Development and Evaluation of a Synthetic Opioid Targeted Gas Chromatography Mass Spectrometry (GC-MS) Method

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Abstract

A method for the targeted confirmation of synthetic opioids and related compounds observed in forensic seized drug analysis was developed and evaluated. An 11-component test solution was used to develop a method that focused on minimizing overlapping retention time acceptance windows and understanding the influence of instrument parameters on reproducibility and sensitivity. Investigated settings included column type, flow rate, temperature program, inlet temperature, source temperature, and tune type. Using a DB-200 column, a 35-minute temperature ramped method was created. It was evaluated against a suite of 222 synthetic opioids and related compounds, and successfully differentiated all but four compound pairs based on retention time or mass spectra. Compared to a general confirmatory method, the targeted method was up to 25 times more sensitive and provided at least a two-fold increase in retention time differences. Analysis of case extracts successfully demonstrated utility of the method and showed no instance of carryover, although the high polarity column required wider retention time windows than other columns. Development of the targeted method is part of a larger effort to better understand the challenges and benefits of different analytical workflows in seized drug analysis.

Highlights

- A method for targeted confirmation of synthetic opioids and related compounds was developed and evaluated.
- The resulting method utilizes a DB-200 column and a 35-minute runtime.
- A total of 222 compounds have been analyzed and four pairs were not differentiable.
- The targeted method was more sensitive and provided better separation than a general confirmatory method.

Keywords: Opioids; GC-MS; Seized Drug; NPS; Method Development

Introduction

Opioids are one of the most frequently encountered compound classes in seized drug analysis [1] and present a number of analytical challenges. The rise of synthetic opioids, like fentanyl and fentanyl analogs, over the last decade[2,3] has brought with it a wide variety of compounds that must be identified, confirmed, and differentiated. Many of these compounds are chemically and structurally similar and there are numerous isobaric and isomeric species. A further complication is the high potency of synthetic opioids leading to street samples that are often heavily cut with inert diluents, heroin, and other controlled substances. These low concentrations result in detection challenges. Increased prevalence of the U series opioids[4] and nitazenes[5] represent other subclasses of compounds that can be difficult to confirm, aside from fentanyl.

Generic analytical methods used in seized drug analysis have become less effective for synthetic opioids, necessitating the development of new approaches and methodologies. A large body of research has grown around presumptive analysis of these compounds, either in the laboratory or the field. A number of ambient ionization mass spectrometry (AI-MS) techniques have been demonstrated for opioid analysis, including direct analysis in real time (DART-MS)[6], direct sample analysis (DSA-MS)[7], and paper spray mass spectrometry[8]. Portable mass spectrometers, using a range of ionization techniques, have also been shown[9–11]. Using similar principles, ion mobility spectrometry has been reported to separate and identify some fentanyl compounds[12,13]. Surface enhanced Raman spectroscopy (SERS) has shown potential for opioid analysis[14], as has, for more concentrated samples, Fourier transform infrared (FTIR) [15]. Other novel approaches for presumptive analysis of fentanyl include lateral flow immunoassays (LFIs)[15,16], electrochemistry[17], and electrophoresis[18].

Several techniques for confirmatory analysis of opioids have also been demonstrated including gas chromatography vacuum ultraviolet (GC-VUV)[19,20], GC coupled with infrared detection (GC-IRD)[21], liquid chromatography mass spectrometry (LC-MS)[22], and low-field nuclear magnetic resonance (NMR)[23]. In addition to new instrument-based approaches, several interesting data interpretation techniques and statistical analyses have also been developed. These techniques, which typically utilize mass spectral data, have provided greater insight into fragmentation pathways[24], isobaric fragment ions[25], and profiling capabilities[26–28].

A potential drawback of many of the previously noted tactics is they require the adoption of new technology, which can be challenging in a forensic laboratory setting due to time and financial constraints. A more pragmatic approach to address challenges in the short-term is to develop new methodologies (instrument-based or data-focused) that leverage existing platforms common to the field. Gilbert *et al.* demonstrated this by highlighting how principal component analysis can be used for the classification of fentanyl analogues based on their gas chromatography mass spectrometry (GC-MS) spectra[29]. Moorthy *et al.* showed the ability to use GC-MS data combined with hybrid similarity searching[30] and mass spectral similarity mapping to identify previously unseen analogs[31].

In this work, we look at complimenting these data-focused approaches by establishing a GC-MS instrumental method specifically for the confirmation of synthetic opioids. The method was developed and evaluated using a previously deployed framework[32] with the goals of (1) minimizing overlapping retention time acceptance windows, (2) understanding how to alter sensitivity of the method without effecting reproducibility, and (3) measuring and documenting the limitations of the method in regards to compound discrimination. Using a test solution consisting of eleven compounds, the method was developed by investigating column type, flow rate, temperature program, inlet, and mass spectrometer source conditions. Once established, the method was evaluated by analyzing over 200 additional opioids and related compounds in addition to a suite of case samples to capture limitations of this approach. Comparisons to an existing general-purpose GC-MS method were also made. Implementation of a targeted method is envisioned to occur alongside information-rich screening tools (such as DART-MS[33], DSA-MS, or Raman spectroscopy[34]) or in instances where there are known compound confirmation difficulties using general-purpose methods.

Materials and Methods

Approach for Developing Targeted Methods

A previously published framework for the development and evaluation of the targeted synthetic opioid method was used for this work[32]. Briefly, method development began with investigation of the six different column stationary phases to establish which phase provided the best balance of retention time differentiation, sensitivity, and total analysis time (Step 1). Once a stationary phase was chosen, additional studies were completed to evaluate different oven temperature programs and flow rates to attempt to shorten analytical runtimes while maintaining sufficient retention time differences (Step 2). A design of experiments was then used to investigate inlet temperature, split ratio, injection volume, and MS source temperature, and tune types (stune vs atune) to understand tradeoffs with sensitivity and reproducibility (Step 3).

The above studies resulted in a preliminary targeted method to use for a series of evaluation studies, the first of which involved expansion of compounds analyzed using the method. Over 200 synthetic opioids and related compounds were analyzed to determine the effectiveness of the method to separate and detect different compounds (Step 4). The targeted method was then compared to a general confirmatory method currently used at the Maryland State Police Forensic Sciences Division (MSP-FSD) to understand the strengths and weaknesses of the targeted method over a currently used method (Step 5). Finally, a series of adjudicated or mock case samples were analyzed to evaluate usability on the type of extracts encountered in a forensic laboratory (Step 6).

Chemicals

Unless otherwise stated, all analyses used a test solution custom-made by Cayman Chemical (Ann Arbor, MI, USA). The solution contained eleven compounds: m-fluoroisobutyryl fentanyl (m-FIBF), p-fluoroisobutyryl fentanyl, (p-FIBF), fentanyl, cyclopropyl fentanyl, methoxyacetyl fentanyl, crotonyl fentanyl, carfentanil, furanyl fentanyl, etizolam, benzodioxole fentanyl, and noscapine. Each compound was present at a nominal concentration of 100 µg mL^{-1}, in methanol. The solution was received as individual 1 mL ampoules, and a fresh ampoule was used each day studies were run. In addition to the test solution, individual compounds in methanol (at a nominal concentration of

100 µg/mL) were also analyzed using standards obtained from Cayman Chemical. A Fentanyl Analog Screening Kit (FAS Kit) from Cayman Chemical was also used to investigate a broad range of synthetic opioids once the targeted method was developed.

Instrument, Consumables, and Data Analysis

All analyses were completed using an Agilent 7890/5977B GC-MS (Agilent Technologies, Santa Clara, CA, USA). Method parameters varied throughout the project and are discussed throughout the text. Six columns were investigated, all of which had dimensions of 30 m x 0.25 mm x 0.25 µm, with stationary phases of DB-1UI, DB-5, DB-5UI, DB-35, DB-200, and VF-1701ms (Agilent Technologies). Unless otherwise noted, the standard spectral tune (stune) was used along with an MS scan range of *m/z* 50 to *m/z* 450, a scan speed of N = 2, and a threshold of 150 counts.

Data analysis protocols from [32] were employed. MassHunter (Agilent Technologies) was used for chromatographic peak integration and AMDIS (NIST, Gaithersburg, MD, USA) for chromatogram deconvolution and mass spectral quality analysis. Using data from the Scientific Working Group for Seized Drug Analysis (SWGDRUG) MS Library (version 3.6), a custom library consisting of mass spectra of all compounds in the test solution was created for assessing the quality of experimentally collected mass spectra. The full SWGDRUG MS Library was used for Step 6. Additional data analysis parameters can be found elsewhere[32] and in the Supplemental Information. The min-max mass spectral similarity test was used to establish instances where mass spectra of closely eluting compounds could be differentiated. Details of the min-max test are provided elsewhere[32] and in the Supplemental Information.

Results and Discussion

Column Comparison (Step 1)

Comparison of the six stationary phases was completed by analyzing the test solution, in triplicate, on all columns using the same instrumental parameters (Supplemental Table 1). Peak area, peak height, retention time, and peak width (FWHM) for each compound were extracted along with peak purity measurements (from AMDIS) and mass spectral quality matches (from AMDIS). The 11 compounds in the test solution were also analyzed individually on each column to aid in identifying occurrences of overlapping peaks and changes in elution order. It should also be noted that column dependent variation in elution order was observed and is highlighted in Supplemental Figure 1.

A summary of the results obtained from the different columns are shown in Figure 1. Significant differences in peak areas (Figure 1B) were observed across the six columns, with VF-1701ms providing the lowest integrated peak areas, on average, and DB-35 the greatest. Peak purity, defined as the percentage of the total ion signal within a chromatographic peak that can be attributed to the analyte, also showed differences across the column types (Figure 1C). Purity tended to decrease with later eluting compounds, which was expected due to increased column bleed at higher oven temperatures. The DB-200 and VF-1701ms columns were found to have the most consistent peak purities (lowest standard deviations) across the components of the test mixture, followed by the DB-35 column. Representative chromatograms of the test solution analyzed on all columns are shown in Supplemental Figure 1.

