THE UNIVERSITY OF CENTRAL OKLAHOMA

Edmond, OK

Jackson College of Graduate Studies

Development of a Solid Phase Extraction Method for Fentanyl Analogs in Biological Matrices for Analysis by LC-MS/MS

A THESIS

SUBMMITTED TO THE GRADUATE FACULTY

In partial fulfillment of the requirements

For the degree of

MASTER OF SCENCE

By

Alli Timmons

Edmond, OK

2021

Development of a Solid Phase Extraction Method for Fentanyl Analogs in Biological Matrices for Analysis by LC-MS/MS

By

Alli Timmons, A-ABFT

A THESIS

APPROVED FOR THE FORENSIC SCIENCE INSTITUTE

2021

By _____

Thomas H. Jourdan, Ph. D. Committee Chairperson

_____

James Creecy, Ph. D. Committee Member

_____

Sanjeewa Gamagedara, Ph. D. Committee Member

_____

Dallas New, Ph. D. Committee Member

Acknowledgements

I would like to express my gratitude to the University of Central Oklahoma and its faculty for their support in preparing me for this project, both as an undergraduate and graduate student. I would not have the career I have now without the opportunities and education I received at this fine establishment. I sincerely believe it is the best undergraduate facility for budding forensic scientists.

To my thesis committee, I am forever grateful for your time and investment in my future. Your input has made this project the best it can be. To my committee chair, Dr. Jourdan, thank you for your support, every step of the way. Your guidance has been the greatest help during this process.

Above all, I would like to express my deepest appreciation to the Oklahoma State Bureau of Investigation and the members of the Toxicology unit, who I am honored to call my colleagues. They have helped me throughout this entire project and I could not be more grateful to work with such an amazing team. To Danielle, my technical manager, this project would have been much harder without your leadership. Thank you for allowing me your thesis to aid me in writing mine. To Mya, thank you for taking the time to edit this document. I greatly appreciate your experience and wisdom.

To my family and friends, thank you for believing in me. I hope I have made you all proud. To my husband Joseph, thank you for everything else and more, from now until the end of time. I could not have done it without you.

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Abstract

To combat the looming Opioid Crisis, the federal government has allotted funds to local and state law enforcement laboratories to research methods to incorporate emerging opioids into their reporting capabilities. Fentanyl and its analogs (fentalogs) are the most significant threat amongst these compounds, with some having many hundreds to thousands of times the strength of morphine. Because of their immense potency, more and more sensitive methods are needed for their detection. The Oklahoma State Bureau of Investigation (OSBI) has invested in the validation of a more robust solid-phase extraction (SPE) method to improve their current capabilities, as well as expand them. Several fentanyl analogs were chosen from an extensive review of the literature to determine which are most prevalent. In addition to two isotopically labeled internal standards, 14 compounds including parent fentanyl were developed into a new liquid chromatography tandem mass spectrometry (LC-MS/MS) method. This method was validated for whole blood and urine matrices. The validation was performed in compliance with ISO/IEC 17025:2017, ANAB standards and guidelines, and the current OSBI Toxicology Unit Quality Manual. It was overseen by the current Toxicology Technical Manager (TM) and managed by the OSBI Forensic Science Center (FSC) Quality Manager. It was successfully validated following the completion of five studies: interference, carry over, ion suppression/enhancement, limit of detection, and stability. Once brought online, this method will be used in toxicology casework. If a sample screens presumptively positive for fentanyl-related compounds, this method will be used for confirmation testing. Given the success with which this method was validated, more of the current liquid-liquid extractions, such as that for benzodiazepines, will likely be converted to the new SPE paradigm in the future.

Introduction

Pain is an unfortunate part of the human experience. Medications to treat it can be both a blessing and a curse. Opium and its derivatives have been around for centuries as a solution for pain. Tragically, these drugs are highly addictive and impairing to the user. Heroin, an opioid, was previously the most damaging of this drug class. Now, fentanyl has entered the global arena as one of the deadliest drugs of the 21st century. At over 200 times the potency of morphine, fentanyl is incredibly effective at treating severe pain, but unfortunately, more likely to result in an overdose.

Illegal drug dealers and manufacturers recognize that they can get a much "bigger bang for their buck" using fentanyl in place of heroin. Heroin is listed as a Schedule I drug[1] by the U.S. Drug Enforcement Administration (DEA) and Food & Drug Administration (FDA). Fentanyl, however, is Schedule II and available in hospitals and pharmacies. Fentanyl's purity makes it highly coveted by those in the illicit drug trade. Heroin users believe they are buying their regular drug of choice when in reality, it has been laced with a comparably tiny amount of fentanyl and a much larger amount of often useless additives. By contrast, if someone gets their hands on fentanyl and thinks they can use the same amount as they do heroin, they will likely die of an overdose.

Credit: New Hampshire State Police Forensic Lab

Figure 1: Lethal Dose of Heroin vs. Lethal Dose of Fentanyl

[1] The DEA classifies drugs into five categories or "schedules." Schedule I is defined as "drugs with no currently accepted medical use and a high potential for abuse." Schedule II also has high abuse potential but possesses a valid medicinal purpose. Schedules III-V have decreasing abuse potentials. (U.S. Drug Enforcement Administration, n.d.)

The number of fentanyl-related overdoses 2016-2017 is captured under the category "Synthetic opioids other than methadone" in Table 1 below prepared by the Center for Disease Control (CDC). The high influx of fentanyl and synthetic opioids has led to what we now call "The Opioid Crisis." This war is being fought on two fronts: medical personnel who over-prescribe these dangerously addictive drugs and the illicit drug market.

Table 1: CDC - Annual Number & Age-adjusted Rate of Drug Overdose Deaths by Gender

	All opioids						Prescription opioids					
	2016		2017		Change from 2016 to 2017¶¶		2016		2017		Change from 2016 to 2017¶¶	
Decedent characteristic	No.	Rate	No.	Rate	Absolute rate change	% Change in rate	No.	Rate	No.	Rate	Absolute rate change	% Change in rate
All	42,249	13.3	47,600	14.9	1.6***	12.0***	17,087	5.2	17,029	5.2	0.0	0.0
Sex												
Male	28,498	18.1	32,337	20.4	2.3***	12.7***	9,978	6.2	9,873	6.1	-0.1	-1.6
Female	13,751	8.5	15,263	9.4	0.9***	10.6***	7,109	4.3	7,156	4.2	-0.1	-2.3

	Heroin						Synthetic opioids other than methadone					
	2016		2017		Change from 2016 to 2017¶¶		2016		2017		Change from 2016 to 2017¶¶	
Decedent characteristic	No.	Rate	No.	Rate	Absolute rate change	% Change in rate	No.	Rate	No.	Rate	Absolute rate change	% Change in rate
All	15,469	4.9	15,482	4.9	0.0	0.0	19,413	6.2	28,466	9.0	2.8***	45.2***
Sex												
Male	11,752	7.5	11,596	7.3	-0.2***	-2.7***	13,835	8.9	20,524	13.0	4.1***	46.1***
Female	3,717	2.4	3,886	2.5	0.1	4.2	5,578	3.5	7,942	5.0	1.5***	42.9***

*** Statistically significant (P-value <0.05).

(Wilson, 2020)

Due to the high potency and power of this drug class, law enforcement and drug labs across the globe have had an increasingly more difficult time detecting the small amounts that it

takes for users to fulfill their high. More and more sensitive methods for extracting them from body fluids as well as instrumentation to detect them has become highly coveted in the scientific arena.

One of the most sensitive methods available for the detection of low concentration drugs of abuse is liquid chromatography tandem mass spectrometry (LC-MS/MS). This instrument allows for sub-nanogram detection of a wide variety of compounds. It operates under the same principles as any chromatographic mass spectral process in that samples are separated into individual components, ionized, and fragmented[2] into characteristic pieces, referred to here as ions. These ions are collectively analyzed to distinguish a single drug from all others.

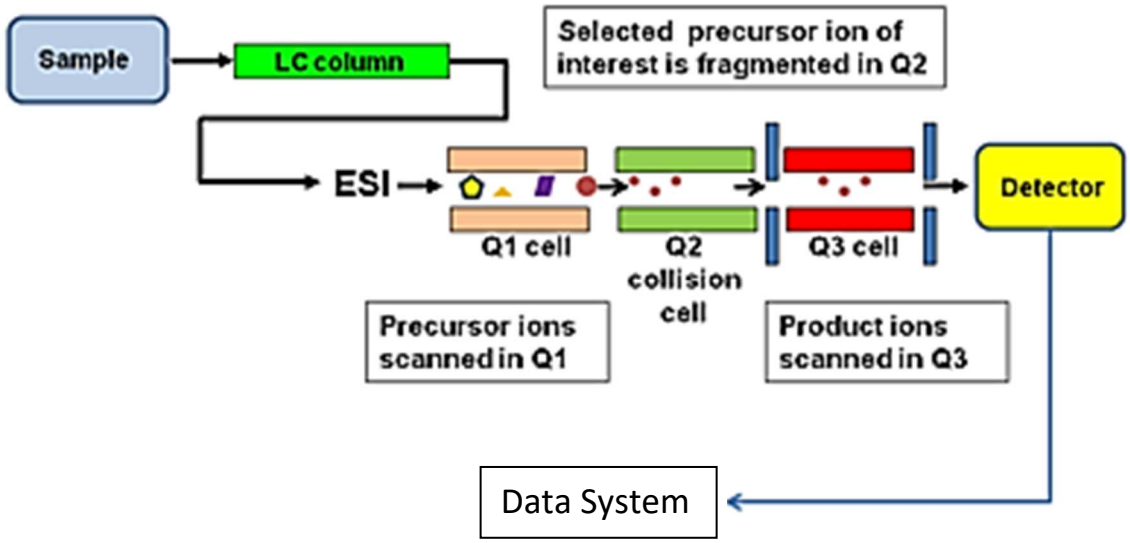


Figure 2: LC-MS/MS Schematic Diagram

LC-MS/MS includes two[3] mass spectrometers linked in tandem. Compounds with a specific molecular weight are selected and allowed to pass into the second where fragmentation occurs. The third is used to select particular fragments allowed to pass on to the final detector.

[2] Collision induced dissociation is one of several fragmentation methods used in mass spectrometry. This occurs when ions collide with an inert gas, in this case, argon. (Pitt, 2009).
[3] LC-MS/MS is often referred to as "triple-quadrupole." The first and third are used as traditional mass selectors while the middle quadrupole is used for fragmentation.

Ionization is achieved by electro spray ionization (ESI). This turns the sample into a fine mist of ions to be filtered or scanned by the subsequent mass spectrometers. The fragmentation from precursor ion to product ion is called a transition. The ratio of the abundances of the product ions is used to individualize compounds. Regarding the example below, the blue peak represents the abundance of the first transition, 316.10 to 174.90, and the red peak represents the second transition, 316.10 to 212.00. Each value represents the number of atomic mass units (amu) of each fragment, which can be thought of as the weight of the ion.

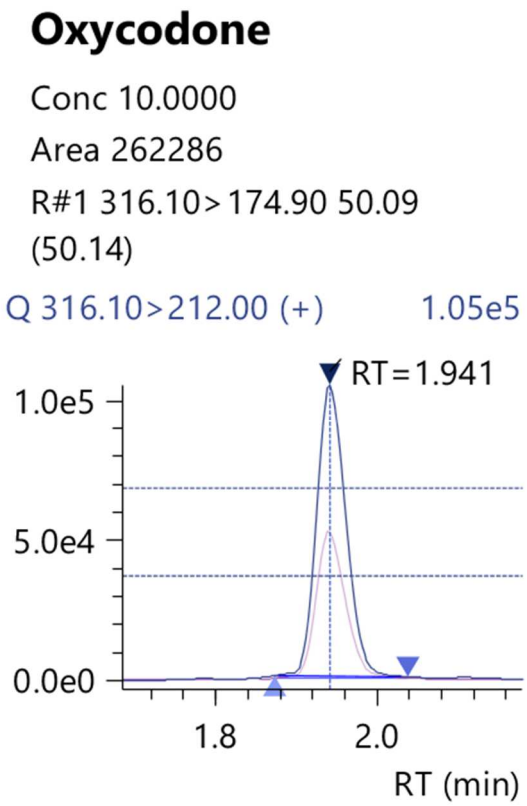


Figure 3: Example of an LC-MS/MS Result

Because LC-MS/MS is so sensitive, the sample must be purified to the greatest extent possible before introducing it to the instrument. One of the best sample preparation methods for this type of work is by solid-phase extraction (SPE.) This methodology employs a small tube packed with a filter medium that retains the analyte of interest and allows contaminants and other

compounds to pass through. A solvent is then used to release the analytes from the filter media. Generally, the sample is then dried down under nitrogen gas and reconstituted in a solvent suitable for the LC column.

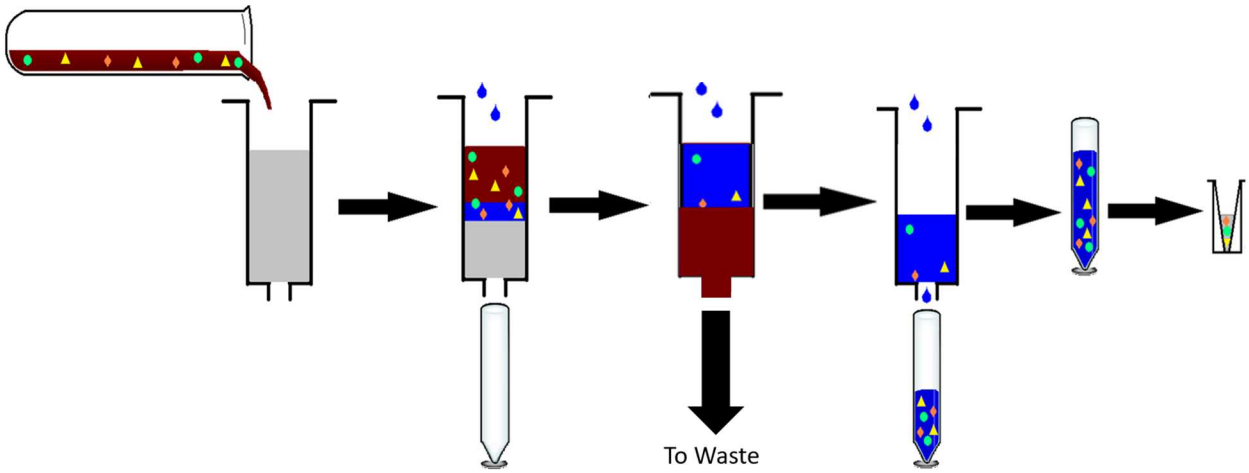


Figure 4: Solid Phase Extraction Schematic Diagram

(Courtesy, in part, of Melissa Brous – OSBI)

The Oklahoma State Bureau of Investigation (OSBI) is one of those labs seeking more sensitive methods for the detection of these compounds. Currently in the OSBI toxicology unit we are able to detect codeine, morphine, 6-monoacetylmorphine (6-MAM, a metabolite of heroin,) hydrocodone, hydromorphone, oxycodone, oxymorphone, and fentanyl as part of our opiates method. The goal of this project is to develop and validate a more robust solid-phase extraction (SPE) to replace the OSBI's current liquid-liquid extraction (LLE) protocol in order to incorporate these harder and harder to detect compounds, as well as develop a LC-MS/MS method for their detection in whole-blood as well as urine. Another aim of this project is to increase the number of compounds the instrument is able to detect and validate them for future casework alongside those already identified by toxicologists. The following compounds were amongst those most highly considered for inclusion in this method.

- Desomorphine (Krokodil)
- Buprenorphine (Suboxone, Buprex)
- Norbuprenorphine (primary metabolite of buprenorphine)
- Norfentanyl (primary metabolite of fentanyl and several fentalogs)
- (±)-cis-3-Methylfentanyl (3-methylfentanyl, methylfentanyl)
- 4-ANPP (despropionyl fentanyl, metabolite of fentanyl & several fentalogs)
- Acetyl fentanyl
- Acryl fentanyl
- AH-7921
- Alfentanil (Rapifen)
- Butyryl fentanyl
- Carfentanil (4-carbomethoxyfentanyl)
- Crotonyl fentanyl (a common contaminant for cyclopropyl fentanyl)
- Cyclopropyl fentanyl
- Fluoro-isobutyryl fentanyl (FIBF, p-FIBF, 4-FIBF)
- Furanyl fentanyl (a.k.a. “grey death”)
- Isobutyryl fentanyl
- Methoxyacetyl fentanyl
- MT-45 (1-cyclohexyl-4-(1,2-diphenylethyl) piperizine a.k.a. “IC-6”)
- Ocfentanil
- *ortho*-fluorofentanyl (o-flourofentanyl)
- *para*-fluorobutyrylfentanyl (PFBF, p-FBF)
- *para*-fluorofentanyl (PFF, p-FF, FF)

- Remifentanil (Ultiva)
- Sufentanil (Sufenta)
- Tetrahydrofuranyl fentanyl (THFF, tetrahydrofuran fentanyl)
- U-47700 (a.k.a. “pink”)
- Valeryl fentanyl (a.k.a Valerie)
- α-Methylfentanyl (alpha methyl fentanyl)
- β-Hydroxythiofentanyl (beta-hydroxythiofentanyl)

Desomorphine, buprenorphine, and its metabolite norbuprenorphine will be added to the original TX40 Opiates protocol the OSBI already has in place. They will be analyzed using the current LC-MS/MS method, with the addition of these compounds. This is due to the similarity in structure and chemical properties these compounds share with morphine and other opiates/opioids in the OSBI’s current protocol. These structures may be found in Figure 5 below. The conversion of the current liquid-liquid extraction to a SPE will be a source of future research. It was the hope that all opiates, opioids, and fentanyl analogs could be extracted via one SPE method, but the non-fentanyl-related compounds require a different elution solvent. The rest of the list above, (the fentalogs) were considered for a new LC-MS/MS method that would become OSBI Toxicology Unit Protocol “TX42,” although not all made it through to the final stage of the validation for reasons explain herein. All of the compounds listed above were mentioned at least four times in one or more of the following academic outlets:

- The Journal of Analytical Toxicology (JAT) Jan 2018-May 2019
- The American Chemical Society (ACS)
- The 2018 Opioid Crisis Webinar hosted by ThermoFisher Scientific
- The Biotage© webinar on opioids which was foundational to this method

- National Medical Services (NMS) labs' current screening methods
- The current list of U.S. scheduled drugs and candidates compiled by the DEA
- The U.S. & European Union (EU) early warning systems[4]
- The United Nations Office on Drugs and Crime (UNODC)

as well as various conferences, published research, and OSBI toxicology & drug chemistry unit[5] case history. This method was developed and validated using the OSBI's current policies and procedures.

Literature Review

Opiates and their semi-synthetic counterparts are derived from the parent compound opium, a natural plant extract or alkaloid, from the poppy plant, *Papaver somniferum*. Opium has been used as a analgesic for thousands of years, and morphine since 1806 (Baselt, 2017.) This drug forms the basis for all of opiates, opioids, and synthetic opioids, whether in terms of effect, structure, or both. Opiates come directly from the opium plant and include morphine, codeine, thebaine and papaverine. Semi-synthetic opioids, or simply, opioids, are similar in structure to morphine but are manmade; these include oxycodone, oxymorphone, hydrocodone, hydromorphone, and heroin. Lastly, fully synthetic opioids, or simply, synthetic opioids, are not similar in structure to morphine but are very much so in effect. These compounds include methadone, meperidine, tramadol, and fentanyl along with its many analogs. The characteristic opiate has a chemical structure similar to morphine that possesses several characteristic functional groups including: five rings, three in the same plane and two protruding at right

[4] Early warning systems, in this instance, are sources for new and emerging drugs. Law enforcement use the information presented here to prepare themselves for emergence of drugs in their area. The US system is managed by the National Institute on Drug Abuse (NIDA), while the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) manages the EU system.

5 The OSBI drug chemistry unit may also be referred to as the seized drugs unit, controlled substances unit, forensic chemistry unit, or simply the drug lab.

angles, one being aromatic, and a quaternary carbon linked to a tertiary amine. The following three opioids were considered for addition to the OSBI's current Opiates/Opioids protocol for the reasons given in each compound's section.

Desomorphine

Desomorphine was once sold in Switzerland for medical use under the brand name "Permonid" (DEA, 2019) but was quickly pulled from the market due to its high toxicity and addictive properties. It has been categorized by the DEA as Schedule I since 1936 (DEA, 2019). It is more commonly known by the Russian name "Krokodil," which is the crude, illicit form of the drug. Krokodil is a deadly compound with an average life span of two years for addicts after their first use, according to Cerilliant[6]. It has claimed the lives of thousands of people in Europe, with the greatest number being in Russia.

Time Magazine (as well as local outlets) reports the first death in the United States from Krokodil was presumably an Oklahoma man, though it was never conclusively determined (Roy, 2013). The Oklahoma Bureau of Narcotics (OBN) representative who was interviewed at the time could not say the death was from Krokodil, though the condition of the deceased was reminiscent of those already claimed by the drug in Russia. It can eat flesh down to the bone at the injection site. It is synthesized rather easily from codeine in a process similar to that of methamphetamine. The drug itself is not the flesh-eating component; the harsh solvents, such as gasoline, and red phosphorous used in the illicit synthesis damage the skin and underlying tissue. It is often referred to in America as the "Zombie Drug" because of the decaying effect it has on the skin and the user in general.

[6] Cerilliant is a metrology laboratory used by research labs to obtain certified reference materials.

Buprenorphine

Buprenorphine has been requested on multiple cases submitted to the OSBI for toxicological analysis. It has been added to the toxicology unit's gas chromatography mass spectrometry (GC-MS) library but the drug is scarcely seen by this method at the typical concentrations found in the body. It has been reported in two cases since this addition, but only in urine specimens where the concentration is much higher than in blood. The OSBI toxicology unit rarely deals with urine, primarily in drug-facilitated sexual assault cases, which constitutes approximately 5% of all cases they work. The majority of casework is driving under the influence (DUI) cases, which use blood specimens.

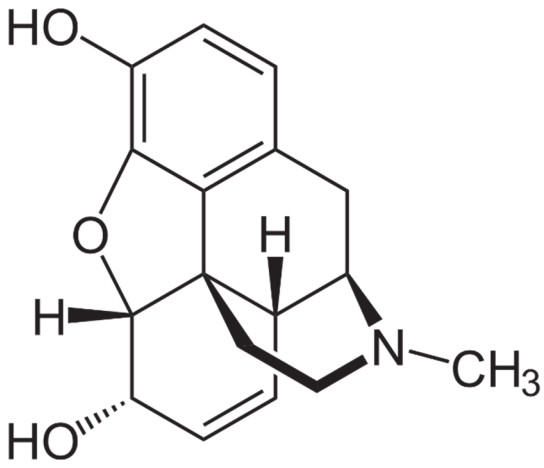
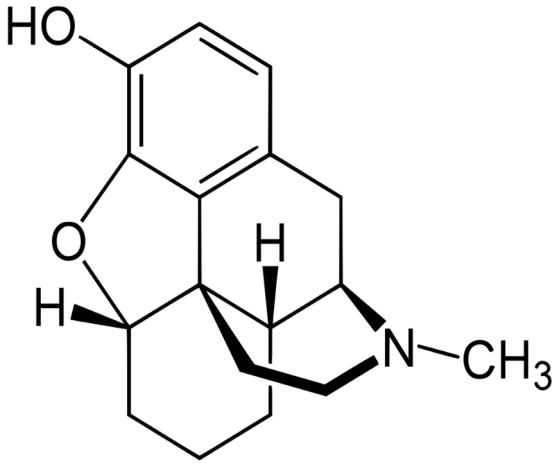
Buprenorphine is 25-40 times the potency of morphine (Baselt, 2019), therefore very little is needed to reach the desired effect. Seemingly contradictory to this, buprenorphine is prescribed to ease addicts' pain while helping with their addiction. This has earned it Schedule III status in the United States (DEA, 2021). Because of its low activity at the μ-opioid receptor, the effects of buprenorphine reach a maximum and do not continue to increase, known as "the ceiling effect" in pharmacology (CAST, 2021). Consequently, an overdose of buprenorphine is less likely to cause fatal respiratory depression than a full opioid agonist like morphine. As a partial agonist, buprenorphine has sufficient activity that addicts subjectively feel "normal" while using significantly less than their drug of choice, and at significantly lower risk of overdose (CAST, 2021).

This analyte is screened for by NMS labs at a cut-off of 0.5 ng/mL. It was reported in 182 cases by the OSBI's drug chemistry unit in 2018 and 118 times in 2019. Its presence is readily apparent and the need for a toxicological method capable of detecting this compound in human specimens is a top priority. To bring the OSBI toxicology unit in line with the current

toxicology organization of scientific area committee (OSAC)[7] guidelines (Appendix 1) for impaired driving investigations, buprenorphine must be part of the scope of testing. This served as the main driving force in validating this compound.

Norbuprenorphine

Norbuprenorphine is the primary active metabolite of buprenorphine and is also screened for by NMS labs at a cut-off of 0.5 ng/mL. Should the parent drug be suspected in a person's system but is not seen due to the passage of time, norbuprenorphine could be pursued in its place. This metabolite has a higher chance of detection; it has a half-life 2-3 times longer than its predecessor. This metabolite is currently unscheduled in the US (DEA, 2021).

Morphine	Desomorphine
	

[7] OSACs are committees formed by the most knowledgeable representatives of each forensic discipline. They provide suggested guidelines and practices for each area of forensic science. They replaced the Scientific Working Groups, the "SWGS," previously established for the same purpose.

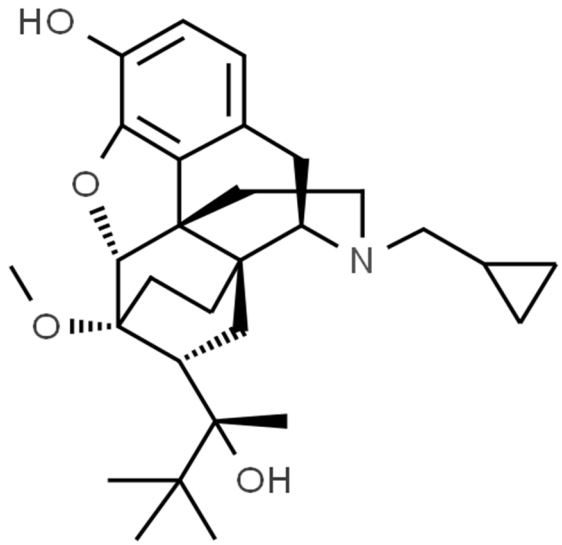
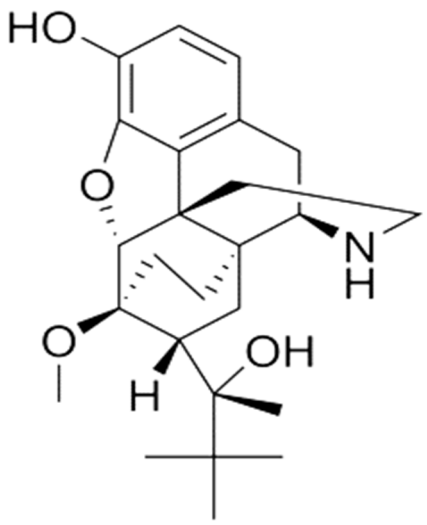
Buprenorphine	Norbuprenorphine
	

Figure 5: Chemical Structures of Morphine and Related Compounds

As mentioned previously, the three drugs just discussed will not be part of the fentalog method of primary concern here. The remaining compounds are all related to fentanyl. Fentanyl is different from morphine in terms of structure but as far as mechanism of action and effect, they are the same animal, though Fentanyl is a much larger bear, over 200 times stronger. Fentanyl was developed in 1960 for the treatment of breakthrough and chronic pain, specifically associated with cancer, and as a surgical anesthetic. Even with its many legitimate uses, fentanyl is highly dangerous and has single-handedly accelerated the opioid crisis over the years.

