Finding the Proverbial Needle: Non-targeted Screening of Synthetic Opioids in Equine Plasma

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Synthetic opioids are a class of compounds that are of particular concern due to their high potency and potential health impacts. With the relentless emergence of new synthetic opioid derivatives, non-targeted screening strategies are required that do not rely on the use of library spectra or reference materials. In this study, product ion searching, and Kendrick mass defect analysis were investigated for non-targeted screening of synthetic opioids. The estimated screening cut-offs for these techniques ranged between 0.05 and 0.1 ng/mL. These techniques were designed to not be reliant on a particular vendor's software, meaning that they can be applied to existing drug screening protocols, without requiring the development and validation of new analytical procedures. The efficacy of the developed techniques was tested through blind trials, with spiked samples inserted amongst authentic plasma samples, which demonstrated the usefulness of these methods for high-throughput screening. The use of a non-targeted screening workflow that contains complementary techniques can increase the likelihood of detecting compounds of interest within a sample, as well as the confidence in detections that are made.

Keywords: synthetic opioids; fentanyl; new psychoactive substances; non-targeted screening; HRMS

1. Introduction

Opioids are compounds that produce analgesic effects through the binding to specific opioid receptors [1]. These compounds emulate the effects of endogenous opioid peptides, such as endorphins, enkephalins and dynorphins, which are present in the central and peripheral nervous systems as neurotransmitters and neuromodulators [2]. Fentanyl is probably the most well-known synthetic opioid and has been used as a medication for pain management since the 1960s [3]. Fentanyl and its analogues, however, have often been misused in place of heroin due to its lower cost [4], and there is an increasing trend of fentanyl and its analogues, and other synthetic opioids, being mixed into heroin or sold as prescription opioids [5]. Alongside the fentanyl analogues, there are a number of other novel synthetic opioids (NSOs) emerging onto the illicit drug market. The most prolific of these NSOs are the AH and U series', which were originally developed as new opioid agonists, but were never brought to the market for human use [6]. Synthetic opioids have recently emerged as a drug class of particular concern due to the health issues caused by their incredibly high potency. The proliferation of fentanyl analogues, along with other NSOs, also poses a significant challenge for toxicologists, with these compounds accounting for 29% of newly identified new psychoactive substances (NPS) in 2017 [7].

In addition to the significant human health risks posed by these compounds, it has been found that opioids have a propensity for causing stimulant-like effects in horses, in conjunction with their conventional pain depressing properties [8-10]. This stimulant effect was found to be common to all narcotic analgesics [8], however it has been theorized that κ-opioid agonists should provide analgesia without stimulation [11]. The effect of the κ-agonist U-50488 on horses was evaluated and it was found that, while there was slight stimulation, it was markedly less than potent μ-agonists, such as fentanyl [11]. The stimulant effects have been confirmed to be the result of action at the opioid

receptors, as administration of opioid receptor antagonists, such as naloxone, supress the stimulation [8,12]. This combination of analgesic and stimulant properties makes synthetic opioids a target of importance from an equine anti-doping perspective. Currently in Australia, there is only an established cut-off concentration for Butorphanol at 0.01 ng/mL in plasma. The presence of any other synthetic opioid, regardless of concentration, is prohibited in equine racing. In 2015, the New York Equine Drug Testing Program reported its first positive detection of AH-7921 in a post-race sample [13]. This shows that novel synthetic opioids have started to encroach into the racing industry and reinforces the importance of employing up-to-date screening techniques in anti-doping laboratories around the world.

The continual evolution of the illicit drug market necessitates the development of non-targeted screening strategies. While there have been numerous analytical methods developed to detect synthetic opioids [3,14-19], these methods are often designed using multiple reaction monitoring (MRM), which needs to be optimised using certified reference materials (CRMs) for the specific analytes included in the assay. There is often a considerable delay between early use of new substances and the availability of CRMs, therefore these strategies need to be able to function without relying on CRMs or library spectra [20]. Previous collision-induced dissociation (CID) work has shown that different groups of synthetic opioids demonstrate certain class-specific fragmentation patterns [21], which provides an interesting avenue for the development of non-targeted screening strategies. Noble et al. [22] demonstrated that this approach shows promise, through the development of a data-independent screening strategy for 50 fentanyl analogues, taking advantage of these class-specific cleavages. Preliminary work has also been conducted to expand this strategy to the other classes of synthetic opioids [21]. Data-independent acquisition (DIA) has an advantage over traditional data-dependent acquisition (DDA) methods in that it can collect the product ions of all present precursor ions in a single scan, providing full scan MS/MS data [20,22]. While this can lead to the production of 'chimeric spectra', where the software cannot associate a product ion to a particular precursor ion [23], the presence of characteristic product ions can be used to identify suspicious samples for further analysis.

In order to interrogate the large amount of data provided by high-resolution mass spectrometry (HRMS), various filtering and extraction methods are required to highlight peaks of potential interest. The use of mass defect filtering (MDF) was first reported by Grabenauer *et al.* who applied it to the analysis of synthetic cannabinoids in herbal products and were able to detect a compound that was not visible in the total ion chromatogram (TIC) [24]. The mass defect of a particular compound is defined as the difference between its exact mass and its nominal integer mass [25,26]. Compound classes often have similar mass defects, or specific trends with increasing mass, and therefore MDF can be used to selectively filter out related compounds [25]. A sub-type of MDF, known as Kendrick mass defect (KMD), may also be beneficial for the development of non-targeted screening methods. The Kendrick mass scale allows for the recognition of a group of compounds that differ by a specific repeating mass unit [25,27].

The use of KMD filtering has also been investigated for drug screening. Anstett *et al.* [28] explored the application of KMD filtering to the screening of different phenethylamine classes, including 2C-, aminopropylbenzofuran and 2,5-dimethoxy-*N*-(2-methoxybenzyl) phenethylamines. The authors successfully implemented KMD filters that were able to distinguish between compounds from the different classes of phenethylamines that were analysed [28]. If similar filters could be developed for the different classes of synthetic opioids, this technique could prove a valuable asset

in the development of potentially complementary non-targeted screening methods for the detection of these compounds.

This study presents a proof-of-concept for the development of a toolbox of complementary techniques demonstrating potential for the non-targeted screening of synthetic opioids in equine plasma, which may also be applicable to other biological matrices. These techniques focus on the 'back end' data processing and data mining techniques, which allows them to be used in conjunction with routine acquisition methods, without the need for laboratories to develop and validate new instrumental analyses.

2. Experimental

2.1. Solvents and Reagents

All solvents used were liquid chromatography-mass spectrometry (LC-MS) grade. Acetonitrile, ethyl acetate and methanol were obtained from Merck (Darmstadt, Germany). Ammonium acetate and trichloroacetic acid were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Acetic acid was obtained from Ajax Chemicals (Sydney, NSW Australia). Ultrapure-grade water (18.2 MΩ.cm) was obtained from a Smart2Pure ultrapure water system (Thermo Scientific, Langenselbold, Hungary).

Fentanyl citrate was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Hydrochloride salts of acetyl fentanyl and AH-7921, along with a neat solid of U-50488, manufactured by Cayman Chemical (Ann Arbor, MI, USA), were purchased from Sapphire Bioscience (Redfern, NSW, Australia). Carfentanil citrate was purchased from Janssen Pharmaceuticals (North Ryde, NSW, Australia).

2.2. Sample Preparation

Blank plasma was obtained from blood samples collected in Lithium Heparin Vacutainers purchased from BD (Mississauga, ON, Canada) from thoroughbred horses following approval of the Racing NSW Animal Care and Ethics Committee (ARA 71).

