Fast & Fluorinated – Development and validation of a rapid benchtop NMR approach and other routine screening methods for the detection and quantification of synthesised fluorofentanyl derivatives

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Highlights

- 8 Fluorofentanyl analogues have been synthesized and fully characterized.
- GC-MS did not allow the full separation of target analogues.
- Benchtop ^{19}F-NMR separated the analogues and facilitated their quantification within regioisomeric mixtures or in the presence of heroin.
- Both techniques can be used orthogonally for regioisomer identification and quantification.

Abstract

Fluorinated fentanyl regioisomers have become increasingly common in forensic casework. This study reports the synthesis of eight fluorinated fentanyl derivatives as pure reference materials and their complete NMR, infrared and mass spectral characterisation. Presumptive colour tests and TLC results are presented as an initial screening method for these compounds. The fully validated GC–MS method (employing SIM mode) allows the quantification of the target analytes (LOD = 9 – 20 ng/mL, LOQ = 31 – 67 ng/mL), fentanyl, heroin, acetaminophen and caffeine, within 13 minutes. In most cases, the regioisomeric fluorofentanyls were resolved from each other except for the 3'- and 4'-fluorinated derivatives (**5b** and **5c**). To achieve full separation, an orthogonal benchtop ^{19}F NMR method allows the identification and quantification of target analogues (LOD = 74 – 400 µg/mL, LOQ = 290 – 1340 µg/mL). The

^{19}F NMR method allows the detection of fluorinated fentanyl analogues at a low concentration (2.4% *w/w*) in heroin.

Keywords: Forensic; illicit drugs; synthetic opioids; fentanyl analogues; regioisomers; GC-MS; benchtop NMR

1. Introduction

Since the 1980s, fentanyl abuse has grown to pose a considerable threat to public health. [1-4] Fentanyl use has been linked to a significant increase in drug-related overdoses, especially in North America, and this risk of overdose is compounded by the occurrence of fentanyl as an adulterant in heroin and other drugs of abuse. [1, 5-10] The rise in fentanyl abuse has been associated with the appearance of novel fentanyl analogues (or "fentalogues"): in 2013-2019, the United Nations Office on Drugs and Crime (UNODC) reported more than 75 New Psychoactive Substances (NPS) with opioid effect (including fentanyl analogues) in its Early Warning Advisory. [11]

Among these fentalogues, a variety of fluorinated derivatives have been encountered in casework. Most commonly, fluorine substitution occurs on the aniline ring of the fentanyl molecule. For instance, *ortho*-, *meta*- and *para*-fluorofentanyl have been reported across Europe and, in some cases, have been linked to overdoses or have been reported in seizures and acute poisonings (see **Figure 1**, **1a-c**). [12-15] Similar derivatives such as 4-fluorobutyrylfentanyl (**2**), [16, 17] 4-fluoroisobutyrylfentanyl (**3**) [18] and ocfentanyl (**4**) [19] have also been reported.

Fluorination in other positions has yet to be frequently observed. One difluorinated fentanyl, 2'-fluoro-*ortho*-fluorofentanyl (**6**), was identified within a seizure in China. [20] Although this was the first example of fluorination on the phenethyl moiety of fentanyl, it highlights the possibility that 2'-, 3'- and 4'-fluorinated derivatives (**5a-c**) could emerge. Spahn *et al*. reported that 3-fluorofentanyl (**7**, also known as NFEPP) only targeted inflamed tissue in mice, and did not produce effects sought from opioid abuse (sedation, euphoria). [21]

The emergence of regioisomeric derivatives of known synthetic drugs is a constant challenge in forensic casework. The availability of regioisomeric starting materials renders the synthesis of regioisomeric derivatives extremely simple. These compounds tend to exhibit similar chemical and chromatographic properties, and their mass spectra are often equivalent. This complicates the identification of specific drug regioisomers, hence the requirement for specific analytical methods to identify regioisomers of synthetic cannabinoids, [22-25] fluoroamphetamines, [26] chloroamphetamines, [27] cathinones, [28] etc. Identification of drug regioisomers may also require the use of multivariate analysis in conjunction with mass spectral data. [29-31]

The same challenge applies to the identification of fluorofentanyl regioisomers, which cannot be discriminated by conventional mass spectral databases, without more sophisticated multivariate approaches. [30, 32] Using reference material synthesized in-house, this study shows that presumptive tests and GC-EI-MS are unable to fully discriminate between a variety of fluorinated regioisomers (**1a-c**, **5a-c**, **6**, **7**). As benchtop NMR has previously been used to identify and quantify illicit drugs, [33-36] it is proposed as an alternative technique for the identification and quantification of these compounds.