The weighted mass spectral comparison scores, from AMDIS, for all compounds were also extracted and, in nearly all instances, were equal to or exceeded 85 a.u., out of a possible 100 a.u. Mass spectra obtained using the VF-1701ms column had the lowest average similarity scores and highest standard deviation, at 86.7 a.u. (±25.1 a.u.), while the spectra from the DB-1 and DB-35 columns performed the best (Supplemental Table 2). Peak widths (Supplemental Table 2) were consistent across all columns, though slightly wider for the VF-1701ms column.

To compare chromatographic separability, the percent differences in retention times between neighboring peaks (%RTD) were measured, as defined in Equation 1, and are plotted in Figure 1A. The VF-1701ms column was superior as it was the only one that achieved greater than 1 % retention time difference (%RTD) between all sets of neighboring peaks that eluted – though noscapine and benzodioxole fentanyl did not elute during the 30 min runtime. Elution of all compounds was achieved on the remaining five columns, and all had at least one instance of co-eluting, or nearly co-eluting peaks. On the DB-1UI column, co-elution was observed with furanyl fentanyl and etizolam, and there were four additional pairs of nearly co-eluting peaks. The same five pairs of compounds were problematic on the DB-5 and DB-5UI columns.

$$\%RTD = \frac{|(Retention\ Time\ Compound\ 1) - (Retention\ Time\ Compound\ 2)|}{(Retention\ Time\ Compound\ 1)} * 100 \quad \textbf{(Eqn. 1)}$$

The DB-1UI, DB-5UI, and DB-5 columns were not explored further due to poor chromatographic separability. The VF-1701ms column was not explored further due to the inability to elute all compounds in the test solution and the high variability in mass spectral library scores. The DB-35 and DB-200 columns both had acceptable mass spectral quality and only two pairs of compounds with %RTDs of less than 1 %. Both columns produced mass spectra with acceptable quality (a similarity score of greater than 90 a.u. when compared to the SWGDRUG library). While both columns were deemed suitable for the development of a targeted method, the DB-200 column was chosen as it has been previously shown to be optimal for the analysis of synthetic cannabinoids[32] and therefore the same column could be employed for both methods.

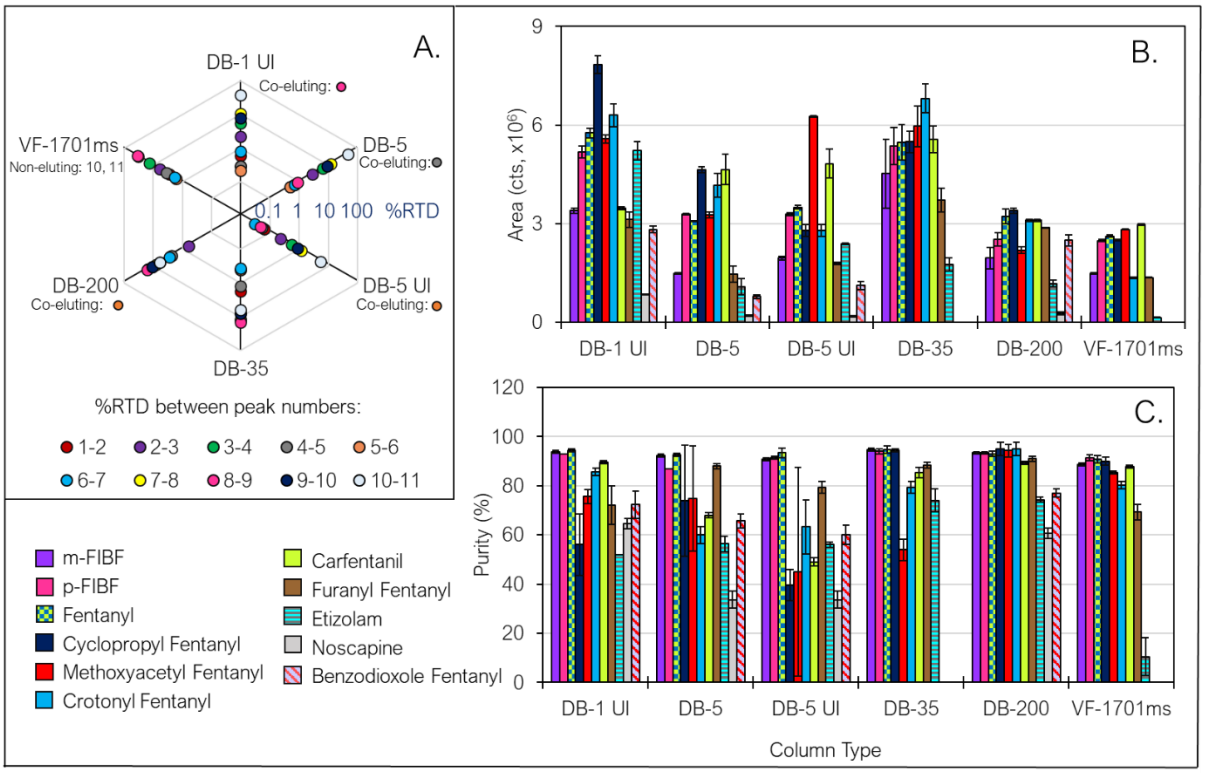

Figure 1. Results of the column comparison study (Step 1). The radar plot (A.) shows the percent retention time difference (%RTD) for neighboring peaks in the test mixture (sequentially numbered 1 through 11 because of differences in elution order). Points further out on the web indicate better separation. Note the plot is log scale. Average peak areas (B.) and peak purities (C.) for each compound analyzed on each column are also shown. Uncertainties represent the standard deviation of triplicate measurements. Compounds are listed in the elution order when using a DB-5 column. For the DB-35 column, detection of noscapine and benzodioxole fentanyl was only possible using extracted ion chromatograms and therefore they are not included in (B.) or (C.)

Maximizing Retention Time Differences Through Temperature and Flow Parameters (Step 2)

Once a column was chosen, different carrier gas flow rates and oven temperature programs were assessed to identify conditions that allowed for maximized differences in retention times for of test solution compounds while maintaining a reasonable runtime. Isothermal and ramped (single ramps and multiple ramps) temperature programs were evaluated, along with flow rates ranging from 0.8 mL min^{-1} to 2.0 mL min^{-1}, as summarized in Table 1. The isothermal temperature programs at 250 ºC and 290 ºC were unable to sufficiently separate all compounds in the test solution as shown in Table 1 and Supplemental Figure 2. A slow ramp rate (2 ºC min^{-1}) did provide sufficient retention time differences (%RTD of approximately 1 % or greater) for all components in the test solution, regardless of the flow rate used. Additionally, while the non-isothermal studies initially had a starting temperature of 200 ºC, it was found that this could be increased to 230 ºC without affecting compound resolution, allowing for a reduction in runtime. The slow temperature program was found to be necessary to achieve a %RTD of at least 1 % between the seven earliest eluting compounds. Attempts to reduce the overall runtime by using a multi-step ramp were investigated but did not lead to faster elution of the late eluting compounds. The single 2 ºC min^{-1} temperature ramp, starting at 230 ºC, was chosen for the targeted method. A mid-range flow rate of 1.2 mL min^{-1} was selected to allow for flexibility in flow rate that could occur when locked retention times were implemented.

Table 1. Summary of the temperature programs and flow rates studied as well as their respective results. The notation "CEP" denotes instances where there were co-eluting peaks. Uncertainties represent the standard deviation of triplicate measurements.

Temperature Program	Flow Rate (mL min^{-1})	Minimum %RTD (%)	Maximum Retention Time (min)
250 °C Isothermal	2.0	0.15 (±0.05)	33.14
290 °C Isothermal	0.8	CEP	11.50
290 °C Isothermal	2.0	CEP	7.75
200 °C – 290 °C ramping at 2 °C min^{-1}	2.0	0.97 (±0.03)	38.02
230 °C – 290 °C ramping at 2 °C min^{-1}	1.2	0.96 (±0.07)	24.13
230 °C – 290 °C ramping at 2 °C min^{-1}	2.0	1.10 (±0.01)	21.45
230°C, hold 2 min Ramp 1 °C min^{-1} to 240 °C Hold 0.5 min Ramp 5 °C min^{-1} to 290 °C	1.2	1.28 (±0.03)	23.91
230°C, hold 2 min Ramp 1 °C min^{-1} to 240 °C Hold 0.5 min Ramp 5 °C min^{-1} to 290 °C	2.0	1.35 (±0.05)	22.23

Assessing Sensitivity and Reproducibility (Step 3)

Once the column type, flow rate, and temperature program were established, a design of experiments (DOE) was completed to determine the effect, if any, of MS source temperature, split ratio, injection volume, and inlet temperature on reproducibility and sensitivity. A two-level (2^{4-1}) DOE was used, as outlined in Supplemental Table 3, with the experimental levels outlined in Figure 2. Sensitivity was measured using peak area and peak height while reproducibility was measured using the percent relative standard deviation (%RSD) of peak area, peak height, retention time, and %RTD across triplicate analyses. The average responses for each measurement were then compared using a Student's T-Test (95 % confidence) to determine which parameters elicited statistically significant differences. The results of the DOE study (Figure 2) showed that MS source temperature and split ratio did not lead to significant differences in sensitivity or reproducibility. A significant difference in peak area ($p = 0.046$) was found for the injection volume, as expected, though the differences were not significant for peak height or for any of the reproducibility measures. Inlet temperature produced statistically different results for reproducibility (%RSD) measures of peak height ($p = 0.038$) and retention time ($p = 0.0002$). In both instances, poorer reproducibility, defined as a higher %RSD, was obtained using a 200 ºC inlet temperature. A 300 ºC inlet temperature was therefore chosen for the targeted method. A source temperature of 280 ºC was also chosen to minimize potential build-up on the source. Since synthetic opioids are typically present as low weight percentages in street samples, a split ratio of 10:1 was chosen to increase sensitivity. An injection volume of 1 µL was chosen to maintain sensitivity without overloading the liner. The split ratio and injection volume, however, could be changed to adjust sensitivity without affecting reproducibility.

