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Utility of gas chromatography infrared spectroscopy (GC-IR) for the differentiation of positional isomers of fentanyl related substances

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ABSTRACT

Forensic laboratories routinely use gas chromatography mass spectrometry (GC–MS) in the identification of controlled substances using both retention time and electron impact ionization (EI) mass spectra. Certain drugs such as positional isomers of some fentanyl related substances (FRS) can produce indistinguishable EI mass spectra but may be differentiated using retention time. The core structure of fentanyl consists of an amide group, a piperidine ring, an aniline ring, and an N-alkyl chain, each providing opportunities for points of substitution that create FRS and corresponding positional isomers. For this study, the analysis by GC coupled to a vapor-phase infrared spectroscopy detector (GC-IR) was used as a complementary technique to GC–MS for the identification of positional isomers of FRS. The result is a novel fentanyl library consisting of 212 different FRS reference compounds. A collaboration among three different laboratories yielded correct identifications of twenty blind samples when searched against the GC-IR FRS library created at Florida International University (FIU). The expected limits of detection for fentanyl using GC-IR range between 0.10 and 0.19 mg/mL, depending on the sample introduction (injector) method and other instrumental parameters. The newly created GC-IR library and its GC–MS counterpart of 212 FRS are shared in the supplementary materials for future use by researchers and practitioners.

Introduction

The number of fentanyl-related drug overdoses in the United States has seen an approximate eight-fold increase over the past few years surpassing deaths from illicit use of prescription opioids and heroin [1]. In 2017, 59.8% of opioid-related deaths involved fentanyl and/or fentanyl related substances (FRS) compared to 14.3% in 2010 [2]. It is estimated that approximately ninety Americans die each day as a result of opioid abuse, and this number continues to increase at alarming levels [3]. In the past 5 years, there has been a 10-fold increase in the reporting of seized fentanyl and FRS [4]. According to the National Forensic Laboratory Information System (NFLIS) 2020 mid-year report, fentanyl is the fourth most identified drug reported by NFLIS-participating forensic laboratories, up from fifth in previous years [5].

The opioid crisis has amplified the need for identification of fentanyl and FRS. To assist with compound identification, libraries created from reference materials are needed. Forensic laboratories routinely use GC–MS as the gold standard for confirmation of seized drugs. For the fentanyl isomers, the typical electron impact ionization (EI) mass spectra results form no unique molecular ions and/or indiscriminate fragmentation for a large number of FRS positional isomers [6–8]. This makes identification challenging if not coupled with other separatory and confirmatory analytical methods. The core structure of fentanyl consists of an amide group, a piperidine ring, an aniline ring, and an *n*-alkyl chain, each of which provides regions for substitutions that alter the structure. These substitution regions are exploited by producers on the illicit market and contribute to the diversity of FRS [6].

Implementation of standard libraries has been vital to drug identification within the field of forensic chemistry. The rapidly evolving seized drugs entering the illicit market require the development and continuous maintenance of libraries containing searchable spectra of reference material. Commercial libraries exist for a wide variety of analytical techniques

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Table 1
Gas chromatography and mass spectrometry instrumental parameters and experimental conditions for the three participating laboratories.

Instrumental and Analytical Conditions	FIU Research Laboratory	DEA Special Testing and Research Laboratory	Pinellas County Forensic Laboratory
Gas Chromatography			
GC Instrument Model	Agilent 7890A	Agilent 7890B	Agilent 7890A
Carrier Gas	Helium	Helium	Helium
Gas Flow Rate	1.5 mL/minute	1.5 mL/minute	1.0 mL/minute
Injector Split Ratio	5:1	25:1	50:1
Column Type	Agilent Technologies DB-5MS (30 m × 0.25 mm with 0.25 μm film coating)	Agilent Technologies DB-5 (30 m × 0.25 mm with 0.25 μm film coating)	Agilent Technologies DB-5MS (20 m × 0.18 mm with 0.18 μm film coating)
Initial Temperature	100 °C no hold	100 °C for 1 min	120 °C for 2 min
Ramp Rate	30 °C/minute	12 °C/minute	35 °C/minute
Final Temperature	310 °C	280 °C	320 °C
Hold Time	9 min	9 min	3.3 min
Inlet Temperature	270 °C	280 °C	250 °C
Injection Volume	1 μL	1 μL	1 μL
Mass Spectrometry			
MS Instrument Model	Agilent Technologies 5975C	Agilent Technologies 5977A	Agilent Technologies 5975C
Source Temperature	230 °C	230 °C	230 °C
Quadrupole Temperature	150 °C	150 °C	150 °C
Scan Range	34–600 amu	30–550 amu	40–500 amu
EI voltage	70 eV	70 eV	70 eV

Table 2
Gas chromatography and infrared instrumental parameters and experimental conditions for the three participating laboratories.

Instrumental and Analytical Conditions	FIU Research Laboratory	DEA Special Testing and Research Laboratory	Pinellas County Forensic Laboratory
Gas Chromatography			
GC Instrument Model	Agilent 6890N	Agilent 7890B	Agilent 7890B
Carrier Gas	Helium	Helium	Helium
Gas Flow Rate	1.5 mL/minute	2.0 mL/minute	1.7 mL/minute
Injector Split Ratio	Splitless	2:1 Split	Splitless
Column Type (All used 30 m × 0.32 mm with 0.25 μm film coating)	Ohio Valley Specialty Company OV-5	Agilent HP-5	Agilent HP-5
Initial Temperature	100 °C no hold	65 °C for 1.5 min	80 °C for 1.2 min
Ramp Rate	30 °C/minute	20 °C/minute	30 °C/minute
Final Temperature	310 °C	310 °C	320 °C
Hold Time	9 min	5 min	4 min
Inlet Temperature	270 °C	280 °C	250 °C
Injection Volume	3 μL	2 μL	3 μL
Infrared Spectroscopy			
IR Instrument Model	ASAP IRD3	ASAP IRD3	ASAP IRD3
Light Pipe Temperature	250 °C	280 °C	250 °C
Transfer Line Temperature	250 °C	280 °C	275 °C
Spectral Range	500–4000 cm^{-1}	550–4000 cm^{-1}	500–4000 cm^{-1}
Spectral Resolution	8 cm^{-1}	8 cm^{-1}	8 cm^{-1}

Table 3
The identity of the 20 FRS used in the blind study along with the rationale of why these were selected.

Currently in the NIST Library
1. Sufentanil
2. Alfentanil
Not in the NIST Library but readily distinguishable by MS spectra
3. Thiophene fentanyl
4. Remifentanil
5. Ocfentanil
6. Fentanyl carbamate
7. α-methyl Butyryl fentanyl
8. 2, 3-seco-Fentanyl
9. Acrylfentanyl
10. Senecioylfentanyl
Not in the NIST Library and not readily distinguishable by MS spectra but readily distinguishable by IR spectra
11. Furanyl fentanyl
12. *ortho*-methyl Furanyl fentanyl
13. Valeryl fentanyl
14. *meta*-Fluorofentanyl
15. *para*-methyl Fentanyl
16. *para*-Chlorobutyryl fentanyl
17. FIBF
18. Crotonyl fentanyl
19. *ortho*-methyl Cyclopropyl fentanyl
20. *ortho*-methyl Acetyl fentanyl

such as GC–MS, infrared (IR), nuclear magnetic resonance (NMR), and Raman spectroscopy [7,8]. Methods complementary to GC–MS, such as GC-IR, are being introduced into forensic laboratories as cost-effective means to distinguish FRS and other novel psychoactive substances (NPS), including cathinones and synthetic cannabinoids. GC-IR is a confirmatory technique that typically results in a unique infrared spectra, whereby no two compounds, though similar, will have the same absorbance characteristics [9]. GC-IR has been reported for the identification of FRS [10,11] isomeric ethoxyphenethylamines and methoxymethcathinones [12], methylenedioxybenzylpiperazines (MDBPs) and methoxymethylbenzylpiperazines (MMBPs) [13], and methamphetamine and regioisomeric substances [14]. Although GC-IR is successful in the differentiation of positional isomers, this technique is more limited is terms of sensitivity compared to GC–MS [15]. Mass loadings of more than 25 ng on column for GC-IR analysis are needed to produce acceptable spectra, in comparison to sub-ng mass loadings needed for a typical GC–MS analysis [10]. GC–MS analysis with a library search of reference standards has been the routine approach for controlled substance identification in complex mixtures [16]. One recent study incorporated a targeted GC–MS method that was able to differentiate all but four compound pairs based on nonoverlapping retention time and acceptance windows or objectively different mass spectra [17].

