Application Note

Clinical Research

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A Comprehensive Approach to Targeted and Untargeted Screening Methodology for Emerging Synthetic Fentanyl Analogues

High Resolution Accurate Mass Spectrometry

Authors

Dr. Julie Cichelli,
Carrie J. Adler, and
Dr. Jim Lau
Agilent Technologies, Inc.

Abstract

In recent years, laboratories have been struggling to keep up with new synthetic fentanyl analogues being introduced faster than they can adapt. As new and more potent opioids are appearing in society every day, it is imperative to have analytical strategies to analyze for these compounds in both a targeted and untargeted manner. Using the Agilent Poroshell 120 Phenyl Hexyl column for isobaric separation, and the Agilent 6546 LC/Q-TOF MS for acquisition and data analysis, a workflow for approximately 150 new synthetic fentanyl analogues and 4-ANPP was developed.

Introduction

Opioid abuse is an ever-expanding crisis in the United States and beyond. It is estimated that 100 deaths from overdoses occur every day in the U.S.[1] These deaths are most often not being caused by historically abused opiates, such as heroin, but by more potent opioids such as fentanyl and its many analogues, which are cut into the classic opiates. Due to the ever-changing synthetic fentanyl analogue portfolio, clinical research laboratories are struggling to keep up with the changes. A high-resolution accurate mass LC/Q-TOF acquisition and data analysis workflow for a list of approximately 150 new synthetic fentanyl analogues and 4-ANPP, the precursor scheduled by the DEA, will be described. Targeted and untargeted approaches for identification will be discussed to keep up with emerging unknown compounds and capitalize on retrospective data analysis.

Another challenge presented by many of these analogues is the number of isobaric compounds within this compound group. Example structures are shown in Figure 1. The Poroshell Phenyl Hexyl column was paramount in the separation of these isobaric compounds, along with the use of the 6546 LC/Q-TOF. This instrument combines excellent performance in every analytical facet, including an extended dynamic range, stable mass accuracy over long periods of time, higher resolution, and performance that is unaffected by acquisition rate.

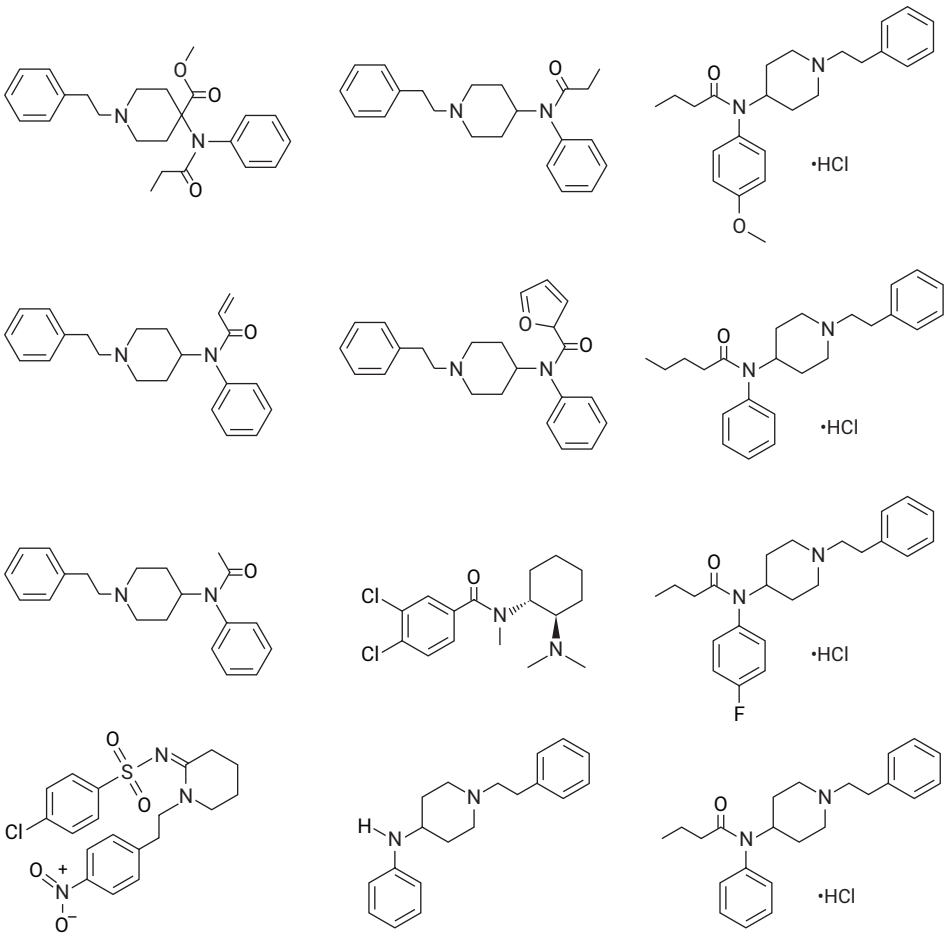


Figure 1. Example structures of fentanyl analogues.

Experimental

LC Configuration and parameters

Configuration	
Agilent 1290 Infinity II Binary pumps (G7120A)	
Agilent 1290 Infinity II Multisampler (G7167A)	
Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)	
Needle Wash	5 seconds in wash port; 100% MeOH
Autosampler Temperature	6 °C
Injection Volume	2 µL (injector loop 20 µL)
Draw Speed	100 µL/min
Eject Speed	100 µL/min
Analytical Column	Agilent Poroshell 120 Phenyl Hexyl, 2.1 × 100 mm, 2.7 µm, LC column (p/n 695775-912)
Column Temperature	55 °C
Mobile Phase A	H_2O + 5 mM Ammonium formate + 0.01% formic acid
Mobile Phase B	Methanol + 0.01% formic acid
Flow Rate	0.35 mL/min
Gradient	Eluent pump Time (min) %B 0.00 30 2.00 35 3.00 37 4.50 40 5.00 43 6.00 47 8.00 47 12.00 95 14.00 98
Stop Time	14.00 minutes
Post Time	1.50 minutes