Despite the risky and addictive nature of the drug, fentanyl is Schedule II in the U.S. and readily prescribed for extreme pain (DEA, 2021). PremierTox. (2017) lists several trade names including: Abstral, Actiq, Duragesic, Fentora, Lazanda, Sublimaze, Sybsys. This is not including its many street names. It can be taken orally as a pill or buccal tablets and lozenges, directly injected, via intrathecal administration, as an extended release transdermal patch, and even as lollipops (Stanley, 1989). Detection can be up to 3 days in the urine, with approximately 5%

excreted unchanged, and 55% excreted as its inactive metabolite, norfentanyl. Typical concentrations are much lower than most analgesics, only about 0.5-2 ng/mL (PremierTox, 2017). This makes it extremely difficult to detect, hence the need for a more robust solid phase extraction and LC-MS/MS method for fentanyl and its counterparts.

According to the UNODC, fentanyl belongs to the phenylethylpiperidine family of drugs, and possesses multiple sites for the addition or substitution of various chemical functional groups to produce compounds with similar or greater analgesic and/or toxic effects.

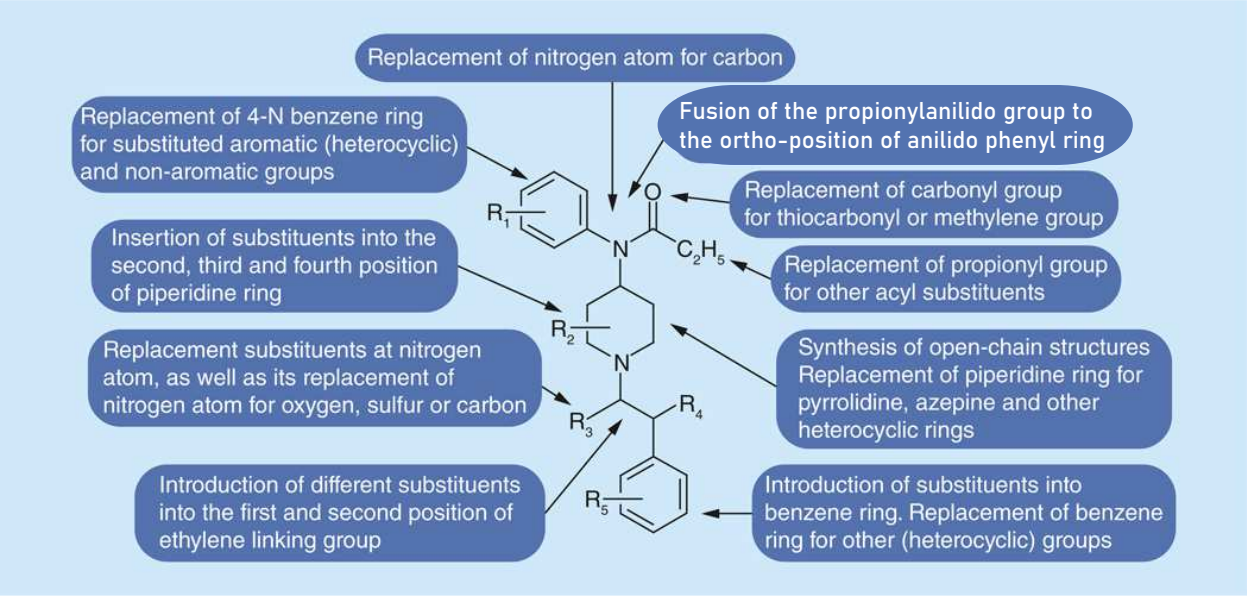


Figure 6: Possible Modifications to the Structure of Fentanyl to Produce an Analog

(Vardanyan, 2014)

Despite the obvious differences between fentanyl and morphine, their chemical make-up is still similar enough to act on the same μ-receptors involved in pain signaling. Drugs acting on these sites, aptly called the "opioid receptors," have the unfortunate side effect of causing respiratory depression, which can lead to death with a high enough dose. This is the main cause of overdose deaths with these types of drugs, each of which is explained in further detail in the following passages.

Norfentanyl

Norfentanyl is the major metabolite of fentanyl, though it is inactive. It is also the primary metabolite of several fentalogs, including beta-Hydroxythiofentanyl and alpha-Methylfentanyl, according the UNODC. Its inclusion in this validation will allow for lengthier detection windows for the parent compound and associated analogs. It is currently a Schedule II compound in the United States (DEA, 2021). This compound was featured in several JAT articles between 2018 & 2019 (Fogarty, 2018; Goggin, 2018; Guerrieri, 2018; Nash, 2018; Partridge, 2018; Salomone, 2018; Seymour, 2018; Sofalvi, 2019).

(±)-cis-3-Methylfentanyl

(±)-cis-3-Methylfentanyl a.k.a. 3-Methylfentanyl (3-MF), or simply, methylfentanyl is second only to carfentanil as far as potency in this family of drugs (UNODC). It is said to be over 5000 times as potent as morphine, and thus several hundred times as potent as fentanyl (UNODC). Deaths as early as the 1970's have been attributed to this compound. It is amongst those screened by NMS Labs (2018). According to the DEA, this compound is a Schedule I drug (DEA, 2021). It has appeared in multiple JAT articles from 2018-2019 (Goggin, 2018; Partridge, 2018; Seymour, 2018).

4-ANPP

4-ANPP, also referred to as despropionyl fentanyl, is an intermediate and subsequent impurity formed during the synthesis of illicit fentanyl. It is a metabolite of fentanyl and several fentalogs including acetyl fentanyl, acryl fentanyl, butyryl fentanyl, furanyl fentanyl, and tetrahydro furanyl fentanyl (THFF) (Cayman Chemical, n.d.). It has long been used as a precursor of fentanyl and, as such, is regulated as a Schedule II drug in this country (DEA, 2021). It is the second most abundant inactive metabolite of fentanyl after norfentanyl. 4-ANPP

is screened for by NMS labs (NMS Labs, 2018) and listed in the previously mentioned Biotage© method (Biotage©, 2019, April 1.). The detection of 4-ANPP in illicit fentanyl seizures in the absence of benzylfentanyl is evidence it was created via the Siegfried Method, a popular favorite of clandestine laboratory cooks for its relative simplicity. This compound has been mentioned in several JAT articles between 2018 and 2019 (Fogarty, 2018; Salomone, 2018; Sofalvi, 2019.)

Acetyl fentanyl

Acetyl fentanyl is included in NMS labs' screening methods (NMS Labs, 2018) and part of the original Biotage© method from which this one stems (Biotage©, 2019, April 1.) It currently has no accepted medicinal use in the U.S., and therefore a Schedule I drug (DEA, 2021). According to UNODC, it is approximately 16x as potent as morphine. This drug entered the global arena in 2013 with the report of 14 deaths in Rhode Island and several subsequent deaths spreading to other states, including 41 in Pennsylvania (Pearson, 2015). Acetyl fentanyl has also spread to the EU with over 30 deaths in 2015, and 34 confirmed in Sweden alone between 2015 and 2016 (UNODC). It incidentally appeared on the 2016 EU Early Warning System (European Monitoring Centre, 2018) and has since been seen in seven JAT articles from 2018-2019 (Finkelstein, 2019; Kahl, 2018; Goggin, 2018; Guerrieri, 2018; Partridge, 2018; Salomone, 2018; Sofalvi, 2019). This compound was seen in Oklahoma in 2019 when it was reported in one case by the OSBI drug chemistry unit.

Acryl fentanyl

Acryl fentanyl a.k.a. acryloylfentanyl is included in NMS labs' screening methods (NMS Labs, 2018) and amongst those validated by Biotage© (Biotage©, 2019, April 1). It first emerged in Denmark in 2016 where it was detected in over 20 cases of non-fatal intoxication, as well as 43 lethal cases in Sweden (UNODC). Acryl fentanyl was confirmed in several deaths in Estonia

that year as well, along with Finland, Latvia, and Slovenia. It was reported by the 2017 EU Early Warning System (European Monitoring Centre, 2018,) and currently a Schedule I drug in the U.S. (DEA, 2021). It has been included in six JAT articles from 2018-2019 (Fogarty, 2018; Goggin, 2018; Guerrieri, 2018; Partridge, 2018; Seymour, 2018; Sofalvi, 2019). According to UNODC, it is approximately 170x as potent as morphine.

AH-7921

This substance emerged as a drug of abuse in 2013 according to Baselt[8] and was included on the 2014 EU Early Warning System (European Monitoring Centre, 2018). It is an isomer of the notorious U-47700 (discussed below). Interestingly, it is an active ingredient in synthetic cannabis in Japan, according to Cerilliant. AH-7921 has appeared in three JAT articles between 2018-2019 (Goggin, 2018; Partridge, 2018; Salomone, 2018) and currently listed as Schedule I in the U.S. (DEA, 2021).

Alfentanil

With over a quarter the potency of fentanyl, this compound is still a formidable sedative (UNODC). Alfentanil is extremely fast acting, making it the perfect pre-surgery anesthetic. It is available in hospitals, and consequently, a Schedule II drug in the U.S. (DEA, 2021). It was also among those originally validated by Biotage© (Biotage©, 2019, April 1). Alfentanil was mentioned in four JAT publications between 2018-2019 (Partridge, 2018; Salomone, 2018; Seymour, 2018; Sofalvi, 2019) and is in NMS Labs' screening protocols (NMS Labs, 2018).

[8] Baselt is a text titled "Disposition of Toxic Drugs and Chemicals in Man" by Randall C. Baselt. It is often referred to as "The Toxicology Bible" amongst toxicologists. It is essentially an encyclopedia of drugs providing research, typical concentrations, and suggested analytical procedures for their detection.

α-Methylfentanyl

alpha-Methylfentanyl (AMF) is over 50 times as potent as morphine according to UNODC, and therefore almost a quarter the strength of fentanyl itself. It has the same major metabolite as fentanyl, norfentanyl. Deaths have been attributed to this drug as early as the 1970's. It was the first major fentalog of note and made its entrance to the illicit drug market under the tradename "China white." It was introduced as super potent heroin, though many heroin users made the mistake of using the same amount as they always had with their old drug of choice. This resulted in a rash of overdoses that flooded the underground. Recently, it has been mentioned in two JAT articles between 2018-2019 (Fogarty, 2018; Seymour, 2018) and is a Schedule I compound in the U.S. (DEA, 2021)

β-Hydroxythiofentanyl

beta-Hydroxythiofentanyl was identified in nine fatality cases in Florida between 2015-2016 (UNODC) and has been seen in three JAT articles between 2018-2019 (Goggin, 2018; Kahl, 2018.) According to UNODC, it also primarily metabolizes to norfentanyl, and is considered a "key analog" by the association. It is a Schedule I substance in this country (DEA, 2021).

Butyryl fentanyl

According to UNODC, this fentalog is approximately 7x as potent as morphine, and therefore not nearly as potent as fentanyl. It is considered a "key analog" by their organization. Butyryl fentanyl emerged in 2013 and reported by the EU Early Warning System after seizures in Poland (European Monitoring Centre, 2018). In 2014, it was seen as part of drug seizures in Sweden and from there, the United States that same year (UNODC). It was then seen in five JAT articles between 2018-2019 (Fogarty, 2018; Goggin, 2018; Kahl, 2018; Partridge, 2018;

Sofalvi, 2019). This analog is screened by NMS Labs and currently listed as a Schedule I drug in this country (NMS Labs, 2018; DEA, 2021).

Carfentanil

This compound has been cited by the Centers for Disease Control (CDC) as "the most potent fentanyl analog detected in the U.S.; [it] is estimated to be 10,000 times more potent than morphine." UNODC corroborates its incredible strength. It has been used as a tranquilizer for large animals, particularly elephants, since 1975; its only legal use in the US is for veterinary purposes. It was supposedly the chemical agent used in the 2002 Moscow theater incident that killed the 40 Chechen hostage-takers, but also 131 innocents (Feasel, 2016).

Carfentanil did not make its way into the illicit drug trade until 2013 when it was identified as part of a drug seizure in Latvia. It appeared in the U.S. drug scene in 2016 in combination with heroin. The drug was responsible for over 500 deaths in that year alone in Ohio and Florida. It was listed on the 2017 EU Early Warning System (European Monitoring Centre, 2018) and has appeared in seven JAT articles between 2018-2019 (Fogarty, 2018; Guerrieri, 2018; Kahl, 2018; Partridge, 2018; Salomone, 2018; Seymour, 2018; Sofalvi, 2019). It is amongst those screened by NMS labs (NMS Labs, 2018) and part of the original method validated by Biotage© from which this project was developed (Biotage©, 2019, April 1). It is currently a Schedule II drug in the United States, but only for use on large animals (DEA, 2021).

Crotonyl fentanyl

Crotonyl fentanyl is a common contaminant in the synthesis of its isomer cycloproyl fentanyl (discussed below). It is listed as Schedule I in the U.S. (DEA, 2021). This compound has not been seen on the illicit drug market but it does bring up valid concerns related to false positives. It will be included as part of the interference study portion of this validation.

Cyclopropyl fentanyl

Cyclopropyl fentanyl was listed on the 2018 EU Early Warning System, and is currently an analyte included in NMS labs' screening methods (European Monitoring Centre, 2018; NMS Labs, 2018). It has also been reported by OSBI's drug lab four times in 2018-2019 and mentioned in five JAT articles in that same period of time (Fogarty, 2018; Guerrieri, 2018; Partridge, 2018; Seymour, 2018; Sofalvi, 2019). It is listed as a Schedule I drug in the United States (DEA, 2021). Cyclopropyl fentanyl has been evaluated for cross-reactivity with the OSBI's fentanyl drug-screen assay. It did not produce a positive result on the Immunalysis© enzyme linked immunosorbent assay (ELISA) test. As mentioned previously, its isomer, crotonyl fentanyl, will be evaluated for interference.

4-Fluoro-isobutyryl fentanyl

4-Fluoro-isobutyryl fentanyl a.k.a. *para*-fluorisobutyryl fentanyl or 4-FIBF was reported in 22 fatality cases in Florida 2015-2016. It was confirmed in 14 cases in Sweden during that time (UNODC). Subsequently, it was included on the 2017 EU Early Warning System (European Monitoring Centre, 2018) and appeared in five JAT articles 2018-2019 (Fogarty, 2018; Guerrieri, 2018; Kahl, 2018; Partridge, 2018; Sofalvi, 2019). It is an analyte in the NMS labs' fentalog screen (NMS Labs, 2018,) part of the original Biotage© method (Biotage©, 2019, April 1,) and Schedule I in the United States (DEA, 2021)

Furanyl fentanyl

This fentanyl analog has an unfortunate street name of "grey death." Between 2015 and 2016, it was seen in 10 confirmed cases across the U.S. It was then listed on the 2017 EU Early Warning System, and monitored by NMS labs (European Monitoring Centre, 2018; NMS Labs, 2018). Furanyl fentanyl has appeared in nine JAT articles from 2018-2019 and identified in one

case by the OSBI's drug lab during that timeframe (Fogarty, 2018; Goggin, 2018; Guerrieri, 2018; Kahl, 2018; Nash, 2018; Partridge, 2018; Salomone, 2018; Seymour, 2018; Sofalvi, 2019). It was one of the original compounds validated by Biotage©. Currently, furanyl fentanyl is a Schedule I compound in this country (DEA, 2021). Its potency is similar to its cousin, butyryl fentanyl (UNODC), which is also Schedule I (DEA, 2021).

Isobutyryl fentanyl

This compound is slightly more potent than morphine, between 2-7x as strong, according to UNODC. It was made a Schedule I compound in February of 2018 (DEA, 2021). Isobutyryl fentanyl has been featured in two JAT articles from 2018-2019 (Fogarty, 2018; Seymour, 2018) is currently screened for by NMS labs (NMS Labs, 2018,) and was part of the method from which this one initially stemmed (Biotage©, 2019, April 1).

Methoxyacetyl fentanyl

Methoxyacetyl fentanyl has appeared in four JAT articles published 2018-2019 (Fogarty, 2018; Partridge, 2018; Seymour, 2018; Sofalvi, 2019). It was reported by the 2018 EU Early Warning System (European Monitoring Centre, 2018), is included as part of NMS labs' screening methods (NMS Labs, 2018), the aforementioned Biotage© method (Biotage©, 2019, April 1), and currently Schedule I in the U.S. (DEA, 2021).

MT-45

MT-45, or 1-cyclohexyl-4-(1,2-diphenylethyl) piperizine, holds the highest scheduling status in both the U.S. and the United Kingdom (U.K.) (DEA, 2021). This drug is banned in the Czech Republic and has several recognized fatalities in Sweden (UNODC). It was included on the 2014 EU Early Warning System (European Monitoring Centre, 2018) and has appeared in

five JAT articles published between the years of 2018 and 2019 (Goggin, 2018; Partridge, 2018; Seymour, 2018; Salomone, 2018).

Ocfentanil

Ocfentanil, originally called A-3217, was synthesized in the early 1990's by the pharmaceutical company Anaquest in an effort to create an opioid with less harmful respiratory and cardiovascular effects than fentanyl (F.E. Dussy, 2016). The drug happened to be more than twice as potent, and thus never approved for medical use. It first appeared as an illicit substance in Belgium in 2015 during the autopsy of a 17-year-old male (Coopman, 2016,) and just recently in 2019, it was identified in powder marketed as heroin (Degreef, 2019.) The powder was submitted for testing in preparation of the Belgium Early Warning System. Ocfentanil has been mentioned in four JAT articles between 2018-2019 (Goggin, 2018; Guerrieri, 2018; Partridge, 2018; Seymour, 2018) and currently listed as a Schedule I drug in the United States (DEA, 2021)

ortho-Flourofentanyl

A report of two men overdosing on this compound was reported out of Norway in 2016; they responded to Naloxone[9] but the one tragically passed away days later (Arne, 2017). Four cases have been reported in California as of 2017, along with three others in Virginia (UNODC). It appeared in a JAT article in 2018 (Fogarty, 2018) and placed on a temporary scheduling order as Schedule I in 2017. This order expired in October of 2019 and as of 2021, this drug is still a Schedule I drug in the US (DEA, 2021). *ortho*-Fluorofentanyl was part of the original Biotage© method (Biotage©, 2019, April 1) and is amongst those screened by NMS labs (NMS Labs, 2018).

[9] Naloxone is an injected or nasally administered compound that counteracts the effect of opiates/opioids. It is carried by first responders to stop an overdose.

para-Fluorobutyryl fentanyl

para-Fluorobutyryl fentanyl (p-FBF, or PFBF) appeared for the first time in a fatality case in Sweden in 2015 (UNODC) and was subsequently mentioned in two JAT articles (Goggin, 2018; Partridge, 2018.) It is currently part of the fentanyl analog screening method employed by NMS labs (NMS Labs, 2018) and listed as Schedule I in the United States (DEA, 2021).

para-Fluorofentanyl

para-Fluorofentanyl (p-FF or PFF) has been mentioned in four JAT articles from 2018-2019 (Fogarty, 2018; Goggin, 2018; Guerrieri, 2018; Sofalvi, 2019) and is currently screened for by NMS labs (NMS Labs, 2018.) It is a Schedule I drug in this country (DEA, 2021). According to UNODC, it shares the same potency as acetyl fentanyl.

Remifentanil

According to UNODC, this compound is on par with fentanyl as far as potency; that being said, it is much more potent than morphine, and is listed as a "key analog" in the organization's publication on the analysis of this group of drugs. Remifentanil is a Schedule II compound in the United States because it is sometimes used as a surgical anesthetic and analgesic (DEA, 2021). This compound was mentioned in a JAT article by Salomone in 2018.

Sufentanil

With upwards of 20 times the potency of fentanyl and 4500x that of morphine (UNODC), this drug has been regularly used as a surgical anesthetic since 1976, and therefore Schedule II (DEA, 2021.) It has been mentioned in three JAT articles between 2018-2019 (Salomone, 2018; Seymour, 2018; Sofalvi, 2019.) It is part of the original Biotage© method (Biotage©, 2019, April 1,) and currently screened for by NMS labs (NMS Labs, 2018.)

Tetrahydrofuranyl fentanyl

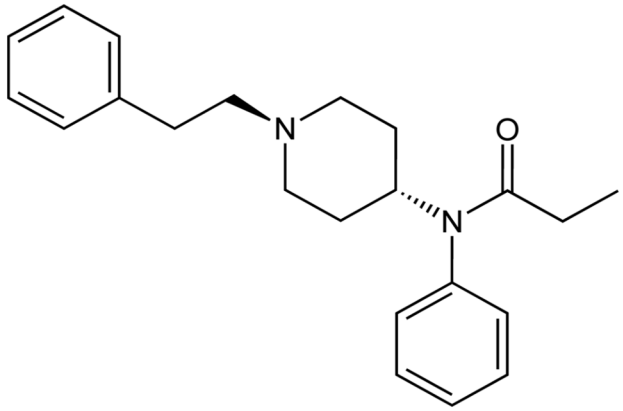
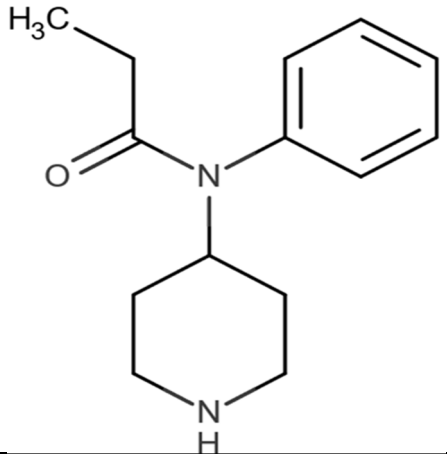
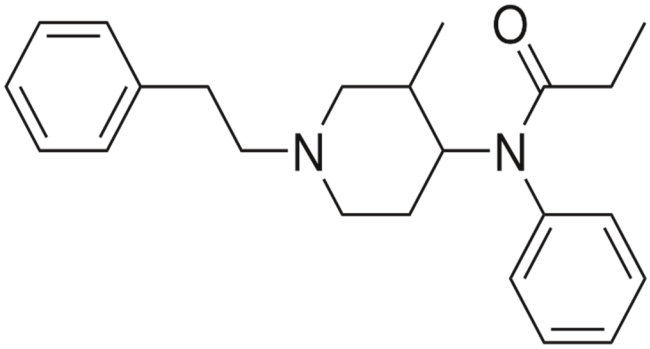
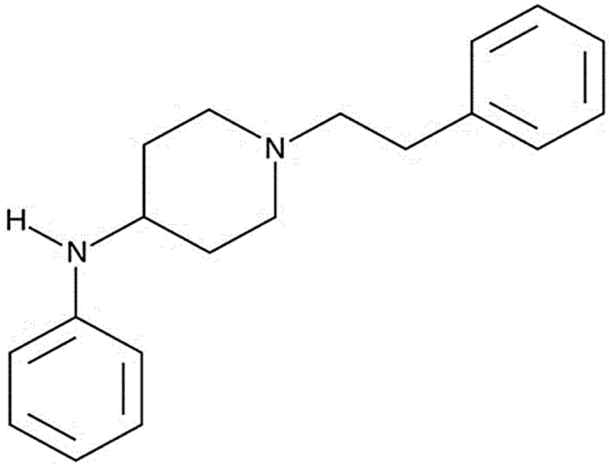
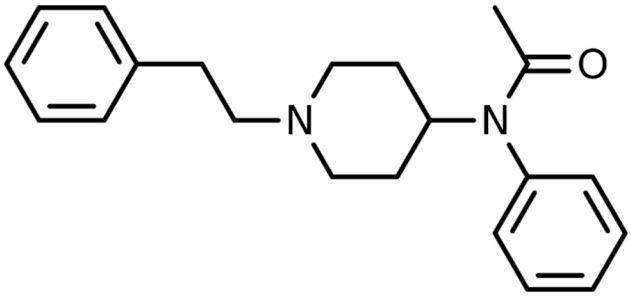
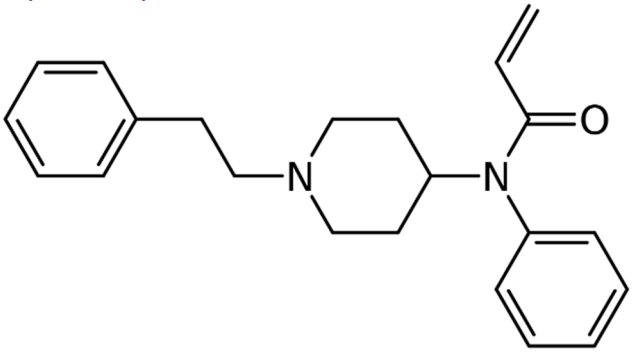
Tetrahydrofuranyl fentanyl, also encountered as tetrahydrofuran fentanyl, THFF was seen in 5 confirmed deaths in Sweden between 2015-2016 (UNODC). It then appeared on the 2017 EU Early Warning System (European Monitoring Centre, 2018) and has been seen in two different JAT articles between 2018-2019 (Fogarty, 2018). THFF is included on NMS labs' screening methods (NMS Labs, 2018) and listed as a Schedule I drug in this country (DEA, 2021).

