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Development of an Analytical Method and Sample Preparation Technique for the Analysis of Sulfur-Containing Fentanyl Analogs by UPLCMSMS, and the Application to Forensic Science

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Abstract

In February 2018, the US Drug Enforcement Agency (DEA) released a statement of the emergency scheduling (Schedule 1) of all illicit fentanyl analogs not already regulated by the Controlled Substances Act due to an alarming increase in overdose deaths linked to synthetic opioids. Fentanyl analogs are pharmacologically similar to fentanyl, but often more potent. This increased potency can create problems with proper dosing of fentanyl analogs and can lead to untoward effects including an increase in overdoses and deaths. Since 2018, there has been a 38.4% increase in illicitly manufactured fentanyl overdose deaths leading the *Centers for Disease Control* and Prevention (CDC) to make available the Fentanyl Analog Screening Kit (FAS Kit) and Emergent Panels containing previously unavailable fentanyl analog reference materials. A limited number of published methods for the identification of multiple fentanyl analogs are available, none of which present a full analog class. Therefore, there is a need for analytical methods capable of identifying isomeric fentanyl analogs. Within the isomeric classes inter/intraclass isobars exist creating the issue of the same molecular weight and transitions, thus the mass spectrometer alone cannot identify the analogs. Given the increase in overdose deaths, an analytical method for identification and separation of all classes of fentanyl analogs is needed to help the clinical and forensic communities overcome this epidemic.

Presented is a three-aim study including the development of a triple quadrupole mass spectral method, an ultra-pressure and high-performance liquid chromatographic method, and an extraction procedure capable of separation and detection of twelve sulfur-containing fentanyl analogs. Validation of the methods developed followed ANSI/SWGTOX guidelines including selectivity, carryover, precision, and limit of detection (LOD).

Keywords: Method Development; Fentanyl Analog; Thiofentanyl; HPLC; UPLC-MSMS

Introduction

In 1984, the US Drug Enforcement Agency (DEA) amended the controlled substances act, the Comprehensive Crime Control Act (CCCA). The CCCA allows DEA administrators to place a substance temporarily into Schedule 1 if the intent is to avoid an imminent hazard to the public health. The emergency scheduling is permitted on substances not currently controlled but are currently abused and a high risk for the public. In February 2018, the DEA released a statement of the emergency scheduling (Schedule 1) of all illicit fentanyl analogs not already regulated by the controlled substances act due to an alarming increase in overdose deaths linked to synthetic opioids.[1] Fentanyl analogs such as beta-hydroxythiofentanyl, acetyl fentanyl, sufentanil, carfentanil, and others have a similar chemical structure to fentanyl creating a similar physiological effect, but with higher potency than fentanyl[2], creating a dangerous problem for users who unknowing ingestion fentanyl analogs in their heroin/cocaine/fentanyl mixture.

Only controlled medicinal fentanyl analogs are regulated by the Food and Drug Administration making the identification of illicit analog substances difficult. An increase in confiscation of fentanyl and fentanyl analogs by law enforcement agencies points to illicit manufacturing as a fueling source. The challenge comes from a lack of universal methodology applying to readily available instruments on which a general toxicology screen is performed. The newly emerging synthetic opioids are appearing on the street as not only illicitly manufactured substances but also diverted research chemicals so analytical information on separation and identification is difficult to gather. Given the 10-fold increase in overdose deaths from 2013-2017[3] and the 38.4% increase in synthetic opioid overdose deaths (specifically illicitly manufacture fentanyl) from May 2019-May 2020[4], it is important novel psychoactive substance (NPS) compounds be accurately identified in biologically matrices to have a better understanding

of the drugs.[5] Much of the current knowledge concerning fentanyl analogs comes from postmortem toxicology findings caused by a previous lack of fentanyl analog class standards; U-47700, acetyl fentanyl, carfentanil, norfentanyl, furanyl fentanyl, and beta-hydroxythiofentanyl are amongst the most frequently encountered analogs.

The Center for Disease Control and Prevention (CDC) contracted with Cayman Chemical Corporation and Cerilliant Corporation to release fentanyl-analog screen kits (FAS kit) and a traceable opioid material kit (TOM kit) to allow researchers the opportunity to examine over 200 fentanyl analogs via reference material. The CDC's recent release of a fentanyl analog screening kit has allowed for the possibility of full analog class research. Currently, there are only two publications[5,6] that directly address the CDC kits. These publications only include a small subset of the over 200 analogs provided in the kits and only one[6] has a method for testing and involves a liquid chromatography quadrupole time-of-flight (LC-QTOF) which is not available in many laboratories.

Several publications have discussed poly-analog analysis[6–9], all of which discuss separation and detection of analogs structurally similar to fentanyl (2-Furanyl fentanyl, 3-methylfentanyl, 4- ANPP, acetyl fentanyl, acryl fentanyl, alpha-methylfentanyl, alfentanil, butyrylfentanyl, carfentanyl, cyclopropylfentanyl, fentanyl, methoxy acetyl fentanyl, norfentanyl, ocfentanil, parafluoroisobutyrylfentanyl, remifentanil, sufentanil, and tetrahydrofuranylfentanyl), none of which separate and quantitate structurally similar analogs such as the sulfur-containing class of fentanyl analogs. Tabarra et al[8] provides a review of sample preparation techniques including extraction methods such as liquid-liquid extraction (LLE), solid-phase extraction (SPE), and a hybrid method (Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS)). LLE is most common followed by SPE, but one publication

involving the identification of acetyl fentanyl[10] showed the QuEChERS method to be the only method to reach acceptable extraction criteria for three fentanyl analogs when compared to LLE and SPE.

Several extraction techniques are commonly used for drug extraction from tissue including SPE, supported liquid extraction (SLE), and LLE. SPE is a preparation technique utilizing an absorbent disk to filter select species from the matrix-analyte solution. UCT, Waters, and Biotage specifically have developed several SPE columns commonly used in drug extraction from complex matrixes such as clean screens, FASt columns, Xcel Columns, QuEChERS, μElution plate, Water's Oasis Prime HLB[11], and Isolute+ PLD. SLE and LLE are extraction techniques reliant on the target analyte and the matrix interferences have a varying polarity as two immiscible layers (polar and nonpolar) will form, allowing for the discard of the matrix layer and further filtering of the analyte layer.

Due to the structural similarity of fentanyl analogs, most ELISA (enzyme-linked immunosorbent assay) immunoassays demonstrate positive cross-reactivity showing ELISA immunoassays are valid for presumptive identification of target analogs in various matrices and confirmatory testing such as a mass spectral method must follow. Confirmatory methodologies for poly-analog detection are currently mostly liquid chromatography-tandem mass spectrometry (LCMS). UPLC (ultra-pressure liquid chromatography) systems utilize smaller particle size columns[12] which increases efficiency and decreases analysis time compared to typical LC systems. Only a small number of publications[7,13] include the UPLC-MS (UPLC tandem mass spectrometry) systems utilized in the identification of fentanyl analogs when compared to the publications utilizing the LCMS system.

Postmortem analysis of fentanyl analogs[9,13,14] appear more often in the literature compared to antemortem analysis. This is believed to be caused by either the increased potency of the analogs or an increased need for clinical identification via urine or plasma. Poklis et al.[13,15] described two case studies where a fentanyl immunoassay presented as a presumptive positive, but after analysis on GC-MS (gas chromatography-tandem mass spectrophotometry) and GC headspace, the sample was negative for fentanyl. The absence of fentanyl led to a targeted screen (acetyl fentanyl, and butyryl fentanyl) to find the presence of butyryl fentanyl in both cases.

Current areas that need more research are poly-fentanyl analog methodology[2] for UPLC systems that can detect and separate structurally similar fentanyl analogs. The methodology must be transferable to an LC system for when a UPLC system is not available. Therefore, there is currently a need for analytical methods capable of separating and detecting poly-drug presence in biological matrices as most drug users are poly-drug users. Unsuccessful identification of all compounds present in a poly-analyte toxicology screen arises when similar analytes co-elute creating poor chromatography. Methodology for identification and separation of structurally similar analogs such as thiofentanyl, thienyl fentanyl, alpha-methyl thiofentanyl, cis/trans-3-methyl thiofentanyl, sufentanil, and norsufentanil creates an elimination of isobaric-interferences and poor chromatography during the analysis.

Research Materials and Methods

The fentanyl analog screen (FAS) kit[16] obtained from Cayman Chemical (Ann Arbor, Michigan) contained ten of the twelve analogs analyzed (α-Methyl Thiofentanyl, β-Hydroxythioacetylfentanyl, cis-3-Methyl Thiofentanyl, Norsufentanil, Sufentanil,

Tetrahydrothiophene Fentanyl, Thienyl Fentanyl, Thiofentanyl, Thiophene Fentanyl, trans-3-methyl Thiofentanyl), while the opioid CRM Kit[17] obtained from Cerilliant (Round Rock, Tx) contained β-hydroxythiofentanyl and the isotopically labeled internal standard β-hydroxythiofentanyl (13C6) (Group 1; part number: CSQ-25819A-1EA). LC-MS grade acetonitrile (ACN) was obtained from Fischer Scientific (Waltham, MA); LC-MS grade methanol (MeOH) was obtained from Honeywell (Muskegon, MI); LC-MS grade water was obtained from Sigma-Aldrich (St. Louis, MO). A working stock solution was prepared in MeOH at a concentration of 5μg/mL.

Each analog was diluted in MeOH to obtain a standard working stock solution for analysis.

Aim 1: **Develop and optimize a mass spectral method for the detection of sulfur-containing fentanyl analogs on a triple quadrupole mass spectrometer with ANSI/SWGTOX acceptable interferences.**

The analogs were divided into five groups based on m/z and further diluted to 50ng/mL in 50:50 MeOH: LCMS water. Ammonium formate (Acros Organic, New Jersey) was added to each sample group before manual infusion. Manual infusion and compound optimization of the analogs was completed on both a Water's ACQUITY UPLC-TQs-micro and an AB Sciex triple quadrupole 5500; declustering potential (dp), entrance potential (ep), collision energy (ce), cxp (collision cell exit potential), and cone voltage were optimized for each sulfur-containing fentanyl analogs (SFA) to identify unique daughter ions. The mass spectral fragments will be utilized to determine the fragmentation patterns of the compounds. The two most abundant and

identifiable daughter ions from each analog were chosen as the target fragments for positive identification during the separation method development.

***Aim 2*: Develop and optimize an ultra-high pressure and high performance liquid chromatographic method for separation of sulfur-containing fentanyl analogs on a Water's ACQUITY UPLC-TQs micro and a Shimadzu LC20AD XR coupled to AB Sciex triple quad 5500.**

A final solution group containing the eleven sulfur-containing fentanyl analogs and the internal standard was diluted to 50ng/mL. The six samples were analyzed in each trial method. The aqueous mobile phase for all chromatographic injections was 0.1% formic acid in water and the organic mobile phase (OMP) for all chromatographic injections was 0.1% formic acid in ACN. Initial chromatographic conditions were based on Sofalvi et al.[9] on the HPLC-triple quadrupole system utilizing a PFPP column (Selectra PFPP 100 x 2.1mm 3mm) with a flow rate of 0.4mL/minute and a total injection time of 5 minutes.

Chromatographic conditions such as injection time (5-20minutes), gradient (linear and curve), and column chemistry (pentafluorophenylpropyl (PFPP), BiPhenyl, C18) were then varied to obtain optimal separation and chromatography.

***Aim 3:* Evaluate extraction and detection methods to identify the 11 analogs and one isotopic analog from a blood serum matrix following ANSI/SWGTOX guidelines for matrix effects (ME), process efficiency (PE), and absolute recovery (RE).**

Several extraction methods were analyzed including a mixed phase (MCX) micro elution plate, an MP3 SPE column, a UCT DAU Purple (micro) column, and an alkaline LLE. Extraction on the Waters Oasis PRiME microelution plate[3] included a pH adjustment with 4% phosphoric acid, a wash of 0.1M 2% FA (formic acid) in ammonium formate and MeOH, followed by an elution buffer of ACN: MeOH containing 5% ammonia. Extraction on the MP3 SPE column[15] included a pH adjustment of phosphate buffer (pH 6), a column condition of MeOH and 100mM phosphate buffer, a wash of DI water and acetic acid, and an elution buffer of dichloromethane: isopropanol: ammonia (78:20:2). Extraction on the UCT DAU Purple micro plate[18] included a pH adjustment with 100mM pH 6 phosphate buffer, a wash of DI H_2O/acetic acid/hexane, and an elution buffer of dichloromethane: isopropanol: ammonia (78:20:2). The MP3 and UCT DAU required a final drying before reconstitution in the mobile phase. Extraction utilizing an alkaline LLE[19] utilizes the addition of ammonium hydroxide and N-butyl chloride/ACN (4:1), centrifuging and rotating of the solution, removal of the organic layer, drying of the aqueous phase, and reconstitution in the mobile phase. Extraction methods were initially analyzed at a 1ng/mL to assess which method resulted in the most acceptable PE, RE, and ME.

Validation of the preferred method followed SWGTOX[20] guidelines: interference, carryover, and limit of detection determination. Interferences evaluated consisted of 10 different sources of plasma, one blank containing IS, one 10ng SFA sample, and endogenous/exogenous potential interferences such as BioRad TDM3 (pharmaceuticals), BioRad C4 (illicit substances), BioRad Unassayed Multiqual (abnormal serum), and three classes of fentanyls (acryl, butyryl, and furanyl). Carryover was identified by injecting consecutive blank samples after the high control (10ng SFA). An empirical limit of detection was identified using a nine-point linear

curve. Precision of the cutoff value was evaluated in a 5-extraction triplicate of the empirical cutoff including an 50% below and 50% above sample.

Research Results and Discussion

Aim 1: MS method

The mass to charge ratio chosen for each analog was centered around the molecular weight determined from the chemical formula. The product ions are unique to the m/z for each analog and were gathered from manual infusion optimization of the dp, ce, cxp, and cone voltage on the Waters and Sciex systems, the analytes were ionized via collision induced dissociation (CID). Mass spectral parameters and daughter ions of each analog including the internal standard are listed in Table 1. The ions identified were the most abundant and stable products discovered from the manual infusion and optimization (Figure 1-11).

Aim 2: LC method

The changes in LC method consisted of injection time, gradient type, and column chemistry. Column chemistry variation consisted of dipole moments, pi-pi interactions, and polarity, all of which were found in the literature.

Waters ACQUITY TQs micro

Four columns with various gradients were evaluated on the Water's UPLC system: Restek Biphenyl, UCT C18, Waters CSH C18, and Waters UPLC BEH C18. Initial separation was attempted using pi-pi interactions from the biphenyl column combined with dipole moments

from the ACN (Figure 12). The twelve compounds eluted from the biphenyl column in three-time intervals resulting in three broad, unsymmetrical peaks (1-2min; 2-3.5min; 4.5-5.5min) meaning the pi-pi interactions of the biphenyl column were unsuccessful in the separation of the twelve SFA.

Next, a polar column- UCT C18- using a linear method was evaluated; two initial concentrations of 0.1% FA in ACN were evaluated, 5% and 20%. The 5% initial concentration of OMP was linearly ramped to 95% to assist in the identification of the mobile phase ratio elution occurred on the C18 column. The 5-95 linear ramp resulted in 7 unresolved and unseparated peaks between 5 and 7 minutes (Figure 13). The organic to aqueous mobile phase ratio in which the SFA eluted from the C18 column was around 30% organic, 70% aqueous; Therefore, the initial starting condition of the OMP was raised to 20% and the linear gradient was reevaluated (Figure 14). The increase in starting conditions of the OMP resulted in an increased separation in less time of the SFA, but sufficient separation and ideal chromatography were not found.

The third column evaluated was the Waters CSH C18. While still a nonpolar C18 column, each manufacture's columns vary to increase/decrease the specificity of various functional groups, therefore a second nonpolar column from Waters was chosen to increase the specificity of the SFA functional groups. The same linear gradient (20-95% OMP over 9 minutes) was evaluated on the CSH C18 and resulted in 5 sharp, tall, narrow peaks within the first 5 minutes of the injection (Figure 15). While fewer overall peaks were identified using the CSH C18 in comparison to the UCT C18, the elution bands obtained from the CSH C18 are fully resolved and resemble a more ideal chromatographic shape. The initial OMP concentration was then decreased (15,10,5%) while maintaining the nine-minute linear gradient to assess if an

increased OMP slope would increase separation. Decreasing the initial concentration of OMP to 5% (Figure 16) increased the separation of the analogs in the column resulting in the addition of a sixth peak. While the 5% allowed for increased separation of the analogs, peaks lost the ideal shape resulting in broad, unsymmetrical peaks meaning the slope obtained from a linear 5-95% OMP was to step to obtain narrow bands in the column. Increasing the OMP to 10% (Figure 17) resulted in more ideal chromatography and a slow enough increase in 0.1% FA in ACN that separation using dipole and polar interactions was partially achieved. Although the 10% initial OMP resulted in the best combination of separation and chromatography of the twelve SFA thus far, the three isobars a-methyl/cis/trans-3-methyl thiofentanyl were not chromatographically separated. Due to the isobars having the same molecular weight and transitions, the isobars cannot be mass spectrally separated so chromatographic separation is the only mode for separation. For this reason, a 15% initial (Figure 18) OMP was evaluated; 10 peaks can be fully identified from 0-6 minutes including chromatographic separation of the three methyl thiofentanyl isobars.

