



Retrospective screening of synthetic cannabinoids, synthetic opioids and designer benzodiazepines in data files from forensic post mortem samples analysed by UHPLC-QTOF-MS from 2014 to 2018

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ABSTRACT

The introduction of new psychoactive substances (NPS) on the illicit drug market has led to major challenges for the analytical laboratories. Keeping screening methods up to date with all relevant drugs is hard to achieve and the risk of missing important findings in biological samples is a matter of concern. Aiming for an extended retrospective data analysis, diagnostic fragment ions from synthetic cannabinoids ($n=251$), synthetic opioids ($n=88$) and designer benzodiazepines ($n=26$) not included in our original analytical method were obtained from the crowdsourced database HighResNPS.com and converted to a personalized library in a format compatible with the analytical instrumentation. Data files from the analysis of 1314 forensic post mortem samples with an Agilent 6540 ultra high pressure liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) performed in our laboratory from January 2014 to December 2018 were retrieved and retrospectively processed with the new personalized library. Potentially positive findings were grouped in two: The most confident findings contained MS/MS data for library match (category 1) whereas the less confident findings lacked such data (category 2). Five new category 1 findings were identified: Flubromazepam in two data files from 2015 and 2016, respectively, phenibut (4-amino-3-phenylbutyric acid) in one data file from 2015, fluorofentanyl in one data file from 2016 and cyclopropylfentanyl in one data file from 2018. Retention time matches with reference standards further strengthened these findings. A list of 35 presumably positive category 2 findings was generated. Of these, only one finding of phenibut was considered plausible after checking retention times and signal-to-noise ratios. This study shows that new compounds can be detected retrospectively in data files from QTOF-MS using an updated library containing diagnostic fragment ions. Automatic screening procedures can be useful, but a manual re-evaluation of positive findings will always be necessary.

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1. Introduction

In recent years, there has been a continuously increasing number of new psychoactive substances (NPS) appearing on the European illicit drug market [1]. The diversity and high number of new compounds pose challenges for clinical and toxicological laboratories who strive to keep their drug screening methods

updated. Synthetic cannabinoids, i.e. compounds acting as cannabinoid receptor agonists and produced as alternatives to Δ -9-tetrahydrocannabinol (THC) represent the largest and most structurally diverse group [2]. New synthetic opioids, and in particular the fentanyl analogues, have been of mounting concern because of their formidable toxic potential [3–6]. Designer benzodiazepines is another group in focus due to the high prevalence of use, at least in our country [7], compared to other groups of NPS.

To develop, establish and maintain a screening method capable of detecting all drugs relevant at any given time is a major challenge. The use of high resolution mass spectrometry (HR-MS)

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e.g. quadrupole time of flight mass spectrometry (QTOF-MS) instrumentation has proven to be an applicable tool when searching for drugs of abuse in biological samples [8–13]. The detection of unknown compounds is time consuming and hardly feasible on a routine basis with a large number of samples. Consequently, the method must encompass a screening or targeted approach, based on an extensive and comprehensive database containing multiple types of data for identification. Such data can be retention times (RTs) and fragmentation data from collision-induced dissociation (CID), *in silico* or other theoretical evaluations, in addition to the molecular formula of the substance. The database can be created and maintained “in-house” by the laboratory. This requires access to a high number of well-defined reference compounds. Procurement of reference standards is costly, particularly if a database should be up to date with as many new and relevant compounds as possible. Databases are also commercially available from suppliers of MS instruments (e.g. the Forensic Toxicology Personal Compound Database and Library from Agilent), but users are dependent on the frequency of new releases and/or additions being up to date. There are also examples of commercial operators offering free databases (e.g. the mzCloud from ThermoFisher). Another opportunity is crowdsourced databases with information submitted by global HR-MS users. One such example is HighResNPS.com [14]. When performing CID on a certain compound, different instrument configurations tend to generate the same diagnostic fragment ions even though the relative abundance may vary. Thus, fragment data acquired on one instrument can then be used as identification across platforms [14–16]. In principle, the same is true for a crowdsourced database with diagnostic fragments acquired by instruments from different manufacturers, providing that the added fragment masses are converted to theoretical values.

In contrast to analytical methods based on single ion monitoring or multiple reaction monitoring, HR-MS full-spectrum data remain available and permit the identification of non-target compounds and retrospective analysis, also called post-target analysis. For data from HR-MS instrumentation with fragmentation capabilities, e.g. QTOF-MS or linear ion trap Orbitrap, fragmentation data are also available. In principle, all compounds are available for investigation at a certain level, but the data available are limited by sample extraction recovery, chromatographic selectivity and the degree of ionization and fragmentation. Depending on which acquisition mode is used, the QTOF-MS data also contain fragment ions originating from the molecular ions generated in the ion source. Based on new knowledge, post-targeted analysis of data can generate new findings in a specific toxicological or clinical sample and ultimately change the conclusion in a particular case. A retrospective study is also important as an internal quality check for the laboratory to assess whether the screening repertoire used is comprehensive and relevant. In addition, new trends in drug abuse can be identified, as exemplified in the study by Kriikku et al. where the toxic lifespan of U-47700 was explored [17].

The number of studies applying such a retrospective approach in a forensic or clinical toxicology setting are limited. Noble et al. processed 2339 forensic samples retrospectively with a targeted screening method to detect 50 4-anilidopiperidine-related fentanyl analogues [18]. In another case study U-47700, diclazepam and flubromazepam were detected in retrospect [19]. Mollerup et al. applied a post-targeted approach when developing a screening method for valproate using positive ionisation mode [20]. Retrospective analysis of urine samples has been used to detect metabolites of pesticides [21]. Post-targeted analysis of data has also been used for detection of drugs and pesticides in non-human matrices including sewage water, surface water and food [22–26].

Since December 2013, our laboratory has utilized a workflow based on ultra-high performance liquid chromatography (UHPLC)

coupled to a 6540 QTOF-MS from Agilent (Santa Clara, CA, USA) for therapeutic drugs and drugs of abuse in post mortem blood samples. The same UHPLC and MS method has been applied from 2014 to the present. A commercial database supplied with entries added manually after analysing reference materials has been used for identification. However, in order to detect a new or previously unknown drug in a biological sample, additional information connected to the case or sample (e.g. a seizure) has to be available. In our experience, such information is rarely available, and this may increase the risk of missing detection of NPS. The consistency of the screening method enables retrospective analysis so that new compounds can be found. The use of HighResNPS for identifying compounds in samples analysed on Agilent QTOF-MS has previously been shown, but only files from data independent acquisition (DIA) could be investigated with this approach [14]. Our method was based on data dependent acquisition (DDA) which, as opposed to DIA, involves acquiring of MS/MS spectra after selection of precursor ions isolated by the quadrupole. A thorough explanation of the differences between DIA and DDA can be found e.g. in the papers of Sundström et al. [27] and Broecker et al. [8]. To be able to use HighResNPS, diagnostic fragment information from the database had to be converted to spectra in the format accepted by the Agilent MassHunter Qualitative searching tool. In Agilent terminology, a library is the sum of compounds in a database containing MS/MS spectra and these databases and libraries are called Personal Compound Database and Library (PCDL).