Spiked equine plasma samples were prepared at varying concentrations to determine the effectiveness of the examined detection methods in an authentic biological matrix. A mixed standard containing fentanyl, acetyl fentanyl, carfentanil, AH-7921 and U-50488 was prepared at a concentration of 200 ng/mL in methanol. The representative panel of compounds used in this study were chosen to demonstrate the potential of the developed techniques across the range of synthetic opioid classes. AH-7921 and U-50488 were chosen specifically due to their relevance to the racing industry, with AH-7921 being the first NSO reported in an equine plasma sample and U-50488 being previously studied to determine the effects of κ-opioid agonists in horses [11]. The chemical structures of the different opioids used in this study can be found in Figure 1.

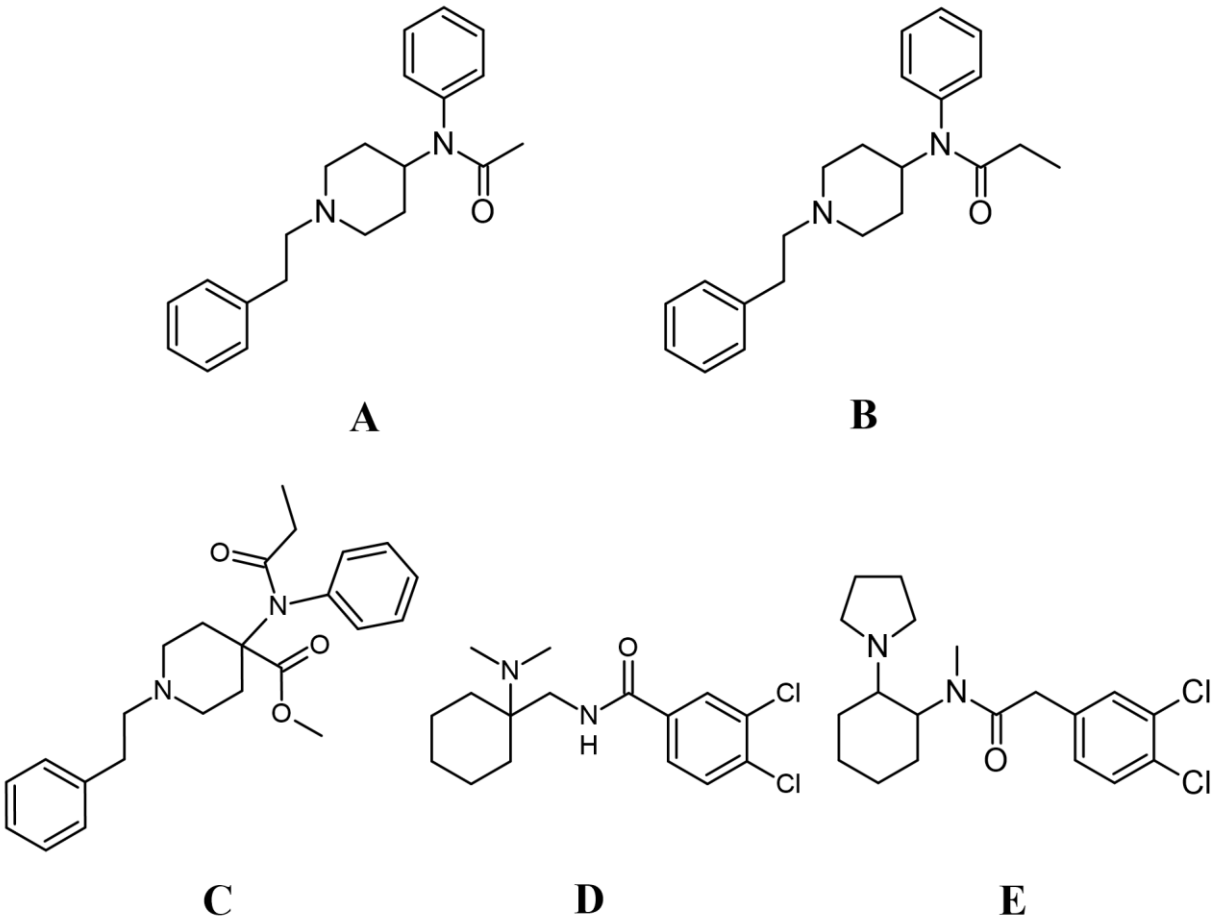


Figure 1. Chemical structures of opioids included in the study including acetyl fentanyl (A), fentanyl (B), carfentanil (C), AH-7921 (D) and U-50488 (E)

Two millilitres of blank equine plasma was spiked with the mixed standard to produce a concentration range of 0.01 to 10 ng/mL across 13 different samples. This concentration range was used to estimate limits of detection (LOD) and screening cut-off levels for the developed techniques. Blank samples were also prepared alongside the spiked samples.

Once an effective screening cut-off level was estimated for each technique, further replicates were prepared to verify the consistency of these cut-offs at concentrations of 0.05 and 0.1 ng/mL. For each concentration, three sets of samples were prepared from a pooled blank plasma source, with seven replicates in each set (n = 21). The sample sets were analysed over different days to account for intra-day variability.

Protein precipitation was performed through the addition of 200 µL of 10% (v/v) trichloroacetic acid to each of the samples. The pH of the samples was then adjusted to 3 – 3.5 using hydrochloric acid and 3 – 3.5 mL of ultrapure water after which they were centrifuged at 1500 x *g* for 10 minutes. Solid-phase extraction (SPE) was completed using XtrackT® Gravity Flow DAU Extraction Columns (3 mL column, 200 mg sorbent, UCT Inc., Bristol, USA). The cartridges were first conditioned with 3 mL of methanol, followed by 3 mL of ultrapure water, after which the samples were loaded (~6 mL). The samples were washed with 3 mL of 0.1 M acetic acid and dried using a positive pressure manifold at 70 psi (UCT Inc., Bristol, PA, USA). The cartridges were washed with 3 mL of methanol and dried under positive pressure. The analytes were eluted from the cartridges using 3 mL of ethyl acetate containing 3% ammonia and 0.5% methanol.

Following SPE, one drop of 0.1 M methanolic hydrochloric acid was added to each of the eluents using a Pasteur pipette (approximately 20 µL) before the solvent was evaporated under a gentle stream of N_2 at 60 °C. The samples were then reconstituted in one drop of methanol from a Pasteur pipette and 100 µL of 10 mM ammonium acetate buffer (pH 3.9), before being transferred to vials for LC-HRMS analysis. All samples were stored at 4 °C until analysis.

2.3. Instrumental Analysis

Chromatographic separation was achieved on an Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity II UHPLC, consisting of a high-speed pump (G7120A), multisampler (G7167B, 18 °C) and thermostat and column compartment (G1316A, 35 °C) coupled to an Agilent Technologies 6545 quadrupole time-of-flight mass spectrometer (QTOF). All data acquisition was performed using Agilent Technologies MassHunter Workstation (Version B.06.01). A sample volume of 1 µL was injected onto a Phenomenex (Torrance, CA, USA) Gemini 110 Å C_{18} LC column (2 x 50 mm, 5 µm particle size) using a gradient elution method with a flow rate of 0.5 mL/min and a total analysis time of 11.5 min. Mobile phase A consisted of a 10 mM ammonium acetate buffer (pH 9) and mobile phase B consisted of 0.1% (v/v) acetic acid in acetonitrile. Initial mobile phase composition was 99% A, which was held for 2 min before being decreased linearly to 20% A over 6.5 min. The mobile phase was then returned to 99% A over 3 min.