Based upon literature searches, this appears to be the first reporting of a blind study using a FRS-specific vapor-phase infrared spectra library and -as a FRS specific mass spectra of library for 212 different FRS compounds. Studies to determine the limits of detection, limits of quantitation, and the results of library searches for the differentiation of

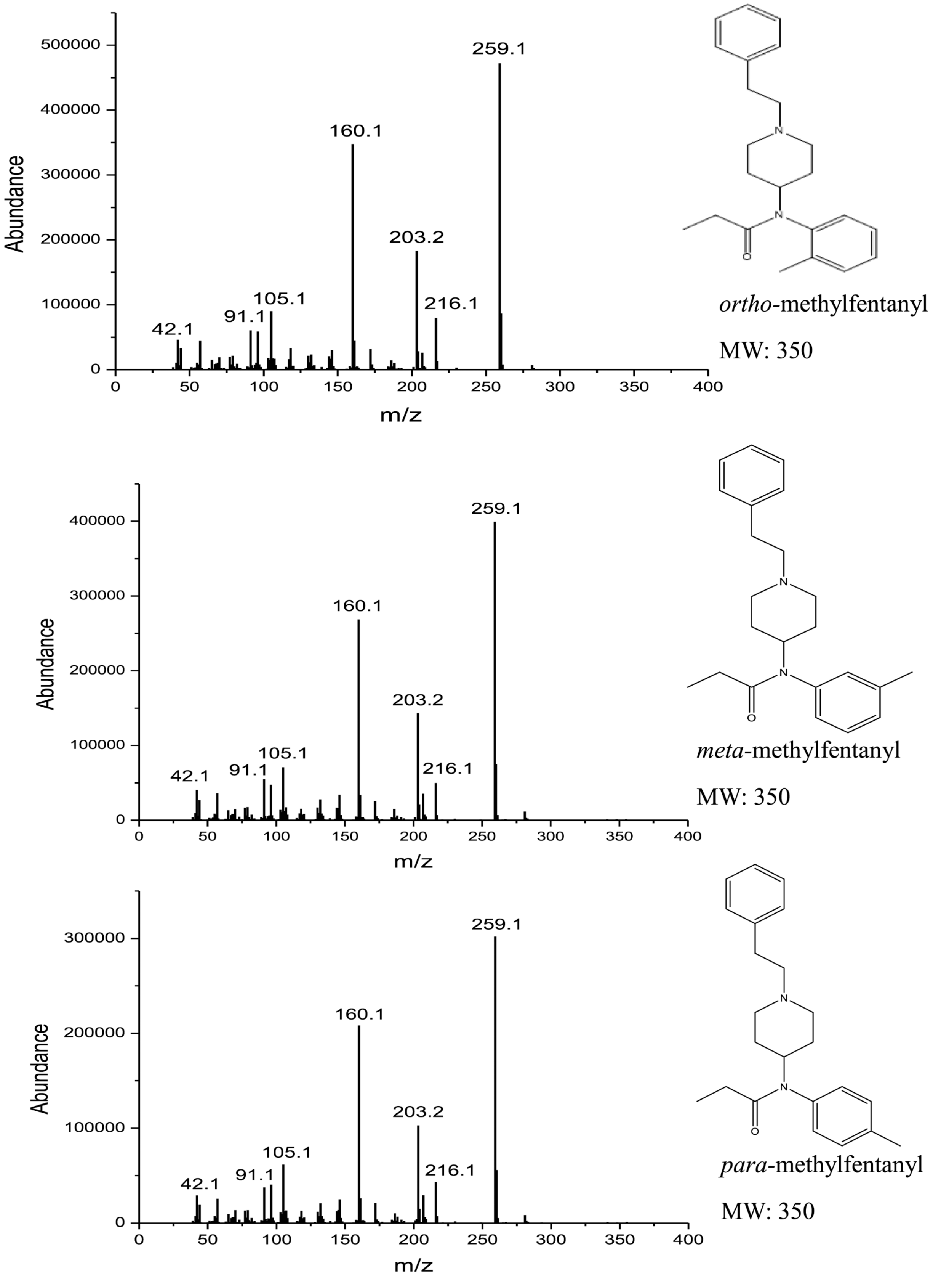

Fig. 1. Mass spectra of *ortho*-methylfentanyl, *meta*-methylfentanyl, and *para*-methylfentanyl.

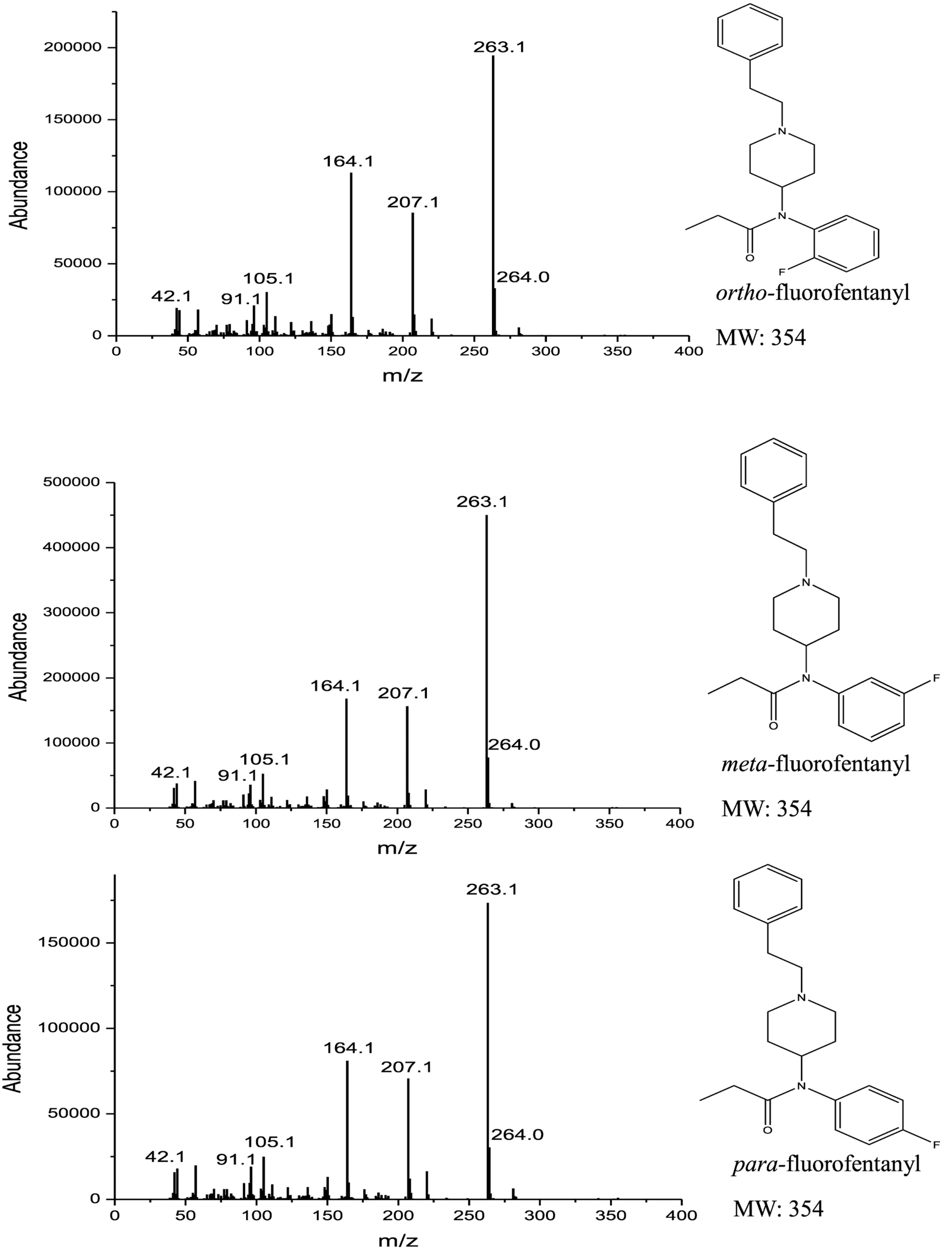

Fig. 2. Mass spectra of *ortho*-fluorofentanyl, *meta*-fluorofentanyl, and *para*-fluorofentanyl.