While either chromatographic or mass spectral separation was achieved for all twelve isobars utilizing the linear gradient of 15-95% OMP on the CSH C18 column (Figure 18), ideal chromatography was not achieved for all 10 peaks- RT 1.14 is broader than the remaining 9 analog peaks showing norsufentanil and thienyl fentanyl did not achieve full separation in the elution. In an attempt to overcome the insufficient separation of norsufentanil and thienyl fentanyl when utilizing the CSH C18 column, a Waters UPLC BEH C18 was evaluated. While both columns are intended for separation based on polarity, the BEH and CSH have differing specificity and sensitivity. The BEH C18 column is packed with is C18 particles with bridged ethanes in the silicon matrix, whereas the CSH is packed with BEH particles that are coated in a

charged surface followed by the C18 end cap. Waters produced the BEH column for reliability and reproducibility, while the CSH was produced to maximize selectivity.[21] The CSH C18 and BEH C18 columns not only differ in silicon matrix, they also differ in particle size of the matrix- the CSH particle size is 2.5μm, while the BEH particle size is 1.7μm. The decrease in particle size increases the number of theoretical plates and requires a higher-pressure system for analysis to occur. The increase in theoretical plates and pressure allowed for a tighter analyte band in the column resulting in a narrow, tall symmetrical peak on the chromatogram (Figure 19-21) compared to the broader peaks observed when the same gradient was evaluated on the CSH column (Figure 15-18). Three of the gradients evaluated on the CSH were reevaluated on the BEH column: 5%, 10%, and 15% (Figure 19). Of the three gradients reevaluated, the same conclusions were made as with the CSH C18 column. The 5 and 10% OMP initial concentration did not provide a slow enough change in OMP to produce separation of the SFA, while the 15% OMP resulted in 10 baseline separated peaks.

While baseline separation with the most ideal chromatography obtained of all columns and gradients was achieved using the linear gradient from 15-95% OMP on the BEH column, increased baseline separation was desired. Knowing the SFA eluted from the column between 25-35%, the injection time was doubled (20 minutes) and a gentle curve of 7 and 8 from 15-40% over 17 minutes was evaluated (Figure 20). Doubling the time provided more time at each mobile phase composition resulting in a tighter band of each analyte within the column. The tighter band results in a narrower elution time of each analyte allowing for tall narrow peaks decreasing potential for interferences and increasing ease in quantitation. The curve of 7 resulted in more ideal chromatography while the curve of 8 lost baseline separation between BOH thiofentanyl/13C6 and thienyl fentanyl and resulted in more broad, shorter peaks.

The presence of tall, sharp, narrow, baseline separated peaks including the baseline separation of the methyl thiofentanyl isobars in the 20 minute, 15-60% 0.1FA in ACN, curve of 7 (Figure 21 and Table 2) gradient applied to the Waters BEH C18 column proves to be the best method evaluated on the UPLC system.

Shimadzu LC AD 20 coupled AB Sciex 5500 MS

Initially, dipole and pi-pi interactions were analyzed in a step-type gradient[22] using a PFPP column. The step type gradient combined with the dipole and pi-pi interaction of the PFPP column on the HPLC system resulted in separation of the Norsufentanil and 13C6 BOH Thiofentanyl while the remaining SFA eluted from the column between 18 and 19 minutes (Figure 22).

Upon identification of insufficient separation and chromatography of the SFA using dipole and pi-pi interactions, separation based more on polarity was analyzed. Three variations of a nonpolar column UCT C18, Restek C18, and Water's CSH C18 (charged surface hybrid) were analyzed. The UCT C18 column was initially analyzed on the HPLC system with the same step-type gradient evaluated on the PFPP and resulted in similar chromatography as the PFPP. Elution of most analogs was within the last minute of the injection run and only one analog separated with broad unsymmetrical peaks (Figure 23).

After additional thought into the unique structural aspect of each SFA structure, the UCT C18 was evaluated again on the HPLC system but with a linear gradient of 5-95% OMP (Figure 24) to identify the percentage of 0.1% FA in ACN the structurally similar SFA eluted at. The linear gradient resulted in all twelve analogs eluting from the column between 5.5 and 7 minutes with peaks that exhibited more optimal shape: tall, narrow, and symmetrical. Elution of the SFA

analogs was identified at approximately 30% OMP (ACN). The initial OMP concentration was then varied (10,15,20%) to assess chromatography and separation of the SFA. The 20% OMP (Figure 25) and 10% OMP (Figure 26) resulted in similar chromatography with all analogs eluting between 5.8 and 7 minutes and no baseline separation between the tall narrow peaks. The 15% OMP resulted in comparable peak shape and resolution but with a shorter retention time, all analogs eluted from the UCT C18 column between 5 and 7 minutes.

At variable initial concentrations of OMP, the UCT C18 was unable to achieve baseline separation of the SFAs, so a Restek C18 column was evaluated. Two concentrations of initial OMP were evaluated, 5 and 20%. The linear gradient of 5-95% (Figure 28) and 20-95% (Figure 29) OMP on the Restek C18 column both resulted in fewer overall peaks compared to those observed utilizing the UCT C18, and the few peaks observed were broad and unseparated.

The absence of ideal chromatography combined with the lack of separation of the peaks observed when the linear gradient was applied to the Restek C18 column led to the evaluation of the Waters CSH C18. The 5% initial (Figure 30) OMP resulted in unresolved narrow peaks while the 10% (Figure 31) and 15% (Figure 32) showed resolution of the methyl thiofentanyl isobars. While both the 10-95% and 15-95% linear OMP were the only chromatographic methods evaluated that resulted in ideal peak shape and separation of the methyl thiofentanyl isobars, the 15-95% linear method on the CSH C18 resulted in the best separation of the isobar peaks of the methods evaluated.

Of the methods evaluated on the HPLC system, the 15-95% linear gradient on the Waters CSH C18 column resulted in the most ideal chromatography and separation of the methyl thiofentanyl isobar but did not achieve baseline separation of all SFA. In an attempt to achieve similar separation to that achieved on the UPLC system with the Waters BEH C18, a curved

gradient was evaluated from 15-60% OMP. The curve of 3 (Figure 33) – similar exponential increase to that identified on the UPLC system- allowed for a gradual increase between 15 and 40% OMP resulting in each analog eluting from the column before the next analog eluted.

The presence of tall, sharp, narrow, baseline separated peaks including the baseline separation of the methyl thiofentanyl isobars in the 20 minute, 15-40% 0.1FA in ACN, curve of 3 (Figure 21 and Table 3) gradient applied to the Waters CSH C18 column proves to be the best method evaluated on the HPLC system

Of the columns tested, the ultra-pressure BEH C18 resulted in the best chromatographic separation of the eleven analogs on the UPLC-MS/MS system. The BEH C18 column available was an ultra-pressure column, therefore the backpressure of the HPLC system was too high for the BEH C18 to be used. The CSH C18 resulted in the best chromatic separation of the analogs on the HPLC-MS/MS system. Both the CSH and BEH C18 columns resulted in a narrow elution time of the analogs producing a narrow, sharp peak.

Separation of Structurally Similar Analog Classes

Potential interferences from three other fentanyl classes (acryl fentanyl, butyryl fentanyl, and furanyl fentanyl) were analyzed against the mass spectral method for the SFA using the optimized UPLCMSMS method- two interferences were detected: fluoro furanyl fentanyl and methyl furanyl fentanyl. These interferences were overcome when separation was based on pi-pi bonding using a biphenyl column (kinetics core shell 2.6 μm 100x3mm) (Figure 34). Utilization of the biphenyl column resulted in a longer retention time for the thio-ring analogs and a short retention time for the furanyl analogs. The greater affinity for the biphenyl stationary phase can

be attributed to the phenyl group substitution of the thiophene and the propanamide substitution of the furanyl.

Aim 3: Plasma Extraction Method

The ideal PE and RE is 100% while the ideal ME is 0% each with an acceptable error rate of 25%. Peak height and peak area were evaluated across the four extractions to determine the most efficient extraction method. Of the three extraction methods evaluated, the alkaline LLE[19] resulted in over ideal ME and lower than ideal RE/PE (Table 5). The MCX μelution[3] SPE resulted in over ideal RE/PE, but ME that was within the ideal range (Table 6). While the MCX μelution proved to have less ME than the alkaline LLE, ion suppression of BOH thiofentanyl and BOH thiofentanyl 13C6 was noted resulting in the evaluation of an additional extraction technique. The MP3 proved to not be as consistently out of ideal PE, RE, and ME (Table 7). The MP3 extraction technique provided ideal RE, PE, and ME for BOH thioacetylfentanyl and thienyl fentanyl as well as ideal RE was achieved for all analogs other than BOH thiofentanyl 13C6 and tetrahydrothiophene fentanyl. The MP3 extraction resulted in overall more ideal RE, PE, and ME, but the non-ideal ME and PE were inconsistent and resulted in some analogs such as BOH thiofentanyl and thiophene fentanyl with lower than ideal ME and some analogs such as tetrahydrothiophene fentanyl and alpha-methyl thiofentanyl with higher than ideal ME.

The inconsistencies observed when the SFA were extracted using the MP3 column resulted in the evaluation of an additional extraction technique found in the literature: UCT DAU Purple micro-extraction columns. While not perfect, the UCT purple extraction method resulted in consistent RE, PE, and ME allowing eight of the twelve analogs to achieve ideal RE, PE, and

ME (Table 8). Norsufentail, BOH thiofentanyl, and tetrahydrothiophene fentanyl were observed to have low recovery and processing efficiency, where BOH thiofentanyl 13C6 only observed lower than ideal recovery. Due to the consistency and overall increased ideal RE, PE, and ME across all twelve analogs, the UCT Purple microextraction was chosen as the extraction method of choice.

To determine specificity of the screening method developed, 10 patient serum samples (Figure 35), common drugs of abuse (BioRad C4, Figure 36), common pharmaceuticals (BioRad TDM3, Figure 37), common abnormalities in serum (BioRad Liquid Unassayed Multiqual, Figure 38), and three fentanyl classes (acryl, butyryl, and furanyl) were evaluated. All five potential interference groups evaluated resulted in the presence of only the added internal standards fentanyl D5, norfentanyl D5, and 13C6 BOH thiofentanyl (Figure35-38). The absence of peaks beyond the internal standards shows the screening method including the mass spectral method (Figure 1), the liquid chromatographic method (Figure 21), and the UCT DAU extraction method are specific for the twelve SFA class identified in this study and other potentially present compounds will not appear as a false positive.

The determination of a cutoff concentration post-preparative consisted of extracting nine concentrations varying from 10pg/mL to 1000pg/mL (0.01-1ng/mL) to evaluate a linear curve. Cutoff concentration was determined by the lowest concentration where 50% and 150% of target concentration can be definitively distinguishable. Figures 38 and 39 show the difference in including and excluding the higher concentrations (250-1000pg/mL). When the upper limit concentrations are included in the linear curve (Figure 39), the lower limit concentration linearity is distorted, whereas exclusion of the upper limit values (Figure 40) allows for the lowest linear portion of the curve to be identified. After evaluation of the lower linearity across all twelve

analogs, 50pg/mL was identified as the empirical cutoff due to the distinct separation and linearity at between 25, 50, and 75pg/mL.

The final SWGTOX qualitative validation is precision at the decision point meaning consistency at the empirical cutoff value. The precision was evaluated in a 5-extraction n=3 at 25, 50, and 75pg/mL with Fentanyl D5 as the internal standard for post-preparative concentration determination (Figure 41). Fentanyl D5 was chosen as the internal standard in place of 13C6 BOH Thiofentanyl as Fentanyl D5 is more stable and did not coelute with any of the analogs. Figure 40 highlights the separation of the cutoff concentration from the negative and positive concentrations. Ten of the eleven SFA evaluated for precision at the cutoff value achieved distinction of the 50% above (75pg/mL) and 50% below (25pg/mL). Based on the indistinction of the cutoff concentration from the 50 above and 50% below concentration concerning Norsufentanil, the screening method provided could not be validated for this analog. All remaining ten analogs achieved the desired distinction of the cutoff from the 50% below and 50% above concentrations across the five-extraction triplicate validating the screening method provided with the use of Fentanyl D5 as the internal standard.

Conclusion

A qualitative screening method for SFA including a mass spectral, liquid chromatographic, and extraction method have been validated for the separation and identification of eleven of the twelve analogs evaluated: a-Methyl Thiofentanyl, b-Hydroxythioacetylfentanyl, b-Hydroxythiofentanyl, b-Hydroxythiofentanyl 13C6, cis-3-Methyl Thiofentanyl, Sufentanil, Tetrahydrothiophene Fentanyl, Thienyl Fentanyl, Thiofentanyl, Thiophene Fentanyl, and trans-3-methyl Thiofentanyl. Validation included selectivity, carryover, sensitivity, and precision outlined by SWGTOX.

The mass spectral method developed included manual optimization of the ce, dp, cone voltage, and cxp for all analogs followed by manual identification of the most stable unique daughter ions of each analog. The mass spectral method developed includes unique product ions for all SFA allowing for unique identification of all twelve analogs including the me-thiofentanyl isobars.

Three column types of several manufactures including biphenyl, PFPP, and C18 were evaluated at various mobile phase gradients including step type, linear, and curved with a variation in injection time to achieve baseline separation of the SFA. The gradient and injection time to achieve the desired chromatographic profile of the SFA was a 20.5-minute injection with a curved and linear gradient. A gentle exponential increase in OMP from 15-40% over 16 minutes on both the UPLC and HPLC system on Waters C18 columns resulted in optimal separation of all twelve analogs including baseline separation of the a-methyl, cis-3-methyl, and trans-3-methyl thiofentanyl isobars. The increased backpressure capable in the UPLC system allowed for the use of the BEH C18 column with sub 2 micro particle size, while the HPLC system required a larger particle size to accommodate for the lower backpressure of the system.

The Waters CSH C18 and BEH C18 had similar separation, but due to the increased theoretical plates in the BEH C18 column the BEH C18 resulted in overall sharper peaks. The decreased possible system backpressure in the HPLC system did not allow for use of the BEH C18 and therefore the best chromatographic separation was achieved using the Waters CSH C18 column.

Several extraction methods were evaluated including an alkaline LLE, Waters Oasis melution, MP3, and the UCT DAU Purple micro. Of the four extraction methods evaluated, the UCT DAU column utilizing the wash buffers LCMS H_2O, 100mM acetic acid, and hexane followed by the elution slolvent dicholormethane: isopropanol: ammonium hydroxide (DCM:IPA: NH_4OH) (78:20:2) resulted in the most consistent and overall ideal conditions of less than 15% variation from the mean ME, PE, and RE.

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Appendix A: Tables

Table 1.

		Sciex		Waters
Analog	**Transitions**	**ce**	**dp**	**ce**
Norsufentanil	277>184	18	57	18
	277>245	18		18
Thienyl Fentanyl	329>97	30	60	30
	329>180	25		25
Thiofentanyl	343>111	26	62	34
	343>194	32		24
β OH Thioacetylfentanyl	345>192	29	51	29
	345>327	23		23
α-Me Thiofentanyl	357>125	35	65	40
	357>259	23		34
trans-3-Me Thiofentanyl	357>208	33	48	24
	357>259	33		34

		Sciex		Waters
Analog	**Transitions**	**ce**	**dp**	**ce**
cis-3-Me Thiofentanyl	357>111	35	60	32
	357>208	31		24
β OH Thiofentanyl	359>146	33	56	24
	359>192	28		22
β OH Thiofentanyl 13C6	365>152	25	68	22
	365>192	31		22
Sufentanil	387>238	27	75	27
	387>355	27		27
Thiophene Fentanyl	391>105	26	63	26
	391>188	26		26
Tetrahydrothiophene Fentanyl	395>105	33	70	33
	395>188	33		33

Table 1 SFA transitions and respective voltages utilized for the identification of the twelve analogs on both the UPLC MS/MS and AB Sciex-5500 triple quad mass spectrometer. Cone voltage for all transitions was held at 16V.