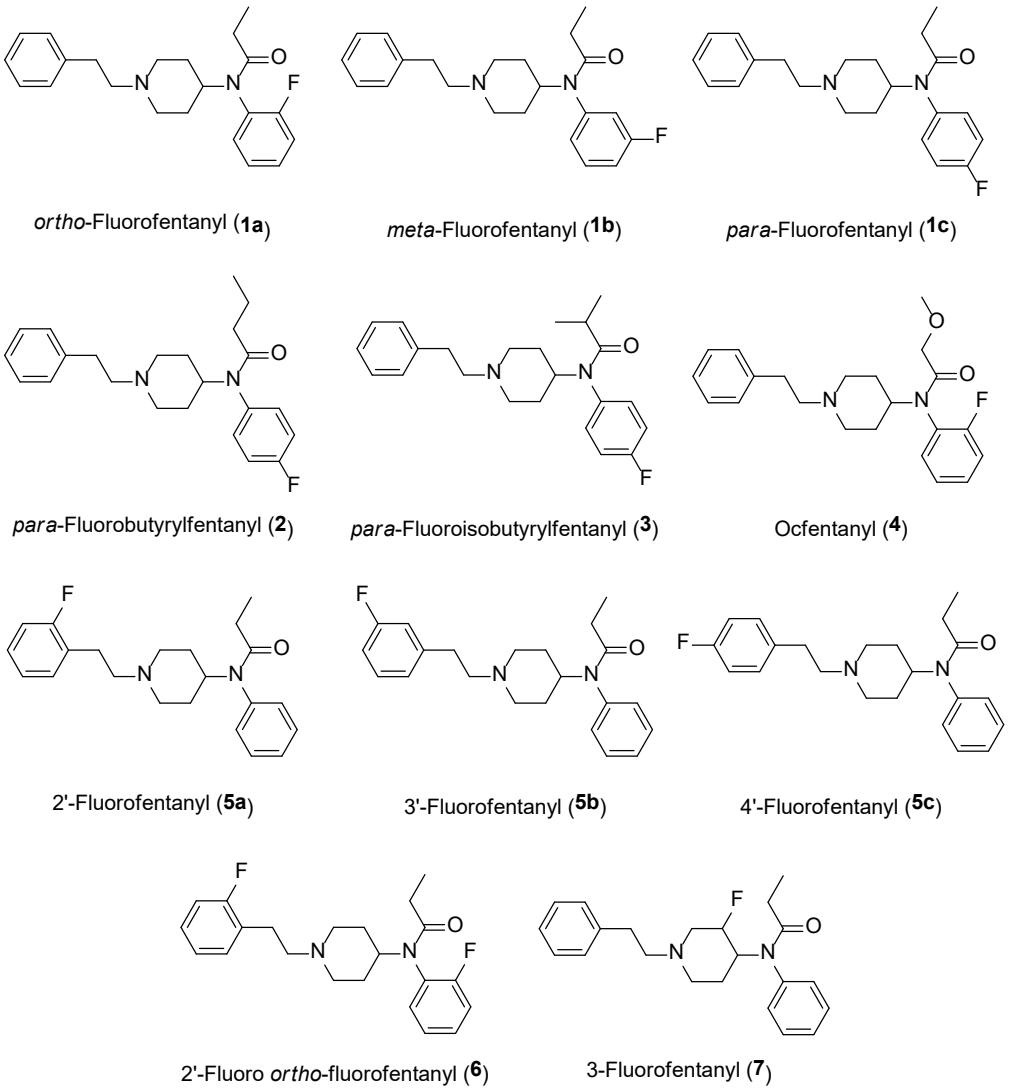

Figure 1. Chemical structure of common fluorinated fentalogues.

2. Materials and methods

All reagents were of commercial quality (Sigma-Aldrich, Gillingham, UK or Fluorochem Limited, Hadfield, UK) and used without further purification. Solvents (Fisher Scientific, Loughborough, UK) were dried, where necessary, using standard procedures. [37] ^{1}H NMR (10 mg in 600 µL in d_6-DMSO) and ^{13}C{^{1}H} NMR spectra (20 mg in 600 µL in d_6-DMSO) were acquired on a JEOL JMN-ECS-400 (JEOL, Tokyo, Japan) NMR spectrometer operating at a proton resonance frequency of 400 MHz and referenced to the residual solvent peak (d_6-DMSO: ^{1}H NMR $\delta = 2.50$ ppm, ^{13}C{^{1}H} NMR $\delta = 39.52$ ppm respectively). [38] ^{19}F{^{1}H} NMR spectra (10 mg/600 µL in d_6-DMSO containing 0.03% v/v trifluoroacetic acid, TFA) for compounds were acquired on the same instrument and referenced to TFA (^{19}F{^{1}H} NMR, $\delta = -76.55$ ppm). [39] Low resolution NMR spectra were acquired on an Oxford Instruments Pulsar benchtop NMR Spectrometer (Oxford Instruments, Oxford, UK) operating at a proton resonance frequency of 60 MHz and referenced to the residual solvent peak (^{1}H NMR) or TFA (^{19}F NMR). Infrared spectra were obtained in the range 4000 – 400 cm^{-1} using a Thermo Scientific Nicolet iS10ATR-FTIR instrument (Thermo Scientific, Rochester, USA). High-resolution mass spectrometry (HRMS) data were obtained on an Agilent 6540 LC-QToF spectrometer in positive electrospray ionization mode.

2.1 Synthesis

The hydrochloride salts of fluorofentanyls (**1a-c**, **5a-c**, **6**, **7**) were synthesized and purified, as per standard procedures (see Supplementary Information), and obtained as stable, off-white powders (6-67% overall yields). All compounds were determined to be soluble (10 mg/mL) in methanol and DMSO and the purity of all samples was confirmed to be >99.5% (by NMR and HRMS) in all cases. The NMR purity was calculated using the relative concentration determination method described by Pauli *et al*. [40] The products were fully characterized by ^{1}H NMR, ^{13}C{^{1}H} NMR, ^{19}F{^{1}H} NMR, FTIR and HRMS (see Supplementary Information).