The QTOF-MS was operated in positive electrospray ionisation mode (ESI+) with capillary and fragmentor voltages of 3500 V and 100 V, respectively. An All Ions MS/MS (DIA) data acquisition mode was used with a mass-to-charge (*m/z*) range of 35 – 1000. Spectra were obtained with an acquisition speed of 10 spectra/s and collision energies (CE) of 10, 20 and 40 eV for CID. The chromatographic and MS conditions were chosen to mimic the routine analytical method employed at the Australian Racing Forensic Laboratory (ARFL) to evaluate the effectiveness of the developed detection techniques for an operational purpose.

2.4. Data Analysis

All extracted ion chromatograms (EICs) for the product ion searching (PIS) and KMD analysis methods were generated using Agilent Technologies MassHunter Qualitative Analysis Software (Version B.10.0, Build 10.0.10305.0).

2.4.1. Targeted Compound Extraction Limit of Detection (LOD)

The suitability of the analytical method was assessed by performing a targeted extraction on the spiked compounds using MassHunter Quantitative Analysis Software (Version 10.0, Build 10.0.707.0). Spiked samples of decreasing concentrations (10 to 0.01 ng/mL) were evaluated to estimate LODs using the targeted extraction method. Precursor and product ion EICs were assessed within a mass error of ±10 ppm and a retention time tolerance of ±0.2 min of their known retention times [29]. Where an EIC was assessed for a targeted compound, the signal-to-noise ratio (*S/N*) was obtained to estimate the LOD where the $S/N > 3$ [30]. These estimated values were used for qualitative comparison to the proposed non-targeted screening methods to demonstrate the effectiveness of these methods.

2.4.2. Product Ion Searching

For the PIS technique, EICs were created from several diagnostic product ions identified in a previous CID study [21] to screen for potential synthetic opioids present in the samples. The

diagnostic product ions used for each compound class can be found in Table 1. The masses used represent the theoretical masses of each product ion. All EICs were generated with a mass tolerance of ±10 ppm. Product ion masses were extracted at the CE (10, 20 or 40 eV) which gave the greatest peak intensity. This CE was determined automatically by the data analysis software.

Table 1. Diagnostic product ions from each compound class used for screening [21].

Compound Class	Diagnostic Product Ions (*m/z*)		
	1	**2**	**3**
Fentanyls	105.0704	188.1439	-
AH Series	172.9561	189.9827	284.0609
U Series	158.9768	218.0140	298.0766

2.4.3. Kendrick Mass Defect

When calculating KMD, a normalisation factor is used to 'correct' the exact mass of a particular compound and give the Kendrick mass. Most commonly, the mass of the CH_2 group is used as the correction factor. Essentially, the mass of a CH_2 group is set to be exactly 14.00000, allowing the Kendrick mass of a compound to be calculated (Equation 1) [27].

$$Kendrick\ mass = IUPAC\ mass \times \frac{14}{14.01565} \quad \text{(Equation 1)}$$

In this study, the KMD values for each compound were calculated as the difference between the Kendrick exact mass and the integer Kendrick mass [25]. For example, the Kendrick mass of fentanyl is 336.8509, in this case the KMD is 0.8509, as the integer Kendrick mass is 336.

KMD analysis was conducted using a custom-built program developed by Pasin in the Visual Basic for Applications (VBA) environment for Microsoft Excel (named *DefectDetect*) [31]. An averaged mass spectrum over a retention time range (0.5 – 8 min) was created for each sample and an *m/z* and intensity list was generated as a Microsoft Excel comma-separated value (.csv) file from these spectra. This retention time range was chosen as it is the range within which the analytes of interest were expected to elute, based on the representative compounds analysed and the chromatographic conditions. The program then calculated the Kendrick masses and mass defects using a normalisation factor of CH_2 and filtered the results based on a mass range of *m/z* 200 – 500, a KMD tolerance of ± 0.005 Da and mass defect filter of 0.1 – 0.27 Da. Table 2 shows the different KMD filters that were used in this study. Examples of the compounds belonging to the different fentanyl groups can be found in the Supporting Information (Figure S1). A minimum *m/z* value was populated for each filter and the program only considered masses that varied by a multiple of 14 Da from the minimum *m/z* to account for the difference of CH_2 units. An example of the *DefectDetect* interface can be found in the Supporting Information (Figure S2). A copy of *DefectDetect* can be obtained on request from the authors.

Table 2. Kendrick mass defect (KMD) filters used for compound detection.

Name	KMD	Minimum *m/z*
Fentanyl (amide chain)	0.8510	323
Fentanyl (cyclic)	0.8380	335
Fentanyl (F analogues)	0.8215	355
Carfentanil	0.7916	395
AH + U Series	0.7510	329
AH Series	0.8276	295

2.4.4. Non-targeted Workflow

For each sample, EICs were extracted for the common product ions identified in Table 1, followed by the extraction of masses identified by the KMD analysis. The generated EICs from each individual technique were first reviewed independently. If a peak of interest was identified by either technique, a comparison was made to the results obtained from the complementary technique to verify concurrence.

2.5. Blind Trial Setup

Blind trials were performed to validate the effectiveness of the detection methods under pseudo-operational conditions. Single compound spikes were prepared for fentanyl and acetyl fentanyl at concentrations of 0.05 ng/mL and 0.5 ng/mL (n = 4). Routine plasma samples from the ARFL were included to make a batch containing 20 samples. The 'Randbetween' function in Excel was used to randomise the positioning of the spiked samples within the worklist, and this randomisation was completed by a third party to ensure that the analyst was unaware of which samples were spiked. All samples in the worklist were then analysed using the instrument parameters outlined in 2.3 and the data was processed using the PIS and KMD analysis methods detailed above. These blind trials were conducted on two separate occasions.

3. Results and Discussion

3.1. Implementation of Non-targeted Workflow

Once the data from each sample has been processed using the developed techniques, the results from each technique can first be reviewed independently to determine the presence of any possible analytes of interest. The results can then be compared with the complementary technique to provide further evidence of detection. Observation of retention time-matched peaks in MS^1 (KMD) and MS^2 (PIS) may provide strong evidence for the presence of a particular subclass of synthetic opioid. Observation of retention time-matched peaks in MS^2 that is not corroborated by an extracted MS^1 peak may indicate the presence of a synthetic opioid containing the same core structure with novel modifications outside of the determined KMD filters. By implementing screening workflows that contain complimentary techniques, it increases confidence that one technique identifies a feature of interest and provides stronger evidence for detection when both techniques provide corroborative results.

3.2. Application of Screening Techniques

The applicability of each developed technique to non-targeted screening was evaluated to ensure that they were fit-for-purpose. The sensitivity of the techniques to the presence of a spiked compound in equine plasma was evaluated to propose an appropriate screening cut-off level. At concentrations below this proposed cut-off, it may be possible to detect an analyte of interest, however the reliability of detection would be reduced. Additionally, the specificity of these techniques was considered to ensure that a sample containing an analyte of interest could easily be distinguished from blank plasma samples.

3.2.1. Product Ion Searching

In a general screening workflow, detection and putative identification are performed by comparison to a spectral library. In some cases, where the fragmentation patterns of novel compounds are sufficiently similar to known compounds, these techniques may be used to detect new analogues. When increased structural diversity is present within groups of analogues, or only a limited number of common product ions are present, simply screening samples against spectral libraries may not be sufficient. To improve the situation, the applicability of PIS for the detection of synthetic opioids in plasma has been demonstrated previously [21].