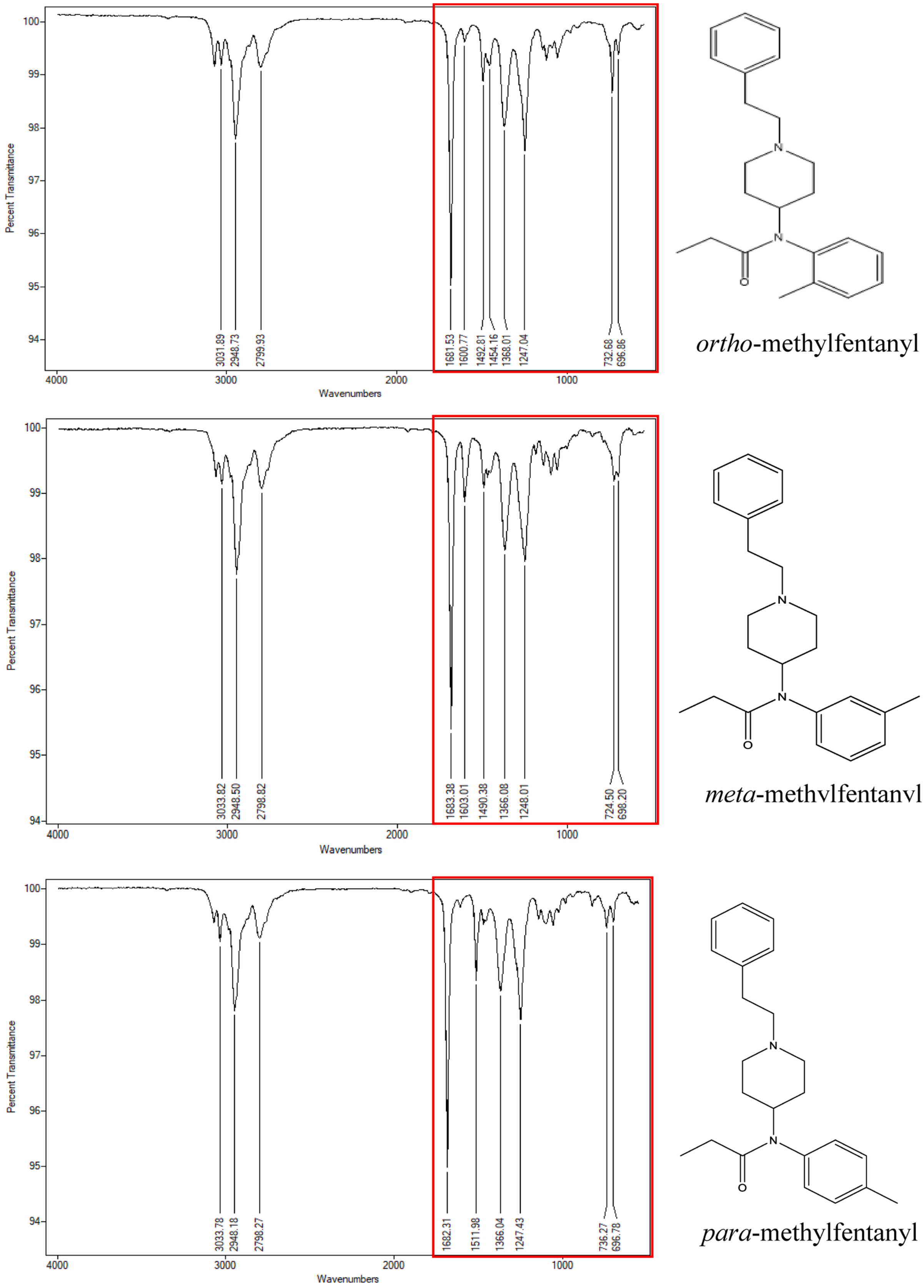

Fig. 3. Vapor phase infra-red spectra of *ortho*-methylfentanyl, *meta*-methylfentanyl, and *para*-methylfentanyl. The red square depicts the “fingerprint” region of the IR spectra. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

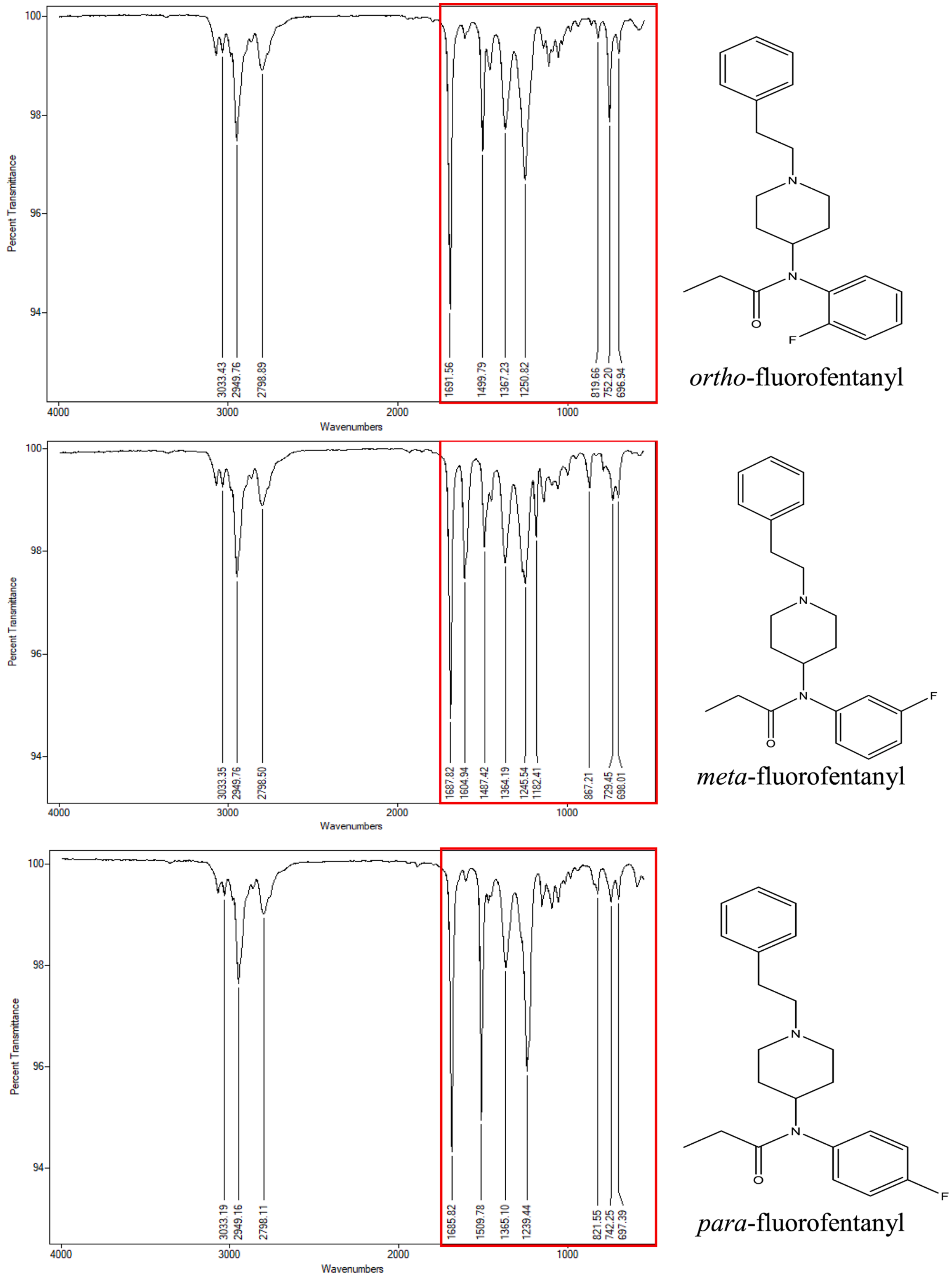

Fig. 4. Vapor phase infra-red spectra of *ortho*-fluorofentanyl, *meta*-fluorofentanyl, and *para*-fluorofentanyl. The red square depicts the “fingerprint” region of the IR spectra. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
The library search results for *para*-methylfentanyl using FIU and EUCLiD libraries.