2.2 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was performed on aluminum-backed SiO_2 plates (Merck, Germany) using a mobile phase of dichloromethane-methanol (9:1 *v/v*) containing 1% triethylamine. The solvent system was selected to directly relate the chromatographic data of these novel fluorinated fentanyl derivatives with previously reported fentalogues. [41] The

developed plates were observed under UV light (254 nm) and developed with modified Dragendorff-Ludy-Tenger reagent. [42] Six replicate tests were conducted for each compound to calculate the average retention factor (R_f) and relative retention factor (RR_f, with respect to fentanyl, **18**).

2.3 Presumptive Tests

Presumptive color tests were carried out according to the United Nations recommended guidelines. [43, 44] The standard Marquis, nitric acid and Eosin Y tests were used in this study. The preparation of each reagent and the corresponding test procedure have been reported previously. [45, 46] Negative controls were used in each test. For each analyte six repetitive tests were carried out.

2.4 Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was performed using an Agilent 7890B GC and a MS5977B mass selective detector (Agilent Technologies, Wokingham, UK). The mass spectrometer was operated in the electron ionization mode at 70 eV. Separation was achieved with a capillary column (HP-5MS, 30 m length, 0.25 mm i.d., 0.25 µm film thickness) with helium as the carrier gas at a constant flow rate of 1.2 mL/min. The following oven temperature program was used: 100 to 200 °C at 30 °C/min, 200 to 230 at 10 °C/min, 230 to 260 at 30 °C/min, 260 to 265 at 1 °C/min, hold for 1 min for a 13.3 min total runtime. A 2 µL aliquot of the samples was injected with a split ratio of 50:1. The injector and the GC interface temperatures were both maintained at 280 °C respectively. The MS source and quadrupole temperatures were set at 230 °C and 150 °C. Mass spectra were obtained in full scan mode (50–550 amu). For qualitative analysis all compounds were dissolved at 1 mg/mL in methanol without derivatization, using eicosane (0.5 mg/mL) as an internal standard. Compounds were analyzed individually to acquire representative mass spectra, and in combination with fentanyl (**18**), heroin (**19**) and two adulterants, acetaminophen (**20**) and caffeine (**21**).

2.5 GC-MS calibration standards

To avoid peak overlap between analytes with indistinguishable m/z values, target compounds were organized into three groups, each validated separately from the others (Group I: 3'-

fluorofentanyl **5b**, fentanyl **18**, heroin **19**, acetaminophen **20** and caffeine **21**; Group II: *ortho*-fluorofentanyl **1a**, *meta*-fluorofentanyl **1b**, *para*-fluorofentanyl **1c** and 4'-fluorofentanyl **5c**; Group III: 2'-fluorofentanyl **5a**, 2'-fluoro-*ortho*-fluorofentanyl **6** and 3-fluorofentanyl **7**). Analytes were weighed accurately (10.0 mg) into 10.0 mL class A volumetric flasks and diluted to volume with methanol to give a 1 mg/mL solution. This solution was then further diluted with methanol and 200 µL of eicosane (50 µg/mL in methanol) to produce calibration standards containing 5.0 µg/mL, 10.0 µg/mL, 15.0 µg/mL, 20.0 µg/mL and 25.0 µg/mL of each analyte and the internal standard at 10.0 µg/mL.

2.6 GC-MS method validation

GC-MS method validation was achieved using the instrument and parameters detailed in Section 2.4. Analysis was performed in selected ion monitoring (SIM) mode, with one quantitative and two qualitative ions for each analyte (Table 1). The GC-MS method was validated in accordance with the ICH guidelines [47] using the following parameters: linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). *Linearity and precision*: six replicate injections of the calibration standards was achieved under the same conditions. The %RSD was calculated for each replicate test sample and the linearity (r^2) of the calibration was determined. *Accuracy (percentage recovery study)*: determined from spiked samples prepared in triplicate at three levels over a range of 80-120% of the target concentration (15 µg/mL). The percentage recovery and %RSD were calculated for each of the replicate samples. *Limits of detection and quantification:* six replicate injections of the calibration standard (5.0 mg/mL) were performed. The limits of detection and quantification were determined based on a signal-to-noise ratio of 3:1 and 10:1 respectively. [47] Signal-to-noise ratios were measured using the auto-root-mean-squared (Auto-RMS) algorithm from the Agilent MassHunter Qualitative Analysis software.

2.7 ^{19}F NMR calibration standards

Each analyte was weighed accurately (75.0 mg) into a 5.0 mL class A volumetric flask. Concentrated TFA was added (0.5 µL) before diluting to volume with d_6-DMSO to produce a 15 mg/mL solution containing TFA at 0.01%. This solution was then further diluted with d_6-DMSO (containing 0.01% TFA) to produce calibration standards containing 5.0 mg/mL,

8.0 mg/mL, 10.0 mg/mL, 12.0 mg/mL and 15.0 mg/mL of the analyte and the internal standard at 0.01%.

2.8 ^{19}F NMR method validation

^{19}F NMR method validation was performed using an Oxford Instruments Pulsar benchtop NMR Spectrometer (Oxford Instruments, Oxford, UK) operating at a proton resonance frequency of 60 MHz and referenced to TFA. ^{19}F experiments were run using 16 scans, a relaxation delay of 15 s and a filter of 5000 Hz for a total runtime of 5.7 min. A 10000 Hz filter is required to analyze 3-fluorofentanyl (**7**) for a 4.9 min runtime. The qNMR method was validated in accordance with the ICH guidelines (see Section 2.6 GC-MS method validation). [47] Signal-to-noise ratios were measured using the MestReNova software algorithm.

