Enhancing NPS characterization using electron-activated dissociation (EAD)

Using the ZenoTOF 7600 system powered by SCIEX OS software

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Here, electron-activated dissociation (EAD) fragmentation on the ZenoTOF 7600 system was used to confirm the detection of multiple classes of structurally similar and isobaric novel psychoactive substances (NPS), including newly emerging fentanyl opioids, halogenated fentanyl analogs, novel synthetic opioids (NSO) and synthetic cannabinoids.[1] The combination of the Zeno trap with EAD provides the MS/MS sensitivity and selectivity to improve confidence in NPS identification and to differentiate isomeric species otherwise indistinguishable using collision-induced dissociation (CID)-based MS/MS methodologies. EAD is a powerful, reagent-free, tunable orthogonal fragmentation technique that can generate unique diagnostic fragment ions to differentiate between structurally similar compounds (Figure 1) and has the potential to provide in-depth characterization of those substances that do not generate unique fragment ions when subjected to CID.

The growing number of NPS emerging on the recreational drug market continues to pose safety concerns for public health and law enforcement officials. NPS are a diverse group of synthetic substances designed to mimic the action and psychoactive effects of controlled substances and are often used as adulterants in heroin and counterfeit preparations. Newly emerging fentanyl opioids, NSO and fentanyl analogs share similar structure and composition, adding additional complexity.

Traditionally, NPS analysis performed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) has used CID for compound fragmentation. In most cases, CID produces unique diagnostic fragment ions that can be used to confidently identify NPS. However, certain classes of NPS, such as isomeric species, do not produce unique fragment ions with CID. Thus, as structurally related NPS have become more prevalent to evade regulations, the challenges to analytically characterize these substances have also increased.

Advantages of EAD on the ZenoTOF 7600 system for NPS characterization

- Zeno data-dependent acquisition (DDA) with EAD provides the specificity and sensitivity required for the characterization of low-level analytes in complex biological matrices, such as discarded postmortem case samples

- Zeno EAD DDA results in increased unique diagnostic fragments, enabling in-depth characterization of NPS and the differentiation of isomeric and structurally related analytes that were previously indistinguishable using CID

- Compatible with drug screening workflows using fast DDA in SCIEX OS software
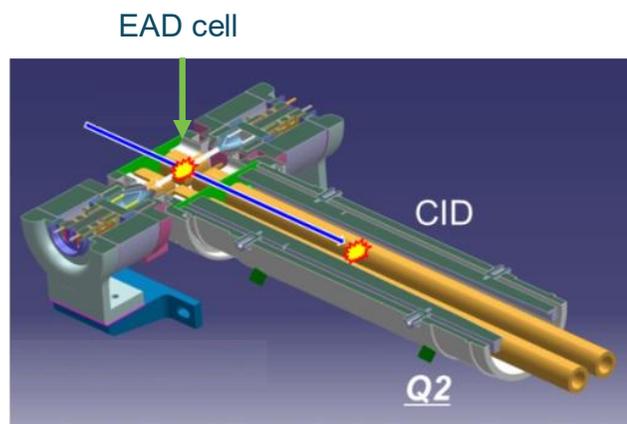

EAD cell

Figure 1. Schematic of the EAD cell on the ZenoTOF 7600 system. EAD provides reproducible and unique fragment ions that enhance the characterization of NPS.

Experimental details

Target analytes: An NPS panel including newly emerging fentanyl opioids, halogenated fentanyl analogs, synthetic opioids and synthetic cannabinoids was selected for method development. Standards were purchased from Cerilliant Corporation (Round Rock, TX) and Cayman Chemical Company (Ann Arbor, MI). Each standard was injected individually twice to generate custom-built CID and EAD MS/MS spectral libraries of high-quality TOF MS/MS spectra for comparison.

Authentic postmortem case samples: Analytes were extracted from human whole blood using a liquid-liquid extraction (LLE) procedure summarized in Figure 2.
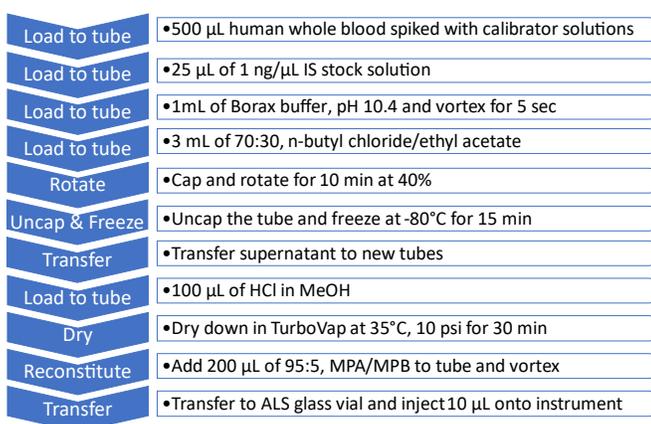
Load to tube	•500 µL human whole blood spiked with calibrator solutions
Load to tube	•25 µL of 1 ng/µL IS stock solution
Load to tube	•1mL of Borax buffer, pH 10.4 and vortex for 5 sec
Load to tube	•3 mL of 70:30, n-butyl chloride/ethyl acetate
Rotate	•Cap and rotate for 10 min at 40%
Uncap & Freeze	•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, MPA/MPB to tube and vortex
Transfer	•Transfer to ALS glass vial and inject 10 µL onto instrument

Figure 2. Liquid-liquid extraction (LLE) procedure for human whole blood samples. A 10-step extraction protocol was used to selectively extract drugs from human whole blood samples for analysis with the ZenoTOF 7600 system.

