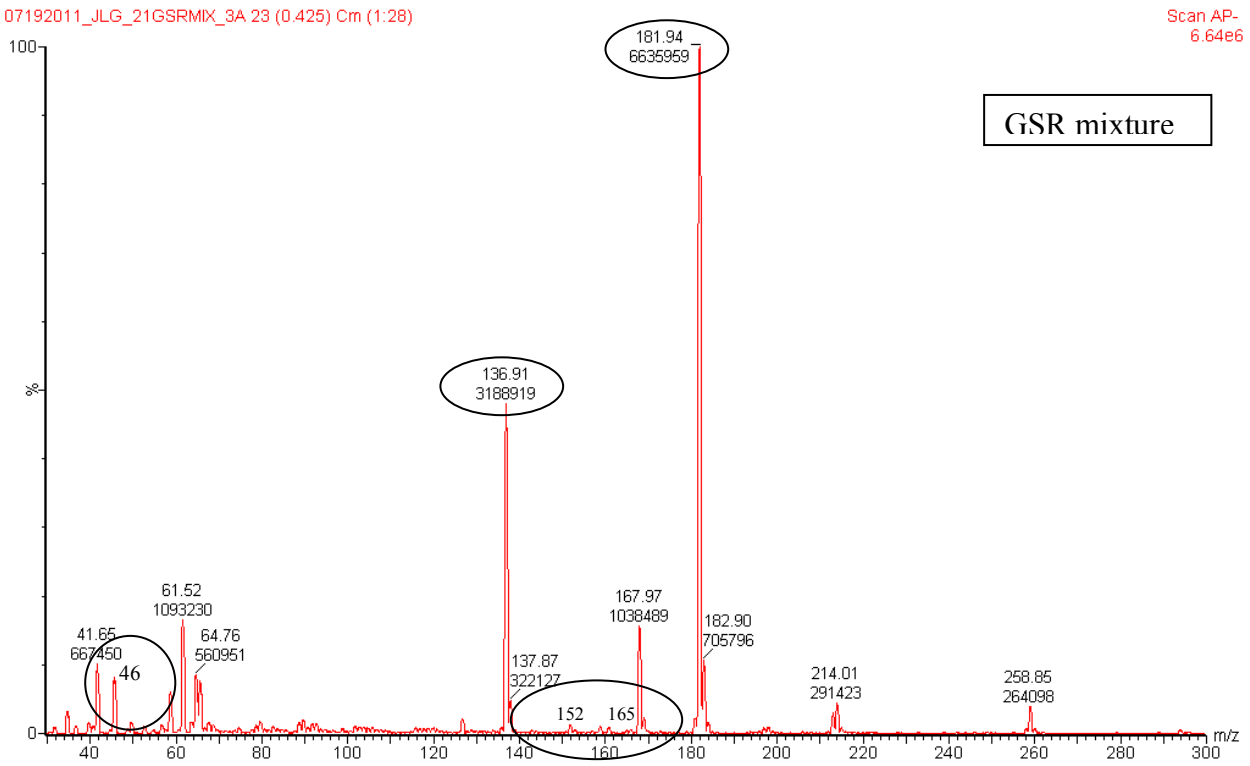


Figure 3.28. MS scan in negative APCI mode. In the spectrum, we see the parent ion of nitrotoluene at m/z 137, the parent ion of dinitrotoluenes at m/z 182, and the common daughter ion of them both at m/z 46 peak, which is indicative of NO_2. We also identified other ions used detected for dinitrotoluenes (circled in spectrum; m/z 46, m/z 152, m/z 165).





The next step was to apply the infusion method to the analysis of hand samples. The tape samples were chosen for this study because compounds have been detected by UPLC on the tape months after collection. These samples were refrigerated to minimize decomposition. The hand samples were collected after shooting by swabbing the fronts and backs of the shooter's hand. For the samples below, three shots were fired and then a hand sample was collected for gunshot residue. Blank samples were also obtained prior to shooting and after hand washing.

The organic compounds on the tape samples were extracted with methanol in a 2mL amber vial. After 15 minutes, the tape was removed from the vial and the liquid was dried under a stream of nitrogen gas. The dried extract was then reconstituted in sample dilutor and analyzed via direct infusion into the mass spectrometer. To increase MS detection of the extracted compounds, lower temperatures and gas flows were used (Table 3.26). A 30 second run was

performed in three ionization modes: ESI+, ESI-, and APCI-. Each spectrum was then examined for the parent compound of the smokeless powder additive. The results can be seen in Figures 3.29-3.32.

Table 3.26. MS conditions for infusion experiments of hand samples.

Condition	Value	Condition	Value
ESI capillary voltage (+)	3.20	Low mass resolution	15
ESI capillary voltage (-)	4.30	High mass resolution	15
APCI current (-)	20.0	Ion energy	0.6
Extractor	3 V	Entrance	50
RF lens	0.1 V	Collision	3
Source temperature	125 ºC	Exit	50
Desolvation temperature	225 ºC	LM resolution 2	14
Desolvation gas flow	425 L/hr	HM resolution 2	14
Cone gas flow	50 L/hr	Ion energy 2	0.6
Collision pirani	~$3.6x10^{-3}$ mbar	Multiplier	650

Figure 3.29. MS scan in positive ESI mode of GSR standard mixture. The cone voltage was set to 15. All of the parent ions were visible for those compounds that ionize in ESI+ mode.

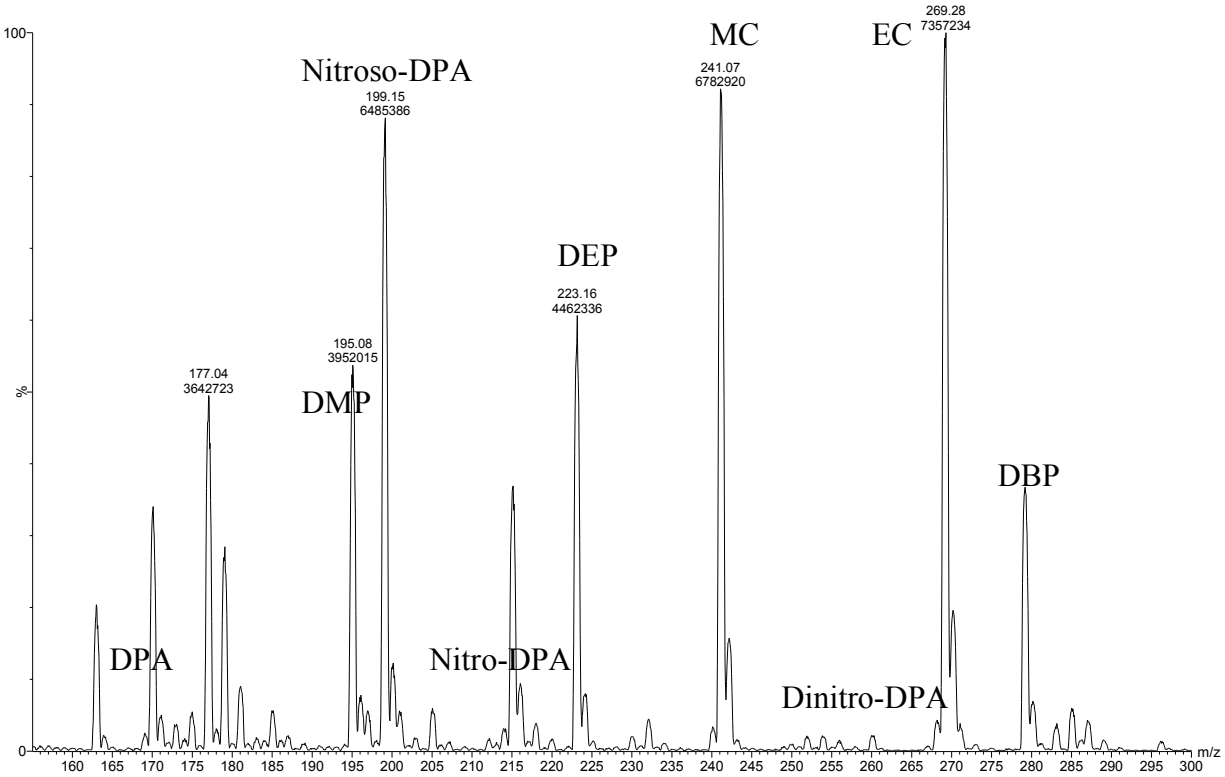


Figure 3.30. MS scan in negative ESI mode of GSR standard mixture. A cone voltage of 10 was used for this experiment. For nitroglycerin, we can see the chlorine adduct at m/z 262 and the two fragments used in the MRM method: m/z 46 peak (NO_2) and m/z 62 (NO_3). For 2-naphthol, we can see the parent ion at m/z 143 and its fragments at m/z 65 and m/z 115.

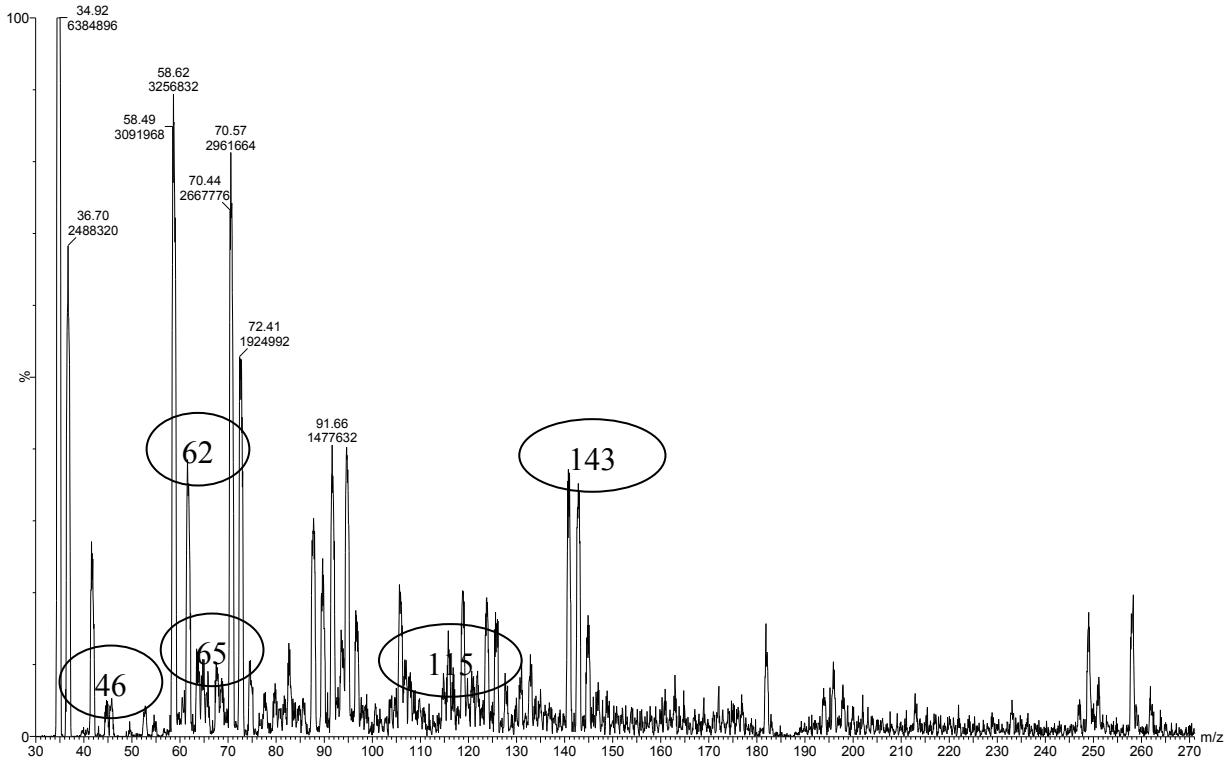


Figure 3.31. MS scan in negative APCI mode of GSR standard mixture. A cone voltage of 15 was used for this experiment. In the spectrum, we see the parent ion of nitrotoluene at m/z 137, the parent ion of dinitrotoluenes at m/z 182, and the common daughter ion of them both at m/z 46 peak, which is indicative of NO_2. We also see some of the ions used for the dinitrotoluenes in the MRM method, such as m/z 152.