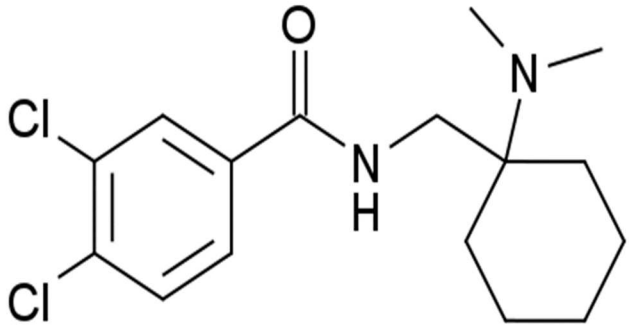
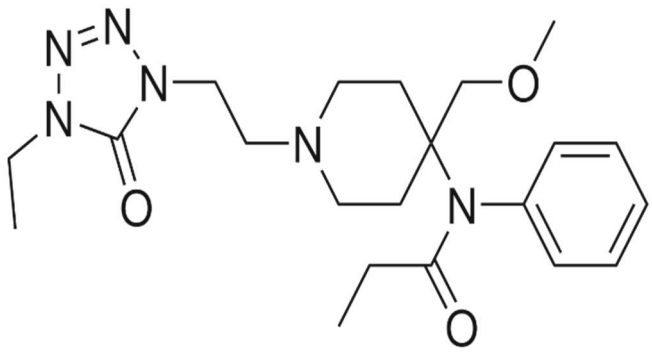
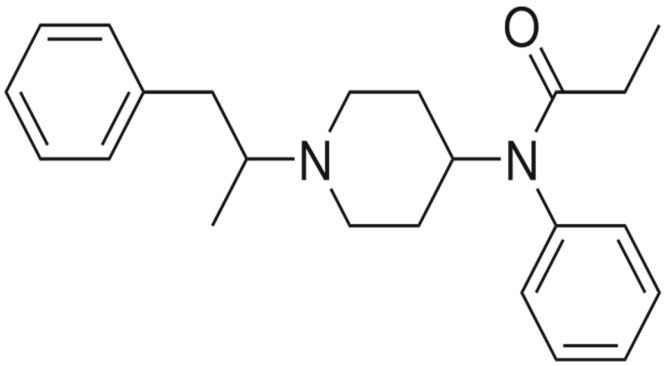
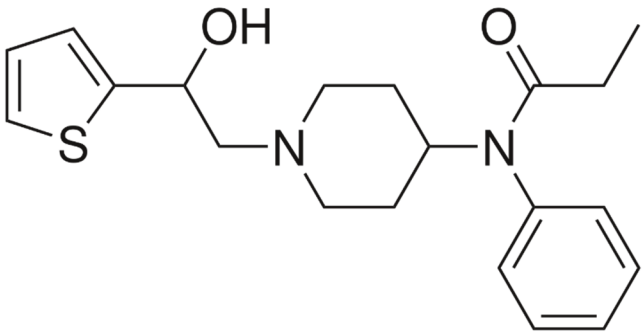
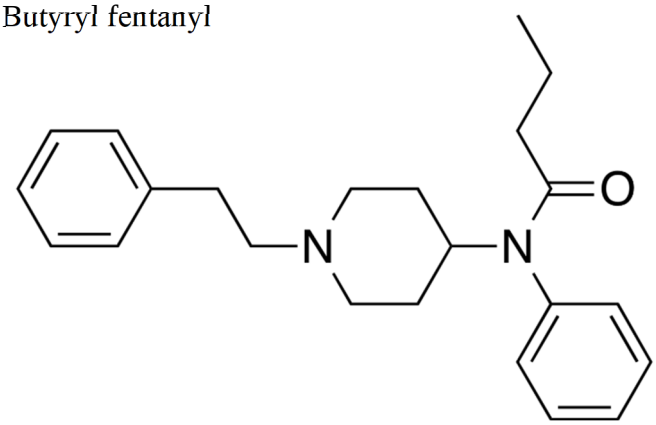
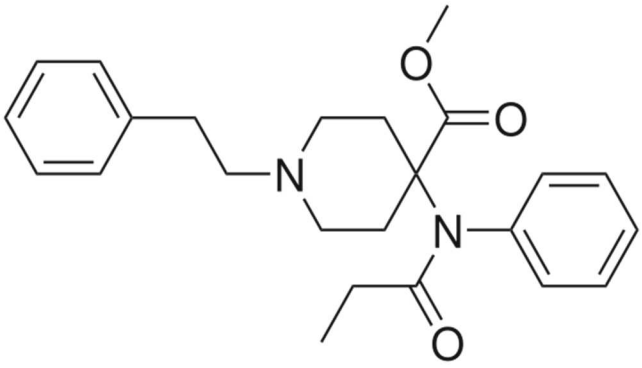
U-47700

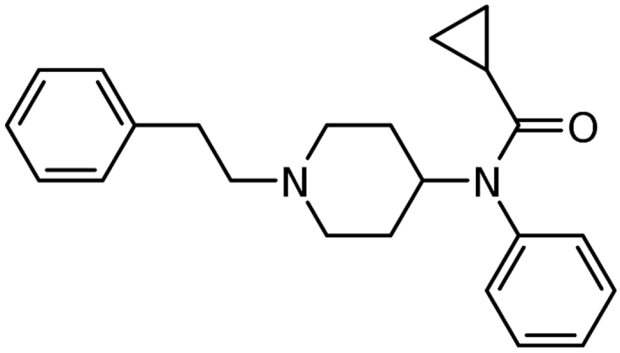
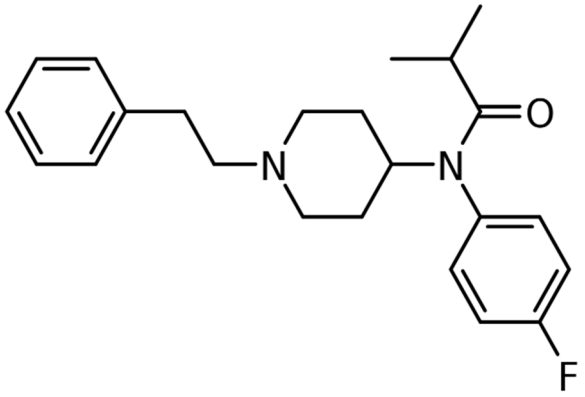
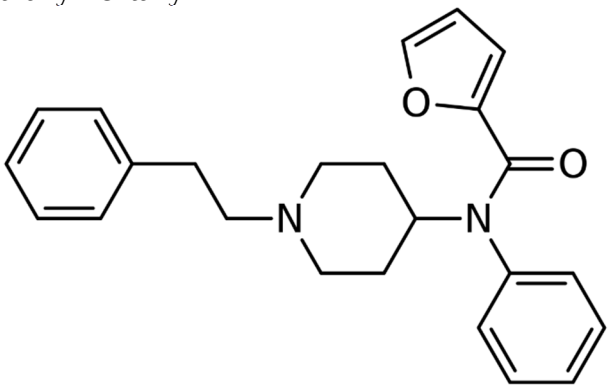
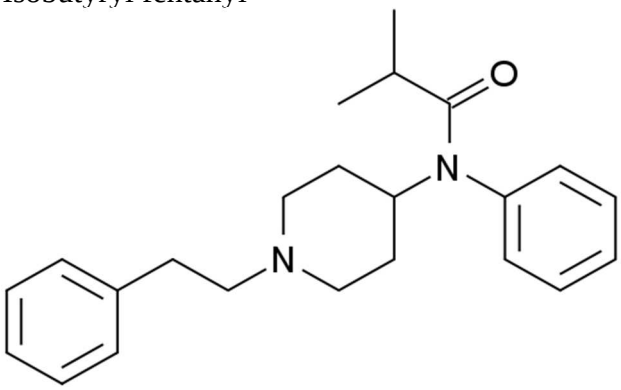
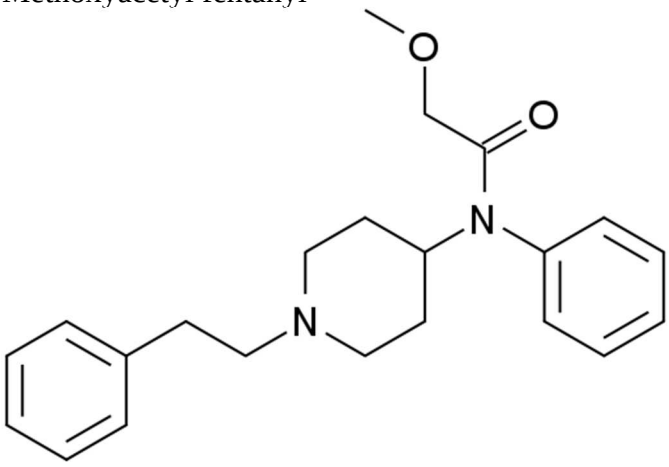
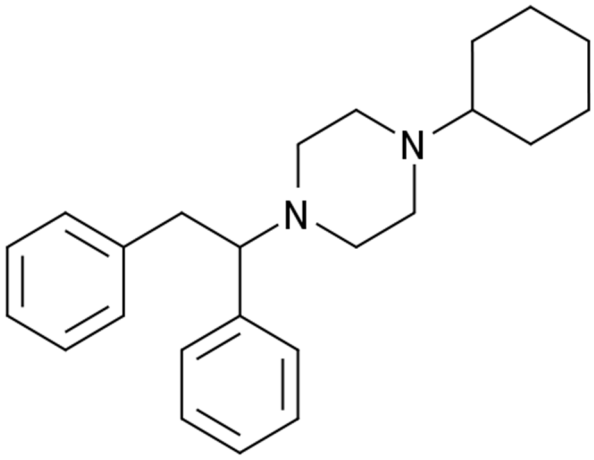
It is estimated that this synthetic opioid has eight times the potency of morphine (Baselt, 2019). It emerged as a drug of abuse in 2015 and has continued to give rise to its own set of variants. It was seen in four fatality cases in Florida between 2015-2016 and featured in five JAT articles between 2018-2019 (Partridge, 2018; Salomone, 2018). U-47700 is an analyte included on NMS labs' screening methods (NMS Labs, 2018), amongst those from the foundational Biotage© method (Biotage©, 2019, April 1,) and currently a Schedule I drug in the U.S. (DEA, 2021).

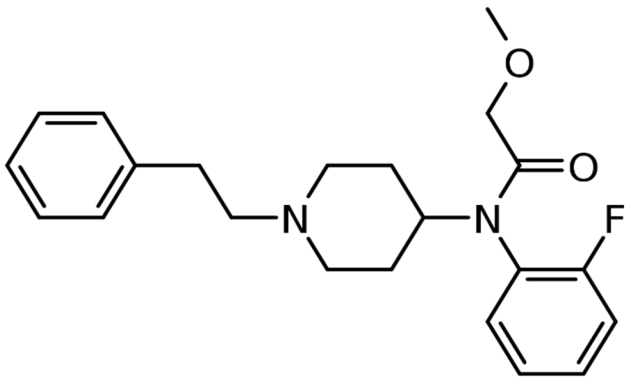
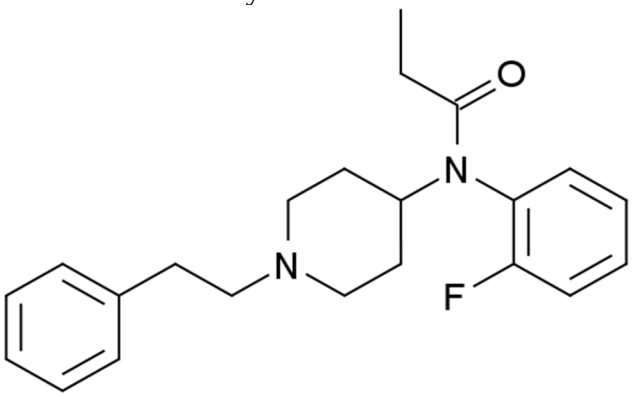
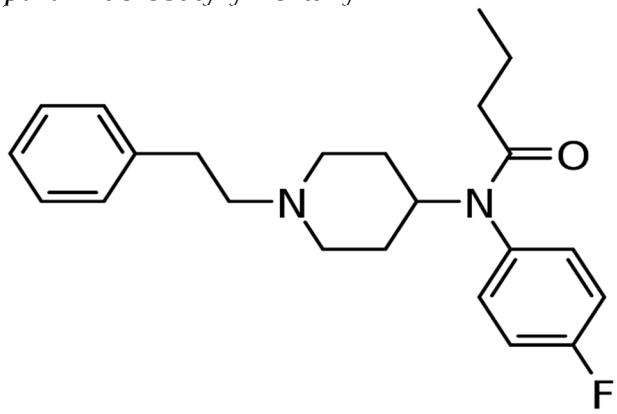
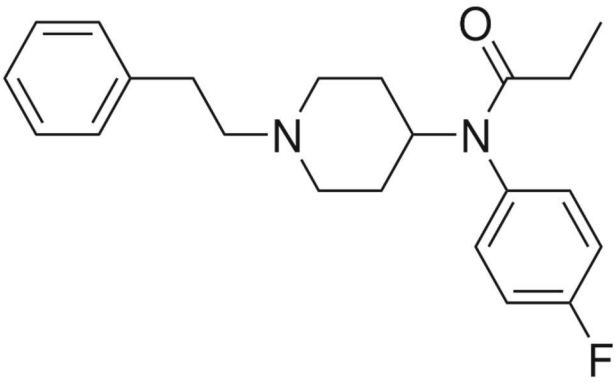
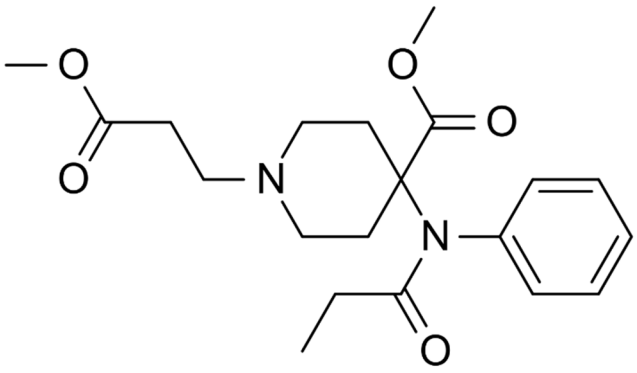
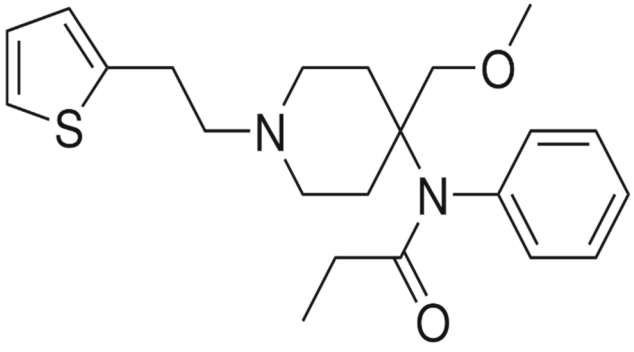
Valeryl fentanyl

Valeryl fentanyl was part of the Biotage© method from which this validation stemmed, is part of NMS labs' fentalog panel (NMS Labs, 2018), and has been featured in two JAT articles between 2018-2019 (Goggin, 2018; Guerrieri, 2018). It is a Schedule I drug in the United States (DEA, 2021). The December 2021 issue of The American Journal of Forensic Medicine and Pathology will include an article from Michigan discussing 13 deaths attributed to this drug. The article calls for an expansion of opioid testing. The OSBI shares this notion.

Fentanyl 	Norfentanyl 
3-Methylfentanyl 	4-ANPP 
Acetyl fentanyl 	Acryl fentanyl 

AH-7921 	Alfentanil 
α-Methylfentanyl 	*β*-Hydroxythiofentanyl 
Butyryl fentanyl 	Carfentanil 

Cyclopropyl fentanyl 	4-Fluoro-isobutyryl fentanyl 
Furanyl fentanyl 	Isobutyryl fentanyl 
Methoxyacetyl fentanyl 	MT-45 

Ocfentanil 	*ortho*-Flourofentanyl 
para-Fluorobutyryl fentanyl 	*para*-Fluorofentanyl 
Remifentanil 	Sufentanil 

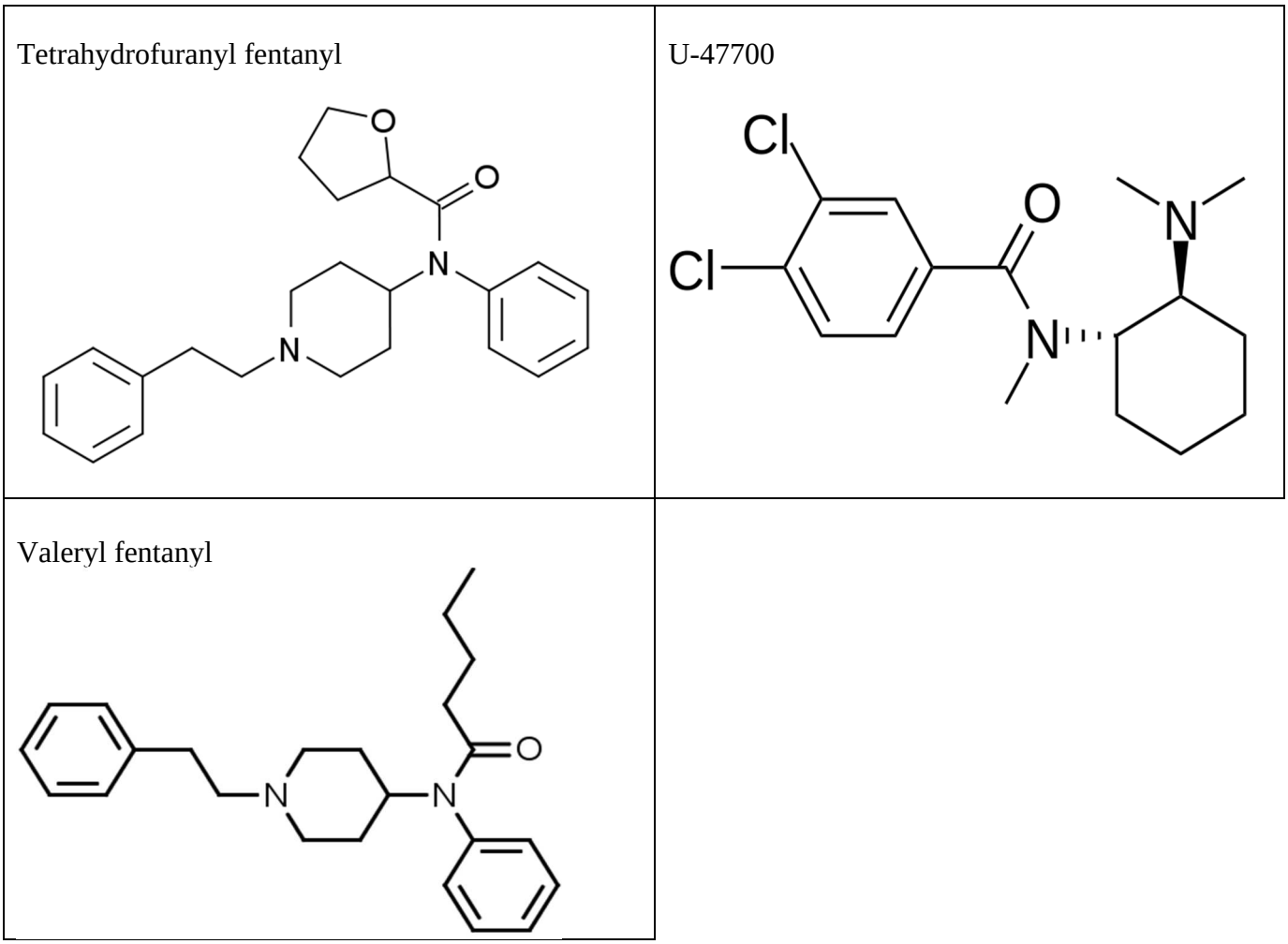


Figure 7: Chemical Structures of Fentanyl and Related Compounds

Several of the listed compounds have yet to be seen in Oklahoma via the OSBI drug chemistry unit. They are none-the-less included in this method for completeness, and to prepare in the event of their emergence. These drugs were chosen due to their prevalence throughout the world. Not all are in the drug chemistry unit's mass spectral library, so there is the potential for false negatives. This is where a drug is not identified because the method used to identify it is unable to detect it, causing it to be falsely reported as negative despite being present. The present method will be put in place in the toxicology unit to reduce the instance of false negatives.

The method from which the present one was developed was published in a paper written by Biotage© as part of the application notes for their products (Biotage©, 2019.) This company is dedicated to providing academic, research, environmental, and forensic facilities with the equipment needed to fulfill their goals. Their free online webinar hosted by SeparationSciences showcased one of their product categories, SPE cartridges. Two different elution solvents were tested with two different SPE cartridges and one supported liquid extraction (SLE) cartridge. The recoveries for each iteration were evaluated and presented in the webinar. The combination with the highest recovery was chosen as a starting point for this validation: the EVOLUTE EXPRESS CX SPE cartridge with 78:20:2 dichloromethane (DCM): isopropanol (IPA): ammonium hydroxide (NH_4OH) as the elution solvent. The method is described in the following figure, as it appeared in the webinar.

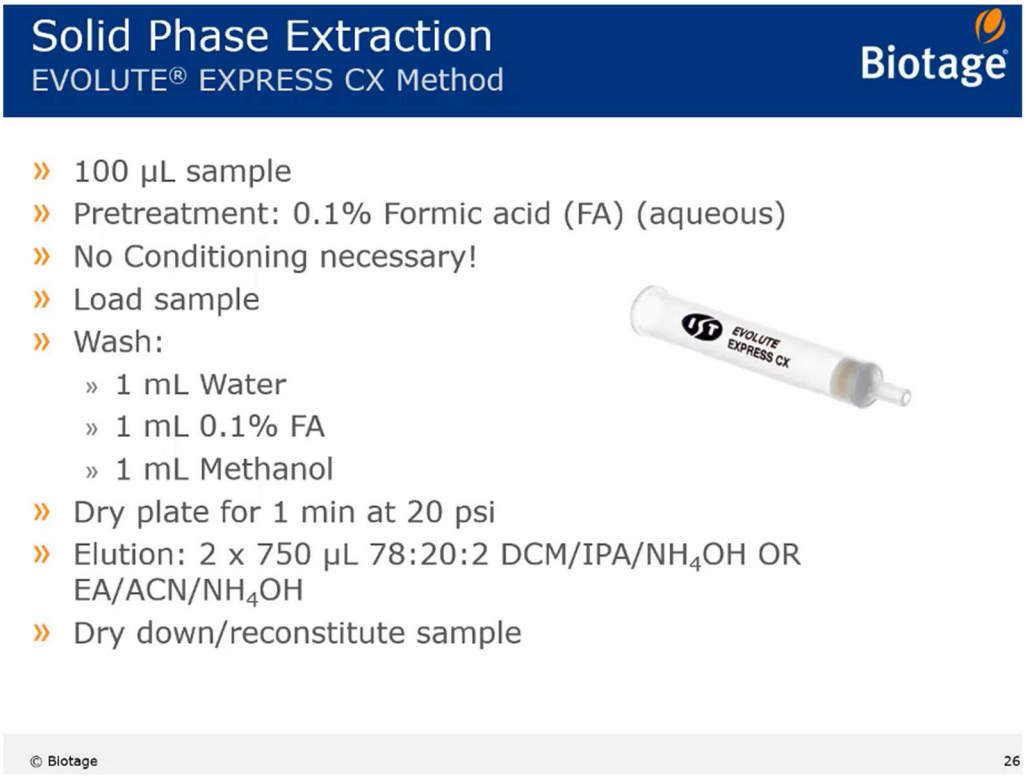


Figure 8: Sample Preparation for Fentanyl Analogs in Whole-Blood

(Biotage©, 2019, April 1)

Seymour et. al. used an extraction solvent of 1 mL 50:50 methanol (MeOH): acetonitrile (ACN), and a reconstitution solvent of 100 µL 90:10 0.1% aqueous formic acid (FA):ACN (Seymour, 2018). This method published in the JAT explained the analysis of fentanyl analogs in dried blood samples (Seymour, 2018). All of the reagents are currently available for use in the OSBI toxicology lab. Though this was not a SPE method given the samples were dried blood, the solvents were still considered for use.

Fogerty et. al. also published a method for detecting fentanyl analogs in the JAT; it uses a 500 µL sample volume with 50 µL internal standard solution (ISTD), a pre-treatment of 2 mL pH 6 phosphate buffer followed by centrifugation for 5 minutes (Fogerty, 2018). The supernatant is then loaded onto the SPE column, and washed with 1.5 mL of deionized water, 0.5 mL of 0.1 M acetic acid, and finally 1.5 mL of MeOH. The elution solvent used for this method was 78:20:2 ethyl acetate: ACN: NH_4OH with a 200 µL reconstitution solvent composed of 60:40 5 mM ammonium formate: 0.1% FA in MeOH. Ammonium formate is not a reagent kept on hand in the OSBI toxicology lab and therefore a substitution, likely one of the mobile phases, would be made unless a solvent with similar properties could be found.

Kahl et.al. published their SPE LC-MS/MS method in the JAT for the quantitation of six fentanyl analogs in various post-mortem specimens, including tissue homogenates (Kahl, 2018). Their method consisted of 500 µL sample volumes with 50 µL ISTD pretreated with 4 mL of pH 6 phosphate buffer. The samples were allowed to stand for 15 minutes then centrifuged for 10 minutes. The supernatant was then loaded onto the column and washed with 3 mL of deionized water, 1 mL of 1M acetic acid, 2 mL of hexane, 3 mL hexane: ethyl acetate (1:1), and 3 mL MeOH before being eluted w/ DCM: IPA: NH_4OH (78:20:2) with 50 µL of 0.1% aqueous FA as the reconstitution solvent. This method seems promising in that it has been validated for

multiple sample matrices including liver & brain homogenates, as well as both whole blood and serum. With a resulting limit of detection (LOD) of at least 0.5 ng/mL for all validated analogs, 0.2 ng/mL for carfentanil; it definitely shows promise.

The following method performed by Winborn & Kerrigan was also published in the JAT, and involves the detection of desomorphine (a.k.a Krokodil) in urine by LC-MS/MS (Winborn, 2019). It is noteworthy because it involves an opioid, not a fentanyl analog. It also addresses the other obstacle, urine as a sample matrix. If this method works universally for both opioids and fentanyl-related drugs, it would be extremely useful. The method uses a 500 µL sample volume with an added 50 µL of ISTD at a concentration of 0.25 µg/mL. A 1 mL 0.1M HCl fortification or pre-treatment solvent is used followed by washing with deionized water, 0.1 M hydrochloric acid (HCl), MeOH, and ethyl acetate all at 1 mL respectively. The elution solvent used was two aliquots of 500 µL of 4% concentrated NH_4OH in ethyl acetate. The samples were reconstituted in 30 µL of 92:8 Mobile Phase A: B, with A being 0.1% aqueous FA and B 0.1% FA in Acetonitrile. These happen to be the same mobile phases used by our lab and so no changes to the method in that regard would need to be made.

Finkelstein et. al. published an article in the JAT describing their fentanyl protocol and extraction (Finkelstein, 2019). Though the analysis uses GCMS, the extraction involves SPE, and thus still offers valuable information. In addition, the matrices involved were both blood and urine and resulted in low LODs for both, 0.5 ng/mL and 0.75 ng/mL respectively. The validated extraction technique consisted of a sample volume of 1 mL treated with 2 mL of pH 6 buffer, vortexed, centrifuged, and loaded onto the SPE column. The supernatant is then washed with 2 mL of deionized water, 1 mL of 100mM acetic acid, 3 mL of MeOH and dried for 5 minutes before being eluted with 1200 µL of 78:20:2 DCM: IPA: NH_4OH. The eluent is finally dried &

reconstituted in 50 µL of ethyl acetate. This method uses the same elution solvent and several of the same washes as the original method developed by Biotage©.

The last method comes from UNODC, though it would be looked at only in the event all other variations above had been exhausted given the fact it is more time-consuming and several of the reagents are not readily available in the OSBI toxicology lab. It is noteworthy in that it is approved for use in both blood and urine and was suitable for 16 different fentalogs, suggesting it is fairly universal. It involves the use of a 500 µL sample treated with 2 mL of potassium phosphate buffer, which does not happen to be in the OSBI toxicology lab already. The pretreated sample is sonicated for 15 minutes which has been unique to this extraction thus far. The OSBI laboratory does have a sonicator, but it is not used in any other protocol currently in policy. After sonication, the sample is centrifuged for 10 minutes, which is twice the longest time for any other current protocol. After it is spun down, the samples are loaded onto SPE cartridge with a sorbent thicker than that used here, 35 mg. The samples are washed with 2 mL of deionized water and 100 mM acetic acid each and dried. After drying, it is washed again this time with 1 mL of MeOH and ethyl acetate respectively. Finally, the sample is eluted with 1.2 mL of ethyl acetate: MeOH: NH_4OH (93:5:2) in two aliquots of 600 µL.

UNODC actually offers three different SPE methods all validated with the same LC-MS/MS method with LODs below that of any other mentioned thus far, less than 0.04 ng/mL for six different opioids. The other two UNODC SPE extractions were not considered for the following reasons. The first uses a reverse phase ion exchange column which is not available for use at this time and could not be considered for this project. Further, this SPE method was only used for U-47700 and does not appear to be universal to all fentalogs. The second is similar to those mentioned thus far. Both of these are for use in blood and not applicable to urine. The

washes and elution solvents from the second method may be considered after all above have been tested, as it is very similar already.

Taking into account all of the methods listed, the Biotage© SPE extraction was modified and adapted for use by the OSBI toxicology unit. The LC-MS/MS analytical method was developed from the OSBI's current one for Opiates, and not that published by Biotage©. The finalized method was validated using OSBI's Toxicology Quality Manual, which follows OSAC recommended guidelines for the validation of methods prepared by American National Standards Institute/American Academy of Forensic Science Standards Board (ANSI/ASB, 2019).

Materials and Methods

Chemicals and Reagents

Certified reference materials purchased or provided by Cerilliant served as primary standards (Cerilliant Inc., Round Rock, TX.) beta-Hydroxythiofentanyl-$^{13}C_6$ and fentanyl-$^{13}C_6$ were used as internal standards. They were provided by Cerilliant. All Cerilliant standards were commercially prepared in methanol at concentrations between 50 µg/mL – 1 mg/mL. This ensured all inherently dangerous drugs were safe to handle in the laboratory. It allows the OSBI toxicology lab to comply with *de minimus* level regulations, even though it has a DEA license for possession of controlled substances. All standards possessed a certificate of analysis. A few certified reference materials from the original list were obtained through Cayman Chemical, (Ann Arbor, MI) but none of the finalized compounds were purchased from this manufacturer.

The following LCMS reagent grade items were purchased through ThermoFisher Scientific (Pittsburgh, PA): acetonitrile (ACN), concentrated ammonium hydroxide (NH_4OH), ethyl acetate (EA), 0.1% formic acid in acetonitrile (Mobile Phase B), 0.1% formic acid in water (Mobile Phase A), isopropanol, and methanol. Deionized water is on tap at the OSBI FSC. A

solution consisting of 50:50 Mobile Phase A: Mobile Phase B served as the reconstitution and dilution solvent for the method.