Liquid chromatography: HPLC separation was performed on an ExionLC system using a Phenomenex Kinetex C18 column (50 × 3.0 mm, 2.6 µm, 00B-4462-Y0). Mobile phase A (MPA) and mobile phase B (MPB) were ammonium formate (pH 5) and formic acid in methanol and acetonitrile, respectively. The flow rate was 0.4 mL/min and the total LC runtime was 15.5 minutes. The injection volume was 10 µL.

Mass spectrometry: MS and MS/MS data were collected twice for each sample using Zeno DDA with CID and Zeno DDA with EAD on the ZenoTOF 7600 system. Data acquisition consisted of a TOF MS scan to collect accurate mass precursor ions from 100 to 700 Da, followed by a full scan TOF MS/MS with the Zeno trap activated, with mass range of 25 to 700 Da to ensure all fragments were captured for identification. For each cycle, a maximum of 16 candidate ions were selected for MS/MS. Data were acquired using SCIEX OS software, version 2.0.1.

Data analysis: Data were processed using SCIEX OS software, version 2.0.1. Detection and integration of the peaks from the background were accomplished using the MQ4 algorithm in the Analytics module of the software. Quantitative and qualitative analyses were then performed. Positive analyte identification was accomplished based on confidence criteria, as previously described.[2] The 4 main confidence criteria used included mass error (M), retention time (R), isotope ratio difference (I) and library score (L). Two separate in-house libraries of CID and EAD MS/MS spectra were generated from standards and used to perform spectral library matching and identification of the drugs present in the discarded authentic postmortem case samples.
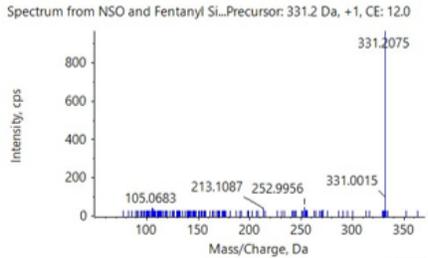
Optimized EAD conditions for reproducible and comprehensive fragment information

Individual neat standard solutions were injected to optimize the EAD parameters, including electron kinetic energy (KE), electron beam current and reaction time. A series of injections were performed with various parameter combinations to achieve optimal sensitivity, reproducibility and selectivity of the generated fragment ions. The collected TOF MS/MS spectra were reviewed individually to determine the optimized EAD parameter values used for the rest of the experiments. These parameters included 10 eV electron KE, 700 nA electron beam current and 35 ms reaction time. These values were used to collect TOF MS and TOF MS/MS spectra of each of the neat standards. The TOF MS/MS spectra were used to build an in-house EAD spectral library that was compared with that generated using CID data.

Zeno MS/MS for improved sensitivity

Average sensitivity gains of ~9x in the TOF MS/MS data have been reported for drugs and metabolites positively identified in discarded postmortem case samples analyzed using CID with the Zeno trap.[3] Here, the use of the Zeno DDA to improve TOF MS/MS sensitivity was investigated using EAD as the fragmentation mechanism. Figure 3 shows representative TOF MS/MS spectra acquired with and without the Zeno trap activated for 3 drugs positively identified in discarded postmortem case samples, including ADB-PINACA, ortho-chlorofentanyl and norbuprenorphine, which are a synthetic cannabinoid, NSO and synthetic opioid, respectively. Without the Zeno trap activated, analysis of these case samples resulted in low-intensity TOF MS/MS spectra. The use of the Zeno trap increased the TOF MS/MS sensitivity of the low abundance fragments, improving compound identification confidence for low levels of drugs and metabolites. Overall, when the Zeno trap was
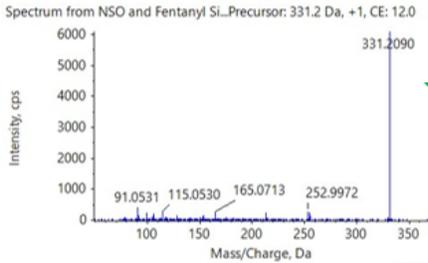
Figure 3. TOF MS/MS sensitivity gains when combining Zeno DDA with EAD for representative NPS. An average of ~8x gain in TOF MS/MS sensitivity was observed across all the analytes positively identified in the discarded postmortem case samples when the Zeno trap was used in combination with EAD.

used, an average 8x improvement was observed in sensitivity across the TOF MS/MS spectra that were positively identified in the discarded postmortem case samples. When combined with EAD, this improvement in sensitivity enabled confident characterization and identification of NPS and metabolites at levels that were not previously achievable.

Comprehensive characterization of synthetic opioids

Some analytes, such as buprenorphine and its main active metabolite norbuprenorphine, are known to fragment poorly when subjected to CID-based fragmentation. As seen in the CID MS/MS spectra shown in Figure 4 (bottom spectra), buprenorphine (A) and norbuprenorphine (B) only produced low-intensity fragment ions that were unreliable for compound characterization and quantification. When EAD was used as the fragmentation technique on the ZenoTOF 7600 system, richer TOF MS/MS spectra containing unique diagnostic fragment ions were generated. As seen in Figure 4, the Zeno EAD MS/MS spectra generated for buprenorphine and norbuprenorphine showed unique diagnostic fragments at m/z 410.2352 and 378.2074 and m/z 356.1843, 338.1772 and 324.1574, respectively. The molecular formulas and the corresponding structures of the identified fragment ions are shown in Figure 4. These results demonstrate that EAD provides richer fragmentation by generating unique diagnostic fragment ions that enable in-depth characterization of these 2 analytes. These identified fragment ions can then be used for downstream development of targeted methods for the sensitive and specific quantification of these analytes.