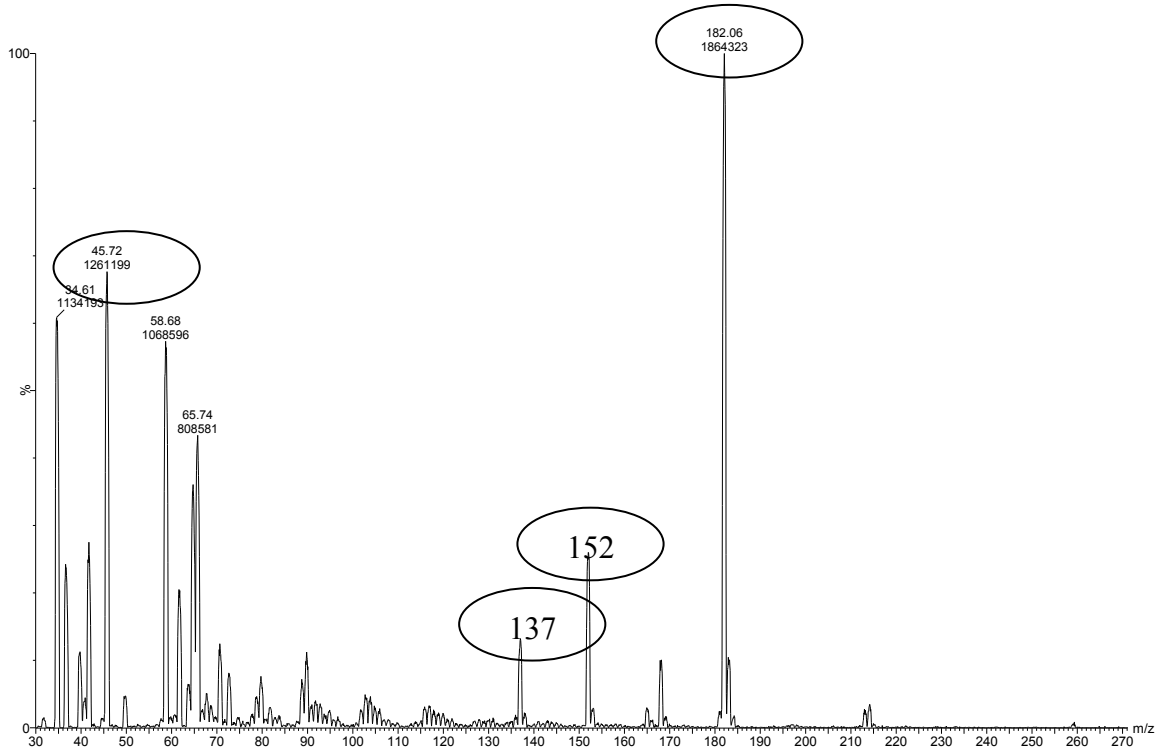


Figure D.2.7: MS scan in positive ESI mode of a firing sample collected using tape. No peak at m/z 170 (DPA) was seen in the spectrum. However, possible reaction product peaks for Nitroso-DPA (m/z 199) and Nitro-DPA (m/z 215) are present. Peaks are also visible in the spectrum at the same masses as EC (m/z 269) and DBP (m/z 279). Fragmentation must be done on this sample to determine if these parent peaks are actually smokeless powder additives.

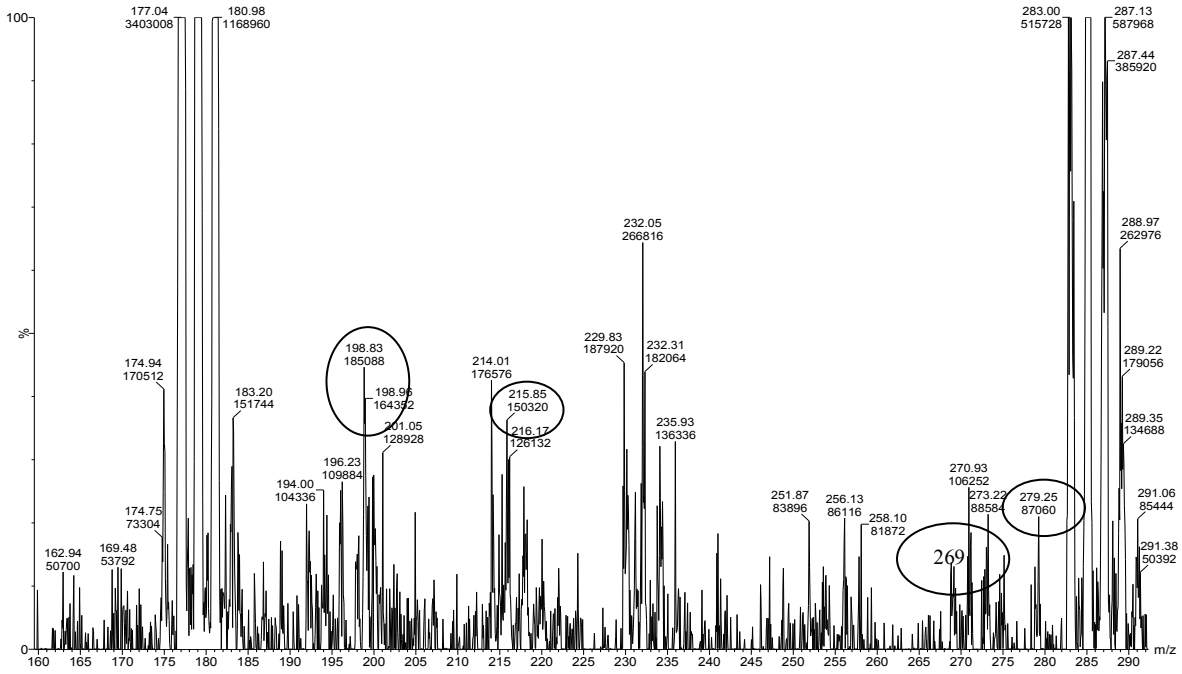
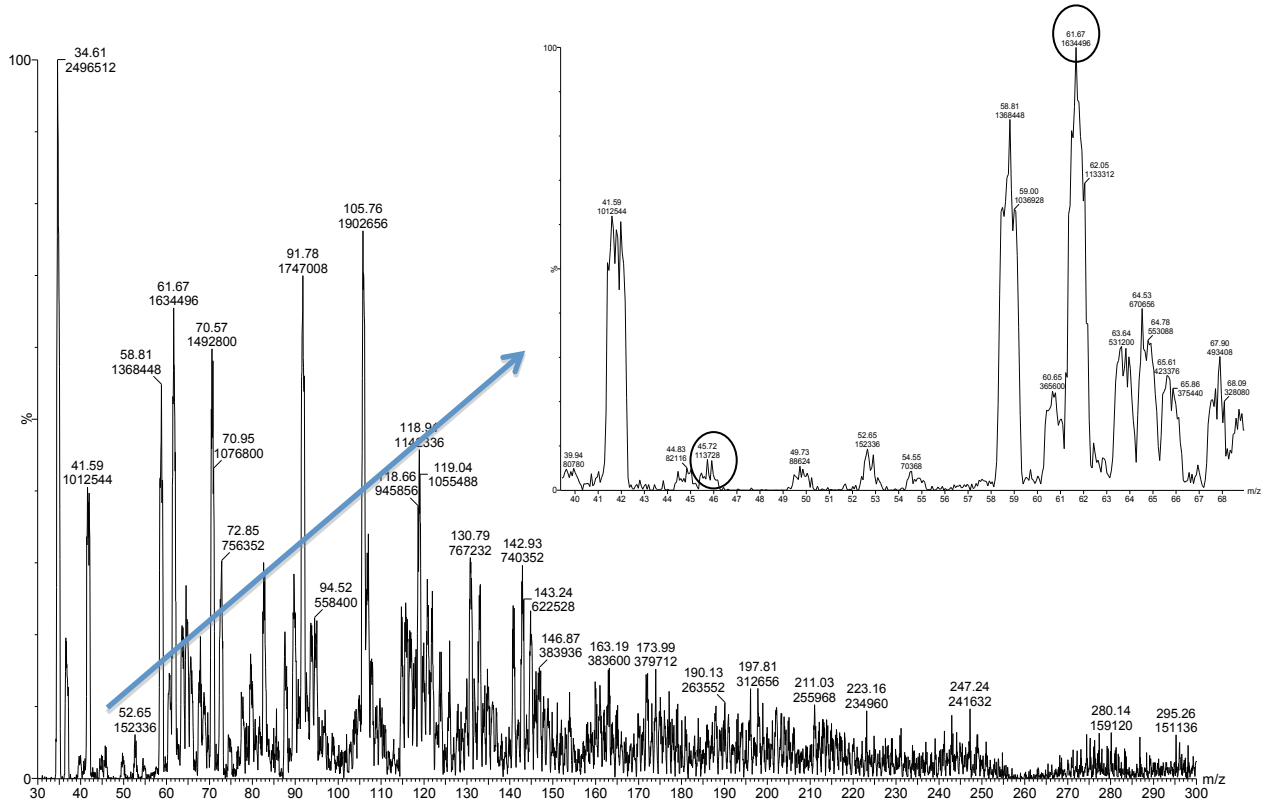


Figure 3.32. MS scan in negative ESI mode of the same firing sample collected using tape. The parent ion of nitroglycerin cannot be seen in the chromatogram. Peaks at m/z 46 and 62 may be due to the fragmentation of NG. The peak at m/z 143 is the internal standard added to the sample.



Summary: Based on the results from the tape samples, it appears that the organic GSR is poorly detected by this technique. This may be due to very dilute samples, higher source temperatures, or problems with the extraction technique. Further samples must be tested in order to determine the feasibility of direct MS infusion for rapid screening.

3. Analysis of live-fire residue samples by UPLC/MS/MS - cotton

Below are results from live-fire residue samples that were collected using cotton swabs. Samples were collected from live firing exercises using a Smith and Wesson revolver with Remington ammunition and cotton swabs. The cotton was wet with a 75:25 solution of isopropanol and water. The tips of the swab were cut and placed in a centrifuge tube containing a 0.22 μm Nylon filter following collection. The organic compounds were then extracted with 500 uL of acetone and centrifuged for 5 minutes. The liquid was transferred to a small sample vial, evaporated to dryness under a stream of nitrogen gas, and then reconstituted in the sample dilutor.

