

# Rapid Identification and Quantification of Novel Psychoactive Substances in Human Whole Blood Using SWATH<sup>®</sup> Acquisition

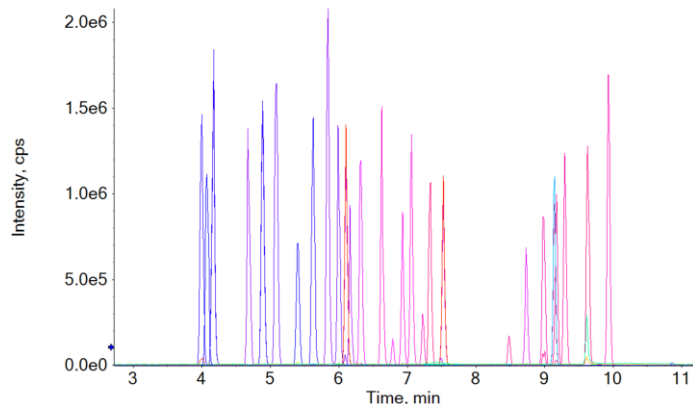
## Using Data Independent Acquisition on the SCIEX TripleTOF<sup>®</sup> 5600<sup>+</sup> LC-MS/MS System

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Every year, the Drug Enforcement Administration (DEA) Special Testing and Research Laboratory publishes a yearly Emerging Threat Report listing the Novel Psychoactive Substances (NPS) the agency has seized and analyzed.<sup>1</sup> This concise report is meant to provide an annual snapshot of current and emerging NPS markets in the United States. As the surge of novel synthetic opioids and other synthetic drug classes continue to pose serious public health and safety problems, timely and comprehensive drug screening approaches are critically needed in the forensic laboratory to quickly and accurately identify these emerging novel substances.

The combination of LC separation coupled to tandem mass spectrometry detection (e.g., LC-MS/MS, LC-QTOF-MS) provides forensic investigators the speed and confidence required to reliably identify novel drugs of abuse and other toxicology compounds present in a variety of complex matrices. In addition, acquisition of accurate mass data and analyte-specific MS/MS fragment spectra provides increased confidence in compound identification. More specifically, high-resolution mass spectrometry (HRMS) offers forensic laboratories a powerful tool for the detection and identification of NPS by reliably obtaining comprehensive MS/MS spectral fragment information on every detectable component in the sample at low analyte concentration with high levels of selectivity and sensitivity.

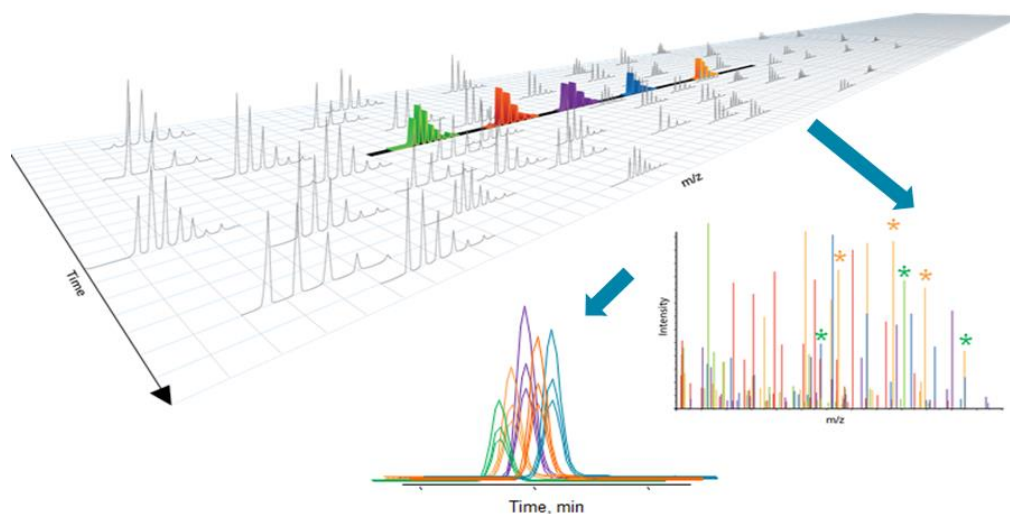


**Figure 1: Chromatographic Profile of the NPS Panel by LC-MS Analysis.** Total Ion Chromatogram (TIC) resulting from total or near baseline separation of 42 compounds in a 15-minute runtime.