Sample	Metric	Name	Library	Entry
1	1.000000	020521 *Para*-Methylfentanyl 1: 3ul 400 ppm	FIU	102: 020521 *Para*-Methylfentanyl 1.spc
2	0.999647	020521 *Para*-Methylfentanyl 2: 3ul 400 ppm	FIU	103: 020521 *Para*-Methylfentanyl 2.spc
3	0.999012	020521 *Para*-Methylfentanyl 3: 3ul 400 ppm	FIU	104: 020521 *Para*-Methylfentanyl 3.spc
4	0.978178	020521 4′-Methyl Fentanyl 2: 3ul 400 ppm	FIU	85: 020521 4′-Methyl Fentanyl 2.spc
5	0.978083	020521 4′-Methyl Fentanyl 1: 3ul 400 ppm	FIU	84: 020521 4′-Methyl Fentanyl 1.spc
6	0.977495	020521 4′-Methyl Fentanyl 3: 3ul 400 ppm	FIU	86: 020521 4′-Methyl Fentanyl 3.spc
7	0.973950	032321 *Para*-Methyl Butyryl Fentanyl 3: 3ul 400 ppm	FIU	341: 032321 *Para*-Methyl Butyryl Fentanyl 3.spc
8	0.973020	020821 Orth0-Methylfentanyl 1: 3ul 400 ppm	FIU	120: 020821 *Ortho*-Methylfentanyl 1.spc
9	0.972718	020821 *Ortho*-Methylfentanyl 2: 3ul 400 ppm	FIU	121: 020821 *Ortho*-Methylfentanyl 2.spc
10	0.972533	020821 *Ortho*-Methylfentanyl 3: 3ul 400 ppm	FIU	122: 020821 *Ortho*-Methylfentanyl 3.spc

FRS at three different laboratories are also included in this study.

Experimental section

Materials

The Fentanyl Analog Screening (FAS) Kit including Emergent Panels 1–3, (see supplementary tables S1–S5 for a complete listing), contained 200 μg of each of the 212 FRS in individual vials and was obtained from Cayman Chemical (Ann Arbor, MI). A 500 μL aliquot of HPLC grade methanol (Fisher Scientific, Atlanta, GA) was pipetted into each vial to create an approximate 400 μg/ml (or 400 ppm) concentration. Each vial was vortexed at medium speed for a minimum of 15 min and stored at 0 °C.

Instrumentation

GC-IR studies were conducted using three different instruments, an Agilent Technologies 6890 N Network GC System coupled to an IRD3 detector obtained from Analytical Solutions and Providers (ASAP) at Florida International University (FIU), an Agilent Technologies 7890B GC System coupled to an IRD3 detector obtained from Analytical Solutions and Providers (ASAP) at the Drug Enforcement Administration's (DEA) Special Testing and Research Laboratory, and an Agilent 7890B GC with an ASAP IRD 3 detector obtained from Analytical Solutions and Providers (ASAP) at the Pinellas County Forensic Laboratory.

The GC at FIU was operated in splitless mode with high purity helium as the carrier gas at a flow rate of 1.5 mL/min. The column used was a 30 m × 0.32 mm coated with 0.25 μm (OV-5) purchased from Ohio Valley Specialty Company (Marietta, OH). The oven program consisted of an initial temperature of 100 °C without hold ramped by a rate of 30 °C/min to a final temperature of 310 °C and a final hold of 9 min. The GC inlet temperature was 270 °C with an injection volume of 3 μL (for a total amount of 1.2 μg of analyte mass loading). The IRD detector at FIU was operated at light pipe and transfer lines temperatures of 250 °C and all spectra were collected in the range 500–4000 cm^{-1} with a resolution of 8 cm^{-1}. Each library standard was analyzed in triplicate using an autosampler.

The Special Testing and Research Laboratory's GC-IR used a 30 m by 0.32 mm column with 0.25 μm HP-5 stationary phase and was operated using a 2:1 split ratio with a 2.0 mL/min constant flow of high purity helium as the carrier gas. The oven program consisted of a starting temperature of 65 °C with an initial 1.5-minute hold ramped at a rate of 20 °C/min to a final temperature of 310 °C and a final hold of 5 min. The GC inlet temperature was 280 °C with an injection volume of 2 μL. The IRD detector was operated at light pipe and transfer lines temperatures of 280 °C and all spectra were collected from 550 to 4000 cm^{-1}.

The GC at the Pinellas County Forensic Laboratory was operated in splitless mode with high purity helium as the carrier gas at a flow rate of 1.7 mL/min with a splitless hold time of 1.2 min. The column used was a 30 m × 0.32 mm Agilent HP5 with a film thickness of 0.25 μm. The oven program consisted of an initial temperature set at 80 °C for 1.2 min ramped at 30 °C/min to 320 °C with a final hold time of 4 min. The GC inlet temperature was 250 °C with an injection volume of 3 μL. The IRD light pipe was operated at 250 °C and the transfer lines at 275 °C and all spectra were collected in the range 500–4000 cm^{-1} with a resolution of 8 cm^{-1}. Each library standard was analyzed in triplicate using an autosampler.

GC–MS studies at FIU were conducted using an Agilent Technologies 7890A GC System coupled to an Agilent Technologies 5975C inert mass spectrometer (MS). The GC was operated using a 5:1 split with high purity helium as the carrier gas at a flow rate of 1.5 mL/min. A 30 m by 0.25 mm column coated with 0.25 μm-thick sorbent DB-5MS column from Agilent Technologies was used. The oven program consisted of the same used for GC-IR studies. The GC inlet temperature was 270 °C with an injector volume of 1 μL (for a total amount of 400 ng of analyte mass loading). The MS was operated in scan range 34–600 amu with the electron ionization at 70 eV while the source temperature was set at 230 °C, and the quadrupole temperature was set to 150 °C. Each library standard was analyzed in triplicate.

At the Special Testing and Research Laboratory, the analysis was performed using two GC–MS systems – an Agilent Technologies 7890B GC System coupled to an Agilent Technologies 5977A MS using high purity helium as the carrier gas and an Agilent Technologies Intuvo 9000 GC System coupled to an Agilent Technologies 5977B MS using high purity hydrogen as the carrier gas.

The 7890B GC used a 30 m by 0.25 mm column with a 0.25 μm DB-5 film thickness and was operated using a 25:1 split with at a constant flow rate of 1.5 mL/min. The oven program started at 100 °C with an initial 1-

Table 5
Results of the EUCLiD library search for *para*-methylfentanyl.

Sample	Metric	Name	Library	Entry
1	0.961873	*para*-methyl Butyryl Fentanyl	Project_Euclid_v1-0-0	1167
2	0.961541	Fentanyl	Project_Euclid_v1-0-0	741
3	0.955077	N-methyl Norfentanyl	Project_Euclid_v1-0-0	1059
4	0.950822	Benzyl Fentanyl	Project_Euclid_v1-0-0	520
5	0.942833	Butyryl fentanyl	Project_Euclid_v1-0-0	558
6	0.938878	Valeryl Fentanyl	Project_Euclid_v1-0-0	1379
7	0.926652	*para*-Bromofentanyl	Project_Euclid_v1-0-0	1162
8	0.914583	Isobutyryl Fentanyl	Project_Euclid_v1-0-0	802
9	0.900042	4-Methoxy-butyryl fentanyl	Project_Euclid_v1-0-0	273
10	0.891177	*para*-Fluorofentanyl	Project_Euclid_v1-0-0	1164