2.9 ^{19}F NMR heroin mixture test solution

Heroin (**19**) was weighed accurately (22.5 mg) and dissolved in 0.5 mL d_6-DMSO (containing 0.01% TFA) to produce a solution containing heroin at 45 mg/mL. A 420 µL aliquot of this solution was mixed with 30 µL of a solution containing *ortho*-fluorofentanyl (**1a**) at 15 mg/mL and TFA at 0.01%. The resulting 450 µL solution contained 42 mg mL^{-1} heroin (**19**) and 1 mg/mL (**1a**), corresponding to a **1a**/**19** ratio of 2.4% *w/w*. This concentration was based on previously reported studies of seized samples within Greater Manchester. [41] Due to the small sample size (n = 7) in these previous studies, the results may not truly reflect the typical levels of fentanyl contained in heroin samples nationally. However, this concentration was chosen to demonstrate that the proposed low-field qNMR method is suitable for the routine screening of suspect samples which may contain fentanyl derivatives at trace levels. The solution was analyzed by ^{1}H and ^{19}F NMR using the Pulsar benchtop NMR spectrometer.

3. **Results and discussion**

3.1 Synthesis

The target fluorofentanyls (**1a-c, 5a-c, 6, 7**) were prepared, as their hydrochloride salts, using adapted procedures reported in the literature (**Figure 2**; see Supplementary Information for detailed procedures and purification). Starting from *N*-phenethylpiperidone (**8**), aniline

derivatives bearing a fluorine at different positions were introduced by reductive amination with sodium triacetoxyborohydride to obtain fluorinated 4-ANPP derivatives (**9a-c**) in 65 – 68% yield. Subsequent acylation with propionyl chloride and reaction with ethereal HCl afforded the target compounds (**1a-c**) in 25 – 43% yield. Synthesis of (**5a-c**) derivatives was initiated through acylation of 1-Boc-4-phenylaminopiperidine (**10**) to obtain compound (**11**) in 95% yield. Sequential Boc deprotection, alkylation with the corresponding regioisomeric fluorophenethyl bromides and salt formation with HCl gave compounds (**5a-c**) in 60 – 70% overall yield from (**11**). Synthesis of (**6**) was achieved in a similar way. Reductive amination of 1-Boc-4-piperidone (**12**) with 2-fluoroaniline gave compound (**13**, 73% yield). Subsequent acylation with propionyl chloride gave compound (**14**, 48% yield). Sequential Boc deprotection, alkylation with 2-fluorophenethyl bromide and salt formation with HCl gave target compound (**6**) in 53% overall yield from (**14**). Finally, synthesis of 3-fluorofentanyl (**7**) started with the reductive amination of 1-Boc-3-fluoro-4-piperidone (**15**) with aniline, which gave compound (**16**) in 39% yield. Boc deprotection and alkylation with phenethyl bromide gave compound (**17**) in 31% yield over the two steps. Acylation of (**17**) with propionyl chloride and salt formation with HCl gave target compound (**7**) in 82% yield. All compounds were determined to be soluble (10 mg/mL) in methanol and DMSO and the purity of all samples was confirmed to be >99.5% (by NMR and HRMS) in all cases. The products were fully characterized by ^{1}H NMR, ^{13}C{^{1}H} NMR, ^{19}F{^{1}H} NMR, FTIR and HRMS (see Supplementary Information).