## Key Features of SWATH Acquisition for NPS Identification and Quantitation

- The NPS quantitation panel consisted of 30 representative NPS listed in the DEA's Emerging Threat Report, as well as 12 internal standards. The NPS qualitative panel consisted of more than 600 NPS and NPS metabolites
- The high scanning speed (up to 100 Hz for single collision energy) of the TripleTOF 5600<sup>+</sup> system allowed detection of all target analytes in the NPS panel
- SWATHAcquisition generated comprehensive and high-quality MS/MS spectra with no loss in sensitivity, enabling confident drug identification using spectral library searching
- Analyte extraction recoveries were demonstrated to be greater than 80%, allowing sub ng/mL detection limits of these drugs in a complex biological matrix while maintaining correlation and precision for all compounds across the calibration range
- The TripleTOF 5600<sup>+</sup> System enabled simultaneous quantitation and confirmation of the NPS by utilizing the more selective MS/MS information as well as using both ion ratio and MS/MS library searching for confident identification of all the NPS used in this workflow



**Figure 2: MS/MS<sup>ALL</sup> with SWATH Acquisition for Screening of Novel Psychoactive Substances (NPS).** In SWATH Acquisition, Q1 isolation windows are stepped across the mass range in an LC timescale, transmitting populations of analytes for fragmentation, and high-resolution composite MS/MS spectra are acquired at each step. This produces a richer MS/MS spectrum which is a composite of all the analytes within that Q1 m/z window. MS/MS acquisition is performed across the full m/z range of target analytes, ensuring MS/MS fragment data is collected on every detectable compound. Because the fragment ions are generated using high-resolution acquisition, detected compounds can be accurately identified through extraction of the specific, accurate mass product ions (in addition to extract of precursor ions from the TOF-MS scan).

## Experimental Details

**Sample Preparation:** A total of 30 target analytes were selected for quantitation representing all of the drug classes reported in the DEA's Emerging Threat Report, in addition to the >600 NPS incorporated into the screening workflow. These NPS consisted of 7 synthetic cannabinoids, 2 dissociatives, 3 hallucinogens, 6 stimulants, 5 benzodiazepines and 7 opioids. A full list of the 30 NPS used in this method is detailed in Table 1, including accurate mass information and fragment ion masses used for ion ratio determinations. Extraction of whole human blood samples was performed according to the liquid-liquid extraction (LLE) procedure shown in Figure 3.

**Internal Standards:** Methylone-D3, Alpha-PVP-D8, Ketamine-D4, PCP-D5, Diazepam-D5, Alprazolam-D5, U-47700-D6, Fentanyl-D5, JWH-018-D9, Cyclopropyl Fentanyl-D5, AM-2201-D5, and AB-FUBINACA-D4 were used as internal standards.

**Calibrator Preparation:** A 10 ng/μL stock standard solution mixture containing all the NPS used for quantitation in this study was prepared via dilution of 1 mg/mL stock standards with methanol. A series of calibration solutions were prepared using the 10 ng/μL stock standard solution mixture to evaluate the dynamic range. These, in turn, were used to spike 500 μL of human whole blood in order to prepare a calibration series covering concentrations ranging from 1 ng/mL to 100 ng/mL. Finally, 25 μL of the 1 ng/μL IS spiking stock solution was added to each human whole blood mixture.

Load to tube	•500 μL human whole blood spiked with calibrator solutions
Load to tube	•25 μL of 1 ng/μL IS stock solution
Load to tube	•1mL of Borax buffer, pH 10.4 and vortex for 5 sec
Load to tube	•3 mL of 70:30 n-butyl chloride : ethyl acetate
Rotate	•Cap and rotate for 10 min at 40%
Uncap & Freeze	•Uncap the tube and freeze at -80°C for 15 min
Transfer	•Transfer supernatant to new tubes
Load to tube	•100 μL of HCl in MeOH
Dry	•Dry down in TurboVap at 35 °C, 10 psi for 30 min
Reconstitute	•Add 200 μL of 95:5 A:B to tube and vortex
Transfer	•Transfer to ALS glass vial and inject 10 μL onto instrument

**Figure 3. Liquid-Liquid Extraction (LLE) Workflow for Human Whole Blood.** A 10-step extraction protocol was used for selectively extracting the 30 target analytes and internal standards from human whole blood samples for analysis with the TripleTOF 5600+ System.