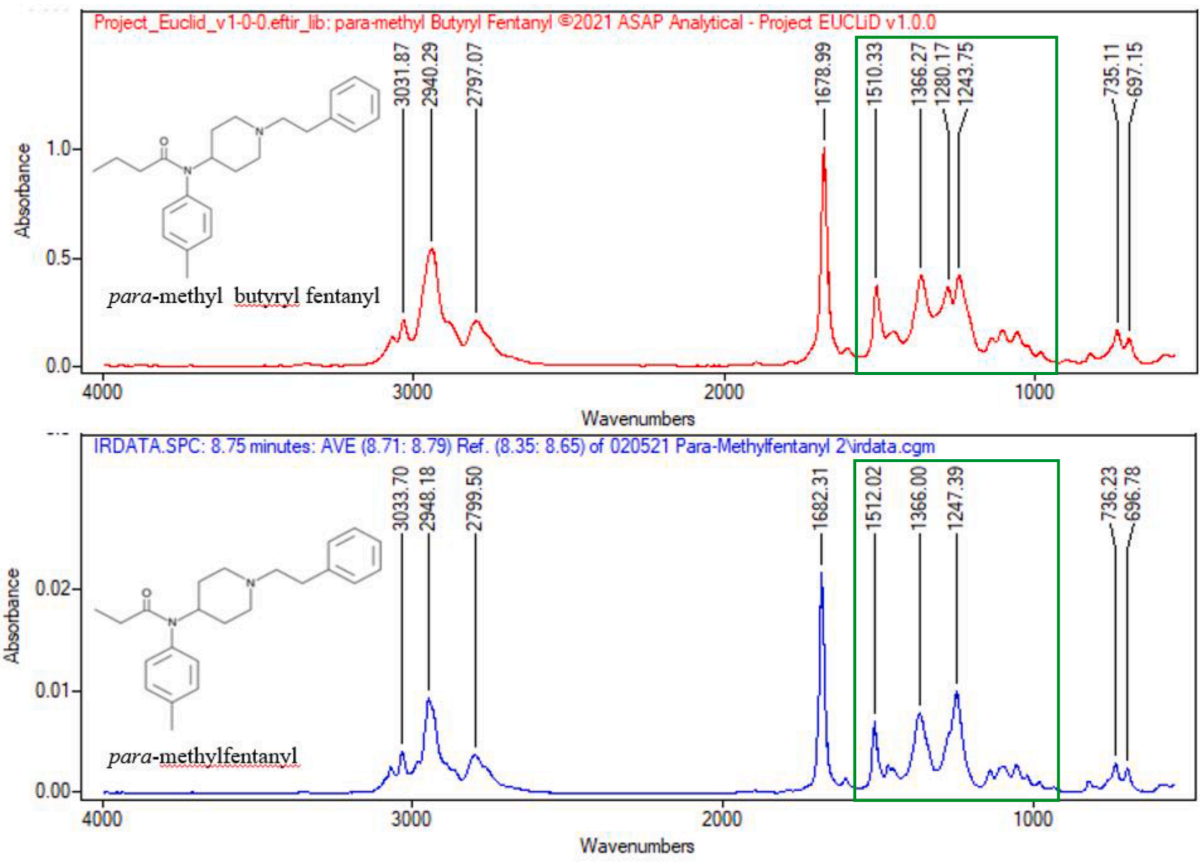

Fig. 5. Comparison of spectra of *para*-methyl butyryl fentanyl (red) and *para*-methylfentanyl (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 6
The library search results for meta-fluorofentanyl using FIU and EUCLiD library for fentanyl related substances (FRS).

Sample	Metric	Name	Library	Entry
1	1.000000	020821 *Meta*-Fluorofentanyl 1: 3ul 400 ppm	FIU	117: 020821 *Meta*-Fluorofentanyl 1.spc
2	0.995785	020821 *Meta*-Fluorofentanyl 2: 3ul 400 ppm	FIU	118: 020821 *Meta*-Fluorofentanyl 2.spc
3	0.995247	020821 *Meta*-Fluorofentanyl 3: 3ul 400 ppm	FIU	119: 020821 *Meta*-Fluorofentanyl 3.spc
4	0.989161	*meta*-Fluorofentanyl	Project_Euclid_v1-0-0	947
5	0.969818	012621 *Meta*-Fluorobutyryl Fentanyl 2: 3ul 400 ppm	FIU	70: 012621 *Meta*-Fluorobutyryl Fentanyl 2.spc
6	0.969783	012621 *Meta*-Fluorobutyryl Fentanyl 3: 3ul 400 ppm	FIU	71: 012621 *Meta*-Fluorobutyryl Fentanyl 3.spc
7	0.968145	012621 *Meta*-Fluorobutyryl Fentanyl 1: 3ul 400 ppm	FIU	69: 012621 *Meta*-Fluorobutyryl Fentanyl 1.spc
8	0.950108	012621 *Meta*-Fluoroisobutyryl Fentanyl 3: 3ul 400 ppm	FIU	74: 012621 *Meta*-Fluoroisobutyryl Fentanyl 3.spc
9	0.949963	012621 *Meta*-Fluoroisobutyryl Fentanyl 2: 3ul 400 ppm	FIU	73: 012621 *Meta*-Fluoroisobutyryl Fentanyl 2.spc
10	0.949947	012621 *Meta*-Fluoroisobutyryl Fentanyl 1: 3ul 400 ppm	FIU	72: 012621 *Meta*-Fluoroisobutyryl Fentanyl 1.spc

minute hold then ramped at a rate of 12 °C/min to a final temperature of 280 °C holding for 9 min. The GC inlet temperature was 280 °C with an injector volume of 1 μL. The MS was operated in scan range 30–550 amu with the electron ionization at 70 eV while the source temperature was set at 230 °C and the quadrupole temperature was set to 150 °C.

The 9000 Intuvo GC used a 20 m by 0.18 mm column with a 0.18 μm HP-5MS film thickness and was operated using a 60:1 split with a constant flow rate of 1.0 mL/min. The oven program started at 80 °C holding for 0.5 min, then ramped at a rate of 80 °C/min to 180 °C with a 2-minute hold, then ramped 40 °C/min to 270 °C with a 1-minute hold, and finally ramped at 100 °C/min to 310 °C with a 2 min hold. The GC inlet temperature was 260 °C with an injector volume of 1 μL. The MS was operated in scan range 40–500 amu with the electron ionization at 70 eV while the source temperature was set at 230 °C and the quadrupole temperature was set to 150 °C.

GC–MS studies at the Pinellas County Laboratory were conducted using an Agilent Technologies 7890A GC System coupled to an Agilent Technologies 5975C inert mass spectrometer (MS). The GC was operated using a 50:1 split with high purity helium as the carrier gas at a flow rate of 1.0 mL/min. A 20 m × 0.18 mm (DB-5MS) column coated with 0.18 μm-thick sorbent from Agilent Technologies was used. The oven program consisted of an initial temperature of 120 °C with an initial hold time of 2 min, ramped to 320 °C at a rate of 35 °C/min, with a final hold time of 3.3 min. The GC inlet temperature was 250 °C with an injection volume of 1 μL. The MS was operated in scan range 40–500 AMU with the electron ionization at 70 eV while the source temperature was set at 230 °C, and the quadrupole temperature was set to 150 °C. Each library standard was analyzed in triplicate.

Table 1 lists the GC–MS instrumental parameters and analytical conditions for the three participating laboratories in this study. Table 2 lists the gas chromatography and infrared instrumental parameters and experimental conditions for the three participating laboratories.