Figure 2 displays EICs from the targeted extraction of selected synthetic opioid precursor ions in a spiked plasma sample (0.05 ng/mL), as well as EICs for the common product ions of each opioid subclass in both the spiked plasma sample and a matrix blank. It can be seen from the spiked sample that there are distinct retention time aligned peaks for each set of common product and precursor ions, indicating the presence of an analyte of interest belonging to each of these subclasses. In the matrix blank, however, there are no clear peaks detected, which demonstrates specificity for this technique. The matrix blank displayed for EICs corresponding to the fentanyl analogues showed higher noise compared to the respective EICs for the other two subclasses. This is most likely due to the fact that these masses, belonging to the AH and U series opioids, have mass defects that are indicative of molecules containing halogens, which are less likely to be present in the matrix background than the masses of the common fentanyl product ions. In this study, all EICs were extracted within a mass error of ±10 ppm. Given that the masses being extracted for the PIS method correspond to the theoretical masses of each ion, this tolerance can be altered on a case-by-case basis depending on the mass accuracy required for specific assays.

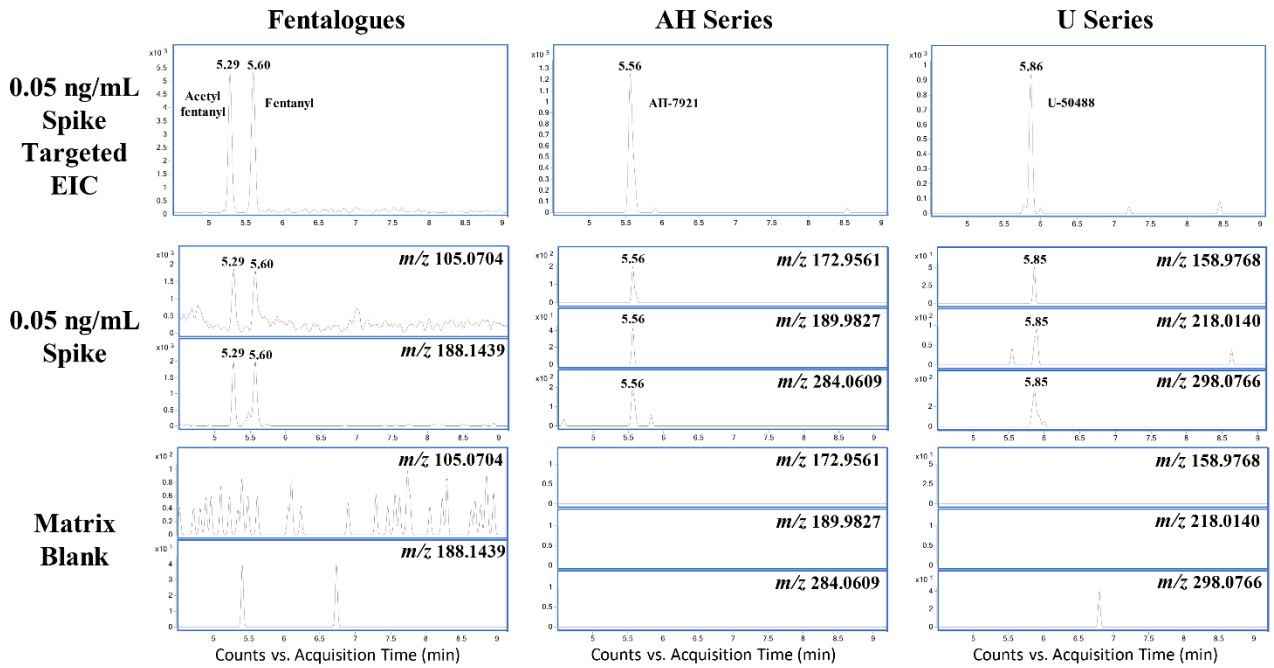


Figure 2. EICs showing the targeted extraction of known precursor masses showing the detection of the spiked compounds in a 0.05 ng/mL spike. The common product ions used for PIS screening also showed detection of the same peaks and could be easily distinguished from the matrix blank, showing the specificity of the technique.

From a routine screening perspective, the EICs generated from the common product ions can be combined (i.e. a single chromatographic trace that represents multiple *m/z* values) in MassHunter to reduce the amount of data that the analyst must review. If a peak is detected in a sample however, separate EICs should be created for that sample to ensure that multiple characteristic ions are present. In some instances, a single, high abundance ion can cause a peak to be displayed in the combined EIC. Without the presence of other diagnostic product ions, it cannot be confidently stated whether that ion is present from a compound of interest or if it is extraneous from an unrelated background compound. Alternatively, overlaid EICs for the individual product ions can be viewed for the same purpose. Figure 3 shows an example of such a case, where the overlaid EIC for common product ions for the U series show detection of all 3 ions, with decreasing intensities, within the same peak (left). On the other hand, Figure 3b shows a large peak at *m/z* 158.9768, while the EICs at *m/z* 218.0140 and 298.0766 only show minor peaks at significantly lower abundances barely noticeable on the overlaid chromatogram, which do not have the same retention time and, therefore, cannot be attributed to the same compound. In this case, the lack of corroborating ions means that the peak at *m/z* 158.9768 cannot be confidently attributed to a U series compound, as there may be a background compound present in the equine plasma with this mass.

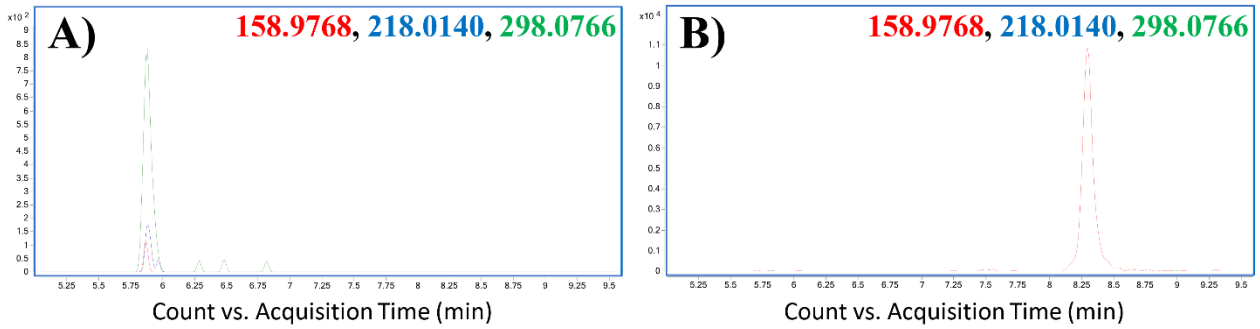


Figure 3. Example overlaid EICs for common U series product ions m/z 158.9768 (red), 218.0140 (blue) and 298.0766 (green) from a spiked plamsa sample with 0.05 ng/mL U-50488 (A) and a blank sample (B)