**Liquid Chromatography:** UHPLC separation was performed on a Phenomenex Kinetex C18 column (50 × 3 mm, 2.6μm, 00B-4462-Y0) on the Shimadzu Nexera system. Mobile phase A (MPA) and mobile phase B (MPB) were ammonium formate with formic acid and formic acid in methanol and acetonitrile, respectively. The injection volume was 10 μL and the total LC runtime was 15.5 minutes.

**Mass Spectrometry:** MS and MS/MS data were collected using SWATH Acquisition on the SCIEX TripleTOF 5600+ System with Analyst 1.7 software. Each SWATH Acquisition scan began with a TOF-MS experiment (50-510 Da).

## Optimized Liquid-Liquid Extraction (LLE) Procedure Leads to High NPS Recovery

One of the main challenges associated with the detection of analytes extracted from a biological matrix, such as whole blood, is the presence of complex matrix contents, some of which are structurally similar to the analytes in this panel. As a result, robust and reliable extraction procedures are critical in achieving the desired reproducibility, linear response, and limits of quantitation (LOQ). To assess the efficiency of the LLE procedure used in this experiment, the recovery (RE) was calculated using 80 ng/mL of each NPS. The average (n=3) peak area ratio (analyte area to internal standard area) of a pre-spike sample can be depicted as "C" and the peak area ratio of a post-spike sample as "B"; therefore, the RE value for each analyte can be calculated as follows:

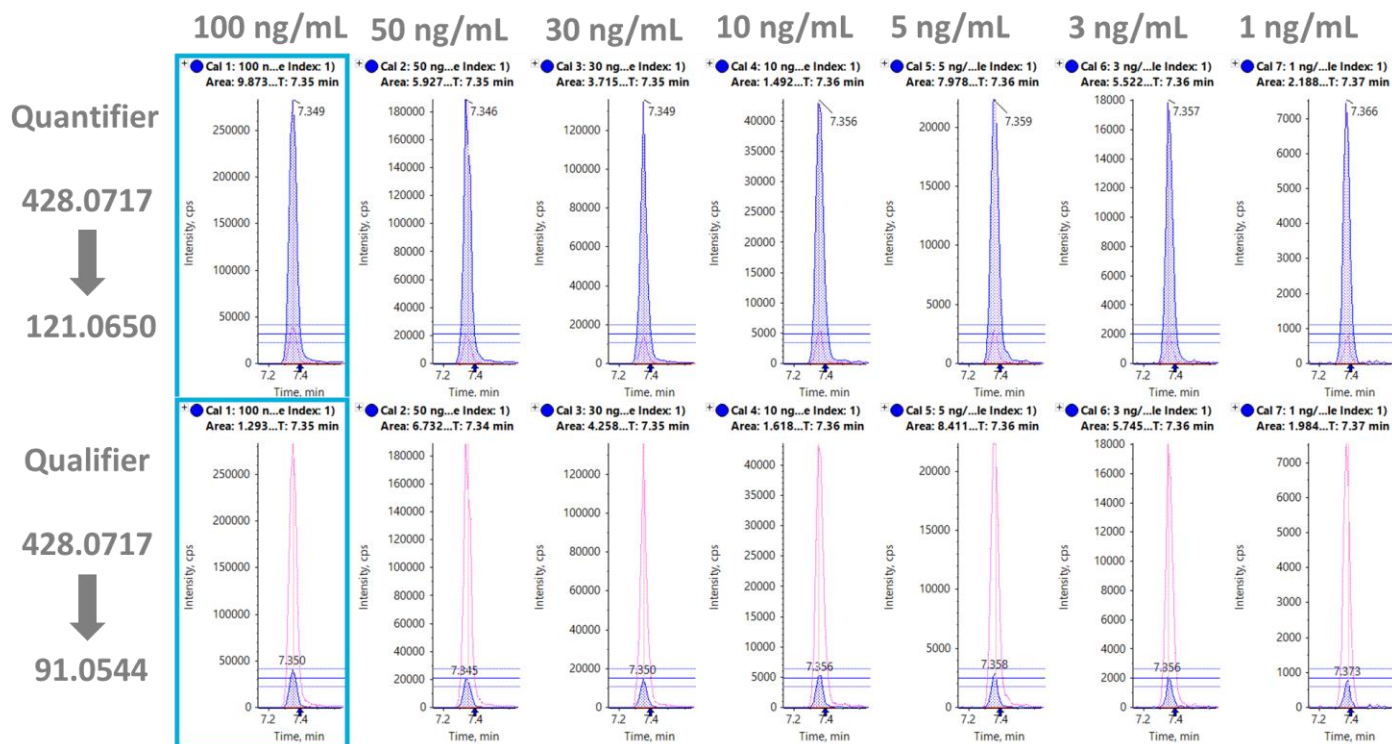
$$RE (\%) = C/B \times 100 \quad (1)$$

The LLE procedure used in this experiment demonstrated excellent recoveries of the analytes used in this panel (>80%). The RE values for each NPS are summarized in Table 1. The high recovery values for the analytes in the panel allowed reliable quantitation, which is only possible through the implementation of an optimized, universal LLE procedure.

## Analytical Sensitivity of the TripleTOF 5600+ LC-MS/MS System

Following the LLE procedure, 10 µL of reconstituted solution were injected for each calibrator sample. A processing method for the 30 target analytes and 12 internal standards was developed to review the SWATH Acquisition data in SCIEX OS Software 1.5. Detection and integration of the peaks was easily accomplished using the MQ4 algorithm in Analytics, which facilitated selection of the correct peak within the viewing window of closely eluting isobaric analytes. As this data was acquired using SWATH Acquisition, peak area ratio (PAR) and ion ratio (IR) determinations can be performed by extraction of accurate mass fragment ions the MS/MS spectra. Quantitation was achieved by plotting the PAR of the analyte to internal standard. The IR was calculated by dividing the area of the two most intense fragments, the quantifier ion and qualifier ion.

Figure 4 shows the extracted ion chromatogram (XIC) traces for both the quantifier (428.0717 Da → 121.0650 Da) and qualifier (428.0717 Da → 91.0544 Da) ions for 25I-NBOMe. The XIC traces for all the compounds showed a high level of consistency and precision across the calibration series. Quantitation results were processed using SCIEX OS Software, which allowed fast data processing with less manual intervention and streamlined reporting tools.



**Figure 4: Streamlined Data Processing of 25I-NBOMe Using SCIEX OS 1.5 Software.** XIC traces and peak integration of 25I-NBOMe using the quantifier (428.0717 Da → 121.0650 Da) and qualifier (428.0717 Da → 91.0544 Da) ions with the ion ratio line overlay displayed. The XIC traces demonstrate reliable quantitation from 1 to 100 ng/mL.

Calibration curves were generated to evaluate the response, dynamic range and quantification performance (precision and accuracy) of the assay. Figure 5 shows the results from the calibration series performed for 251-NBOMe including the calibration curves for both ions and statistics of quantitation generated using this comprehensive method. Seven levels of calibrators ranging from 1 to 100 ng/mL were used to determine the ion ratio criteria for the quantifier and qualifier ions of 251-NBOMe. The results demonstrated excellent correlation of the generated regression curves covering a broad dynamic range from 2 to 3 orders of magnitude. The assay showed excellent precision (<15%) and accuracy (>95%), and the  $R^2$  values for the quantifier and qualifier ions were 0.99965 and 0.99967, respectively. Full quantification was achieved with SCIEX OS 1.5, designed for quick, intuitive and streamlined data processing with accurate and reliable results.