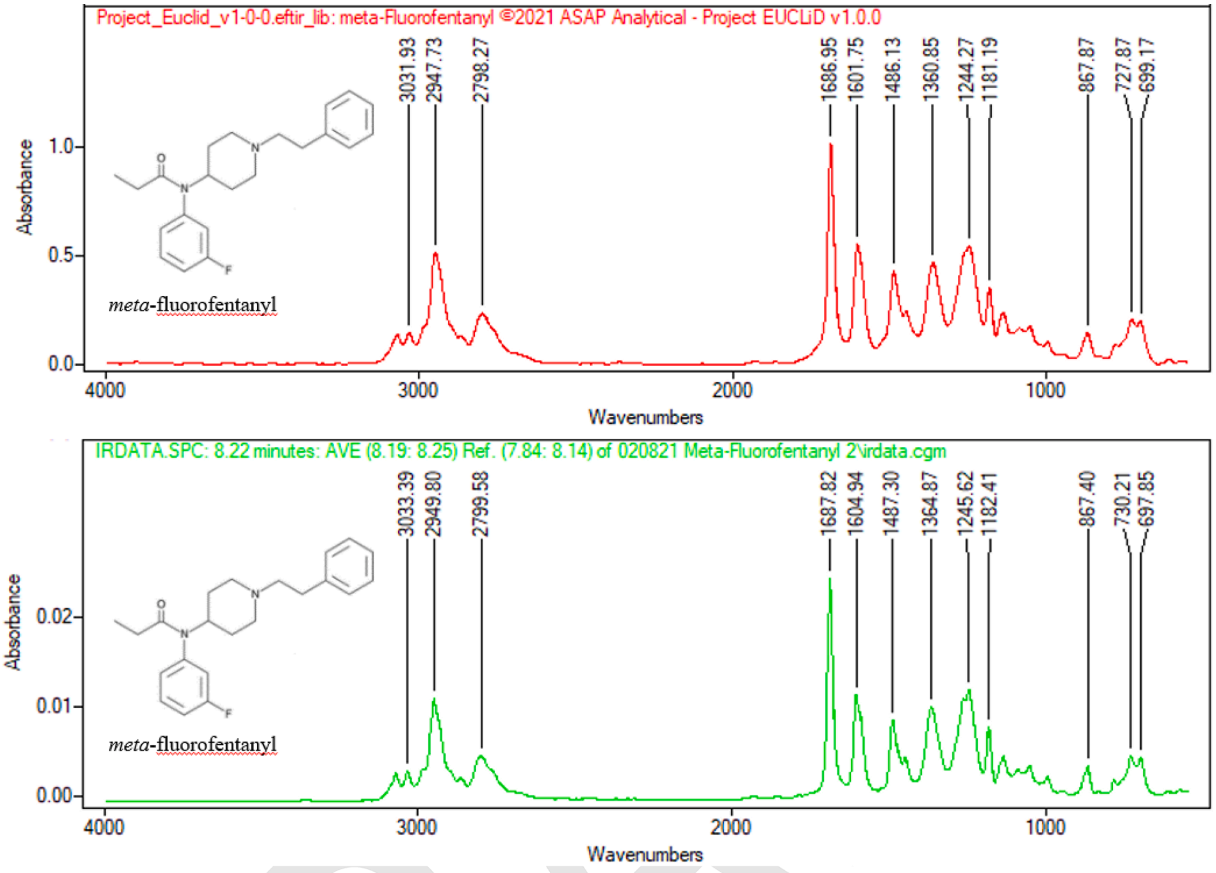

Fig. 6. Comparison of spectra from EUCLiD library search (top spectra) the new FIU Fentanyl library search (bottom spectra) for *meta*-fluorofentanyl.

Table 7
Comparison of the search results for an existing NIST GC–MS library search, a search of the new FIU MS library, the existing EUCLiD GC-IR library and the new FIU Fentanyl IR library created at the FIU laboratory.

Library	Number of Matches in the Top 5 Search Results	Percentage of Correct Matches (%)
GC–MS NIST Library	10	4.7
New FIU Fentanyl GC–MS Library	190	89.6
EUCLiD GC-IR Library (2021)	32	15.1
New FIU GC-IR FRSLibrary	212	100.0

FIU Fentanyl library construction for MS spectra

The MS spectra generated from all 212 compounds were used to create a library using Agilent's MSD Chemstation version E.02.01.1177 at FIU and shared with the DEA Special Testing and Research Laboratory and the Pinellas County Forensic Laboratory. This library consists solely of spectra from FRS. All spectra were collected in triplicate.

Library construction for FTIR spectra using Analytical Solutions and Providers (ASAP) Analytical software

All spectra acquired by the GC-IR method were converted to .spc files and a library of all the analyzed FRS was created at FIU and shared with the DEA Special Testing and Research Laboratory and the Pinellas County Forensic Laboratory. All spectra were collected in triplicate. ASAP's End User Contributed Library (EUCLiD) [18] was also obtained and utilized in the search studies at FIU. The EUCLiD library consists of 1,400 spectra of different compounds contributed by end users, including 32 FRS compounds contained in the FAS-Kit.

Library evaluation performed by FIU

The FIU MS Library, the NIST08 MS library, the EUCLiD library, and the FIU Fentanyl IR libraries were assessed. All 212 FRS were searched, in triplicate, using each of the two IR libraries (the EUCLiD library and the new FIU vapor-phase IR Fentanyl library) for a total of 636 searches. The NIST08 MS library and the FIU MS Library were searched in the same manner. The following criteria were followed to ensure spectra of good quality were added to the library:

(i) Chromatographic peaks with a signal to noise ratio of equal to or greater than three (3) produced mass spectra with clearly defined base peaks and reproducible fragmentation for MS and smooth (not noisy) absorption peaks in the IR.
(ii) The spectral searches were deemed correctly identified after they were searched in triplicate against the created library and the resulting "match" was number one in the match for at least two out of the three searches.
(iii) The quality score needed to be equal to or greater than 90 for the MS and equal to or greater than 0.95 for the IR.

Additional library evaluation occurred when the Special Testing and Research Laboratory analyzed 20 blind samples. The GC-IR analyst was instructed to select the best library spectra using the FIU generated vapor phase IR library only. A GC–MS analyst, independent of the GC-IR analyst, was instructed to select the best library spectra on mass spectral data only using in house available libraries (NIST20 library, the CaymanSpectralLibrary_092220 library) and the FIU generated mass

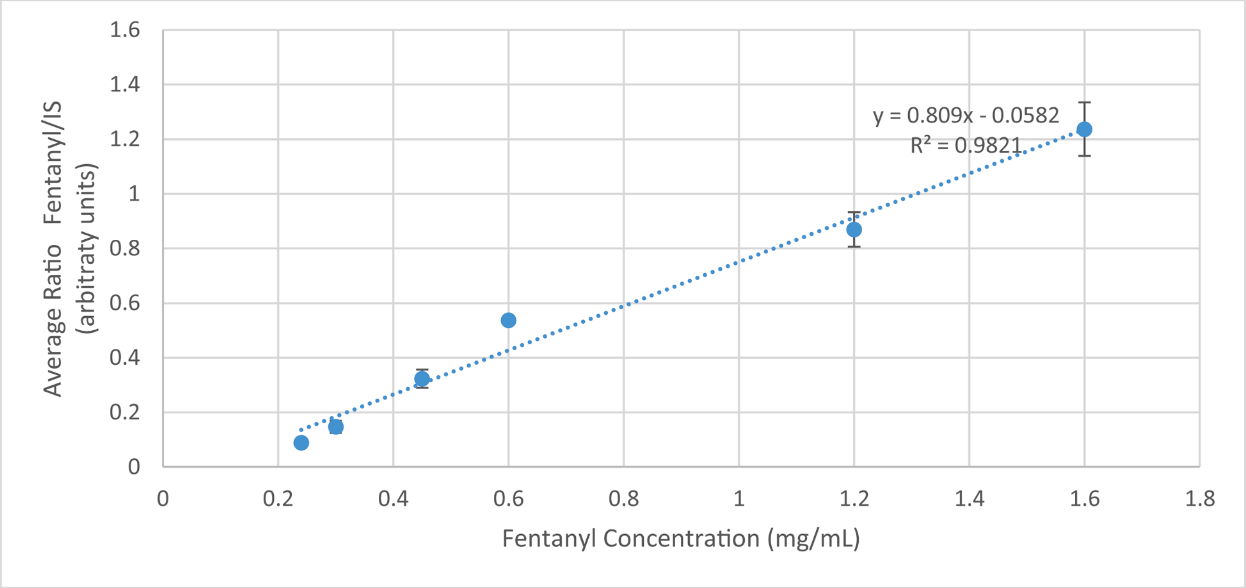

Fig. 7. Calibration curve for fentanyl showing concentration (mg/mL) vs. average integrated peak area (arbitrary units).

Table 8
Results of the blind study in the identification of 20 FRS using GC–MS.

Lab Number	Number of Compounds Correctly Identified
1	20/20
2	16/20
3	16/20

Table 9
Results of the blind study in the identification of 20 FRS using GC-IR.

Lab Number	Number of Compounds Correctly Identified
1	20/20
2	20/20
3	20/20

spectral library.