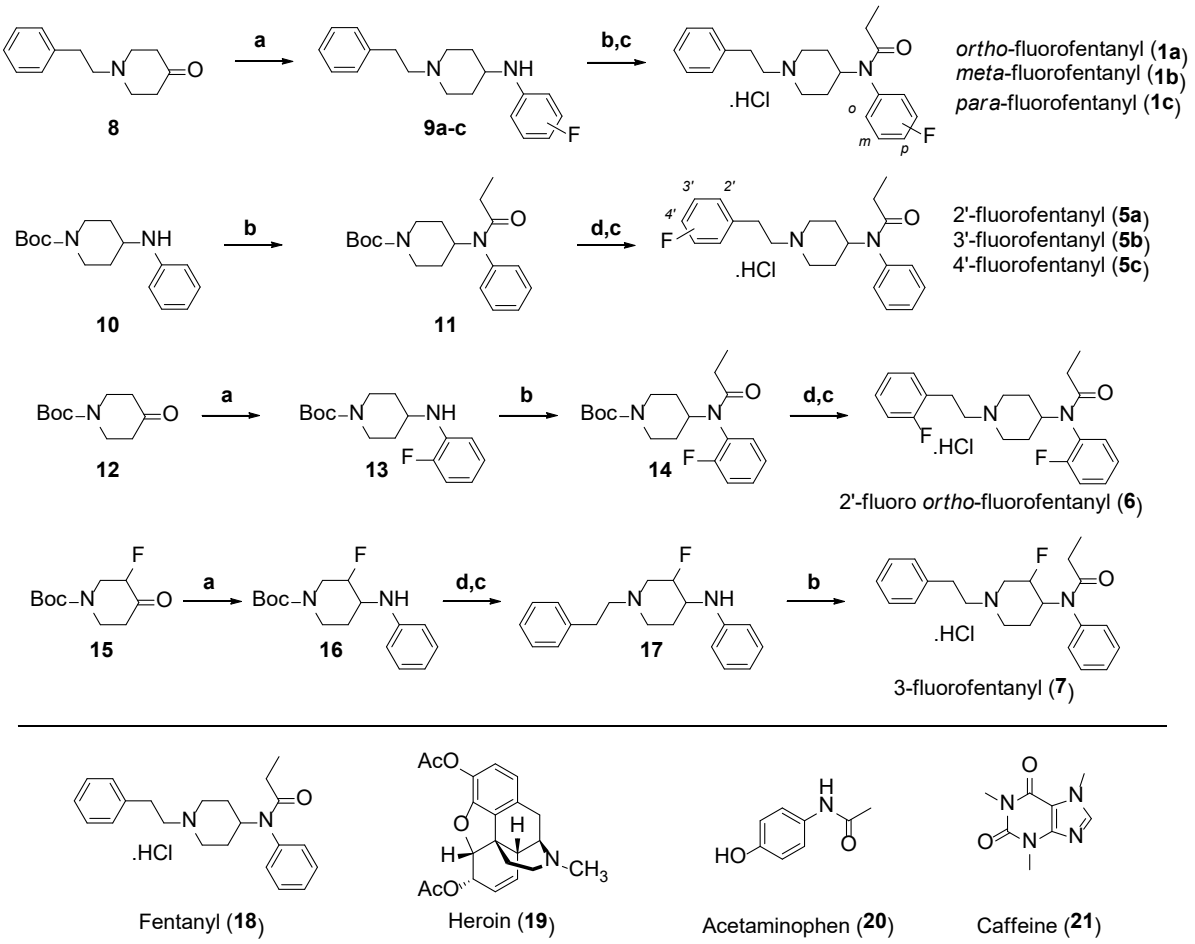

Figure 2. Synthesis of target fluorofentanyls (**1a-c, 5a-c, 6, 7**) and structure of fentanyl (**18**), heroin (**19**), acetaminophen (**20**) and caffeine (**21**). *Reagents and conditions*: (a) $ArNH_2$, AcOH, $NaBH(OAc)_3$, DCE, r.t., 48 h; (b) Propionyl chloride (2.0 eq.), $^{i}Pr_2NEt$ (2.0 eq.), DCM (0.1 M), 0 °C to r.t., 2 h; (c) HCl (3 M in CPME, 1.0 eq.), Et_2O or acetone (0.1 M), r.t., 2 h; (d) 1. TFA/DCM (1:3) (0.3 M), 0 °C to r.t., 1 h, 2. $Ar(CH_2)_2Br$, Cs_2CO_3, ACN, reflux, 5 h.

3.2 Thin layer chromatography

TLC analysis of the target compounds was carried out using an eluent of dichloromethane-methanol (9:1 *v/v*) containing 1% triethylamine. The plates were developed with modified Dragendorff-Ludy-Tenger reagent. [42] TLC data, including Retention Factor (R_f) and Relative Retention Factor (RR_f, with respect to fentanyl, **18**) are reported in Table S1 (see Supplementary Information). The R_f values showed clear separation of the *ortho*/*meta*/*para* series (**1a-c**, R_f = 0.52, 0.49 and 0.46, respectively). Separation was less defined between 2'-fluoro *ortho*-fluorofentanyl (**6**, R_f = 0.50) and fentanyl (**18**, R_f = 0.51). *para*-Fluorofentanyl (**1c**, R_f = 0.46) co-eluted with 2'-fluorofentanyl (**5a**, R_f = 0.46) and 3'-fluorofentanyl (**5b**, R_f =

0.47). 4'-fluorofentanyl (**5c**, R_f = 0.44) and 3-fluorofentanyl (**7**, R_f = 0.64) were clearly separated from all other derivatives. Despite their similar chromatographic properties, partial discrimination of the target fluorofentanyls was possible under these TLC conditions.

3.3 Presumptive tests

Fluorinated fentalogues, fentanyl and heroin were submitted to the Marquis, nitric acid and Eosin Y colorimetric tests; results are reported in the Supplementary Information (Table S2). Heroin (**19**) could easily be differentiated from fentanyl (**18**) and its fluorinated analogues (**1a-c**, **5a-c**, **6** and **7**) using the nitric acid or Marquis tests.

Heroin produces a green color with nitric acid, whereas fentalogues do not react. Heroin reacts with Marquis reagent to produce a deep violet color. As for fentalogues, compounds (**1a-c**) and (**7**) reacted with Marquis reagent in the same fashion to fentanyl, producing a dark brown color. Interestingly, fluorination of the phenethyl ring (**5a-b**, **6**) appeared to hinder the reaction. This is most likely because it proceeds through formylation of the aromatic ring by formaldehyde, catalyzed by sulfuric acid. [44] The presence of a fluorine deactivates the ring for electrophilic aromatic substitution. This reveals an important observation about the potential mechanism of the reaction between fentalogues and the Marquis reagent: formylation mostly occurs at the phenethyl ring, not the aniline ring. Any modification of this moiety is therefore likely to prevent a conclusive color change from appearing, leading to a false negative result. This illustrates how these commonly used presumptive color tests can prove unreliable when testing for emerging drug analogues.

The Eosin Y test allows effective discrimination between fentanyl analogues and common drugs and adulterants. This also applied to fluorinated fentalogues, except for (**1c**) and (**7**), which produced a false negative result.