LC/Q-TOF mass spectrometer configuration and parameters

Instrument and Source Conditions		
Agilent 6546 Quadrupole Time of Flight Mass Spectrometer		
Ionization Mode	AJS ESI, Positive	
Drying Gas Temperature	275 °C	
Drying Gas Flow	12 L/min	
Nebulizer Pressure	35 psi	
Sheath Gas Temperature	350 °C	
Sheath Gas Flow	11 L/min	
Capillary Voltage	3500 V	
Nozzle Voltage	0 V	
MS Parameters		
Acquisition Mode	**Auto MS/MS**	**MS**
Isolation Width	Medium (4 amu)	N/A
Fragmentor Voltage	135 V	135 V
MS Scan Rate	6 spectra/sec	3 spectra/sec
MS/MS Scan Rate	3 spectra/sec	N/A
Fixed Collision Energies	10, 20, 40 V	N/A

Chemicals and reagents

Optima grade methanol was from Fisher Scientific (Hampton, NH), and ammonium formate and formic acid were purchased from MilliporeSigma (Saint Louis, MO). Clinical Laboratory Reagent Water (CLRW) was from a MilliQ Advantage A10 system manufactured by MilliporeSigma. A Fentanyl Analog Screening (FAS) Kit was purchased from Caymen Chemical (Ann Arbor, MI). A reference mass solution and a low-concentration tuning mix were from Agilent Technologies (Santa Clara, CA).

Standards

Standards were spiked into starting mobile phase conditions (70% 5 mM ammonium formate/0.01% formic acid in water:30% 0.01% formic acid in methanol) at a concentration of 200 ng/mL. Two microliters were injected into the LC/Q-TOF system.

Data analysis

Data acquisition was performed using Agilent MassHunter Q-TOF Acquisition Software (version 10.1). Data were analyzed using MassHunter Qualitative Analysis Software (version 10.1). Two separate peak picking algorithms were used to approach the data processing, including Molecular Feature Extractor (MFE) for an untargeted approach and Find by Formula for a targeted approach.

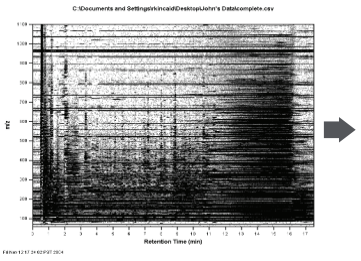
Table 1. Fentanyl analogues.