## Full Scan MS/MS with SWATH Acquisition Leads to Accurate Compound Identification

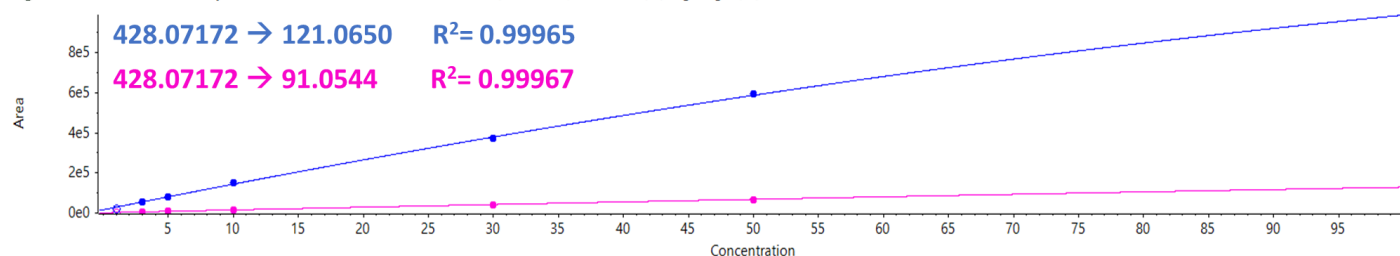
SWATH Acquisition is a non-targeted method that provides a comprehensive qualitative and quantitative analysis of complex samples by acquiring full scan high resolution MS and MS/MS data on all detectable compounds eluting during an LC run. It provides selective MS/MS detection of compounds extracted from biological samples where matrix interference is common.

A great advantage to the use of the SCIEX OS Software is that it allows for simultaneously quantification using ion ratio determination and library searching confirmation. This processing is performed at the same time and allows for the results to be displayed within one window. Figure 6 (Bottom) shows the peak integration for an extracted ion chromatogram (XIC) of valeryfentanyl, as well as the TOF MS and TOF MS/MS spectra with library search match. A library fit score of 100 for the identification of valeryfentanyl provides excellent confidence for a definitive detection of this NPS. Also displayed in Figure 6 (Top) are the results from the calibration curves resulting from the peak area integration of the compound at concentrations ranging from 1 to 100 ng/mL. Overall, excellent linearity and reproducibility was observed across the entire concentration range assessed for all the compounds in this panel. Using this comprehensive method, the 30 NPS used in this study were quantitatively identified and confirmation was achieved using MS/MS library searching with excellent library scores (>80% for all NPS analytes).

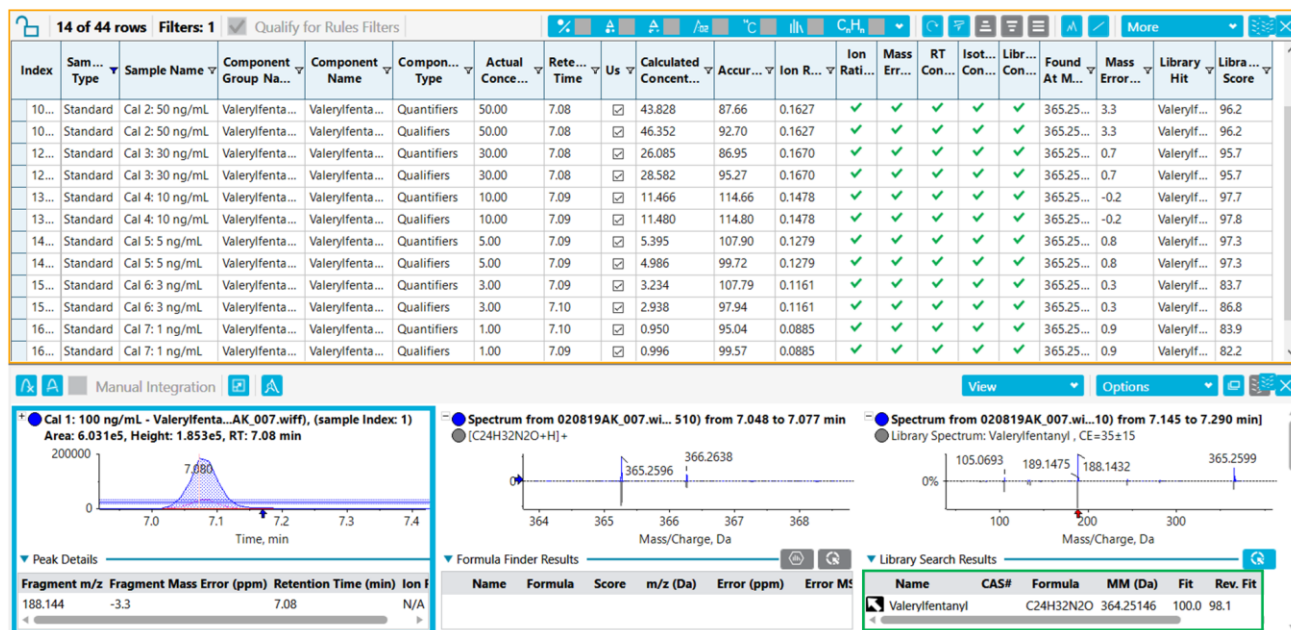
The processing method used in this workflow was optimized on the SCIEX TripleTOF 5600+ System. However, it is worth noting that the same processing method can be easily transferred to the SCIEX X500R QTOF System and used for additional screening. This feature allows data sets from other panels to be re-processed when newly identified forensic targets are discovered.