Differentiation of AP-series NSO

The recent scheduling of fentanyl analogs has sparked the emergence of new classes of NSO. Among those, the cinnamylpiperazine analogs, also known as the AP series isomers, have recently emerged on the recreational drug market. These isomeric species are similar in structure and composition, as they all contain a piperazine core and a cinnamyl moiety. These similarities therefore make their characterization and differentiation analytically challenging. An alternative fragmentation technique such as EAD has the potential to provide additional fragment ions that would enable differentiation of these analogs from one another.

Figure 5 shows the CID MS/MS spectra of AP-238 (top), 2-methyl AP-237 (middle) and para-methyl AP-237 (bottom). The TOF MS/MS spectra for these 3 AP series isomers are similar and share common fragment ions at m/z 131.0848, 117.0692, 115.0536 and 91.0536. The bottom panels in Figure 5 show the

EAD MS/MS spectra of the same 3 AP series isomers. Each spectrum contains unique fragments and spectral differences that enable the differentiation of the 3 isobaric synthetic opioids, as circled in red. These unique spectral features highlight the ability of EAD to provide complementary and unique fragment ions for the in-depth characterization of isomeric compounds, such as the cinnamylpiperazine analogs. The use of EAD also enabled the formation of unique lower molecular weight fragments that enabled the differentiation of these analogs.

In-depth characterization of synthetic cannabinoids

Synthetic cannabinoids are a class of NPS that are designed to mimic the active ingredient of cannabis, delta-9-tetrahydrocannabinol (THC). In recent years, these substances have gained popularity and rapidly emerged on the recreational drug market. Most synthetic cannabinoids have an indole or indazole core structure, which makes them challenging to identify since they share similar structures and identical masses to the corresponding indazole analogs. As a result, an alternative fragmentation technique such as EAD can potentially be used to characterize and identify synthetic cannabinoids.