Table 2.

Time	%A	%B	Curve
0	85	15	6
1	85	15	6
17	60	40	7
17.8	5	95	6
18.5	5	95	6
19	85	15	6
20.5	85	15	6

Table 2. Most effective time table gradient to separate the SFA on the Waters BEH C18 column.

Table 3.

Time	%A	%B	Curve
0	85	15	0
1	85	15	0
17	40	60	3
17.8	5	95	0
18.5	5	95	0
19	85	15	0
20.5	85	15	0

Table 3. Most effective time table gradient to separate the SFA on the Waters CSH C18 column.

Table 4.

Analog	RT UPLC (min)	RT HPLC (min)
BOH Thioacetylfentanyl	2.92	5.16
Norsufentanil	5.07	8.41
BOH Thiofentanyl	5.64	9.32
BOH Thiofentanyl (13C6)	5.64	9.32
Thienyl Fentanyl	5.86	9.51
Thiofentanyl	7.42	11.19
a-methyl Thiofentanyl	8.16	11.87
trans-3-methyl Thiofentanyl	8.76	12.48
cis-3-methyl Thiofentanyl	9.14	12.78
Tetrahydrothiophene Fentanyl	10.48	13.99
Thiophene Fentanyl	10.97	14.43
Sufentanil	11.01	14.48

Table 4. Retention times of all twelve analogs on both UPLC and HPLC systems when gradient from Table 2 and 3 are applied to the BEH and CSH C18 column respectively.

Table 5

		BOH Thioacetylfentanyl	Norsufentanil	BOH Thiofentanyl	13C6 BOH Thiofentanyl	Thienyl Fentanyl	Thiofentanyl	a-me Thiofentanyl	trans-3-me Thiofentanyl	cis-3-me Thiofentanyl	Tetrahydrothiophene Fentanyl	Thiophene Fentanyl	Sufentanil
Peak Area	ME	236	29	57	185	162	158	195	183	268	1456	488	307
	RE	42	30	5	35	31	31	45	27	28	31	18	24
	PE	142	39	7	101	82	79	13	76	76	488	108	97

Table 5. Percent matrix effect (ME), recovery (RE), and processing efficiency (PE) calculated from peak area after extraction using the alkaline LLE technique. The values in red are outside of the ideal error rate of 25%.

Table 6

		BOH Thioacetylfentanyl	Norsufentanil	BOH Thiofentanyl	13C6 BOH Thiofentanyl	Thienyl Fentanyl	Thiofentanyl	a-me Thiofentanyl	trans-3-me Thiofentanyl	cis-3-me Thiofentanyl	Tetrahydrothiophene Fentanyl	Thiophene Fentanyl	Sufentanil
Peak Area	ME	-22	31	NA	NA	-1	-21	4	-4	4	-1	23	-51
	RE	173	84	NA	NA	135	174	162	138	148	160	128	91
	PE	134	109	NA	NA	134	137	168	132	154	158	159	45

Table 6. Percent matrix effect (ME), recovery (RE), and processing efficiency (PE) calculated from peak area after extraction using the Waters Oasis µelution technique. The values in red are outside of the ideal error rate of 25%.

Table 7

		BOH Thioacetylfentanyl	Norsufentanil	BOH Thiofentanyl	13C6 BOH Thiofentanyl	Thienyl Fentanyl	Thiofentanyl	a-me Thiofentanyl	trans-3-me Thiofentanyl	cis-3-me Thiofentanyl	Tetrahydrothiophene Fentanyl	Thiophene Fentanyl	Sufentanil
Peak Area	ME	31	26	-83	58	22	55	165	61	74	181	75	26
	RE	95	113	364	103	106	105	86	98	97	68	84	113
	PE	124	142	61	162	129	162	227	157	169	190	147	142

Table 7. Percent matrix effect (ME), recovery (RE), and processing efficiency (PE) calculated from peak area after extraction using the MP3 SPE technique. The values in red are outside of the ideal error rate of 25%.

Table 8

		BOH Thioacetylfentanyl	Norsufentanil	BOH Thiofentanyl	13C6 BOH Thiofentanyl	Thienyl Fentanyl	Thiofentanyl	a-me Thiofentanyl	trans-3-me Thiofentanyl	cis-3-me Thiofentanyl	Tetrahydrothiophene Fentanyl	Thiophene Fentanyl	Sufentanil
Peak Area	ME	-1	-8	19	31	-10	-2	-3	0	-4	2	-3	16
	RE	69	18	48	56	69	70	63	65	65	55	67	55
	PE	68	16	57	73	62	68	61	65	63	56	65	64

Table 8. Percent matrix effect (ME), recovery (RE), and processing efficiency (PE) calculated from peak area after extraction using the UCT DAU (purple) micro technique. The values in red are outside of the ideal error rate of 25%.

Appendix B: Figures

Figure 1.

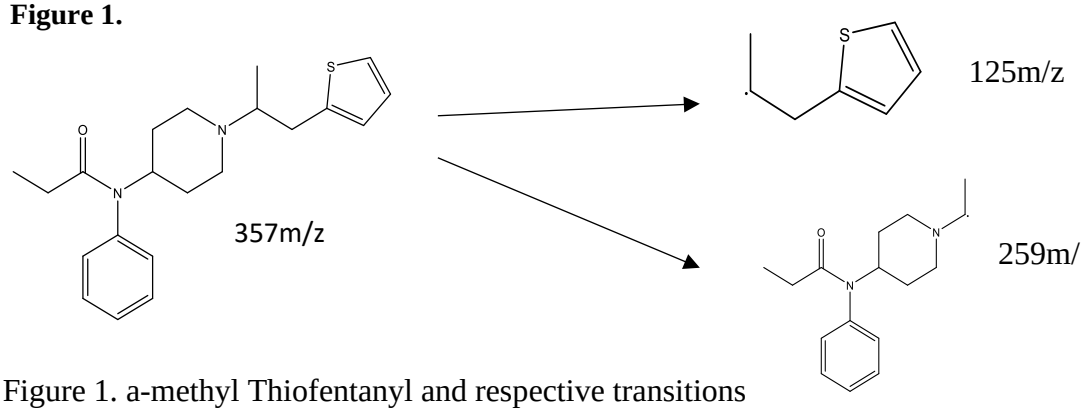

Figure 1. a-methyl Thiofentanyl and respective transitions

Figure 2.

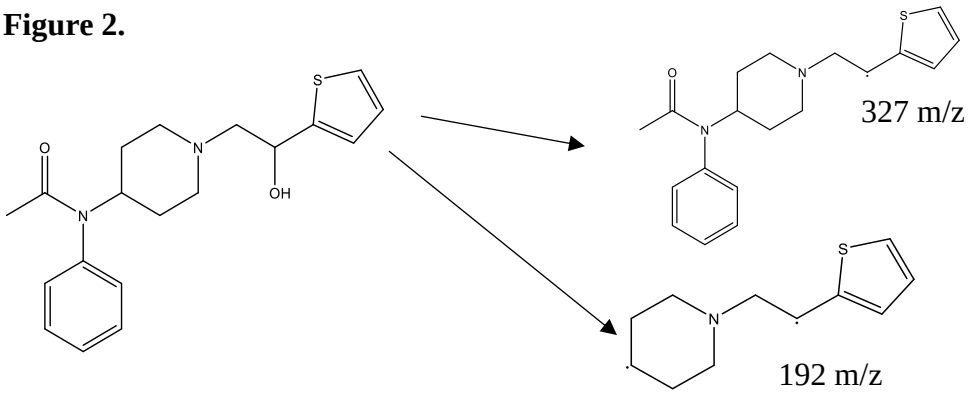

Figure 2. BOH Thioacetylfentanyl and respective transitions

Figure 3.

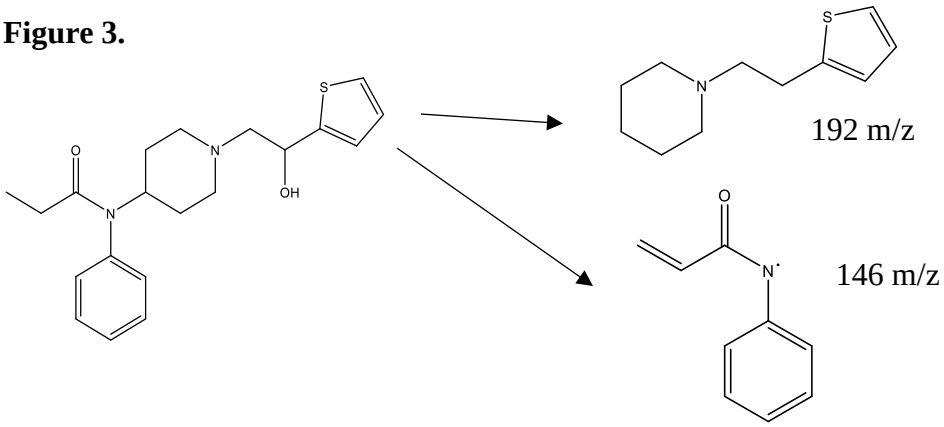

Figure 3. BOH Thiofentanyl and respective transitions

Figure 4.

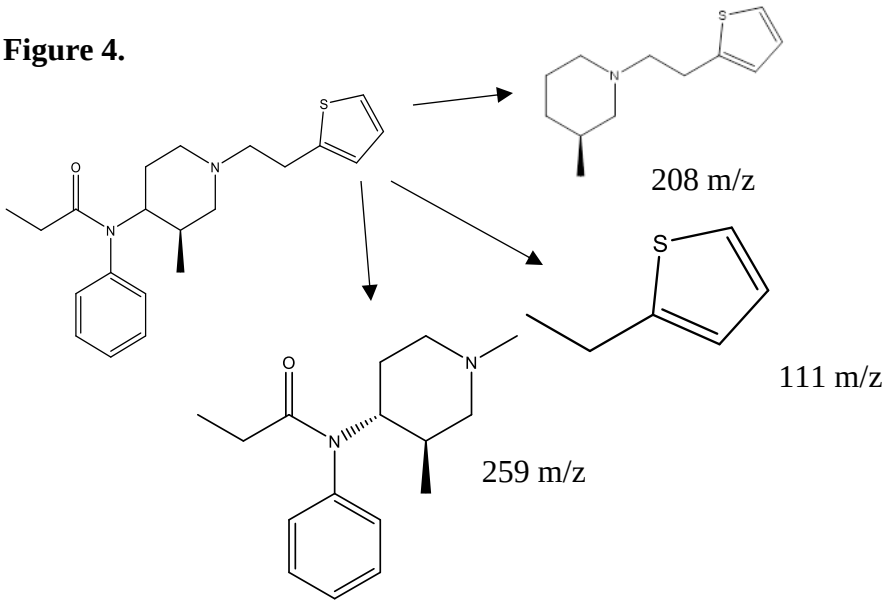

Figure 4. cis/trans-3-methyl Thiofentanyl and respective transitions

Figure 5.

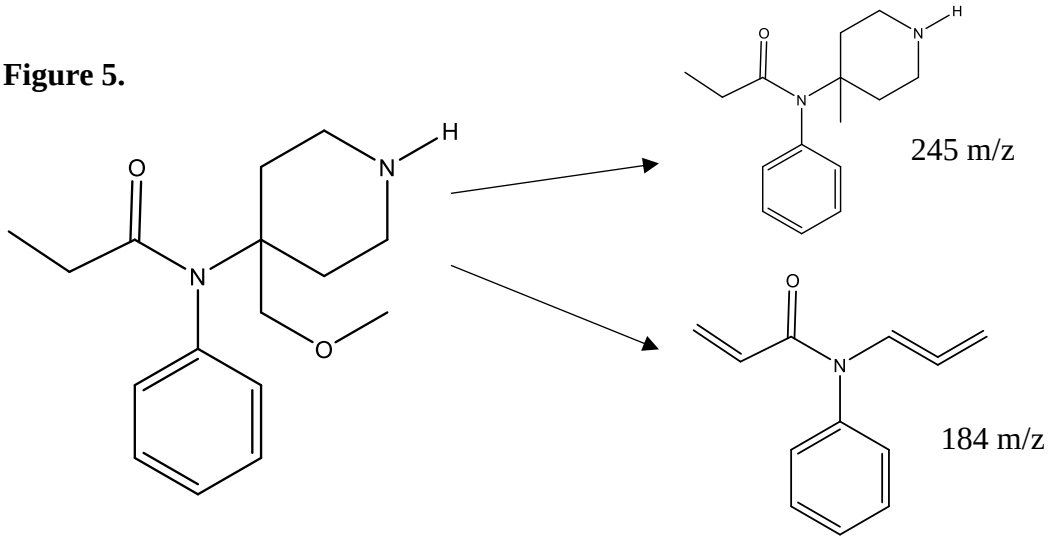

Figure 5. Norsufentanil and respective transitions

Figure 6.

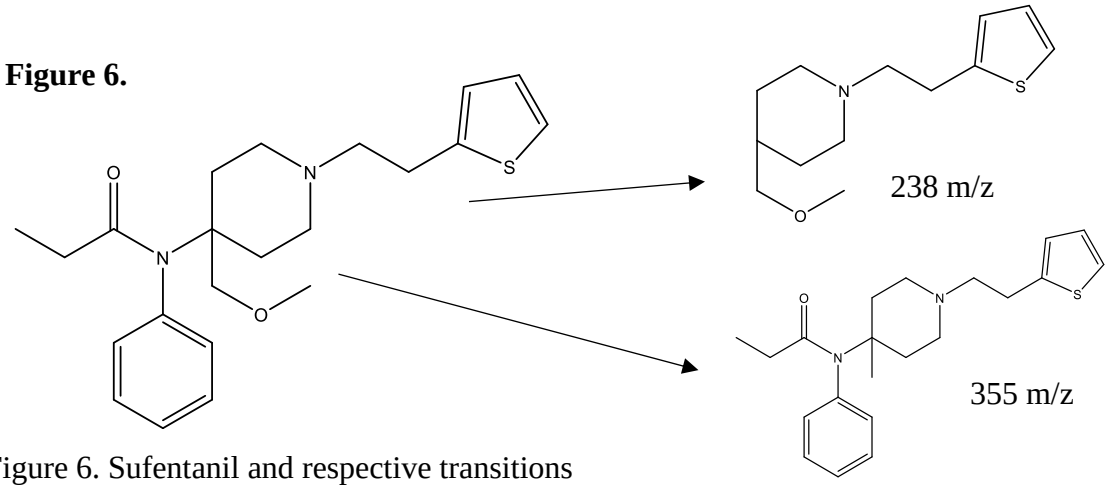

Figure 6. Sufentanil and respective transitions

Figure 7.

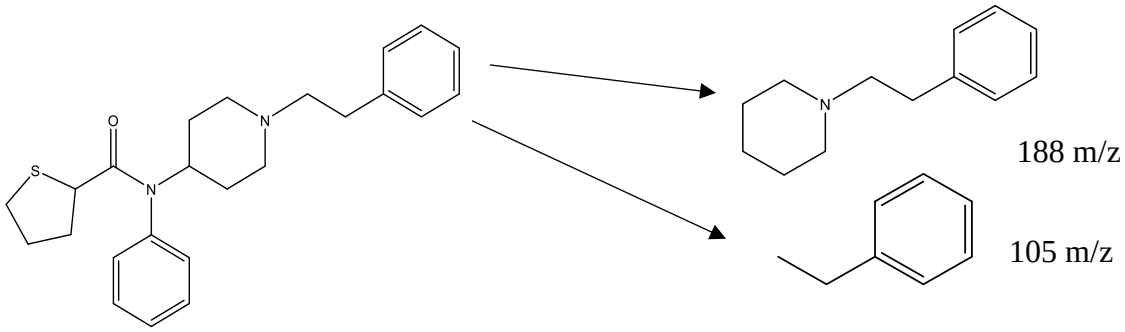

Figure 7. Tetrahydrothiophene and respective transitions

Figure 8.