Index	Sample Type	Sample Na...	Component Name	Compon... Type	Component Group Na...	Actual Conce...	Reten... Time	Us	Calculated Concent...	Accur...	Ion R...	Ion Rati...	Mass Err...	RT Con...	Isot...	Libr...	Found At M...	Mass Error...	Libra... Hit	Libra... Score
728	Standard	Cal 1: 100 ng...	251-NBOMe 2	Quantifiers	251-NBOMe	100.00	7.35	<input checked="" type="checkbox"/>	99.904	99.90	0.1310	✓	✓	✓	✓	✓	428.07...	1.9	251-NB...	100.0
729	Standard	Cal 1: 100 ng...	251-NBOMe 3	Qualifiers	251-NBOMe	100.00	7.35	<input checked="" type="checkbox"/>	100.352	100.35	0.1310	✓	✓	✓	✓	✓	428.07...	1.9	251-NB...	100.0
970	Standard	Cal 2: 50 ng/...	251-NBOMe 2	Quantifiers	251-NBOMe	50.00	7.35	<input checked="" type="checkbox"/>	50.534	101.07	0.1136	✓	✓	✓	✓	✓	428.07...	-0.2	251-NB...	97.4
971	Standard	Cal 2: 50 ng/...	251-NBOMe 3	Qualifiers	251-NBOMe	50.00	7.34	<input checked="" type="checkbox"/>	49.191	98.38	0.1136	✓	✓	✓	✓	✓	428.07...	-0.2	251-NB...	97.4
10...	Standard	Cal 3: 30 ng/...	251-NBOMe 2	Quantifiers	251-NBOMe	30.00	7.35	<input checked="" type="checkbox"/>	29.278	97.59	0.1146	✓	✓	✓	✓	✓	428.07...	2.6	251-NB...	100.0
10...	Standard	Cal 3: 30 ng/...	251-NBOMe 3	Qualifiers	251-NBOMe	30.00	7.35	<input checked="" type="checkbox"/>	30.128	100.43	0.1146	✓	✓	✓	✓	✓	428.07...	2.6	251-NB...	100.0
12...	Standard	Cal 4: 10 ng/...	251-NBOMe 2	Quantifiers	251-NBOMe	10.00	7.36	<input checked="" type="checkbox"/>	10.399	103.99	0.1085	✓	✓	✓	✓	✓	428.07...	-0.5	251-NB...	98.5
12...	Standard	Cal 4: 10 ng/...	251-NBOMe 3	Qualifiers	251-NBOMe	10.00	7.36	<input checked="" type="checkbox"/>	10.516	105.16	0.1085	✓	✓	✓	✓	✓	428.07...	-0.5	251-NB...	98.5
13...	Standard	Cal 5: 5 ng/mL	251-NBOMe 2	Quantifiers	251-NBOMe	5.00	7.36	<input checked="" type="checkbox"/>	4.896	97.91	0.1054	✓	✓	✓	✓	✓	428.07...	-1.3	251-NB...	98.6
13...	Standard	Cal 5: 5 ng/mL	251-NBOMe 3	Qualifiers	251-NBOMe	5.00	7.36	<input checked="" type="checkbox"/>	4.871	97.43	0.1054	✓	✓	✓	✓	✓	428.07...	-1.3	251-NB...	98.5
14...	Standard	Cal 6: 3 ng/mL	251-NBOMe 2	Quantifiers	251-NBOMe	3.00	7.36	<input checked="" type="checkbox"/>	2.986	99.54	0.1040	✓	✓	✓	✓	✓	428.07...	1.7	251-NB...	100.0
14...	Standard	Cal 6: 3 ng/mL	251-NBOMe 3	Qualifiers	251-NBOMe	3.00	7.36	<input checked="" type="checkbox"/>	2.948	98.26	0.1040	✓	✓	✓	✓	✓	428.07...	1.7	251-NB...	100.0