No.	Compound
1	(±)-*cis*-3-Methyl butyrylfentanyl (hydrochloride)
2	(±)-*cis*-3-Methyl fentanyl (hydrochloride)
3	(±)-*cis*-3-Methyl-thiofentanyl (hydrochloride)
4	(±)-*trans*-3-Methyl fentanyl (hydrochloride)
5	(±)-*trans*-3-Methyl thiofentanyl (hydrochloride)
6	2,2,3,3-Tetramethyl-cyclopropyl fentanyl (hydrochloride)
7	2,3-*seco*-Fentanyl (hydrochloride)
8	2-Fluoro MT-45 (hydrochloride)
9	2'-Fluoro *ortho*-fluorofentanyl (hydrochloride)
10	3'-Methyl fentanyl (hydrochloride)
11	4'-Fluorofentanyl
12	4'-Fluoro-isobutyryl fentanyl (FIBF)
13	4-ANPP
14	4'-Fluoro, *para*-fluoro (±)-*trans*-3-methyl fentanyl (hydrochloride)
15	4'-Fluorofentanyl (hydrochloride)
16	4'-Methyl acetylfentanyl (hydrochloride)
17	4'-Methyl fentanyl (hydrochloride)
18	4-Phenyl fentanyl (hydrochloride)
19	Acetyl fentanyl (hydrochloride)
20	Acetyl norfentanyl (hydrochloride)
21	Acrylfentanyl (hydrochloride)
22	AH 7921
23	Alfentanil (hydrochloride)
24	Benzodioxole fentanyl
25	Benzyl acrylfentanyl (hydrochloride)
26	Benzyl carfentanil (hydrochloride)
27	Benzyl fentanyl (hydrochloride)
28	Butyryl fentanyl (hydrochloride)
29	Butyryl norfentanyl (hydrochloride)
30	Carfentanil
31	Crotonyl fentanyl
32	Cyclobutyl fentanyl (hydrochloride)
33	Cyclohexyl fentanyl (hydrochloride)
34	Cyclopentenyl fentanyl (hydrochloride)
35	Cyclopentyl fentanyl (hydrochloride)
36	Cyclopropyl fentanyl (hydrochloride)
37	Ethoxyacetyl fentanyl (hydrochloride)
38	Fentanyl
39	Fentanyl carbamate
40	Fentanyl methyl carbamate
41	Furanyl fentanyl (hydrochloride)
42	Furanyl fentanyl 3-furancarboxamide isomer (hydrochloride)
43	Furanyl norfentanyl (hydrochloride)
44	Furanylethyl fentanyl (hydrochloride)
45	Heptanoyl fentanyl (hydrochloride)
46	Hexanoyl fentanyl (hydrochloride)
47	Isobutyryl fentanyl (hydrochloride)
48	Isopropyl U-47700
49	Isovaleryl fentanyl (hydrochloride)
50	*meta*-Fluoro methoxyacetyl fentanyl (hydrochloride)
51	*meta*-Fluorobutyryl fentanyl (hydrochloride)
52	*meta*-Fluorofentanyl (hydrochloride)
53	*meta*-Fluoroisobutyryl fentanyl (hydrochloride)
54	*meta*-Methyl cyclopropyl fentanyl (hydrochloride)
55	*meta*-Methyl furanyl fentanyl (hydrochloride)
56	*meta*-Methyl methoxyacetyl fentanyl (hydrochloride)
57	*meta*-Methylfentanyl (hydrochloride)
58	Methacrylfentanyl
59	Methoxyacetyl fentanyl (hydrochloride)
60	MT-45 (hydrochloride)
61	N-(3-Ethylindole) norfentanyl
62	N,N-Dimethylamido-despropionyl fentanyl
63	N-benzyl furanyl norfentanyl (hydrochloride)
64	N-Desmethyl U-47700
65	N-Methyl cyclopropyl norfentanyl (hydrochloride)
66	N-Methyl norcarfentanil (hydrochloride)
67	Norcarfentanil (hydrochloride)
68	Norfentanyl
69	Norsufentanil
70	Ocfentanil (hydrochloride)
71	*ortho*-Fluoro acrylfentanyl (hydrochloride)
72	*ortho*-Fluoro furanyl fentanyl (hydrochloride)
73	*ortho*-Fluorobutyryl fentanyl (hydrochloride)
74	*ortho*-Fluorofentanyl (hydrochloride)
75	*ortho*-Fluoroisobutyryl fentanyl (hydrochloride)
76	*ortho*-Isopropyl furanyl fentanyl
77	*ortho*-Methoxy butyryl fentanyl (hydrochloride)
78	*ortho*-Methoxy furanyl fentanyl
79	*ortho*-Methyl acetylfentanyl (hydrochloride)
80	*ortho*-Methyl acrylfentanyl (hydrochloride)
81	*ortho*-Methyl cyclopropyl fentanyl (hydrochloride)
82	*ortho*-Methyl furanyl fentanyl
83	*ortho*-Methyl methoxyacetyl fentanyl (hydrochloride)
84	*ortho*-Methyl phenyl fentanyl (hydrochloride)
85	*ortho*-Methylfentanyl (hydrochloride)
86	*para*-Chloro acrylfentanyl (hydrochloride)
87	*para*-Chloro cyclobutyl fentanyl (hydrochloride)
88	*para*-Chloro cyclopentyl fentanyl (hydrochloride)
89	*para*-Chloro cyclopropyl fentanyl (hydrochloride)
90	*para*-Chloro furanyl fentanyl
91	*para*-Chloro methoxyacetyl fentanyl (hydrochloride)
92	*para*-Chloro valeryl fentanyl (hydrochloride)
93	*para*-Chlorobutyryl fentanyl (hydrochloride)
94	*para*-Chlorofentanyl (hydrochloride)
95	*para*-Chloroisobutyryl fentanyl (hydrochloride)
96	*para*-Fluoro acrylfentanyl
97	*para*-Fluoro crotonyl fentanyl
98	*para*-Fluoro cyclopentyl fentanyl (hydrochloride)
99	*para*-Fluoro cyclopropyl fentanyl (hydrochloride)
100	*para*-Fluoro furanyl fentanyl (hydrochloride)
101	*para*-Fluoro furanyl fentanyl 3-furancarboxamide (hydrochloride)
102	*para*-Fluoro methoxyacetyl fentanyl (hydrochloride)
103	*para*-Fluoro tetrahydrofuran fentanyl (hydrochloride)
104	*para*-Fluoro valeryl fentanyl (hydrochloride)
105	*para*-Fluoroacetyl fentanyl (hydrochloride)
106	*para*-Fluorobutyryl fentanyl (hydrochloride)
107	*para*-Fluorofentanyl (hydrochloride)
108	*para*-Methoxy acetylfentanyl (hydrochloride)
109	*para*-Methoxy acrylfentanyl (hydrochloride)
110	*para*-Methoxy butyryl fentanyl (hydrochloride)
111	*para*-Methoxy furanyl fentanyl (hydrochloride)
112	*para*-Methoxy methoxyacetyl fentanyl (hydrochloride)
113	*para*-Methoxy tetrahydrofuran fentanyl (hydrochloride)
114	*para*-Methoxy valeryl fentanyl (hydrochloride)
115	*para*-Methoxyfentanyl (hydrochloride)
116	*para*-Methyl acetyl fentanyl (hydrochloride)
117	*para*-Methyl acrylfentanyl (hydrochloride)
118	*para*-Methyl butyryl fentanyl (hydrochloride)
119	*para*-Methyl cyclopentyl fentanyl (hydrochloride)
120	*para*-Methyl cyclopropyl fentanyl (hydrochloride)
121	*para*-Methyl furanyl fentanyl (hydrochloride)
122	*para*-Methyl isobutyryl fentanyl (hydrochloride)
123	*para*-Methyl methoxyacetyl fentanyl (hydrochloride)
124	*para*-Methyl tetrahydrofuran fentanyl (hydrochloride)
125	*para*-Methylfentanyl (hydrochloride)
126	Phenoxyacetyl fentanyl (hydrochloride)
127	Phenylfentanyl (hydrochloride)
128	Phenylacetyl fentanyl (hydrochloride)
129	Pivaloyl fentanyl (hydrochloride)
130	Remifentanil (hydrochloride)

No.	Compound
131	Senecioylfentanyl
132	Sufentanil
133	Tetrahydrofuran fentanyl (hydrochloride)
134	Tetrahydrofuran fentanyl 3-tetrahydrofurancarboxamide (hydrochloride)
135	Tetrahydrothiophene fentanyl
136	Thienyl fentanyl (hydrochloride)
137	Thiofentanyl (hydrochloride)
138	Thiophene fentanyl (hydrochloride)
139	U-47700
140	U-48800 (hydrochloride)
141	U-49900
142	Valeryl fentanyl (hydrochloride)
143	W-18
144	α'-Methoxy fentanyl (hydrochloride)
145	α-Methyl acetyl fentanyl (hydrochloride)
146	α'-Methyl butyryl fentanyl (hydrochloride)
147	α-Methyl butyryl fentanyl (hydrochloride)
148	α-Methyl fentanyl (hydrochloride)
149	α-Methyl thiofentanyl (hydrochloride)
150	β-Hydroxy fentanyl (hydrochloride)
151	β-Hydroxythioacetylfentanyl
152	β-Hydroxythiofentanyl (hydrochloride)
153	β-Methyl acetyl fentanyl (hydrochloride)
154	β-Methyl fentanyl (hydrochloride)
155	β'-Phenylfentanyl