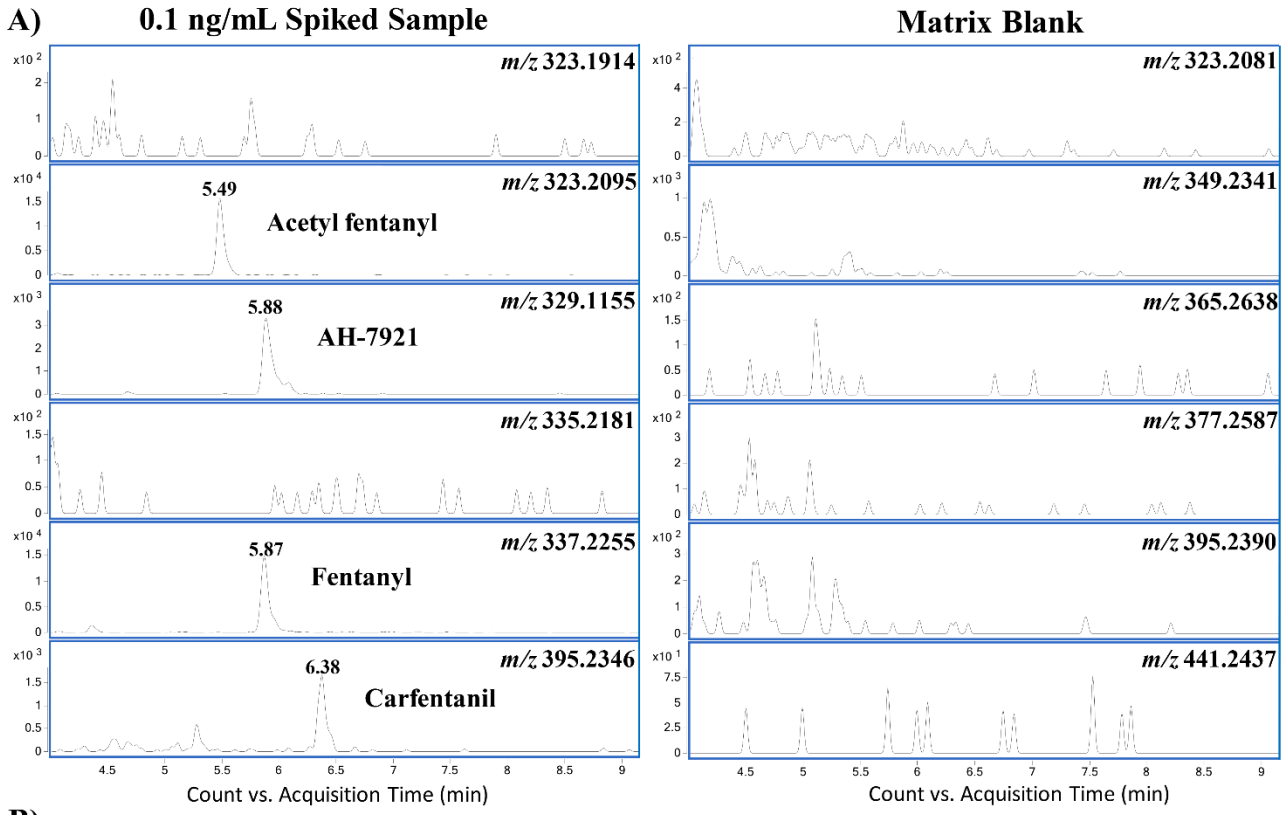
The screening cut-off of this technique was found to be 0.05 ng/mL [21]. This cut-off level was determined by the concentration at which the common product ions for the majority of analytes in a particular class had a *S/N* > 3. Below this concentration, some product ions may still be detected in a sample, which can give some indication that an opioid may be present. Some product ions, however, were not observed. If the EICs generated from a sample only reveal a single product ion, then a lower confidence in the detection occurs. Some of the product ions that were found to be common to the different subclasses of synthetic opioids are not specific to these compounds alone. For example, the fentanyl product ion at *m/z* 105.0704 will likely be present in any compound containing a phenylethyl group. While this ion constitutes a good marker for fentanyl analogues in combination with other common product ions, its presence alone would not necessarily be indicative of these compounds. Therefore, care should be taken when interpreting results of samples that may have concentrations below this cut-off, where some common product ions are not detected.

3.2.2. Kendrick Mass Defect Analysis

KMD analysis using the *DefectDetect* program operates using an imported mass list containing *m/z* and intensity values generated from an averaged mass spectrum over a given retention time range. The program will then calculate the Kendrick mass and KMD of each identified *m/z* value and filter the results based on the user-defined parameters. An example of the results table can be found in the Supporting Information (Figure S3). Once the filtered list has been generated, EICs can be created for the selected masses to determine if there is a peak present or if the mass has arisen from background noise. It is possible to include intensity filters in the *DefectDetect* parameters, however it was decided that the absolute intensity can be variable depending on the instrument and analytical method used and, as such, this filter was not included in the analysis.

Figure 4 shows example EICs for some of the filtered masses detected by KMD analysis of a plasma sample spiked with 0.1 ng/mL of a mixed standard, as well as the results table generated for this sample. The results tables generated by the *DefectDetect* program are colour coded to correspond to the individual filters used in the analysis. It can be seen in these chromatograms that the masses correlating to the spiked samples show well-defined peaks at relatively high abundances, in the order of 10^3 and 10^4. On the other hand, the EICs at *m/z* 323.1914 and 335.2181 have much lower abundances and significantly more noise along the baseline, indicating that they could be present due to the matrix background. Additionally, Figure 4 shows the top six masses identified by performing KMD analysis on a blank matrix sample. Once again, the EICs generated are noisy and have quite a low abundance, suggesting that there is no meaningful detection of an analyte of

interest. This demonstrates the specificity of KMD analysis for analytes of interest, as they can be easily differentiated from background noise.



B)

Mass-to-charge ratio (m/z)	Intensity (counts)	Even m/z?	Mass defect (Da)	Kendrick Mass	Kendrick Mass Defect	Compound Class
323.1914	33	FALSE	0.1914	322.8293	0.8293	AH
323.2095	96	FALSE	0.2095	322.8474	0.8474	Fentanyl (AC)
329.1155	32	FALSE	0.1155	328.7468	0.7468	AH + U
335.2181	19	FALSE	0.2181	334.8426	0.8426	Fentanyl (Cy)
337.2255	113	FALSE	0.2255	336.8478	0.8478	Fentanyl (AC)
343.1329	4	FALSE	0.1329	342.7485	0.7485	AH + U
349.2317	50	FALSE	0.2317	348.8405	0.8405	Fentanyl (Cy)
355.2146	260	FALSE	0.2146	354.8167	0.8167	Fentanyl (FA)
357.1465	10	FALSE	0.1465	356.7465	0.7465	AH + U
363.2445	32	FALSE	0.2445	362.8376	0.8376	Fentanyl (Cy)
365.2401	4	FALSE	0.2401	364.8309	0.8309	AH
365.2649	7	FALSE	0.2649	364.8558	0.8558	Fentanyl (AC)
369.2336	33	FALSE	0.2336	368.8200	0.8200	Fentanyl (FA)
371.1658	26	FALSE	0.1658	370.7500	0.7500	AH + U
377.2583	23	FALSE	0.2583	376.8357	0.8357	Fentanyl (Cy)
383.2547	20	FALSE	0.2547	382.8254	0.8254	Fentanyl (FA)
385.1780	10	FALSE	0.1780	384.7465	0.7465	AH + U
395.2346	51	FALSE	0.2346	394.7919	0.7919	Carfentanil
427.2274	18	FALSE	0.2274	426.7488	0.7488	AH + U

Figure 4. EICs for some of the identified masses from DefectDetect showing selectivity to the spiked compounds in comparison to the blank (A) and the results table given by DefectDetect for the spiked sample (B). The detected class for each mass in the results table is given according to the filters listed in Table 2. AC: Amide Chain, Cy: Cyclic, FA: F analogues.

A number of different KMD tolerances were trialled in order to determine the best balance between reliability of detection and total number of filtered results. It is important to note that this technique operates using a list of averaged masses for each sample, therefore the accuracy of the calculated

KMD values is dependent on the accuracy of the instrument used. In this case, the tolerance used for routine KMD analysis may require adjustment to suit instrument capabilities. For this study, it was determined that a tolerance of ± 0.005 Da was fit for purpose. When optimising the parameters for this detection technique, a reliability of ≥ 95% for each spiked compound at a concentration of 0.1 ng/mL was deemed to be a suitable cut-off. The reliability of detection was evaluated by analysing three sets of spiked samples, each containing seven replicates (n = 21), and ensuring that the spiked compounds were being detected by the KMD filters applied. In addition, an arbitrary threshold of 20 filtered results was decided upon to limit the amount of data that needed review. It is important to note that, for the purpose of this proof-of-concept study, the retention times of the peaks identified by the KMD analysis were compared to the known retention times of the spiked compounds (within a tolerance of ± 0.2 min [29]) to ensure that the suspected compound was being correctly identified.