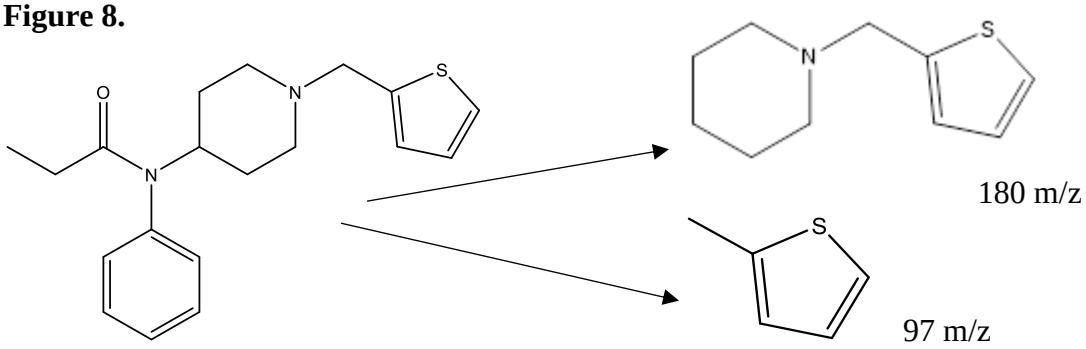

Figure 8. Thienyl Fentanyl and respective transitions

Figure 9.

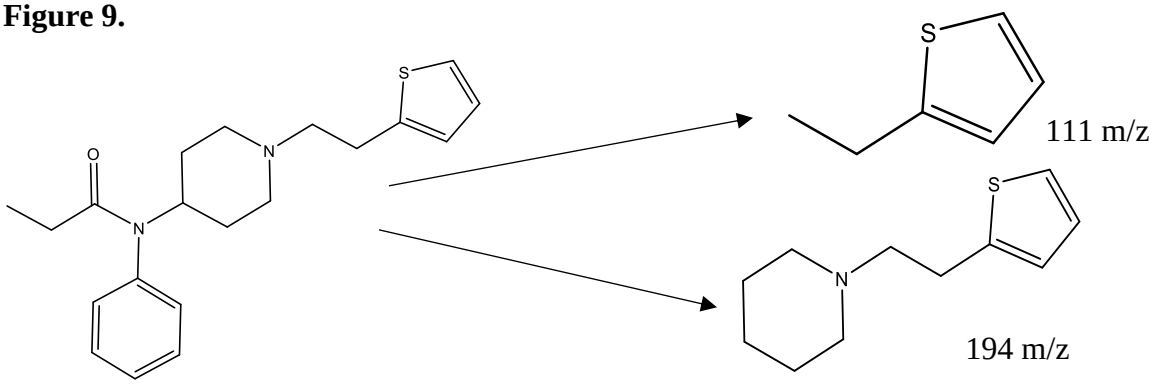

Figure 9. Thiofentanyl and respective transitions

Figure 10.

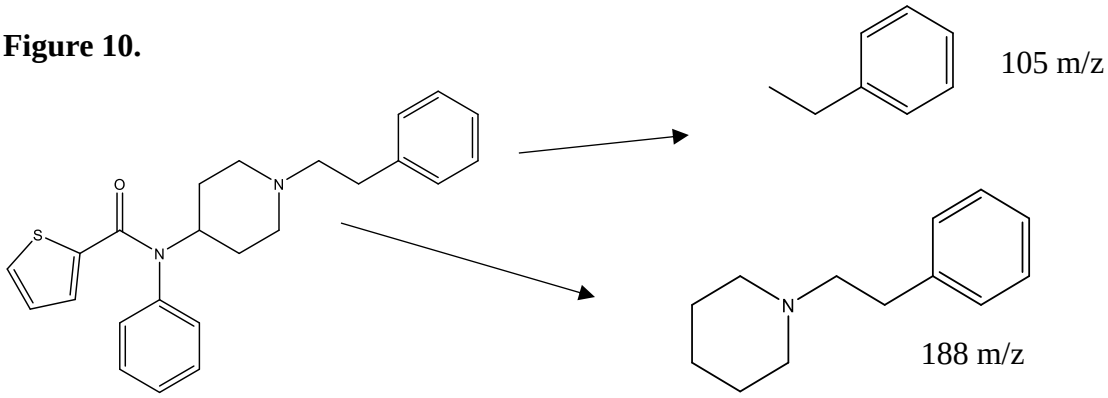

Figure 10. Thienyl Fentanyl and respective transitions

Figure 11.

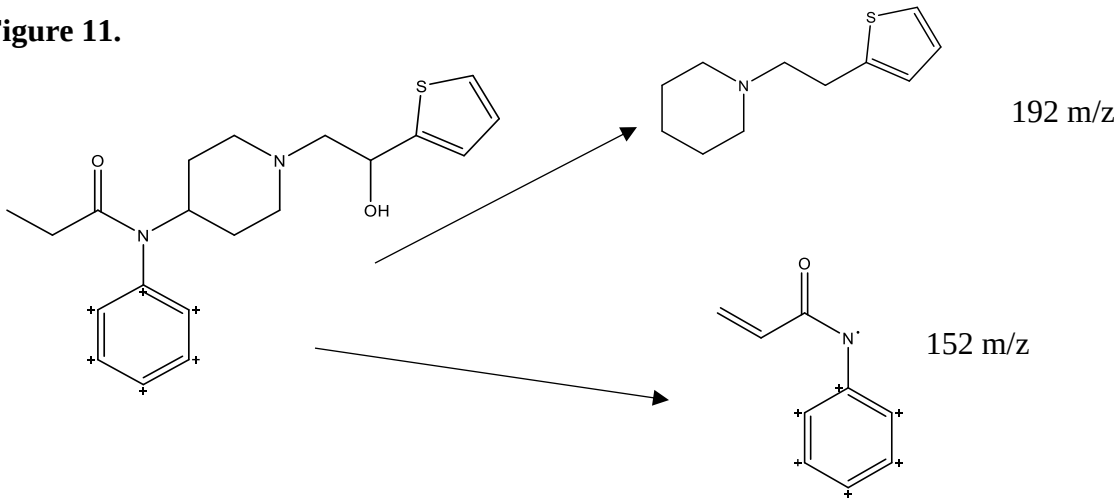

Figure 11. 13C6 BOH Thiofentanyl and respective transitions

Figure 12.

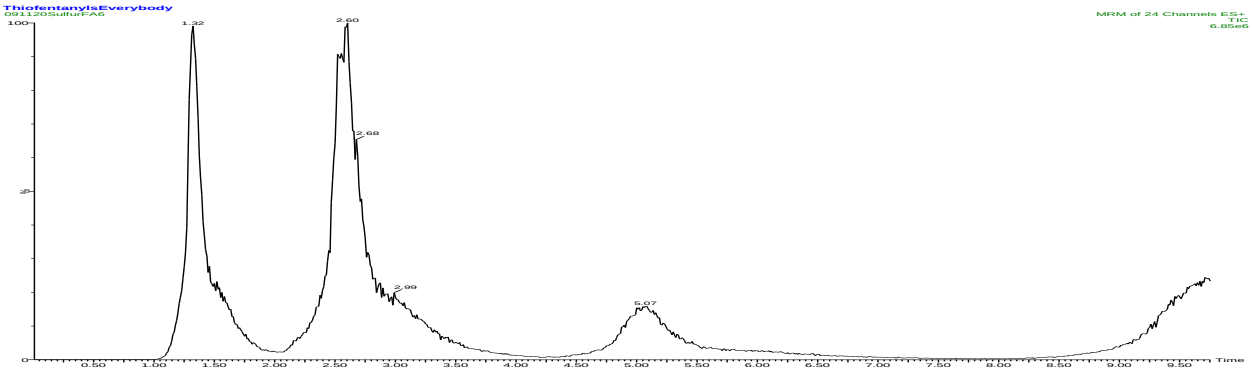

Figure 12. Chromatography of all twelve SFA eluting off the Biphenyl column when a curved, step-type gradient[22] was applied using 0.1% FA in water (aqueous) and 0.1% FA in ACN (OMP). 3 nonsymmetrical, broad, and not baseline resolved peaks were identified to contain the twelve SFA. Instrument: Waters ACQUITY TQs UPLC.

Figure 13.

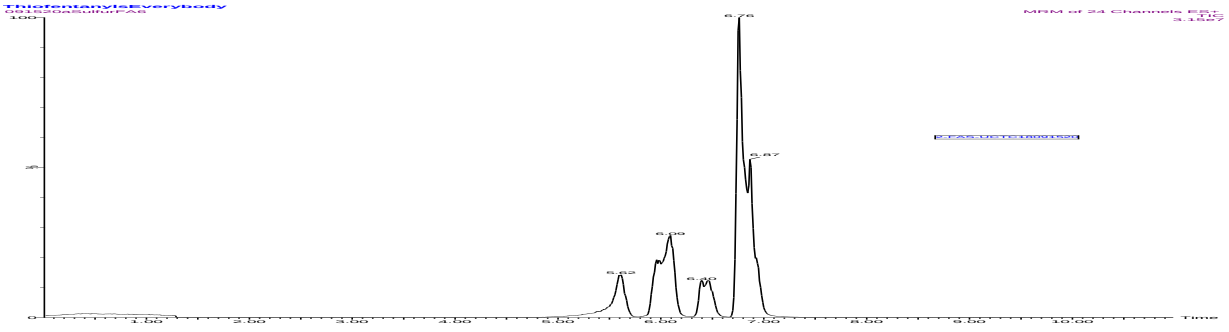

Figure 13. Chromatography of all twelve SFA eluting from the UCT C18 column when a linear gradient of 5-95% 0.1% FA in ACN over nine minutes was applied. All twelve analogs eluted from the column between 5 and 7 minutes resulting in 7 unresolved, broad, short peaks. Instrument: Waters ACQUITY TQs UPLC.

Figure 14.

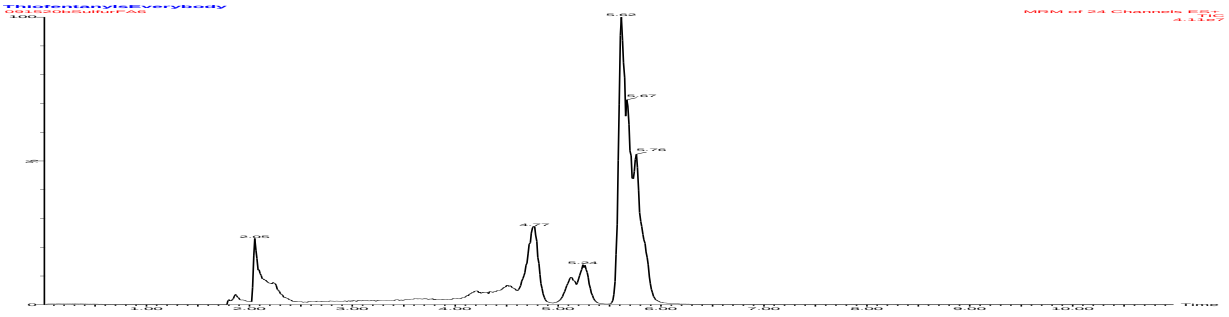

Figure 14. Chromatography of all twelve SFA eluting from the UCT C18 column when a linear gradient of 20-95% 0.1% FA in ACN over nine minutes was applied. All twelve analogs eluted from the column between 1.5 and 6 minutes resulting in 7/8 unresolved, broad peaks. Instrument: Waters ACQUITY TQs UPLC.

Figure 15.

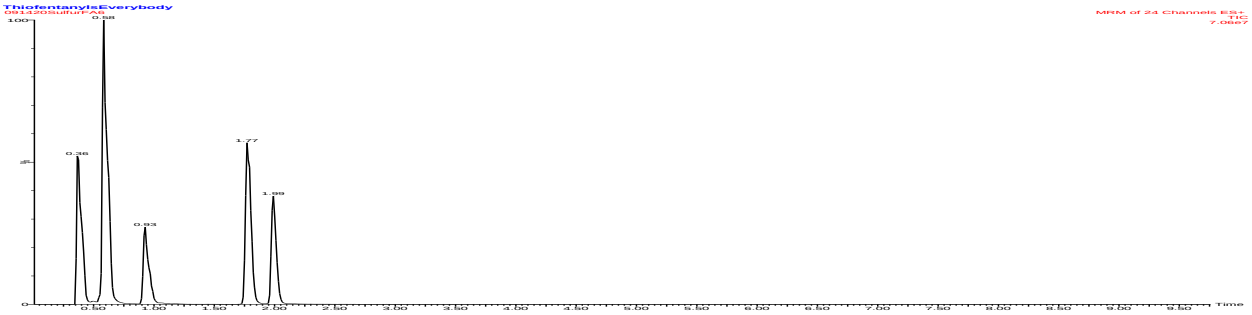

Figure 15. Chromatogram of all twelve SFA eluting off the Waters X-Select C18 column when a linear gradient of 20-95% 0.1%FA in ACN over 9 minutes was applied. All analogs eluted from the column between 0 and 2 minutes with five defined peaks. The five peaks resulted in narrow, symmetrical, tall, almost baseline separated peaks. Instrument: Waters ACQUITY TQs UPLC**.**

Figure 16

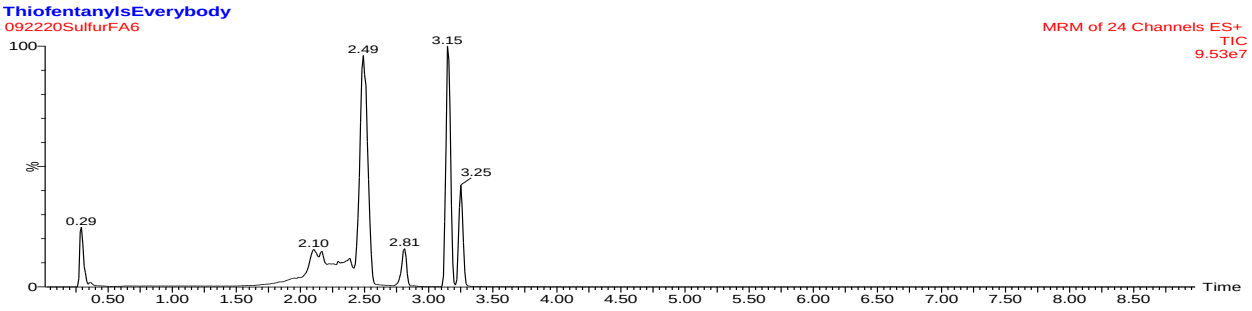

Figure 16. Chromatogram of all twelve SFA eluting off the Waters X-Select C18 column when a linear gradient of 5-95% 0.1%FA in ACN over 9 minutes was applied. All analogs eluted from the column between 0 and 3.5 minutes with four defined peaks and one large broad unresolved several analog elution from 1.5minutes to 2.5 minutes. Instrument: Waters ACQUITY TQs UPLC.

Figure 17.

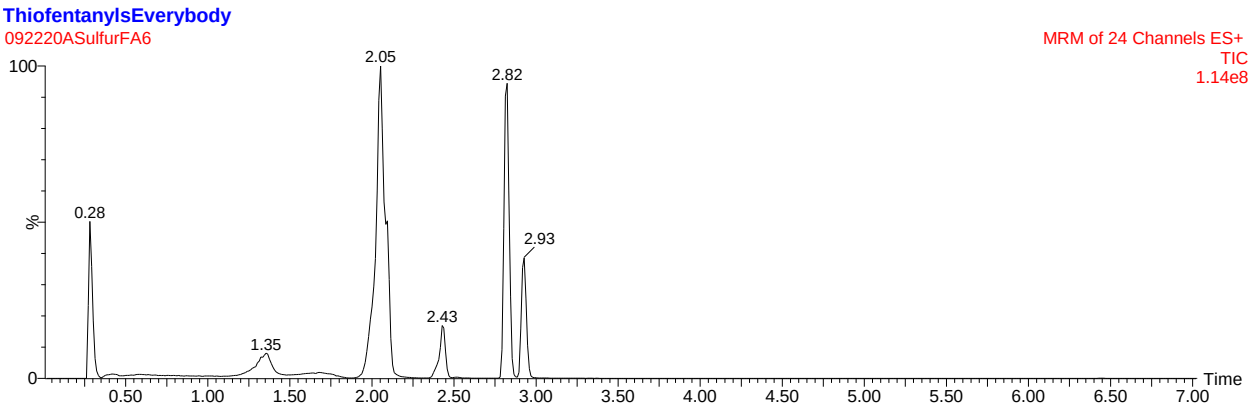

Figure 17. Chromatogram of all twelve SFA eluting off the Waters X-Select C18 column when a linear gradient of 10-95% 0.1%FA in ACN over 9 minutes was applied. All analogs eluted from the column between 0 and 3 minutes with six defined peaks. The six peaks resulted in narrow, symmetrical, tall, almost baseline separated peaks. Instrument: Waters ACQUITY TQs UPLC.

Figure 18.