Figure 3.33 gives three different UV chromatograms for the cotton swabs. By comparing the samples collected after shooting to the standard GSR mixture, we can identify different organic GSR compounds present. Slight variations in retention time were observed to a problem with pump seals. However the application of mass spectrometry confirms the presence of the compounds. Background peaks from the cotton swab and hand can also be seen in the samples but do not interfere with peak identification. The firing sample (Sample A) was positive for nitroglycerin, N-nitrosodiphenylamine, ethyl centralite, and dibutyl phthalate (Figure 3.34). The N-NsDPA only exhibited one MRM transition due to low amounts present in the sample.

Figure 3.33 Comparison of hand samples collected with cotton swabs. The UV chromatograms are for (A) Sample collected after firing, (B) Blank cotton swab, and (C) Standard GSR mixture.

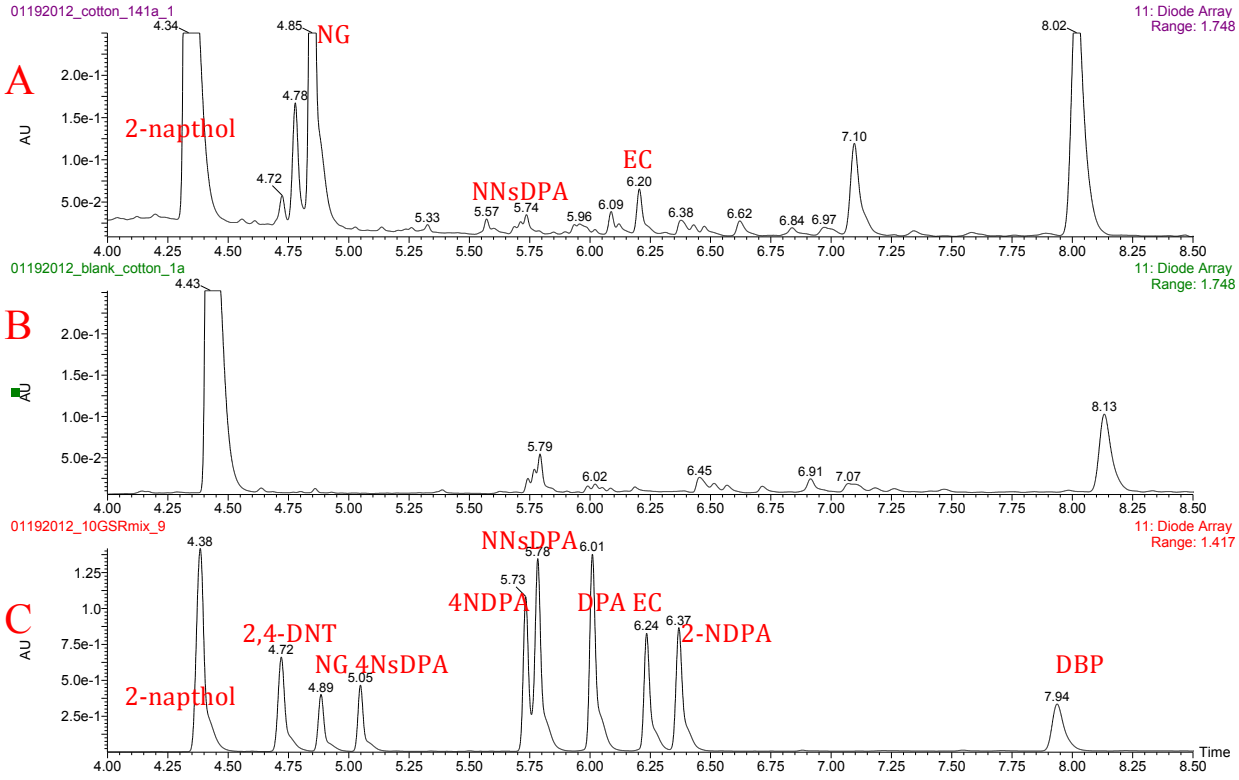
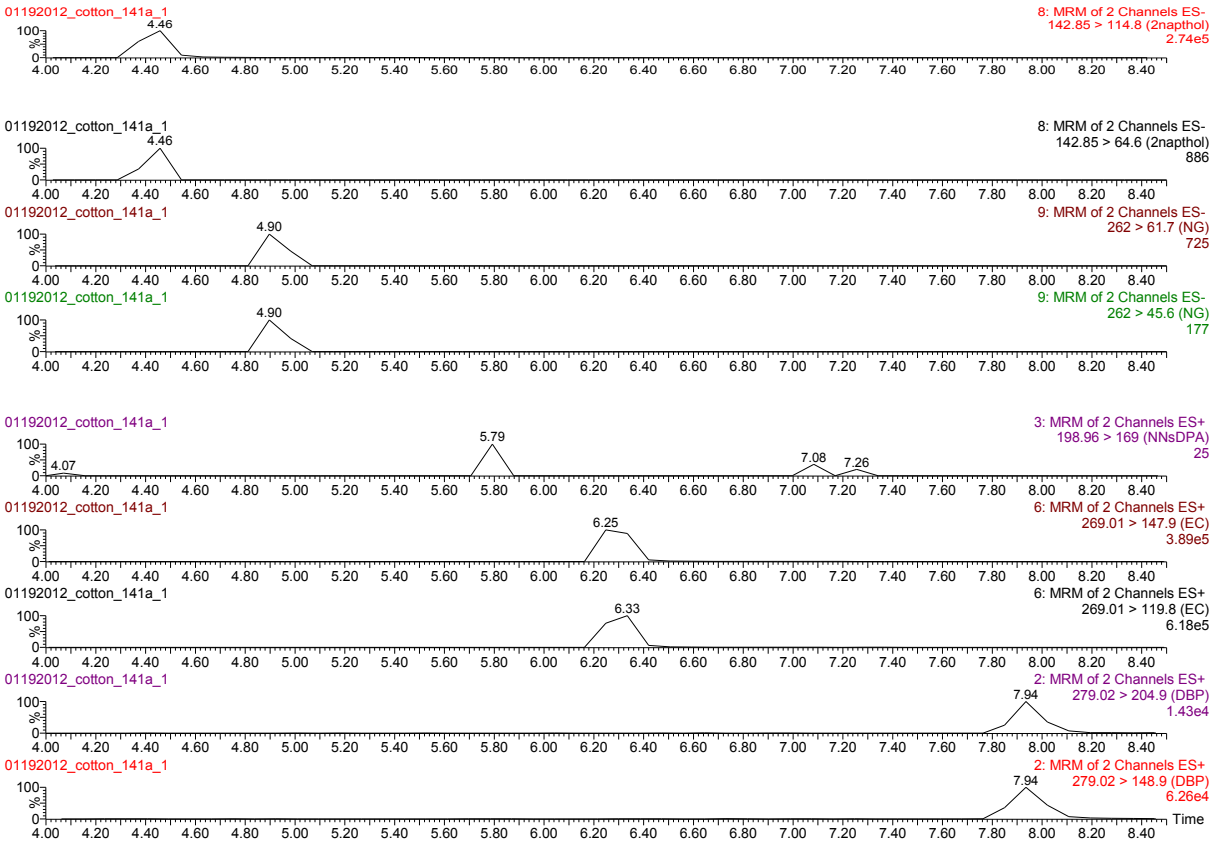


Figure 3.34. MRM chromatograms from an extracted cotton swab sample (Sample A in Figure 3.33).



One final study was done using the weapons and ammunition in Table D.3.1. MDPD personnel cleaned both weapons prior to firing each type of ammunition. Other studies were done but inconsistent and poor results were obtained and therefore, not presented. The sample collection process is given below. Cotton swabs with wooden handles were used to collect blank and firing samples off of the shooter's hands. These swabs were moistened with a 75:25 mixture of isopropanol and water to increase recovery of the organic GSR.

1. The shooter washed and dried his hands and a blank sample was collected using a wet cotton swab.

2. The shooter entered the indoor firing range and fired 3 rounds of one type of ammunition.

3. Samples were collected from the shooter's right and left hands (2 different swabs) with a wet cotton swab.

4. Each swab was cut and placed into a clean centrifuge tube.

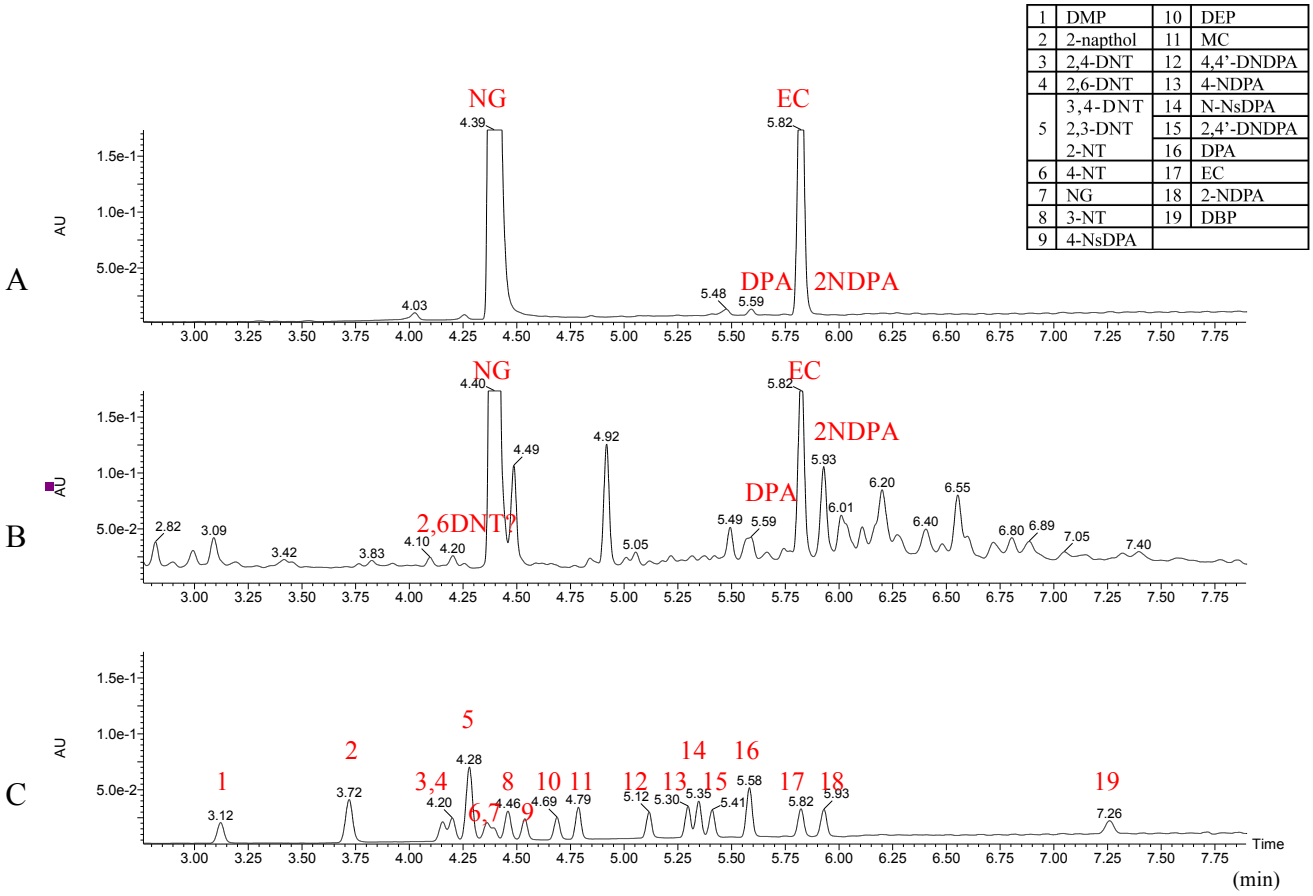
*This collection process was repeated five times for each ammunition type. All of the samples were then transferred back to the laboratory for analysis.