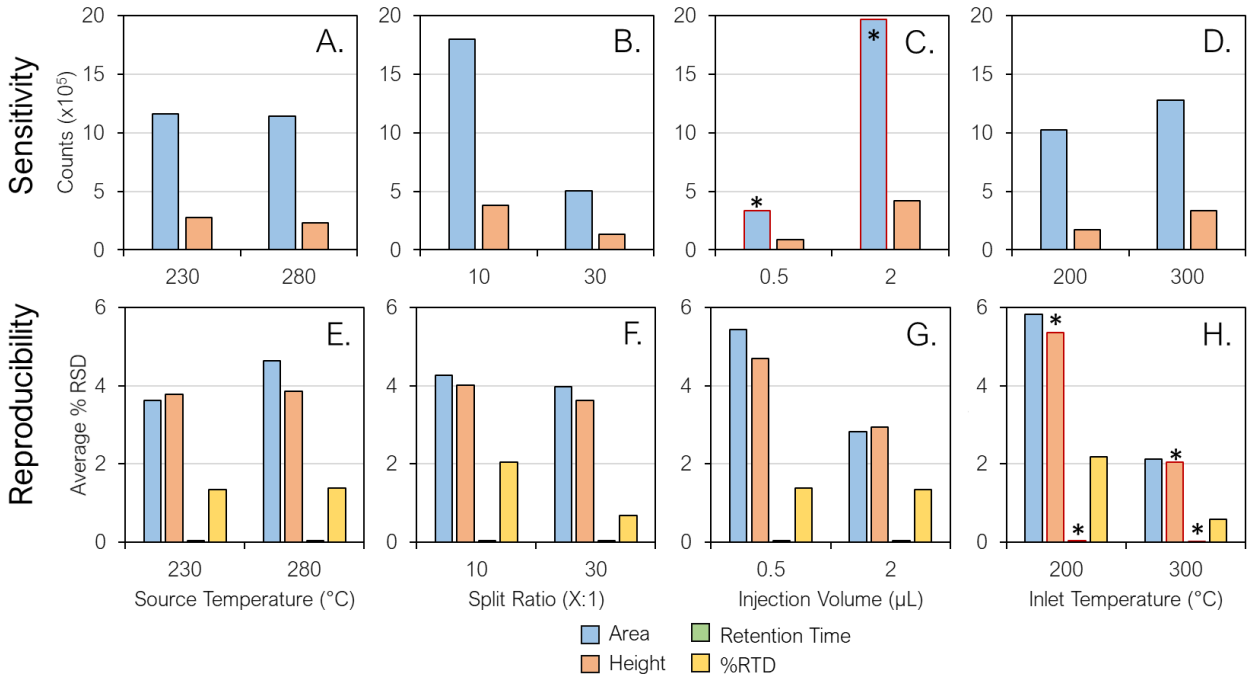

Figure 2. Results of the DOE experiment for source temperature (A. and E.), split ratio (B. and F.), injection volume (C. and G.), and inlet temperature (D. and H.). Peak area (blue) and peak height (orange) are shown in A. through D. while the average %RSD of peak area (blue), peak height (orange), retention time (green), and %RTD (yellow) across triplicate injections are shown in E. through H. Boxes with asterisks (*) indicate those where the observed difference was statistically significant at the 95 % confidence level.

The two available tune options, stune and atune, were also examined. Triplicate injections of the test solution were completed using identical methods (consisting of the settings chosen above) except for tune type. Peak areas, shown in Supplemental Figure 3, were nearly identical for all compounds in the test solution for both tune types. The weighted mass spectral scores, obtained by comparing the resulting mass spectra to the SWGDRUG GC-MS library spectra using AMDIS, produced a score of 94 a.u. (out of 100 a.u.) or greater for all components regardless of the tune type used. Since no obvious advantage was shown for either tune type, stune was chosen as it is currently used for casework at the laboratory and is the tune type used for creation of the SWGDRUG library. The final settings chosen for the targeted method are listed in Table 2.

Table 2. Settings for the targeted method. Split ratio and injection volume could be altered, as necessary, to achieve the desired sensitivity. Settings in parentheses indicate settings that were changed after evaluation of the method to address the need for longer runtimes to elute non-volatile compounds (Step 4) and the need to reduce sensitive for the analysis of case samples (Step 6).

Column	DB-200 30 m x 0.25 mm x 0.25 µm
Temperature Program	1) 230 °C for 0.0 min 2) Ramp at 2 °C min^{-1} to 290 °C 3) Hold 0.0 min (5.0 min)
Flow Rate	1.2 mL min^{-1}
Injection Volume	1.0 µL
Inlet Temperature	300 °C
Split Ratio	10:1 (20:1)
Transfer Line	300 °C

Quad Temperature	150 °C
Source Temperature	280 °C
Tune Mode	Stune
Solvent Delay	1.3 min
Mass Scan Range	*m/z* 40 – *m/z* 550
Threshold	150
Scan Speed	N = 2
Total Runtime	30.0 min (35.0 min)

Evaluation of the Method with Additional Compounds (Step 4)

To evaluate and characterize the utility and limitations of the targeted method, an extended panel (Supplemental Table 4) consisting of a wide range of synthetic opioids and commonly co-observed compounds were analyzed. First, each compound was run as single component solution to establish an approximate retention time and to determine if any method modifications were necessary due to elution difficulties. Three fentanyl analogs did not elute in the 30 min run, resulting in the need to extend the method by an additional 5 min.

After extending the method, it was retention time locked using fentanyl (8.847 min) as the lock compound. The single component solutions were combined into forty mixtures, each containing compounds that had significantly different retention times. Each mixture was measured three times to establish locked retention times and to obtain replicate mass spectra in order to apply the min-max test to closely eluting peaks, as described below. Since some laboratories utilize retention indices, an alkane ladder was also incorporated into the sequence so that retention indices could be calculated. Retention times and retention indices for a subset of the commonly encountered compounds is presented in Table 3, while the full list is presented in Supplemental Table 4.

In addition to establishing retention times and indices, the full dataset was also used to identify which compounds could not be differentiated by retention time or mass spectra. While laboratories use different retention time windows to establish when two compounds are sufficiently separated, a conservative %RTD window of 2 % was used to identify the largest number of potentially indistinguishable pairs. A total of 599 pairs of compounds (out of 24531 unique pairs of compounds) had retention times within 2 % of one another. The ability to differentiate this subset of compound pairs based on their mass spectra was then examined using the min-max test. While described in detail elsewhere[32], the min-max test compares the similarity scores obtained from replicate mass spectra of two unique compounds to themselves to the similarity scores obtained from mass spectra of the two unique compounds to each other. The result of this approach is a *min-max index* which represents the difference between the minimum similarity score when comparing replicate spectra of Compound 1 or Compound 2 and the maximum similarity score between spectra from Compound 1 and Compound 2. Possible min-max indices range from -999 a.u. to 999 a.u, where a non-positive value indicates that the two compounds are not differentiable based on their mass spectra.

Of the 599 pairs of compounds that had %RTDs less than or equal to 2 %, only four pairs had non-positive min-max indices (Table 4), indicating that their spectra are not differentiable. Three of the compound pairs (m-methyl Cyclopropyl fentanyl | o-methyl Cyclopropyl Fentanyl, p-fluoro Furanyl Fentanyl 3-furancarboxamide | p-fluoro

Furanyl Fentanyl, and m-Methylfentanyl | o-Methylfentanyl) were found to be positional isomers and the fourth pair was the acid and free-base forms of Remifentanil. While a non-positive min-max index definitively indicates that the two compounds do not have distinguishable mass spectra, pairs of compounds with index values less than 100 may also produce mass spectra that would be too difficult to differentiate visually. Of the 599 compound pairs that had %RTDs less than or equal to 2 %, an additional 22 pairs, also listed in Table 4, had positive indices less than 100. In nearly all instances, the pairs were positional isomers of compounds that are not frequently encountered. All but one pair (etodesnitazene | isodesnitazene) were fentanyl analogs. While a %RTD of 2 % was used here to provide a conservative evaluation of the method, laboratories that have tighter windows would have fewer compound pairs that are not differentiable.

Table 3. Retention time (RT), percent retention time difference (%RTD, as defined by Equation 1), retention time difference (RTD, defined as the difference between a compound and the one listed below), and retention index (RI) for a subset of the frequently seen compounds analyzed by the targeted method. Retention times and indices are the average of three replicates of an approximately 100 µg mL^{-1} solution. Uncertainties represent the standard deviation of three replicates.