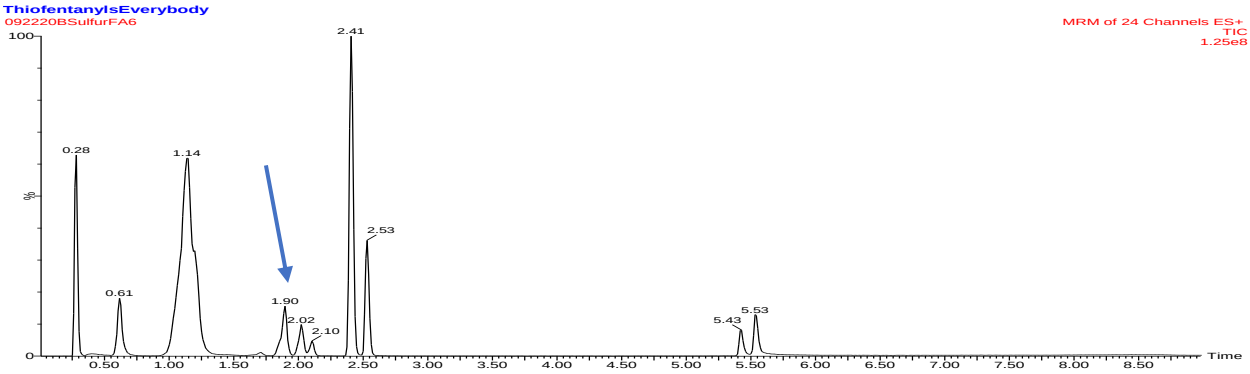

Figure 18. Chromatogram of all twelve SFA eluting off the Waters X-Select C18 column when a linear gradient of 15-95% 0.1%FA in ACN over 9 minutes was applied. All analogs eluted from the column between 0 and 6 minutes with ten defined peaks including chromatic separation of the a-methyl thiofentanyl (RT: 1.90), trans-3-methyl thiofentanyl (RT: 2.02), and cis-3-methyl thiofentanyl (RT:2.16). The ten peaks resulted in narrow, symmetrical, tall, almost baseline separated peaks. Instrument: Waters ACQUITY TQs UPLC.

Figure 19.

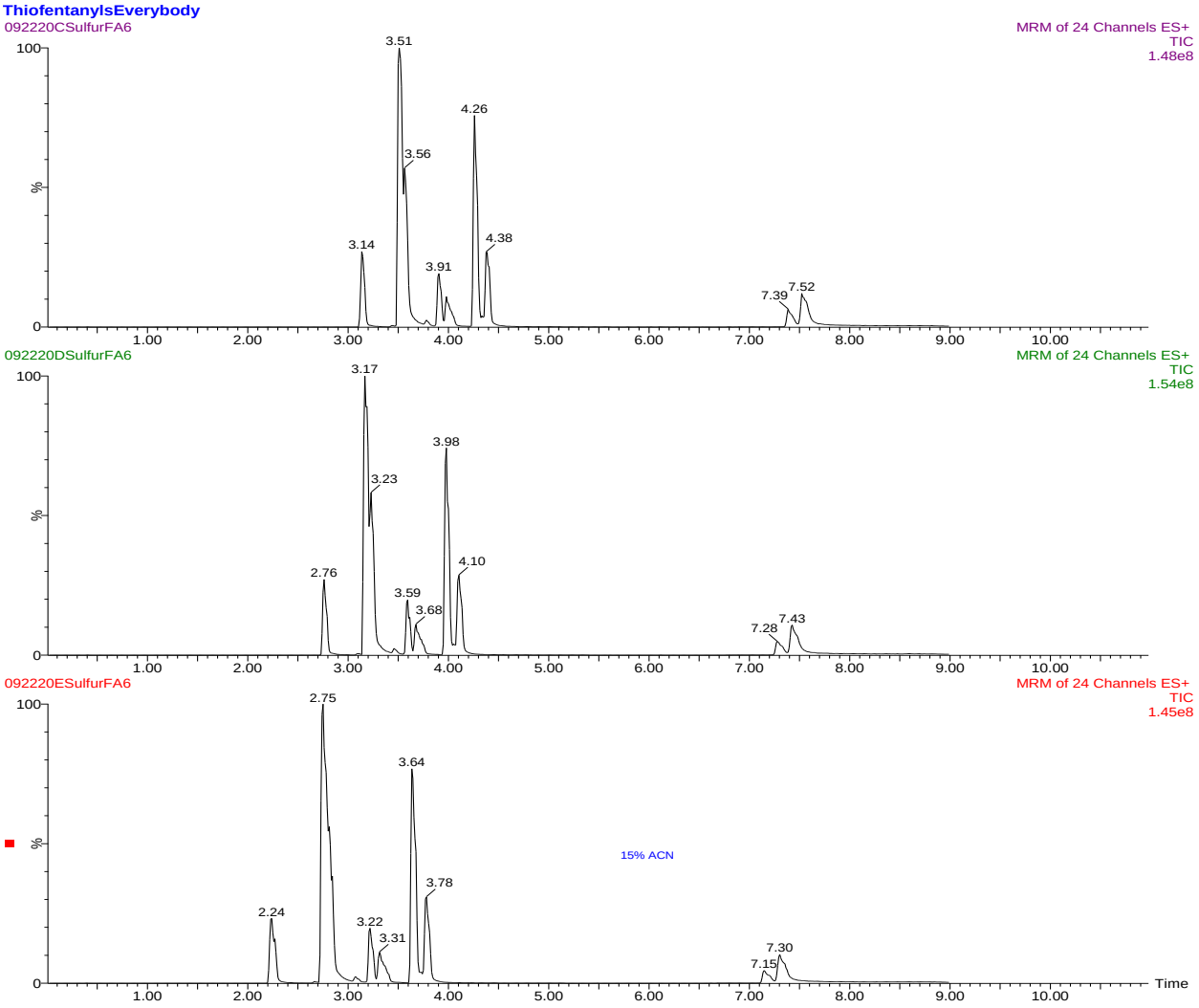

Figure 19. Three chromatograms showing the elution of all twelve SFA when three different mobile phase gradients were applied to the column. The top chromatogram shows 5-95% FA in ACN over a linear gradient, the middle chromatogram shows 10-95% FA in ACN over a linear gradient, the bottom chromatogram shows 15-95% FA in ACN over a linear gradient. While the resolution of the analogs does not change over the three gradient conditions, the elution of the SFA is about 0.5 minutes quicker as the initial concentration of the OMP increases by 5%. Only eight peaks can be identified in the chromatograms of the 5,10,15% initial OMP conditions. Instrument: Waters ACQUITY TQs UPLC.

Figure 20.

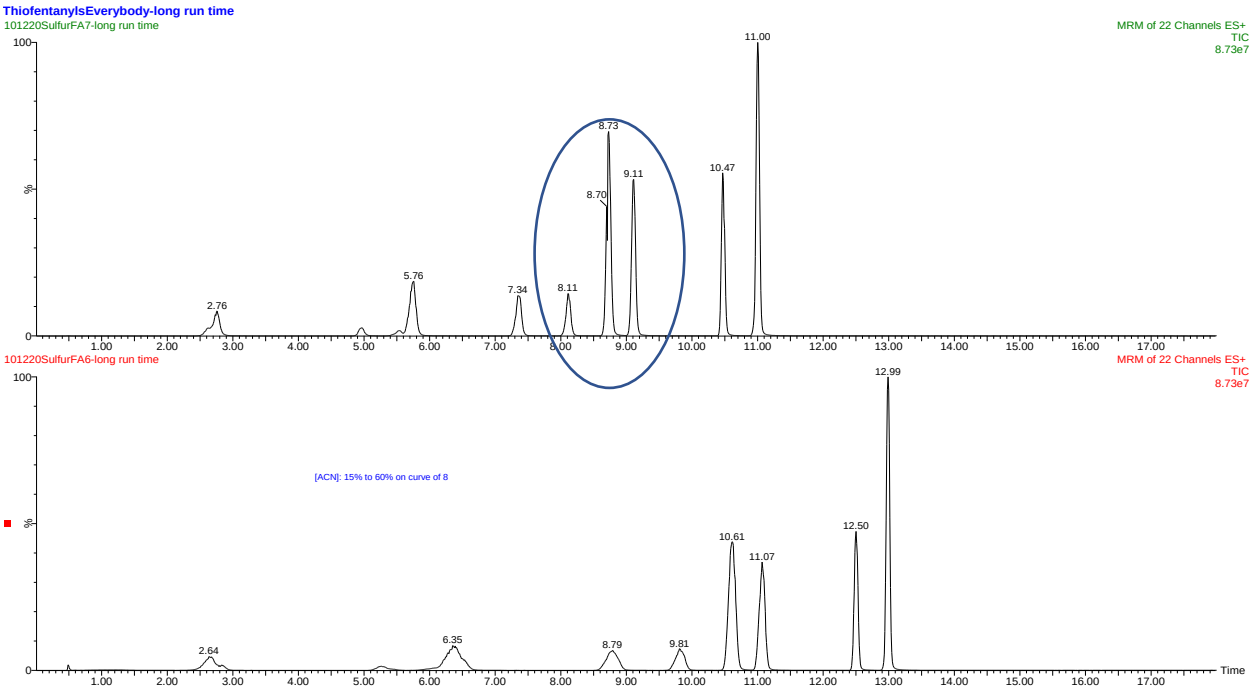

Figure 20. The injection time was increased to 20 minutes and the addition of a curve was added to the gradient. Top chromatogram: curve of 7 from 15-60% OMP over 17 minutes followed by a linear ramp to 95% OMP for 1.5 minutes. Bottom Chromatogram: curve of 8 from 15-60% OMP over 17 minutes followed by a linear ramp to 95% OMP for 1.5 minutes. The addition of the curve allowed for 10 peaks to be defined and resolved. Analogs eluted from the column as follows: BOH thioacetylfentanyl, norsufentanil, BOH thiofentanyl/BOH thiofentanyl 13C6, thienyl fentanyl, thiofentanyl, alpha-methyl thiofentanyl, trans-3-methyl thiofentanyl, cis-3-methyl thiofentanyl, tetrahydrothiophene fentanyl, thiophene fentanyl, and sufentanil. Instrument: Waters ACQUITY TQs UPLC.

Figure 21.

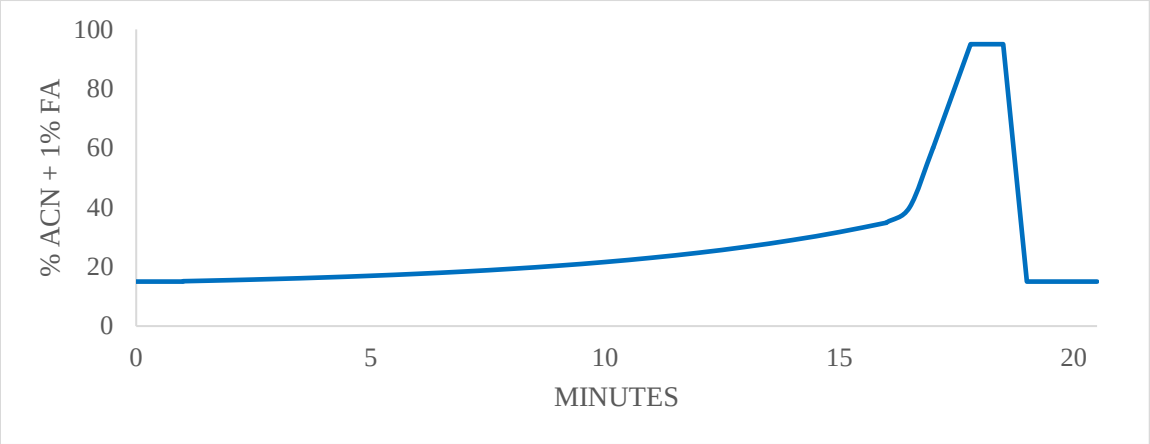

Figure 21. Graphical representation of the most effective gradient to separate the SFA on the Waters BEH C18 column and the Waters CSH C18 column.

Figure 22.

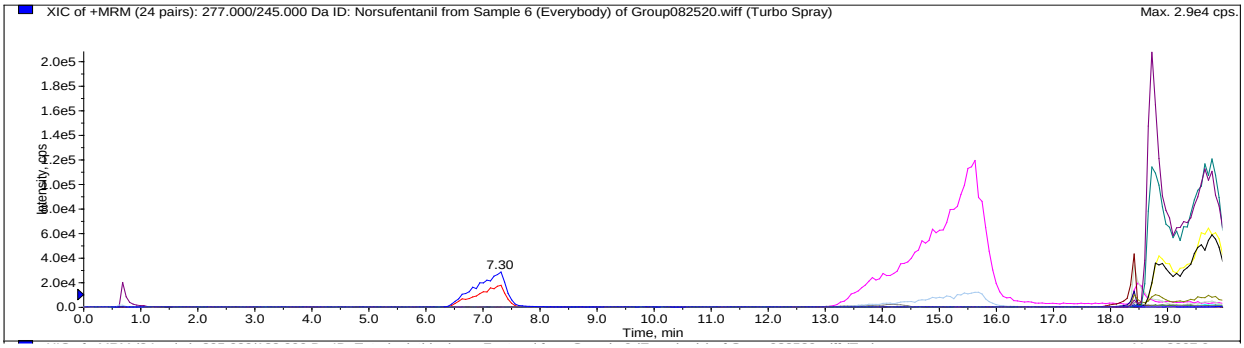

Figure 22. Chromatogram of all twelve SFA eluting off the PFPP column when Sofalvi et al.[22] gradient was applied with 0.1 FA in Water (aqueous) and 0.1 FA in ACN (OMP. R.T. 7.30 minutes: Norsufentanil. 13C6 BOH Thiofentanyl was identified as the broad peak with a W_{base} of 3 minutes at 15.5minutes. The remaining 10 analogs eluted from the PFPP column between 18 and 19 minutes. Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 23.

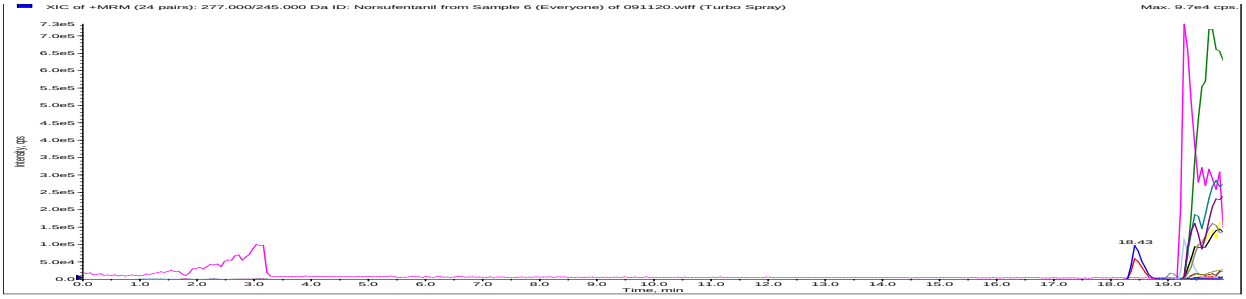

Figure 23. Chromatogram of all twelve SFA eluting off the UCT C18 column when Sofalvi et al.[22] gradient was applied using 0.1% FA in water (aqueous) and 0.1% FA in ACN (OMP). R.T. 18.43: Norsufentanil. Remaining 11 analogs eluted from UCT C18 column after 19 minutes. Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 24.

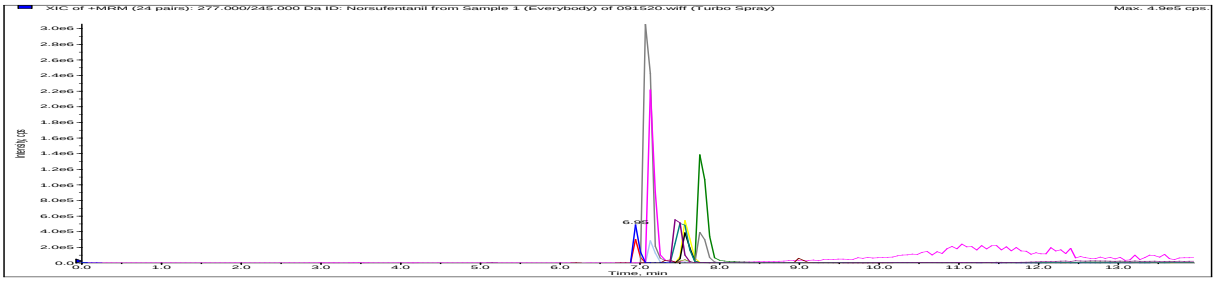

Figure 24. Chromatogram of all twelve SFA eluting off the UCT C18 column when a linear gradient of 5-95% 0.1% FA in ACN over nine minutes was applied. All twelve analogs eluted from the column between 7-8 minutes and resulted in unseparated sharp narrow peaks. Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 25.