Table D.3.1: Weapons and ammunition tested in firearms study.

Weapon	Ammunition
Smith & Wesson 10-8	Federal 38 special (+P) 158 grain
Glock 19	American Eagle 9mm Luger 147 grain

The general extraction process used to recover the organic compounds off of the swab is as follows. To each tube, 500uL of acetonitrile was added. The tubes were sonicated for 15 minutes and then centrifuged for 5 minutes at 9 rpm. The extract was transferred to a clean amber vial, dried under nitrogen gas, and reconstituted in 1mL of a 60:40 mixture of water and acetonitrile with ammonium acetate and ammonium chloride. For the spent cartridges, a wet swab was passed along the inside of the cartridge, placed in a centrifuge tube, and extracted using the same process described above. The smokeless powders were also extracted the same way but by placing 5mg of the powder in the centrifuge tube instead. This was done so that each type of sample went through a similar extraction process. The results are shown in Figures 3.35 – 3.38.

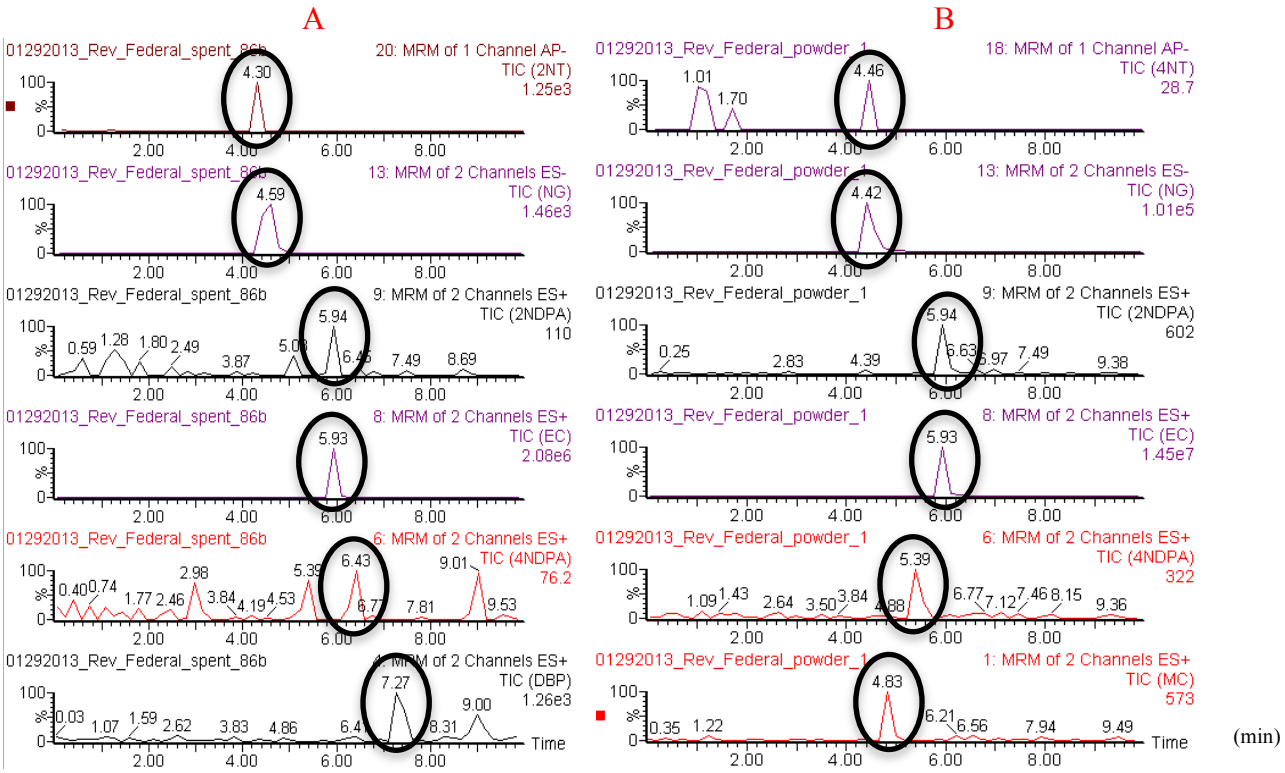
Figure 3.35. UV chromatograms of the (A) Federal 38 special smokeless powder, (B) Federal 38 special spent cartridge, and (C) 10ppm standard GSR mixture. The Smith& Wesson was used for firing.



The Federal 38 special fired with the Smith & Wesson firearm produced high levels of ethyl centralite (EC) and nitroglycerine (NG). These were both identified and confirmed by mass spectrometry in the spent cartridge samples and the smokeless powder samples pulled from the bullet (Figure D.3.3). Lower levels of 2-nitrodiphenylamine (2NDPA) and 4-nitrodiphenylamine (4NDPA) were also seen in the samples due to the decomposition of the diphenylamine (DPA). However, the DPA wasn't confirmed in any of the MS samples. The manufacturer may only use a small amount of DPA in the Federal 38 special powder and therefore, it may be difficult to detect by MS.

Some differenes were noticeable between the spent cartridge and powder samples. The spent cartridges appear to have 2NT and DBP, whereas the powder samples show 4NT and MC (Figure 3.36). The presence of nitrotoluene may be due to the decomposition of 2,6DNT; however, DNT could only be confirmed by UV. Methyl centralite (MC) showed up in the MS of the powder sample. It is very interesting to have found both ethyl and methyl centralite in the powder samples, as only one is commonly seen in each powder.

Figure 3.36. MRM chromatograms of the (A) Federal 38 special spent cartridge and (B) Federal 38 special smokeless powder confirming the presence of each compound. The Smith& Wesson was used for firing.



For the American Eagle ammunition used with the Glock 19, lower levels of nitroglycerin, ethyl centralite and diphenylamine were seen in the spent cartridge and smokeless powder samples when compared to the Smith & Wesson Federal Ammunition (Figures 3.37-3.38). The Federal brand also showed nitrotoluene products in the MRM chromatograms, none of which were detected in the American Eagle ammunition.

In the American Eagle 9mm Luger ammunition, decomposition products of DPA were identified by UV in both the spent cartridge and smokeless powder samples. These included 4-nitrodiphenylamine (4NDPA), N-Nitrosodiphenylamine (NNsDPA), 2NDPA, and 4NDPA. Only the DPA, 4NDPA, and NNsDPA were confirmed by MS, in addition to EC and DBP. NG was

seen in the MRM of the powder but not in the spent cartridge sample. Even though American Eagle is now part of Federal ammunition, differences are visible between ammunition types.

Figure 3.37 UV chromatograms of the (A) American Eagle 9mm Luger smokeless powder, (B) American Eagle 9mm Luger spent cartridge, and (C) 10ppm standard GSR mixture. The Glock 19 was used for firing.

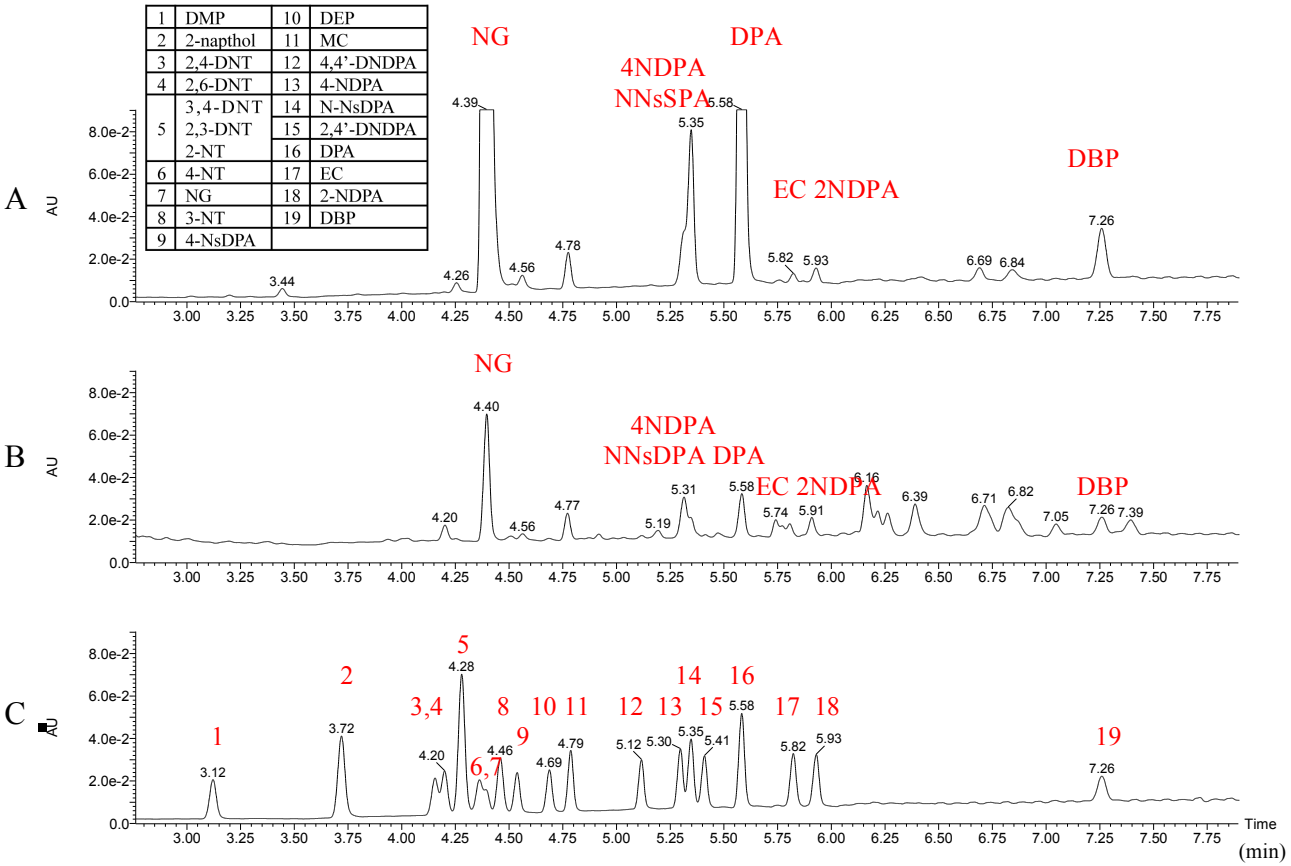
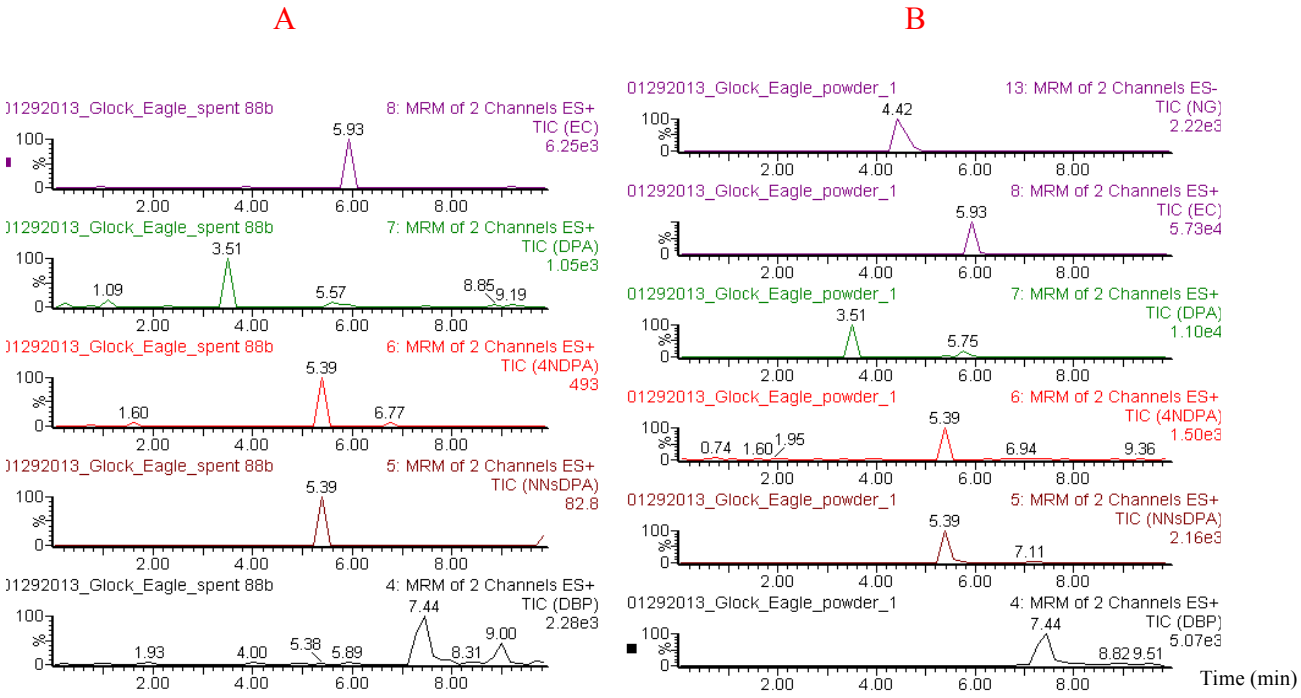