The aim of this study was to re-process data files of forensic post mortem samples acquired from January 2014 to December 2018 in a PCDL-facilitated search for NPS belonging to the sub-groups synthetic cannabinoids, synthetic opioids and designer benzodiazepines.

2. Materials and method

2.1. Chemicals and reagents

Reference substances used in the experiments to calculate recoveries and matrix effects and explore instrument sensitivities were purchased as solid material or stock solutions from either of the following sources: Cayman Chemicals (Ann Arbor, MI, USA), Chiron AS (Trondheim, Norway), Sigma Aldrich (St. Louis, MO, USA) and Lipomed (Arlesheim, Switzerland). Individual stock solutions in the range from 0.2 to 1.0 mg/mL were prepared and combined into working solutions which were spiked into blood. For confirmation of tentative findings, reference substances of tilidine, phenibut (4-amino-3-phenylbutyric acid) and JWH-167 were purchased from Sigma Aldrich, Chiron AS and Cayman Chemicals respectively. LC-MS quality acetonitrile, methanol, LiChrosolve[®] water and ARISTAR[®] formic acid were all purchased from VWR Chemicals (Oslo, Norway). Ammonium acetate of LC-MS grade was from Sigma Aldrich (St. Louis, MO, USA). A solution of the internal reference standards codeine-d₃, morphine-d₃, benzoylecgonine-d₃ and griseofulvin was prepared by diluting stock solutions in 20% methanol (v/v) in water to a final concentration of 200 ng/mL. D₃-codeine, d₃-morphine and d₃-benzoylecgonine were from Lipomed whereas griseofulvin was from Janssen Chimica (Geel, Belgium).

2.2. Validation of original screening method

2.2.1. Instrument sensitivity and limit of identification

The same UHPLC-QTOF-MS instrumental method, sample preparation and internal reference standard concentration were used for all the samples throughout the period. The peak area results and RTs of the internal reference standards in one data file

per batch were extracted in order to illustrate the variation in response over time. Limit of identification (LOI) was evaluated for a selection of synthetic cannabinoids (MDMB-CHMICA, AB-CHMINACA, BB-22, JWH-018, PB-22 and THJ-018), synthetic opioids (fentanyl, remifentanyl, cyclopropylfentanyl, para-fluorofentanyl, furanylfentanyl, acetylfentanyl) and designer benzodiazepines (deschloroetizolam, diclazepam, etizolam, flubromazepam, flubromazolam, pyrazolam and meclonazepam). Blood samples were spiked at 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10 and 20 ng/mL, and prepared in triplicates with the same method as described for the post mortem samples. LOI was defined as the minimum concentration where the compound was identified and at least one MS/MS spectrum was acquired for library search in all three parallels (see Section 2.5 for details on identification).

2.2.2. Recovery and matrix effects

Recoveries (REs) and matrix effects (MEs) were calculated for the same compounds as used in the LOI experiment. Subsamples of pooled whole blood were spiked after (B) or before (C) extraction to a final concentration of 0.1 µg/mL. The peak areas in neat standard solution of the same concentration (A), sample B and C were used to calculate RE and ME (Eqs. (1) and (2)). An ME below 100% indicates ion suppression whereas a value above 100% indicates ion enhancement.

$$RE (\%) = \frac{C}{B} \times 100 \quad (1)$$

$$ME (\%) = \frac{B}{A} \times 100 \quad (2)$$

2.3. Original analysis of the blood samples

Data files included in this study were from the analyses of post mortem blood samples from forensic autopsies sent to our laboratory in the period from January 2014 to December 2018. In a limited number of cases where blood was not available, spleen tissue was used. Samples from a total of 1314 cases were analysed in this period. Permission to re-process the data files (in this context meaning opening the data file and run the algorithm with the new PCDL) was given by the Regional Committee of Medical and Health Research Ethics in Mid Norway (approval No. 2018/2157). The data files were anonymized and the analyst had no information about the original findings when doing the re-processing. A second person compared the new findings with the analytical report originally attached to the relevant cases. According to the permission granted from the ethics committee, re-analysis of the sample specimens as such could not be performed. The samples were originally processed with the commercially available *Forensic Toxicology Personal Compound Database and Library* from Agilent (Santa Clara, CA, USA) with more than 3000 compounds containing MS/MS spectra complemented with between 250 and 300 compounds with RTs.

2.3.1. Sample preparation

Each blood sample was thawed at room temperature and 200 mg was weighed into a micro tube and 50 µL solution of internal reference standard and 800 µL ice-cold acetonitrile were added. The tube was then mixed on a vortex mixer for 30 s and centrifuged at 7000 g for 10 min. before 500 µL of the supernatant was transferred to a 96-well plate, evaporated to dryness and reconstituted in 50 µL of 30% acetonitrile (v/v) in 0.03 mg/mL ammonium formate. In the cases where only spleen was available, sample preparation was adjusted according to the condition of the tissue. If a blood-like material could be obtained from the spleen, it

was handled as a blood sample. In the other cases a subsample of tissue material was homogenized with an equal volume of H₂O, and 200 mg of this material were processed like a blood sample. The samples were prepared in weekly batches by the same procedure throughout the period.

2.3.2. Instrumentation

Instrumental analysis was performed using a 6540 QTOF-MS (Agilent, Santa Clara, CA, USA) with electrospray ionization (ESI) coupled with a 1290 Infinity UHPLC system from Agilent equipped with an Acquity HSS T3 column (100 mm × 2.1 mm, 1.8 µm) from Waters (Milford, MA, USA). An injection volume of 2 µL was used. Separation was achieved using a mobile phase consisting of 0.05% formic acid in 10 mM ammonium formate (A) and 0.05% formic acid in acetonitrile (B). A gradient with a flow of 0.50 ml/min starting at 5% B increasing to 50% in 10 min. and continuing to 100% over the next 6 min. was used. After a 4-minute hold at 100% B the column was re-equilibrated for 2 min. at 5% B, giving a total cycle time of 22 min. Autosampler and column temperatures were set to 10 °C and 50 °C, respectively.