3.4 GC-MS Analysis

Compounds (**1a-c, 5a-c, 6** and **7**) were analyzed individually to acquire representative mass spectra (**Figure 3**).

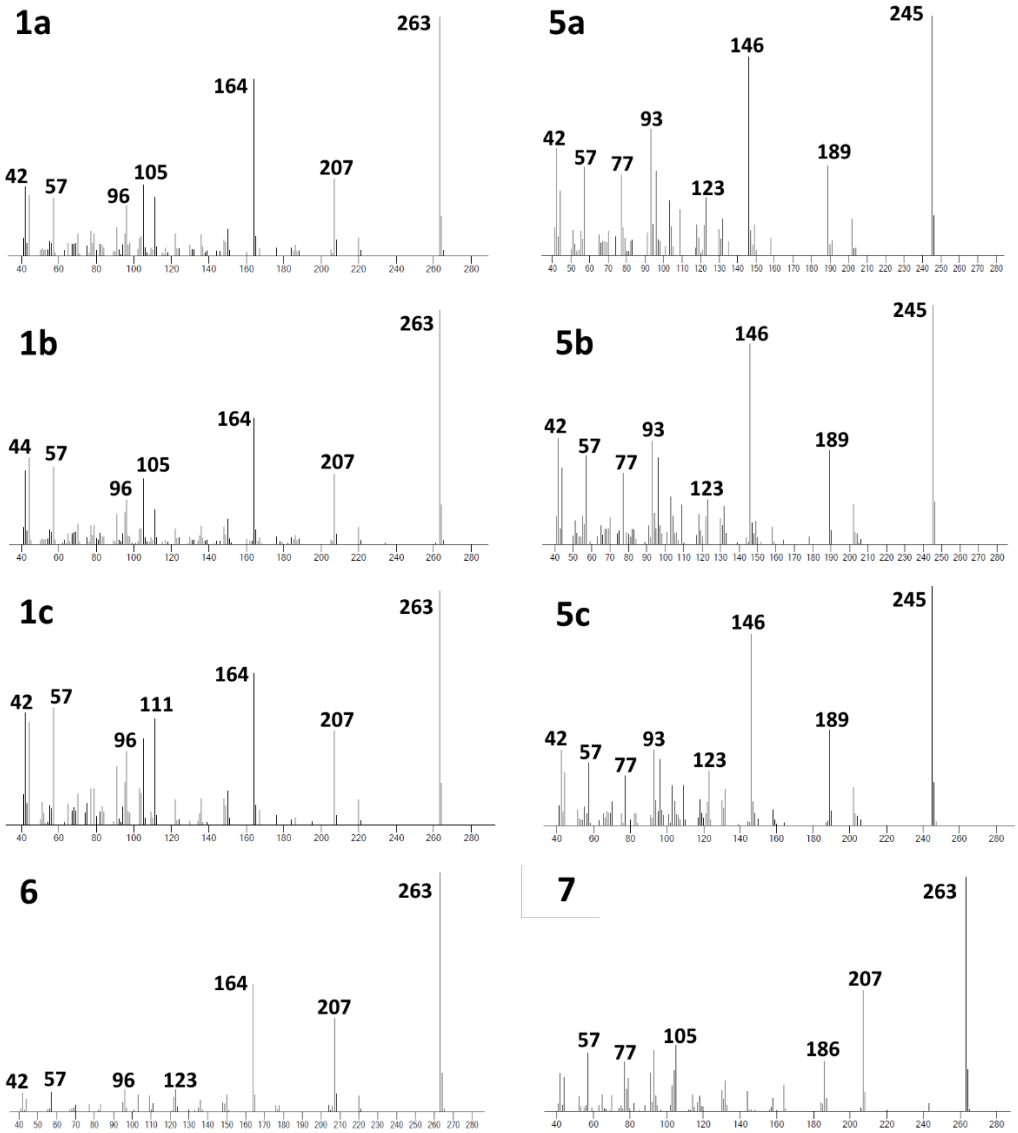

Figure 3. Electron ionisation mass spectra of fentalogues (**1a-c, 5a-c, 6** and **7**).

The general EI-MS fragmentation pattern of fentanyl is represented in **Figure 4**. The main fragment at *m/z* = 245 results from the loss of a benzyl radical. This is followed by a constriction of the piperidine ring and/or cleavage of the amide bond, resulting in three fragments of *m/z* = 202, 189 and 146.