Two certified drug-free blank matrices were used including bovine blood from Lampire Biological Laboratories (Pipersville, PA) and synthetic urine from Immunalysis (Pomona, CA). Human blood obtained from prior casework that had reached its maximum retention was also used if it was reported as "No drugs detected." Use of case specimens is allowed by OSBI policy for research purposes only. An institutional review board (IRB) consisting of UCO faculty and staff was consulted for the use of human specimens. Their use was found to be acceptable because no identifying information would be retained or published for the individuals' samples used in this study.

Supplies and Equipment

The following consumables were purchased from ThermoFisher Scientific (Pittsburgh, PA): 5 mL conical centrifuge tubes with polytetrafluoroethylene (PTFE) lined screw caps, limited volume inserts, microcentrifuge tubes, Pasteur pipettes, pipette tips, and silicone auto-sampler vial caps with rubber septum. Glass autosampler vials were purchased from Phenomenex (Torrance, CA.) The following laboratory equipment was also purchased through ThermoFisher Scientific (Pittsburgh, PA): calibrated volumetric flasks, centrifuge, Eppendorf pipettes (adjustable and fixed), nitrogen evaporator (N-Evap), pneumatic positive pressure manifold, and vortex mixer.

The following specialty equipment was purchased from the listed manufacturer: EVOLUTE EXPRESS CX 30 mg 1 mL solid phase extraction cartridges (Biotage©, Uppsala, Sweden), LCMS 8050 triple quadrupole system (Shimadzu, Columbia, MD), and a Raptor™

Biphenyl HPLC column with dimensions of 100×2.1 mm and 2.7 µm particle size (Restek, Bellefonte, PA.) Nitrogen was supplied by a nitrogen generator (Peak Scientific, Billerica, MA.)

Preparation of Standard Solutions

The standard solution preparation below is worded and formatted in line with the OSBI toxicology unit's current opiates protocol.

High Positive Control (HPC)

Secondary High Positive Control Solution (5:1 µg/mL): Transfer the appropriate amount of each 1 mg/mL primary standard (50 µL or 10 µL) as shown in the table below to a 10 mL volumetric flask and fill to the mark with dilution solvent; refrigerate.

Table 2: Volume of Certified Reference Materials Used to Prepare Standard Solutions

Compound	Volume (µL)
4-ANPP	50
Acryl fentanyl	10
Butyryl fentanyl	10
Cyclopropyl fentanyl	50
Fentanyl	50
***para*-Fluorofentanyl**	10
Furanyl fentanyl	10
Methoxyacetyl fentanyl	50
Norfentanyl	50
Valeryl fentanyl	50

Tertiary High Positive Control Solution (500:100 ng/mL): Transfer 1 mL of secondary high positive control, 50 µL of each 100 µg/mL primary standard (Sufentanil, alpha-Methylfentanyl, and 4-FIBF) and 100 µL of each 50 µg/mL primary standard (acetyl fentanyl) to a 10 mL volumetric flask and fill to the mark with dilution solvent; refrigerate.

Working High Positive Control Solution (50:10 ng/mL): Transfer 1 mL of tertiary high positive control to a 10 mL volumetric flask and fill to the mark with dilution solvent; refrigerate.

Low Positive Control (LPC)

TX42 Working Low Positive Control Solution (5:1 ng/mL): Transfer 1 mL of working HPC to a 10 mL volumetric flask and fill to the mark with dilution solvent; refrigerate.

Table 3: Final Concentrations of Controls in 100 µL of Sample

Compound	LPC (ng/mL)	HPC (ng/mL)
4-ANPP	0.5	5
4-FIBF/PFBF	0.5	5
Acetyl fentanyl	0.5	5
Acryl fentanyl	0.1	1
alpha-Methylfentanyl	0.5	5
Butyryl fentanyl	0.1	1
Cyclopropyl/Crotonyl fentanyl	0.5	5
Fentanyl	0.5	5
Fluorofentanyl	0.1	1
Furanyl fentanyl	0.1	1
Methoxyacetyl fentanyl	0.5	5
Norfentanyl	0.5	5

Sufentanil	0.5	5
Valeryl fentanyl	0.5	5

Pre-Treatment Solution/Internal Standard (ISTD)

Secondary Internal Standard Solution (5 µg/mL): Transfer 10 µL of each 1 mg/mL carbon-13 labeled primary internal standard to a 2 mL volumetric flask and dilute with Mobile Phase A; refrigerate.

Pre-treatment Solution/Working Internal Standard Solution (1 ng/mL): Transfer 10 µL of secondary internal standard solution to a 50 mL volumetric flask and dilute with Mobile Phase A; refrigerate.

Elution Solvent

Combine Ethyl Acetate, Acetonitrile, and concentrated Ammonium Hydroxide in a 39:10:1 EA/ACN/NH_4OH ratio and store at room temperature. This must be made the day of use.

LC-MS/MS Conditions

The following conditions are listed as they will appear in the OSBI toxicology unit's official protocol for this method.

Gradient Elution

Mobile Phase A: 0.1% Formic Acid in Water

Mobile Phase B: 0.1% Formic Acid in Acetonitrile

Initial Composition: 95% A, 5% B, Total Flow 0.60 mL/min

0.2 – 3.5 min: % B increased to 50%

3.5 – 4.25 min: % B increased to 95%

4.25 – 5.45 min: % B is held at 95%

5.45 – 6.50 min: % B decreased to 5%

Column/Oven Temperature: 50°C

Column Type: Restek Biphenyl 100 × 2.1 mm and 2.7 µm particle size

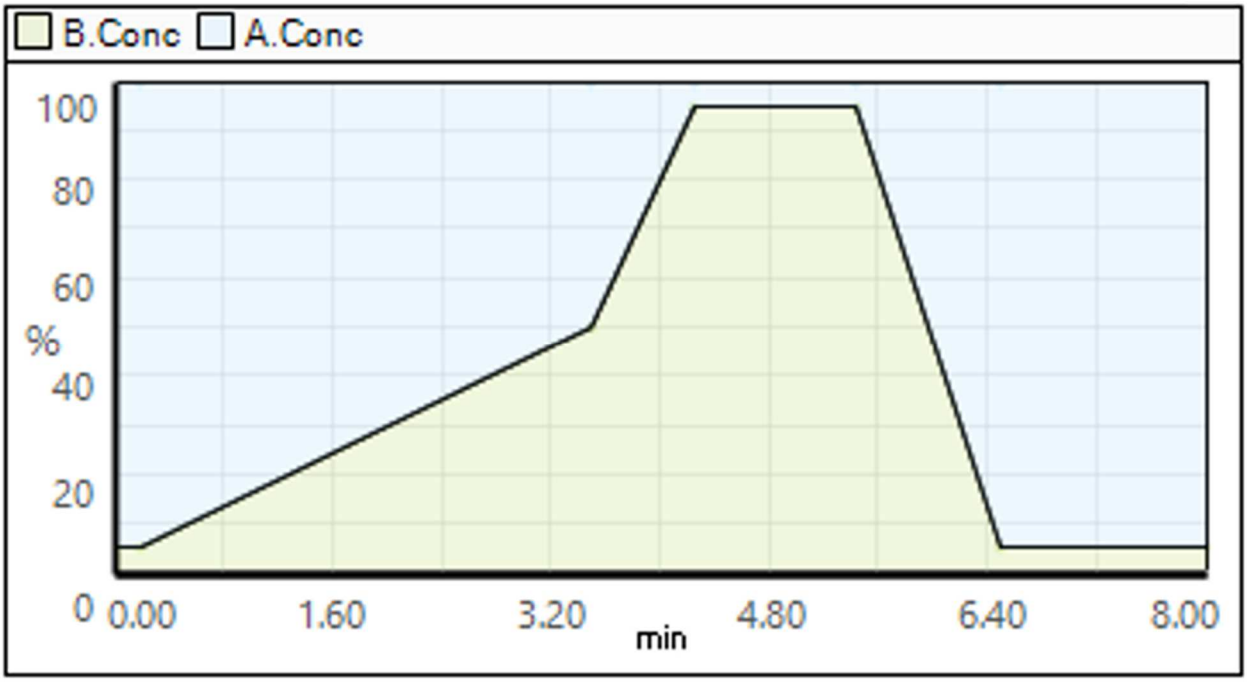


Figure 9: LC-MS/MS Gradient Elution Time Program

Autosampler

Injection Volume: 3 µL, may be adjusted down as needed

Sampling Speed: 5 µL/s

Cooler Temperature: 15°C

Interface

Electro-spray Ionization (ESI)

Nebulizing & Drying Gas: Nitrogen

Nebulizing Gas Flow: 2.0 L/min

Drying Gas Flow: 15.0 L/min

Collision Induced Dissociation (CID) Gas: Argon 230 kPa

Desolvation Line (DL) Temperature: 250°C

Heat Block Temperature: 400°C

Table 4: LC-MS/MS Parameters

Compound Name	Molecular Weight[10] (amu[11])	Retention Time (min)	MRM Transitions[12] (m/Z)	Q1 Voltage (Volts)	Collision Energy (Volts)	Q3 Voltage (Volts)
Norfentanyl	232.32	1.95	233.10>84.10	-12	-21	-21
			233.10>55.00	-12	-36	-22
beta-Hydroxythiofentanyl - $^{13}C_6$	364.45	2.62	365.00>347.30	-18	-17	-26
			365.00>192.05	-25	-24	-21
			365.00>110.95	-18	-40	-21
Methoxyacetyl fentanyl	352.47	2.63	353.00>188.10	-20	-25	-20
			353.00>105.15	-19	-40	-20
Acetyl fentanyl	322.44	2.70	323.00>188.10	-20	-25	-20
			323.00>105.15	-20	-40	-20
4-ANPP	280.41	2.92	281.00>188.15	-20	-19	-20
			281.00>105.20	-20	-30	-20
Acryl fentanyl	334.45	2.94	335.00>188.20	-20	-25	-20
			335.00>105.20	-20	-40	-20
Fentanyl	336.47	2.97	337.20>188.00	-20	-25	-20
			337.20>105.00	-20	-40	-20
Fentanyl – $^{13}C_6$	342.43	2.97	343.00>188.20	-20	-25	-20
			343.00>105.15	-18	-40	-20
Fluorofentanyl	354.46	3.03	355.00>188.15	-20	-25	-20
			355.00>105.20	-20	-40	-20

[10] All molecular weights were obtained from the certificate of analysis and are listed here as the base.
[11] Atomic mass units
[12] These were obtained from UNODC and various other sources with the allowance for the instrument to fine tune them during optimization.

alpha-Methylfentanyl	**350.50**	**3.12**	**351.30>91.10**	**-18**	**-40**	**-20**
			351.30>202.00	-13	-24	-20
			351.30>119.20	-13	-28	-13
Cyclopropyl fentanyl	348.48	3.12	349.00>188.15	-20	-25	-20
			349.00>105.20	-19	-40	-20
Furanyl fentanyl	374.48	3.12	375.00>188.10	-20	-25	-20
			375.00>105.15	-20	-40	-20
Butyryl fentanyl	350.50	3.19	351.00>188.10	-20	-25	-20
			351.00>105.15	-20	-45	-20
4-FIBF/PFBF	368.49	3.20	369.30>188.15	-20	-25	-20
			369.30>105.05	-19	-40	-20
Sufentanil	386.55	3.31	387.10>238.15	-23	-23	-26
			387.10>111.10	-14	-39	-20
			387.10>355.05	-14	-21	-18
Valeryl fentanyl	364.52	3.44	365.10>188.15	-20	-25	-20
			365.10>105.15	-20	-40	-20

(Ross-Carr, 2017)

Sample Preparation

The sample preparation scheme below is worded and formatted in line with the OSBI toxicology unit's current opiates protocol.

1. Label a clean, disposable micro-centrifuge tube, conical centrifuge tube, and autosampler vial for each control and case sample.
2. Rotate & thoroughly vortex blood samples before pipetting.
3. Prepare the low positive control by adding 10 µL of working low positive control solution and 90 µL of drug-free whole blood to the low positive micro-centrifuge tube.
4. Prepare the high positive control by adding 10 µL of working high positive control solution and 90 µL of drug-free whole blood to the high positive micro-centrifuge tube.

5. Add 100 µL of drug-free whole blood to the negative control micro-centrifuge tube.
6. Add 100 µL of each case specimen to the appropriately labeled micro-centrifuge tubes.
7. Add 100 µL of pretreatment/working internal standard solution and vortex.
8. Load each sample onto a separate SPE cartridge previously placed onto the sample plate of a positive pressure manifold.
9. Apply the necessary pressure to elute the sample into the waste trough, ~85 psi for blood (full-flow) and ~10-20 psi for urine (regulated-flow.)
10. Wash each cartridge with each of the following reagents, eluting into the waste trough before moving on to the next wash: 1 mL of deionized water, 1 mL of 0.1% FA (Mobile Phase A), and 1 mL of methanol.
11. Dry the cartridges for ~1 min at 20 psi or switch to full flow.
12. Elute into labeled conical centrifuge tubes by switching the waste trough for the sample rack and washing with two aliquots of 760 µL of elution solvent.
13. Evaporate to dryness at approximately 40°C with a steady stream of nitrogen.
14. Add 50 µL of reconstitution solvent to each conical.
15. Vortex briefly and centrifuge to collect the sample in the bottom of conical.
16. Transfer sample to appropriately labeled autosampler vials.
17. Centrifuge at 2800 – 3000 rpm as needed.
18. Begin each run with the following sequence: low positive control, high positive control, negative control.
19. Inject 3 µL of sample, injection volume may be adjusted down as needed. If a different injection volume is used, it should be documented in the case record. The same injection volume must be used for entire sequence. Utilize “TX42.lcm” method.

Method Development

The first challenge that arose when attempting this method was that it was created specifically for fentalogs, and not the more common opiates. This resulted in extremely high recoveries for fentanyl and very low recoveries for the rest of the compounds in the OSBI's current TX40 Opiates Protocol.

The second challenge was finding a method that not only worked for both opiates & opioids alongside fentanyl-related compounds, but also effective for blood and urine specimens. To obtain higher recoveries for all compounds from both matrices, several different combinations of pretreatment, reconstitution, and elution solvents were tested using the general Biotage© method. 200 30 mg/mL 1-mL EVOLUTE EXPRESS CX cartridges from Biotage© were provided for preliminary trials, free of charge. The 30 mg refers to the amount of sorbent bed packed into the cartridges, and 1-mL is the total volume of the cartridge.

After exploratory testing and discussion with Biotage©, it was determined the traditional opiates would require a different elution solvent than that of the fentalogs. This shifted the project from incorporating the opiates and fentalogs into one method to a fentalog only endeavor.

This method was first developed on a Shimadzu LCMS 8030+ using a C-18 column. This did not display adequate separation of the structurally similar fentalogs and sensitivity was poor. An acceptable method was developed under these conditions but a new column and instrument were purchased. This provided adequate separation for all compounds in the final method, as evidenced by Figure 10 on the following page. The method was moved to the new instrument, re-optimized, developed, and validated on the LCMS 8050 system using the biphenyl column listed previously.

Optimization was performed by preparing 50 or 500 ng/mL solutions by adding 1 µL of each primary standard (100 µg/ml or 1 mg/mL) to 2 mL of dilution solvent in an autosampler vial. Ion transitions from literature were used as starting points with the allowance for them to be modified by the instrument's optimization program. Voltages and collision energies for each compound was calculated for maximum recovery and specificity. These values in volts may be found in Table 4.

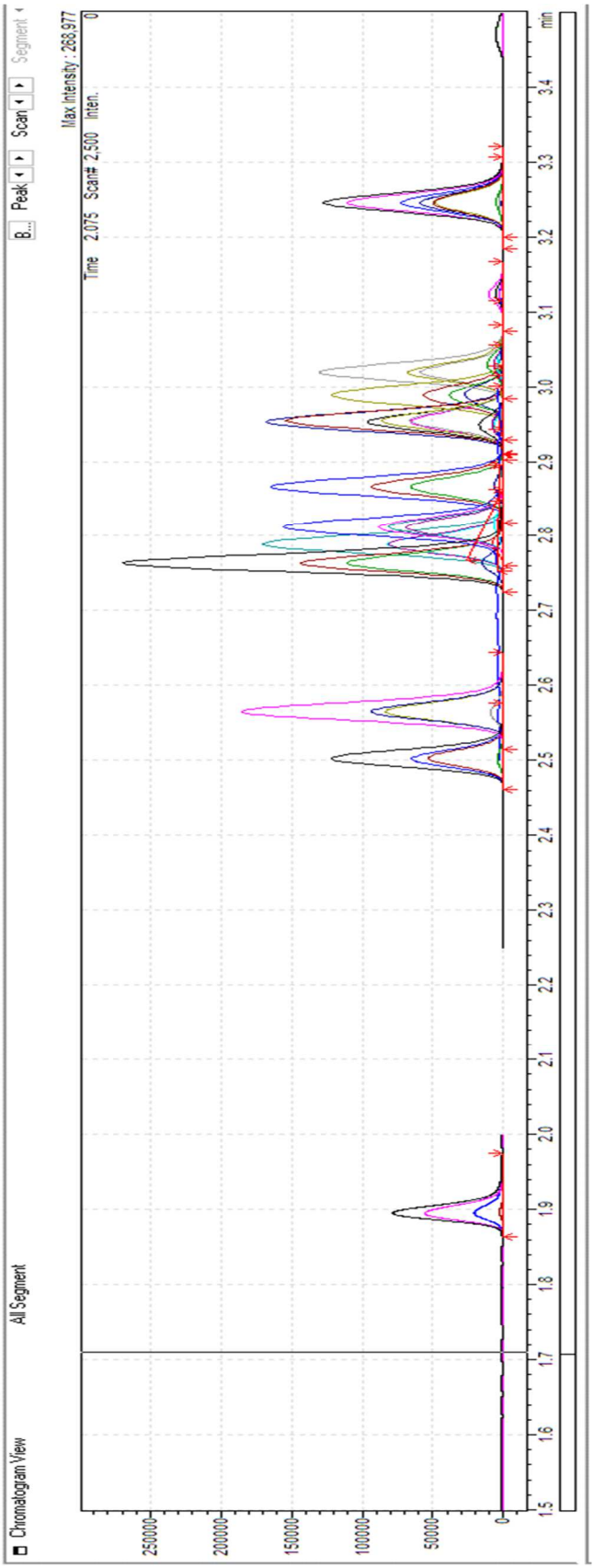


Figure 10: Chromatographic Overview - Overlaid Ion Chromatograms for All Compounds

All of the aforementioned compounds found in Figure 7 were optimized on the instrument. This means they will be detected should they appear in the sample. However, not all were validated for reporting. The final list was pared down to the top 15 compounds in addition to two internal standards. This was an administrative decision by the OSBI laboratory hierarchy to optimize analyst time and laboratory resources. Those optimized but not validated may undergo full validation if they are identified in a sample using a version of the analytical method that contains their optimized transitions. Only compounds successfully validated will be included in the low and high controls used for identification and reporting. The list of compounds considered for full validation is as follows.

- Norfentanyl
- 4-ANPP
- Acetyl fentanyl
- Acryl fentanyl
- Carfentanil
- Cyclopropyl/Crotonyl fentanyl
- Fentanyl
- Furanyl fentanyl
- Butyryl fentanyl
- Methoxyacetyl fentanyl
- 4-Fluoro-isobutyryl fentanyl/*para*-Fluorobutyryl fentanyl/ (4-FIBF/PFBF)
- Fluorofentanyl
- Sufentanil
- Valeryl fentanyl

- α-Methylfentanyl
- Fentanyl – $^{13}C_6$
- β-Hydroxythiofentanyl – $^{13}C_6$

The following compounds were not included in the final method but they will be added to the OSBI toxicology unit's mass spectral library for identification by full-scan gas chromatography mass spectrometry (GCMS), although it is unlikely any will be seen by this less sensitive method. Those underlined were mentioned in the previously listed sources at least twice and were considered for inclusion in this method due to their availability and prevalence in the literature.

Benzyl fentanyl	4'-methyl Acetyl fentanyl	Norcarfentanil
U-48800	U-49900	U-51754

The following compounds were mentioned in the previously listed sources at least twice and were considered for inclusion in this method, but were not provided or purchased for this validation.

Butyryl norfentanyl	*para*-Methoxybutyryl fentanyl	Norsufentanil
Cyclopentenyl fentanyl	Furanyl norfentanyl	

The following isotopically labeled compounds were provided by Cerilliant. Those underlined were considered as internal standards for this method.

Fentanyl-$^{13}C_6$	β-Hydroxythiofentanyl-$^{13}C_6$	Valeryl fentanyl-$^{13}C_6$
Acetyl fentanyl-$^{13}C_6$	Acryl fentanyl-$^{13}C_6$	4-ANPP-$^{13}C_6$
Butyryl fentanyl-$^{13}C_6$	Cyclopropyl fentanyl-$^{13}C_6$	Furanyl fentanyl-$^{13}C_6$

para-Fluorobutyryl fentanyl-$^{13}C_6$	*para*-Fluorofentanyl-$^{13}C_6$	Methoxyacetyl fentanyl-$^{13}C_6$
4'-Methylacetyl fentanyl-$^{13}C_6$	Remifentanil-$^{13}C_6$	U-47700-$^{13}C_3$,$^{15}N_2$
Carfentanil-$^{13}C_6$	Benzyl fentanyl-$^{13}C_6$	Norfentanyl-$^{13}C_6$
Norcarfentanil-$^{13}C_6$	U-48800-$^{13}C_3$,$^{15}N_2$	U-49900-$^{13}C_5$

Fentanyl-d5 had been previously purchased by the OSBI toxicology unit and was compared to fentanyl-$^{13}C_6$. Upon optimization of both, no difference could be seen. The literature speaks of the "deuterated shift" and the superiority of the C-13 labeled standards, therefore, these were chosen over those labeled with deuterium (Landvatter, 2017). β-Hydroxythiofentanyl – $^{13}C_6$ was chosen because its unlabeled counterpart was the earliest eluting compound in the method and valeryl fentanyl – $^{13}C_6$ was likewise the latest. Fentanyl – $^{13}C_6$ was chosen as a mid-eluter to cover the full range of the method and ensure recovery at the beginning, middle, and end of the analytical run.

Validation Results

Table 5: Validation Parameters Evaluated and Results

Parameter	Acceptable Limit	Result
Carryover	No analyte carryover may be observed above the LOD. Post-mortem concentrations from literature and previous case history will be used to determine a suitable testing limit.	- Valeryl fentanyl displayed carry-over at the highest concentration tested but did not meet reporting criteria. - No other analytes in the finalized list displayed carryover at 28 ng/mL (280x and 56x LPC)

Interference	Evaluate all compounds from other validated methods, as well as other drugs commonly identified in the toxicology laboratory. Ten blank samples of each matrix will be analyzed to verify no matrix interference is present.	- See Table 6 for interferences tested. - The following pairs of compounds were found to be indistinguishable: • Cyclopropyl & crotonyl fentanyl • Butyryl & isobutyryl fentanyl • 4-FIBF & PFBF • *ortho*- & *para*- Fluorofentanyl. - Valeryl fentanyl-13C6 appeared to be contaminated with unlabeled compound and was not included in the final method.
Ionization Suppression/ Enhancement	< 25% suppression or enhancement and < 20% CV due to matrix (if not, evaluate impact on LOD by tripling the number of matrix sources used for evaluation)	- The following compounds were above the specified limits: • 4-ANPP, acryl fentanyl, and furanyl fentanyl in blood and urine • Butyryl fentanyl and cyclopropyl fentanyl in blood - The impact on the LOD was evaluated.
Limit of Detection (LOD)	Defined as the decision point (Ross-Carr, 2017.) Policy allows this parameter to be administratively set (Stillwell, 2020.) A minimum of nine samples per run of each fortified matrix sample at the concentration of the decision point shall be analyzed over three runs to demonstrate all detection and identification criteria are met and to evaluate the impact of ISE.	- Carfentanil did not meet acceptance criteria and was not included in the final method. - All criteria were met at the decision point for all other analytes in both matrices.

(Stillwell, 2020)

Discussion

Carry-Over

Carry-over occurs when a substance from a previous sample is falsely detected in a subsequent one. This could potentially lead to false positives for the sample in which the substance is not present. The analytical instrumentation should be able to clean itself effectively between samples to keep this from happening. If carry-over is observed, the instrumental parameters are changed to avoid this issue.

Carry over was evaluated by analyzing neat samples at 28 ng/mL followed by extracted blanks of each matrix. Each was analyzed in triplicate with blanks after each injection to show no carry-over. 28 ng/mL is more than five times that of the decided HPC. According to Pearson (2015), the average fatal concentration when combined with heroin is 18 ng/mL for fentanyl, 2 ng/mL for norfentanyl, and 8 ng/mL for acetyl fentanyl. Baselt (2017) lists an average blood fentanyl concentration of 8.3 ng/mL in fatalities attributed to this drug. Because the OSBI only performs ante-mortem toxicology, it is unlikely any samples will have concentrations this high in living people. With this in mind, 28 ng/mL is more than high enough to evaluate carry-over.

Valeryl fentanyl displayed carry-over at this concentration though the signal did not meet reporting criteria when evaluated against controls. No other compounds displayed significant carry-over.

Interference

Interference occurs when one substance is falsely identified as another. Compound interference was evaluated by analyzing neat samples of each drug individually to see if they gave signals for other compounds in the method. No compounds in the final method gave signals for others; however, known isomers of the selected compounds were tested and found to

be indistinguishable. This includes cyclopropyl & crotonyl fentanyl, butyryl & isobutyryl fentanyl, 4-FIBF & PFBF, *ortho*- & *para*-fluorofentanyl.

To remedy these identical compounds, certain adjustments were made. For cyclopropyl and crotonyl fentanyl, the two will be reported as one result with a "/" between their names, "cyclopropyl/crotonyl fentanyl." For the same reason, 4-FIBF and PFBF will be reported in this fashion as well.

For butyryl & isobutyryl, the two will be reported as simply "butyryl Fentanyl" with the knowledge that it could be either the straight chain or branched compound if asked in court. The reasoning for this is the two do not have completely separate names as with crotonyl and cyclopropyl fentanyl. "Isobutyryl/Butyryl fentanyl" would be needlessly overcomplicated and redundant. For the same reasons as with butyryl fentanyl, fluorofentanyl will be reported without a prefix.

Valeryl fentanyl – $^{13}C_6$ did not pass the interference study. This was due to contamination by its unlabeled counterpart. This could lead to false positives in the future. Only beta-Hydroxythiofentanyl– $^{13}C_6$ and fentanyl– $^{13}C_6$ would be included in the final method. This would nonetheless provide adequate coverage over the length of the method.

Non-fentalog compound interferences were evaluated using drugs routinely encountered by the OSBI toxicology unit. All HPCs from all protocols were analyzed as separate neat solutions. One neat solution was made containing drugs not present in HPCs but commonly encountered. This solution was prepared from standards already on hand, purchased from Cerilliant, Inc. (Round Rock, TX.)