* = QC analyte (#) = isobaric compounds

MFE is capable of finding and associating mass signals in very complex data files to all the different compounds present in a sample. The algorithm maps all mass signals in the three-dimensional space of time, mass, and intensity. As shown in the first illustration of Figure 2, this could be 1,000 to 100,000 signals in an LC/MS run with high chromatographic resolution and the high resolution of a TOF instrument. The algorithm then removes areas that only contain noise and leaves only those mass signals that show a peak-like intensity change during the run, as shown in the middle illustration of Figure 2. In the next step, the algorithm identifies all mass signals with a common retention time, as well as the chemical relation to each other as a "molecular feature", which represents a compound. Related ions, which are user-configured, can be isotopes, adducts, and dimers or higher charge states. Finally, the algorithm creates extracted compound chromatograms and compound mass spectra from all the ions associated to each molecular feature or compound and creates a molecular feature/compound list. This algorithm can be used in both MS and MS/MS experiments. When analyzing MS/MS data, MFE is used in conjunction with scrutinizing neutral loss and reporter ions, to help identify and align analytes with common features.[2]



Compound list or Molecular feature list

RT	*m/z*	Abund.
–	–	–
–	–	–
2.11	195.1745	21,000
2.11	257.2566	33,550
2.34	224.2134	11,784
–	–	–
–	–	–

Figure 2. The Agilent Molecular Feature Extractor algorithm process.

Find by Formula (FBF) is an algorithm used to analyze MS data files and determine if they contain any evidence of the presence of specified compounds. This is a targeted approach as the algorithm only searches for specific formulas that are user-defined by manual entry or by use of a personal compound database and library (PCDL), Figure 3. There are many parameters that a user can set to achieve the best data. The parameters used in this study are shown in Figure 4. This approach of only showing results for specific compounds can have its advantages and disadvantages. For MS/MS data files, the Find by Auto MS/MS peak picking algorithm was used, in conjunction with FBF.

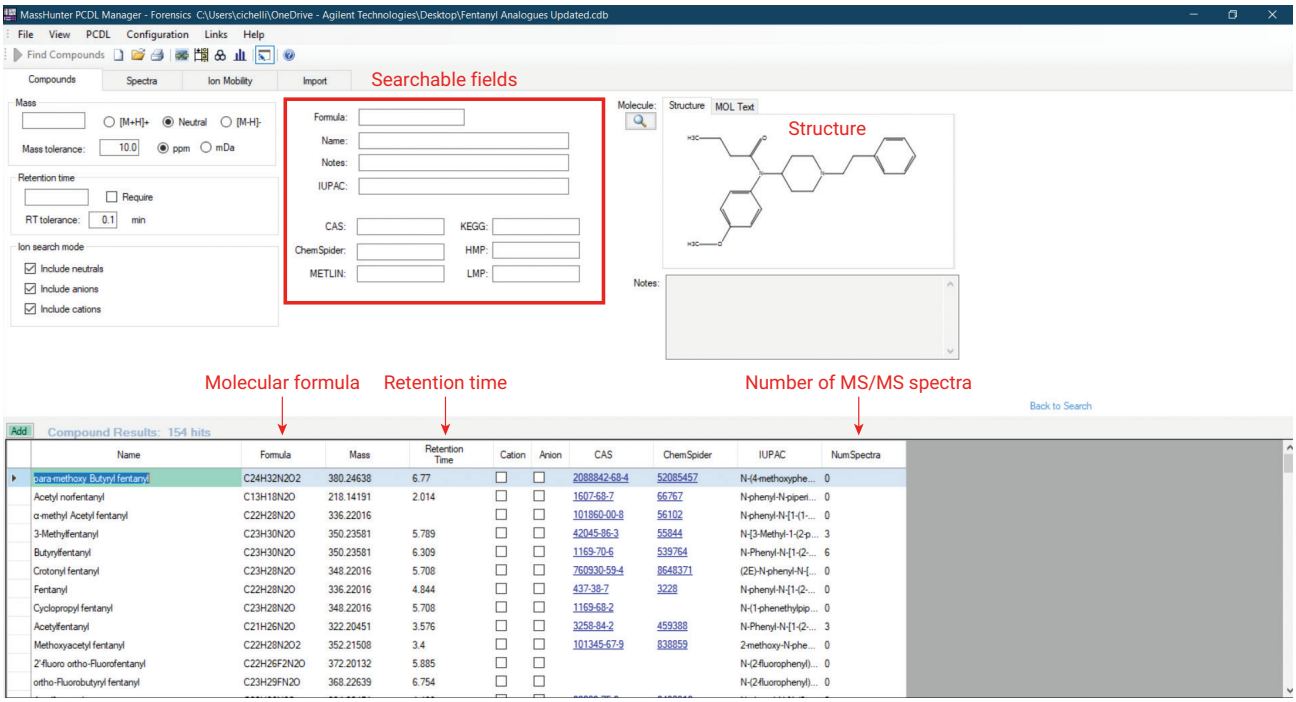


Figure 3. Comprehensive Fentanyl Analogues PCDL.