Positive ESI was used and with fragmentor voltage at 120 V, capillary voltage at 3500 V, gas temp at 320 °C, gas flow at 8 L/min, nebulizer pressure at 40 psig and sheath gas temperature at 380 °C. Data was acquired in data dependent Auto MS/MS mode. MS spectra and MS/MS spectra were both acquired in the mass range of 50–1000 *m/z* at a rate of 6 Hz. The detector operated in 2 GHz extended dynamic range giving a resolution (*m/Δm* at FWHM) of approx. 20,000 at *m/z* 322.0481. Precursor selection was based on abundance and an intensity threshold of 1000 counts was applied. After one spectrum from a precursor was acquired, this specific precursor was excluded for 0.03 min. Precursors were fragmented in the collision cell using an electron voltage according to Eq. (3):

$$\text{Collision energy (eV)} = 4 + (0.06 \times m/z \text{ of precursor}) \quad (3)$$

The computer controlling the instrument was equipped with the MassHunter Acquisition software (Acq) B.05.01 (Agilent, Santa Clara, CA, USA). The acquired data files consisted of MS1 (full spectrum MS-only) of all ionized compounds and MS/MS spectra of the precursors selected for fragmentation. The *m/z* masses of 121.0509 and 922.0098 were applied for automated mass correction in all MS spectra. A daily performance sample of amphetamine (0.74 ng/mL), diazepam (0.35 ng/mL), 7-amino-flunitrazepam (0.35 ng/mL), morphine (0.35 ng/mL) and Δ⁹-tetrahydrocannabinol (0.5 ng/mL) in MeOH was injected at the beginning of every analytical run to monitor important instrument parameters. Samples were not analysed if large deviations in RTs (more than 0.2 min.), mass accuracies (more than 5 ppm) or peak areas from the historical averages were observed for the compounds in the daily performance sample.

2.4. Creating a new PCDL

HighResNPS (highresnps.com) is a free, online, spreadsheet-format, crowdsourced HR-MS database for NPS-screening initiated and managed by a group of researchers at Section of Forensic Chemistry at the University of Copenhagen [14]. Several contributors worldwide submit fragmentation data when new drugs (reference standards or seizures etc.) are detected and analysed by a HR-MS instrument. Also, diagnostic ions derived from theoretical dissociations of the molecules are supplied. From this HighResNPS database (total number of entries in May 2019 was 1782 including duplicates, and 1304 contained at least one diagnostic fragment ion), 374 unique compounds with minimum one diagnostic fragment primarily belonging to the drug classes synthetic cannabinoids, synthetic opioids or designer benzodiazepines were selected. NPS already present in the screening method

Table 1
Number of new compounds included in the HighResNPS subset Personal Compound Database and Library (PCDL) grouped according to drug class and source of diagnostic fragment ions.

	Synthetic cannabinoids	Synthetic opioids	Designer benzodiazepines	Total
Library spectra based on diagnostic ions from standards	126	47	22	195
Library spectra based on diagnostic ions from theoretical evaluation	116	40	0	156
Library spectra based on diagnostic ions from seizures	4	2	4	10
Library spectra based on diagnostic ions from RESPONSE project ^a (seizures or test purchase on-line)	13	–	–	13
Total number of unique compounds (database entries)	259	89	26	374

^a A European project named Response to challenges in forensic drug analysis. https://www.policija.si/apps/nfl_response_web/seznam.php.

implemented in 2014 were filtered out. Based on this selection a PCDL was developed. For this purpose each compound was added as an individual database entry. Then the software tool “Spectrum Generator” created by Broeckers Solutions (Berlin, Germany) was used to convert the text-based information of diagnostic ions from the HighResNPS database into the Agilent “cef” file format which allows an import of library spectra for each PCDL entry. Table 1 shows the resulting HighResNPS subset PCDL content. By this approach the diagnostic fragment ions were stored as a library spectrum. Relative abundance of the ions was not taken into account even though this would be possible by the software “Spectrum Generator”. The collision energy of the library spectra was chosen by the software as 20 eV just to have any value in the PCDL. An example of the library entry of flubromazepam is shown in Fig. 1. A complete list of the 374 unique compounds is given in the supplementary material (Table S1).

2.5. Data processing

Of the 1314 data files available, batches of approx. 250 were re-processed using MassHunter DA Reprocessor software B.09.00 (Agilent, Santa Barbara, CA, USA). The re-processing was relatively fast, approximately 1 min. per sample, when using a computer equipped with a 2.67 GHz processor and 8 GB of RAM. This process was running in the background allowing re-processed data files to be opened and evaluated in batches of 50–80 simultaneously in MassHunter Qualitative Analysis Software (version 10.0) (Agilent, Santa Clara, CA, USA).

The qualitative method used in the re-processing was based on the algorithm “Find by formula” together with a library search, both using the HighResNPS subset PCDL. The “Find by formula” search lead to positive findings that were based on MS1 spectral information. The criterion was a mass error less than ± 5 ppm and a score above 80 where the scoring was taking the mass match, isotope spacing and isotope abundance into account. In the case when MS/MS spectra were acquired for the precursor ion of a detected compound, these MS/MS spectra were compared with those in the PCDL. The comparison was done both by reverse search (the peaks in the PCDL are compared with the MS/MS

spectra) and by forward search (the peaks in the MS/MS spectra are compared with the PCDL). The threshold library match was set to 1 (of max. 100) for both forward and reverse score. As the maximum number of fragment ions per library spectrum was three, the lowest resulting reverse score of a match was 33.

A filter in the software was applied in order to distinguish compounds with MS/MS spectra (category 1) and without MS/MS spectra (category 2). Category 1 compounds found by the algorithm “Find by Formula” could be evaluated further by comparing the acquired MS/MS with the library spectrum. If there was no agreement based on the MS/MS comparison the compound was considered a false positive. If there was a match, a visual evaluation comparing the acquired spectrum with the library spectrum was undertaken to rule out false positive matches due to fragments of low abundance e.g. from contaminants. The LOIs estimated for the compounds selected in the validation applies for category 1 compounds.

For category 2 compounds, no MS/MS data had been acquired and fragment confirmation could not be done. Thus, only the MS signal could be used to evaluate the quality of the findings. Without the MS/MS spectra identification parameter the number of potential positives would have been large and included noisy signals and bad peak shapes. A peak area threshold of 5×10^4 was applied to limit the number of findings to investigate. Consequently, higher detection limits were expected for these compounds compared to category 1 compounds. In order not to miss any important findings, a mass accuracy limit of ± 10 ppm and mass match score above 80 was first applied (criterion a). This was tested with 42 random data files and gave 74 findings. After investigating the results and filtering out findings due to interferences and background signal, only compounds with mass accuracy better than ± 5 ppm and mass match score above 95 were left. These two thresholds were consequently used as criterion b. Finally, a third factor was added to criterion b, an RT restriction of 1.5 min, as the compounds in the groups under investigation are highly likely to elute after this time period (criterion c). The number of findings in the 42 random data files as a function of criterion a, b or c are illustrated in Fig. 2. Criterion c (mass accuracy better than ± 5 ppm, mass match score higher than 95 and RT 1.5 min. or more) was applied for all category 2 compounds. A compound appearing in several data files in the same batch was considered an isomer originating from the chemicals used or as endogenous molecules with equal theoretical masses. The risk of accepting false positives is higher for category 2 than for category 1 findings, especially if thresholds and limits are set too wide.