Table 6: Commonly Encountered Drugs Evaluated for Interference

11-Hydroxy-Δ9-tetrahydrocannabinol (THC-OH)	Diphenhydramine	N-desmethylcitalopram
11-Nor-9-carboxy-Δ9-tetrahydrocannabinol (THCA)	Doxepin	N-desmethyl-tramadol
3-4 methylenedioxy methamphetamine (MDMA)	Etizolam	Nordiazepam
5-Fluoro-ADB	Flualprazolam	Nordoxepin (Desmethyldoxepin)
5-Fluoro-AMB	Flubromazolam	Nortriptyline
6-monoacetylmorphine	Flunitrazepam	O-desmethylvenlafaxine
AB Chminaca	Flurazepam	Oxazepam
AB-Fubinaca	FUB-PB-22	Oxycodone
AB-Pinaca	Gabapentin	Oxymorphone
ADB Pinaca	Hydrocodone	PB-22
Alprazolam	Hydromorphone	Pentobarbital
AM1248	JWH-018	Phenazepam
AM2201	JWH-073	Phencyclidine (PCP)
Amitriptyline	JWH-081	Phenobarbital
Amobarbital	JWH122	Phentermine
Amphetamine	JWH-210	Prazepam
Benzoylecgonine	JWH-250	Secobarbital
Butalbital	Ketamine	Sertraline

Cannabinol	Lorazepam	Temazepam
Carisoprodol	MAB-Chminaca	Topiramate
Chlordiazepoxide	MAM2201	Tramadol
Clonazepam	Meprobamate	Trazodone
Cocaine	Methadone	Triazolam
Codeine	Methamphetamine	UR-144
Cyclobenzaprine	Methylone	XLR11
Dextromethorphan	Midazolam	Zolpidem
Diazepam	Morphine	Δ9-tetrahydrocannabinol (THC)

Matrix interference was evaluated by analyzing ten extracted blank blood samples from previously worked cases and ten synthetic urine samples all from different lot numbers. The blood samples were analyzed for casework purposes and shown to contain no drugs or alcohol. They had been labeled as destroyed in the evidence tracking system as they had reached their four-month retention date. The synthetic urine samples were included with Immunalysis ELISA kits used for casework. They were labeled as "drug-free synthetic urine with preservatives."

Ion Suppression/Enhancement (ISE)

ISE occurs when an ion from one substance causes a false enhancement or suppression of the signal for another. This would cause the ion ratios for the enhanced/suppressed compound to appear different than if the interfering compound were not present. ISE was evaluated by extracting 20 blank samples of each matrix and fortified post-extraction with reconstitution solvent spiked with either the LPC or HPC concentration. Ten of the samples were reconstituted

at the LPC concentration and the other ten at the HPC concentration for each matrix. Area counts from the fortified blanks were compared to those of six neat samples at each of the two concentrations to evaluate the impact from other compounds in the sample.

The following compounds did not meet the <25% ISE and/or the <20% coefficient of variation (CV) requirement: 4-ANPP, acryl fentanyl, & furanyl fentanyl in blood and urine, butyryl fentanyl and cyclopropyl fentanyl in blood only. Carfentanil failed in both matrices as well but it was not included in the final method due to issues during the limit of detection (LOD) study, discussed below. ISE charts for each compound in both matrices may be found in the appendix.

The OSBI Toxicology Quality Manual required <15% CV at the time the validation plan was written. The OSAC recommended standards published by ANSI/ASB were updated shortly after with the <20% CV (ANSI/ASB, 2019.) A new version of the quality manual was put in place with this change and the new value was used for the validation.

For all instances where the data did not meet the ISE requirements, the farthest outlying data point was evaluated using a statistical Q-test[13]. If its Q-value was higher than that for a data set of 10 samples, it was not considered. One data point for 4-ANPP in urine was deemed an outlier although its removal did not affect the result, it still failed to meet criteria.

The impact on the LOD for those compounds listed above was evaluated by tripling the number of matrix sources used in the evaluation as dictated in the OSBI Toxicology Quality Manual.

[13] The Dixon's Q-test is a statistical evaluation to determine whether a data point in a set can be classified as an outlier and thus considered invalid. If the result is higher than the Q value for a specific number of data points, it may be eliminated. A table of Q values must be consulted. Mathematically, Q = gap/range (Libretexts, 2020). The range is the difference of the highest data point in the set from the point in question, while the gap is the difference of the lowest data point.

Limit of Detection (LOD)

Table 7: Decision Points for Limit of Detection of Each Compound in Both Matrices Tested

Compound	LOD (ng/mL) Blood	LOD (ng/mL) Urine
4-ANPP	0.5	0.5
4-FIBF/PFBF	0.5	0.25
Acetyl fentanyl	0.125	0.125
Acryl fentanyl	0.05	0.05
alpha-Methylfentanyl	0.5	0.5
Butyryl fentanyl	0.1	0.05
Cyclopropyl/Crotonyl fentanyl	0.125	0.125
Fentanyl	0.125	0.125
Fluorofentanyl	0.1	0.1
Furanyl fentanyl	0.1	0.1
Methoxyacetyl fentanyl	0.125	0.125
Norfentanyl	0.5	0.5
Sufentanil	0.25	0.125
Valeryl fentanyl	0.125	0.125

The decision points were established by performing the first day of the LOD study with three different matrix sources, extracted in duplicate, at the LPC concentration, 50% of the LPC concentration, and 25%. A serial dilution was performed on the LPC to prepare the other two concentrations. The samples were prepared in the same fashion as the LPC in the protocol. 90 µL of blank matrix was combined with 10 µL of each of the three LPC solutions separately. The

dilution solvent used was the same as that used to prepare all standard solutions. 38 samples were extracted the first day to establish the decision points. Subsequently, 19 solutions were extracted each day for the next two days for confirmation.

The concentration that most consistently met acceptance criteria was set as the decision point. Acceptance criteria is described in the OSBI Toxicology Quality Manual. It includes symmetrical peak shape, retention time within 0.15 minutes of that of the LPC, and ion ratios within 30% of the average of the LPC & HPC. To establish the ion ratios, HPCs of each blank matrix type was extracted with the LOD samples. Because there were multiple LPCs, the average of them all was used to set the retention time. After the decision point was established and approved by the TM, the following two days of the LOD study were the same only the different matrix sources were not extracted in duplicate.

Carfentanil was not included in the the finalized method after the LOD study showed inconsistent ion ratios at the LPC concentration. 4-ANPP also exhibited issues during the LOD study, in that the abundance was very low and chromatography was consistently poor. Discussions to increase the LPC concentration from 0.1 ng/mL to 0.5 ng/mL for both compounds ensued. 4-ANPP would be increased while carfentanil would not and would be dropped form the final method. According to Tiscione (2018), "reports have demonstrated that methods with limits of detection of 100 pg/mL or more will fail to detect many instances of carfentanil use in PM samples." Because the LPC concentration was already set at this recommended concentration, anything higher was deemed pointless, especially for the ante-mortem work performed at the OSBI.

Stability

The stability of extracted samples was evaluated across a typical five-day workweek. Ten aliquots of each blank matrix was extracted, five at the HPC concentration and five at the LPC concentration. Bovine blood and synthetic urine served as the blank matrix sources. The five samples of like concentration and matrix type were combined to ensure a homogenous sample and then redistributed into autosampler vials. One of each concentration and type was injected per day. Each sample was injected in triplicate. The samples were refrigerated when not being used.

The results of the stability study showed no significant difference in concentration across the five-day study. Although this study was not on the original validation plan and thus not required, it was performed none-the-less.

Bias, Precision, Calibration Model, and Limit of Quantitation

As seen in "Appendix 2 – OSBI Toxicology Unit TX42 Validation Plan," bias, precision, calibration model, and limit of quantitation were not required components of this validation. This is because this method is entirely qualitative in nature; no quantitative data will be produced from this procedure. In the OSBI toxicology unit, we routinely quantitate alcohol, but scarcely do so with drugs. Only six compounds have validated quantitation methods: alprazolam, methamphetamine, codeine, oxycodone, hydrocodone, and morphine. It is the policy of the OSBI to quantitate drugs only under specific circumstances, by request or court order of the state prosecuting attorney, and prior supervisor approval. This is due to the many decades correlating a blood alcohol concentration (BAC) with impairment, and very little in the way of drugs. In addition, state law dictates impairment is irrelevant in regards to alcohol. Above a BAC of 0.08 g/100 mL of whole blood, a person is *per se* impaired no matter the circumstances.

When we criminalists must appear in court, our testimony is the same whether we quantitate a drug or qualitatively identify it. When asked on the stand about how a drug may affect a person, we give generalizations about the possible effects it may have on an average person. We will not provide statements about how a specific individual may be affected by a particular drug. This is why we rarely quantitate, and have protocols for quantitating so few drugs. We feel we cannot provide meaning to the number produced by quantitation, and feel it could be misleading to the trier of fact (judge or jury). If someone sees a seemingly large or small number without a reference, we feel it could inadvertently taint their judgment. We do not feel comfortable providing such a circumstance by quantitating drugs in our casework.

Future Research

In 2019, ten authentic case specimens suspected to contain fentalogs were retained for future testing. They screened positive by ELISA for fentanyl but when LC-MS/MS analysis was performed, fentanyl did not appear to be present. ELISA is a non-specific test that can be triggered positive by drugs similar in structure to the target. Theoretically, the ELISA could have been triggered by a fentalog. LC-MS/MS is a highly specific test and would only be able to detect fentanyl itself. With this new method, these specimens could be analyze in order to test this theory.

Upon completion of this project and its successful institution for casework, the current opiates method will begin its conversion from a liquid-liquid “crash and shoot” sample preparation to a solid phase one. This converted method will be the same as that explained herein with one difference, the elution solvent. This is to account for differences in molecular structure and polarity of opiates and opioids in contrast to fentalogs. After this new opiates method is instituted as well, the benzodiazepines will also be converted to the new paradigm.

According to Biotage©, the same extraction method as the fentalogs has purportedly proven successful for the benzodiazepines. This is another avenue for exploration. By extension, this could mean all basic drugs could be recovered via this extraction. Potentially, this would mean the OSBI could convert their outdated (circa 1976) alkaline drug screen performed on GC-MS to a much more sensitive LC-MS/MS method. This would drastically decrease the amount of time for extraction. It is the hope of the OSBI Toxicology unit that all LC-MS/MS methods be converted to solid phase sample preparations. This will extend the life of the instrument by increasing its sensitivity for the detection of all drugs, no matter the method.

Another aim is to use less sample without sacrificing sensitivity. In the event of a fatality or serious injury collision, the subject is often taken to the hospital. When they arrive, several vials of blood are drawn for their testing purposes. Officers often obtain these vials under search warrant and submit them in lieu of a state issued blood kit for toxicology testing. These vials are significantly smaller and contain very little sample. Methods that only require 100 µL of sample are highly coveted in these circumstances. The OSBI can detect over 30 compounds via LC-MS/MS using only 100 µL of sample. With the validation of this method, this number will increase by almost 50%. The paradigm shift from LLE to SPE for all alkaline drug extractions would reduce the required amount of sample from 2 mL to 100 µL, and increase the number of reportable drugs using this sample amount from less than 50 to over 250.

This validation was met with several analytical, financial, and technical challenges over the two years it took to complete. This was primarily due to the need to balance casework while still finding time to work on this project. The instrument was down for periods of time and often unavailable due to the priority of casework over validations. Despite the challenges faced, it was imperative this method be validated. As of 2021, fentanyl has made its way into the top ten

drugs reported by the OSBI toxicology unit. The number of fentanyl cases reported by the OSBI drug chemistry unit doubled from 2020 to 2021. This goes to show, the opioid crisis is far from over.

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Appendices

Appendix 1 – OSAC Guidelines for DUI Cases

Table 1: Required Minimum Analytical Scope and Sensitivity1 for Toxicology Testing in Impaired Driving Investigations

Compound[1]	Blood Screen	Blood Confirmation[2]	Urine Screen	Urine Confirmation[2]
Ethanol				
Ethanol	0.01 g/dL	0.01 g/dL	0.01 g/dL	0.01 g/dL
Cannabinoids				
THC	-	1	-	N/A
Carboxy-THC	10 ng/mL	5 ng/mL	20 ng/mL	5 ng/mL
11-OH-THC	-	1	-	N/A
CNS Stimulants				
Amphetamine	20 ng/mL	20 ng/mL	200 ng/mL	50 ng/mL
Methamphetamine	20 ng/mL	20 ng/mL	200 ng/mL	50 ng/mL
MDA	-	20 ng/mL	-	50 ng/mL
MDMA	-	20 ng/mL	-	50 ng/mL
Cocaine	-	10 ng/mL	-	20 ng/mL
Cocaethylene	-	10 ng/mL	-	20 ng/mL
Benzoylecgonine	50 ng/mL	50 ng/mL	150 ng/mL	50 ng/mL
CNS Depressants				
Carisoprodol	500 ng/mL	500 ng/mL	500 ng/mL	500 ng/mL
Meprobamate[3]	-	500 ng/mL	-	500 ng/mL
Zolpidem	10 ng/mL	10 ng/mL	20 ng/mL	20 ng/mL
Low Dose Benzodiazepines[3]	10 ng/mL	-	50 ng/mL	-
Alprazolam	-	10 ng/mL	-	50 ng/mL
αOH-alprazolam	-	N/A	-	50 ng/mL
Clonazepam	-	10 ng/mL	-	50 ng/mL
7-aminoclonazepam	-	10 ng/mL	-	50 ng/mL
Lorazepam	-	10 ng/mL	-	50 ng/mL
High Dose Benzodiazepines	50 ng/mL	-	100 ng/mL	-
Diazepam	-	20 ng/mL	-	50 ng/mL
Nordiazepam	-	20 ng/mL	-	50 ng/mL
Oxazepam	-	20 ng/mL	-	50 ng/mL
Temazepam	-	20 ng/mL	-	50 ng/mL
Narcotic Analgesics				
Morphine	10 ng/mL	10 ng/mL	200 ng/mL	50 ng/mL
Codeine	-	10 ng/mL	-	50 ng/mL
6-acetylmorphine	-	5 ng/mL	-	10 ng/mL
Hydrocodone	-	10 ng/mL	-	50 ng/mL
Hydromorphone	-	5 ng/mL	-	50 ng/mL
Oxycodone	10 ng/mL	10 ng/mL	100 ng/mL	50 ng/mL
Oxymorphone	-	5 ng/mL	-	50 ng/mL
Methadone	50 ng/mL	20 ng/mL	300 ng/mL	50 ng/mL
Fentanyl	1 ng/mL	0.5 ng/mL	1 ng/mL	0.5 ng/mL
Buprenorphine	1 ng/mL	0.5 ng/mL	5 ng/mL	1 ng/mL
Norbuprenorphine	-	0.5 ng/mL	-	1 ng/mL
Tramadol	100 ng/mL	50 ng/mL	100 ng/mL	50 ng/mL
o-desmethyltramadol	-	50 ng/mL	-	50 ng/mL

[1]ng/mL is equivalent to g/L

[2]Confirmation is based on free drug concentrations

Appendix 2 – OSBI Toxicology Unit TX42 Validation Plan

VALIDATION PLAN

TOXICOLOGY UNIT // OSBI-FSC Laboratory

Analyst: Alli Timmons Date: 05/06/20

Scope: Validate new solid-phase extraction protocols and create new LC-MS/MS methods for the detection of synthetic opioids, fentanyl analogs, and other opioids not already validated for detection in whole blood and urine.

Matrix(ces):	Whole blood (bovine and human) & urine (water and synthetic)
Analyte(s):	Opioids, fentanyl analogs, and synthetic opioids
Instrumentation:	LC-MS/MS
Analytical Method(s):	Qualitative
Sample Preparation:	Solid-phase extraction

Acceptable Limits

☐ Bias (accuracy):	N/A
☐ Calibration Model:	N/A
☒ Carryover:	No analyte carryover may be observed above the method limit of detection.
☒ Interference Studies:	Evaluate interference from compounds currently in TX40 as well as other drugs commonly identified in the toxicology laboratory. Ten blank samples of each matrix will be analyzed to verify that no matrix interference is present.
☒ Ionization Suppression/Enhancement:	Less than 25% suppression or enhancement and < 15% CV due to matrix (if not evaluate impact on LOD)
☒ Limit of Detection:	A minimum of three samples per run of a fortified matrix sample at the concentration of the decision points shall be analyzed over three runs to demonstrate that all detection and identification criteria are met. Decision point concentrations attached as an appendix to this document.
☐ Limit of Quantitation:	N/A
☐ Precision:	N/A
☐ Processed Sample Stability:	N/A
☐ Dilution Integrity (if applicable):	N/A

Other Information: The newly developed method and all validated compounds will be assessed for adherence to the above criteria using the current Toxicology Quality Manual.

Technical Manager Approval: _____ Date: 05/07/20

(09/24/18)

ISO/IEC 17025:2017(E) 6.2.6 – Analysts authorized to perform a validation or verification for the aforementioned scope include: Alli Timmons, Melissa Cavazos, Melissa Brous, Danielle Ross-Carr, Sean Mize, Kourtney Heard, Jeff Hickerson, Torrance Anderson, and Garry Metcalfe.

Goals/Objectives: The current opiates method (TX40) employed by the OSBI Forensic Toxicology Unit contains morphine, 6-monoacetyl morphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, and fentanyl. One goal of this project will be to expand the TX40 opiates protocol to include desomorphine, buprenorphine, and norbuprenorphine. There have been several customers that have requested buprenorphine testing, but we do not currently have a method sensitive enough for its detection. Buprenorphine is also recommended by the Organization of Scientific Action Committees (OSAC) and listed as a tier I drug by the National Safety Council's Alcohol, Drugs and Impairment Division. Tier I drugs are listed as mandatory in the OSAC recommendations. There has also been an increase in use noticed by the toxicology community. Finally, our new drug-screen instrumentation, Randox, will be able to test for it as well as desomorphine, and it is strongly recommended by the toxicology scientific community that a presumptive test should not be performed if a subsequent confirmation cannot also be performed.

Another goal is to take fentanyl out of TX40 and create a new fentanyl method with the additional analogues and synthetics listed in the attached table. This new method will allow for the detection of 25 additional compounds not previously available to our customers, in addition to the three compounds that will be added to the current TX40 method. By removing fentanyl from the TX40 protocol, a more comprehensive and targeted confirmatory test will be available for cases that screen positive for fentanyl. Although the ELISA presumptive screen analyzes for fentanyl and some fentanyl analogs, our current method is validated for fentanyl only. The new method and addition of fentanyl analogs will reduce the possibility of unconfirmed presumptive positive cases. Both protocols will have a new, more robust solid-phase extraction for sample preparation. The two will be nearly identical and will only differ by elution solvent.

Financial Impact: All needed materials and necessary equipment have already been purchased on a grant providing law enforcement the needed funds to combat the opioid crisis in the United States. There may be a slight increase in solvent usage, but all necessary solvents are in use by the unit in other protocols. Due to the inclusion of additional compounds, more standards will need to be routinely ordered to maintain a supply of unexpired standards for preparation of the controls and internal standards required to complete both analyses. Based on a survey of eight fentanyl analogs used for this new method, the average standard costs approximately $90 and lasts several years. More solid-phase extraction cartridges will need to be purchased in the future, although a large amount were bought with this grant, which will likely last several years. Cartridges cost approximately $250 for a pack of 100 and one cartridge is used per sample.

Evaluation Process: The new methods will be evaluated using the above mentioned criteria for carryover, interferences, ionization suppression/enhancement, and limit of detection. FTU QM 7.2, which was developed from SWGTOX recommended method validation guidelines, will be used to determine all steps needed to complete the validation along with the recently released Standard Practices for Method Validation in Forensic Toxicology *from the American Academy of Forensic Sciences' (AAFS) Standards Board. The latter will be referenced for any additional acceptance criteria.*

(09/24/18)

Compound	Cut-off (ng/mL)
Buprenorphine	0.5
Norbuprenorphine	0.5
Desomorpine	See Fentanyl
Cyclopropyl Fentanyl	0.5
Carfentanil	0.1
Alfentanil	See Fentanyl
Norfentanyl	See Fentanyl
Furanyl Fentanyl	0.1
U-47700	1
(+/-)-cis-3-mthyl Fentanyl	0.1
4-ANPP	0.1
MT-45	See Fentanyl
AH-7921	See Fentanyl
Ocfentanil	See Fentanyl
Remifentanil	See Fentanyl
Sufentanil	1.0
Acetyl Fentanyl	0.5
Isobutyryl Fentanyl	0.1
para-Fluorofentanyl	0.1
ortho-Fluorofentanyl	0.1
para-Flurobutyryl Fentanyl	0.1
Fluoro-isobutyryl Fentanyl	0.1
Methoxy Acetyl Fentanyl	0.5
Acryl Fentanyl	0.1
Valeryl Fentanyl	0.5
Tetrahydro Furanyl Fentanyl	0.2
α-methyl Fentanyl	See Fentanyl
β-hydroxythio Fentanyl	See Fentanyl
Fentanyl	0.5
Morphine	10
Oxymorphone	2.5
Hydromorphone	10
Codeine	10
Oxycodone	10
6-monoacetylmorphine	2.5
Hydrocodone	10

The last eight compounds in the chart above were taken from the current OSBI TX40 protocol. The remaining cut-off limits were taken from "NMS Labs Designer Opioids Screen Reporting Limits." These values are from a validated LC-MS/MS screening method. Those that state "See Fentanyl" could not be found in any reputable source and thus the cut-off for fentanyl was used.

Blood

Low Concentration	
Neat	**beta-OH thio Fentanyl-13C6**
Set 1 - 1	65905
Set 1 - 2	65565
Set 1 - 3	66417
Set 1 - 4	64675
Set 1 - 5	67453
Set 1 - 6	65084
Recon Avg	65849.83333
STDEV	994.2563888
%CV	1.51
Extracted	
Set 2 - 1	39970
Set 2 - 2	61628
Set 2 - 3	61962
Set 2 - 4	55996
Set 2 - 5	40341
Set 2 - 6	56206
Set 2 - 7	61552
Set 2 - 8	71445
Set 2 - 9	60369
Set 2 - 10	60860
Matrix Avg	57032.9
STDEV	9836.661171
%CV	17
%Suppression /Enhancement	-13

High Concentration	
Neat	**beta-OH thio Fentanyl-13C6**
Set 1 - 1	62862
Set 1 - 2	57756
Set 1 - 3	56086
Set 1 - 4	60264
Set 1 - 5	52930
Set 1 - 6	61352
Recon Avg	58541.66667
STDEV	3678.805766
%CV	6.28
Extracted	
Set 2 - 1	63195
Set 2 - 2	50840
Set 2 - 3	64083
Set 2 - 4	63601
Set 2 - 5	50456
Set 2 - 6	74436
Set 2 - 7	70811
Set 2 - 8	70843
Set 2 - 9	58188
Set 2 - 10	63241
Matrix Avg	62969.4
STDEV	8050.536065
%CV	13
%Suppression /Enhancement	8

Urine

Low Concentration	
Neat	**beta-OH thio Fentanyl-13C6**
Set 1 - 1	65905
Set 1 - 2	65565
Set 1 - 3	66417
Set 1 - 4	64675
Set 1 - 5	67453
Set 1 - 6	65084
Recon Avg	65849.83333
STDEV	994.2563888
%CV	1.51
Extracted	
Set 2 - 1	74208
Set 2 - 2	58983
Set 2 - 3	51382
Set 2 - 4	61023
Set 2 - 5	63189
Set 2 - 6	80003
Set 2 - 7	44881
Set 2 - 8	66254
Set 2 - 9	66434
Set 2 - 10	72904
Matrix Avg	63926.1
STDEV	10593.34892
%CV	17
%Suppression /Enhancement	-3

High Concentration	
Neat	**beta-OH thio Fentanyl-13C6**
Set 1 - 1	62862
Set 1 - 2	57756
Set 1 - 3	56086
Set 1 - 4	60264
Set 1 - 5	52930
Set 1 - 6	61352
Recon Avg	58541.66667
STDEV	3678.805766
%CV	6.28
Extracted	
Set 2 - 1	59494
Set 2 - 2	33507
Set 2 - 3	57246
Set 2 - 4	67462
Set 2 - 5	75110
Set 2 - 6	65858
Set 2 - 7	73734
Set 2 - 8	67781
Set 2 - 9	65888
Set 2 - 10	67768
Matrix Avg	63384.8
STDEV	11821.06257
%CV	19
%Suppression /Enhancement	8

Blood

Low Concentration	
Neat	Fentanyl
Set 1 - 1	117665
Set 1 - 2	114125
Set 1 - 3	103537
Set 1 - 4	109218
Set 1 - 5	109221
Set 1 - 6	95702
Recon Avg	108244.6667
STDEV	7806.436028
%CV	7.21
Extracted	
Set 2 - 1	62421
Set 2 - 2	95452
Set 2 - 3	96889
Set 2 - 4	92040
Set 2 - 5	56029
Set 2 - 6	86055
Set 2 - 7	111724
Set 2 - 8	99874
Set 2 - 9	99810
Set 2 - 10	90509
Matrix Avg	89080.3
STDEV	17220.79519
%CV	19
%Suppression /Enhancement	-18

High Concentration	
Neat	Fentanyl
Set 1 - 1	1072277
Set 1 - 2	1011931
Set 1 - 3	939080
Set 1 - 4	1034455
Set 1 - 5	984425
Set 1 - 6	1037102
Recon Avg	1013211.667
STDEV	46554.39212
%CV	4.59
Extracted	
Set 2 - 1	981402
Set 2 - 2	738619
Set 2 - 3	980732
Set 2 - 4	1013226
Set 2 - 5	921912
Set 2 - 6	1201285
Set 2 - 7	1029964
Set 2 - 8	1154201
Set 2 - 9	998604
Set 2 - 10	1078522
Matrix Avg	1009846.7
STDEV	127235.281
%CV	13
%Suppression /Enhancement	0

Urine

Low Concentration	
Neat	Fentanyl
Set 1 - 1	117665
Set 1 - 2	114125
Set 1 - 3	103537
Set 1 - 4	109218
Set 1 - 5	109221
Set 1 - 6	95702
Recon Avg	108244.6667
STDEV	7806.436028
%CV	7.21
Extracted	
Set 2 - 1	100745
Set 2 - 2	84105
Set 2 - 3	80086
Set 2 - 4	96457
Set 2 - 5	103390
Set 2 - 6	115872
Set 2 - 7	68365
Set 2 - 8	97715
Set 2 - 9	108880
Set 2 - 10	105865
Matrix Avg	96148
STDEV	14516.68318
%CV	15
%Suppression /Enhancement	-11

High Concentration	
Neat	Fentanyl
Set 1 - 1	1072277
Set 1 - 2	1011931
Set 1 - 3	939080
Set 1 - 4	1034455
Set 1 - 5	984425
Set 1 - 6	1037102
Recon Avg	1013211.667
STDEV	46554.39212
%CV	4.59
Extracted	
Set 2 - 1	1022999
Set 2 - 2	594032
Set 2 - 3	1058392
Set 2 - 4	1160069
Set 2 - 5	1300192
Set 2 - 6	1123115
Set 2 - 7	1249055
Set 2 - 8	1084056
Set 2 - 9	1054777
Set 2 - 10	1077456
Matrix Avg	1072414.3
STDEV	190116.5341
%CV	18
%Suppression /Enhancement	6

Blood

Low Concentration	
Neat	**Fentanyl-13C6**
Set 1 - 1	215340
Set 1 - 2	219462
Set 1 - 3	214695
Set 1 - 4	230279
Set 1 - 5	214119
Set 1 - 6	201509
Recon Avg	215900.6667
STDEV	9289.585021
%CV	4.30
Extracted	
Set 2 - 1	121566
Set 2 - 2	212138
Set 2 - 3	200751
Set 2 - 4	189303
Set 2 - 5	111934
Set 2 - 6	161524
Set 2 - 7	190423
Set 2 - 8	194876
Set 2 - 9	218529
Set 2 - 10	188749
Matrix Avg	178979.3
STDEV	36239.05353
%CV	20
%Suppression /Enhancement	-17.1010897

High Concentration	
Neat	**Fentanyl-13C6**
Set 1 - 1	173955
Set 1 - 2	186237
Set 1 - 3	172101
Set 1 - 4	194978
Set 1 - 5	181808
Set 1 - 6	199757
Recon Avg	184806
STDEV	11110.34631
%CV	6.011896969
Extracted	
Set 2 - 1	181544
Set 2 - 2	127602
Set 2 - 3	182842
Set 2 - 4	194442
Set 2 - 5	176833
Set 2 - 6	221103
Set 2 - 7	210903
Set 2 - 8	225424
Set 2 - 9	188955
Set 2 - 10	191786
Matrix Avg	190143.4
STDEV	27633.7566
%CV	15
%Suppression /Enhancement	2.888109693