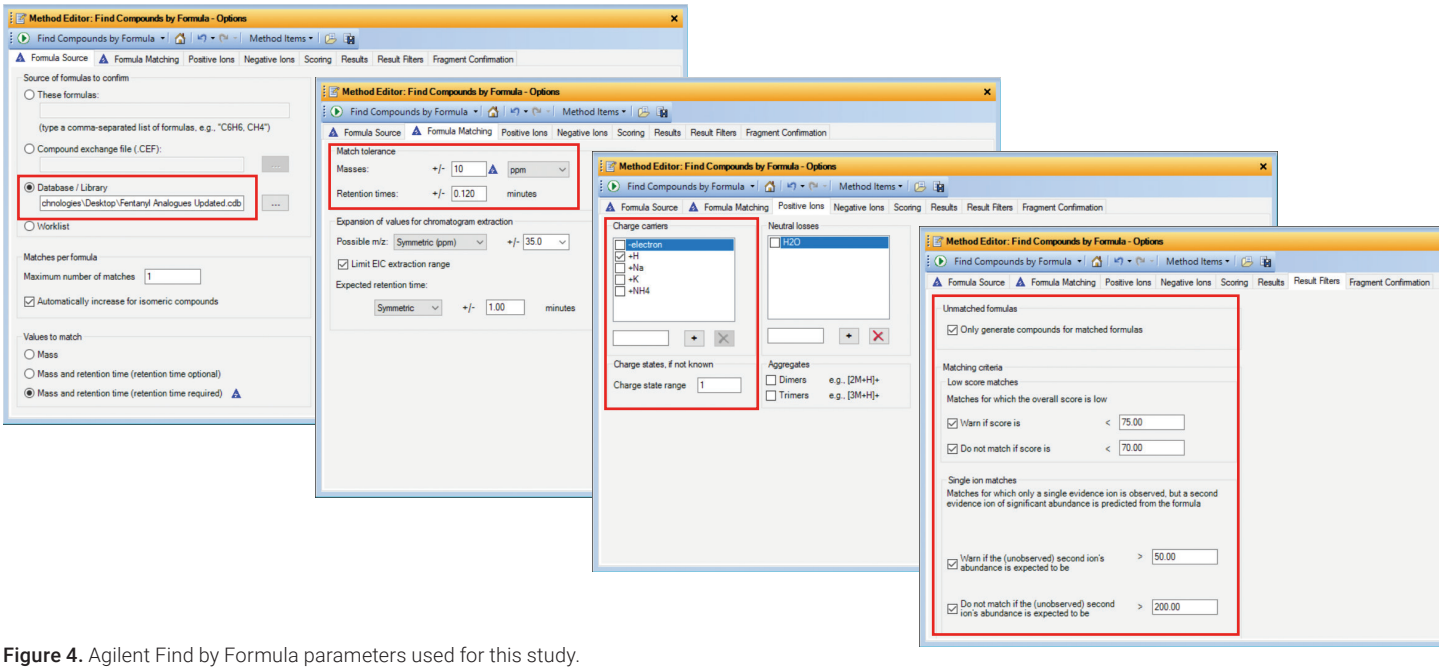


Figure 4. Agilent Find by Formula parameters used for this study.

Results and discussion

Chromatography

Each compound standard was run individually in MS mode, and its formula and retention time were added to the Fentanyl Analogue PCDL. Now, the database generated can be used to screen for all 150 analogues in a single run. Figure 5 shows overlaid extracted ion chromatographs (EIC) of the identified compounds. By executing the FBF algorithm using the parameters shown in Figure 4, accurate detection and identification of each fentanyl analogue in the sample can be achieved.

Targeted analysis

Given the large number of compounds in this study, as shown in Figure 5, along with the fact that approximately 90% of the compounds have isobaric relationships with each other, chromatographic resolution and retention time are highly important to accurately identifying the correct compound. Figure 6 shows an example of the isobaric complexity in this assay; these compounds have the same elemental composition, meaning they have the same exact mass. While the Q-TOF instrument is very powerful with resolution and sensitivity, it cannot distinguish between isobaric compounds. Therefore, both the accurate mass of the precursor and product ions, as well as retention time are required to accurately identify these compounds.

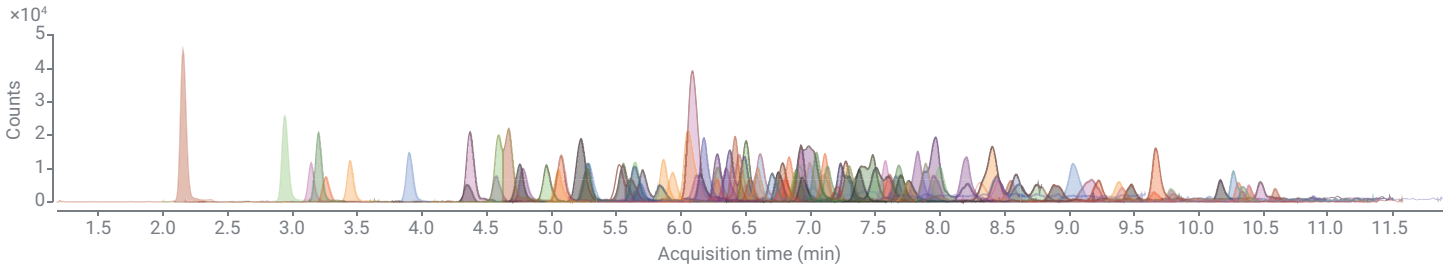


Figure 5. Overlaid EIC of the fentanyl analogues identified.

Name	Formula	Mass	Retention Time	Cation	Anion	CAS	ChemSpider	IUPAC	NumSpectra
α-methyl Fentanyl	C23H30N2O	350.23581	6.596	☐	☐	79704-88-4	56081	N-[1-(1-methyl-2-p...	6
para-Methylfentanyl	C23H30N2O	350.23581	7.483	☐	☐			N-(4-methylphenyl...	6
ortho-Methylfentanyl	C23H30N2O	350.23581	6.973	☐	☐			N-(2-methylphenyl...	6
4'-methyl Fentanyl	C23H30N2O	350.23581	7.42	☐	☐			N-[1-[2-(4-methylp...	6
(±)-cis-3-methyl Fentanyl	C23H30N2O	350.23581	7.003	☐	☐	65814-07-5	23134942	N-[(3S,4S)-3-meth...	6
β-methyl Fentanyl	C23H30N2O	350.23581	7.012	☐	☐	79146-56-8	4931228	N-phenyl-N-[1-(2-...	6
meta-Methylfentanyl	C23H30N2O	350.23581	7.441	☐	☐			N-(3-methylphenyl...	6
Isobutyryl fentanyl	C23H30N2O	350.23581	7.045	☐	☐	119618-70-1	10551382	2-methyl-N-phenyl...	9
Butyrylfentanyl	C23H30N2O	350.23581	7.297	☐	☐	1169-70-6	539764	N-Phenyl-N-[1-(2-...	12
3-Methylfentanyl	C23H30N2O	350.23581	5.789	☐	☐	42045-86-3	55844	N-[3-Methyl-1-(2-p...	9
(±)-trans-3-methyl Fentanyl	C23H30N2O	350.23581	6.79	☐	☐				6

Figure 6. PCDL example of isobaric complexity of the fentanyl analogues.