Figure 6 compares the EAD and CID MS/MS spectra as a mirror image for 3 synthetic cannabinoids, including ADB-BINACA, ADB-PHETINACA and 4F-MDMB-BINACA. The Zeno EAD MS/MS (top) and Zeno CID MS/MS (bottom) spectra for each of the 3 cannabinoids share several fragments. However, EAD provides a much richer fragmentation when compared to CID. As circled in red, the EAD spectra show unique diagnostic fragments that enable in-depth characterization of each of the 3 synthetic cannabinoids. For example, EAD generated 4 unique fragments at m/z 274.1458, 257.1173, 131.0598 and 117.0472 in the TOF MS/MS spectrum of ADB-BINACA and 3 unique fragments at m/z 186.0676, 145.0403 and 91.0529 in the TOF MS/MS spectrum of ADB-PHETINACA. Fragments at m/z 275.1089, 131.0612, 117.0470 and 90.0342 were unique fragments in the TOF MS/MS spectrum of 4F-MDMB-BINACA that were not present in its CID MS/MS spectrum. Also shown are the molecular structures for each of these unique fragment ions generated by EAD. These unique spectral features provided complementary structural information that can be leveraged for in-depth characterization synthetic cannabinoids.
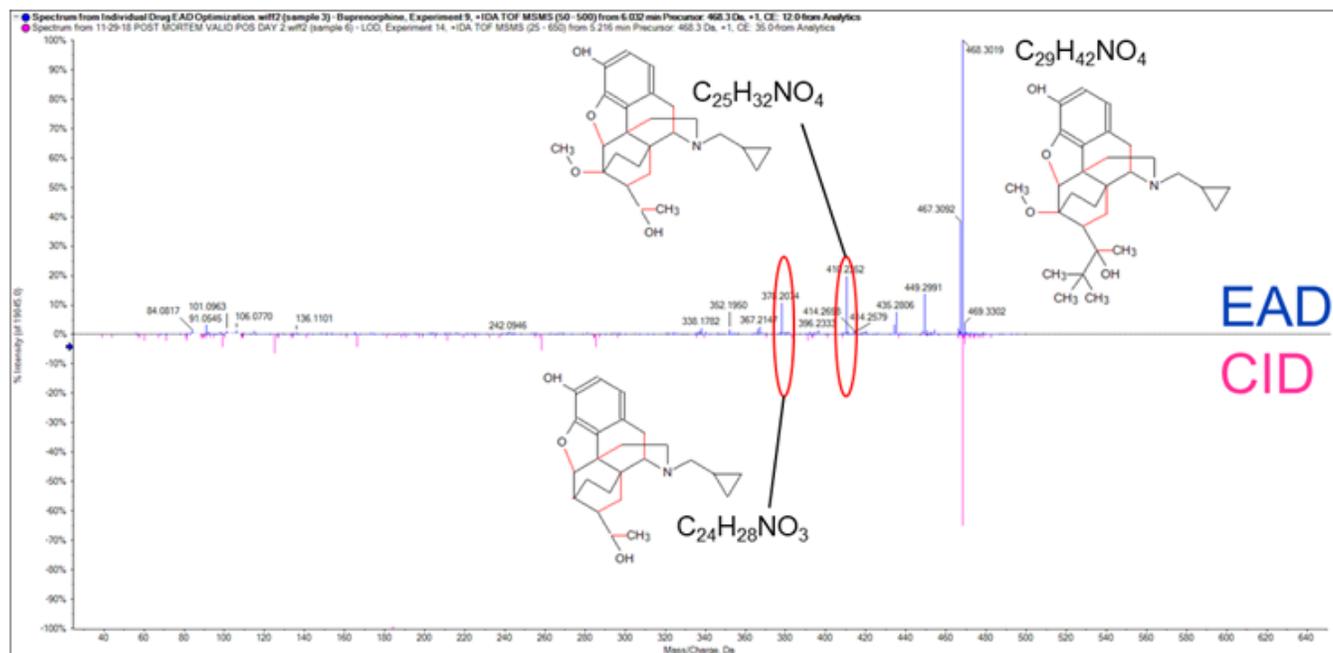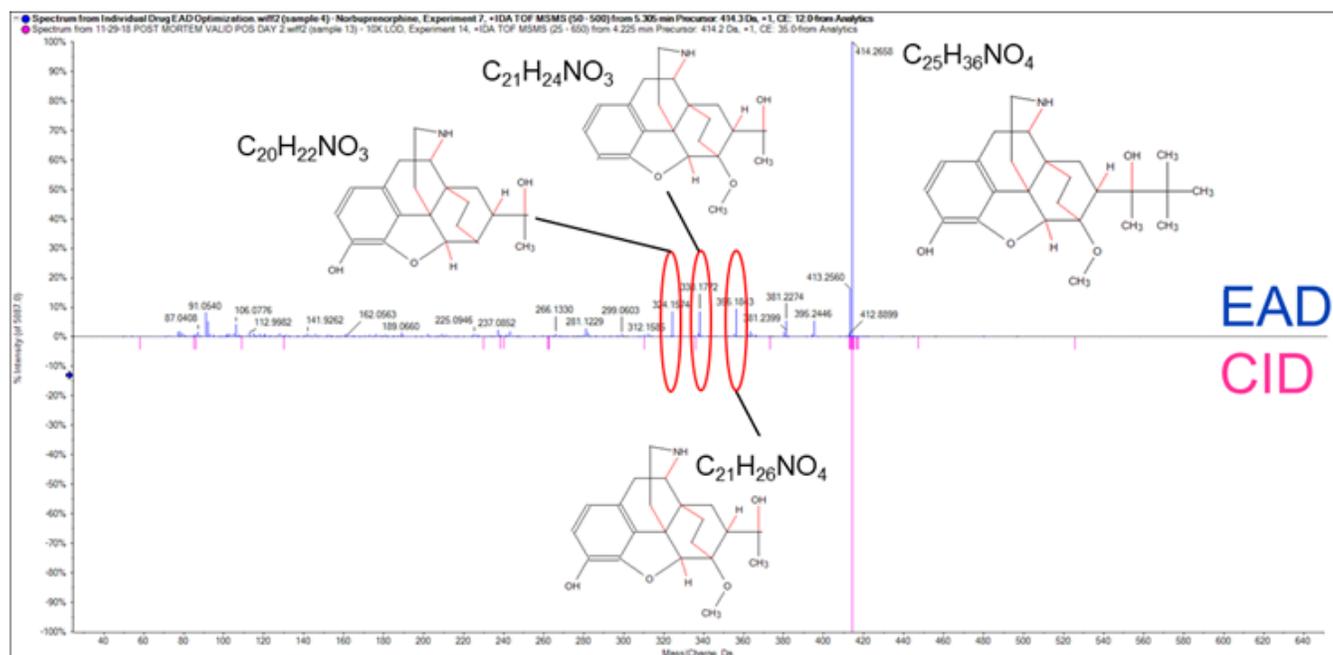
Figure 4. EAD enables in-depth characterization of challenging synthetic opioids. Zeno EAD MS/MS (top) and CID MS/MS (bottom) spectra for A) buprenorphine and B) its main active metabolite norbuprenorphine. The Zeno EAD MS/MS show unique diagnostic fragment ions that enable in-depth characterization of these 2 analytes. Activation of the Zeno trap ensured that high sensitivity was achieved for both MS/MS modes.