Determination of the limit of detection (LOD) and limit of quantitation (LOQ) of the GC- IR

A calibration curve was constructed at both FIU and the Special Testing and Research Laboratory laboratories using peak areas to determine the approximate LOD and LOQ of the GC-IR instrumental systems using the methods described above. Fentanyl was used for the LOD/LOQ studies since it is the parent compound to the fentanyl-related compounds of interest. Seven different concentrations of fentanyl ranging from 10 ppm to 1000 ppm were analyzed in triplicate with 1 μL injections using the same instrument programming as detailed in the section 2.2.

Summary of study

The main goals of this study were to describe the process of the creation of new gas-phase mass spectral and infrared libraries of FRS at FIU, the evaluation of the utility of these libraries to differentiate between positional isomers of FRS at FIU and to evaluate the usefulness of the libraries by two external laboratories (DEA STRL and Pinellas County Forensic Laboratory). The performance of the libraries was evaluated using a blind study of twenty (20) FRS that were distributed to the two external laboratories. The 20 different FRS isomers were selected on the basis of similarity of mass spectral fragmentation patterns and are shown in Table 3 below.

The analysts in the external laboratories were asked to analyze the 20 blind FRS samples and to search the FIU-generated MS and IR libraries to determine compound identity using the criteria defined in section 2.5.

Results and discussion

Gas chromatography mass spectrometry results (GC–MS)

Within the 20 blind samples, six different FRS were selected based on their isomeric nature including two sets of three different structural/positional isomers. Compounds 1–3 are N-(2-methylphenyl)-N-[1-(2-phenylethyl)-4-piperidinyl]-propanamide (commonly referred to as *ortho*-methylfentanyl), N-(3-methylphenyl)-N-[1-(2-phenylethyl)-4-piperidinyl]-propanamide (commonly referred to as *meta*-methylfentanyl), and N-(4-methylphenyl)-N-[1-(2-phenylethyl)-4-piperidinyl]-propanamide (commonly referred to as *para*-methylfentanyl); all have the same molecular weight of 350 amu and produce a similar fragmentation pattern containing the major ions of *m/z* 259, 160, and 203 (Fig. 1). Compounds 4–6 are N-(2-fluorophenyl)-N-[1-(2-phenylethyl)-4-piperidinyl]-propanamide (commonly known as *ortho*-fluorofentanyl), N-(3-fluorophenyl)-N-[1-(2-phenylethyl)-4-piperidinyl]-propanamide (commonly known as *meta*-fluorofentoanyl), and N-(4-fluorophenyl)-N-[1-(2-phenylethyl)-4-piperidinyl]-propanamide (commonly known as *para*-fluorofentanyl; all have the same molecular weight of 354 amu and produce a similar fragmentation pattern containing the major ions of *m/z* 263, 164, and 207 (Fig. 2). Note the lack of a molecular ion within all spectra, a characteristic of many of the FRS. One reason for the similarity of the fragmentation pattern is due to the cleavage of the α and β carbons of the ethyl heterocyclic linker that result in a base peak ion and a loss of the tropylium ion (C_7H_7, 91 amu) [6]. Positional isomers with indistinguishable mass spectra have been previously reported [10,14,19,20].

The blind samples of *meta*-fluorofentanyl, *para*-methylfentanyl, and *ortho*-methyl cyclopropyl fentanyl using the reported GC–MS systems operating with helium carrier gas were not correctly identified using the criteria described in section 2.5. The blind samples fluoroisobutyryl fentanyl and *ortho*-methyl cyclopropyl fentanyl using the reported GC–MS systems operating with hydrogen carrier gas were also not correctly identified using the criteria described in section 2.5. All other blind samples were correctly identified using the criteria defined in section 2.5.

Gas chromatography infrared detection results (GC-IR)

The same positional isomers from the GC–MS study were then subjected to GC-IR analysis. The vapor phase IR spectra of compounds 1–3 are shown in Fig. 3. Similarities are noticeable in the C—H stretch region of ~3000 cm^{-1}, as well as the ~1600 cm^{-1} C═O region. Differences were observed both in the 1400 cm^{-1} to 1350 cm^{-1} ring region and in the "fingerprint region" of 1300 cm^{-1} to 500 cm^{-1} allowing for differentiation.

The IR spectra of compounds 4–6 are shown in Fig. 4 with similar observations for differences in the C—H stretch region, the C═O region, the ring region, as well as the fingerprint region.

Evaluation of the FIU FRS MS library and FIU FRS IR library

FIU FRS MS library search results

MS spectra were searched with the Agilent MSD Chemstation software using both the National Institute of Standards and Technology (NIST) library and the FIU MS FRS library. Some of the triplicate spectra produced had minor ion differences; however, the major ions produced were similar with only slight abundance differences. Each of the 212 triplicate spectra was searched against both libraries, resulting in 212 compounds being searched three times for a total of 636 searches. The search utility uses the Probability Based Matching algorithm developed by Fred McLafferty where the significant ions and abundance spectra of unknown compounds are statistically compared to library spectra [19]. The algorithm verifies that the main peaks in the reference spectrum are also present in the unknown spectrum.

When the NIST MS library was searched, only 10 samples resulted in a primary match to the expected compound (4.7% of the searches). This was attributed to the fact that the NIST library does not contain many of the FRS contained in the FAS-Kit and Emergent Panels. When the new FIU FRS library was searched, 190 analyses resulted in a primary match to the expected compound (89.6% of the searchers). It should be noted that the FIU FRS library does contain all 212 FRS compounds. Misidentifications in the top five results were found ~90% of the time highlighting the similarity of MS spectra for positional isomers. For example, the results for a search of *para*-methylfentanyl yielded matches in the top five followed by its positional isomers *ortho*-methylfentanyl and *meta*-methylfentanyl also in the top five matches. A library search was also conducted for *meta*-fluorofentanyl also resulting in the top three matches, closely followed by its positional isomers *para*-fluorofentanyl and *ortho*-fluorofentanyl in the top five matches. Figs. 1 and 2 shows the similarities in the fragmentation patterns shared among the different positional isomers.

A "perfect match" was not found for ~10% of the positional isomers in the MS library given that the search algorithm utilizes the abundance of the significant ions in the reference spectrum in order to identify the unknown spectrum. Since these isomers have the same peaks due to similar fragmentation, it is difficult to distinguish between positional isomers, even when the reference spectra are included in the library.

FIU Fentanyl IR library search results

The FIU FRS IR library generated from the analysis of the compounds in the FAS Kit (using the Essential FTIR software) and the existing EUCLiD IR Library were also evaluated. There are 32 FRS common to both libraries. When using the combination of both FIU FRS IR and EUCLiD libraries, 100% of the searches resulted in best matches to the correct compound for each of the 212 standards. *Para*-methylfentanyl and *meta*-fluorofentanyl were subsequently searched using both libraries. *Ortho*-fluorofentanyl was not searched because it is common to both the FIU Fentanyl IR Library and the EUCLiD IR library. Additionally, the same positional isomers that were searched using the MS libraries were searched again with the IR libraries. To calculate the similarity scores, ASAP uses the correlation coefficient method. This calculation is similar to the calculation of correlation coefficient in statistics to obtain the R^2 value [20]. The × (wavenumber) and y (percent absorbance/transmittance) are collected at each point individually and calculated. The results are then compared with the reference and the unknown to determine a similarity score. It should be noted that the *para*-methylfentanyl was not present in the EUCLiD library so it was not present in the library results after the search (Table 4).

When *para*-methylfentanyl was searched against EUCLiD, the best match suggested *para*-methyl butyryl fentanyl (Table 5), as *para*-methylfentanyl was not contained within the EULCiD library. A comparison of the spectra of both *para*-methylfentanyl and *para*-methyl butyryl fentanyl is shown in Fig. 5, with data being presented in absorbance units since that is the only data output which EUCLiD offers. Differences are observed in the ~1500 cm^{-1} region. It should be noted that searches with the existing EUCLiD library only return 32/212 (15.1%) matches to the correct compound of the FRS searched. However, this can be explained by the lack of FRS compounds in the EUCLiD library resulting in other FRS being selected as the "best match" but with diminished confidence scores.