Compound	RT (min)	%RTD	RTD (min)	RI (a.u.)
Tramadol	2.085 (±0.006)	12.3	0.257	2265
Xylazine	2.342 (±0.011)	2.5	0.058	2363
o-Desmethyl-cis-Tramadol	2.400 (±0.010)	12.4	0.298	2386
Norfentanyl	2.698 (±0.005)	0.4	0.011	2473
Acetyl norfentanyl	2.709 (±0.003)	70.6	1.912	2483
4-ANPP	4.621 (±0.004)	17.3	0.799	2834
AP-238	5.420 (±0.006)	8.5	0.462	2929
2-Methyl AP-237	5.882 (±0.012)	11.5	0.677	2980
6-Monoacetylmorphine	6.559 (±0.007)	3.8	0.247	3047
U-47700	6.806 (±0.004)	3.0	0.205	3071
U-48800	7.011 (±0.000)	1.4	0.099	3091
Benzyl Fentanyl	7.110 (±0.002)	11.9	0.845	3103
Remifentanil	7.955 (±0.000)	3.2	0.251	3174
m-Fluoroisobutyryl Fentanyl	8.206 (±0.003)	0.4	0.035	3192
U-49900	8.241 (±0.003)	0.4	0.033	3198
Oxycodone	8.274 (±0.008)	3.7	0.306	3198
FIBF	8.580 (±0.004)	1.1	0.098	3222
trans-3-methyl Fentanyl	8.678 (±0.002)	1.9	0.169	3231
Fentanyl	8.847 (±0.009)	1.1	0.094	3243
Acetyl fentanyl	8.941 (±0.006)	0.1	0.005	3249
Acrylfentanyl	8.946 (±0.003)	1.7	0.151	3250
cis-3-methyl Fentanyl	9.097 (±0.002)	1.3	0.117	3266
Heroin	9.214 (±0.014)	0.1	0.012	3269
p-Fluorofentanyl	9.226 (±0.003)	1.3	0.117	3274
Metodesnitazene	9.343 (±0.008)	1.4	0.131	3279
Butyryl Fentanyl	9.474 (±0.002)	3.1	0.293	3289
Cyclopropyl Fentanyl	9.767 (±0.002)	2.2	0.213	3308
Etodesnitazene	9.980 (±0.007)	1.3	0.132	3333
p-Fluorobutyryl Fentanyl	10.112 (±0.000)	0.1	0.014	3324
Isodesnitazene	10.126 (±0.007)	0.8	0.082	3340
Quinine	10.208 (±0.011)	0.1	0.008	3334
4'-methyl Acetyl Fentanyl	10.216 (±0.006)	5.1	0.519	3340
Crotonyl Fentanyl	10.735 (±0.003)	0.5	0.049	3377
Carfentanil	10.784 (±0.000)	0.1	0.016	3385
Valeryl Fentanyl	10.800 (±0.002)	10.7	1.159	3379
Methoxyacetyl Fentanyl	11.959 (±0.004)	8.3	0.997	3461
Cyclopentyl Fentanyl	12.956 (±0.004)	3.9	0.507	3320
p-Methoxyfentanyl	13.463 (±0.005)	7.1	0.961	3551

Tetrahydrofuran Fentanyl	14.424 (±0.005)	2.8	0.403	3607
Furanyl fentanyl 3-furancarboxamide isomer	14.827 (±0.004)	2.8	0.408	3632
Furanyl fentanyl	15.235 (±0.005)	39.3	5.989	3652
Noscapine	21.224 (±0.011)	2.7	0.575	3977
Flunitazene	21.799 (±0.015)	10.5	2.298	4009
Etizolam	24.097 (±0.010)	3.4	0.819	4130
Brorphine	24.916 (±0.023)	8.2	2.035	4173
Bendioxole Fentanyl	26.951 (±0.002)	0.2	0.045	4281
Metonitazene	26.996 (±0.010)	4.0	1.079	4283
Isotonitazene	28.075 (±0.015)	N/A	N/A	4341

Table 4. Compound pairs with a %RTD of 2 % or less that also had a min-max index of 100 or less, indicating similarity in their mass spectra. The %RTD and the min-max index are listed. Compound pairs are listed in order of increasing min-max indices.

Compound 1 (C_1)	**Compound 2 (C_2)**	**%RTD (%)**	**Min-Max Index**
m-methyl Cyclopropyl fentanyl	o-methyl Cyclopropyl Fentanyl	0.66	-12
Remifentanil	Remifentanil Acid	1.03	-2
p-fluoro Furanyl Fentanyl 3-furancarboxamide	p-fluoro Furanyl Fentanyl	0.79	-2
m-Methylfentanyl	o-Methylfentanyl	0.55	0
m-methyl Acetyl fentanyl	o-methyl Acetyl fentanyl	0.04	6
m-methyl Methoxyacetyl Fentanyl	o-methyl Methoxyacetyl Fentanyl	0.36	8
Octfentanil	m-fluoro Methoxyacetyl Fentanyl	1.03	9
o-Fluorofentanyl	m-Fluorofentanyl	1.81	10
o-methyl Furanyl fentanyl	m-methyl Furanyl fentanyl	0.15	13
o-Fluoroisobutyryl Fentanyl	m-Fluoroisobutyryl Fentanyl	0.65	13
N-(2C-T) Fentanyl	N-(2C-T-2) Fentanyl	0.71	26
o-Fluorobutyryl Fentanyl	m-Fluorobutyryl Fentanyl	1.60	26
o-fluoro Furanyl Fentanyl	p-fluoro Furanyl Fentanyl 3-furancarboxamide	1.51	28
2',5'-dimethoxy Fentanyl	N-(2C-D) Fentanyl	1.31	33
Etodesnitazene	Isodesnitazene	1.46	39
N-(2,5-DMA) Fentanyl	N-(DOM) Fentanyl	0.77	41
N-(2C-T-4) Fentanyl	N-(2C-T-2) Fentanyl	1.68	49
N-(2C-T-4) Fentanyl	N-(2C-T) Fentanyl	0.96	49
N-(2C-iP) Fentanyl	N-(2C-E) Fentanyl	0.03	54
o-Fluorofentanyl	2'-fluoro-o-fluorofentanyl	0.14	59
N-(2C-P) Fentanyl	N-(2C-G) Fentanyl	1.69	75
N-(3,4,5-TMA) Fentanyl	N-(DOC) Fentanyl	0.05	75
N-(2C-TFM) Fentanyl	N-(2C-D) Fentanyl	1.17	76
N-(MDA) Fentanyl	N-(6-APB) Fentanyl	1.73	80
2'-fluoro-o-fluorofentanyl	m-Fluorofentanyl	1.67	88
N-(2-APB) Fentanyl	N-(MDA) Fentanyl	0.65	92

Limits of Detection and Comparison to a General Method (Step 5a)

Following the expansion of the compound set, the targeted method was compared to a general confirmation method used at MSP-FSD, parameters of which are shown in Supplemental Table 5. Triplicate measurements of the original 11-compound test solution were completed using the targeted method and the general confirmatory method to establish the differences in separation that the targeted method offered. Additionally, the test solution was diluted, volumetrically, to approximate concentrations of 50 µg/mL, 25 µg/mL, 10 µg/mL, 5 µg/mL, and 1 µg/mL to measure

the approximate limit of detection (LOD), defined as the lowest concentration that provided a chromatographic peak with a signal to noise ratio of at least 3:1 and a mass spectral similarity score (from AMDIS) of at least 80 a.u. The results of these experiments are presented in Supplemental Table 6 and representative chromatograms of both methods are shown in Figure 3.

Using the method in Table 2, the targeted method was found to have approximate LODs between 2 and 25 times more sensitive than the general confirmatory method and approximately an order of magnitude higher peak area. When looking at the representative chromatographs in Figure 3 there are multiple instances where the difference in elution time for neighboring compounds was less than 1 % for the general confirmatory method. Methoxyacetyl fentanyl, cyclopropyl fentanyl, and crotonyl fentanyl co-eluted on the general method as did furanyl fentanyl and etizolam. Benzodioxole fentanyl did not elute within the analysis time of the general method. In nearly all instances, the %RTD between neighboring peaks was larger for the targeted method compared to the general confirmation method.

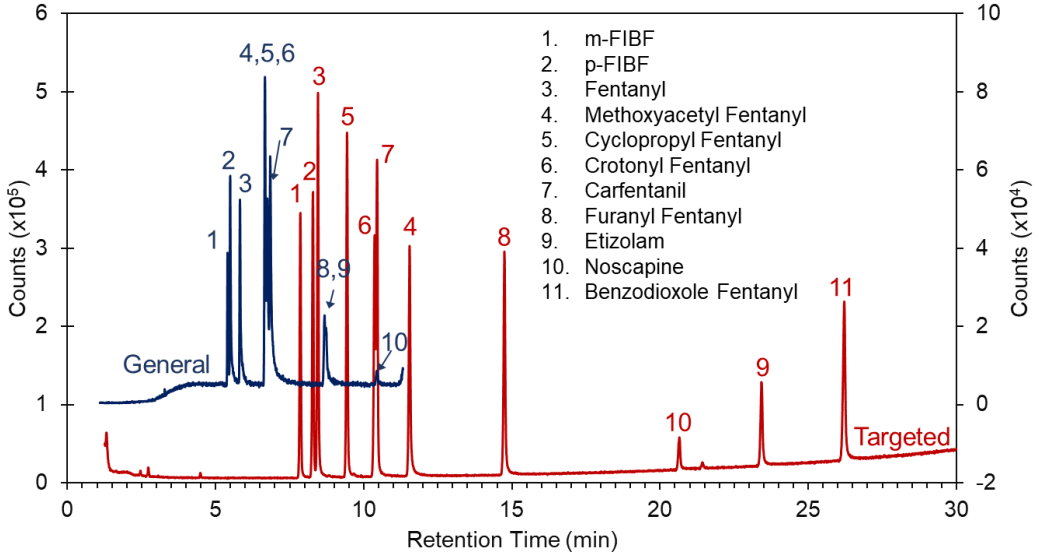

Figure 3. Comparison of the undiluted test solution analyzed using the targeted method (red) and the general confirmatory method (blue). Note the secondary y-axis for the general confirmatory method.