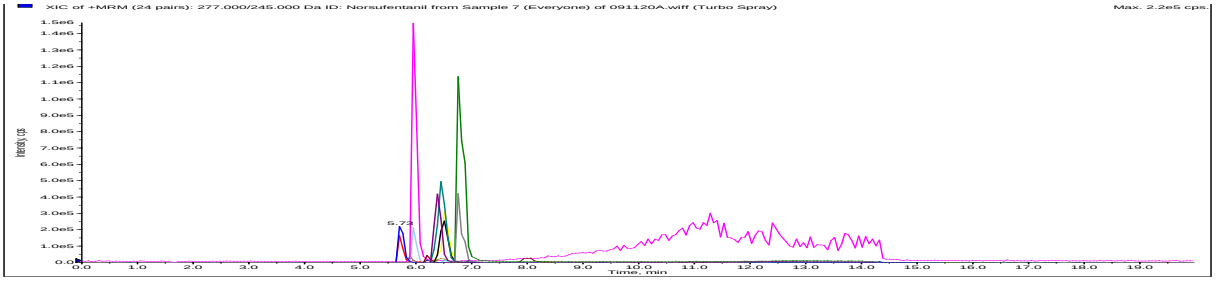

Figure 25. Chromatogram of all twelve SFA eluting off the UCT C18 column when a linear gradient of 20-95% 0.1% FA in ACN over nine minutes was applied. All twelve analogs eluted from the column between 5.5-7 minutes. Retention time of analogs left to right: Norsufentanil (5.73 minutes), Thienyl Fentanyl (5.96), BOH Thiofentanyl/ 13C6 (6.03), Thiofentanyl (6.21), alpha-methyl/trans-3-methyl Thiofentanyl (6.46), Cis-3-methyl Thiofentanyl (6.53), Sufentanil (6.96), Tetrahydrothiophene Fentanyl (6.77). Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 26.

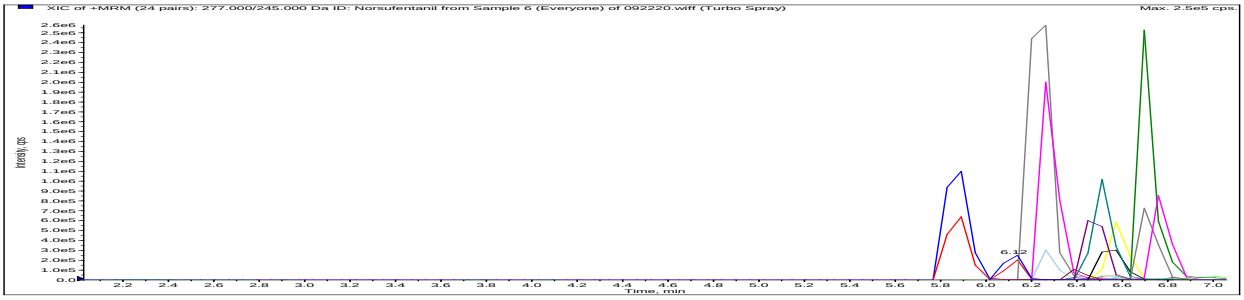

Figure 26. Zoomed in chromatogram of all twelve SFA eluting off the UCT C18 column when a linear gradient of 10-95% 0.1% FA in ACN over 9 minutes was applied. All twelve analogs elute from the column between 5 and 7 minutes and resulted in 4 unresolved peaks. Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 27.

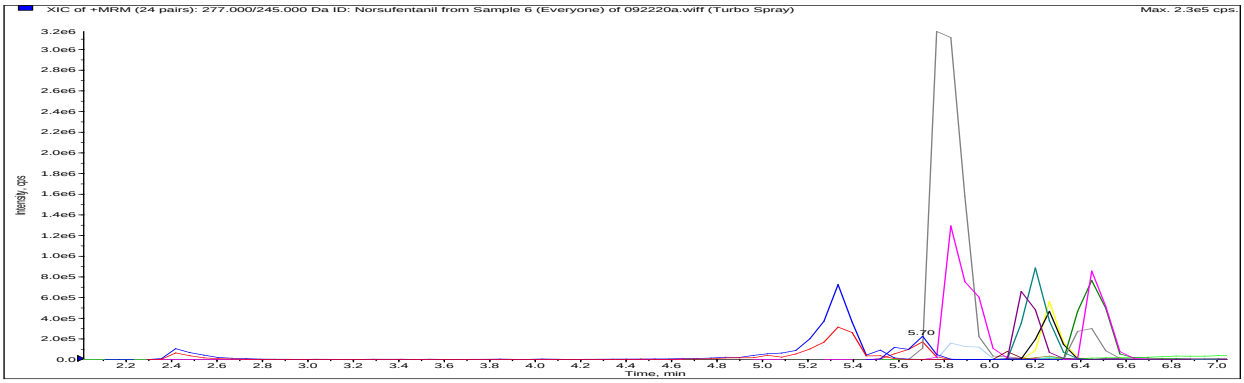

Figure 27. Zoomed in chromatogram of all twelve SFA eluting off the UCT C18 column when a linear gradient of 15-95% 0.1% FA in CAN over 9 minutes was applied. All twelve analogs elute from the column between 5 and 7 minutes and resulted in 4 unresolved peaks. Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 28.

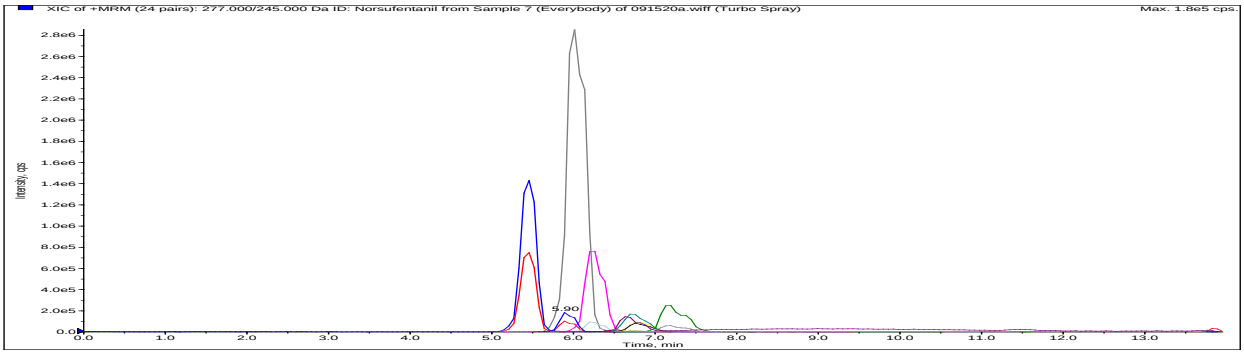

Figure 28. Chromatogram of all twelve SFA eluting off the Restek C18 column when a linear gradient of 5-95% 0.1%FA in ACN over 9 minutes was applied. All analogs eluted from the column between 5 and 7 minutes and resulted in broad, short, and mixture of symmetrical and unsymmetrical peaks. Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 29.

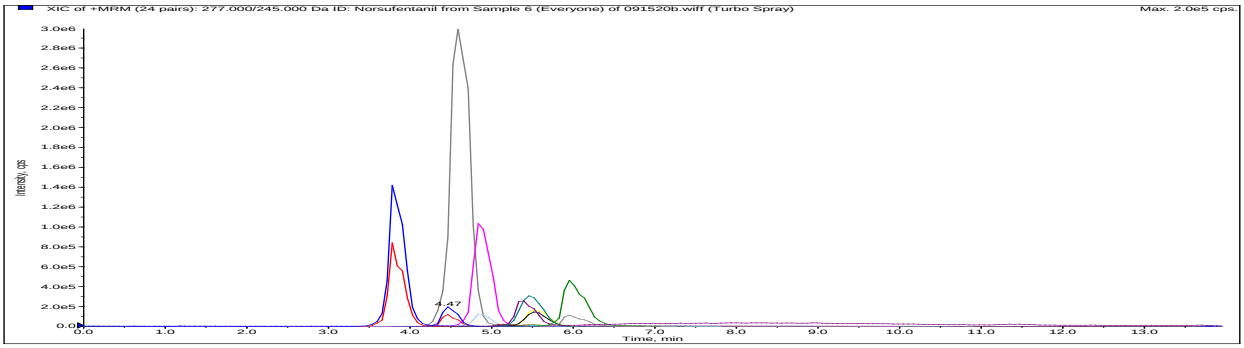

Figure 29. Chromatogram of all twelve SFA eluting off the Restek C18 column when a linear gradient of 20-95% 0.1% FA in ACN over 9 minutes was applied. All analogs eluted from the column between 3 and 7 minutes resulting in broad, short, unresolved peaks. Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 30.

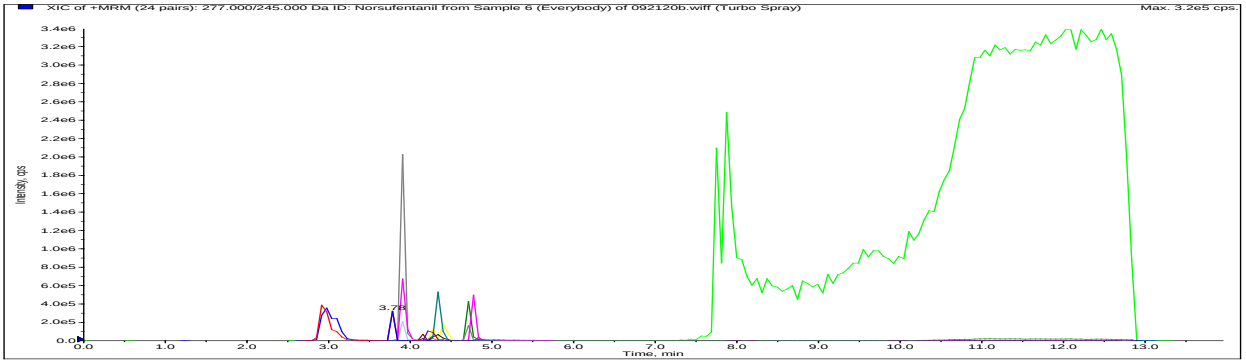

Figure 30. Chromatogram of all twelve SFA eluting off the Waters X-Select C18 column when a linear gradient of 5-95% 0.1%FA in ACN over 9 minutes was applied. All analogs eluted from the column between 2.5 and 5 minutes with 5 defined peaks. All peaks other than BOH Thioacetylfentanyl (RT: 2.5) resulted in narrow, symmetrical, unresolved fully peaks. Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 31.

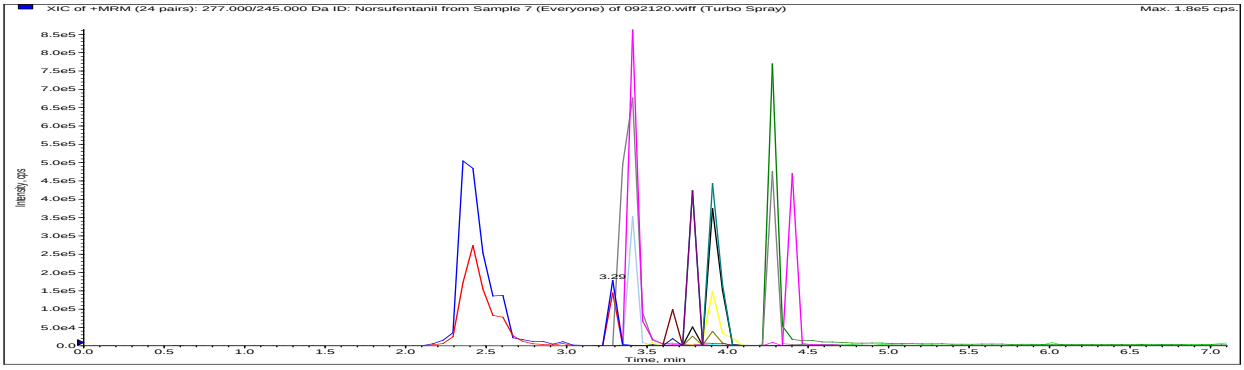

Figure 31. Chromatogram of all twelve SFA eluting off the Waters X-Select C18 column when a linear gradient of 10-95% 0.1%FA in ACN over 9 minutes was applied. All analogs eluted from the column between 2 and 4.5 minutes with 8 defined peaks. All peaks other than BOH Thioacetylfentanyl (RT: 2.5) resulted in narrow, symmetrical, unresolved fully peaks. Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 32.

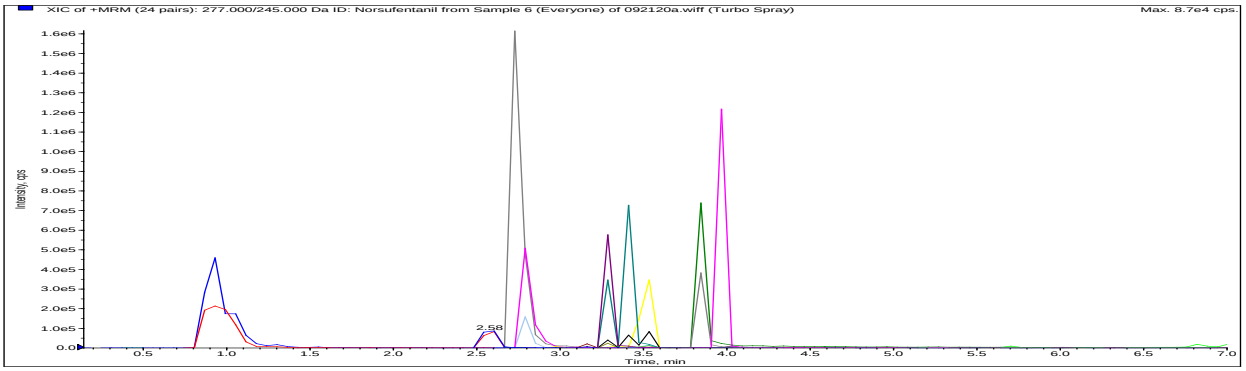

Figure 32. Chromatogram of all twelve SFA eluting off the Waters X-Select C18 column when a linear gradient of 15-95% 0.1%FA in ACN over 9 minutes was applied. All analogs eluted from the column between 1 and 4 minutes with 8 defined peaks. All peaks other than BOH Thioacetylfentanyl (RT: 2.5) resulted in narrow, symmetrical, almost fully resolved peaks. Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 33.

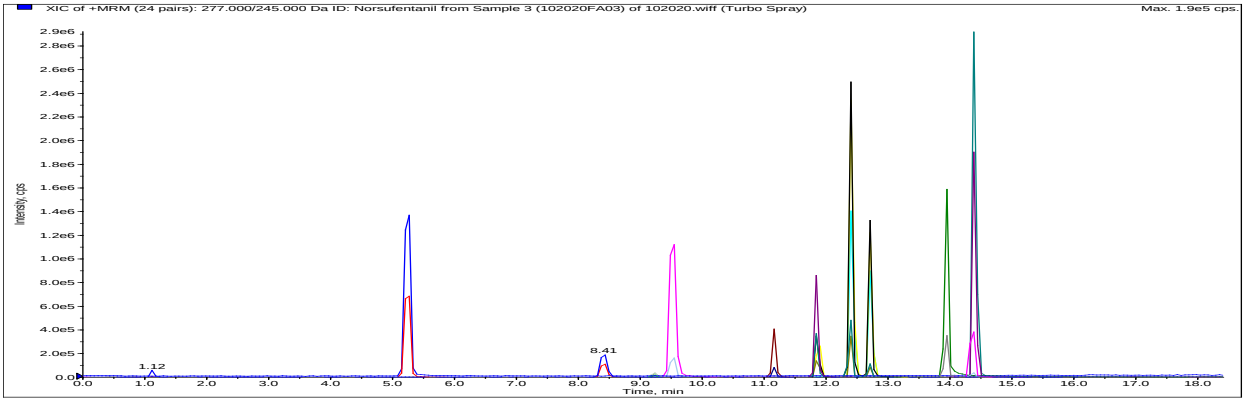

Figure 33. Chromatogram of all twelve SFA eluting off the Waters X-Select C18 column when a curved gradient of 15-60% 0.1%FA in ACN over 16 minutes was applied. All analogs eluted from the column between 2 and 11 minutes with 10 defined peaks. All peaks resulted in narrow, symmetrical, almost fully resolved peaks. Analogs eluted: BOH thioacetylfentanyl, norsufentanil, BOH thiofentanyl/BOH thiofentanyl 13C6, thienyl fentanyl, thiofentanyl, alpha-methyl thiofentanyl, trans-3-methyl thiofentanyl, cis-3-methyl thiofentanyl, tetrahydrothiophene fentanyl, thiophene fentanyl, and sufentanil. Instrument: Shimadzu AD 20 HPLC coupled Sciex AB 5500 triple quadrupole.