Any new finding was further evaluated by comparing acquired MS/MS spectra with other sources (e.g. mzCloud¹) or alternatively by analysing a reference standard, if available at the laboratory. Due to variations in the RTs over the time period the samples were

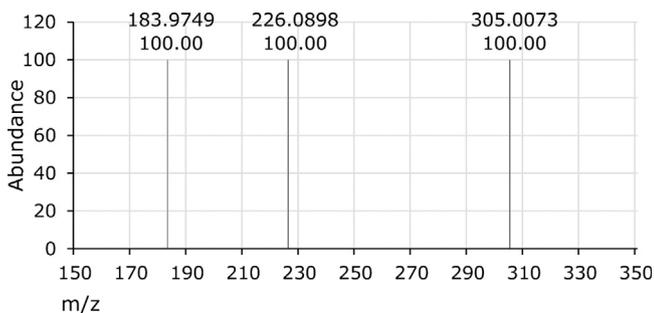


Fig. 1. Library spectrum of flubromazepam.

¹ <https://www.mzcloud.org>.

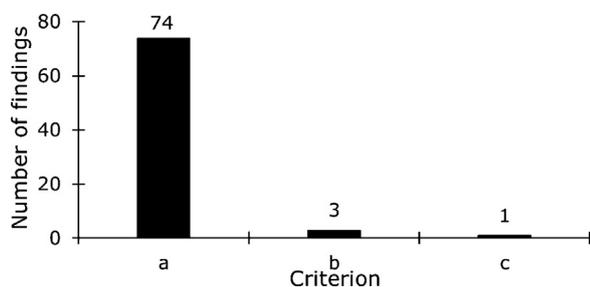


Fig. 2. Number of category 2 findings in 42 random data files as a function of criterion a (peak area threshold of 5×10^4 , mass accuracy limit of ± 10 ppm and mass match score above 80), criterion b (mass accuracy limit reduced to ± 5 ppm and mass match score above 95) or criterion c (mass accuracy better than ± 5 ppm, mass match score higher than 95 and RT 1.5 min. or more).

originally analyzed, RT deviations up to 0.5 min. were tolerated when comparing these samples to reference standards. If a consistency in fragments or RTs was observed, the finding was reported to a person with access to the original case report. If a presumably novel moiety was identified and a reference standard was available, this standard was analysed and RTs and MS/MS spectra were compared.

3. Results and discussion

3.1. Validation of original analytical method

3.1.1. Instrument sensitivity and limit of identification

The instrument response and RT variation over time was expressed by plotting the peak area and RT of the internal reference standards extracted from one calibrator from each analytical run (Fig. S1 in supplementary material). Morphine-d3 showed an RT difference (maximum – minimum) of 0.28 min. and a mean peak area of 2.7×10^5 (standard deviation (SD) 1.3×10^5). Codeine-d3 showed an RT difference of 0.35 min. and a mean peak area of 4.4×10^5 (SD 1.6×10^5). Benzoylcegonine-d3 showed an RT difference of 0.32 min. and a mean peak area of 7.8×10^5 (SD 3.9×10^5). Finally, griseofulvin showed a RT difference of 0.44 min. and a mean peak area of 2.8×10^5 (SD 1.4×10^5). The peak areas of internal reference standards in the data files are not only reflecting the variation in instrument response but also variation in extraction efficiency and matrix effects over time. This gives a more relevant expression compared to a direct injection of a neat performance test sample.

LOIs were estimated for a representative group of synthetic cannabinoids, synthetic opioids and designer benzodiazepines (Table 2). LOIs are unknown for new compounds but the experiment indicated that synthetic cannabinoids could be detected if present above approximately 10–20 ng/mL, synthetic opioids above 1 ng/mL and designer benzodiazepines above 10 ng/mL. Electrospray ionization is best suited for analysis of compounds with medium-to-high polarity but is not optimal for all compounds [28]. The LOIs in Table 2 are only estimates of the instrument sensitivity through the acquisition period. As seen by the results from the internal reference standards, the peak areas varied during the period due to e.g. instrument condition and periodic maintenance. How this in turn affected the LOIs is difficult to determine, as the value is not only a result of signal intensity, but also the automatic selection of precursor ions based on the DDA settings. If the compound still is among the precursors selected for fragmentation it will probably be identified. Given the peak area threshold applied to detect category 2 substances, a higher concentration must be present in order to detect them as compared to category 1 substances. A review of the data files from the LOI experiments shows that a peak area of 5×10^4

Table 2

Retention time (RT), limit of identification (LOI), recovery (RE) and matrix effect (ME) for a selection of compounds in the three groups of new psychoactive substances included in the present study.

Substance	RT [min]	LOI [ng/mL]	RE [%]	ME [%]
Synthetic cannabinoids				
MDMB-CHMICA	14.0	10	68	97
AB-CHMINACA	11.9	20	91	107
BB-22	14.3	10	57	86
JWH-018	14.4	2	51	85
PB-22	13.8	10	68	89
THJ-018	14.8	10	32	69
Synthetic opioids				
Fentanyl	7.1	1	87	132
Remifentanyl	5.4	1	94	123
Cyclopropylfentanyl	7.5	1	82	128
Para-fluorofentanyl	7.2	0.5	88	124
Furanylfentanyl	7.3	0.5	100	124
Acetylfentanyl	6.0	1	100	127
Designer benzodiazepines				
Deschloroetizolam	8.8	5	107	119
Diclozepam	10.7	2	87	110
Etizolam	9.3	2	110	121
Flubromazepam	9.1	10	110	72
Flubromazolam	8.5	5	113	121
Pyrazolam	6.4	10	114	122
Meclonazepam	9.3	10	110	105

generally corresponds to two- or threefold the concentration of the LOI of category 1 substances (see Table S2 in supplementary material). These data also indicate that mass match score of 95 is achieved for most compounds when a peak area around 5×10^4 is measured.

3.1.2. Recovery and matrix effects

Major differences were observed in the estimated RE (%) of the synthetic cannabinoids, with values ranging from 32% (THJ-018) to 91% (AB-CHMINACA) (Table 2). The remaining compounds had REs above 82%. All compounds showed an ME between 69% and 127% demonstrating that both ion-suppression and ion-enhancement occur. ME values with relatively little deviation from 100% for the studied compounds indicate that severe ion suppression is unlikely for other compounds in these groups.

3.2. Retrospective data file analysis

A total number of 1314 data files (242, 252, 273, 242 and 305, respectively, from the years 2014 to 2018) were processed with the new PCDL. The retrospective analysis revealed six new findings of category 1 in addition to two compounds (fluorofentanyl and cyclopropylfentanyl) that had been reported when the data files were processed with the original method, but only after seized material had become available (Tables 3 and 4). In addition there were 35 possible findings of category 2 (Table 5) not reported when the data files were processed with the original method.