In addition to the KMD filters, an MDF can be applied to further reduce the number of filtered results. The mass defect range for a large number of synthetic opioids fell within a range of 0.10 – 0.27 Da, allowing some variation either side to account for mass tolerance and different structures. This range is broad, and by itself would not mean much. When it was applied on top of the other filters that were already in place, however, it significantly reduced the number of results that were obtained for each sample (50 – 63% reduction), while not affecting the reliability of the detection of the spiked compounds. Table 3 provides a summary of one set of the spiked samples showing the reliability and variation in the number of filtered results obtained. These results demonstrate that KMD analysis can reliably detect the presence of an analyte of interest, while still returning a reasonable number of results that need to be reviewed by generating EICs in an operational environment. Across all the 21 samples analysed, the spiked compounds within the defined KMD filters were detected in every sample.

Table 3. KMD analysis of one set of 0.1 ng/mL samples, showing consistent detection of analytes of interest and total number of filtered results for each sample.

Sample	Acetyl fentanyl	Fentanyl	AH-7921	Carfentanil	Filtered Results
A	✓	✓	✓	✓	11
B	✓	✓	✓	✓	15
C	✓	✓	✓	✓	13
D	✓	✓	✓	✓	13
E	✓	✓	✓	✓	14
F	✓	✓	✓	✓	16
G	✓	✓	✓	✓	12

3.3. Blind Trial

In order to evaluate the effectiveness of these techniques under more realistic conditions, the blind trial was completed on two different occasions using the PIS and KMD analysis methods to provide complementary detection. On both of these occasions, the higher concentration spikes (0.5 ng/mL) were quite clearly detectable within the dataset. Figure 5 shows the PIS results for the acetyl fentanyl spikes, along with a negative sample and the matrix blank.

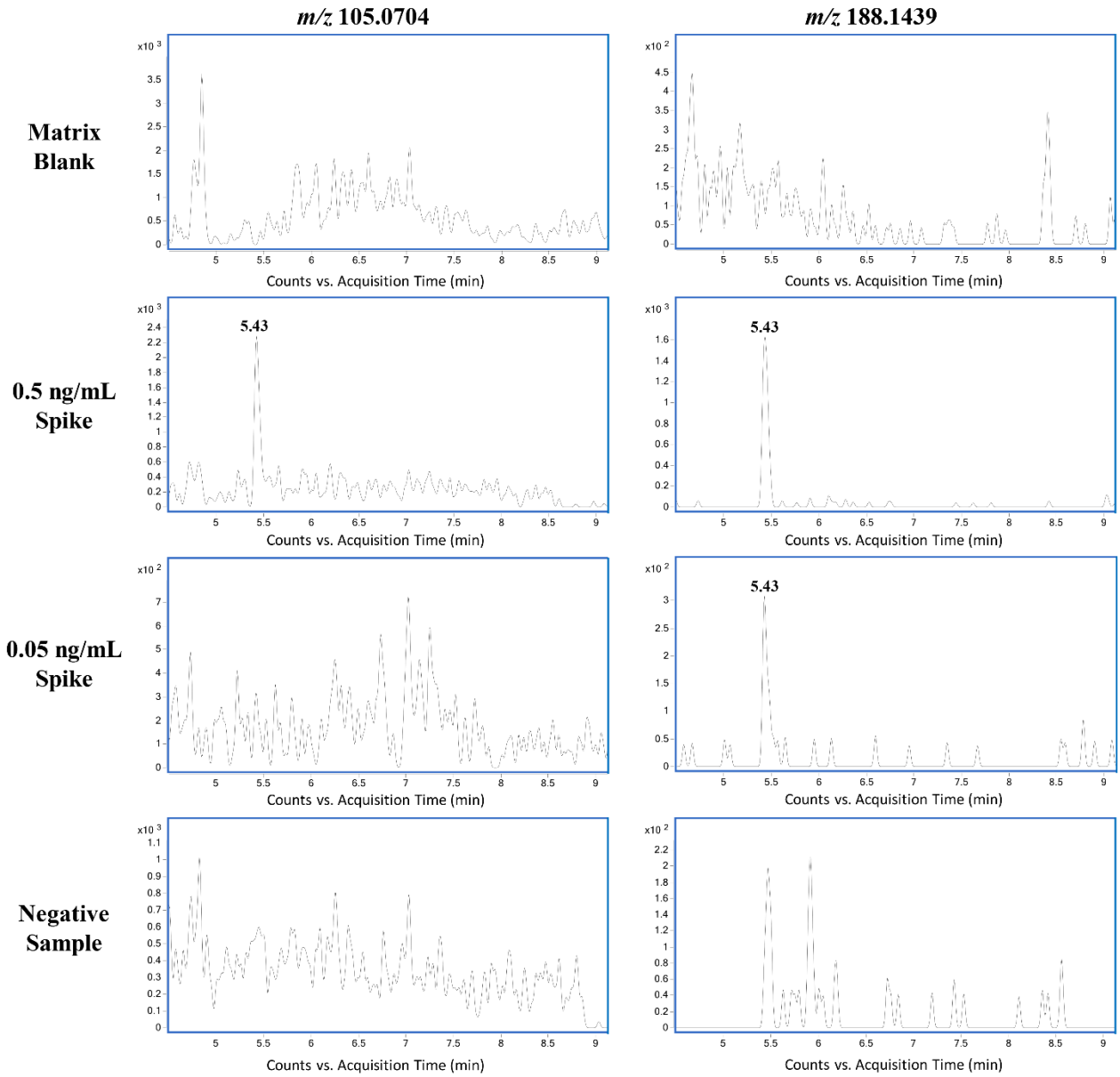


Figure 5. PIS results obtained from the blind trial showing detection of both monitored ions in the 0.5 ng/mL spike and only detection of the m/z 188.1439 ion in the 0.05 ng/mL spike

There are distinct peaks present at relatively high abundances for both the *m/z* 105.0704 and 188.1439 product ions expected from a fentanyl derivative. Importantly, the peaks present in the 0.5 ng/mL spike can be clearly differentiated from both the matrix blank and the negative sample presented in Figure 5. While these peaks could not specifically identify acetyl fentanyl in a routine sample, they could be used to identify the sample as possibly containing a fentanyl derivative for further putative identification.

In the 0.05 ng/mL acetyl fentanyl spike (Figure 5), one of the common product ions at *m/z* 105.0704 was not detected in the sample. Given that the *m/z* 105.0704 ion is common for organic molecules containing a phenethyl moiety, it is reasonable to suggest that this ion may be more commonly present in the background of biological matrices. This is further demonstrated by the fact that EICs generated for *m/z* 105.0704 tend to have a noisier baseline, as seen in Figures 2 and 5. At lower concentrations, therefore, it is possible that the presence of this common fentanyl product ion is masked by the background evident from the sample matrix. This can affect the confidence with

which the presence of a compound of interest can be indicated. This again highlights the benefit of using complementary techniques. In addition to one technique being able to detect compounds that are missed by the other, the results from one detection technique can be used to corroborate the findings from another.