Urine

Low Concentration	
Neat	**Fentanyl-13C6**
Set 1 - 1	215340
Set 1 - 2	219462
Set 1 - 3	214695
Set 1 - 4	230279
Set 1 - 5	214119
Set 1 - 6	201509
Recon Avg	215900.6667
STDEV	9289.585021
%CV	4.30
Extracted	
Set 2 - 1	227866
Set 2 - 2	185963
Set 2 - 3	165534
Set 2 - 4	196366
Set 2 - 5	190842
Set 2 - 6	250187
Set 2 - 7	144571
Set 2 - 8	189859
Set 2 - 9	224393
Set 2 - 10	201503
Matrix Avg	197708.4
STDEV	30726.83128
%CV	16
%Suppression /Enhancement	-8.4262207

High Concentration	
Neat	**Fentanyl-13C6**
Set 1 - 1	173955
Set 1 - 2	186237
Set 1 - 3	172101
Set 1 - 4	194978
Set 1 - 5	181808
Set 1 - 6	199757
Recon Avg	184806
STDEV	11110.34631
%CV	6.011896969
Extracted	
Set 2 - 1	187474
Set 2 - 2	103657
Set 2 - 3	200640
Set 2 - 4	212363
Set 2 - 5	247120
Set 2 - 6	199606
Set 2 - 7	246662
Set 2 - 8	200293
Set 2 - 9	208686
Set 2 - 10	196499
Matrix Avg	200300
STDEV	39548.57635
%CV	20
%Suppression /Enhancement	8.383926929

Blood

Low Concentration	
Neat	α-Methyl Fentanyl
Set 1 - 1	131838
Set 1 - 2	152053
Set 1 - 3	130455
Set 1 - 4	145594
Set 1 - 5	133599
Set 1 - 6	137425
Recon Avg	138494
STDEV	8582.402041
%CV	6.20
Extracted	
Set 2 - 1	75704
Set 2 - 2	126783
Set 2 - 3	117912
Set 2 - 4	119446
Set 2 - 5	84022
Set 2 - 6	100182
Set 2 - 7	129972
Set 2 - 8	101884
Set 2 - 9	124537
Set 2 - 10	106195
Matrix Avg	108663.7
STDEV	18419.96691
%CV	17
%Suppression /Enhancement	-22

High Concentration	
Neat	α-Methyl Fentanyl
Set 1 - 1	1299967
Set 1 - 2	1214007
Set 1 - 3	1150079
Set 1 - 4	1252731
Set 1 - 5	1280146
Set 1 - 6	1290174
Recon Avg	1247850.667
STDEV	57059.3998
%CV	4.572614442
Extracted	
Set 2 - 1	1176156
Set 2 - 2	900164
Set 2 - 3	1210382
Set 2 - 4	1250985
Set 2 - 5	1088466
Set 2 - 6	1094796
Set 2 - 7	1208874
Set 2 - 8	1269363
Set 2 - 9	1168539
Set 2 - 10	1242490
Matrix Avg	1161021.5
STDEV	110139.8443
%CV	9
%Suppression /Enhancement	-7

Urine

Low Concentration	
Neat	α-Methyl Fentanyl
Set 1 - 1	131838
Set 1 - 2	152053
Set 1 - 3	130455
Set 1 - 4	145594
Set 1 - 5	133599
Set 1 - 6	137425
Recon Avg	138494
STDEV	8582.402041
%CV	6.20
Extracted	
Set 2 - 1	141191
Set 2 - 2	123989
Set 2 - 3	104372
Set 2 - 4	122313
Set 2 - 5	126751
Set 2 - 6	152098
Set 2 - 7	99980
Set 2 - 8	123399
Set 2 - 9	137961
Set 2 - 10	141644
Matrix Avg	127369.8
STDEV	16501.47097
%CV	13
%Suppression /Enhancement	-8

High Concentration	
Neat	α-Methyl Fentanyl
Set 1 - 1	1299967
Set 1 - 2	1214007
Set 1 - 3	1150079
Set 1 - 4	1252731
Set 1 - 5	1280146
Set 1 - 6	1290174
Recon Avg	1247850.667
STDEV	57059.3998
%CV	4.572614442
Extracted	
Set 2 - 1	1277003
Set 2 - 2	676850
Set 2 - 3	1354392
Set 2 - 4	1518619
Set 2 - 5	1626089
Set 2 - 6	1368114
Set 2 - 7	1581562
Set 2 - 8	1372975
Set 2 - 9	1345054
Set 2 - 10	1334000
Matrix Avg	1345465.8
STDEV	261977.1148
%CV	19
%Suppression /Enhancement	8

Blood

Low Concentration	
Neat	**Valeryl Fentanyl**
Set 1 - 1	96930
Set 1 - 2	101937
Set 1 - 3	100579
Set 1 - 4	104670
Set 1 - 5	101953
Set 1 - 6	97297
Recon Avg	100561
STDEV	2985.06623
%CV	2.97
Extracted	
Set 2 - 1	56278
Set 2 - 2	93219
Set 2 - 3	90692
Set 2 - 4	87995
Set 2 - 5	56409
Set 2 - 6	80231
Set 2 - 7	95569
Set 2 - 8	82127
Set 2 - 9	82590
Set 2 - 10	80318
Matrix Avg	80542.8
STDEV	13836.68061
%CV	17
%Suppression /Enhancement	-20

High Concentration	
Neat	**Valeryl Fentanyl**
Set 1 - 1	985093
Set 1 - 2	907890
Set 1 - 3	890181
Set 1 - 4	969543
Set 1 - 5	938973
Set 1 - 6	966758
Recon Avg	943073
STDEV	37628.54682
%CV	3.989993014
Extracted	
Set 2 - 1	854721
Set 2 - 2	664610
Set 2 - 3	873476
Set 2 - 4	939325
Set 2 - 5	795659
Set 2 - 6	952116
Set 2 - 7	890622
Set 2 - 8	953988
Set 2 - 9	864351
Set 2 - 10	906846
Matrix Avg	869571.4
STDEV	87160.84292
%CV	10
%Suppression /Enhancement	-8

Urine

Low Concentration	
Neat	**Valeryl Fentanyl**
Set 1 - 1	96930
Set 1 - 2	101937
Set 1 - 3	100579
Set 1 - 4	104670
Set 1 - 5	101953
Set 1 - 6	97297
Recon Avg	100561
STDEV	2985.06623
%CV	2.97
Extracted	
Set 2 - 1	102190
Set 2 - 2	82703
Set 2 - 3	76177
Set 2 - 4	94191
Set 2 - 5	94029
Set 2 - 6	117558
Set 2 - 7	68815
Set 2 - 8	91102
Set 2 - 9	101225
Set 2 - 10	104390
Matrix Avg	93238
STDEV	14392.91198
%CV	15
%Suppression /Enhancement	-7

High Concentration	
Neat	**Valeryl Fentanyl**
Set 1 - 1	985093
Set 1 - 2	907890
Set 1 - 3	890181
Set 1 - 4	969543
Set 1 - 5	938973
Set 1 - 6	966758
Recon Avg	943073
STDEV	37628.54682
%CV	3.989993014
Extracted	
Set 2 - 1	967083
Set 2 - 2	532194
Set 2 - 3	989042
Set 2 - 4	1111335
Set 2 - 5	1181272
Set 2 - 6	1014044
Set 2 - 7	1178791
Set 2 - 8	1034215
Set 2 - 9	1011066
Set 2 - 10	990060
Matrix Avg	1000910.2
STDEV	182223.3604
%CV	18
%Suppression /Enhancement	6

Blood

Low Concentration	
Neat	**Sufentanil**
Set 1 - 1	111455
Set 1 - 2	124692
Set 1 - 3	114128
Set 1 - 4	122651
Set 1 - 5	123809
Set 1 - 6	118347
Recon Avg	119180.3333
STDEV	5472.656454
%CV	4.59
Extracted	
Set 2 - 1	63439
Set 2 - 2	108293
Set 2 - 3	113755
Set 2 - 4	104201
Set 2 - 5	61565
Set 2 - 6	97976
Set 2 - 7	112860
Set 2 - 8	101086
Set 2 - 9	108227
Set 2 - 10	91236
Matrix Avg	96263.8
STDEV	19047.11443
%CV	20
%Suppression /Enhancement	-19

High Concentration	
Neat	**Sufentanil**
Set 1 - 1	1166302
Set 1 - 2	1109957
Set 1 - 3	1074160
Set 1 - 4	1116533
Set 1 - 5	1126693
Set 1 - 6	1152506
Recon Avg	1124358.5
STDEV	32697.15551
%CV	2.908072071
Extracted	
Set 2 - 1	1022867
Set 2 - 2	765243
Set 2 - 3	1016981
Set 2 - 4	1145818
Set 2 - 5	953572
Set 2 - 6	1163396
Set 2 - 7	1075633
Set 2 - 8	1179613
Set 2 - 9	1038348
Set 2 - 10	1093141
Matrix Avg	1045461.2
STDEV	121791.5307
%CV	12
%Suppression /Enhancement	-7

Urine

Low Concentration	
Neat	**Sufentanil**
Set 1 - 1	111455
Set 1 - 2	124692
Set 1 - 3	114128
Set 1 - 4	122651
Set 1 - 5	123809
Set 1 - 6	118347
Recon Avg	119180.3333
STDEV	5472.656454
%CV	4.59
Extracted	
Set 2 - 1	122814
Set 2 - 2	103408
Set 2 - 3	89441
Set 2 - 4	114072
Set 2 - 5	114513
Set 2 - 6	130287
Set 2 - 7	76448
Set 2 - 8	103135
Set 2 - 9	109117
Set 2 - 10	118656
Matrix Avg	108189.1
STDEV	15967.37517
%CV	15
%Suppression /Enhancement	-9

High Concentration	
Neat	**Sufentanil**
Set 1 - 1	1166302
Set 1 - 2	1109957
Set 1 - 3	1074160
Set 1 - 4	1116533
Set 1 - 5	1126693
Set 1 - 6	1152506
Recon Avg	1124358.5
STDEV	32697.15551
%CV	2.908072071
Extracted	
Set 2 - 1	1163298
Set 2 - 2	654705
Set 2 - 3	1222418
Set 2 - 4	1342867
Set 2 - 5	1428989
Set 2 - 6	1208655
Set 2 - 7	1450485
Set 2 - 8	1173979
Set 2 - 9	1180176
Set 2 - 10	1173828
Matrix Avg	1199940
STDEV	220256.3778
%CV	18
%Suppression /Enhancement	7

Blood

Low Concentration	
Neat	Fluorofentanyl
Set 1 - 1	23358
Set 1 - 2	27012
Set 1 - 3	15688
Set 1 - 4	26560
Set 1 - 5	26608
Set 1 - 6	25373
Recon Avg	24099.83333
STDEV	4330.225048
%CV	17.97
Extracted	
Set 2 - 1	10315
Set 2 - 2	24172
Set 2 - 3	14825
Set 2 - 4	17546
Set 2 - 5	15964
Set 2 - 6	19505
Set 2 - 7	19475
Set 2 - 8	23649
Set 2 - 9	25231
Set 2 - 10	23579
Matrix Avg	19426.1
STDEV	4842.08044
%CV	25
%Suppression /Enhancement	-19

High Concentration	
Neat	Fluorofentanyl
Set 1 - 1	217661
Set 1 - 2	229638
Set 1 - 3	203511
Set 1 - 4	225954
Set 1 - 5	204297
Set 1 - 6	201850
Recon Avg	213818.5
STDEV	12267.35715
%CV	5.737275845
Extracted	
Set 2 - 1	208714
Set 2 - 2	166015
Set 2 - 3	221085
Set 2 - 4	236631
Set 2 - 5	184892
Set 2 - 6	244528
Set 2 - 7	194642
Set 2 - 8	249194
Set 2 - 9	206246
Set 2 - 10	222360
Matrix Avg	213430.7
STDEV	26692.11025
%CV	13
%Suppression /Enhancement	0

Q test: 0.302359882

Urine

Low Concentration	
Neat	Fluorofentanyl
Set 1 - 1	23358
Set 1 - 2	27012
Set 1 - 3	15688
Set 1 - 4	26560
Set 1 - 5	26608
Set 1 - 6	25373
Recon Avg	24099.83333
STDEV	4330.225048
%CV	17.97
Extracted	
Set 2 - 1	28360
Set 2 - 2	19950
Set 2 - 3	15007
Set 2 - 4	23452
Set 2 - 5	21773
Set 2 - 6	25802
Set 2 - 7	13901
Set 2 - 8	23988
Set 2 - 9	22835
Set 2 - 10	20954
Matrix Avg	21602.2
STDEV	4471.192031
%CV	21
%Suppression /Enhancement	-10

High Concentration	
Neat	Fluorofentanyl
Set 1 - 1	217661
Set 1 - 2	229638
Set 1 - 3	203511
Set 1 - 4	225954
Set 1 - 5	204297
Set 1 - 6	201850
Recon Avg	213818.5
STDEV	12267.35715
%CV	5.737275845
Extracted	
Set 2 - 1	224140
Set 2 - 2	123168
Set 2 - 3	221467
Set 2 - 4	242455
Set 2 - 5	300020
Set 2 - 6	238015
Set 2 - 7	276703
Set 2 - 8	216386
Set 2 - 9	225175
Set 2 - 10	239348
Matrix Avg	230687.7
STDEV	46085.47998
%CV	20
%Suppression /Enhancement	8

Q test: 0.07649215
0.176914033

Q=0.412 @ 90% for 10 samples

Blood

Low Concentration	
Neat	Methoxy Acetyl Fentanyl
Set 1 - 1	43855
Set 1 - 2	46564
Set 1 - 3	43982
Set 1 - 4	47832
Set 1 - 5	47334
Set 1 - 6	44498
Recon Avg	45677.5
STDEV	1775.341742
%CV	3.89
Extracted	
Set 2 - 1	27630
Set 2 - 2	43225
Set 2 - 3	38970
Set 2 - 4	40855
Set 2 - 5	25959
Set 2 - 6	34719
Set 2 - 7	42193
Set 2 - 8	42792
Set 2 - 9	43521
Set 2 - 10	38781
Matrix Avg	37864.5
STDEV	6421.599009
%CV	17
%Suppression /Enhancement	-17

High Concentration	
Neat	Methoxy Acetyl Fentanyl
Set 1 - 1	457209
Set 1 - 2	424542
Set 1 - 3	411588
Set 1 - 4	447726
Set 1 - 5	430619
Set 1 - 6	443750
Recon Avg	435905.6667
STDEV	16754.20266
%CV	3.843538623
Extracted	
Set 2 - 1	441880
Set 2 - 2	331932
Set 2 - 3	434738
Set 2 - 4	457790
Set 2 - 5	391067
Set 2 - 6	523009
Set 2 - 7	487392
Set 2 - 8	552476
Set 2 - 9	443636
Set 2 - 10	473160
Matrix Avg	453708
STDEV	62753.39569
%CV	14
%Suppression /Enhancement	4

Urine

Low Concentration	
Neat	Methoxy Acetyl Fentanyl
Set 1 - 1	43855
Set 1 - 2	46564
Set 1 - 3	43982
Set 1 - 4	47832
Set 1 - 5	47334
Set 1 - 6	44498
Recon Avg	45677.5
STDEV	1775.341742
%CV	3.89
Extracted	
Set 2 - 1	45858
Set 2 - 2	37278
Set 2 - 3	34190
Set 2 - 4	42021
Set 2 - 5	44613
Set 2 - 6	54272
Set 2 - 7	31862
Set 2 - 8	39599
Set 2 - 9	45466
Set 2 - 10	47628
Matrix Avg	42278.7
STDEV	6725.262408
%CV	16
%Suppression /Enhancement	-7

High Concentration	
Neat	Methoxy Acetyl Fentanyl
Set 1 - 1	457209
Set 1 - 2	424542
Set 1 - 3	411588
Set 1 - 4	447726
Set 1 - 5	430619
Set 1 - 6	443750
Recon Avg	435905.6667
STDEV	16754.20266
%CV	3.843538623
Extracted	
Set 2 - 1	441746
Set 2 - 2	251669
Set 2 - 3	440603
Set 2 - 4	526515
Set 2 - 5	553826
Set 2 - 6	478371
Set 2 - 7	539071
Set 2 - 8	463746
Set 2 - 9	464675
Set 2 - 10	461945
Matrix Avg	462216.7
STDEV	84274.07104
%CV	18
%Suppression /Enhancement	6

Blood

Low Concentration	
Neat	**Furanyl Fentanyl**
Set 1 - 1	32838
Set 1 - 2	39323
Set 1 - 3	31235
Set 1 - 4	30540
Set 1 - 5	39628
Set 1 - 6	45338
Recon Avg	36483.66667
STDEV	5873.91565
%CV	16.10
Extracted	
Set 2 - 1	20543
Set 2 - 2	30309
Set 2 - 3	31306
Set 2 - 4	33032
Set 2 - 5	21098
Set 2 - 6	30163
Set 2 - 7	31302
Set 2 - 8	28260
Set 2 - 9	25186
Set 2 - 10	25669
Matrix Avg	27686.8
STDEV	4382.378624
%CV	16
%Suppression /Enhancement	-24

High Concentration	
Neat	**Furanyl Fentanyl**
Set 1 - 1	329893
Set 1 - 2	325434
Set 1 - 3	324076
Set 1 - 4	352189
Set 1 - 5	353341
Set 1 - 6	351187
Recon Avg	339353.3333
STDEV	14262.4506
%CV	4.202832033
Extracted	
Set 2 - 1	308540
Set 2 - 2	245276
Set 2 - 3	321508
Set 2 - 4	340674
Set 2 - 5	300125
Set 2 - 6	289050
Set 2 - 7	293828
Set 2 - 8	332990
Set 2 - 9	316353
Set 2 - 10	331396
Matrix Avg	307974
STDEV	27984.61996
%CV	9
%Suppression /Enhancement	-9

Urine

Low Concentration	
Neat	**Furanyl Fentanyl**
Set 1 - 1	32838
Set 1 - 2	39323
Set 1 - 3	31235
Set 1 - 4	30540
Set 1 - 5	39628
Set 1 - 6	45338
Recon Avg	36483.66667
STDEV	5873.91565
%CV	16.10
Extracted	
Set 2 - 1	33955
Set 2 - 2	31676
Set 2 - 3	24581
Set 2 - 4	34754
Set 2 - 5	30210
Set 2 - 6	37618
Set 2 - 7	26638
Set 2 - 8	33508
Set 2 - 9	37086
Set 2 - 10	29267
Matrix Avg	31929.3
STDEV	4289.92243
%CV	13
%Suppression /Enhancement	-12

High Concentration	
Neat	**Furanyl Fentanyl**
Set 1 - 1	329893
Set 1 - 2	325434
Set 1 - 3	324076
Set 1 - 4	352189
Set 1 - 5	353341
Set 1 - 6	351187
Recon Avg	339353.3333
STDEV	14262.4506
%CV	4.202832033
Extracted	
Set 2 - 1	344562
Set 2 - 2	202253
Set 2 - 3	369654
Set 2 - 4	385417
Set 2 - 5	427083
Set 2 - 6	361563
Set 2 - 7	435523
Set 2 - 8	355847
Set 2 - 9	364853
Set 2 - 10	376347
Matrix Avg	362310.2
STDEV	63528.31393
%CV	18
%Suppression /Enhancement	7

Blood

Low Concentration	
Neat	**4-FIBF**
Set 1 - 1	140484
Set 1 - 2	130371
Set 1 - 3	128730
Set 1 - 4	147682
Set 1 - 5	127020
Set 1 - 6	130611
Recon Avg	134149.6667
STDEV	8127.154992
%CV	6.06
Extracted	
Set 2 - 1	76138
Set 2 - 2	123660
Set 2 - 3	134988
Set 2 - 4	131232
Set 2 - 5	69217
Set 2 - 6	109681
Set 2 - 7	127814
Set 2 - 8	115179
Set 2 - 9	120234
Set 2 - 10	110697
Matrix Avg	111884
STDEV	22325.04847
%CV	20
%Suppression /Enhancement	-17

High Concentration	
Neat	**4-FIBF**
Set 1 - 1	1282657
Set 1 - 2	1233084
Set 1 - 3	1186885
Set 1 - 4	1314634
Set 1 - 5	1236789
Set 1 - 6	1274775
Recon Avg	1254804
STDEV	45085.58203
%CV	3.5930378
Extracted	
Set 2 - 1	1188249
Set 2 - 2	935987
Set 2 - 3	1148751
Set 2 - 4	1289891
Set 2 - 5	1089181
Set 2 - 6	1328132
Set 2 - 7	1293299
Set 2 - 8	1355718
Set 2 - 9	1149659
Set 2 - 10	1226328
Matrix Avg	1200519.5
STDEV	127114.9708
%CV	11
%Suppression /Enhancement	-4

Urine

Low Concentration	
Neat	**4-FIBF**
Set 1 - 1	140484
Set 1 - 2	130371
Set 1 - 3	128730
Set 1 - 4	147682
Set 1 - 5	127020
Set 1 - 6	130611
Recon Avg	134149.6667
STDEV	8127.154992
%CV	6.06
Extracted	
Set 2 - 1	126970
Set 2 - 2	122511
Set 2 - 3	106522
Set 2 - 4	112330
Set 2 - 5	124617
Set 2 - 6	138301
Set 2 - 7	90103
Set 2 - 8	114616
Set 2 - 9	128137
Set 2 - 10	131371
Matrix Avg	119547.8
STDEV	14015.46099
%CV	12
%Suppression /Enhancement	-11

High Concentration	
Neat	**4-FIBF**
Set 1 - 1	1282657
Set 1 - 2	1233084
Set 1 - 3	1186885
Set 1 - 4	1314634
Set 1 - 5	1236789
Set 1 - 6	1274775
Recon Avg	1254804
STDEV	45085.58203
%CV	3.5930378
Extracted	
Set 2 - 1	1318790
Set 2 - 2	708245
Set 2 - 3	1270226
Set 2 - 4	1485250
Set 2 - 5	1590367
Set 2 - 6	1391925
Set 2 - 7	1574917
Set 2 - 8	1360355
Set 2 - 9	1287072
Set 2 - 10	1391179
Matrix Avg	1337832.6
STDEV	247629.667
%CV	19
%Suppression /Enhancement	7

Blood

Low Concentration	
Neat	Cyclopropyl Fentanyl
Set 1 - 1	105817
Set 1 - 2	110815
Set 1 - 3	103279
Set 1 - 4	108171
Set 1 - 5	112650
Set 1 - 6	101935
Recon Avg	107111.1667
STDEV	4211.2557
%CV	3.93
Extracted	
Set 2 - 1	60715
Set 2 - 2	105967
Set 2 - 3	103949
Set 2 - 4	105198
Set 2 - 5	61212
Set 2 - 6	90182
Set 2 - 7	113099
Set 2 - 8	85182
Set 2 - 9	87869
Set 2 - 10	79938
Matrix Avg	89331.1
STDEV	18333.27226
%CV	21
%Suppression /Enhancement	-17

High Concentration	
Neat	Cyclopropyl Fentanyl
Set 1 - 1	1049091
Set 1 - 2	1047531
Set 1 - 3	969356
Set 1 - 4	1089614
Set 1 - 5	1006688
Set 1 - 6	1010884
Recon Avg	1028860.667
STDEV	41962.86046
%CV	4.078575634
Extracted	
Set 2 - 1	921845
Set 2 - 2	727377
Set 2 - 3	1012841
Set 2 - 4	1058822
Set 2 - 5	882418
Set 2 - 6	829146
Set 2 - 7	897765
Set 2 - 8	981847
Set 2 - 9	912773
Set 2 - 10	1014365
Matrix Avg	923919.9
STDEV	98763.27049
%CV	11
%Suppression /Enhancement	-10

Urine

Low Concentration	
Neat	Cyclopropyl Fentanyl
Set 1 - 1	105817
Set 1 - 2	110815
Set 1 - 3	103279
Set 1 - 4	108171
Set 1 - 5	112650
Set 1 - 6	101935
Recon Avg	107111.1667
STDEV	4211.2557
%CV	3.93
Extracted	
Set 2 - 1	119826
Set 2 - 2	101887
Set 2 - 3	80200
Set 2 - 4	100654
Set 2 - 5	106027
Set 2 - 6	127389
Set 2 - 7	74004
Set 2 - 8	89435
Set 2 - 9	108054
Set 2 - 10	120728
Matrix Avg	102820.4
STDEV	17568.79301
%CV	17
%Suppression /Enhancement	-4

High Concentration	
Neat	Cyclopropyl Fentanyl
Set 1 - 1	1049091
Set 1 - 2	1047531
Set 1 - 3	969356
Set 1 - 4	1089614
Set 1 - 5	1006688
Set 1 - 6	1010884
Recon Avg	1028860.667
STDEV	41962.86046
%CV	4.078575634
Extracted	
Set 2 - 1	1057349
Set 2 - 2	555356
Set 2 - 3	1085590
Set 2 - 4	1231594
Set 2 - 5	1297494
Set 2 - 6	1171825
Set 2 - 7	1270248
Set 2 - 8	1131972
Set 2 - 9	1072810
Set 2 - 10	1079779
Matrix Avg	1095401.7
STDEV	208456.4611
%CV	19
%Suppression /Enhancement	6

Q test: 0.00948763
0.136148442

Q=0.412 @ 90% for 10 samples

Blood

Low Concentration	
Neat	Carfentanil
Set 1 - 1	8913
Set 1 - 2	12736
Set 1 - 3	13392
Set 1 - 4	10684
Set 1 - 5	15546
Set 1 - 6	13998
Recon Avg	12544.83333
STDEV	2388.549553
%CV	19.04
Extracted	
Set 2 - 1	9103
Set 2 - 2	10659
Set 2 - 3	15370
Set 2 - 4	13299
Set 2 - 5	7260
Set 2 - 6	12201
Set 2 - 7	13530
Set 2 - 8	14496
Set 2 - 9	12154
Set 2 - 10	13325
Matrix Avg	12139.7
STDEV	2492.171924
%CV	21
%Suppression /Enhancement	-3

High Concentration	
Neat	Carfentanil
Set 1 - 1	141751
Set 1 - 2	133899
Set 1 - 3	120996
Set 1 - 4	134139
Set 1 - 5	130206
Set 1 - 6	129031
Recon Avg	131670.3333
STDEV	6866.336073
%CV	5.214793567
Extracted	
Set 2 - 1	127390
Set 2 - 2	98214
Set 2 - 3	130126
Set 2 - 4	143756
Set 2 - 5	131154
Set 2 - 6	150977
Set 2 - 7	145159
Set 2 - 8	156393
Set 2 - 9	134869
Set 2 - 10	140625
Matrix Avg	135866.3
STDEV	16215.97399
%CV	12
%Suppression /Enhancement	3

Q test: 0.254698729

Urine

Low Concentration	
Neat	Carfentanil
Set 1 - 1	8913
Set 1 - 2	12736
Set 1 - 3	13392
Set 1 - 4	10684
Set 1 - 5	15546
Set 1 - 6	13998
Recon Avg	12544.83333
STDEV	2388.549553
%CV	19.04
Extracted	
Set 2 - 1	16396
Set 2 - 2	10056
Set 2 - 3	9218
Set 2 - 4	10690
Set 2 - 5	17840
Set 2 - 6	15056
Set 2 - 7	8040
Set 2 - 8	13922
Set 2 - 9	13008
Set 2 - 10	13902
Matrix Avg	12812.8
STDEV	3221.580454
%CV	25
%Suppression /Enhancement	2

High Concentration	
Neat	Carfentanil
Set 1 - 1	141751
Set 1 - 2	133899
Set 1 - 3	120996
Set 1 - 4	134139
Set 1 - 5	130206
Set 1 - 6	129031
Recon Avg	131670.3333
STDEV	6866.336073
%CV	5.214793567
Extracted	
Set 2 - 1	132878
Set 2 - 2	73802
Set 2 - 3	149528
Set 2 - 4	152473
Set 2 - 5	170159
Set 2 - 6	155940
Set 2 - 7	148057
Set 2 - 8	145584
Set 2 - 9	147061
Set 2 - 10	140847
Matrix Avg	141632.9
STDEV	25739.96739
%CV	18
%Suppression /Enhancement	8