3.2.1. Category 1 findings

Flubromazepam was detected in two data files from 2015 and 2016 respectively. There was a mass match score in both data files higher than 95, a mass accuracy better than 3.46 ppm and an RT deviation of less than 0.07 min. The mass match can be visualized by the resemblance of the spectrum of flubromazepam and the theoretical pattern indicated by the boxes in Fig. 3. The three diagnostic fragments in the library spectrum were also found in the MS/MS data acquired from the precursor in the two data files (see Fig. 4A). An additional comparison of the MS/MS spectra from the data file and the analysis of a reference standard showed good

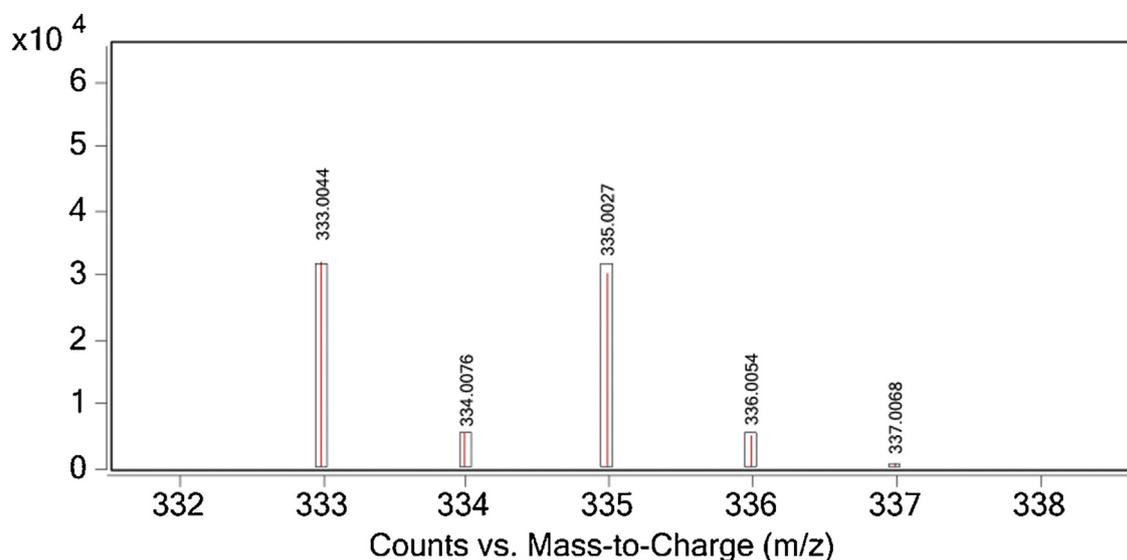


Fig. 3. MS1-spectrum of flubromazepam extracted from a data file (red lines) with theoretical isotopic pattern illustrated by the black boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

agreement also for additional fragment masses (see Fig. 4B). Flubromazepam was first described in 1962 and is a highly potent and incompletely evaluated benzodiazepine structurally related to phenazepam [29,30]. Flubromazepam started to emerge in online shops in Europe in 2012. In Norway it was detected in seized material by the Norwegian National Criminal Investigation (KRIPOS) for the first time in 2013.

Phenibut was detected in a data file from 2015 and showed a mass match score higher than 85, a mass accuracy of -1.63 ppm and an RT deviation of 0.12 min. compared to a reference standard analysed in 2018. Evaluation of the RT over time showed that a deviation up to 0.5 min. could be expected due to change of analytical column lot and tubing. Phenibut is a neuropsychotropic drug with possible cognition enhancing effects that was discovered and introduced into clinical practice in the 1960s Soviet Union [31]. The drug is widely used in Russia and is claimed to have various clinical effects, e.g. to relieve tension and anxiety and to improve sleep. Phenibut can cause dependency. It is not scheduled or classified as a medicinal drug in Norway and is not for legal sale. Private import is prohibited by law. KRIPOS did not detect phenibut in any cases before 2019. Our laboratory reported detection of phenibut in seized material and biological samples for the first time in 2016, and it has since then been part of the routine analytical repertoire at our laboratory.

Fluorofentanyl was detected in one data file from 2016 with a mass match score higher than 97, a mass accuracy of -0.21 ppm and good agreement in the diagnostic ions. Analysis of reference

material showed an RT deviation of less than 0.05 min. Moreover, a compound with molecular formula $C_{23}H_{28}N_2O$ was detected in a data file from 2018 with mass match score higher than 96 and mass accuracy of 2.87 ppm. The diagnostic fragments of m/z 105.0699 and 188.1434 showed that the compound most probably was a fentanyl analogue and the software suggested either cyclopropylfentanyl, methacrylfentanyl or crotonylfentanyl. These three compounds share the same formula and diagnostic fragments. Consequently, they are not possible to distinguish from each other based on category 1 criteria only, but analysis of reference material showed good RT agreement (deviation 0.01 min.) with cyclopropylfentanyl. In fact, fluorofentanyl and cyclopropylfentanyl had already been confirmed by targeted analysis of the data files based upon information from analysis of seizures from the scene requested by the police [32,33]. However, as these compounds would not have been detected originally if we had not known which substances to suspect, they are included in the present material.

Identification of flubromazepam, phenibut, fluorofentanyl and cyclopropylfentanyl (of category 1) was based on the mass accuracy of the monoisotopic MS signal, presence of diagnostic fragment ions and, finally, RT agreement. Fulfilment of these criteria gave the highest level of confidence that can be achieved in a retrospective review when re-analysis of the actual specimen is not possible. Detection and confirmation of compounds with HR-MS can be divided in different levels of confidence based on information available from the data acquisition, as suggested by

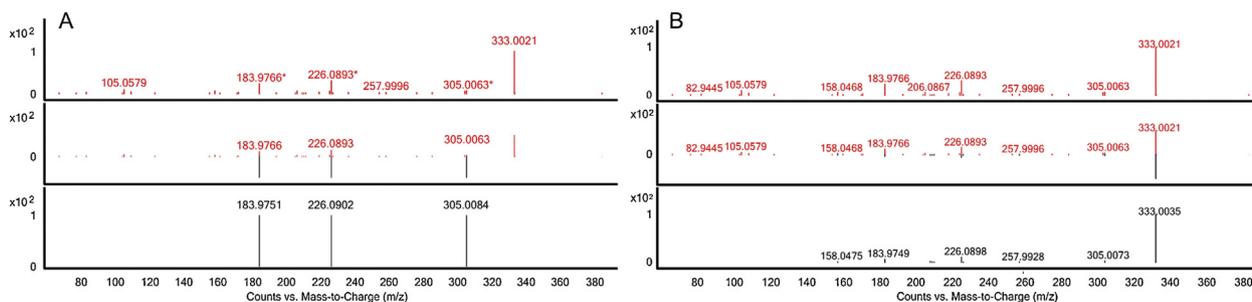


Fig. 4. (A) Acquired MS/MS-spectrum of flubromazepam with diagnostic fragments marked with asterisk (at the top), library spectrum from PCDL (at the bottom) and a comparison (in the middle). (B) Acquired MS/MS-spectrum (at the top), full MS/MS-spectrum from a flubromazepam reference standard (at the bottom) and a comparison (in the middle).