For *meta*-fluorofentanyl, the search results included output from both libraries (Table 6). The FIU Fentanyl IR library results included the top three results correctly as *meta*-fluorofentanyl with confidence scores of 1.00, 0.99, and 0.99 respectively. The EUCLiD library had the fourth match at a score of 0.99. It should be noted, however, that scores do not constitute an identification. The spectral comparison of the FIU Fentanyl IR library and the EUCLiD library for this compound is shown in Fig. 6.

In Fig. 6, the similarity in the IR spectra is evident, particularly in wavenumber region 550–1600 cm^{-1}. A summary of the comparison between the different search tools (MS and IR) is shown in Table 7 including a comparison of the search results between the GC–MS NIST library, the FIU MS library, the existing EUCLiD library and the FIU Fentanyl IR library. It should be noted that the EUCLiD user library will continue to grow, and these results are expected to improve as additional reference standards are added by users including the addition of the FIU Fentanyl IR library in the future.

Lods and LOQs of the ASAP IRD3 detector in the GC-IR studies

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated for the ASAP IRD3 detector using fentanyl as a model compound. Each of the three participating laboratories created calibration curves using peak areas with correlation coefficients (R^2) ranging from 0.9821 to 0.9978. Fig. 7 shows the linear range of the calibration curve between 0.20 mg/mL to 1.2 mg/mL for one laboratory. The calculated LODs from the three laboratories ranged from 0.10 mg/mL to 0.19 mg/mL and the LOQs ranged from 0.30 mg/mL to 0.57 mg/mL. In comparison, the LOD and LOQ for MS calculated at FIU was 0.009 mg/mL and 0.027 mg/mL, respectively, approximately one order of magnitude better detection with the MS.

Blind study results

A blind study was conducted to determine the utility of the MS and IR libraries to forensic laboratories for the identification of FRS. The rationale behind choosing the 20 for investigation is detailed in Table 3. The laboratories are identified by numbers 1 to 3 for this paper. The results of the study are detailed in Tables 8 and 9.

A correct identification criteria is defined in section 2.5. The quality of the match must be equal to or greater than 90 for MS spectral comparison and/or must be equal to or greater than 0.95 for the IR spectral comparison. For the MS studies, laboratory #1 performed better than the other two laboratories in the identification of the compounds in question by correctly identifying all 20 compounds. Lab # 1 reported also taking into account the retention time whereas Labs # 2 and 3 solely relied on the results of the MS library search. It should be noted that when the retention time was taken into consideration for those compounds that were misidentified by MS, correct identifications of the unknown compounds were reported by the other laboratories. The use of the IR spectral comparisons performed better in the identification of the unknowns with 100% correct for all three laboratories.

Conclusion

GC–MS alone may not be sufficient for the identification of FRS; however, GC-IR provides an *orthogonal* confirmatory technique that differentiates between positional isomers of FRS. New GC–MS and GC-IR libraries were created with authentic reference standards, with each reference standard acquired in triplicate, for 212 FRS and are now freely available [21] to the forensic science community. Seventy-nine, or approximately 37% of the 212 analogs, did not produce a molecular ion with electron impact ionization mass spectrometry and, as expected, positional isomers of FRS produce similar fragmentation in their mass spectra when using electron impact GC–MS. A library search of each of the 212 FRS using the NIST library produced 4.7% matches to the correct compound, which is not unexpected as most of the FRS were not included the NIST library. Only 89.6% of the searches resulted in the correct compound within the top five candidates when using the newly created GC–MS library. An existing GC-IR database (EUCLiD) that contained only 32 of the 212 FRS was also searched with 15.1% correct correlation. When the new GC-IR library containing all 212 FRS was searched, 100% identification was achieved. The three laboratories in this study searched 20 blind FRS samples using the FIU FRS Library with 100% correct match correlations.

The LODs and LOQs were also calculated for the GC-IR methods used by the three laboratories ranging in LODs between 0.10 mg/mL to 0.19 mg/mL and the LOQs from 0.30 mg/mL to 0.57 mg/mL. A larger scale interlaboratory study using a double-blind design and simulating real-world case scenarios is planned for the near future.

This study strongly supports the use of GC-IR in the analysis of unknown compounds, especially FRS, but also for all other highly manipulated drug structures with abundant isomer configurations, including cathinones and synthetic cannabinoids. The combination of GC–MS and GC-IR results in the highest confidence of isomer identification of NPS compounds.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.forc.2022.100425.

References

[1] Overdose Death Rates, *National Institute on Drug Abuse*, 2019.

[2] National Institute on Drug Abuse (NIDA), DrugFacts: Fentanyl, no. February 2019. pp. 1–6, 2019.

[3] J. Knestrick, Impact of the opioid crisis, J. Nurse Pract. 13 (9) (2017) A18.

[4] U.S. Department of Justice, Special NFLIS Drug Maps Release: Tracking Fentanyl and Fentanyl-Related Compounds Reported in NFLIS Drug by State, 2018–2019, no. December, 2020.

[5] U.S. Department of Justice, NFLIS-Drug 2020 Mid-Year Report. 2020.

[6] H. Grenke Pierzynski, L. Neubauer, C. Choi, R. Franckowski, S.D. Augustin, D. M. Iula, Tips for interpreting GC-MS fragmentation of unknown substituted fentanyls, Cayman Curr. 28 (2017).

[7] National Institute of Standards and Technology (NIST), Forensic Database Chemistry & Toxicology Table, 2017. [Online]. Available: https://www.nist.gov/oles/forensic-database-chemistry-toxicology-table. [Accessed: 22-Mar-2021].

[8] A. Urbas, T. Schoenberger, C. Corbett, K. Lippa, F. Rudolphi, W. Robien, NPS Data Hub: a web-based community driven analytical data repository for new psychoactive substances, Forensic Chem. 9 (2018) 76–81.

[9] D.L. Pavia, G.M. Lampman, G.S. Kriz, J.R. Vyvyan, Introduction to Spectroscopy, 5th ed., Cengage Learning, 2015.

[10] A. Winokur, L. Kaufman, J. Almirall, Differentiation and identification of fentanyl analogues using GC-IRD, Forensic Chem. 20 (March) (2020).

[11] H. Ohta, S. Suzuki, K. Ogasawara, Studies on fentanyl and related compounds IV. Chromatographic and spectrometric discrimination of fentanyl and its derivatives, J. Anal. Toxicol. 23 (4) (1999) 280–285.

[12] T. Belal, T. Awad, J. DeRuiter, C.R. Clark, GC-IRD methods for the identification of isomeric ethoxyphenethylamines and methoxymethcathinones, Forensic Sci. Int. 184 (1–3) (2009) 54–63.

[13] K.M. Abdel-Hay, T. Awad, J. DeRuiter, C.R. Clark, Differentiation of methylenedioxybenzylpiperazines (MDBPs) and methoxymethylbenzylpiperazines (MMBPs) By GC-IRD and GC-MS, Forensic Sci. Int. 210 (1–3) (2011) 122–128.

[14] T. Awad, T. Belal, J. DeRuiter, K. Kramer, C.R. Clark, Comparison of GC-MS and GC-IRD methods for the differentiation of methamphetamine and regioisomeric substances, Forensic Sci. Int. 185 (1-3) (2009) 67–77.

[15] T.A. Sasaki, C.L. Wilkins, Gas chromatography with Fourier transform infrared and mass spectral detection, J. Chromatogr. A 842 (1–2) (1999) 341–349.

[16] S. Stein, Mass spectral reference libraries: An ever-expanding resource for chemical identification, Anal. Chem. 84 (17) (2012) 7274–7282.

[17] E. Sisco, A. Burns, A.S. Moorthy, Development and evaluation of a synthetic opioid targeted gas chromatography mass spectrometry (GC-MS) method, J. Forensic Sci. 66 (6) (2021) 2369–2380.

[18] ASAP Analytical EUCLiD Database. Covington, KY, 2021.

[19] Agilent Technologies, GC/MSD Libraries for the MSD ChemStation MSD ChemStation General-Purpose Libraries. 2000.

[20] J. Miller, J. Miller, R. Miller, *Statistics and Chemometrics for Analytical Chemistry*. 2018.

[21] J. Almirall, K. Ferguson. FIU Infrared Fentanyl Library, FIU Research Data Portal, 2022, https://doi.org/10.34703/gzx1-9v95/A68TVK.