Figure 7 shows a typical MS data result with good chromatography with retention times, which is a crucial figure of merit, along with accurate mass and isotopic fidelity for confirming a compound.

Once all fentanyl analogue compounds have been analyzed in MS mode and individual retention times have been added to the PCDL, identification by the targeted Find by Formula approach is achieved. Next, acquired MS/MS data are used to create spectral libraries for an extra layer of confirmation and identification, as well as structural elucidation of unknowns using various data analysis techniques. Each fentanyl analogue was acquired in the targeted MS/MS mode using the MS parameters specified previously to obtain MS/MS spectra at three collision energies (10, 20, and 40 V). These acquired product ion spectra are then added to the fentanyl analogue PCDL. Once added, the Find by Auto MS/MS algorithm, in conjunction with FBF, can be used to mine MS/MS data against that library. The combination of retention time, accurate mass, isotopic fidelity, and spectral matching gives the utmost confidence in your targeted identification, as shown in Figure 8.

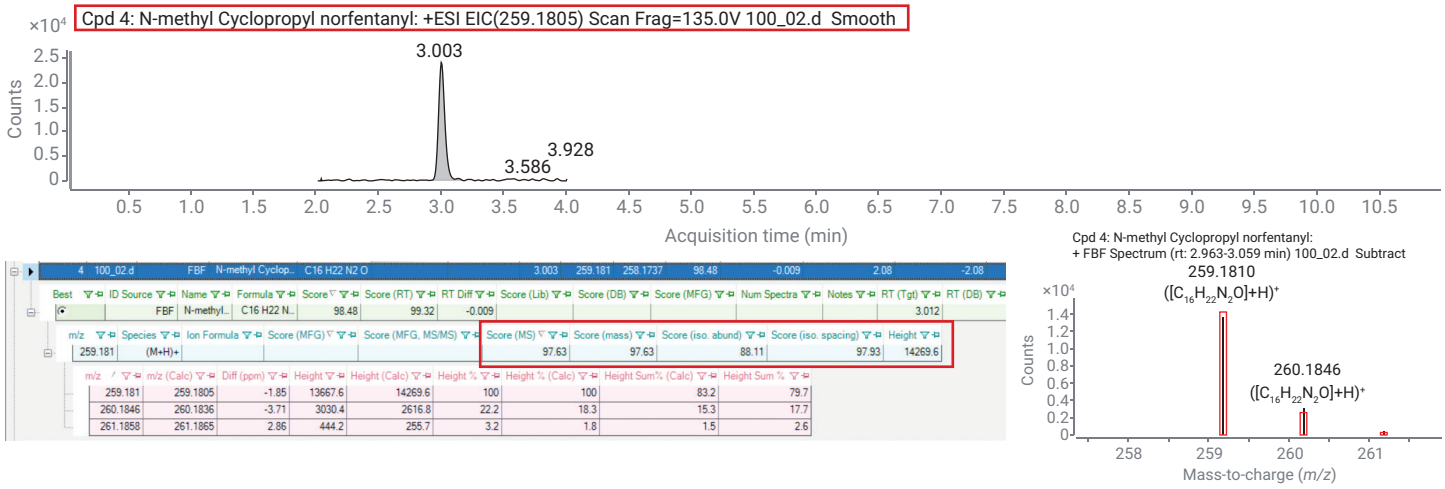


Figure 7. Typical MS result.