Figure 3.38. MRM chromatograms of the (A) American Eagle 9mm Luger spent cartridge and (B) American Eagle 9mm Luger smokeless powder confirming the presence of each compound. The Glock 19 was used for firing.



By comparing the two different ammunition types, clear differences can be seen in the profiles of the spent cartridges and smokeless powders pulled from the bullet. It appears that the Smith & Wesson samples using the Federal ammunition produced more NG and EC when compared to the Glock samples using the American Eagle ammunition. On the other hand, the Glock samples showed DPA and some of its decomposition products that weren't as noticeable in the Smith & Wesson samples. These differences must be measured quantitatively in order to determine whether or not they are significant.

Summary: We have developed a new extraction method using acetonitrile to recover organic GSR compounds from cotton swabs. This method has been applied to spent cartridges, unburned

smokeless powders, and samples collected off the hands of a shooter. We have been successful in identifying differences between ammunition with the acetonitrile extraction. It is important to optimize the swab extraction and apply it to more hand samples to look at differences in powder residue with different ammunition.

4. Analysis of live-fire residue samples by UPLC/MS/MS – tape

The masking tape was cleaned in methanol and allowed to air-dry. The tape was then cut and placed in clean vials for sample collection. Using a tweezers to hold the tape, the shooter's hands were processed for organic GSR. Figure 3.39 compares a standard GSR mixture to a blank tape sample, a blank tape sample of the shooter's hand after washing with soap, and a tape sample collected after firing the Revolver. It is important to emphasize that these tape samples were stored for 4 months prior to extraction and analysis.

Despite the long period of time in between analysis, the tape sample collected after firing (sample A) shows several organic GSR compounds. MRM comparisons of the blank hand sample and the firing sample can be seen in Figure 3.40. The red circles indicate the location of the peak for each compound. This tape sample appears to be positive for nitroglycerine, 2-nitrodiphenylamine, 4-nitrodiphenylamine, ethyl centralite, and dibutyl phthalate. DBP is seen in lower levels of most samples as a background contaminant because it is used in a wide array of materials, including plastics. However, by comparing background levels to levels seen in a GSR sample, one can determine if DBP is actually present, however this is not a particularly informative result.

Figure 3.39. Comparison of hand samples collected with masking tape. The UV chromatograms are given below for several analyses: (A) Sample collected after firing, (B) Blank hand sample, (C) Blank tape sample, and (D) Standard GSR mixture

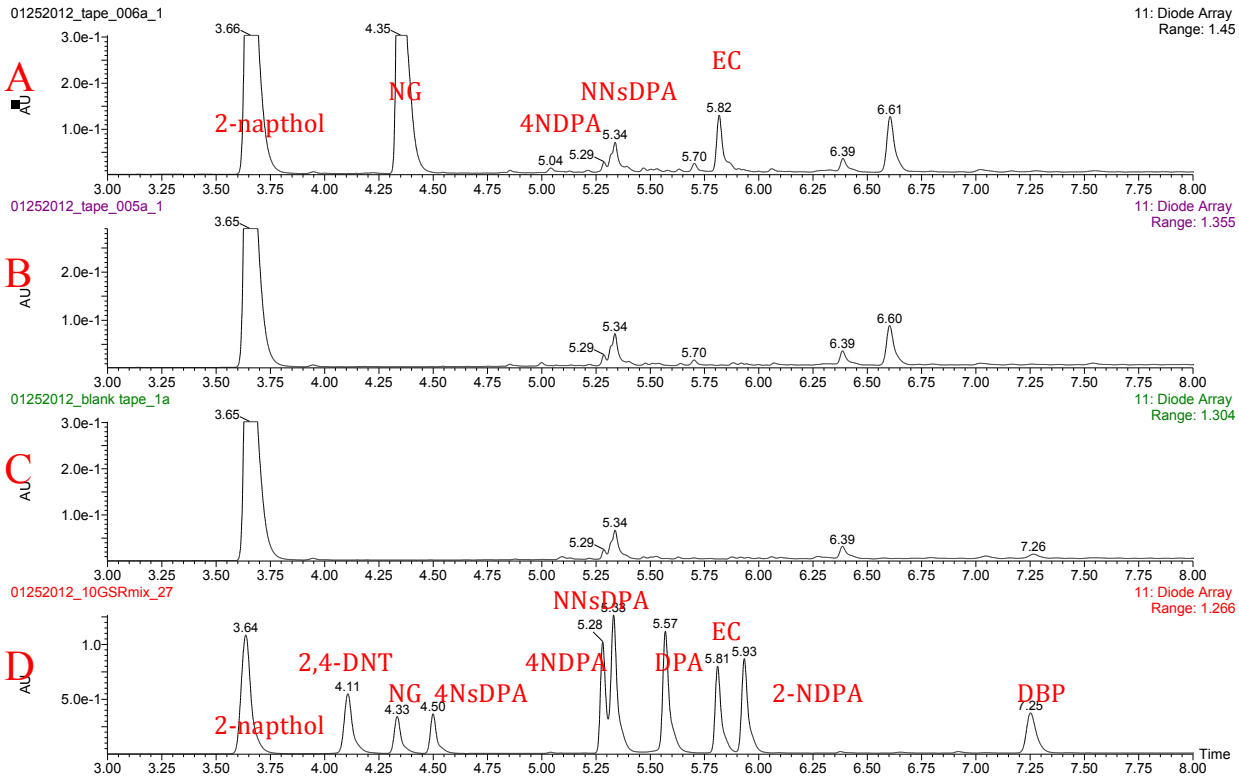
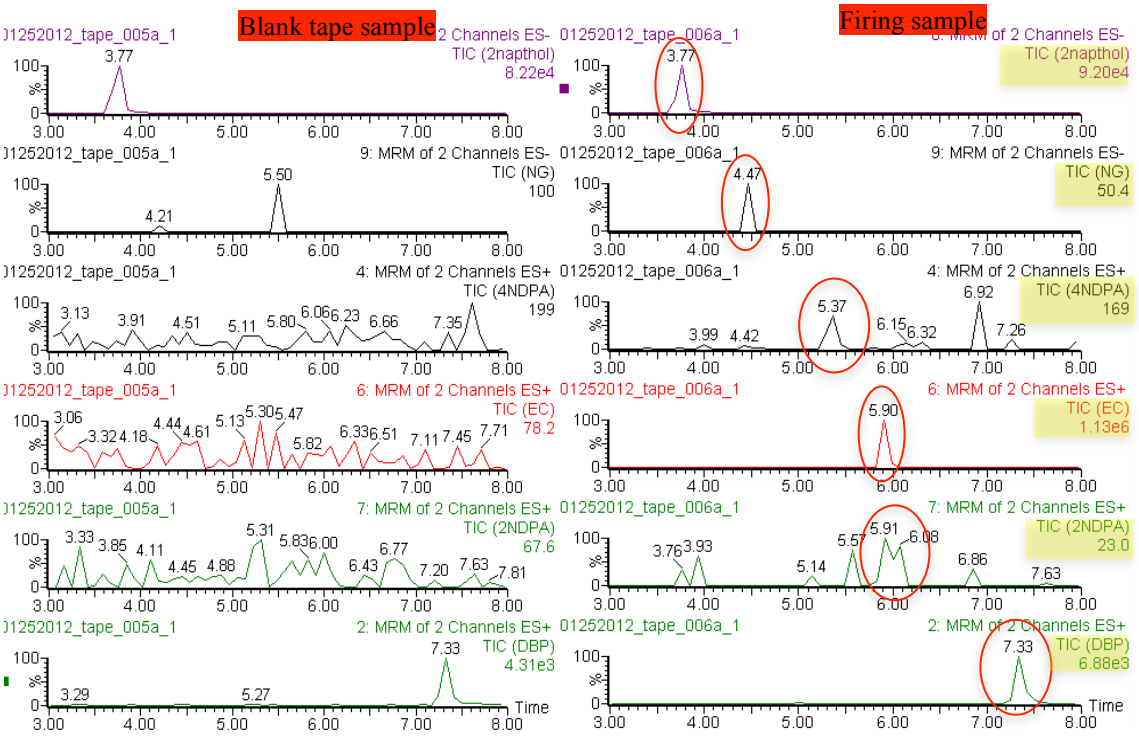


Figure 3.40. MRM chromatograms from extracted masking tape samples A and B in Fig. 3.39).