● Calibration for 251-NBOMe 2:  $y = -34.15313 x^2 + 13131.10399 x + 16309.65975$  ( $r = 0.99983$ ,  $r^2 = 0.99965$ ) (weighting: 1 / x)  
 ● Calibration for 251-NBOMe 3:  $y = -1.23203 x^2 + 1395.87724 x + 1640.84585$  ( $r = 0.99983$ ,  $r^2 = 0.99967$ ) (weighting: 1 / x)



**Figure 5: Accurate and Reliable Quantitation Using SCIEX OS Software.** Calibrations curves resulting from the calibration series for 251-NBOMe from 1 to 100 ng/mL for both the quantifier and qualifier ions. The calibration curves show excellent correlation, precision and accuracy.



**Figure 6. Comprehensive and Streamlined Data Processing Using SCIEX OS Software Leads to Accurate Compound Identification.** Using multiple acceptance criteria enabled the accurate identification of all NPS showcased in this quantitative workflow. (Top) Calibration series for valerylentanyl ranging from 1 to 100 ng/mL for both the quantifier and qualifier ions passing the acceptance criteria (green traffic light) for ion ratio, mass error, isotope and library match. Also included are the mass error and the library score for valerylentanyl (Bottom), including the XIC, TOF-MS spectrum and MS/MS spectrum along with spectral library match.

## Conclusion

A comprehensive drug screening workflow using the TripleTOF 5600+ System for the analysis of NPS listed in the DEA's Emerging Threat Report is described. The results demonstrate the use of SWATHAcquisition in combination with SCIEX OS Software makes this workflow easily adaptable into a forensic toxicology laboratory and compatible with high throughput laboratory screening workflows.

- A targeted drug screening method using SWATH Acquisition was developed for fast, selective, and quantitative detection of low concentration NPS in a single analysis
- A 10-step liquid-liquid extraction protocol was rapidly implemented to extract the analytes from human whole blood
- Analyte extraction recoveries were demonstrated to be greater than 80%, enabling the analytical workflow to obtain sub ng/mL lower limits of quantification (LLOQ) for most analytes in a complex biological matrix.
- The data-independent acquisition strategy allowed ion ratio determinations by extraction of multiple accurate mass fragment ions from the MS/MS spectra in combination with library searching for confident identification
- The workflow showed excellent precision (<15%) and accuracy (>95%), with excellent linearity resulting in R<sup>2</sup> values above 0.990 for all analytes

## References

1. <https://ndews.umd.edu/sites/ndews.umd.edu/files/dea-emerging-threat-report-2017-annual.pdf>

**Table 1. List of the Analytes, (Including Accurate Mass Information and Fragment Ions Used for Ion Ratio Determination), Internal Standard (IS) Used, and Recovery Values For Each of the 32 NPS Quantitatively Confirmed in This Workflow.**