Table 3

New compounds found after applying category 1 criteria, including identification data and case information.

Compound (year)	Molecular formula	Retention time sample/reference standard (Δ min)	Mass match score	Diagnostic fragment	Mass (calculated)	Mass accuracy [ppm]	First reported in Norway	Case information
Flubromazepam (2015)	$C_{15}H_{10}BrFN_2O$	9.08/9.15 (-0.07)	95.55		314.0049	3.46	2013 ^a	Male, approx. 30 yrs. old. History of drug abuse, found dead after drug use. Ethanol, amphetamine, metamphetamine, methylenedioxyamphetamine, methylenedioxyamphetamine, diazepam, desmethyl-diazepam, 7-aminoclonazepam, alprazolam, pregabalin, mephedrone, buprenorphine, norbuprenorphine and gamma-hydroxybutyrate found in blood.
				$C_{14}H_{11}FN_2$	226.0901	-3.29		
				C_7H_7BrN	183.9756	5.25		
				$C_{14}H_{11}N_2FBr$	305.0084	-7.10		
Phenibut ^c (2015)	$C_{10}H_{13}NO_2$	1.53/1.65 (-0.12)	85.36		180.1019	-1.63	2016 ^b	Same subject as above.
				C_9H_9	117.0699	1.81		
				$C_{10}H_9O$	145.0648	-16.67		
Flubromazepam (2016)	$C_{15}H_{10}BrFN_2O$	9.21/9.15 (0.06)	97.45		333.0033	0.24	2013 ^a	Female, approx. 50 yrs. old. History of drug abuse, found dead at home. Ethanol, paracetamol, gabapentin, pregabalin, tramadol, O-desmethyltramadol, amitriptyline, nortriptyline, sertraline and chlorprothixene found in blood.
				$C_{14}H_{11}FN_2$	226.0901	3.48		
				C_7H_7BrN	183.9756	0.95		
				$C_{14}H_{11}N_2FBr$	305.0084	16.4		
Fluorofentanyl ^d (2016)	$C_{22}H_{27}FN_2O$	7.18/7.17 (0.01)	97.73		355.2180	-0.21	2016 ^b	Male, approx. 20 yrs. old. Found dead at home with drug paraphernalia. 7-aminoclonazepam, diazepam, desmethyl-diazepam, alprazolam, tetrahydrocannabinol and gamma-hydroxybutyrate found in blood.
				$C_{13}H_{18}N$	188.1434	-5.42		
				C_8H_9	105.0699	-3.56		
				$C_{14}H_{17}FNO$	234.1289	-9.92		
Cyclopropylfentanyl ^d (2018)	$C_{23}H_{28}N_2O$	7.46/7.47 (-0.01)	96.53		349.2274	2.87	2017 ^a	Male, approx. 30 yrs. old. Found dead at home with pills on site. Morphine, morphine-3-glucuronide, morphine-6-glucuronide, buprenorphine, norbuprenorphine, pregabalin, amphetamine, methylenedioxyamphetamine, methylenedioxyamphetamine, benzoylecgonine, 7-aminoclonazepam and tetrahydrocannabinol found in blood.
				$C_{13}H_{18}N$	188.1434	-2.52		
				C_8H_9	105.0699	1.05		
				$C_{15}H_{18}NO$	228.1383	-4.46		

^a Detected in seized material by the Norwegian National Criminal Investigation.^b Detected in seized material by our department.^c 4-amino-3-phenylbutyric acid.^d Reported originally but included here to illustrate method suitability.

Schymanski et al. [34]. In that approach, level 5 through level 1 requires increasing information from the MS signal to diagnostic fragments and RTs [34]. Findings of category 1 in our retrospective method can be compared to a situation close to level 1. Level 1 requires confirmation with a reference standard, which was the case with our new findings, but as long as the sample and standard are not analysed simultaneously, a definite confirmation is not achieved.

In a retrospective approach co-identification of metabolites can further strengthen the confidence of a finding. Searches for the major metabolites of the detected compounds were done in the relevant data files. Metabolites from published in vivo and in vitro studies were selected [29,35–37]. Neither of the metabolites of fluorofentanyl were detected in the data file containing this compound. In the data file containing cyclopropylfentanyl the *N*-dealkylated metabolite and two hydroxylated metabolites were detected. The metabolites of flubromazepam found in literature to be the most abundant (hydroxylated flubromazepam and debrominated flubromazepam) were not detected in any of the two positive samples. The metabolism of phenibut has to our knowledge not been studied, and no putative target metabolites have been described in the literature.

Three other positive category 1 findings could be refuted after further investigation (Table 4). For methoxyacetylfentanyl the RT deviation compared with the reference standard was significant,

indicating that the compound rather was an isomer of methoxyacetylfentanyl with similar fragmentation patterns. There were no other described fentanyl analogues with identical molecular formula. The presence of fragments of *m/z* 105.0699 and 188.1434 was however a strong indicator that the compound consisted of the piperidine and phenyl moiety characteristic to fentanyl itself as well as many fentanyl analogues. Metabolites of fentanyl hydroxylated at the alkyl or phenetyl moiety have the same monoisotopic mass as methoxyacetylfentanyl and the diagnostic fragments 105.0699 and 188.1434 will be the same (Fig. 5).

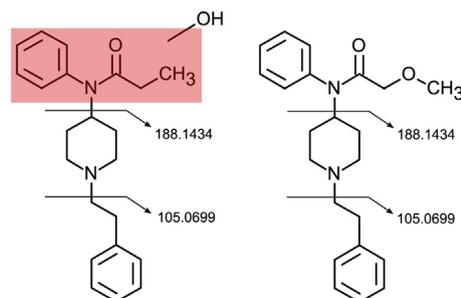


Fig. 5. Fragmentation of hydroxyfentanyl (left) and methoxyacetylfentanyl (right). The superimposed area indicates position of hydroxyl-group.

Table 4
New compounds found after applying category 1 criteria, but refuted as “false positive” findings.