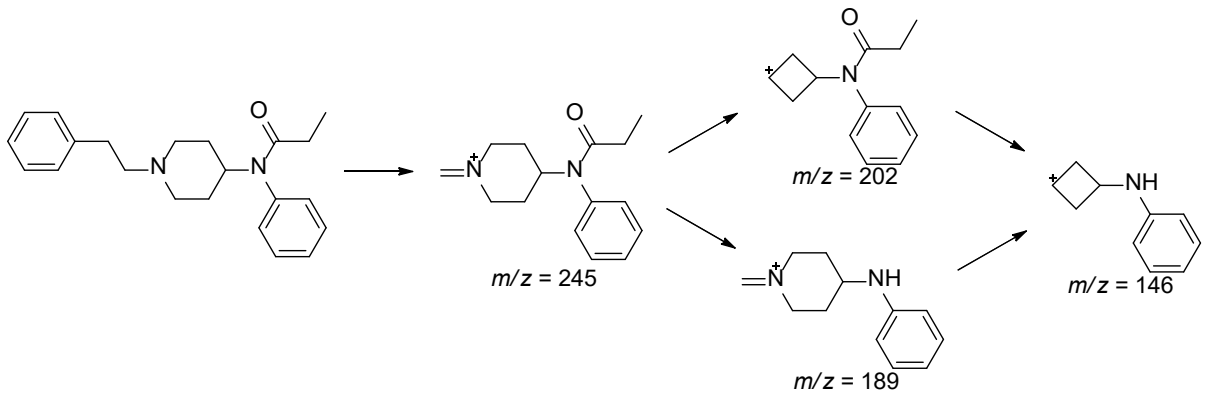

Figure 4. Typical EI-MS fragmentation of fentanyl (**18**).

In the same way, fluorofentanyls (**5a-c**), readily lose a benzyl radical upon fragmentation to form a cation of *m/z* = 245 (**Figure 5**). Their main fragments did not contain a fluorine atom and were therefore equivalent to those of fentanyl. The only difference with fentanyl was an ion of *m/z* = 109, which could be attributed to a fluorinated tropylium ion. In fentanyl, the corresponding tropylium ion has an *m/z* of 91.

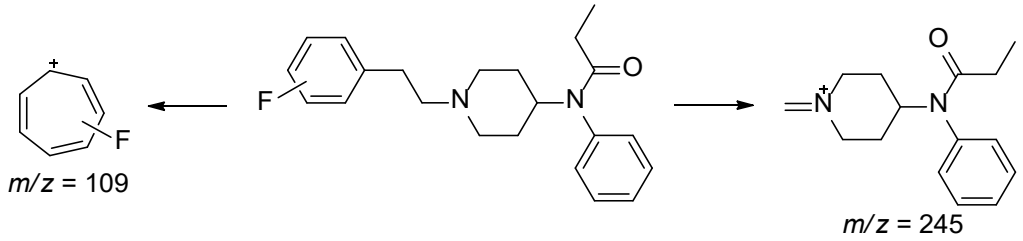

Figure 5. Typical EI-MS fragmentation of 2'-, 3'- and 4'-fluorofentanyl (**5a-c**).

Isomers (**1a-c**) all retained a fluorine atom in their main fragments (see **Figure 6**). They were thus easily differentiated from fentanyl, but not from each other, by their mass spectra (see **Figure 3**).

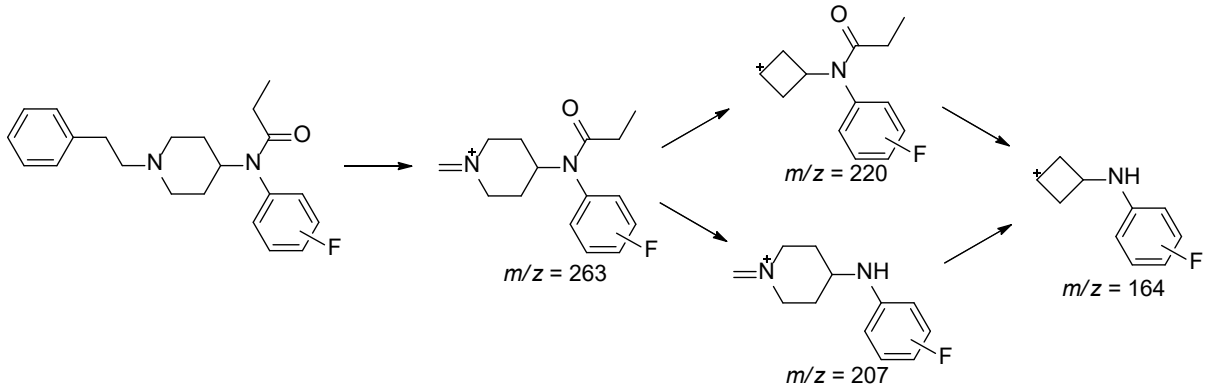

Figure 6. Typical EI-MS fragmentation of *ortho*-, *meta*-, *para*-fluorofentanyl (**1a-c**).

2'-Fluoro-*ortho*-fluorofentanyl (**6**), by losing a fluorobenzyl radical, produced essentially the same fragment ions as compounds (**1a-c**), outlined in **Figure 6**. However, its three largest fragments (*m/z* = 263, 207 and 164) were more abundant than in **1a-c**. 3-Fluorofentanyl (**7**) produced fragment ions with the same mass as those reported in **Figure 6**, with one additional fragment ion (*m/z* = 186), which could tentatively be explained by the loss of a phenyl radical from the ion at *m/z* = 263.

A GC-MS method was optimized to separate the target compounds (see **Figure 7**). Only partial chromatographic resolution was achieved, due to their similar chromatographic properties. Target fentalogues were fully separated from heroin (**19**) and adulterants acetaminophen (**20**) and caffeine (**21**). The method also separated compounds (**1a-c**). Despite an optimization of the temperature program, however, compounds (5**a-c**) co-eluted and could not be resolved by SIM analysis as they produced the same fragment ions. In a similar way, *meta*-fluorofentanyl (**1b**) partially co-eluted with (**6**)**,** and the two produced indistinguishable mass spectra ions. Although 3-fluorofentanyl (**7**) co-eluted with fentanyl (**18**), they could be easily differentiated in SIM mode.