Figure 6 presents the KMD results for each of the spiked acetyl fentanyl standards compared to their PIS results. In both spiked samples, the KMD analysis identified a peak with comparable retention time at the expected molecular ion mass of acetyl fentanyl (within a mass error of ± 10 ppm). From these results, it can also be seen how the other masses identified by the KMD analysis can be eliminated by reviewing their EICs and comparing with the PIS results. While the detection of the common product ions at *m/z* 105.0704 and 188.1439 cannot indicate the specific identity of the compound detected, the supporting KMD results identify the mass of the suspected molecular ion, which can be used for further putative identification work.

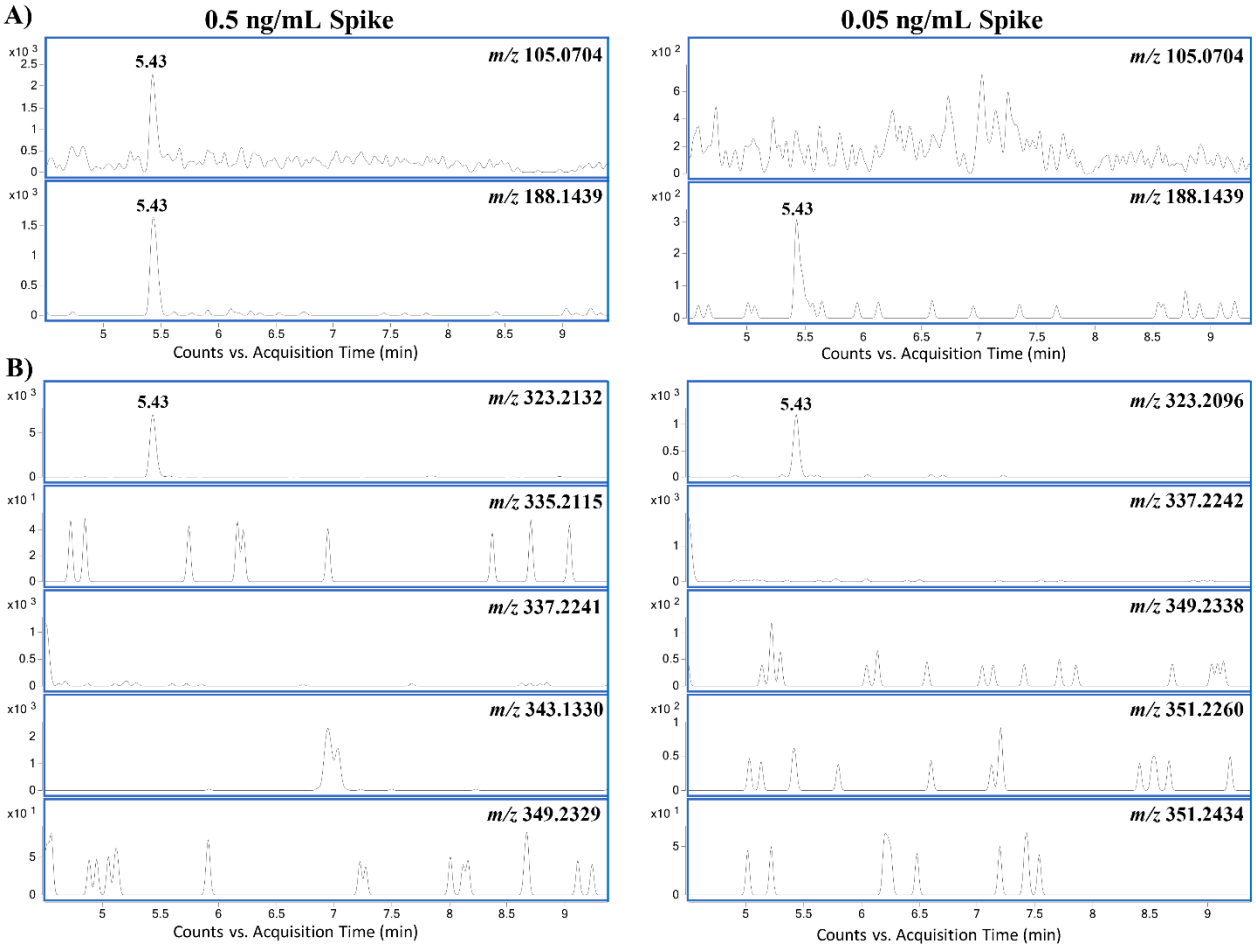


Figure 6. PIS results for the spiked acetyl fentanyl samples (A) compared with the associated KMD results for each sample (B)

Interestingly, in the lower concentration (0.05 ng/mL) samples used for the blind trial, the KMD analysis was able to show the acetyl fentanyl peak even though this sample fell below the proposed screening cut-off. A cut-off of 0.1 ng/mL was proposed as this is the concentration where the reliability of detection with this technique was > 95%. It is, of course, possible that detections may be made below this concentration, as demonstrated in this blind trial, however, the reliability of identifying all samples with compounds of interest at lower concentrations would be affected. The spiked fentanyl samples at both concentrations (0.05 and 0.5 ng/mL) were also identified among the

dataset and it was found that they followed the same trends highlighted by the acetyl fentanyl results presented (Figures S4 and S5).

While the higher concentration samples (0.5 ng/mL) were clearly identified with corroborative evidence from both complementary techniques, the lower concentration samples (0.05 ng/mL) lacked the identification of the common product ion at *m/z* 105.0704. In practice, this can be used to create 'confidence levels' when it comes to the detection of compounds of interest. When all components of a detection workflow are in agreement, as seen in Figure 6 for the 0.5 ng/mL spike, a high degree of confidence can be placed in the detection of a given compound. On the other hand, when no indication of an exogenous substance is provided by either technique, it can be said that the compounds of interest are not present. A compromise position can also exist, where some components of the detection workflow indicate the presence of a compound, but others do not, as seen in Figure 6 for the 0.05 ng/mL spike. In these cases, laboratories can set out a standard operating procedure about what samples should be subject to further analysis.

3.4. Comparison of Techniques

The major limitation of both techniques is the structural diversity of the opioids themselves. While the identified diagnostic product ions cover a large majority of the known structures of synthetic opioids, it is always possible that new structures will arise that will not lead to the formation of these ions. As has been demonstrated previously [21], certain fentanyl analogues that contain additional substitutions on their piperidine ring, such as carfentanil, do not give rise to the same diagnostic product ions as the majority of fentanyl analogues. Carfentanil, in particular, presents the common product ion at *m/z* 105.0704 similar to other fentanyl derivatives (Figure 7), albeit at a lower abundance. This compound, however, does not display the other common product ion at *m/z* 188.1439. This means that screening using the proposed PIS method may not identify samples where this compound is present. It is possible to include further diagnostic product ions that are more specific to different compounds, however this begins to create a large list of possible masses for extraction, comparable to targeting a wide array of theoretical opioid analogue precursor *m/z* values, which introduces difficulties for high-throughput screening. The benefit of using a smaller number of product ions to encompass a larger range of compounds is that there is much less data for the analyst to review, making the screening easier from an operational perspective. While there are certainly limitations to the PIS technique, the broad array of compounds that it does encompass, along with the opportunity for a similar technique to be applied to different compound classes [32], demonstrates the applicability of this approach to a non-targeted screening workflow.