Compound (year)	Molecular formula	Retention time (RT) sample/reference standard (Δ min)	Mass match score	Diagnostic fragment	Mass (calculated)	Mass accuracy [ppm]	Comment
Methoxyacetylfentanyl (2016)	C ₂₂ H ₂₈ N ₂ O ₂	5.13–5.75 (–0.62)	91.36		353.2224	0.20	RT not in agreement with reference standard. Monoisotopic mass and diagnostic fragments suggest fentanyl hydroxylated at the alkyl or phenethyl moiety
				C ₁₃ H ₁₈ N	188.1434	–3.8	
				C ₈ H ₉	105.0699	–17.23	
				C ₉ H ₁₂ N	134.0964	Not found	
JWH-167 (2014)	C ₂₁ H ₂₃ NO	11.82–13.86 (–2.06)	95.32		306.1852	0.90	RT not in agreement with reference standard
				C ₁₄ H ₁₆ NO	214.1226	7.94	
				C ₇ H ₇	91.0542	–9.63	
				C ₁₃ H ₁₈ N ^a	188.1434	–11.05	
Tilidine (2015)	C ₁₇ H ₂₃ NO ₂	9.24–5.56 (3.28)	90.87		274.1802	1.65	RT not in agreement with reference standard
				C ₁₅ H ₁₇ O	229.1223	Not found	
				C ₁₂ H ₁₁	155.0855	–5.88	
				C ₇ H ₇	91.0542	45.1	

^a Diagnostic fragment from mzCloud.

Fentanyl was reported in the original analysis of the sample, which explains the presence of a metabolite. Thus, it could be concluded that the finding was caused by fentanyl intake.

Category 1 findings of JWH-167 and tilidine were detected in one data file each, from 2014 and 2015, respectively. The fragments in the MS/MS spectra were in relatively good agreement with the diagnostic fragments from the library spectrum, and in addition the m/zCloud database was consulted and showed agreement with one additional fragment. Reference standards were acquired to compare RTs and significant RT differences clearly showed that neither JWH-167 nor tilidine were present. These examples of false positive results illustrate the importance of having access to the reference substance in order to check RT conformity.

3.2.2. Category 2 findings

A total of 35 possible category 2 findings was the result when applying criterion c (better than 5 ppm mass accuracy, mass match score higher than 95 and RT 1.5 min. or later). The initial findings are presented in Table 5. A further evaluation of RT, signal-to-noise ratio and chromatographic peak shape for every finding was done. The metabolite AB-FUBINACA M3 (#13–15), carfentanil (#17 and 18), tilidine (#35) and three of four findings of phenibut (#31, 32 and 34) could be disproved due to large RT deviations from reference standards. Based on the RTs of other synthetic cannabinoids analysed with the same chromatographic conditions (see Table 2) findings of synthetic cannabinoids with RTs less than 5 min. were regarded as highly unlikely and removed from the list. This was the case for 5-fluoro-PY-PINACA (#3 and 4), 5-fluoro-3,5-AB-PFUPPYCA (#5), AB-BICA (#9 and 10) and MA-CHMINACA (#20). 5-fluoro-AB-PINACA N-(4-hydroxypentyl) (#2), a metabolite and presumably more polar compound than its parent substance, is likely to have a shorter RT. Still, it will probably not elute as early as 3.6 min. A similar limit of 4 min. was applied on the synthetic opioids which lead to the rejection of 3-fluoro methoxyacetyl fentanyl (or ocfentanyl) (#1) and two findings of N-methyl norcarfentanil (#25 and 26). The signal-to-noise ratio was 3 or less for AB-CHMINACA 3-carboxylindazol (#11 and 12), N-methyl norcarfentanil (#27) and PB-22 3-carboxyindole (#30). The initial finding determined as benzyl carfentanil (#16) was disproved due to poor peak shape. A category 2 compound found in one or more data files, and also found with the same RT in other data files having MS/MS spectra acquired but no library match, was likewise rejected. One such example was ohmefentanyl, which was found in two data files with RTs of 7.8 min. The ion could also be

found in other data files with the same RT but with acquired MS/MS spectra not in agreement with the PCDL. This strongly indicated that the two findings of ohmefentanyl (#28 and 29) were false positives. The same was the case with presumable findings of AB-FUBINACA (#6–8), JWH-200 analog 1 (or A-796260) (#19) and methoxyacetylfentanyl (#21–24).

Thus, after reviewing the 35 suggested category 2 findings, only one finding of phenibut (#33) remained. As no MS/MS spectra were available for library comparison, this finding could, however, not be confirmed with the same degree of confidence as those of category 1.

3.3. Strengths and weaknesses

The PCDL constructed in this study is based on data acquired on instruments from different manufacturers and based on different principles. A previous study has shown that libraries constructed from data acquired on either Orbitrap or QTOF can be used interchangeably by both instruments providing that suitable collision energies are applied [38,39]. An essential feature of the PCDL is the mass accuracy of the diagnostic fragments. In HighResNPS the masses of the fragments are added by either typing the formula, selecting the correct formula from a drop-down list of common fragments or typing the theoretical mass of the acquired fragment. This ensures that mass errors from the acquisition are not transferred to the database. A second important setting is the choice of collision energy applied when acquiring the diagnostic fragments that are added to the database. The collision energy applied can either be discrete (e.g. 10, 20 and 40 eV) or ramped, providing a combined result. Information on the choice of strategy used in the individual entry was not present in the database. In the Auto MS/MS method used in this study, the collision energy was a voltage correlated to the mass of the precursor. Potentially this can result in differences in relative abundance when comparing a library spectrum and an acquired MS/MS spectrum. However, the settings in the retrospective reprocessing algorithm ensure a hit even if only one of the diagnostic fragment ions could be found in the acquired spectrum.

The risk of false negative samples will always be present when searching for compounds that have not been subject to specific evaluation of LOI, which is the case for the majority of the compounds in the PCDL. In addition, the instrument response has been shown to fluctuate to some extent during the period of data acquisition. Due to the relatively high LOIs and low recoveries

Table 5

The 35 suggested findings after applying category 2 criteria, with retention time (RT) and an evaluation of whether the identification was correct or not based on the RT and the signal-to-noise ratio (S/N).