Evaluation of the Method Against Case Samples (Step 6)

The final component of this study looked to evaluate a suite of case extracts using the targeted method to establish whether it was fit-for-purpose or not. Ten previously analyzed case samples were prepared following MSP-FSD protocols whereby approximately 5 mg of powder was dissolved in 1.5 mL of methanol. Any solids were allowed to settle, and a portion of the extract was transferred into a GC-MS vial for analysis. The identities of the samples are provided in Table 5 and representative chromatograms of the case samples are provided in Supplemental Figure 4.

Extracts were initially run on the targeted method using a 10:1 split ratio which resulted in broad and sometime saturated peaks from the diluents. The split ratio was reduced to 20:1 and was found to provide sufficient reduction in diluent signal without compromising detection of the peaks of interest. In all instances, the synthetic opioids were

readily detected and returned mass spectral similarity scores – compared to the SWGDRUG library (v3.6) – of 89 a.u. or greater. Retention times were found to be within 2 % of the measured standards in all instances, even given the wide variations in concentrations and the fact that the standards were measured using the lower, 10:1, split ratio. Retention time variation was greater than has been reported[35] using other methods and is likely due to concentration differences coupled with the use of a higher polarity column. Carryover was not observed. In addition to allowing for detection of opioids, the method was able to readily detect many of the cutting agents and other controlled substances present in the extracts. The results of this study highlight the utility of the method for case analysis, and the laboratory is working on fully validating it for casework.

Table 5. Identities and results from the analysis of representative case samples. Samples originated from powders unless otherwise noted. Standard retention times and MS match scores are provided for the synthetic opioids and related compounds only. MS match scores were obtained by searching against the SWGDRUG Library (v3.6).

Case Contents	Sample RT (min)	Standard RT (min)	%RTD (relative to standard)	MS Match Score (Weighted)	Opioid Peak Height (Count/s)
N-methyl Norfentanyl	2.453	2.470	0.69 %	98	$1.5x10^7$
Mannitol	2.196				$5.8x10^6$
Caffeine	2.427				$1.0x10^6$
Heroin	9.207	9.214	0.08 %	99	$1.1x10^5$
Cyclopropyl Fentanyl	9.740	9.767	0.28 %	97	$6.4x10^5$
Phenyl Fentanyl	17.811	18.022	1.17 %	95	$1.4x10^5$
Mannitol	2.383				$3.4x10^6$
Fentanyl	8.755	8.847	1.04 %	97	$2.7x10^6$
Acetyl Fentanyl	8.906	8.941	0.39 %	94	$1.6x10^6$
Melatonin	11.394				$1.1x10^6$
Mannitol	2.220				$4.5x10^6$
Caffeine	2.424				$2.5x10^7$
6-Monoacetylmorphine	6.556	6.559	0.05 %	98	$3.7x10^6$
FIBF	8.544	8.580	0.42 %	97	$2.9x10^6$
Fentanyl	8.748	8.847	1.12 %	97	$1.0x10^6$
Acetyl Fentanyl	8.899	8.941	0.47 %	94	$4.7x10^6$
Heroin	9.243	9.214	0.31 %	98	$1.1x10^7$
Fentanyl	8.707	8.847	1.58 %	97	$1.5x10^6$
XLR11	9.476				$1.2x10^7$
XLR Degradant	10.216				$7.3x10^5$
Dibutylone	1.893				$2.5x10^5$
Fentanyl	8.724	8.847	1.39 %	97	$3.4x10^5$
JWH-250	15.188				$5.3x10^6$
AP-238	5.454	5.420	0.63 %	97*	$1.7x10^7$
Phenacetin	2.082				$7.9x10^4$
Mannitol	2.150				$6.2x10^5$
Xylazine	2.350				$8.7x10^4$
Caffeine	2.412				$8.4x10^6$
Lidocaine	2.824				$4.4x10^4$
6-Monoacetylmorphine	6.527	6.559	0.49 %	89	$3.9x10^4$
Fentanyl	8.689	8.847	1.78 %	97	$4.2x10^4$
Mannitol	2.144				$4.5x10^5$
Caffeine	2.412				$9.4x10^6$
Cocaine	3.624				$3.7x10^4$
Acetylcodeine	6.061				$1.6x10^5$

6-Monoacetylmorphine	6.527	6.559	0.49 %	98	$3.9x10^5$
FIBF	8.526	8.580	0.63 %	98	$3.5x10^5$
Fentanyl	8.695	8.847	1.72 %	97	$6.6x10^5$
Acetyl Fentanyl	8.858	8.941	0.92 %	94	$2.4x10^5$
Heroin	9.202	9.214	0.13 %	98	$5.0x10^6$
Noscapine	21.150	21.224	0.35 %	96	$4.0x10^4$
N-methyl Cyclopropyl norfentanyl	2.662	2.714	1.91 %	97	$2.4x10^6$

*AP-238 is not in the SWGDRUG Library v3.6. Comparison score was obtained using v3.9.

Conclusion

This paper presents a GC-MS method (Table 2) that was developed specifically for the confirmatory analysis of synthetic opioids and related compounds using a previously established framework. The method was substantially different than the general confirmatory method employed at MSP-FSD and used a different stationary phase, MS source temperature, oven program, and inlet temperature. It was demonstrated that MS source temperature, split ratio, and injection volume could be altered within the ranges studied without affecting the reproducibility of the method. While the method was substantially longer than the general confirmatory method, it did allow for far greater retention time differences and detection of all compounds in the test solution. The developed method was evaluated using a suite of over 200 compounds and only four pairs of compounds were found to not be differentiable by either retention time or mass spectra. Current efforts are looking at identifying whether or not the implementation of targeted methods, such as this one, coupled with screening by direct analysis in real time mass spectrometry (DART-MS) provides measurable benefits over traditional workflows. Other ongoing efforts are focused on the development of additional targeted methods (stimulants, tryptamines, and benzodiazepines) and further study of the min-max spectral comparison test. Additional data to support this work, as well as updates to panel of compounds analyzed, can be found here[36].

Disclaimer

Certain commercial products are identified in order to adequately specify the procedure; this does not imply endorsement or recommendation by NIST, nor does it imply that such products are necessarily the best available for the purpose.

Certain commercial products are identified in order to adequately specify the procedure; this does not imply endorsement or recommendation by the Maryland State Police, nor does it imply that such products are necessarily the best available for the purpose.

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Supplemental Information:

Development and Evaluation of a Synthetic Opioid Targeted Gas Chromatography Mass Spectrometry (GC-MS) Method

Additional Information on Data Analysis

All datafiles were analyzed using MassHunter (Agilent Technologies) for chromatographic analysis and AMDIS (NIST) for mass spectral analysis. Peak integration in MassHunter was completed using the Agile2 integrator. In AMDIS, a "Simple" analysis of the data files was used along with a component width of 12, a minimum match factor of 40, medium resolution, medium sensitivity, and medium shape requirements. For retention index calculation, an alkane list was loaded into the RI Calibration File. From the AMDIS results, the weighted match factor, peak purity, and, when applicable, the retention indices were obtained.

Additional Information on the Min-Max Test

An internally developed min-max match factor comparison test was used as an objective and automated way to classify the mass spectra of closely eluting compounds. The comparison test is first described in (1), but, briefly, the difference between the minimum match factor computed between replicate spectra of the same compounds and the maximum match factor computed between all spectra of the two different compounds is computed as the min-max index. The min-max index employs identity match factors, a numerical estimate of similarity between a pair of mass spectra, as described in (2). Confidence that the two compounds are distinguishable via their mass spectra grows as min-max indices increase. Due to its use of extreme values (min and max), the min-max test may behave undesirably if provided with incorrect measurements of one or both target compounds. This is one of our preliminary implementations of the min-max test; additional development and evaluation is on-going. Alternative methods for classifying spectra may also be appropriate for discrimination, including using a match factor threshold between single spectra representing each compound, clustering using all replicates of each compound, or even visual inspection if automation is not required.