AP-238
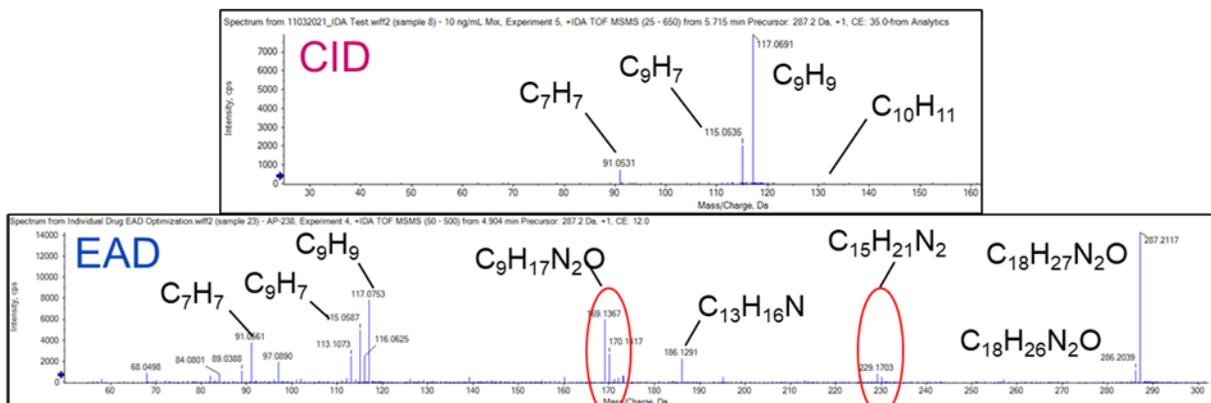
2-METHYL AP-237
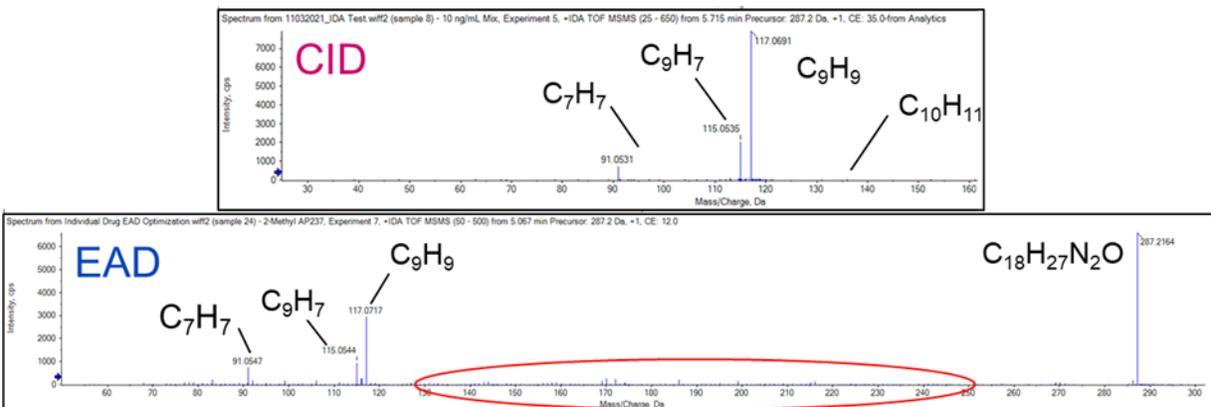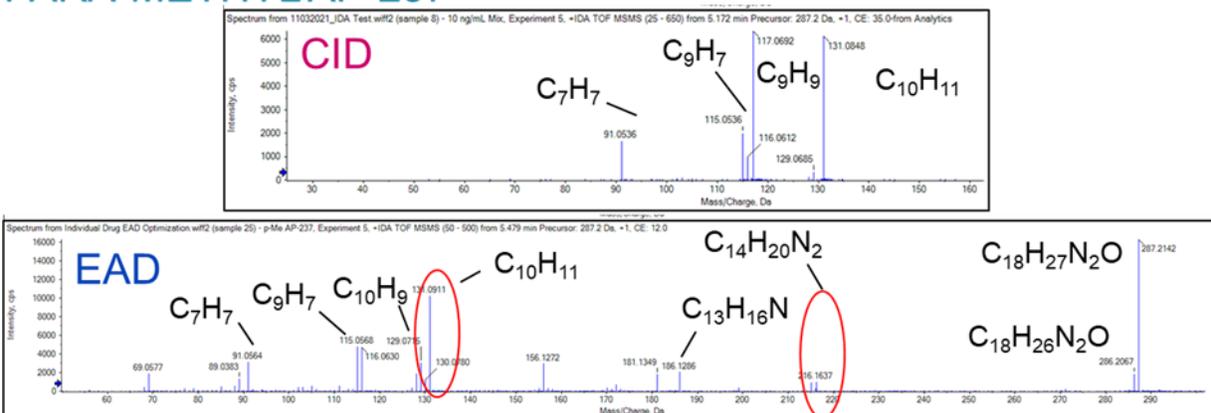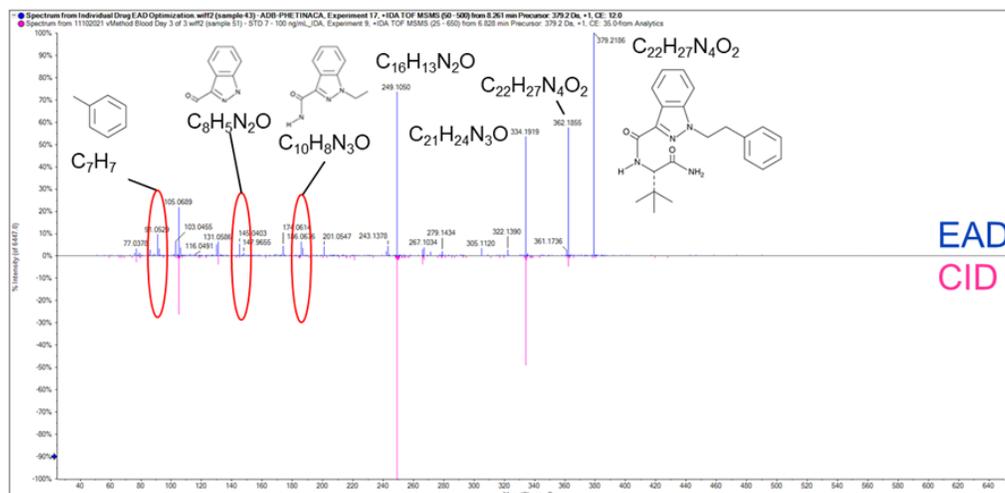