Suggested finding #	Compound	Year	RT [min]	Correct identification?
1	3-fluoro-methoxyacetyl fentanyl (or ofentanyl)	2015	3.77	No, fentanyl analogue with RT under 4 min is not likely
2	5-fluoro-AB-PINACA N-(4-hydroxypentyl)	2018	3.59	No, synthetic cannabinoid with RT under 5 min is not likely
3	5-fluoro-PY-PINACA	2016	2.58	No, synthetic cannabinoid with RT under 5 min is not likely
4	5-fluoro-PY-PINACA	2018	2.63	No, synthetic cannabinoid with RT under 5 min is not likely
5	5-fluoro-3,5-AB-PFUPPYCA	2014	3.52	No, synthetic cannabinoid with RT under 5 min is not likely
6	AB-FUBINACA	2016	10.30	No, reference standard showed RT of 10.3 min. A large number of additional data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
7	AB-FUBINACA	2016	10.31	No, reference standard showed RT of 10.3 min. A large number of additional data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
8	AB-FUBINACA	2017	10.44	No, reference standard showed RT of 10.3 min. A large number of additional data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
9	AB-BICA	2014	3.13	No, synthetic cannabinoid with RT under 5 min is not likely
10	AB-BICA	2017	3.79	No, synthetic cannabinoid with RT under 5 min is not likely
11	AB-CHMINACA 3-carboxylindazol	2014	4.12	No, chromatogram shows S/N < 3
12	AB-CHMINACA 3-carboxylindazol	2014	3.92	No, chromatogram shows S/N < 3
13	AB-FUBINACA M3	2014	4.89	No, reference standard showed RT of 11.0 min
14	AB-FUBINACA M3	2015	4.77	No, reference standard showed RT of 11.0 min
15	AB-FUBINACA M3	2017	3.54	No, reference standard showed RT of 11.0 min
16	Benzyl carfentanil	2015	10.91	No, poor chromatography
17	Carfentanil	2015	11.87	No, reference standard showed RT of 7.7 min
18	Carfentanil	2016	11.77	No, reference standard showed RT of 7.7 min
19	JWH-200 analog 1 (or A-796260)	2017	6.15	No, other data files contain the same ion with same RT but with fragment ions not in agreement with library
20	MA-CHMINACA	2014	2.86	No, synthetic cannabinoid with RT under 5 min is not likely
21	Methoxyacetylfentanyl	2014	6.34	No, reference standard showed RT of 5.7 min. Other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
22	Methoxyacetylfentanyl	2018	6.59	No, reference standard showed RT of 5.7 min. Other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
23	Methoxyacetylfentanyl	2018	6.58	No, reference standard showed RT of 5.7 min. Other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
24	Methoxyacetylfentanyl	2018	6.59	No, reference standard showed RT of 5.7 min. Other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
25	N-Methyl norcarfentanil	2014	2.90	No, fentanyl analogue with RT under 4 min is not likely
26	N-Methyl norcarfentanil	2016	2.72	No, fentanyl analogue with RT under 4 min is not likely
27	N-Methyl norcarfentanil	2018	6.20	No, chromatogram shows S/N < 3
28	Ohmefentanyl	2018	7.84	No, other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
29	Ohmefentanyl	2018	7.85	No, other data files contain the same ion with same RT but with fragment ions not in agreement with library spectra
30	PB-22 3-carboxyindole	2016	6.61	No, chromatogram shows S/N ~ 3.6
31	Phenibut	2014	6.39	No, reference standard showed RT of 1.65 min
32	Phenibut	2014	2.94	No, reference standard showed RT of 1.65 min
33	Phenibut	2016	1.58	Yes, probably since reference standard showed RT of 1.65 min
34	Phenibut	2016	4.11	No, reference standard showed RT of 1.65 min
35	Tilidine	2018	4.34	No, reference standard showed RT of 1.65 min

among the synthetic cannabinoids in the validation, the risk of false negatives appears to be more likely in this group. It should also be emphasised that the two large NPS groups cathinones and phenethylamines were left out of this study in order to limit the extent of investigated compounds.

Applying the method on our data files has shown that identification of ions that were not selected for fragmentation (category 2) clearly requires a manual re-evaluation. The list of category 2 findings was significantly longer than category 1 findings, but still 35 potential positives out of 1314 data files is a manageably low number. The peak area threshold of 5×10^4 was important to keep the number of potential category 2 findings low, but will at the same time result in higher detection limits for these compounds. All except one of the potential category 2 findings could be disproved after a careful evaluation of the RTs and signal-to-noise ratios in the chromatogram. The need for a manual evaluation of category 2 findings is a limitation of the DDA approach. If DIA had been used there would have been few presumable findings where the MS1

signal was detected but no fragment ions were available. DIA, on the other hand, is limited by co-eluting compounds being fragmented at the same time resulting in complicated high energy spectra. The pattern can be even more complex by co-eluting compounds sharing the same fragments. DDA generates MS/MS spectra from a known precursor which minimizes the risk of “contaminating” fragments from co-eluting compounds. On the other hand, there is a limit to the number of co-eluting precursors which can be isolated and fragmented. The many category 2 findings also show the importance of having the fragmentation information in order to do an efficient retrospective analysis. Re-analysis of case samples was not possible in this study due to ethical restrictions. Consequently, the presumable category 2 finding of phenibut could not be confirmed. In real forensic case work the sample could have been re-analysed with a targeted MS/MS method where the precursor ion of phenibut is prioritized for fragmentation experiments. If a match with a library spectrum was achieved the finding would have been of category 1.

4. Conclusion

Data files from UHPLC-QTOF-MS analysis of 1314 forensic post mortem samples from the period 2014 to 2018 were retrospectively re-evaluated. The re-evaluation was performed using a PCDL with compounds within the groups of synthetic cannabinoids, synthetic opioids and designer benzodiazepines. In total, five new substances were identified with the highest degree of confidence possible with a retrospective approach. The identification relied on available MS/MS spectra from the acquisition and matching with the diagnostic fragment ions in the library spectrum. In addition, RT agreement with a reference substance was decisive in order to filter out false positives. The number of new findings were lower than expected and mainly originated from the first half of the time period investigated, indicating that our laboratory has been able to keep the analytical library fairly up to date.

It is important to emphasise that new and highly potent drugs like fluorofentanyl and cyclopropylfentanyl can escape attention if not specifically searched for. Detection in biological samples is in many cases dependent on information about the likely drug candidates - either from indirect sources such as labelling on seized drug packages, which may be imprecise, or preferably from direct analysis of the ingested substance. In Norway, biological samples are analysed by toxicology laboratories, whereas impounded drugs are submitted for analysis at a central police laboratory. There are no organizational connections or traditions for exchange of information between these two types of institutions. If it had not been for the availability of seizures in the two cases involving fluorofentanyl and cyclopropylfentanyl these would not have been detected with our original screening method.

The presented method proved to be a relatively easy and convenient approach to search for new compounds retrospectively. The use is not limited to retrospective analysis and can easily be applied as a supplement to the standard screening method with little extra effort, especially when the routine screening workflow gives a negative result but the circumstances suggest a more thorough investigation. The PCDL can be updated at regular time intervals or when important compounds are added to HighResNPS.com.

Note

After the completion of this study a Public Compound Database and Library version of the complete highresnps database has been made available for download from the website highresnps.com.

CRedit authorship contribution statement

Per Ole M. Gundersen: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Sebastian Broecker:** Software, Methodology, Writing - review & editing. **Lars Slørdal:** Resources, Data curation, Writing - review & editing. **Olav Spigset:** Conceptualization, Supervision, Resources, Writing - review & editing, Project administration. **Martin Josefsson:** Conceptualization, Supervision, Methodology, Writing - review & editing.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <https://doi.org/10.1016/j.forsciint.2020.110274>.

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