5. Recovery of Organic GSR from Spent Cartridges

Spent cartridges were collected after shooting and the insides were swabbed for organic GSR (Figure 3.41-3.44). Wet cotton swabs and polyester swabs were used for sampling. The inside of the cartridges were swabbed and the tip of the swab was cut and placed in a 2mL centrifuge tube. 500 uL of acetone was added to the tube and centrifuged for 5 minutes. The extract was then removed, dried under nitrogen gas, reconstituted in sample dilutor, and analyzed by UPLC/MS/MS.

Figure 3.41. UV chromatograms comparing spent cartridge cotton sample (top) to standard GSR mixture (bottom).

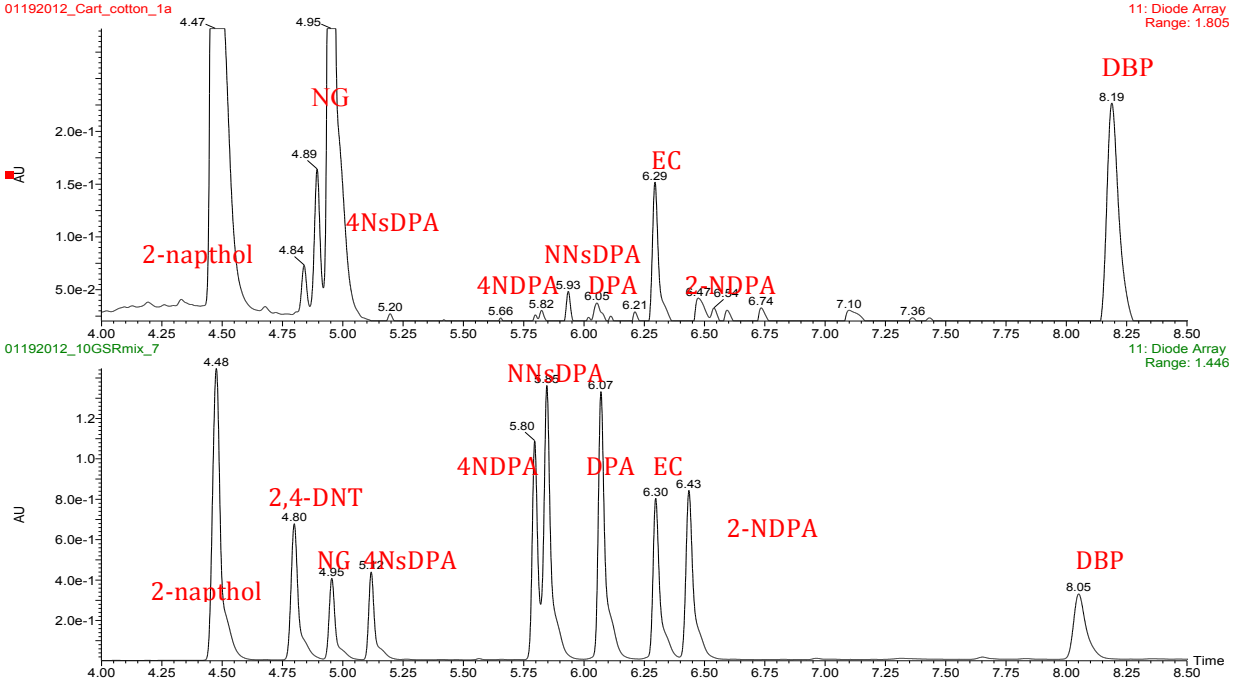


Figure 3.42. MRM chromatograms for the spent cartridge sample obtained using a cotton swab.

In this figure, both transitions are shown for each compound. Red circles are MS peaks.

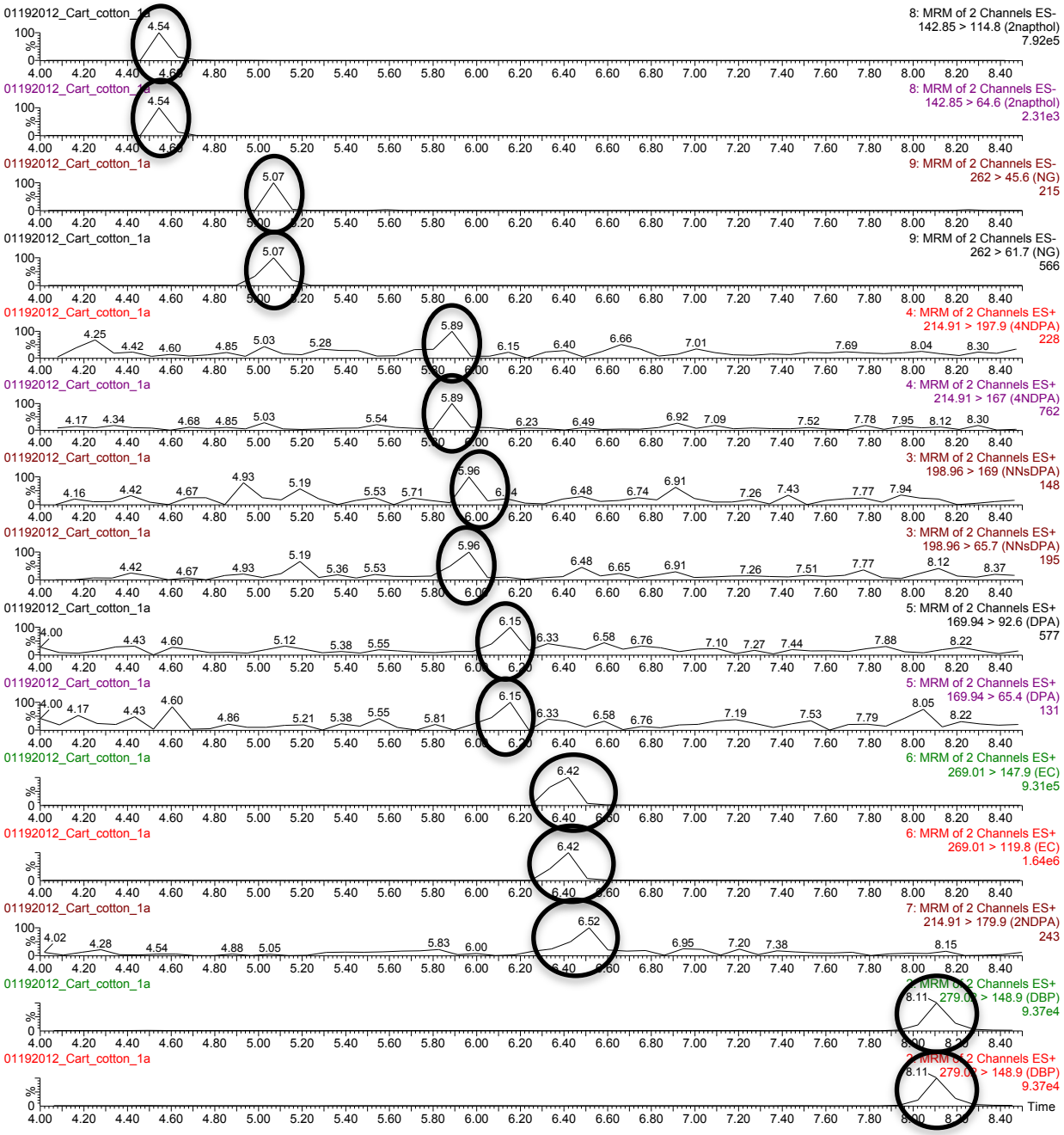


Figure 3.43. UV chromatogram comparing spent cartridge polyester sample to standard GSR mixture. The extraction process for the polyester swabs was the same as the one for the cotton swabs.

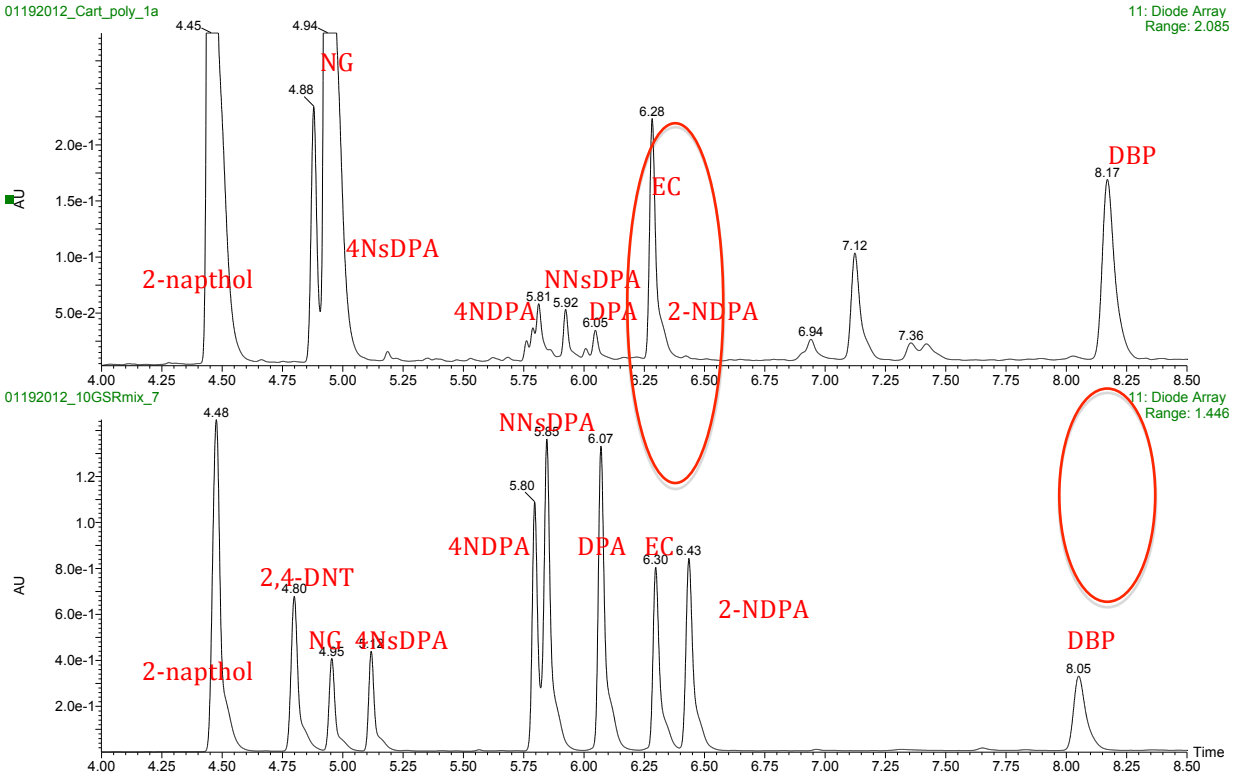
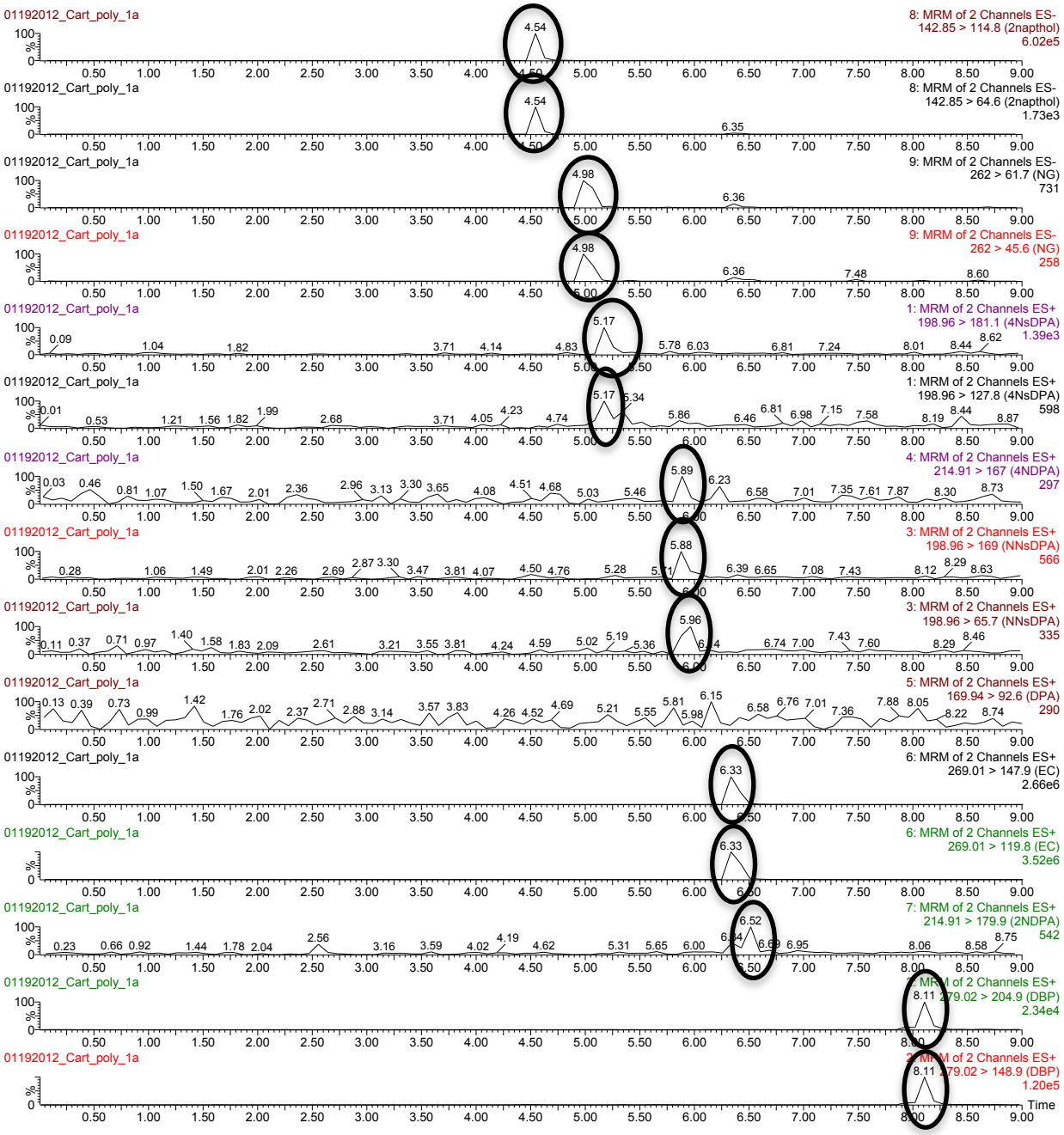