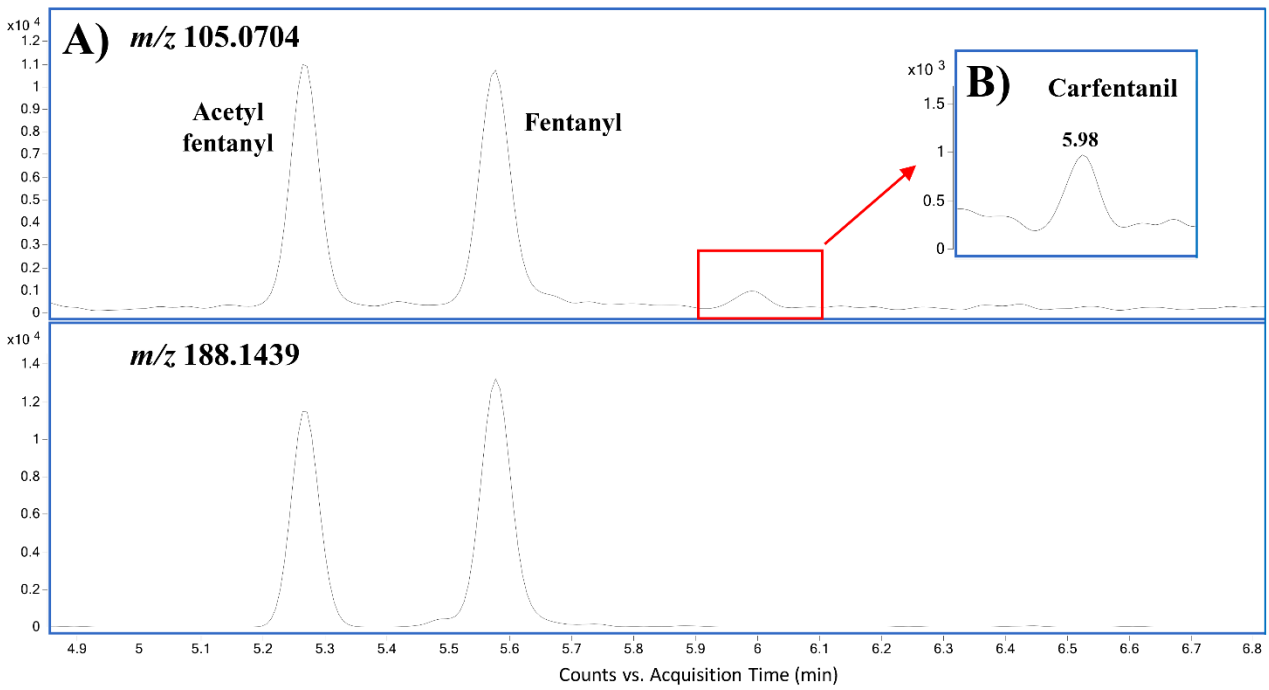


Figure 7. EICs showing common fentanyl product ions at m/z 105.0704 and 188.1439 (A) showing a peak for carfentanil at a lower abundance at m/z 105.0704 (B) but not m/z 188.1439

The structural diversity of the opioids also presents challenges for KMD analysis, which is particularly evident for the fentanyl analogues. This technique works well for compounds that contain repeating units, however if multiple different changes are present in the chemical structure, it will change the KMD value, meaning that it may no longer fall within the filter parameters. This drawback has been somewhat mitigated by the inclusion of multiple filters for different subgroups of fentanyl analogues, which can then capture a representative group of the known compounds in this group. This means that a balance needs to be maintained between targeted and non-targeted approaches by selecting the right number of filters appropriate to a specific application. When screening for high priority compounds, such as carfentanil, specific filters can be added to detect these compounds, however, for the purpose of non-targeted screening, this should be used sparingly. In comparison to the fentanyl analogues, a larger portion of AH and U opioids are covered by a small number of filters, however there are still some compounds, such as U-50488, that may fall outside of these filters.

These drawbacks should not detract from the application of this technique. Given that *DefectDetect* operates using an averaged mass list, it is vendor software agnostic. This means that it can be implemented into a routine workflow regardless of the type of instrument used in a laboratory, so long as an averaged mass list can be generated. In addition, this technique has been previously shown to be useful for other drug classes, namely cathinones and phenethylamines [28,31]. The filters used for the KMD analysis are user-defined, meaning that multiple different drug classes can be combined into a single output, depending on the priorities of the specific laboratory. In this study, KMD values were corrected for a repeating CH_2 unit, which was found to be most appropriate for synthetic opioids, however different correction factors may be applied to more efficiently detect different drug classes. It is important when applying this technique to new compound groups that common structural features are identified and different correction factors are evaluated to determine the optimal filters to use, which encompass the broadest range of compounds.

These non-targeted screening methods were applied to 157 race-day equine plasma samples obtained by the ARFL during the 2019 Autumn Carnival. These samples were analysed using the routine screening method in place at ARFL before being subjected to the non-targeted workflow. While these samples did not return any positive detections with the applied methods, it did allow for both PIS and KMD analysis to be evaluated alongside routine analysis in terms of their ease of use. The PIS method was found to be easy to append to previously employed data analysis workflows. Having an established list of product ions to be extracted made it simple to apply to routine batches containing 30 to 50 samples. KMD analysis, while still easy to perform, proved slightly more time consuming in the generation of EICs for each sample based on the results table provided by *DefectDetect*. The implementation of both PIS and KMD techniques alongside routine workflows can be completed in approximately 2 to 3 hours (in addition to normal data checking procedures) for a routine sequence containing 30 to 50 samples.

Table 4 provides a summary of the performance of the PIS and KMD techniques. While quantitative validation was not completed for this proof-of-concept study, the screening cut-off levels show promise for the application of these techniques to routine screening. The estimated cut-off levels indicate that PIS can detect analytes close to their estimated LODs from validation performed by the ARFL for conventional targeted screening. KMD analysis resulted in a higher screening cut-off level being estimated. This may be due to the averaging of mass spectral data, specifically the averaging of ion intensities, potentially causing low concentration analytes to fall outside of the defined filters.

Table 4. Overview of the estimated limits of detection (LODs) for the different detection techniques applied.

Compound	Estimated Targeted LOD (ng/mL) [30]	Screening Cut-off (ng/mL)	
		PIS	KMD
Acetyl fentanyl	0.01	0.05	0.1
Carfentanil	0.05	-	0.1
Fentanyl	0.01	0.05	0.1
AH-7921	0.01	0.05	0.1
U-50488	0.05	0.05	-

*PIS: Product Ion Searching; KMD: Kendrick mass defect

Given that these techniques are focused on the ‘back-end’ data analysis methods, their sensitivity can also be affected by the instrument and analytical methods used. For both techniques, it is possible that compounds at concentrations lower than the estimated cut-off levels will be detected, however the reliability of detection will be lower.

Due to the structural diversity of the synthetic opioids, there is no one technique that is best for all the compounds analysed. The compounds used for this study were chosen to provide a representative panel of compounds that belong to each group of the synthetic opioids. While there might be other compounds that do not follow the patterns observed in this study, the results obtained demonstrate the importance of using a workflow containing multiple, complimentary

techniques. In this way, the drawbacks of one technique can be made up for by another, providing a more holistic screening for compounds that might be present in a given sample.

This study has demonstrated the potential of the of PIS and KMD analysis for non-targeted screening of synthetic opioids. There is scope for future work to continue to expand the usefulness of these techniques to further derivatives to increase their effectiveness. Newer synthetic opioid classes, such as the benzimidazole opioids recently reported by Blanckaert *et al.* [33], can also be investigated for inclusion in non-targeted screening workflows implementing these techniques. From an operational perspective, further work into the automation of the developed techniques may prove valuable to assist their application to routine analysis.

4. Conclusion

Non-targeted screening of synthetic opioids can be performed by exploiting common fragmentation patterns within the different subclasses of opioids and trends within the KMD of the compounds. The blind trials demonstrated the potential of the PIS and KMD analysis techniques in a realistic, pseudo-operational context to enhance the screening capabilities for fentanyl analogues. In addition, they demonstrated the importance of using the detection techniques as part of a complementary workflow to increase the confidence in the detection of compounds of interest.

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