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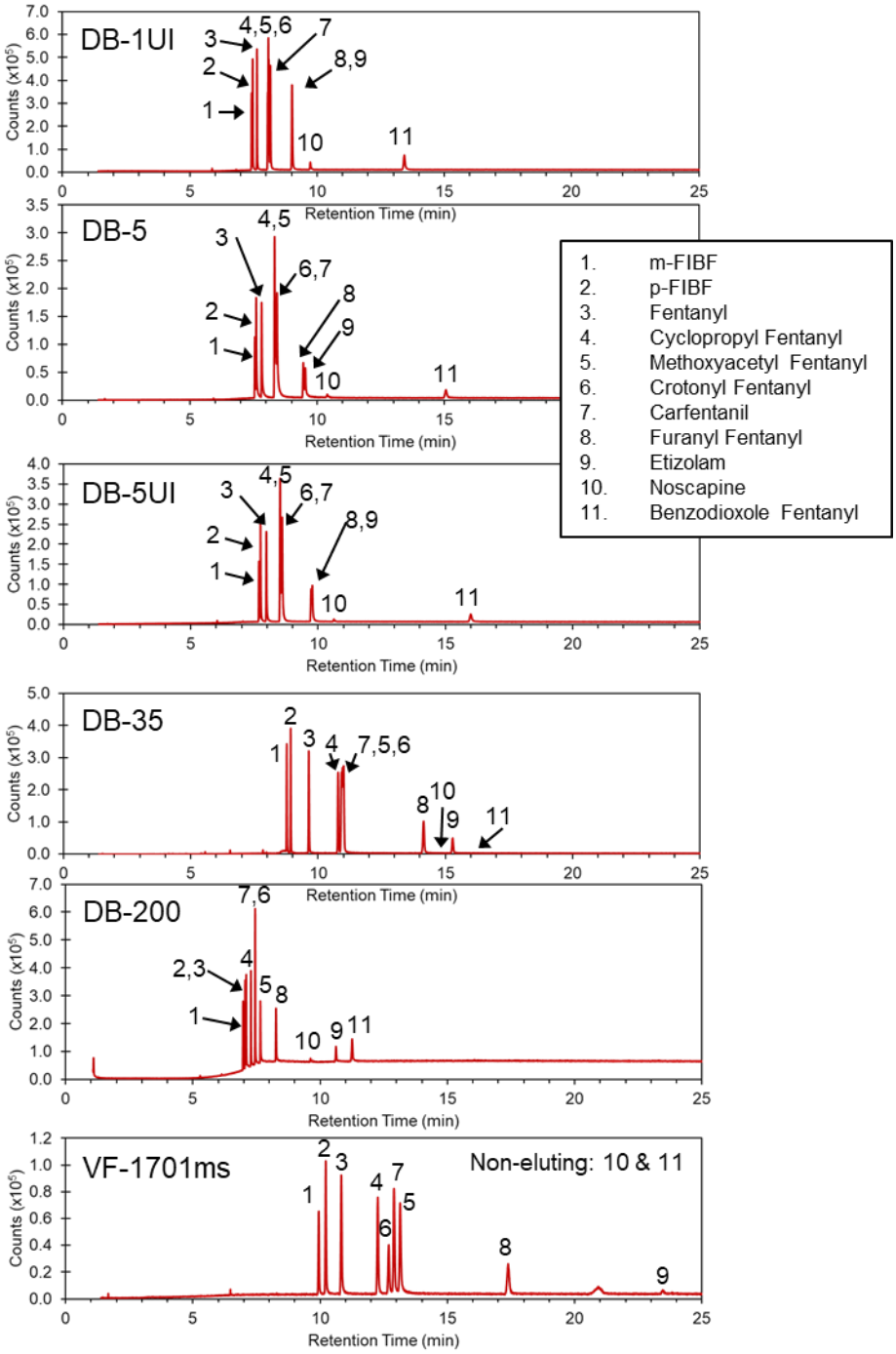
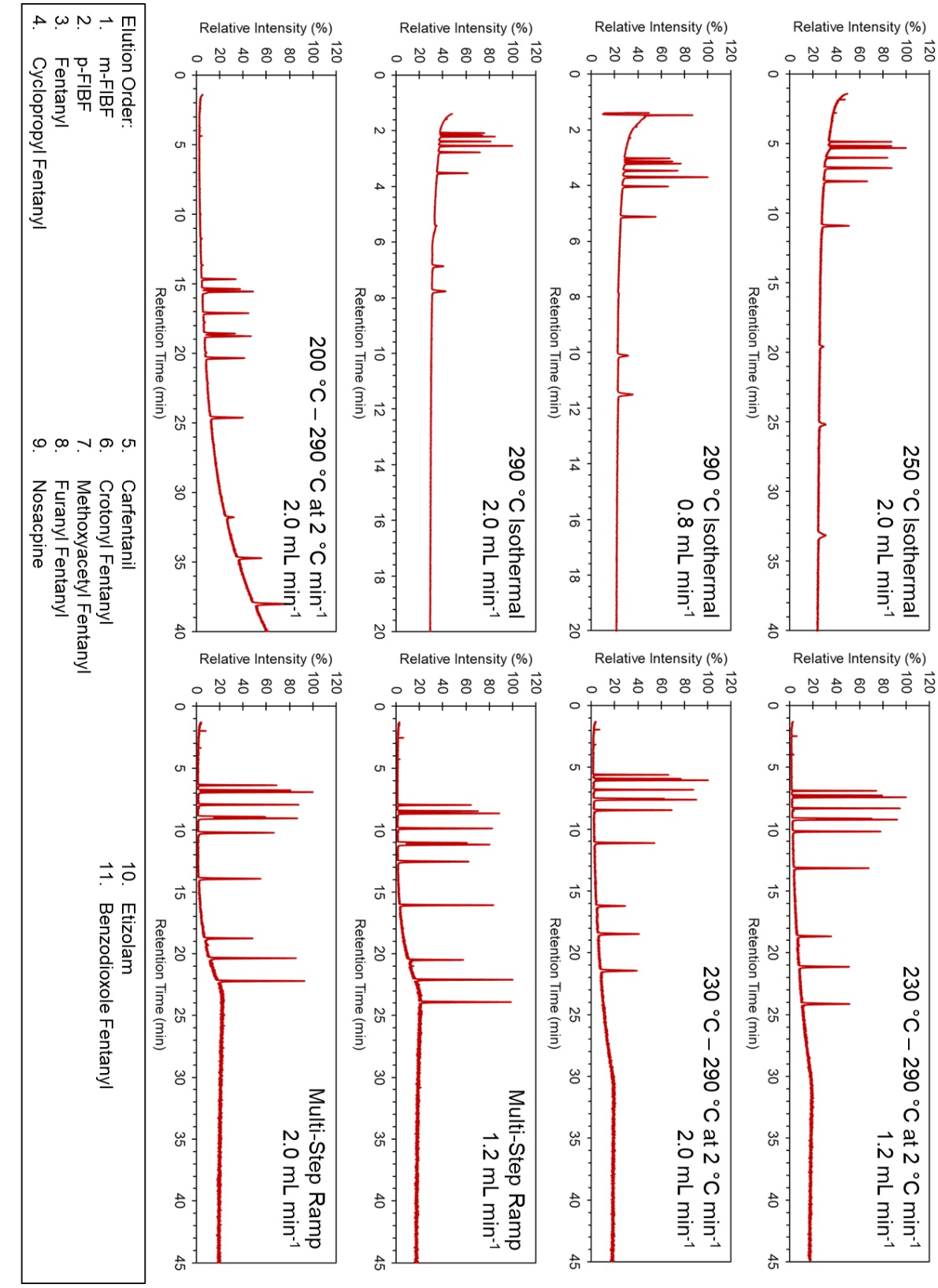
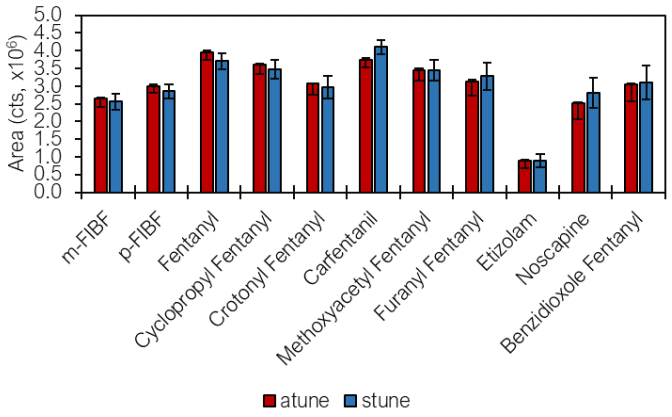


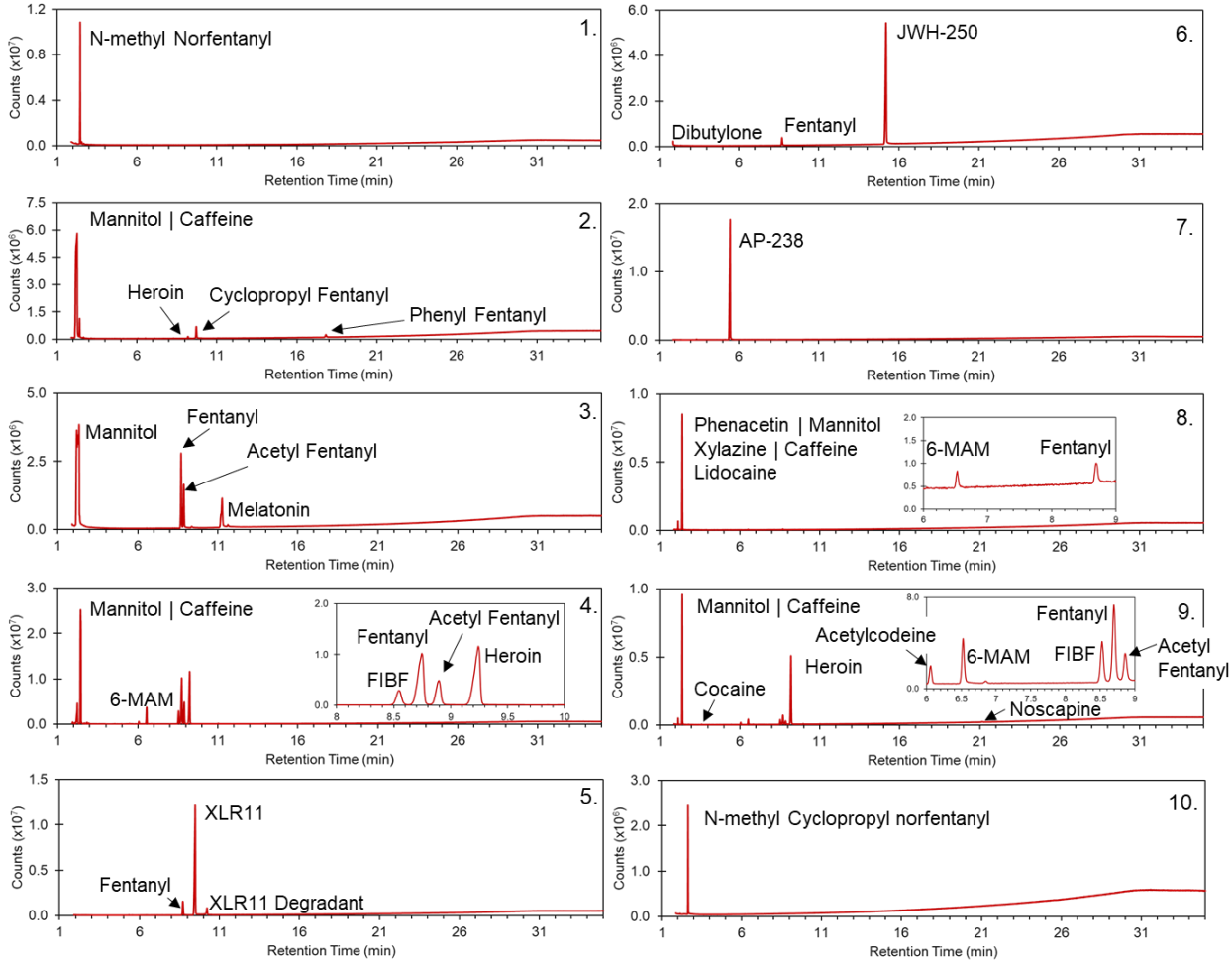
Supplemental Figure 1. Representative chromatograms of the test solution analyzed on each column using the method described in Supplemental Table 1 (Step 1). Note that for the DB-35 column, detection of noscapine and benzdioxole fentanyl was only possible using extracted ion chromatograms.