Analyte Name	Chemical Formula	Precursor Mass (Da)	Fragment Mass (Da) # 1	Fragment Mass (Da) # 2	Retention Time (min)	Internal Standard	Recovery (%)
25I-NBOMe	C18H22INO3	428.07172	121.0650	91.0544	7.39	Methylone-D3	90.1
2F-Deschloroketamine	C13H16FNO	222.12887	163.0924	191.0877	4.21	Ketamine-D4	97.0
4-Cl-alpha-PVP	C15H20ClNO	266.13062	195.0586	125.0158	6.15	Alpha-PVP-D8	93.4
4-MeO-PCP	C18H27NO	274.21654	189.1285	121.065	6.25	PCP-D5	98.8
5F-ADB	C20H28FN3O3	378.21875	233.1086	318.1973	9.62	AM-2201-D5	86.3
5F-CUMYL-PeGACLONE	C25H27FN2O	391.21802	273.1395	119.0851	9.43	JWH-018-D9	92.2
5F-EDMB-PINACA	C21H30FN3O3	392.2344	318.198	233.1095	9.92	AM-2201-D5	80.5
5F-MDMB-PICA	C21H29FN2O3	377.2235	232.1139	144.0452	9.33	Methylone-D3	91.7
ADB-FUBINACA	C21H23FN4O2	383.18778	338.1677	253.0784	8.65	AB-FUBINACA-D4	94.6
Alpha-PHP	C16H23NO	246.18524	140.143	105.0332	5.88	Alpha-PVP-D8	88.5
Clonazepam	C17H12ClN5O2	354.07523	308.0827	326.0575	6.94	AM-2201-D5	91.8
CUMYL-PeGACLONE	C25H28N2O	373.22744	255.1489	119.0854	10.2	JWH-018-D9	84.9
Cyclopropyl Fentanyl	C23H28N2O	349.22744	188.1435	105.0698	6.48	Cyclopropyl Fentanyl-D5	93.7
Deschloroketamine	C13H17NO	204.13829	173.0965	145.1015	4.29	Ketamine-D4	98.4
Dibutylone (bk-DMBDB)	C13H17NO3	236.12812	191.0706	161.0601	4.28	Methylone-D3	95.0
DOM	C12H19NO2	210.14886	193.123	165.0917	5.48	Methylone-D3	102.3
Etizolam	C17H15ClN4S	343.07787	314.0396	309.0926	7.7	Alprazolam-D5	88.6
Fentanyl	C22H28N2O	337.22744	188.1443	105.0705	6.2	Fentanyl-D5	84.9
Flubromazepam	C17H12BrFN4	371.03021	292.1127	343.0125	7.39	Alprazolam-D5	88.8
Flubromazepam	C15H10BrFN2O	333.00333	226.0898	183.9751	7.65	Diazepam-D5	95.6
Fluoroisobutyl Fentanyl	C23H29FN2O	369.23367	188.1441	105.0703	6.88	Fentanyl-D5	91.1
Flutoprazepam	C19H16ClFN2O	343.1008	289.0525	140.0255	9.04	Diazepam-D5	103.1
Isopropyl-U-47700	C18H26Cl2N2O	357.1495	314.0898	312.0926	7.09	U-47700-D6	88.1
Methylenedioxy-alpha-PHP	C17H23NO3	290.17507	219.1025	189.0916	6.01	Alpha-PVP-D8	87.5
Methylenedioxy-U-47700	C17H24N2O3	305.18597	260.1276	149.0232	4.9	U-47700-D6	96.7
MMB-FUBINACA	C21H22FN3O3	384.1718	253.078	324.1516	9.42	AM-2201-D5	88.4
N-Ethyl Hexedrone (Hexen)	C14H21NO	220.16959	202.1597	146.0967	5.81	Methylone-D3	88.9
N-Ethyl Pentylone	C14H19NO3	250.14377	232.1339	202.1234	5.26	Methylone-D3	93.4
Pentylone	C13H17NO3	236.12812	188.1075	218.1182	4.98	Methylone-D	95.4
U-47700	C16H22Cl2N2O	329.1182	284.0623	172.9573	6.21	U-47700-D6	93.7
Valeryl fentanyl	C24H32N2O	365.25874	188.1438	105.0702	7.17	Fentanyl-D5	90.1

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