Q test: 0.120204082

Q=0.412 @ 90% for 10 samples

Blood

Low Concentration	
Neat	Butyryl Fentanyl
Set 1 - 1	37555
Set 1 - 2	34871
Set 1 - 3	34476
Set 1 - 4	33488
Set 1 - 5	34481
Set 1 - 6	39474
Recon Avg	35724.16667
STDEV	2291.066076
%CV	6.41
Extracted	
Set 2 - 1	17238
Set 2 - 2	32428
Set 2 - 3	28772
Set 2 - 4	34534
Set 2 - 5	19064
Set 2 - 6	28764
Set 2 - 7	31048
Set 2 - 8	34801
Set 2 - 9	38040
Set 2 - 10	23105
Matrix Avg	28779.4
STDEV	6939.297486
%CV	24
%Suppression /Enhancement	-19

High Concentration	
Neat	Butyryl Fentanyl
Set 1 - 1	382510
Set 1 - 2	353013
Set 1 - 3	316857
Set 1 - 4	349646
Set 1 - 5	362224
Set 1 - 6	358908
Recon Avg	353859.6667
STDEV	21466.05932
%CV	6.066263364
Extracted	
Set 2 - 1	327876
Set 2 - 2	254665
Set 2 - 3	316520
Set 2 - 4	381461
Set 2 - 5	314875
Set 2 - 6	332475
Set 2 - 7	314096
Set 2 - 8	365123
Set 2 - 9	320285
Set 2 - 10	348561
Matrix Avg	327593.7
STDEV	34338.61006
%CV	10
%Suppression /Enhancement	-7

Urine

Low Concentration	
Neat	Butyryl Fentanyl
Set 1 - 1	37555
Set 1 - 2	34871
Set 1 - 3	34476
Set 1 - 4	33488
Set 1 - 5	34481
Set 1 - 6	39474
Recon Avg	35724.16667
STDEV	2291.066076
%CV	6.41
Extracted	
Set 2 - 1	35516
Set 2 - 2	31113
Set 2 - 3	26334
Set 2 - 4	34649
Set 2 - 5	33040
Set 2 - 6	48190
Set 2 - 7	23459
Set 2 - 8	32856
Set 2 - 9	34766
Set 2 - 10	39715
Matrix Avg	33963.8
STDEV	6813.024908
%CV	20
%Suppression /Enhancement	-5

High Concentration	
Neat	Butyryl Fentanyl
Set 1 - 1	382510
Set 1 - 2	353013
Set 1 - 3	316857
Set 1 - 4	349646
Set 1 - 5	362224
Set 1 - 6	358908
Recon Avg	353859.6667
STDEV	21466.05932
%CV	6.066263364
Extracted	
Set 2 - 1	361537
Set 2 - 2	204085
Set 2 - 3	369610
Set 2 - 4	400109
Set 2 - 5	429709
Set 2 - 6	382618
Set 2 - 7	456789
Set 2 - 8	392752
Set 2 - 9	384580
Set 2 - 10	389826
Matrix Avg	377161.5
STDEV	66976.9878
%CV	18
%Suppression /Enhancement	7

Q test: 0.087780021

0.155706182

Q=0.412 @ 90% for 10 samples

Blood

Low Concentration	
Neat	Acryl Fentanyl
Set 1 - 1	25820
Set 1 - 2	31666
Set 1 - 3	22451
Set 1 - 4	27621
Set 1 - 5	27582
Set 1 - 6	27323
Recon Avg	27077.16667
STDEV	2988.321363
%CV	11.04
Extracted	
Set 2 - 1	12973
Set 2 - 2	25958
Set 2 - 3	20421
Set 2 - 4	25235
Set 2 - 5	11779
Set 2 - 6	18971
Set 2 - 7	27839
Set 2 - 8	22037
Set 2 - 9	29758
Set 2 - 10	24995
Matrix Avg	21996.6
STDEV	6027.7728
%CV	27
%Suppression /Enhancement	-19

High Concentration	
Neat	Acryl Fentanyl
Set 1 - 1	266648
Set 1 - 2	256967
Set 1 - 3	230299
Set 1 - 4	268919
Set 1 - 5	236260
Set 1 - 6	254330
Recon Avg	252237.1667
STDEV	15806.70723
%CV	6.26660513
Extracted	
Set 2 - 1	246708
Set 2 - 2	195632
Set 2 - 3	259004
Set 2 - 4	275277
Set 2 - 5	231855
Set 2 - 6	346329
Set 2 - 7	294025
Set 2 - 8	299108
Set 2 - 9	267892
Set 2 - 10	295901
Matrix Avg	271173.1
STDEV	41618.70949
%CV	15
%Suppression /Enhancement	8

Q test: 0.066410813

Urine

Low Concentration	
Neat	Acryl Fentanyl
Set 1 - 1	25820
Set 1 - 2	31666
Set 1 - 3	22451
Set 1 - 4	27621
Set 1 - 5	27582
Set 1 - 6	27323
Recon Avg	27077.16667
STDEV	2988.321363
%CV	11.04
Extracted	
Set 2 - 1	32047
Set 2 - 2	16354
Set 2 - 3	20300
Set 2 - 4	26927
Set 2 - 5	29230
Set 2 - 6	32477
Set 2 - 7	16335
Set 2 - 8	28249
Set 2 - 9	23262
Set 2 - 10	22729
Matrix Avg	24791
STDEV	5938.525668
%CV	24
%Suppression /Enhancement	-8

High Concentration	
Neat	Acryl Fentanyl
Set 1 - 1	266648
Set 1 - 2	256967
Set 1 - 3	230299
Set 1 - 4	268919
Set 1 - 5	236260
Set 1 - 6	254330
Recon Avg	252237.1667
STDEV	15806.70723
%CV	6.26660513
Extracted	
Set 2 - 1	272708
Set 2 - 2	145250
Set 2 - 3	276797
Set 2 - 4	326453
Set 2 - 5	337263
Set 2 - 6	287753
Set 2 - 7	324356
Set 2 - 8	278700
Set 2 - 9	292848
Set 2 - 10	285845
Matrix Avg	282797.3
STDEV	53549.7353
%CV	19
%Suppression /Enhancement	12

Q test: 0.001177054

Q=0.412 @ 90% for 10 samples

Blood

Low Concentration	
Neat	**Acetyl Fentanyl**
Set 1 - 1	134308
Set 1 - 2	140636
Set 1 - 3	136386
Set 1 - 4	131632
Set 1 - 5	138688
Set 1 - 6	139360
Recon Avg	136835
STDEV	3403.391661
%CV	2.49
Extracted	
Set 2 - 1	80886
Set 2 - 2	127602
Set 2 - 3	125093
Set 2 - 4	123398
Set 2 - 5	82153
Set 2 - 6	112774
Set 2 - 7	127368
Set 2 - 8	119547
Set 2 - 9	125460
Set 2 - 10	114025
Matrix Avg	113830.6
STDEV	17792.57283
%CV	16
%Suppression /Enhancement	-17

High Concentration	
Neat	**Acetyl Fentanyl**
Set 1 - 1	1311519
Set 1 - 2	1240088
Set 1 - 3	1180401
Set 1 - 4	1273216
Set 1 - 5	1283001
Set 1 - 6	1307624
Recon Avg	1265974.833
STDEV	49283.38085
%CV	3.892919476
Extracted	
Set 2 - 1	1257335
Set 2 - 2	946546
Set 2 - 3	1228154
Set 2 - 4	1341395
Set 2 - 5	1119763
Set 2 - 6	1534032
Set 2 - 7	1398795
Set 2 - 8	1473458
Set 2 - 9	1257022
Set 2 - 10	1360607
Matrix Avg	1291710.7
STDEV	171776.0496
%CV	13
%Suppression /Enhancement	2

Urine

Low Concentration	
Neat	**Acetyl Fentanyl**
Set 1 - 1	134308
Set 1 - 2	140636
Set 1 - 3	136386
Set 1 - 4	131632
Set 1 - 5	138688
Set 1 - 6	139360
Recon Avg	136835
STDEV	3403.391661
%CV	2.49
Extracted	
Set 2 - 1	133912
Set 2 - 2	117503
Set 2 - 3	106044
Set 2 - 4	128674
Set 2 - 5	131511
Set 2 - 6	154071
Set 2 - 7	99884
Set 2 - 8	117388
Set 2 - 9	126146
Set 2 - 10	143381
Matrix Avg	125851.4
STDEV	16386.49439
%CV	13
%Suppression /Enhancement	-8

High Concentration	
Neat	**Acetyl Fentanyl**
Set 1 - 1	1311519
Set 1 - 2	1240088
Set 1 - 3	1180401
Set 1 - 4	1273216
Set 1 - 5	1283001
Set 1 - 6	1307624
Recon Avg	1265974.833
STDEV	49283.38085
%CV	3.892919476
Extracted	
Set 2 - 1	1308660
Set 2 - 2	728197
Set 2 - 3	1320365
Set 2 - 4	1477989
Set 2 - 5	1607829
Set 2 - 6	1417603
Set 2 - 7	1599523
Set 2 - 8	1366286
Set 2 - 9	1312419
Set 2 - 10	1343416
Matrix Avg	1348228.7
STDEV	245086.0641
%CV	18
%Suppression /Enhancement	6

Blood

Low Concentration	
Neat	4-ANPP
Set 1 - 1	34111
Set 1 - 2	33148
Set 1 - 3	27301
Set 1 - 4	32842
Set 1 - 5	32294
Set 1 - 6	29516
Recon Avg	31535.33333
STDEV	2589.401141
%CV	8.21
Extracted	
Set 2 - 1	16027
Set 2 - 2	25059
Set 2 - 3	19246
Set 2 - 4	22713
Set 2 - 5	17125
Set 2 - 6	22440
Set 2 - 7	29965
Set 2 - 8	23676
Set 2 - 9	20116
Set 2 - 10	27558
Matrix Avg	22392.5
STDEV	4425.140029
%CV	20
%Suppression /Enhancement	-29

Q test: 0.078777443

0.172693356

High Concentration	
Neat	4-ANPP
Set 1 - 1	311915
Set 1 - 2	297379
Set 1 - 3	299312
Set 1 - 4	315882
Set 1 - 5	276613
Set 1 - 6	297608
Recon Avg	299784.8333
STDEV	13812.63036
%CV	4.607514731
Extracted	
Set 2 - 1	283763
Set 2 - 2	200126
Set 2 - 3	266032
Set 2 - 4	280302
Set 2 - 5	239043
Set 2 - 6	321509
Set 2 - 7	289860
Set 2 - 8	315573
Set 2 - 9	264628
Set 2 - 10	304408
Matrix Avg	276524.4
STDEV	36611.46455
%CV	13
%Suppression /Enhancement	-8

Urine

Low Concentration	
Neat	4-ANPP
Set 1 - 1	34111
Set 1 - 2	33148
Set 1 - 3	27301
Set 1 - 4	32842
Set 1 - 5	32294
Set 1 - 6	29516
Recon Avg	31535.33333
STDEV	2589.401141
%CV	8.21
Extracted	
Set 2 - 1	23876
Set 2 - 2	23333
Set 2 - 3	21264
Set 2 - 4	22757
Set 2 - 5	24134
Set 2 - 6	27285
Set 2 - 7	
Set 2 - 8	25616
Set 2 - 9	28563
Set 2 - 10	23603
Matrix Avg	24492.33333
STDEV	2283.328054
%CV	9
%Suppression /Enhancement	-22

Q test: 0.571453734

dropped 11531

High Concentration	
Neat	4-ANPP
Set 1 - 1	311915
Set 1 - 2	297379
Set 1 - 3	299312
Set 1 - 4	315882
Set 1 - 5	276613
Set 1 - 6	297608
Recon Avg	299784.8333
STDEV	13812.63036
%CV	4.607514731
Extracted	
Set 2 - 1	186394
Set 2 - 2	138259
Set 2 - 3	280679
Set 2 - 4	278832
Set 2 - 5	322582
Set 2 - 6	237234
Set 2 - 7	321786
Set 2 - 8	272301
Set 2 - 9	181596
Set 2 - 10	239850
Matrix Avg	245951.3
STDEV	61467.38201
%CV	25
%Suppression /Enhancement	-18

Q test: 0.235114446

Q=0.412 @ 90% for 10 samples

Blood

Low Concentration	
Neat	**Norfentayl**
Set 1 - 1	41214
Set 1 - 2	44004
Set 1 - 3	40561
Set 1 - 4	43601
Set 1 - 5	43256
Set 1 - 6	42712
Recon Avg	42558
STDEV	1377.245802
%CV	3.24
Extracted	
Set 2 - 1	22688
Set 2 - 2	37784
Set 2 - 3	35985
Set 2 - 4	35309
Set 2 - 5	22892
Set 2 - 6	33303
Set 2 - 7	37949
Set 2 - 8	37987
Set 2 - 9	38358
Set 2 - 10	29588
Matrix Avg	33184.3
STDEV	6423.45553
%CV	19
%Suppression /Enhancement	-22

High Concentration	
Neat	**Norfentayl**
Set 1 - 1	432444
Set 1 - 2	394841
Set 1 - 3	376836
Set 1 - 4	406695
Set 1 - 5	404814
Set 1 - 6	403437
Recon Avg	403177.8333
STDEV	18083.24172
%CV	4.49
Extracted	
Set 2 - 1	374928
Set 2 - 2	284599
Set 2 - 3	363767
Set 2 - 4	395597
Set 2 - 5	330040
Set 2 - 6	457389
Set 2 - 7	411063
Set 2 - 8	444357
Set 2 - 9	367319
Set 2 - 10	397722
Matrix Avg	382678.1
STDEV	51238.87399
%CV	13
%Suppression /Enhancement	-5

Urine

Low Concentration	
Neat	**Norfentayl**
Set 1 - 1	41214
Set 1 - 2	44004
Set 1 - 3	40561
Set 1 - 4	43601
Set 1 - 5	43256
Set 1 - 6	42712
Recon Avg	42558
STDEV	1377.245802
%CV	3.24
Extracted	
Set 2 - 1	42995
Set 2 - 2	34827
Set 2 - 3	31495
Set 2 - 4	39280
Set 2 - 5	40745
Set 2 - 6	45767
Set 2 - 7	29570
Set 2 - 8	35571
Set 2 - 9	40085
Set 2 - 10	40868
Matrix Avg	38120.3
STDEV	5416.770535
%CV	14
%Suppression /Enhancement	-10

High Concentration	
Neat	**Norfentayl**
Set 1 - 1	432444
Set 1 - 2	394841
Set 1 - 3	376836
Set 1 - 4	406695
Set 1 - 5	404814
Set 1 - 6	403437
Recon Avg	403177.8333
STDEV	18083.24172
%CV	4.49
Extracted	
Set 2 - 1	402569
Set 2 - 2	226906
Set 2 - 3	406905
Set 2 - 4	453395
Set 2 - 5	498744
Set 2 - 6	440297
Set 2 - 7	490763
Set 2 - 8	424486
Set 2 - 9	426063
Set 2 - 10	415769
Matrix Avg	418589.7
STDEV	74944.95879
%CV	18
%Suppression /Enhancement	4

Batch File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\LOD.lcb
Method File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\TX42 FINAL 102021.lcm

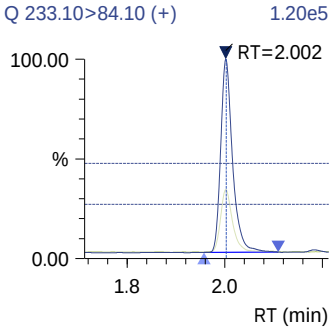
Blood 3 HPC_004

Sample ID: Blood 3 HPC
Date Acquired: 10/20/2021 4:41:32 PM
Acquired by: System Administrator
Method File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\TX42 FINAL 102021.lcm
Data File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\Blood 3 HPC_004.lcd
Vial: 1 | Inj. Volume: 3.0000uL | Tray: 1

Name	Found RT	Area	Ref 1 Range	Ref 1 Actual Ratio	Ref 2 Range	Ref 2 Actual Ratio
Norfentanyl	2.002	201943.620	24.67 - 45.81	32.15	----	----
4-ANPP	2.946	207517.400	83.68 - 155.41	99.55	----	----
Acetyl fentanyl	2.733	656829.994	66.95 - 124.34	96.37	----	----
Acryl fentanyl	2.969	177114.719	63.76 - 118.40	91.21	----	----
Butyryl fentanyl	3.218	182707.607	54.61 - 101.41	81.58	----	----
Cyclopropyl fentanyl	3.145	773985.949	52.79 - 98.04	83.30	----	----
4-FIBF/PFBF	3.226	377800.498	57.22 - 106.26	82.81	----	----
Furanyl fentanyl	3.146	171488.248	68.87 - 127.91	81.03	----	----
Methoxy acetyl fentanyl	2.664	397050.604	71.73 - 133.21	99.27	----	----
Fluorofentanyl	3.050	156785.767	50.32 - 93.45	92.13	----	----
Sufentanil	3.328	815346.528	59.07 - 109.70	89.79	16.01 - 29.74	23.77
Valeryl fentanyl	3.462	797097.317	49.01 - 91.02	73.59	----	----
alpha-Methyl fentanyl	3.145	644475.806	24.19 - 44.93	35.86	20.86 - 38.74	28.18
Fentanyl-13C6	2.992	154179.095	77.96 - 144.78	113.67	----	----
Fentanyl	2.993	647588.318	69.76 - 129.55	100.44	----	----
betahyroxythio-Fentanyl -13C6	2.651	33386.932	48.93 - 90.88	63.56	28.14 - 52.25	36.07

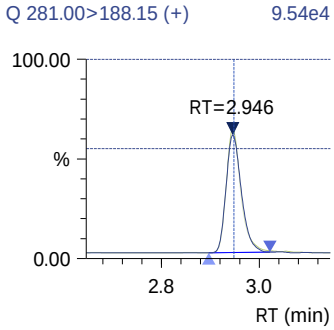
Norfentanyl

Conc 5.0000
Area 201943.620
R#1 233.10>55.00 32.15 (35.24)



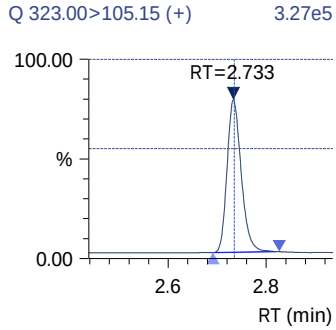
4-ANPP

Conc 5.0000
Area 207517.400
R#1 281.00>105.20 99.55 (119.54)



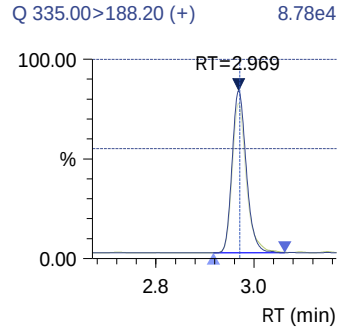
Acetyl fentanyl

Conc 5.0000
Area 656829.994
R#1 323.00>188.10 96.37 (95.65)



Acryl fentanyl

Conc 1.0000
Area 177114.719
R#1 335.00>105.20 91.21 (91.08)

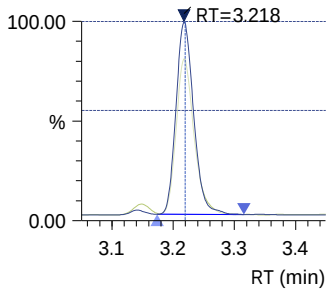


Blood 3 HPC_004 (continued)

Butyryl fentanyl

Conc 1.0000
Area 182707.607
R#1 351.00>105.15 81.58 (78.01)

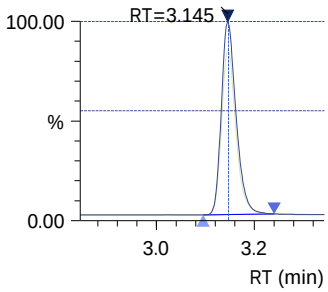
Q 351.00>188.10 (+) 9.07e4



Cyclopropyl fentanyl

Conc 5.0000
Area 773985.949
R#1 349.00>105.20 83.30 (75.41)

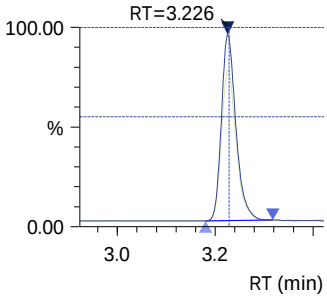
Q 349.00>188.15 (+) 3.68e5



4-FIBF/PFBF

Conc 5.0000
Area 377800.498
R#1 369.30>105.05 82.81 (81.74)

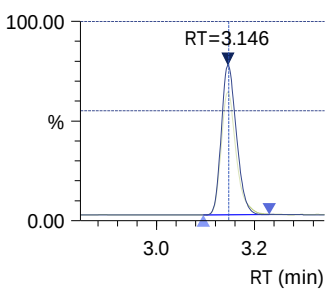
Q 369.30>188.15 (+) 1.84e5



Furanyl fentanyl

Conc 1.0000
Area 171488.248
R#1 375.00>105.15 81.03 (98.39)

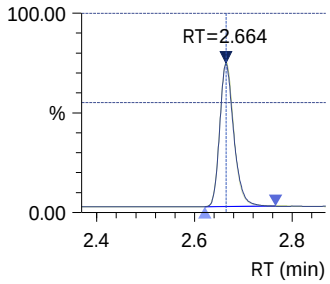
Q 375.00>188.10 (+) 7.94e4



Methoxy acetyl fentanyl

Conc 5.0000
Area 397050.604
R#1 353.00>105.15 99.27 (102.47)

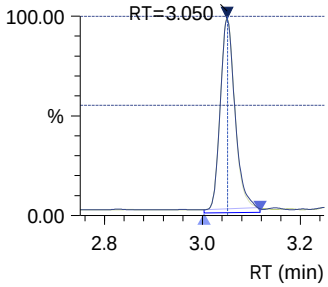
Q 353.00>188.10 (+) 1.98e5



Fluorofentanyl

Conc 1.0000
Area 156785.767
R#1 355.00>105.20 92.13 (71.89)

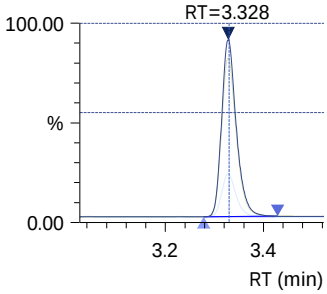
Q 355.00>188.15 (+) 7.07e4



Sufentanil

Conc 5.0000
Area 815346.528
R#1 387.10>111.10 89.79 (84.38)
R#2 387.10>355.05 23.77 (22.87)

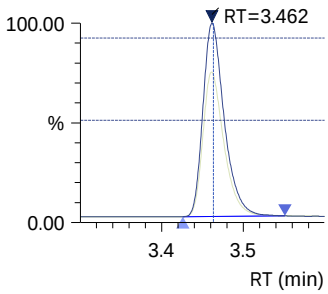
Q 387.10>238.15 (+) 3.91e5



Valeryl fentanyl

Conc 5.0000
Area 797097.317
R#1 365.10>105.15 73.59 (70.01)

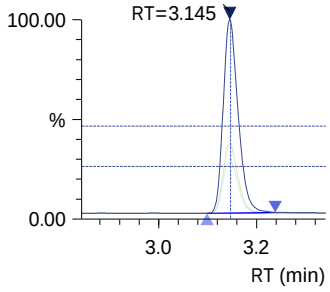
Q 365.10>188.15 (+) 4.39e5



alpha-Methyl fentanyl

Conc 5.0000
Area 644475.806
R#1 351.30>202.00 35.86 (34.56)
R#2 351.30>119.20 28.18 (29.80)

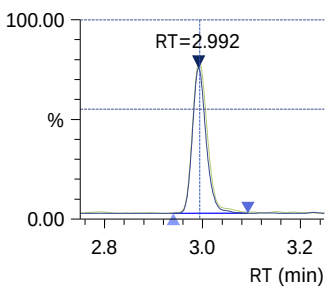
Q 351.30>91.10 (+) 3.05e5



Fentanyl-13C6

Conc 1.0000
Area 154179.095
R#1 343.00>105.15 113.67 (111.37)

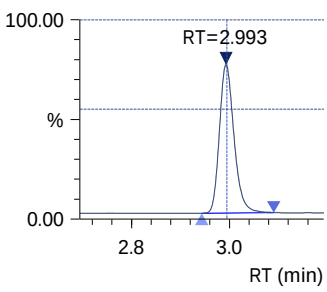
ISTD 343.00>188.20 (+) 7.90e4



Fentanyl

Conc 5.0000
Area 647588.318
R#1 337.20>105.00 100.44 (99.65)

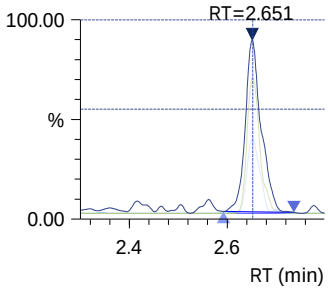
Q 337.20>188.00 (+) 3.14e5



betahyroxythio -Fentanyl-13C6

Conc 1.0000
Area 33386.932
R#1 365.00>192.05 63.56 (69.91)
R#2 365.00>110.95 36.07 (40.19)

ISTD 365.00>347.30 (+) 1.49e4



Batch File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\LOD.lcb
Method File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\TX42 FINAL 102021.lcm

Blood 3 LPC_005

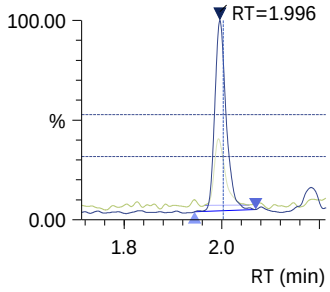
Sample ID: Blood 3 LPC
Date Acquired: 10/20/2021 4:50:28 PM
Acquired by: System Administrator
Method File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\TX42 FINAL 102021.lcm
Data File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\Blood 3 LPC_005.lcd
Vial: 2 | Inj. Volume: 3.0000uL | Tray: 1

Name	Found RT	Area	Ref 1 Range	Ref 1 Actual Ratio	Ref 2 Range	Ref 2 Actual Ratio
Norfentanyl	1.996	14019.096	24.67 - 45.81	33.52	----	----
4-ANPP	2.941	17047.960	83.68 - 155.41	154.81	----	----
Acetyl fentanyl	2.730	53553.237	66.95 - 124.34	88.46	----	----
Acryl fentanyl	2.970	13800.556	63.76 - 118.40	80.64	----	----
Butyryl fentanyl	3.214	10428.684	54.61 - 101.41	93.85	----	----
Cyclopropyl fentanyl	3.141	61339.071	52.79 - 98.04	70.66	----	----
4-FIBF/PFBF	3.220	28338.010	57.22 - 106.26	82.08	----	----
Furanyl fentanyl	3.140	11161.110	68.87 - 127.91	78.83	----	----
Methoxy acetyl fentanyl	2.661	28959.351	71.73 - 133.21	98.55	----	----
Fluorofentanyl	3.049	12332.096	50.32 - 93.45	71.95	----	----
Sufentanil	3.325	59997.047	59.07 - 109.70	90.60	16.01 - 29.74	21.07
Valeryl fentanyl	3.459	61627.484	49.01 - 91.02	70.59	----	----
alpha-Methyl fentanyl	3.141	46158.596	24.19 - 44.93	38.37	20.86 - 38.74	26.89
Fentanyl-13C6	2.988	151464.080	77.96 - 144.78	115.42	----	----
Fentanyl	2.989	47826.829	69.76 - 129.55	94.94	----	----
betahyroxythio-Fentanyl -13C6	2.647	28252.291	48.93 - 90.88	72.35	28.14 - 52.25	42.77

Norfentanyl

Conc 0.3533
Area 14019.096
R#1 233.10>55.00 33.52 (35.24)