PARA-METHYL AP-237

Figure 5. EAD provides rich MS/MS spectral features to enable the differentiation of the isobaric synthetic opioids from the AP series. CID and EAD MS/MS spectra for 3 synthetic opioids: AP-238 (top), 2-methyl AP-237 (middle) and para-methyl AP-237 (bottom). The CID MS/MS spectra are indistinguishable from one another. The Zeno EAD MS/MS spectra have unique spectral features and fragment ions that enable the differentiation of the 3 isobaric species.

A. ADB-BINACA
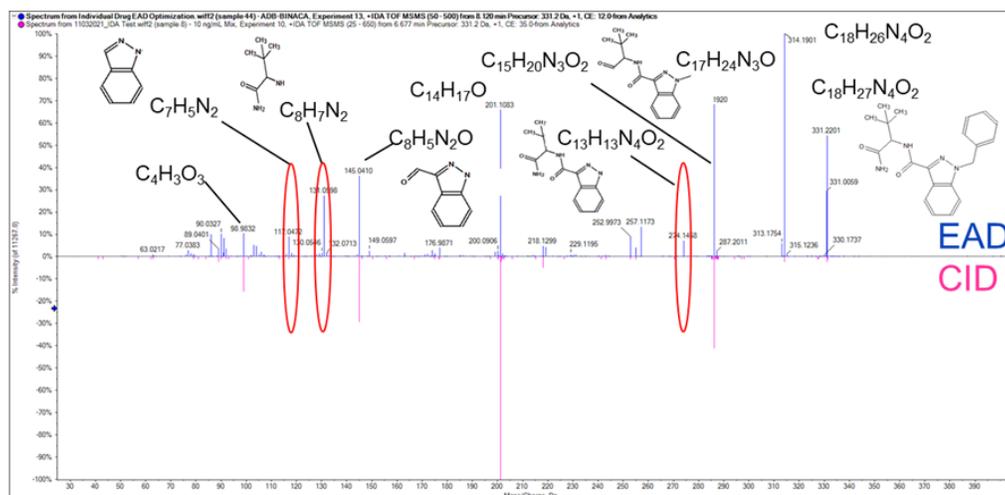
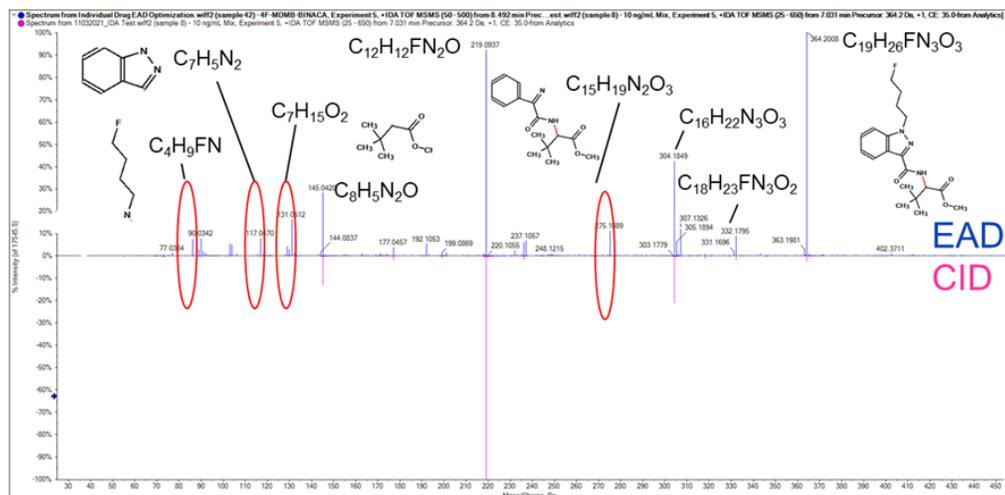## B. ADB-PHETINACA

C. 4F-MDMB-BINACA

Figure 6. EAD enables in-depth characterization of challenging synthetic cannabinoids. Spectral comparisons between Zeno EAD MS/MS (top) and Zeno CID MS/MS (bottom) for 3 synthetic cannabinoids: A) ADB-BINACA, B) ADB-PHENITACA and C) 4F-MDMB-BINACA. EAD provides richer fragmentation in the form of unique fragment ions that enable structural characterization of challenging NPS.
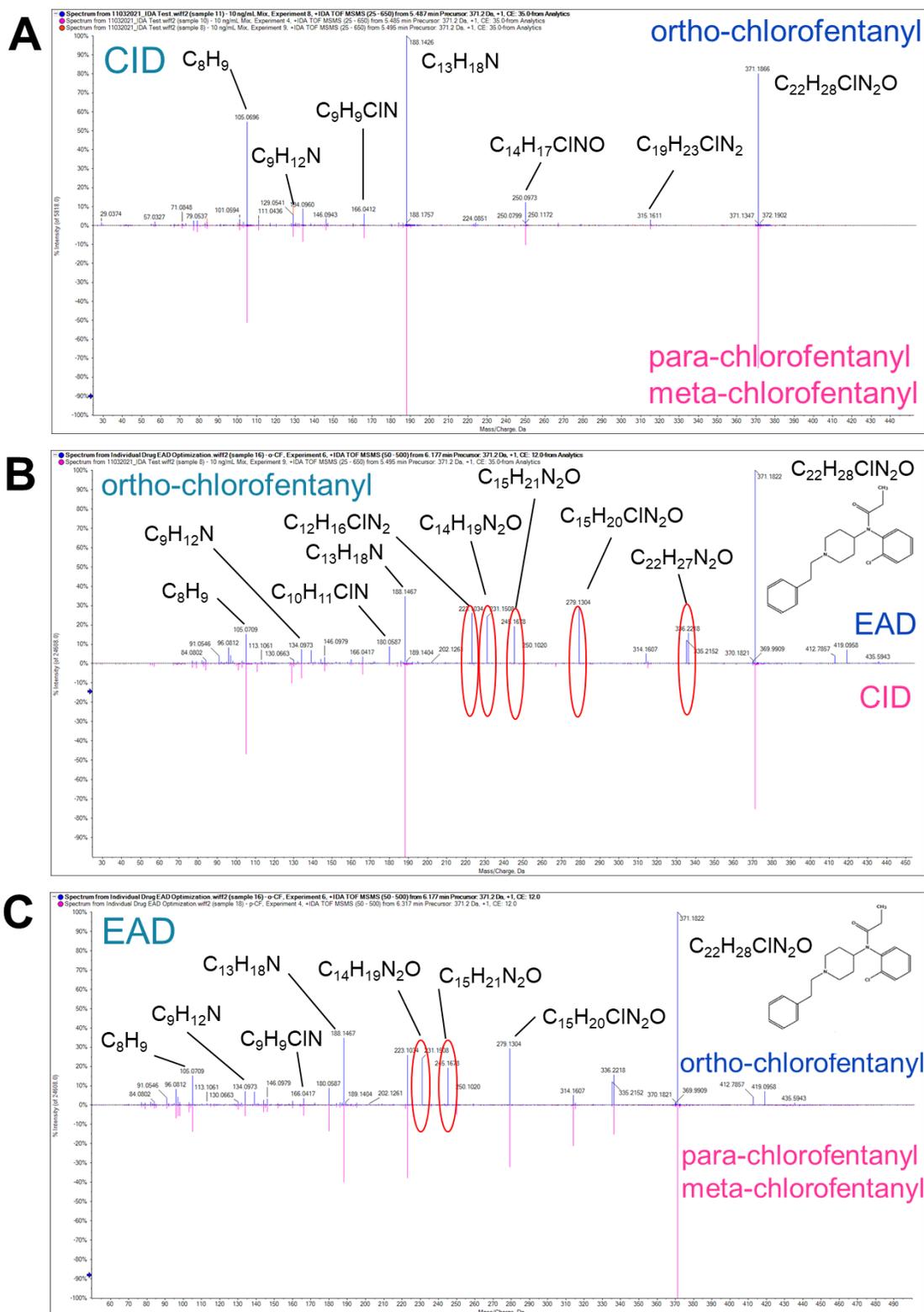
Figure 7. EAD generates unique fragment ions that enable differentiation of isobaric species. A) Zeno CID MS/MS spectra for isobaric NSO ortho-chlorofentanyl (top) and para-chlorofentanyl and meta-chlorofentanyl (bottom) showing no spectral differences, B) spectral comparison between Zeno EAD MS/MS (top) and Zeno CID MS/MS for ortho-chlorofentanyl showing unique fragment ions generated by EAD and C) Zeno EAD MS/MS spectra showing two unique fragments highlighted in red at m/z 231.1034 and 245.1678 in the spectrum of ortho-chlorofentanyl (top) that enable its differentiation from its para- and meta-chlorofentanyl analogs (bottom).