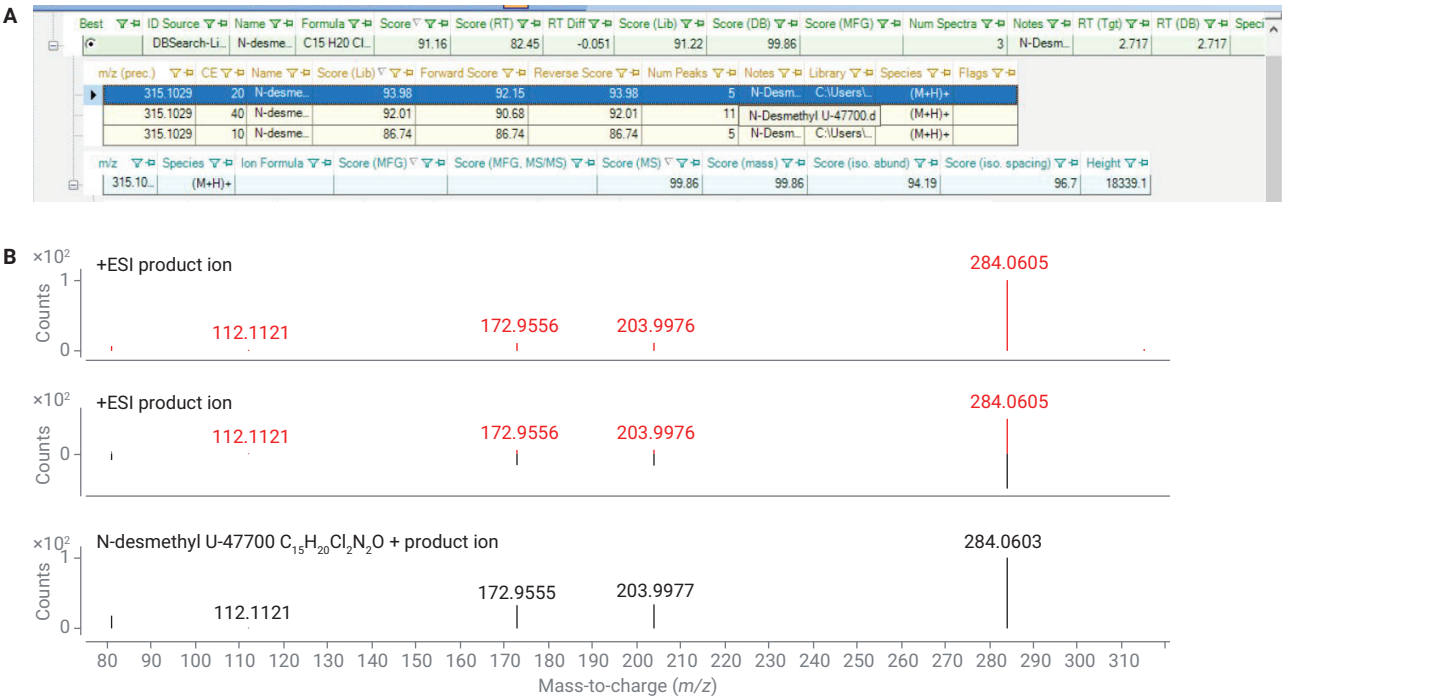


Figure 8. Combined search results for enhanced compound identification.

Untargeted analysis

Due to the ever-changing landscape of fentanyl analogues, untargeted analysis is of high interest in screening samples for these newly emerging compounds. A combined MS/MS approach was used with Agilent Mass Hunter Qualitative Analysis software to identify unknowns using Neutral Loss scan, Reporter Ions or common fragment ions, Molecular Structure Correlation of Product ions, and Variable Mass Defect.

Due to the similar structures and backbone of these fentanyl analogues, as they travel through the Q-TOF and experience fragmentation, they will often follow a similar pattern, which can aid in identification of related compounds. For example, in Figure 9, examples of reporter ions and neutral loss are shown. A fentanyl precursor ion of 337.2278 is shown, followed by the MS/MS spectra containing a constant neutral loss at 149.0800 and common fragment ions of 105 and 188 (fentanyl backbone). These reporter ions of 105.0701 and 188.1433, as well as the neutral loss of 149, are observed consistently based on the structure of these fentanyl analogues and can be used to find compounds that may be derived from, or chemically related to fentanyl, to further investigate and identify unknowns.

This pattern of neutral loss and reporter ions can be observed over and over, helping with identification. When dealing with compounds that are more similar to carfentanyl (methocarbonyl fentanyl), the same neutral loss of 149.0800 will be observed, however the backbone will be different at 246 with the addition of the methocarbonyl group. In this case, the similarities in neutral loss will help to align compounds similar in nature to determine possible unknowns.

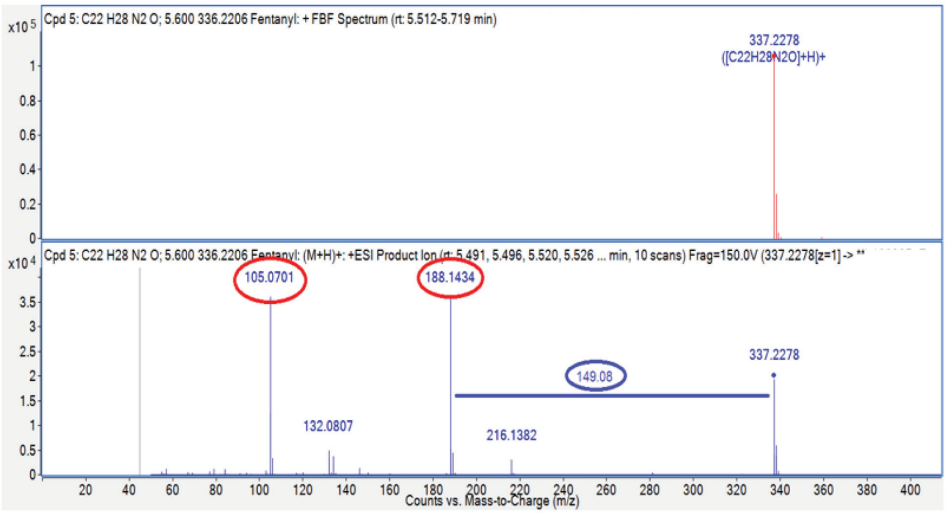


Figure 9. Fentanyl precursor ion spectra and MS/MS spectra, showing a neutral loss of 149.0800, and reporter ions of 105.0701 and 188.1433.