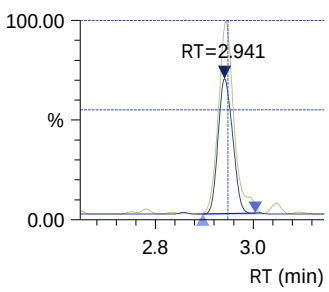
Q 233.10>84.10 (+) 8.76e3



4-ANPP

Conc 0.4181
Area 17047.960
R#1 281.00>105.20 154.81 (119.54)

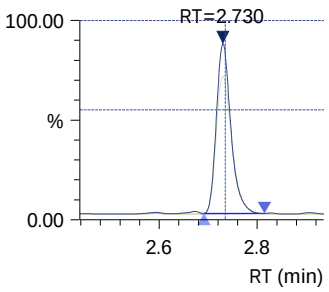
Q 281.00>188.15 (+) 8.31e3



Acetyl fentanyl

Conc 0.4150
Area 53553.237
R#1 323.00>188.10 88.46 (95.65)

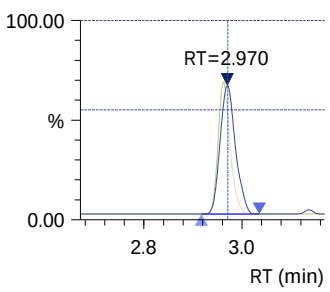
Q 323.00>105.15 (+) 2.61e4



Acryl fentanyl

Conc 0.0793
Area 13800.556
R#1 335.00>105.20 80.64 (91.08)

Q 335.00>188.20 (+) 6.33e3

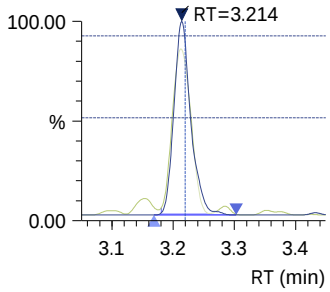


Blood 3 LPC_005 (continued)

Butyryl fentanyl

Conc 0.0675
Area 10428.684
R#1 351.00>105.15 93.85 (78.01)

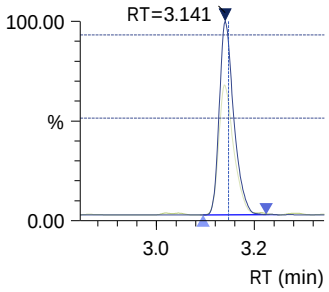
Q 351.00>188.10 (+) 5.44e3



Cyclopropyl fentanyl

Conc 0.4683
Area 61339.071
R#1 349.00>105.20 70.66 (75.41)

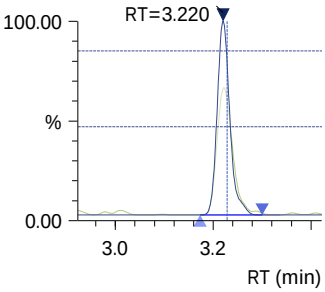
Q 349.00>188.15 (+) 2.99e4



4-FIBF/PFBF

Conc 0.4432
Area 28338.010
R#1 369.30>105.05 82.08 (81.74)

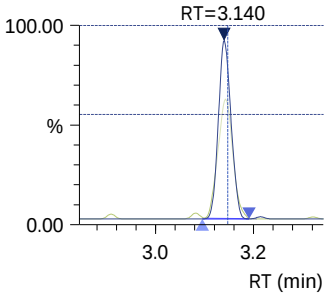
Q 369.30>188.15 (+) 1.57e4



Furanyl fentanyl

Conc 0.0769
Area 11161.110
R#1 375.00>105.15 78.83 (98.39)

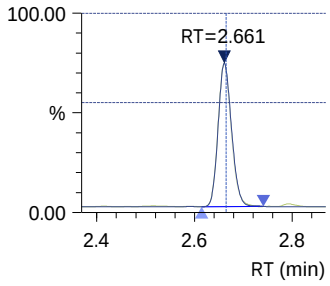
Q 375.00>188.10 (+) 6.18e3



Methoxy acetyl fentanyl

Conc 0.3712
Area 28959.351
R#1 353.00>105.15 98.55 (102.47)

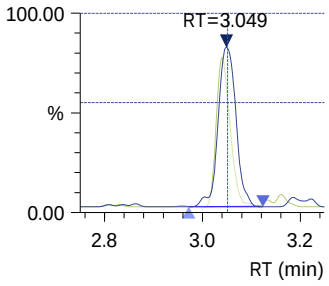
Q 353.00>188.10 (+) 1.51e4



Fluorofentanyl

Conc 0.0930
Area 12332.096
R#1 355.00>105.20 71.95 (71.89)

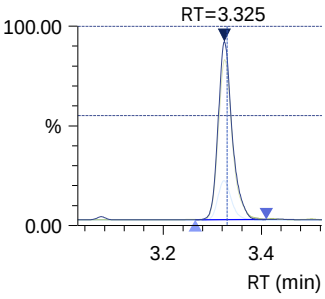
Q 355.00>188.15 (+) 4.86e3



Sufentanil

Conc 0.4348
Area 59997.047
R#1 387.10>111.10 90.60 (84.38)
R#2 387.10>355.05 21.07 (22.87)

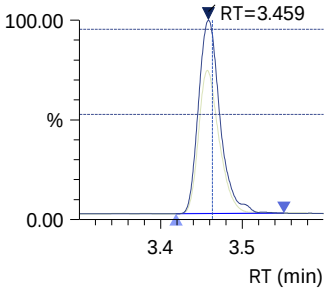
Q 387.10>238.15 (+) 3.01e4



Valeryl fentanyl

Conc 0.4568
Area 61627.484
R#1 365.10>105.15 70.59 (70.01)

Q 365.10>188.15 (+) 3.36e4

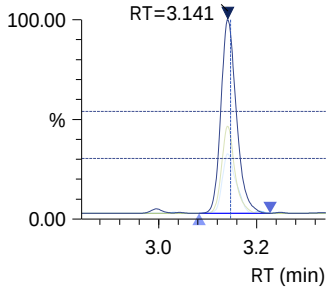


alpha-Methyl fentanyl

Conc 0.4232
Area 46158.596
R#1 351.30>202.00 38.37 (34.56)

R#2 351.30>119.20 26.89 (29.80)

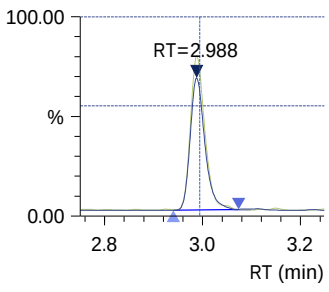
Q 351.30>91.10 (+) 2.09e4



Fentanyl-13C6

Conc 1.0000
Area 151464.080
R#1 343.00>105.15 115.42 (111.37)

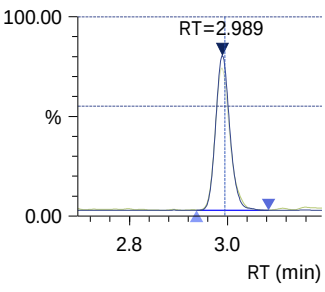
ISTD 343.00>188.20 (+) 7.29e4



Fentanyl

Conc 0.3759
Area 47826.829
R#1 337.20>105.00 94.94 (99.65)

Q 337.20>188.00 (+) 2.37e4

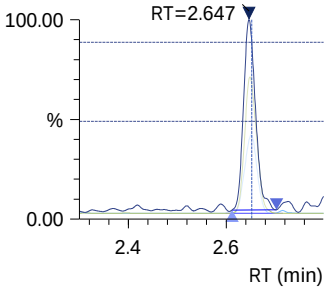


betahyroxythio -Fentanyl-13C6

Conc 1.0000
Area 28252.291
R#1 365.00>192.05 72.35 (69.91)

R#2 365.00>110.95 42.77 (40.19)

ISTD 365.00>347.30 (+) 1.68e4



Batch File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\LOD.lcb
Method File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\TX42 FINAL 102021.lcm

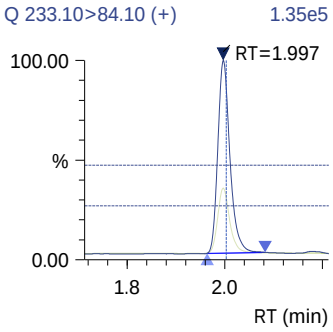
Urine 5 HPC_014

Sample ID: Urine 5 HPC
Date Acquired: 10/20/2021 6:11:07 PM
Acquired by: System Administrator
Method File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\TX42 FINAL 102021.lcm
Data File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\Urine 5 HPC_014.lcd
Vial: 11 | Inj. Volume: 3.0000uL | Tray: 1

Name	Found RT	Area	Ref 1 Range	Ref 1 Actual Ratio	Ref 2 Range	Ref 2 Actual Ratio
Norfentanyl	1.997	231926.610	24.67 - 45.81	33.76	----	----
4-ANPP	2.947	868397.418	83.68 - 155.41	109.59	----	----
Acetyl fentanyl	2.732	977646.089	66.95 - 124.34	100.05	----	----
Acryl fentanyl	2.969	258319.198	63.76 - 118.40	92.79	----	----
Butyryl fentanyl	3.218	303000.406	54.61 - 101.41	72.48	----	----
Cyclopropyl fentanyl	3.146	1312862.408	52.79 - 98.04	76.68	----	----
4-FIBF/PFBF	3.227	606140.583	57.22 - 106.26	77.21	----	----
Furanyl fentanyl	3.146	250637.707	68.87 - 127.91	91.32	----	----
Methoxy acetyl fentanyl	2.663	569257.177	71.73 - 133.21	95.33	----	----
Fluorofentanyl	3.050	240030.926	50.32 - 93.45	79.22	----	----
Sufentanil	3.329	1234122.875	59.07 - 109.70	83.25	16.01 - 29.74	23.53
Valeryl fentanyl	3.463	1308808.989	49.01 - 91.02	69.47	----	----
alpha-Methyl fentanyl	3.146	998670.501	24.19 - 44.93	33.96	20.86 - 38.74	30.07
Fentanyl-13C6	2.993	236795.905	77.96 - 144.78	108.87	----	----
Fentanyl	2.993	943115.183	69.76 - 129.55	100.66	----	----
betahyroxythio-Fentanyl -13C6	2.649	47298.918	48.93 - 90.88	68.79	28.14 - 52.25	34.95

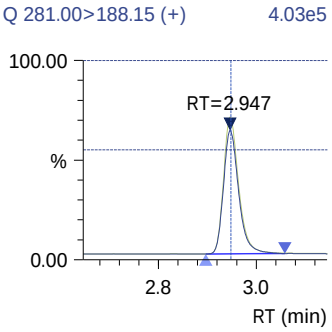
Norfentanyl

Conc 3.7389
Area 231926.610
R#1 233.10>55.00 33.76 (35.24)



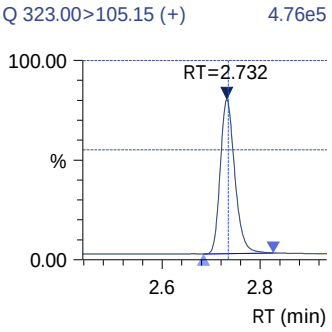
4-ANPP

Conc 13.6234
Area 868397.418
R#1 281.00>105.20 109.59 (119.54)



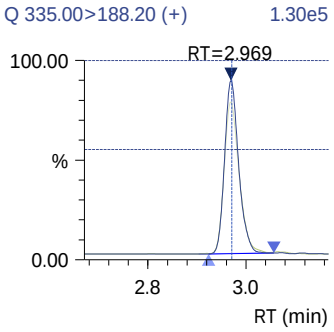
Acetyl fentanyl

Conc 4.8456
Area 977646.089
R#1 323.00>188.10 100.05 (95.65)



Acryl fentanyl

Conc 0.9496
Area 258319.198
R#1 335.00>105.20 92.79 (91.08)

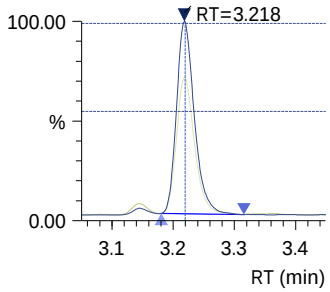


Urine 5 HPC_014 (continued)

Butyryl fentanyl

Conc 1.1706
Area 303000.406
R#1 351.00>105.15 72.48 (78.01)

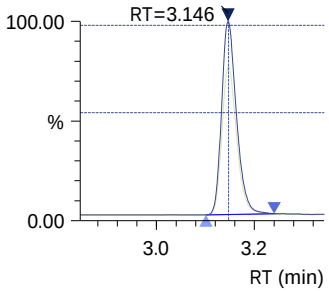
Q 351.00>188.10 (+) 1.51e5



Cyclopropyl fentanyl

Conc 5.9866
Area 1312862.408
R#1 349.00>105.20 76.68 (75.41)

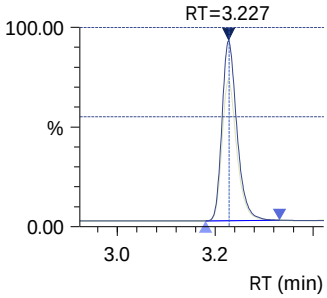
Q 349.00>188.15 (+) 6.24e5



4-FIBF/PFBF

Conc 5.6625
Area 606140.583
R#1 369.30>105.05 77.21 (81.74)

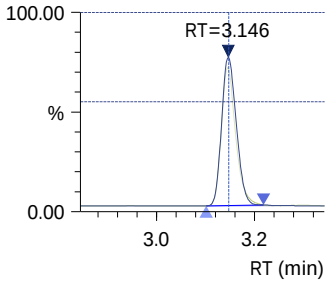
Q 369.30>188.15 (+) 2.86e5



Furanyl fentanyl

Conc 1.0317
Area 250637.707
R#1 375.00>105.15 91.32 (98.39)

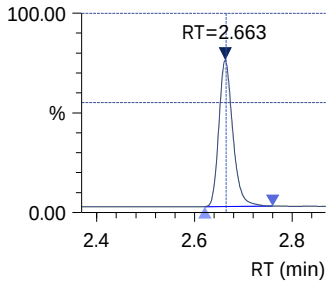
Q 375.00>188.10 (+) 1.20e5



Methoxy acetyl fentanyl

Conc 4.6675
Area 569257.177
R#1 353.00>105.15 95.33 (102.47)

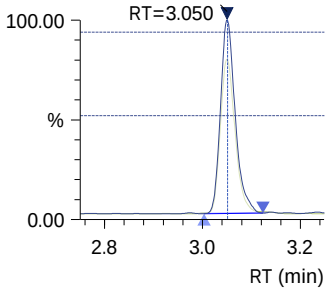
Q 353.00>188.10 (+) 2.87e5



Fluorofentanyl

Conc 1.0807
Area 240030.926
R#1 355.00>105.20 79.22 (71.89)

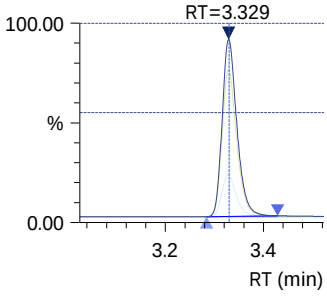
Q 355.00>188.15 (+) 1.14e5



Sufentanil

Conc 5.3421
Area 1234122.875
R#1 387.10>111.10 83.25 (84.38)
R#2 387.10>355.05 23.53 (22.87)

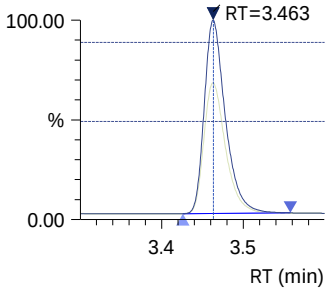
Q 387.10>238.15 (+) 5.91e5



Valeryl fentanyl

Conc 5.7951
Area 1308808.989
R#1 365.10>105.15 69.47 (70.01)

Q 365.10>188.15 (+) 7.29e5

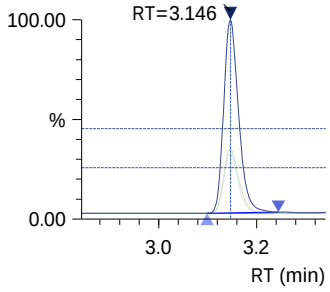


alpha-Methyl fentanyl

Conc 5.4690
Area 998670.501
R#1 351.30>202.00 33.96 (34.56)

R#2 351.30>119.20 30.07 (29.80)

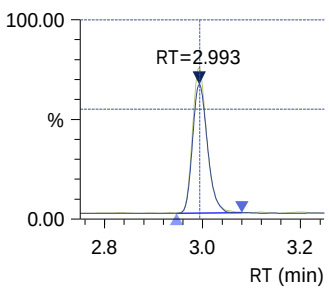
Q 351.30>91.10 (+) 4.73e5



Fentanyl-13C6

Conc 1.0000
Area 236795.905
R#1 343.00>105.15 108.87 (111.37)

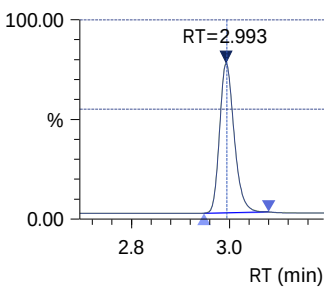
ISTD 343.00>188.20 (+) 1.12e5



Fentanyl

Conc 4.7412
Area 943115.183
R#1 337.20>105.00 100.66 (99.65)

Q 337.20>188.00 (+) 4.59e5

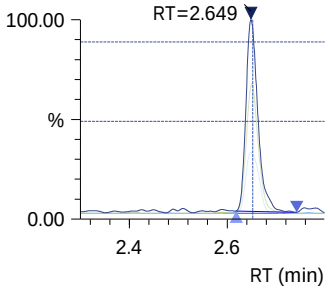


betahyroxythio -Fentanyl-13C6

Conc 1.0000
Area 47298.918
R#1 365.00>192.05 68.79 (69.91)

R#2 365.00>110.95 34.95 (40.19)

ISTD 365.00>347.30 (+) 2.80e4



Batch File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\LOD.lcb
Method File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\TX42 FINAL 102021.lcm

Urine 5 LPC _015

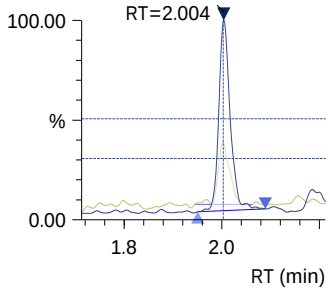
Sample ID: Urine 5 LPC
Date Acquired: 10/20/2021 6:20:05 PM
Acquired by: System Administrator
Method File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\TX42 FINAL 102021.lcm
Data File: C:\LabSolutions\Data\Validation_Verification\TX42\LOD\102021\Urine 5 LPC _015.lcd
Vial: 12 | Inj. Volume: 3.0000uL | Tray: 1

Name	Found RT	Area	Ref 1 Range	Ref 1 Actual Ratio	Ref 2 Range	Ref 2 Actual Ratio
Norfentanyl	2.004	12195.804	24.67 - 45.81	33.45	----	----
4-ANPP	2.953	36506.004	83.68 - 155.41	114.29	----	----
Acetyl fentanyl	2.738	48948.072	66.95 - 124.34	94.10	----	----
Acryl fentanyl	2.976	10038.052	63.76 - 118.40	99.72	----	----
Butyryl fentanyl	3.223	13627.968	54.61 - 101.41	78.16	----	----
Cyclopropyl fentanyl	3.150	74939.783	52.79 - 98.04	69.11	----	----
4-FIBF/PFBF	3.231	29860.092	57.22 - 106.26	93.25	----	----
Furanyl fentanyl	3.151	10824.131	68.87 - 127.91	92.99	----	----
Methoxy acetyl fentanyl	2.668	26753.952	71.73 - 133.21	87.74	----	----
Fluorofentanyl	3.053	13754.051	50.32 - 93.45	59.61	----	----
Sufentanil	3.333	58508.621	59.07 - 109.70	88.11	16.01 - 29.74	23.86
Valeryl fentanyl	3.465	62499.227	49.01 - 91.02	71.19	----	----
alpha-Methyl fentanyl	3.149	49266.517	24.19 - 44.93	30.96	20.86 - 38.74	27.73
Fentanyl-13C6	2.997	128951.474	77.96 - 144.78	106.85	----	----
Fentanyl	2.998	43470.399	69.76 - 129.55	104.04	----	----
betahyroxythio-Fentanyl -13C6	2.653	23824.879	48.93 - 90.88	62.29	28.14 - 52.25	35.48

Norfentanyl

Conc 0.3610
Area 12195.804
R#1 233.10>55.00 33.45 (35.24)

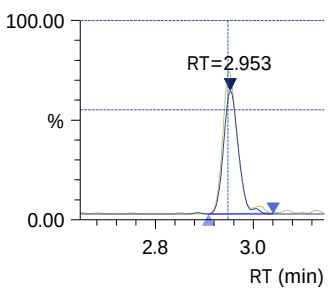
Q 233.10>84.10 (+) 7.64e3



4-ANPP

Conc 1.0517
Area 36506.004
R#1 281.00>105.20 114.29 (119.54)

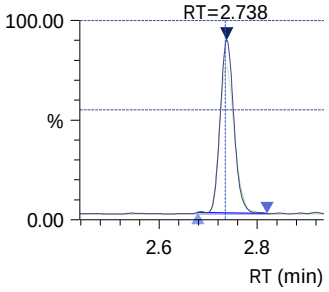
Q 281.00>188.15 (+) 1.72e4



Acetyl fentanyl

Conc 0.4455
Area 48948.072
R#1 323.00>188.10 94.10 (95.65)

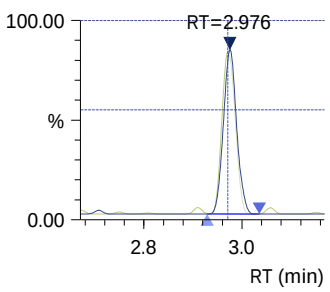
Q 323.00>105.15 (+) 2.54e4



Acryl fentanyl

Conc 0.0678
Area 10038.052
R#1 335.00>105.20 99.72 (91.08)

Q 335.00>188.20 (+) 5.34e3

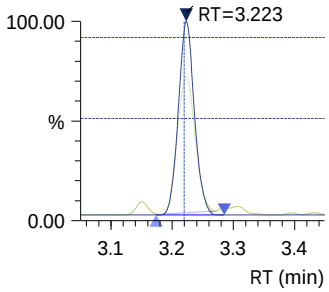


Urine 5 LPC _015 (continued)

Butyryl fentanyl

Conc 0.1045
Area 13627.968
R#1 351.00>105.15 78.16 (78.01)

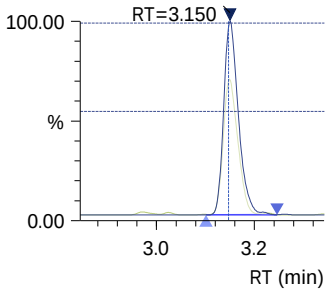
Q 351.00>188.10 (+) 7.49e3



Cyclopropyl fentanyl

Conc 0.6784
Area 74939.783
R#1 349.00>105.20 69.11 (75.41)

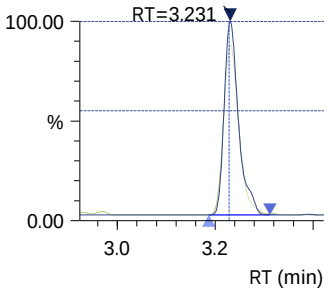
Q 349.00>188.15 (+) 3.59e4



4-FIBF/PFBF

Conc 0.5538
Area 29860.092
R#1 369.30>105.05 93.25 (81.74)

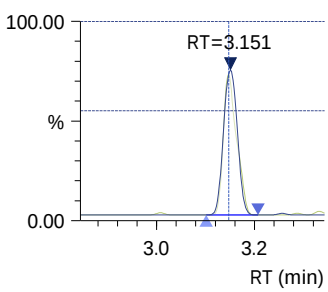
Q 369.30>188.15 (+) 1.46e4



Furanyl fentanyl

Conc 0.0885
Area 10824.131
R#1 375.00>105.15 92.99 (98.39)

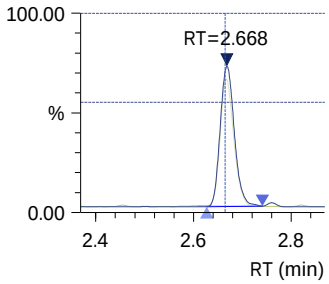
Q 375.00>188.10 (+) 5.47e3



Methoxy acetyl fentanyl

Conc 0.4028
Area 26753.952
R#1 353.00>105.15 87.74 (102.47)

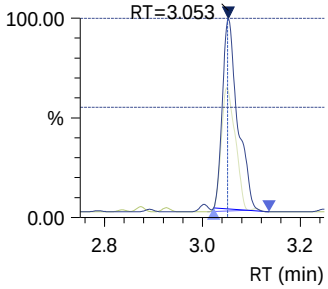
Q 353.00>188.10 (+) 1.33e4



Fluorofentanyl

Conc 0.1229
Area 13754.051
R#1 355.00>105.20 59.61 (71.89)

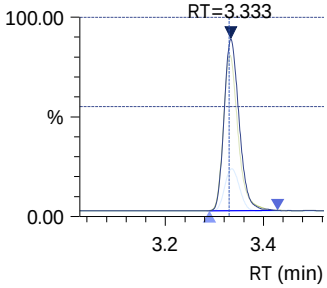
Q 355.00>188.15 (+) 6.40e3



Sufentanil

Conc 0.5028
Area 58508.621
R#1 387.10>111.10 88.11 (84.38)
R#2 387.10>355.05 23.86 (22.87)

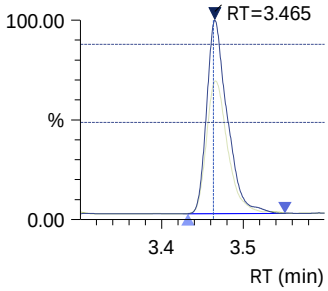
Q 387.10>238.15 (+) 2.75e4



Valeryl fentanyl

Conc 0.5494
Area 62499.227
R#1 365.10>105.15 71.19 (70.01)

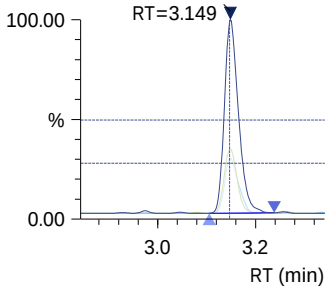
Q 365.10>188.15 (+) 3.56e4



alpha-Methyl fentanyl

Conc 0.5356
Area 49266.517
R#1 351.30>202.00 30.96 (34.56)
R#2 351.30>119.20 27.73 (29.80)

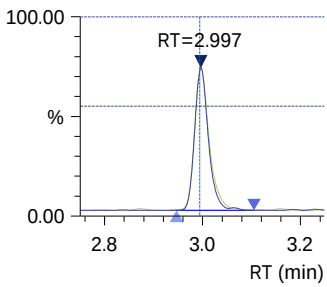
Q 351.30>91.10 (+) 2.47e4



Fentanyl-13C6

Conc 1.0000
Area 128951.474
R#1 343.00>105.15 106.85 (111.37)

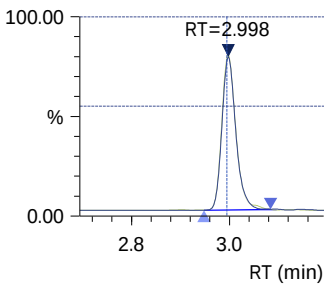
ISTD 343.00>188.20 (+) 6.58e4



Fentanyl

Conc 0.4013
Area 43470.399
R#1 337.20>105.00 104.04 (99.65)

Q 337.20>188.00 (+) 2.21e4



betahyroxythio -Fentanyl-13C6

Conc 1.0000
Area 23824.879
R#1 365.00>192.05 62.29 (69.91)
R#2 365.00>110.95 35.48 (40.19)

ISTD 365.00>347.30 (+) 1.37e4