Supplemental Figure 2. Representative chromatograms of the test solution analyzed at each of the temperature and flow program settings investigated in Table 1 (Step 2).



Supplemental Figure 3. Comparison of peak areas obtained when analyzing the test solution using atune (red) and stune (blue). Uncertainties represent the standard deviation of triplicate measurements (Step 3).



Supplemental Figure 4. Chromatographs of the ten adjudicated or mock case samples (Step 6).

Supplemental Table 1. Parameters of the method used for the column comparison studies (Step 1).

Temperature Program	1) 100 °C for 0 min 2) Ramp at 30 °C/min to 300 °C 3) Hold for 24 min
Flow Rate	1.8 mL/min (Constant Flow)
Injection Volume	1 µL
Inlet Temperature	275 °C
Split Ratio	30:1
Transfer Line	300 °C
Quad Temperature	150 °C
Source Temperature	230 °C
Tune Mode	stune
Solvent Delay	1.30 min
Mass Scan Range	*m/z* 40 to *m/z* 550
Threshold	150
Scan Speed	N = 2
Total Run Time	30.667 min

Supplemental Table 2. Summary results of some key metrics for the different column types examined (Step 1). Uncertainties indicate the standard deviation of averages obtained for each of the detectable compounds in the test solution. The notation "CEP" denotes instances where there were overlapping co-eluting peaks. The notation "NEC" denotes non-eluting compounds.

Column Type	Max RT (min)	Min %RTD (%)	Avg. MS Match Score	Avg. Peak Width (min)
DB-1 UI	13.42	CEP	96.4 (±2.0)	0.09 (±0.07)
DB-5	15.05	CEP	89.5 (±10.4)	0.12 (±0.07)
DB-5 UI	16.00	CEP	93.6 (±3.4)	0.12 (±0.09)
DB-35	16.24	0.4	96.0 (±2.7)	0.14 (±0.06)
DB-200	11.29	CEP	92.2 (±7.4)	0.12 (±0.09)
VF-1701ms	NEC	1.6	86.7 (±25.1)	0.21 (±0.05)

Supplemental Table 3. Experimental settings used in the 2^{4-1} design of experiment (DOE) study (Step 3).

Setting #	MS Source Temperature (°C)	Split Ratio (X:1)	Injection Volume (µL)	Inlet Temperature (°C)
1	230	10	0.5	200
2	280	10	0.5	300
3	230	30	0.5	300
4	280	30	0.5	200
5	230	10	2	300
6	280	10	2	200
7	230	30	2	200
8	280	30	2	300

Supplemental Table 4. Complete list of the 222 opioids and related compounds analyzed using the targeted method along with their retention times and retention indices (Step 4).

Compound	RT (min)	RI	Compound	RT (min)	RI
Tramadol	2.085	2265	Carfentanil	10.784	3379
Xylazine	2.342	2363	3,4-Ethylenedioxy U-47700	10.794	3380
o-Desmethyl-cis-Tramadol	2.400	2386	Valeryl Fentanyl	10.8	3385
N-methyl Norfentanyl	2.470	2409	2,2,3,3-tetramethyl-Cyclopropyl Fentanyl	10.869	3385
Norsufentanil	2.692	2471	p-methyl Cyclopropyl Fentanyl	11.05	3398
Norfentanyl	2.698	2473	3,4-Ethylenedioxy U-51754	11.167	3405
Acetyl norfentanyl	2.709	2483	N-(Phentermine) Fentanyl	11.248	3408
N-methyl Cyclopropyl Norfentanyl	2.714	2487	p-fluoro Crotonyl Fentanyl	11.295	3413
cis-3-methyl Norfentanyl	2.791	2500	α'-methoxy Fentanyl	11.388	3419
Butyryl Norfentanyl	2.902	2526	p-fluoro Valeryl Fentanyl	11.458	3424
N-methyl Norcarfentanil	3.024	2554	p-Chloroisobutyryl Fentanyl	11.493	3426
Norcarfentanil	3.589	2669	Octfentanil	11.609	3434
U-48753E	3.659	2682	m-fluoro Methoxyacetyl Fentanyl	11.729	3441
p-fluoro 4-ANBP	4.026	2743	Methoxyacetyl Fentanyl	11.959	3461
Despropionyl 2'-fluoro o-Fluorofentanyl	4.207	2776	Cyclobutyl Fentanyl	12.14	3467
4-ANPP	4.621	2834	p-fluoro Methoxyacetyl Fentanyl	12.274	3476
Furanyl norfentanyl	4.755	2847	Ethoxyacetyl Fentanyl	12.297	3477
Despropioneyl p-Fluorofentanyl	5.029	2882	Hexanoyl fentanyl	12.332	3479
U-47931E	5.075	2888	p-Chlorofentanyl	12.396	3484
U-48520	5.134	2896	p-chloro Acrylfentanyl	12.437	3486
Despropionyl m-Methylfentanyl	5.268	2912	n-benzyl Furanyl Norfentanyl	12.862	3512
AP-238	5.420	2929	o-methoxy Butyryl Fentanyl	12.95	3517
2,3-seco-Fentanyl	5.821	2973	Cyclopentyl Fentanyl	12.956	3520
2-Methyl AP-237	5.882	2980	m-methyl Methoxyacetyl Fentanyl	12.979	3520
MT-45	5.915	2983	o-methyl Methoxyacetyl Fentanyl	13.026	3523
2-fluoro MT-45	6.060	2999	p-Chlorobutyryl Fentanyl	13.241	3535
Fentanyl Methyl Carbamate	6.480	3039	p-Methoxyfentanyl	13.463	3551
6-Monoacetylmorphine	6.559	3047	p-methyl Methoxyacetyl Fentanyl	13.51	3551
U-47700	6.806	3071	p-chloro Cyclopropyl Fentanyl	13.667	3560
Fentanyl Carbamate	6.812	3071	p-fluoro Cyclopentyl Fentanyl	13.748	3565
Isopropyl U-47700	6.818	3072	p-methoxy Acetyl fentanyl	13.876	3573
U-48800	7.011	3091	p-methoxy Acrylfentanyl	13.906	3575
Propyl U-47700	7.069	3096	Heptanoyl fentanyl	14.069	3584
Benzyl Fentanyl	7.110	3103	p-Bromofentanyl	14.256	3594

Compound	RT (min)	RI	Compound	RT (min)	RI
Isobutyryl Fentanyl	7.215	3109	p-methyl Cyclopentyl fentanyl	14.268	3596
Benzyl Acrylfentanyl	7.302	3116	p-methoxy Butyryl Fentanyl	14.39	3603
3,4-Methylenedioxy U-47700	7.320	3118	Tetrahydrofuran Fentanyl	14.424	3607
cis-Isofentanyl	7.523	3135	p-fluoro Tetrahydrofuran Fentanyl	14.652	3618
Thienyl Fentanyl	7.576	3139	p-chloro Valeryl Fentanyl	14.745	3624
N-benzyl p-fluoro Norfentanyl	7.664	3144	m-fluoro Furanyl Fentanyl	14.821	3631
Remifentanil	7.955	3174	Furanyl fentanyl 3-furancarboxamide isomer	14.827	3632
Remifentanil Acid	8.037	3178	Cyclohexyl Fentanyl	14.914	3634
o-Fluoroisobutyryl Fentanyl	8.153	3188	4-Phenyl fentanyl	14.955	3636
o-fluoro Acrylfentanyl	8.171	3189	4-phenyl U-51754	15.089	3643
AH 7921	8.176	3189	o-fluoro Furanyl Fentanyl	15.124	3645
m-Fluoroisobutyryl Fentanyl	8.206	3192	Furanyl fentanyl	15.235	3652
U-49900	8.241	3198	Cyclopentenyl fentanyl	15.258	3653
Oxycodone	8.274	3198	p-fluoro Furanyl Fentanyl 3-furacncarboxamide	15.353	3659
β-methyl Fentanyl	8.299	3200	N-Benzyl phenyl norfentanyl	15.404	3661
Sufentanil	8.433	3210	p-fluoro Furanyl Fentanyl	15.474	3666
β-methyl Acetyl Fentanyl	8.497	3214	p-chloro Methoxyacetyl Fentanyl	15.631	3675
Pivaloyl Fentanyl	8.579	3221	Alfentanil	15.672	3677
FIBF	8.580	3222	p-methoxy Valeryl fentanyl	15.928	3692
N-benzyl p-fluoro Cyclopropyl norfentanyl	8.596	3225	p-methyl Tetrahydrofuran fentanyl	16.03	3697
U-51754	8.608	3230	o-methyl Furanyl fentanyl	16.173	3706
trans-3-methyl Fentanyl	8.678	3231	m-methyl Furanyl fentanyl	16.197	3707
o-Fluorofentanyl	8.712	3231	p-chloro Cyclobutyl fentanyl	16.319	3714
2'-fluoro-o-fluorofentanyl	8.724	3232	p-methyl Furanyl fentanyl	16.954	3749
Fentanyl	8.847	3243	2',5'-dimethoxy Fentanyl	17.001	3751
m-Fluorofentanyl	8.870	3243	N-(2C-TFM) Fentanyl	17.024	3752
Acetyl fentanyl	8.941	3249	N-(2-APB) Fentanyl	17.03	3753
Benzyl Carfentanil	8.946	3249	N-(MDA) Fentanyl	17.141	3759
Acrylfentanyl	8.946	3250	N-(2C-D) Fentanyl	17.223	3764
trans-3-methyl Thiofentanyl	9.020	3254	p-chloro Cyclopentyl fentanyl	17.397	3773
N,N-Dimethylamido-despropionyl fentanyl	9.039	3255	N-(6-APB) Fentanyl	17.438	3775
Thiofentanyl	9.077	3259	N-(2,5-DMA) Fentanyl	17.444	3777
cis-3-methyl Fentanyl	9.097	3266	Tetrahydrothiophene fentanyl	17.514	3779
p-methyl Isobutyryl Fentanyl	9.150	3267	N-(DOM) Fentanyl	17.578	3782
α'-methyl Butyryl Fentanyl	9.167	3267	p-methoxy Methoxyacetyl fentanyl	17.584	3784
Heroin	9.214	3269	N-(2C-iP) Fentanyl	17.753	3793
p-Fluorofentanyl	9.226	3274	N-(2C-E) Fentanyl	17.759	3793