Figure 3.44. MRM chromatograms for the spent cartridge sample obtained using a polyester swab. In this figure, both transitions are shown for each compound.



It can be seen that both swabs were efficient at recovering different organic compounds from the inside of a spent cartridge. From the preliminary data, it appears that the polyester swab is able to recover more of these compounds, as their MS intensities are greater than the ones for the cotton extractions. Hand samples were also collected using the polyester swabs; however, the ones that have been processed were negative for all of the compounds.

6. Recovery of Organic Chemicals from Smokeless Powder Pulled from a Cartridge

Bullets were pulled apart to retrieve some of the smokeless powder (SP) for analysis. Approximately 5 mg was added to a clean vial and extracted using two different methods:

(i.) Extract for 6 hours in methylene chloride in the absence of light (13)

(ii.) Extract for 15 minutes in methanol (MeOH) with sonication (11)

The extracts were transferred to a clean vial, evaporated to dryness using nitrogen gas, and then reconsituted in sample dilutor. 2-napthol was added to all of the extracts to ensure that the UPLC-MS/MS method was working. The two methods were compared to determine which one provided higher recoveries. The UV chromatograms of the standard GSR mixture and two smokeless powder samples are shown in Figure 3.45. The MRM chromatograms for the powders can be seen in Figures 3.46 and 3.47.

Figure 3.45. Comparison of extracted smokeless powder (SP) samples. The UV chromatograms are: (A) SP extracted with MeOH, (B) SP extracted with methylene chloride, and (C) Standards.

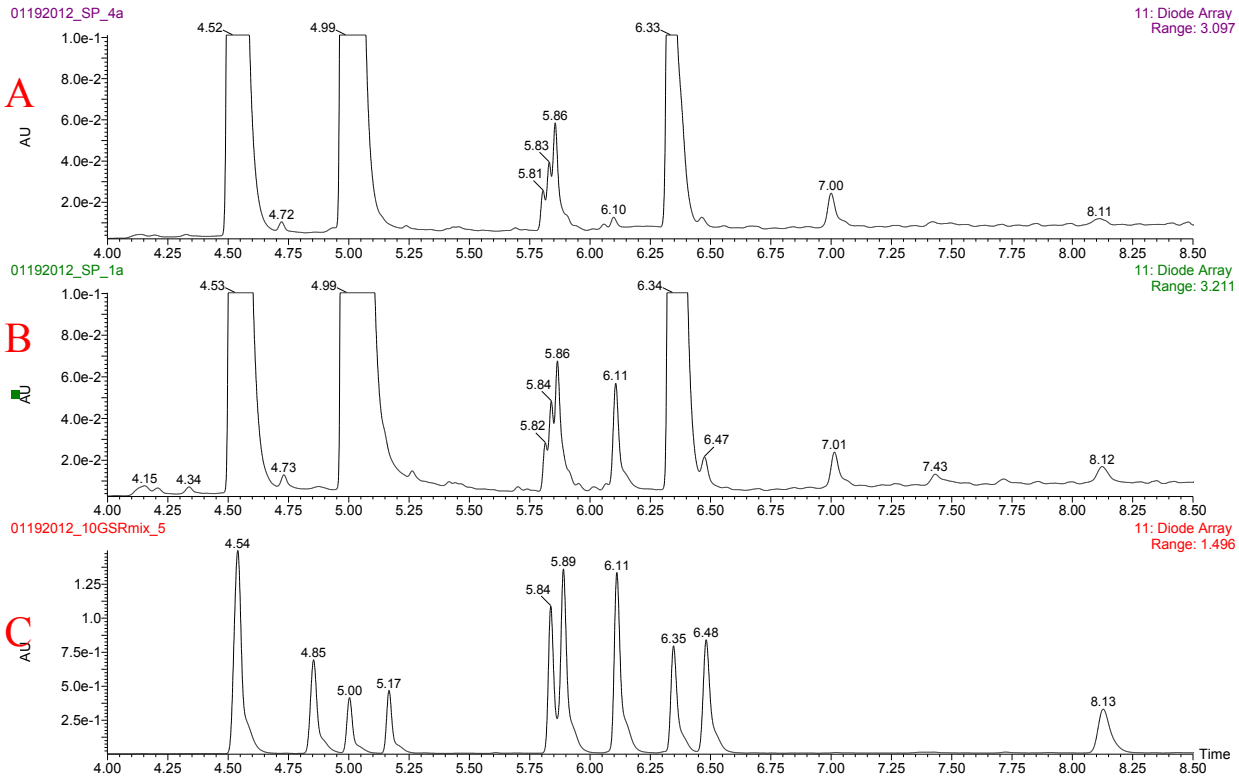


Figure 3.46. MRM chromatograms from an extracted SP sample using the method by Wissinger.

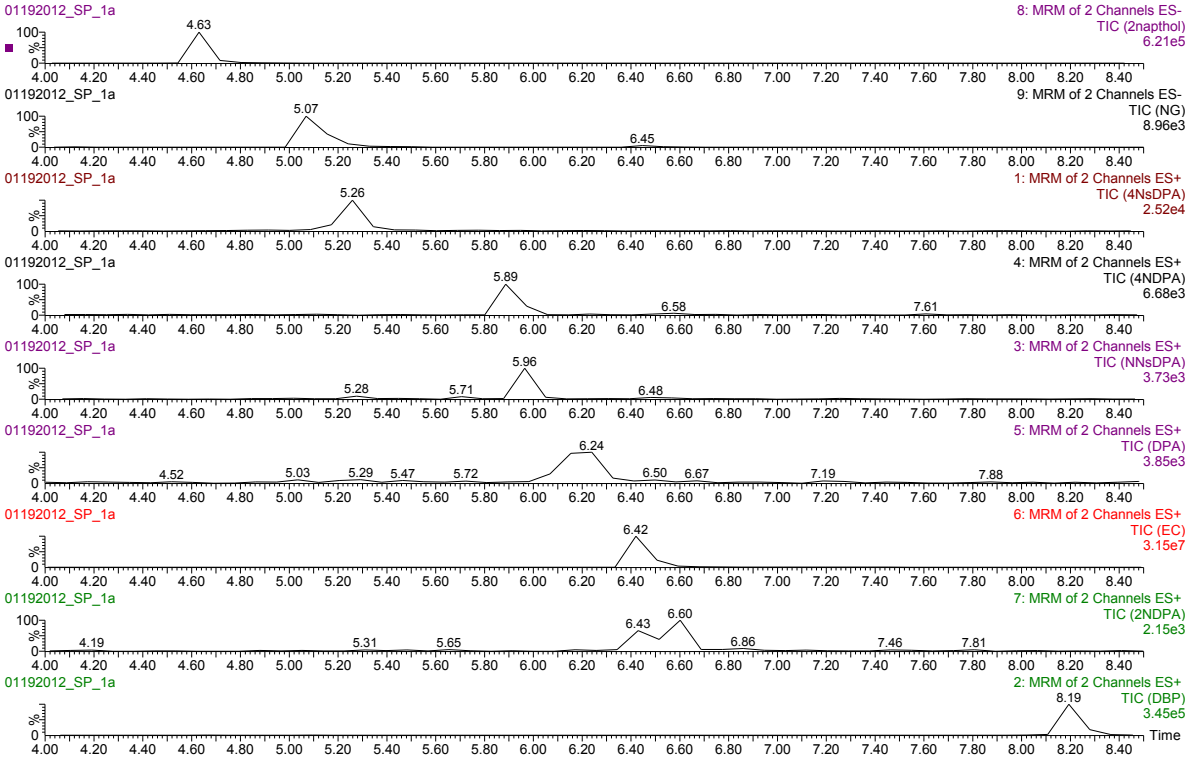
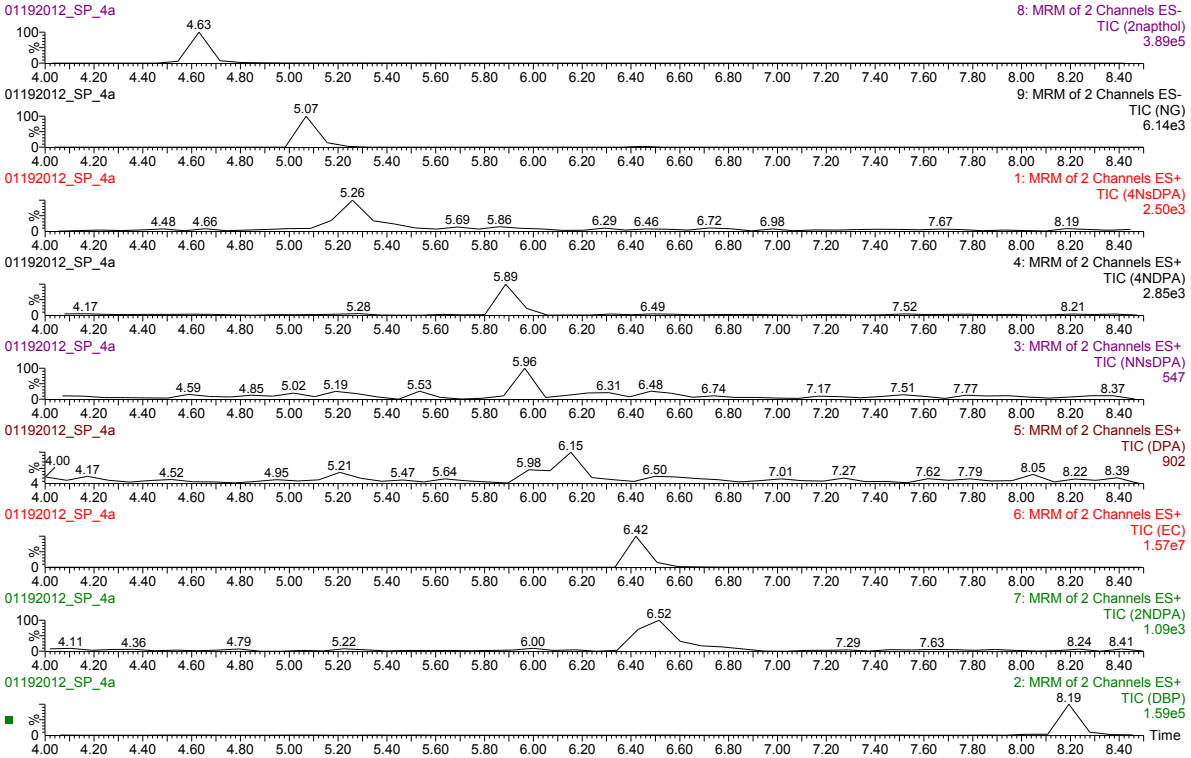