Figure 34.

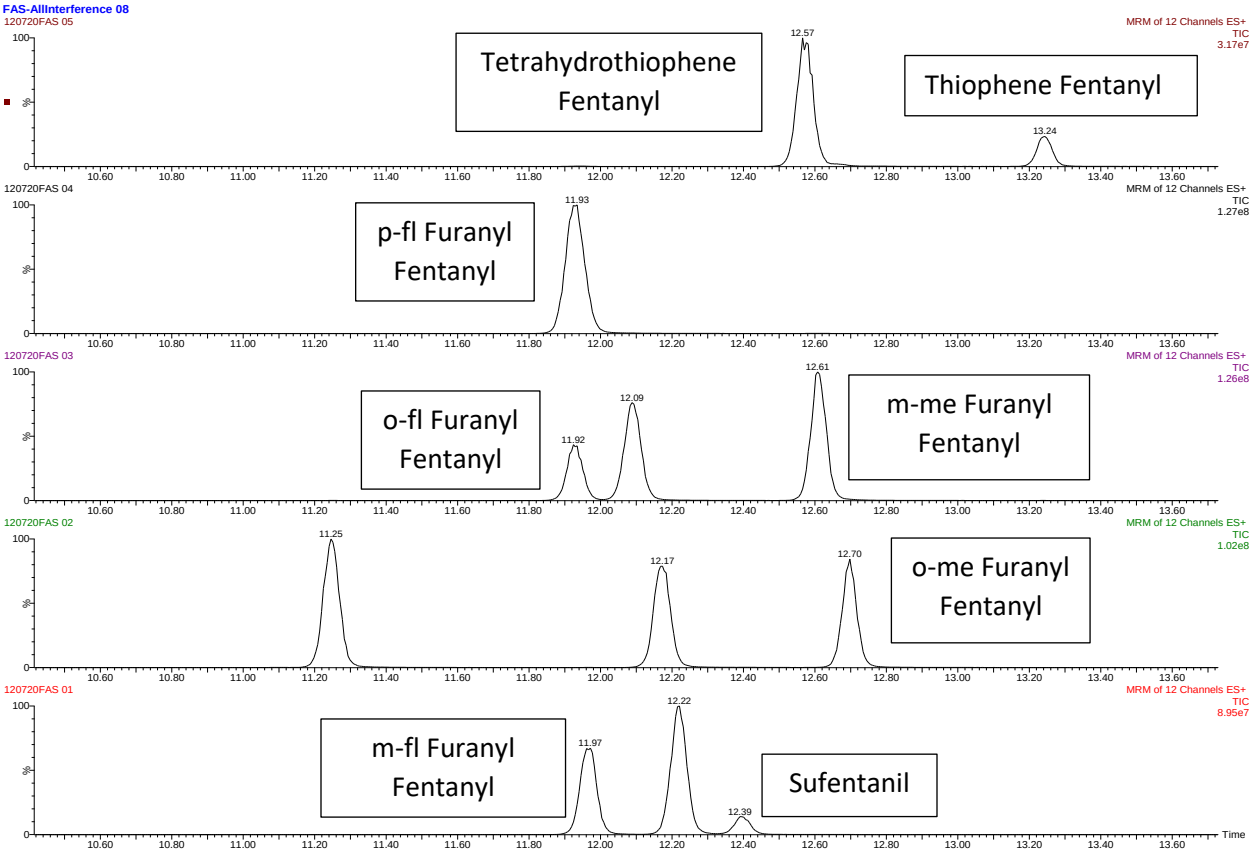

Figure 34. Chromatogram of the structurally similar isobar analogs not chromatographically separated from the butyryl, furanyl, and thiofentanyl class. Utilization of the previously optimized gradient on a Phenomenex biphenyl column resulted in the separation of p-me furanyl fentanyl (MW: 389), Sufentanil (MW: 387), and Thiophene Fentanyl (MW: 391) as well as the separation of p-fl Furanyl Fentanyl (MW: 393)and Tetrahydrothiophene Fentanyl (MW: 395). All other unlabeled peaks are remaining butyryl fentanyl and furanyl fentanyl unresolved from the respective class separation.

Figure 35

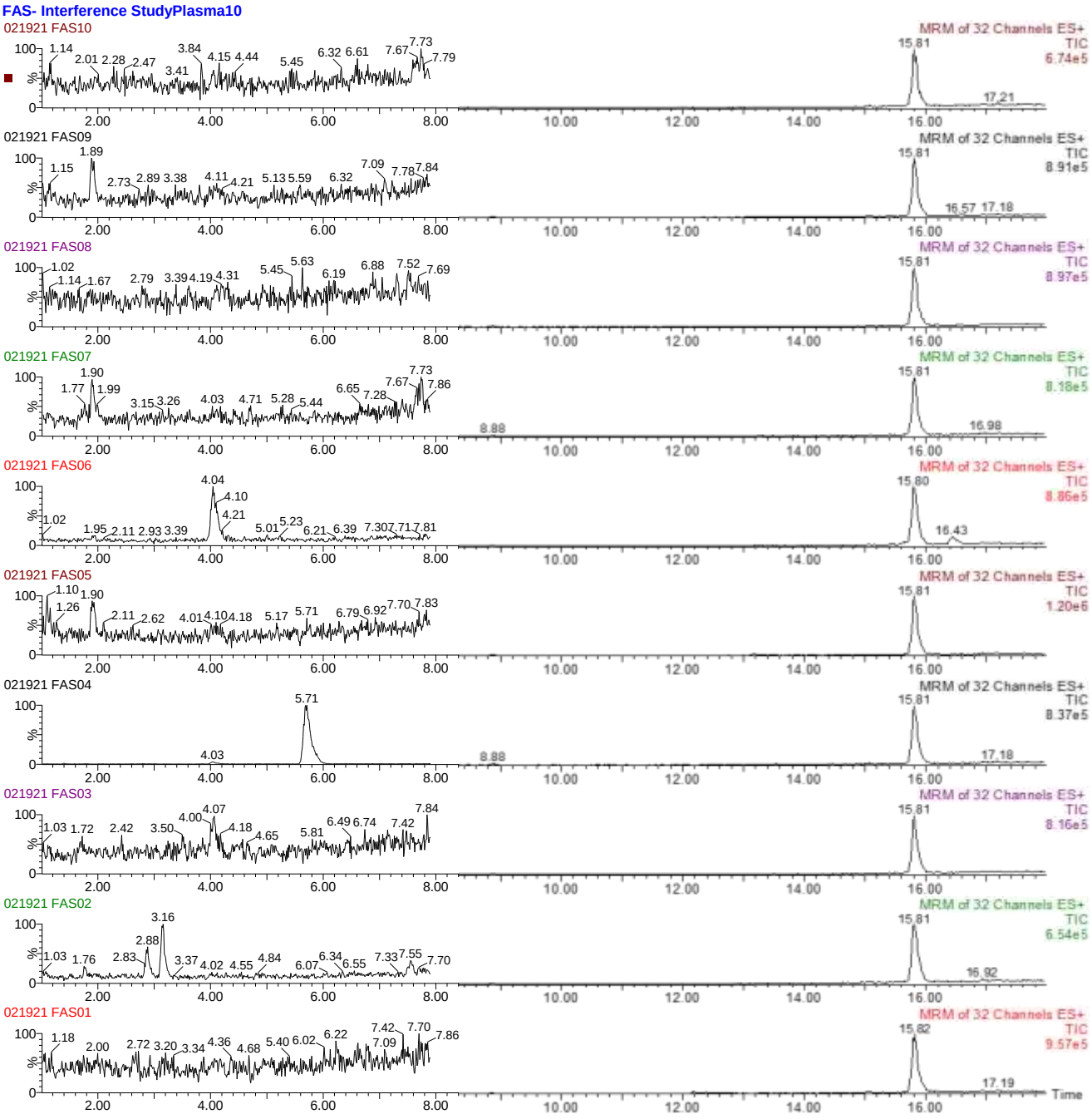

Figure 35. 10 plasma samples evaluated utilizing the optimized mass spectral method. Two mass spectral channels were utilized and the chromatograms are overlayed. MS channel 1 (0-8 minutes). MS channel 2 (8-21.5 minutes).

Figure 36

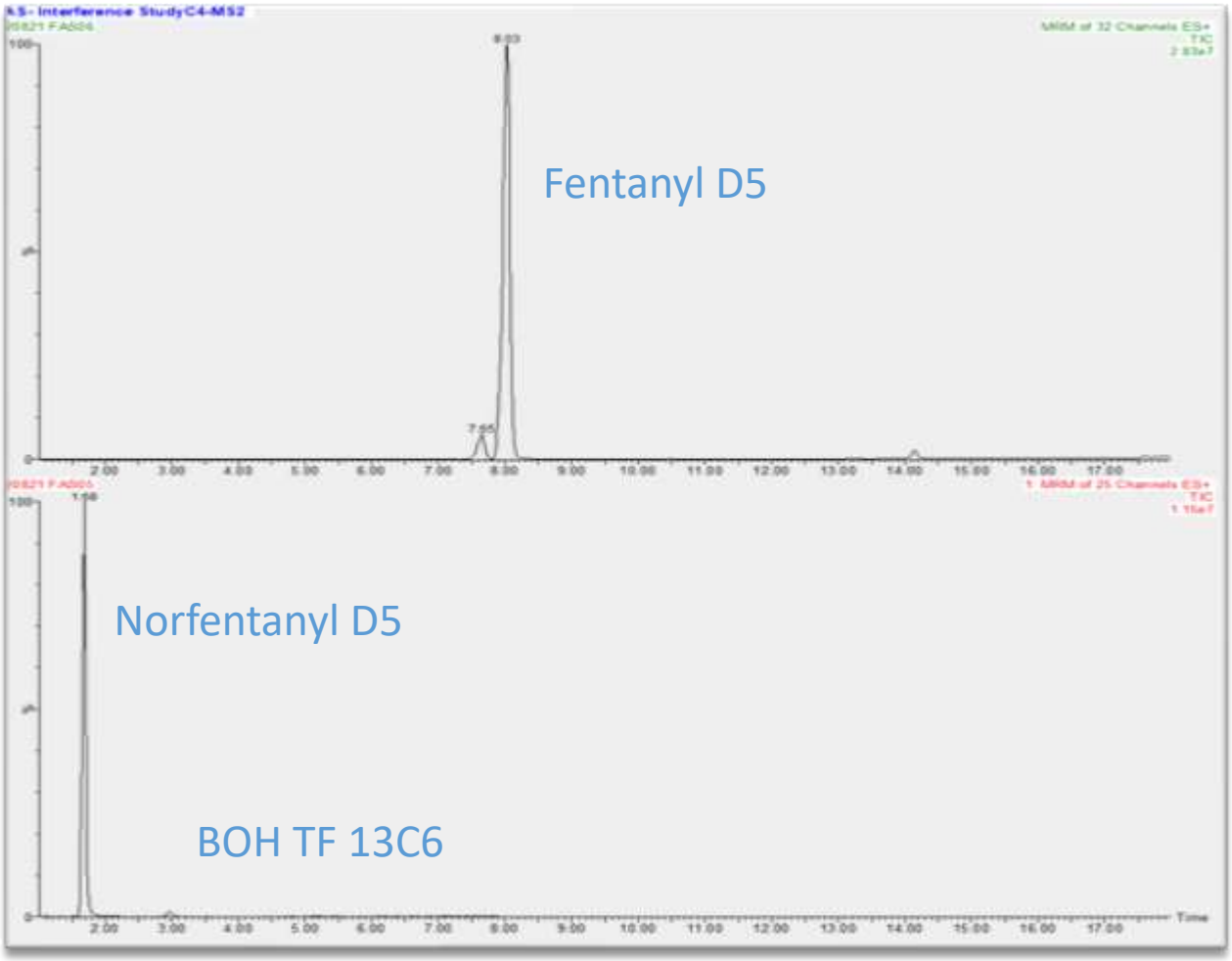

Figure 36. The SFA mass spectral method (Table 1) evaluated against the BioRad C4-common drugs of abuse. Only the transitions of fentanyl, norfentanyl, and BOH thiofentanyl 13C6 were identified in the sample all three of which were added as internal standards.

Figure 37

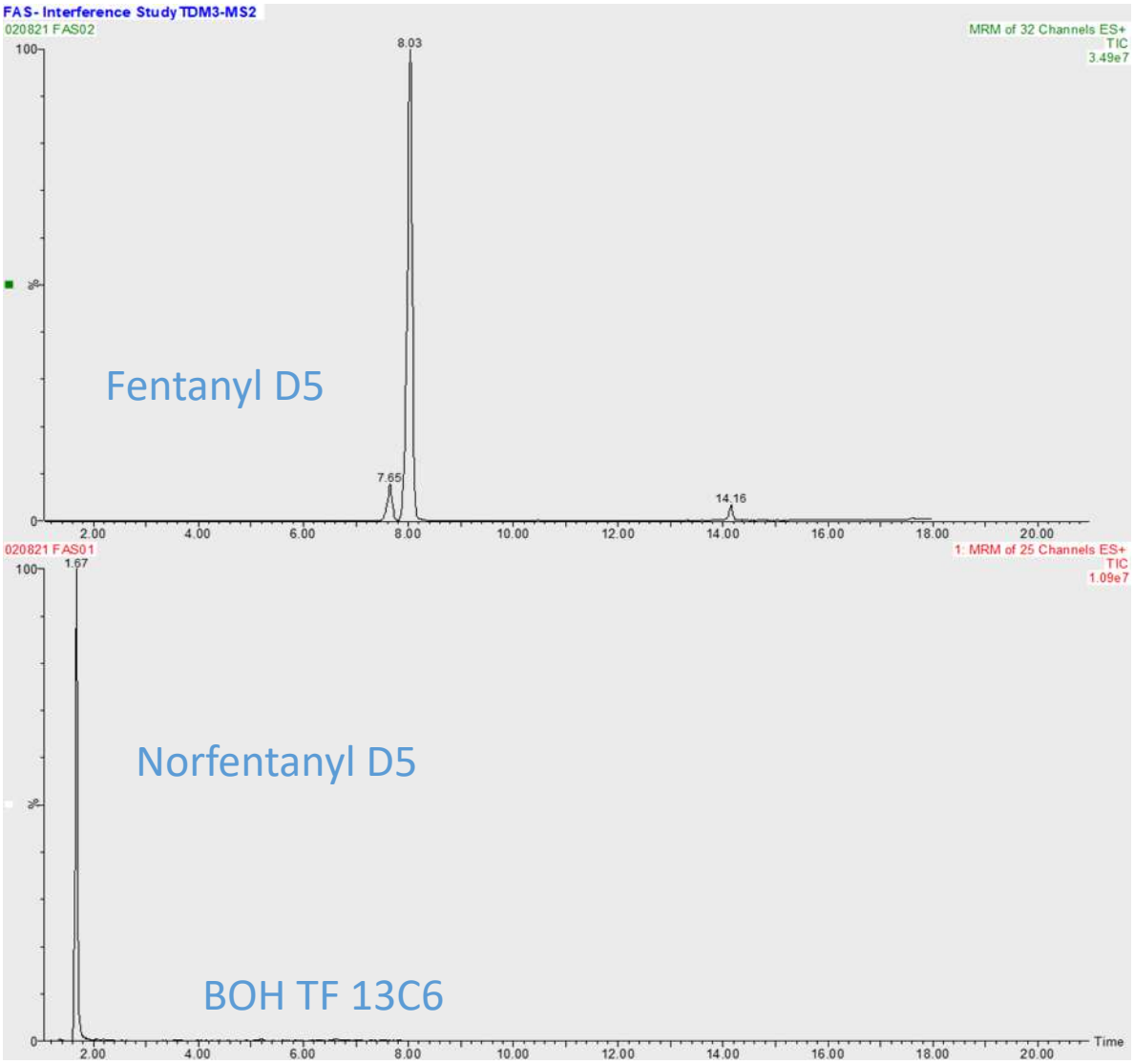

Figure 37. The SFA mass spectral method (Table 1) evaluated against the BioRad TDM3 common pharmaceuticals. Only the transitions of fentanyl, norfentanyl, and BOH thiofentanyl 13C6 were identified in the sample all three of which were added as internal standards.