Differentiation of halogenated fentanyl analogs

Fentanyl analogs have been commonly used as adulterants in heroin and counterfeit preparations due to their high potency. In recent years, several different substituents like halogen atoms, methyl or methoxy groups of the aniline or phenethyl ring have emerged on the recreational drug market.[4] More specifically, the addition of a halogen atom to the phenethyl ring has been shown to increase potency and evade substance-specific regulations. Characterization of these designer drugs has been particularly challenging due to their structural similarities.

Figure 7A shows the Zeno CID MS/MS spectra of ortho-chlorofentanyl (top) and para- and meta-chlorofentanyl (bottom), as a mirror image. The 3 chlorofentanyl isobaric species share common fragment ions and are indistinguishable from one another. Figure 7B compares the Zeno EAD (top) and Zeno CID (bottom) MS/MS spectra as a mirror image for ortho-chlorofentanyl. As seen in the top spectrum, EAD contains many additional and unique fragments that can be used for the in-depth characterization of these isobaric species. As circled in red, EAD generated 5 unique fragments at m/z 336.2218, 279.1304, 245.1678, 231.1508 and 223.1034, which were not generated using CID. The molecular formulas of these unique fragment ions are shown with their molecular structures. Figure 7C shows the Zeno EAD MS/MS spectra of ortho-chlorofentanyl (top) and para- and meta-chlorofentanyl (bottom) as a mirror image. The spectrum of ortho-chlorofentanyl (top) contains 2 unique fragment ions highlighted in red at m/z 231.1034 and 245.1678 that are not present in the spectra of para- and meta-chlorofentanyl (bottom). The presence of these unique fragment ions generated by EAD enabled differentiation between ortho-chlorofentanyl from its para- and meta-chlorofentanyl analogs using standard solutions.

The method applicability to differentiate ortho-chlorofentanyl from para- and meta-chlorofentanyl was demonstrated using a discarded postmortem case sample. Figure 8A shows the results table generated in SCIEX OS software, which showed the positive identification of drugs and metabolites in the discarded postmortem case sample when analyzed using CID. The CID results show the positive identification of 3 compounds, which included tramadol, fentanyl and 1 of the 3 isobaric species. Positive identification determination was accomplished using the 4 confidence criteria and sorted out using the traffic light system. The Smart Confirmation algorithm was used for the spectral library, which scores all the spectra that match precursor m/z, collision energy and other filters. The spectra that match known compound names were preferentially selected and therefore each targeted chlorofentanyl isobar matched its corresponding

name. This approach did not enable ubiquitous identification of the isobar present in the sample. Figure 8B shows the results table for the same samples analyzed using EAD. The table shows that the algorithm matched ortho-chlorofentanyl as the chlorofentanyl isobar present in the sample for all 3 entries. The presence of the 2 unique fragment ions at m/z 231.1034 and 245.1678 in the EAD spectrum provided unambiguous evidence for the identification of ortho-chlorofentanyl in this discarded postmortem case sample, which was not possible using CID.