Figure 3.47. MRM chromatograms from an extracted SP sample using the method by Northrop.



Summary: After comparing the two extraction methods for the smokeless powder samples it appears that the Wissinger method recovers more of the extracted components. The required extraction time is longer though for this method. It will be valuable to test if Northrop's method will recover equal, more, or less organic compounds if allowed to sonicate for the 6-hour period.

IV. CONCLUSIONS

A. Discussion of findings.

A rapid separation and detection method has been developed for the analysis of standard organic compounds that may be present in organic gunshot residue. Standard mixtures were created and separated on a C18 column using ultra performance liquid chromatography (UPLC). The mobile phases were: (A) 90:10 water:acetonitrile and (B) 95:5 acetonitrile:methanol. Both mobile phases contained 2mM ammonium acetate and 0.2mM ammonium chloride to enhance MS ionization and were ran at 0.5mL/min. For detection, a tandem mass spectrometer (MS/MS) with an ESCi® source was utilized for switching between electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) modes at high speeds all within the same source, allowing the detection of a wide array of compounds in a single run. Confirmation was achieved by monitoring two MRM transitions for most of the compounds. Limits of detection for the UV and MRM method ranged from 0.08 to 2.6 ng injected and 0.4 to 64 ng injected, respectively. Based on these results, it can be seen that the method is fast and sensitive for the identification of common smokeless powder additives.

The developed UPLC/MS/MS method was then successfully applied to the analysis of five different brands of smokeless powders. The percent composition was calculated for each component in the sample and powder comparisons showed differences between brands and even lots of the same powder. The results were also consistent with information found in the material safety data sheets obtained from the manufacturer's websites.

In addition to the powders, the developed UPLC/MS/MS method was also applied to the analysis of live-fire residue samples. Different extraction techniques were tested for sample collection and it was found that the cotton swab provided the best recovery thus far. Alcohol

swabs may also be a good alternative for collection but these swabs must undergo further testing to determine recovery percentages. The aluminum stubs used for collecting inorganic GSR produced low recoveries and were challenging to extract. However, it may be beneficial to reevaluate the use of the aluminum stubs to standardize collection for both inorganic and organic analytical techniques. In cases when a lead-free primer is used, organic GSR analysis may provide results that can be used in identifying suspects. The tape was also very challenging to use for sample collection and didn't provide recoveries as high as the cotton swabs in this study.

B. Points to Consider

There are several considerations to keep in mind when analyzing and reporting GSR results. These considerations are listed below for organic GSR analysis. Many of them were highlighted in the literature. This list is not exhaustive of all considerations.

1. The powder residue collected after firing is expected to be chemically identical to the virgin powder (24).
2. It is possible that powder GSR may be contaminated with particles from a previous firing in which a different ammunition type was used and as a result hinders identification (24).
3. One assumes that the manufacturer only uses one kind of smokeless powder to fill the cartridge (24).
4. According to the FBI, "the presence of primer residue on a person's hand is consistent with that person having discharged a firearm, having been in the vicinity of a firearm when it was discharged, or having handled an item with primer residue on it." Conversely, negative GSR reports often contain a qualifying statement, such as "the absence of gunshot residue on a

person's hands does not eliminate that individual from having discharged a firearm." (http://www.fbi.gov/stats-services/publications/law-enforcement-bulletin/may_2011/The%20Current%20Status%20of%20GSR%20Examinations)

5. False positives may be due to transfer of GSR from the arresting officer [25], transfer inside of the police vehicle or at the station, through occupational or environmental exposure [14], or by handling a recently fired weapon.

6. False negatives may be due to washed hands and routine activity post-firing.

C. Implications for policy and practice

The results of this study can be used in a multitude of forensic venues including gunshot residue detection, analysis of spent cartridges, and pipe bomb determinations. The project permits an increase in the accuracy of determination of GSR by providing an alternative to traditional inorganic analysis. The procedure also will assist investigators in situations in which there is limited inorganic residue available. In addition, the quantitative aspects of the results will permit determination of small variations in manufacturing of different lots of powder. The development of optimized methods for gunshot residue analysis is also of direct interest to society. The procedures can help to determine individuals responsible for significant crimes of violence – specifically those involving guns – and assist in the definition of the cause of death by suicide. The project will also enhance the field of separation science and forensic explosives analysis by providing new methods for the analysis of smokeless powders.

V. REFERENCES

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VI. DISSEMINATION OF RESEARCH FINDINGS

An article that outlines the separation and detection method and its application to smokeless powder analysis was published in the May 2013 issue of the Journal of Forensic Sciences. A second article is currently being written that describes the development of UPLC/MS/MS and extraction methods on organic GSR samples collected post-firing. Several presentations have been made on this research project. A list of all of these presentations – both oral and poster – is below. In addition, links are given to different press releases regarding this research project.

In terms of training and professional development, we have been working with the Miami Dade Police Department on collecting GSR samples using their personnel and facilities. In addition, many organizations have found the research relevant enough to provide additional funding and speaking opportunities, including Waters Corporation, NIST, and IAI conference personnel. BATF has requested our separation protocols. Other involved organizations and collaborators include the Washington State Patrol, Florida International University, NATO publication, NOVA Southeastern University (Training Course), NBC news, and the Discovery Channel.

(1.) Publications

1. Thomas, J. L.; McCord, B. R.; Lincoln, D. L. The separation and detection of smokeless powder additives by ultra performance liquid chromatography with tandem mass spectrometry. J Forensic Sci 2013;58:609-615.

(2.) Oral presentations

1. Jennifer Greaux, Dr. Bruce McCord; Method Development for the Rapid Separation and Detection of Organic Gunshot Residue by UPLC/MS; American Academy of Forensic Sciences Annual Meeting, NIJ Grantee Meeting oral presentation, Chicago, IL; February 22, 2011.

2. Jennifer Greaux; Analysis of Smokeless Powders and Gunshot Residue Samples by UPLC-MS/MS; 3rd Annual South Florida Discipline Meeting; Miami Dade Police Department in Miami, FL; February 2, 2012.

3. Jennifer Greaux; The Application of UPLC-MS/MS to the Analysis of Smokeless Powders and Gunshot Residue Samples; AAFS conference; Atlanta GA; February 24, 2012.

4. Jennifer Greaux; The Application of UPLC-MS/MS to the Analysis of Smokeless Powders and Gunshot Residue Samples; 1st Annual FIU Forensic Symposium; Miami FL; March 10, 2012.

5. Jennifer Greaux; The Application of UPLC/MS/MS to Explosive residue detection; Waters corporation Forensic seminar; Springfield VA; May 9, 2012.

6. Jennifer Thomas; A Rapid UPLC/MS/MS Method for the Analysis of Smokeless Powders and Organic Gunshot Residue; IAI Annual International Educational Conference; Providence, Rhode Island; August 2013.

(3.) Poster presentations

1. Jennifer Greaux; Method Development for the Rapid Separation and Detection of Organic Gunshot Residue by UPLC/MS; American Academy of Forensic Sciences Conference - Criminalistics division; Chicago, IL; February 24, 2011.

2. Jennifer Greaux; Method Development for the Rapid Separation and Detection of Organic Gunshot Residue by UPLC/MS; Chemistry Department Graduate Student Visitation Day; Florida International University, Miami, FL; March 4, 2011.

3. Jennifer Greaux; Method Development for the Rapid Separation and Detection of Organic Gunshot Residue by UPLC/MS; Graduate Student Scholarly Forum at Florida International University; Miami, FL; March 29, 2011.

4. Jennifer Greaux; Method Development for the Rapid Separation and Detection of Organic Gunshot Residue by UPLC/MS; FAME; Innisbrook, FL; May 14, 2011.

5. Dr. Bruce McCord; Method Development for the Rapid Separation and Detection of Organic Gunshot Residue by UPLC/MS; NIJ Conference: Translational Criminology-Shaping Policy and Practice with Research; Arlington, VA; June 21, 2011.

6. Jennifer Greaux; Method Development for the Rapid Separation and Detection of Organic Gunshot Residue by UPLC/MS/MS; 1st Annual FIU Forensic Symposium; Miami FL; March 9, 2012.

7. Jennifer Greaux; Method Development for the Rapid Separation and Detection of Organic Gunshot Residue by UPLC/MS; NIJ Trace Evidence Symposium; Kansas City, MO; August 10, 2011 - won the student award for poster presentation

(4.) Press releases

1. FIU press release:
http://news.fiu.edu/2012/06/new-forensic-method-could-help-police-solve-crimes/41111
2. Forensic Magazine article:
http://www.forensicmag.com/news/new-forensic-method-could-help-police-solve-crimes

3. NBC news:
http://www.nbcmiami.com/news/local/Ballistics-Breakthrough-at-FIU-158218815.html