Figure 38

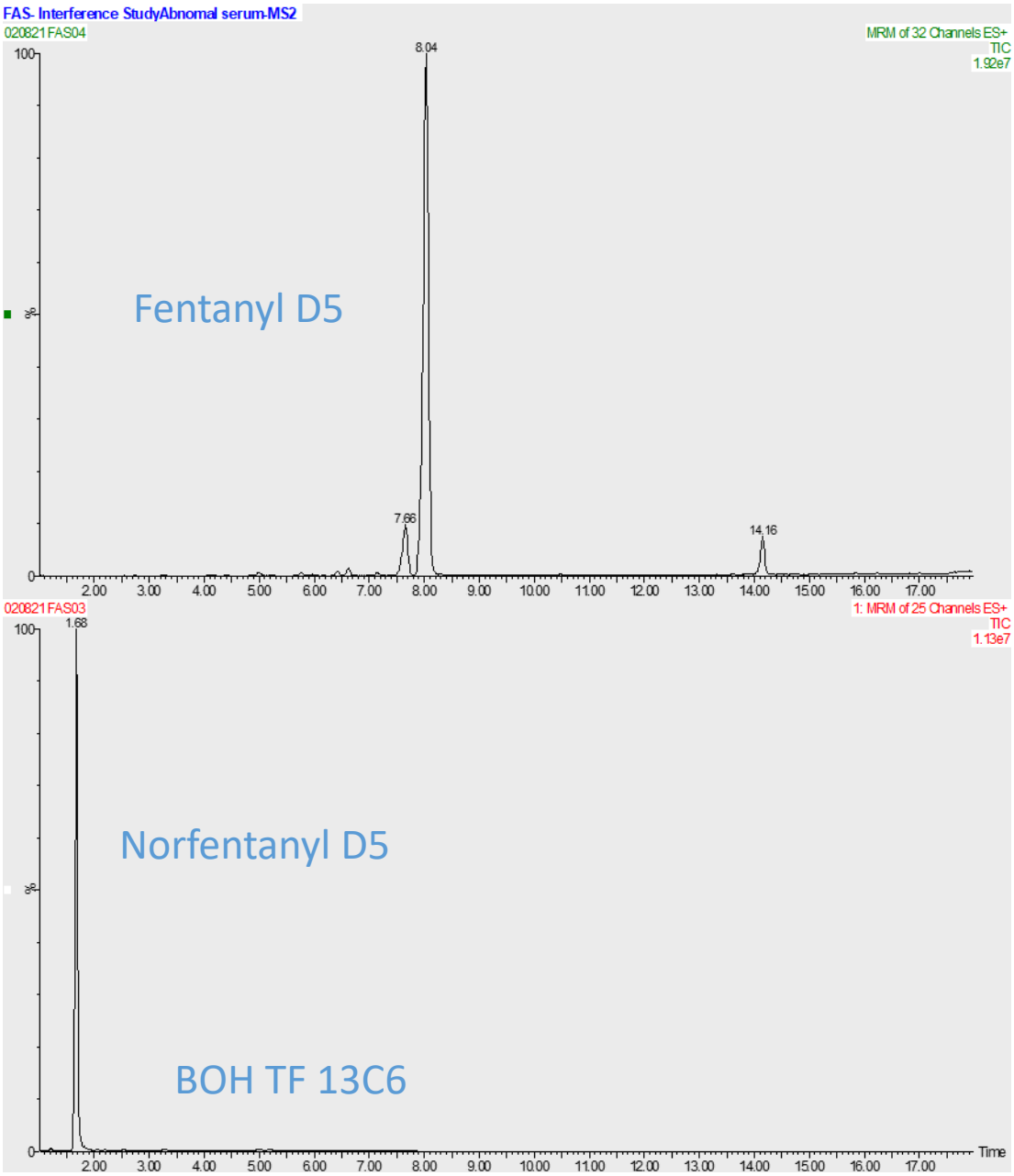

Figure 38. The SFA mass spectral method (Table 1) evaluated against the BioRad Unassayed Multiqual-abnormal serum. The transitions of fentanyl, norfentanyl, and BOH thiofentanyl 13C6 were identified in the sample all three of which were added as internal standards. The peak located at 14.16 was identified as a Furanyl Fentanyl and therefore was not identified in the SFA MS method.

Figure 39

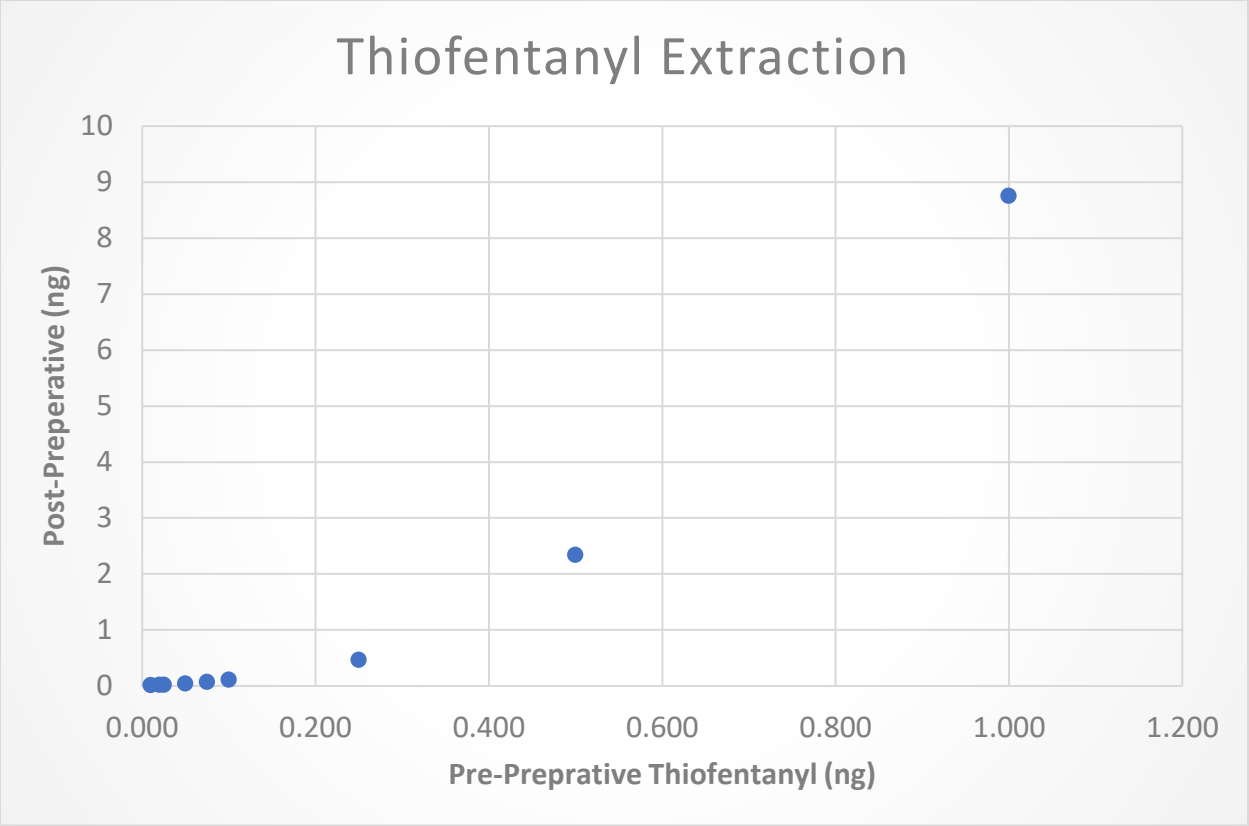

Figure 39. The graph shows the linearity of the 0.01-1ng/mL extraction of thiofentanyl.

Figure 40

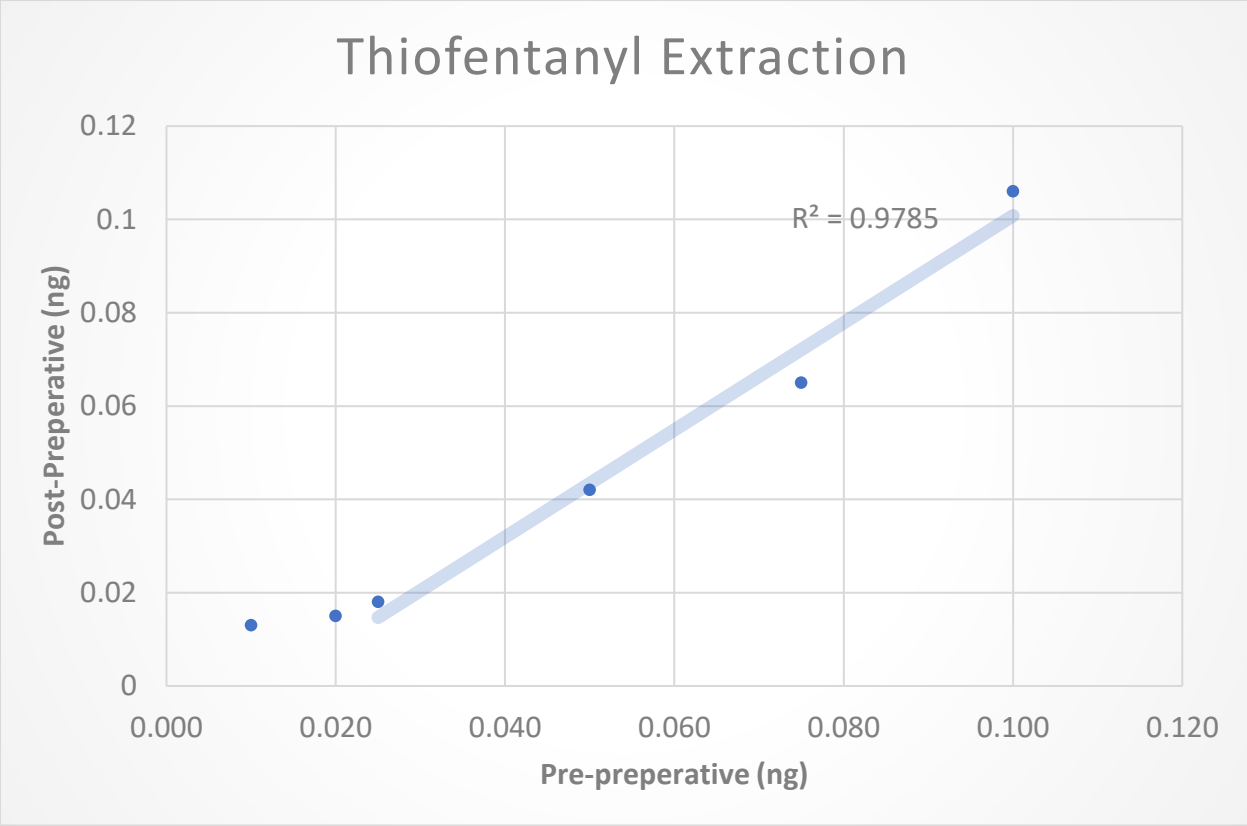

Figure 40. The graph shows the lower concentrations of the linear curve-0.010-.100ng/mL. The explained variation of 0.025-0.100ng/mL is 98% meaning 98% of the change in post-preparative concertation is caused by the change in pre-preparation concentration. The high explained variance (r^2) allows for the use of 0.05ng/mL as the empirical cutoff concentration.

Figure 41

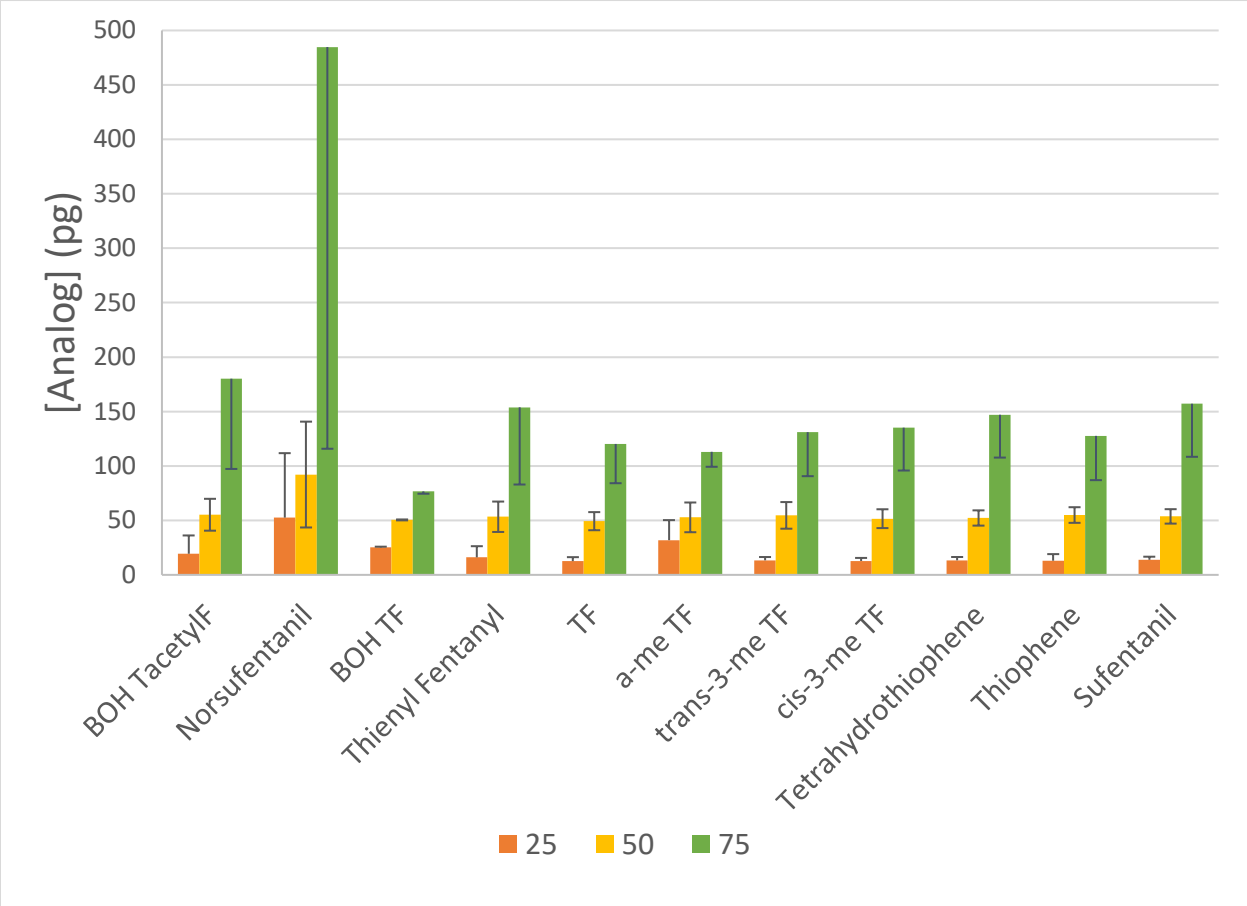

Figure 41. The bar graph shows the precision at the cutoff and allows for visualization of the distinctive three groups, 25pg, 50pg, and 75pg, for the eleven analogs. BOH thiofentanyl 13C6 was removed due to its use as an internal standard. Error bars represent the standard deviation of the post-preparative concentration of each analyte over the 5 extraction triplicate.

Appendix C: Vita

Amber Budmark will graduate earning a Master of Science in Forensic Science in the concentration of Drug Chemistry/Toxicology from Virginia Commonwealth University in May 2021. She graduated Magna Cum Laude earning a Bachelor of Science in Biochemistry from Dakota Wesleyan University in May 2019. In April 2019, she was initiated into the Alpha Sigma Chapter of Sigma Zeta National Science and Mathematics Honor Society as well as awarded the Alumni Medal in Chemistry. While at Dakota Wesleyan, Amber was named a Dakota Wesleyan Scholar Athlete (2017-2019) and an NAIA Scholar Athlete (2018 and 2019). She assisted in the Chemistry Department as a laboratory teaching assistant and department tutor. She is listed as a contributing author on the published abstract "Single Nucleotide Variations within and around microRNA-binding sites" Kofman, A. (2018). She has presented this research in the Toxicology poster session at the 2021 American Academy of Forensic Science meeting where her abstract earned the Forensic Science Foundation Student Scholarship Award. In addition AAFS this research was also presented in the Toxicology poster session at 2021 Pittcon. She is currently a student affiliate of the American Academy of Forensic Science (AAFS) and Society of Forensic Toxicologists (SOFT).