Compound	RT (min)	RI	Compound	RT (min)	RI
Metodesnitazene	9.343	3279	Tetrahydrofuran Fentanyl 3-THFcarboximamide	17.8	3796
p-fluoro Acrylfentanyl	9.389	3282	o-isopropyl Furanyl fentanyl	17.974	3805
o-Fluorobutyryl Fentanyl	9.453	3287	Phenyl fentanyl	18.022	3807
Butyryl Fentanyl	9.474	3289	N-(DOET) Fentanyl	18.173	3815
p-Fluoro acetyl Fentanyl	9.482	3289	N-(2C-P) Fentanyl	18.971	3858
4'-Fluorofentanyl	9.482	3289	p-chloro Furanyl fentanyl	18.989	3859
cis-3-methyl Thiofentanyl	9.534	3293	N-(6-APDB) Fentanyl	19.07	3864
m-Methylfentanyl	9.569	3298	o-methoxy Furanyl Fentanyl	19.14	3867
m-Fluorobutyryl Fentanyl	9.604	3298	Thiophene fentanyl	19.245	3873
o-Methylfentanyl	9.622	3300	N-(2C-G) Fentanyl	19.292	3875
Isovaleryl Fentanyl	9.627	3300	o-methyl Phenyl fentanyl	19.373	3880
α-methyl Fentanyl	9.715	3306	Phenylacetyl fentanyl	19.834	3904
Cyclopropyl Fentanyl	9.767	3308	p-Toluoyl fentanyl	20.079	3917
o-methyl Acrylfentanyl	9.773	3310	p-methoxy Tetrahydrofuran fentanyl	20.364	3932
α-methyl Acetyl Fentanyl	9.913	3320	β'-Phenyl fentanyl	20.86	3959
m-methyl Acetyl fentanyl	9.919	3320	N-(DOBU) Fentanyl	20.982	3965
3'-methyl Fentanyl	9.919	3320	Noscapine	21.224	3977
o-methyl Acetyl fentanyl	9.923	3321	p-methoxy Furanyl fentanyl	21.431	3989
cis-3-methyl Butyryl Fentanyl	9.930	3321	Flunitazene	21.799	4009
p-Methylfentanyl	9.971	3323	N-(2C-C) Fentanyl	22.008	4019
Etodesnitazene	9.980	3324	N-(3,4,5-TMA) Fentanyl	22.392	4040
α-methyl Thiofentanyl	10.012	3326	N-(DOC) Fentanyl	22.404	4040
4'-fluoro, p-fluoro-trans-3-methyl Fentanyl	10.059	3329	N-(2C-B) Fentanyl	23.931	4121
p-Fluorobutyryl Fentanyl	10.112	3333	Etizolam	24.097	4130
Isodesnitazene	10.126	3334	Phenoxyacetyl fentanyl	24.188	4135
4'-methyl Fentanyl	10.129	3334	N-(DOB) Fentanyl	24.31	4141
3'-methyl Acetyl fentanyl	10.141	3335	Brorphine	24.916	4173
Senecioylfentanyl	10.146	3336	N-(3-ethylindole) Norfentanyl	24.934	4174
Quinine	10.208	3340	N-(2C-T-4) Fentanyl	25.4	4199
4'-methyl Acetyl Fentanyl	10.216	3340	N-(2C-T) Fentanyl	25.644	4212
p-methyl Acetyl Fentanyl	10.234	3342	N-(2C-T-2) Fentanyl	25.826	4221
p-methyl Acryl Fentanyl	10.246	3342	N-(2C-I) Fentanyl	25.867	4222
p-fluoro Cyclopropyl Fentanyl	10.415	3354	N-(DOI) Fentanyl	26.175	4240
α-methyl Butyryl Fentanyl	10.420	3354	Benzodioxole fentanyl	26.951	4281
2'-methyl Fentanyl	10.496	3360	Metonitazene	26.996	4283
m-methyl Cyclopropyl fentanyl	10.560	3364	N-(2C-T-7) Fentanyl	27.044	4285
o-methyl Cyclopropyl Fentanyl	10.630	3368	Isotonitazene	28.075	4341
p-methyl Butyryl fentanyl	10.636	3369	N-(2C-B-fly) Fentanyl	32.752	4601
2'-methyl Acetyl fentanyl	10.724	3375	N-(3C-B-fly) Fentanyl	33.205	4623
Crotonyl Fentanyl	10.735	3377	N-(2C-N) Fentanyl	33.665	4654

Supplemental Table 5. Instrumental parameters for the general confirmation method used for comparison purposes (Step 5).

Column	DB-5
Temperature Program	1) 180 °C starting temperature 2) Ramp 30 °C min^{-1} to 280 °C 3) Hold 8 min
Flow Rate	1) 1.8 mL min^{-1}, hold for 5 min 2) Ramp 0.5 mL min^{-1} to 2.0 mL min^{-1} 3) Hold at 2.0 mL min^{-1}
Injection Volume	1.0 µL
Inlet Temperature	250 °C
Split Ratio	50:1
Transfer Line	280 °C
Quad Temperature	150 °C
Source Temperature	230 °C
Tune Mode	stune
Solvent Delay	1.0 min
Mass Scan Range	*m/z* 40 – *m/z* 550
Threshold	150
Scan Speed	N = 2
Total Run Time	11.33 min

Supplemental Table 6. Metrics for the comparison of the targeted method to the general screening method (Step 5). Uncertainties represent the standard deviation of triplicate measurements. The notation "NEP" indicates a non-eluting peak. Compounds are listed in the order they elute on the DB-5 column (general confirmatory method). The number in parenthesis to right of the name is the elution order on the DB-200 column.

	Approximate LOD (µg mL^{-1})		**%RTD Between Neighboring Compounds**		**Peak Area (Counts/s)**	
	Targeted	**General**	**Targeted**	**General**	**Targeted**	**General**
m-FIBF (1)	1	10	5.45 (±0.04)	1.29 (±0.07)	$1.1x10^6$ ($±3.6x10^4$)	$2.0x10^5$ ($±3.3x10^4$)
p-FIBF (2)	1	10	2.02 (±0.04)	4.60 (±0.00)	$1.2x10^6$ ($±3.9x10^4$)	$2.5x10^5$ ($±2.1x10^4$)
Fentanyl (3)	1	25	11.63 (±0.04)	9.89 (±0.01)	$1.6x10^6$ ($±5.1x10^4$)	$3.2x10^5$ ($±2.3x10^4$)
Methoxyacetyl Fentanyl (7)	10	25	27.73 (±0.02)	0	$1.2x10^6$ ($±3.3x10^4$)	$3.4x10^5$ ($±7.0x10^3$)
Cyclopropyl Fentanyl (4)	1	25	9.84 (±0.03)	0	$1.5x10^6$ ($±5.3x10^4$)	$3.1x10^5$ (±4.1 $x10^3$)
Crotonyl Fentanyl (5)	10	25	10.49 (±0.00)	1.32 (±0.04)	$1.5x10^6$ ($±6.2x10^4$)	$3.1x10^5$ ($±4.1x10^3$)
Carfentanil (6)	10	25	0.83 (±0.03)	17.55 (±1.68)	$1.1x10^6$ ($±3.5x10^4$)	$2.7x10^5$ ($±1.0x10^4$)
Furanyl Fentanyl (8)	1	25	40.01 (±0.05)	0.76 (±0.05)	$1.2x10^6$ ($±3.1x10^4$)	$2.9x10^5$ ($±8.3x10^2$)
Etizolam (10)	25	50	13.45 (±0.03)	40.09 (±8.09)	$2.0x10^5$ ($±6.0x10^4$)	$1.2x10^5$ ($±1.1x10^4$)
Noscapine (9)	10	50	11.91 (±0.04)	N/A	$1.1x10^6$ ($±2.6x10^4$)	$2.5x10^5$ ($±1.7x10^4$)
Benzodioxole Fentanyl (11)	25	NEP	N/A	N/A	$5.3x10^5$ ($±2.0x10^4$)	NEP