Conclusions

The use of EAD as an alternative fragmentation mechanism to generate unique, diagnostic fragment ions for the in-depth characterization and identification of challenging NPS was demonstrated. The results show that the robustness and reproducibility of EAD can provide forensic toxicologists with a unique tool for the characterization, identification and differentiation of structurally similar and isobaric NPS. The spectra acquired using Zeno EAD MS/MS contained much richer fragmentation with unique spectral features that enabled differentiation of isobaric species that were not previously distinguishable using Zeno CID MS/MS. Combining EAD with Zeno DDA provided the ability to automatically generate high-intensity diagnostic fragment ions that enabled the confident characterization and identification of challenging and low-level NPS in discarded postmortem case samples. Overall, the technological enhancements of the ZenoTOF 7600 system provided a high degree of sensitivity, selectivity and confidence for MS/MS-based characterization experiments for the forensic toxicologist.

References

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2. vMethod Application – Single-Injection Screening of 664 Forensic Toxicology Compounds on a SCIEX X500R QTOF System.

3. Highly sensitive MS/MS detection for confident identification of potent novel synthetic opioids and their metabolites. SCIEX technical note, RUO-MKT-02-13303-B.

4. UNODC. UNODC Early Warning Advisory (EWA) on New Psychoactive Substances (NPS).
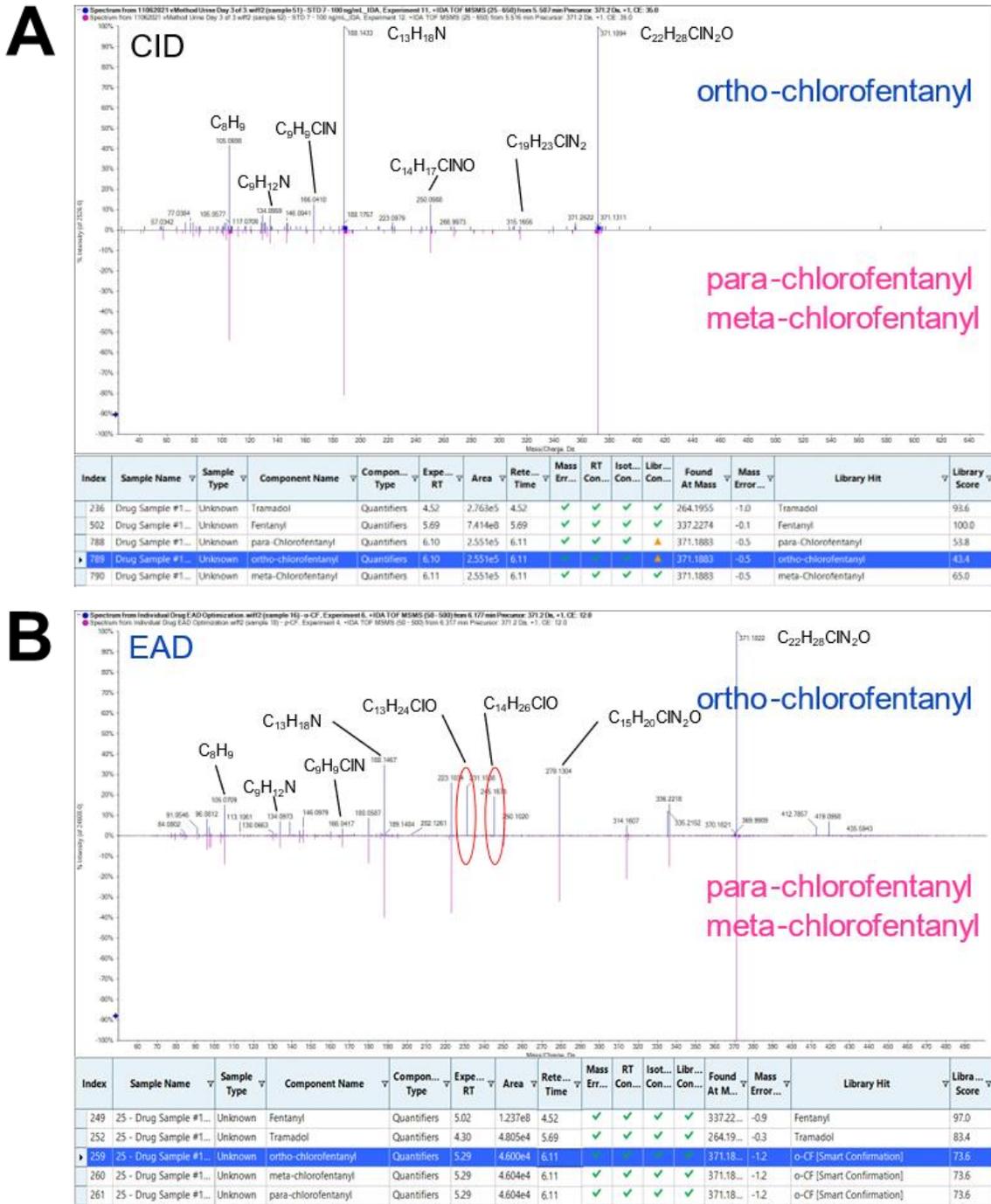
Figure 8. EAD enabled the identification of the correct chlorofentanyl isobar in a discarded postmortem case sample. A) The SCIEX OS software results table showing positive identification of 3 analytes acquired using CID, including a chlorofentanyl isobar. The acquired Zeno CID MS/MS spectra were identical for the 3 possible chlorofentanyl isobars and did not enable correct identification. B) The SCIEX OS software results table for the same sample analyzed using EAD. The results showed the positive identification of ortho-chlorofentanyl as the chlorofentanyl isobar present in the sample for all 3 entries. The acquired Zeno EAD MS/MS spectra showed 2 unique fragment ions that provided unambiguous evidence for the identification of ortho-chlorofentanyl.

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