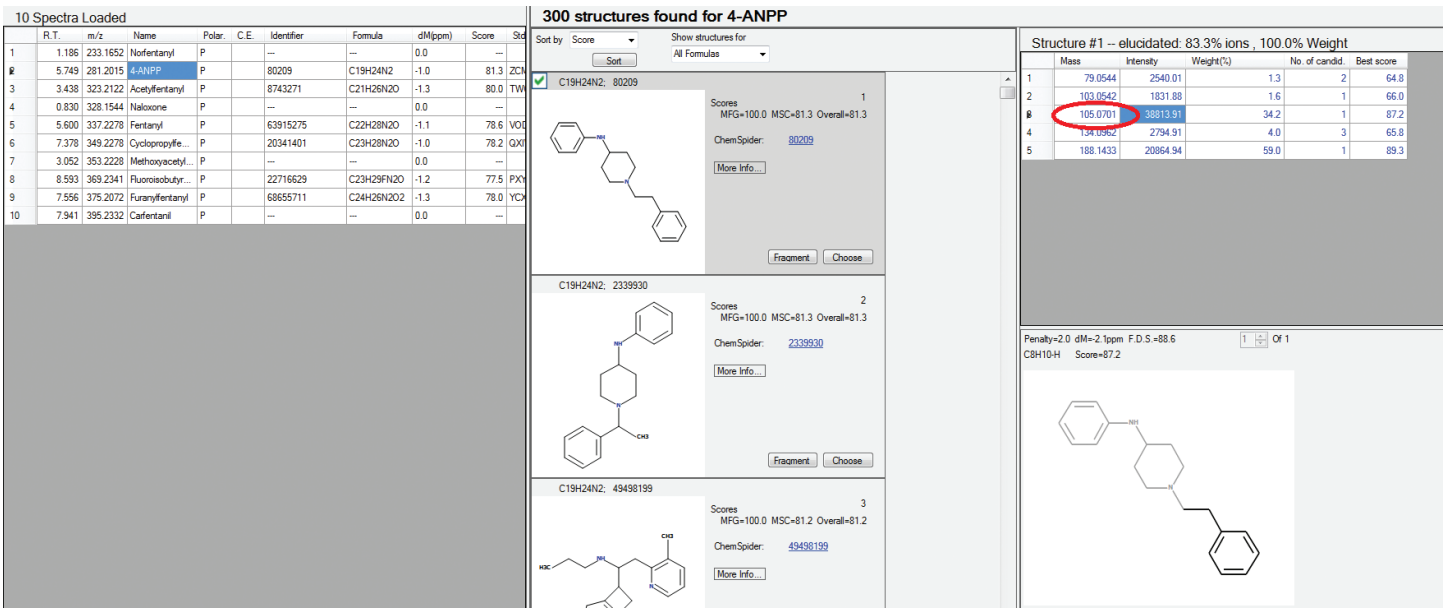


Figure 10. Agilent Molecular Structure Correlator Software.

Once you have identified common reporter ions, the Agilent Molecular Structure Correlator (MSC) software can determine the similarity of the ion to the fentanyl analogues as well as predict product ions based on precursor structure, and prediction of how compounds will fragment based on a precursor structure. MSC software can also be used to determine neutral loss fragments.

The last technique used to determine unknowns, which uses the power of high-resolution accurate mass analysis, is finding similar compounds by mass defect. Mass defect is the difference between the mass of an isotope and its nominal mass. This is accomplished using a tool within the Qualitative Analysis software in conjunction with the MFE algorithm. Mass Defect can be used in both variable or constant mass defect mode.

Variable Mass Defect (VMD) is used to hone in on compounds that are related fentanyl type analytes, without unrelated chemical noise that can be captured using Constant Mass Defect (CMD). A linear equation is used, as shown by the red line in Figure 11. The linear regression is calculated by monitoring the change in mass defect over the change in nominal mass, narrowing the window based on the trend line, and thus eliminating unwanted chemical noise that would have been included with CMD filtering. In Figure 11, the black box of plotted fentanyl analogue compounds detected are those compounds that contain Cl or F, dropping the expected mass defect out of the calculated regression. In this case, a second pass would be necessary to address the halogenated compounds.

When using CMD, it is possible to introduce too much chemical noise, as shown in Figure 12. The results could include compounds that are not related to any sort of fentanyl analogue, but are still able to pass through the filter.

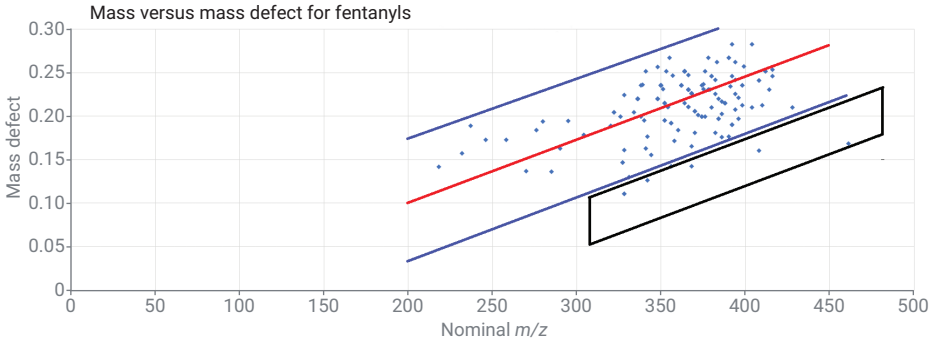


Figure 11. Variable mass defect.

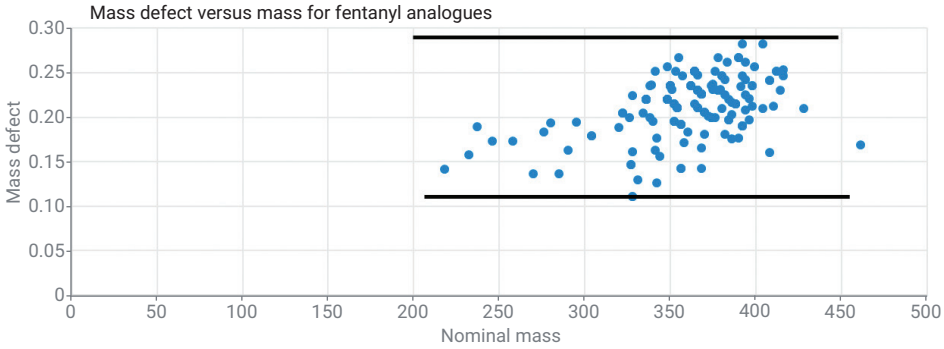


Figure 12. Constant mass defect.

Conclusion

In this new landscape of opioid abuse and ever-changing synthetic fentanyl analogue compounds, it is beneficial to have both a targeted and untargeted approach to analysis. The High Resolution Accurate Mass LC/Q-TOF acquisition and data analysis workflow of approximately 150 new synthetic fentanyl analogues and 4-ANPP shown here is able to identify these compounds with confidence.

Reference

1. Seth, P.; *et al.* Overdose Deaths Involving Opioids, Cocaine, and Psychostimulants –United States, 2015-2016. *MMWR MorbMortal WklyRep*. March **2018**, *67(12)*, 349–358.
2. Krajewski, L. C. *et al.* Application of the Fentanyl Analog Screening Kit Toward the Identification of Emerging Synthetic Opioids in Human Plasma and Urine by LC-QTOF. *Toxicology Letters* **2020**, *320,* 87–94.

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