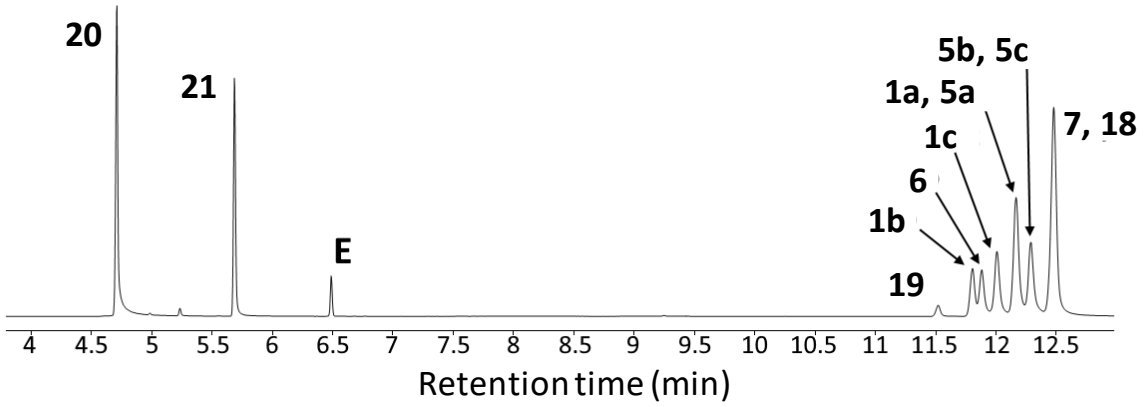

Figure 7. GC-MS chromatogram of target fluorofentanyls (**1a-c, 5a-c, 6, 7**), fentanyl (**18**), heroin (**19**), acetaminophen (**20**), caffeine (**21**) and eicosane (internal standard, E).

3.5 GC-MS Validation

To avoid peak overlap between analytes which produced indistinguishable SIM ions, target compounds were organized into three groups, each validated separately from the others (Group I: 3'-fluorofentanyl **5b**, fentanyl **18**, heroin **19**, acetaminophen **20** and caffeine **21**; Group II: *ortho*-fluorofentanyl **1a**, *meta*-fluorofentanyl **1b**, *para*-fluorofentanyl **1c** and 4'-fluorofentanyl **5c**; Group III: 2'-fluorofentanyl **5a**, 2'-fluoro-*ortho*-fluorofentanyl **6** and 3-fluorofentanyl **7**).

The quantitative GC-MS method (SIM mode) was developed and validated in accordance with the ICH guidelines. [47] Three ions were monitored for each analyte; the base peak was used for quantification, and two additional ions were used to confirm the identification of target compounds. The relative intensities of confirmatory ions (relative to the base peak) are reported in Table S3 (see Supporting Information).

Results of the method validation are reported in **Table 1**. Calibration standards were prepared and all 9 fentalogues demonstrated a linear response (r^2 = 0.998–0.999) over a 5.0–25.0 µg/mL range with satisfactory repeatability (RSD = 0.3 – 3.9 %, n = 6). The limits of detection (LOD) and quantification (LOQ) for the analytes (in bulk samples) were of 9 – 20 and 31 – 67 µg/mL respectively, based on signal-to-noise ratio. The method was also suitable for the detection and quantification of heroin and two of its common adulterants (acetaminophen and caffeine). Linear response (r^2 = 0.993 – 0.998) was observed over the same concentration range with reasonable repeatability (RSD = 1.1 – 7.4%, n = 6). The limits of detection and quantification were 34 – 620 and 110 – 2100 ng/mL, respectively.

SIM analysis proved significantly more sensitive than scan mode: LOD and LOQ in scan mode were 0.5 – 23.0 and 1.6 – 76.5 μg/mL, respectively (see Supplementary Information, Table S4). This difference in sensitivity must be considered when screening samples in scan mode, as low concentrations of fentalogues may not be detected. [41]

The accuracy (percentage recovery study) of the method was determined using a percentage recovery study (see Supplementary Information, **Table S5**). Spiked samples were prepared in triplicates at three concentration levels over a range of 80–120% of a target concentration (15 μg/mL). The result of these injections is fed back into the calibration curve and the experimental concentration is compared with the theoretical concentration (assay recovery). The relative error shows how the mean assay recovery diverges from an expected 100%. Acceptable recoveries (100 ± 3%) were obtained for all analytes. The precision (inter- and intraday precision) was calculated from six replicate injections of a spiked sample (10 μg/mL) and analysed on two consecutive days (see Supplementary information, **Table S6**). Most analytes showed acceptable precision, with intraday RSDs between 1.7 and 4.6%, apart from acetaminophen (interday: 6.5%).

Although the GC-MS analysis of fluorinated fentalogues was fully validated, co-elution of compounds with identical *m/z* ions (**5a-c**) prevented the identification of specific regioisomers. Identification of regioisomers would have to be confirmed using a different technique, such as NMR. ^{1}H and ^{19}F NMR spectroscopy are proposed as alternative detection and quantification techniques to resolve this issue.

3.6 NMR Analysis

It has been shown previously that ^{1}H NMR was suitable for the qualitative identification of certain fluorofentanyls (**1a-c** and **7**). [36] All studied regioisomers (**1a-c**, **5a-c**, **6** and **7**) could be discriminated based on the aromatic region of their ^{1}H NMR spectra (**Figure 8**).

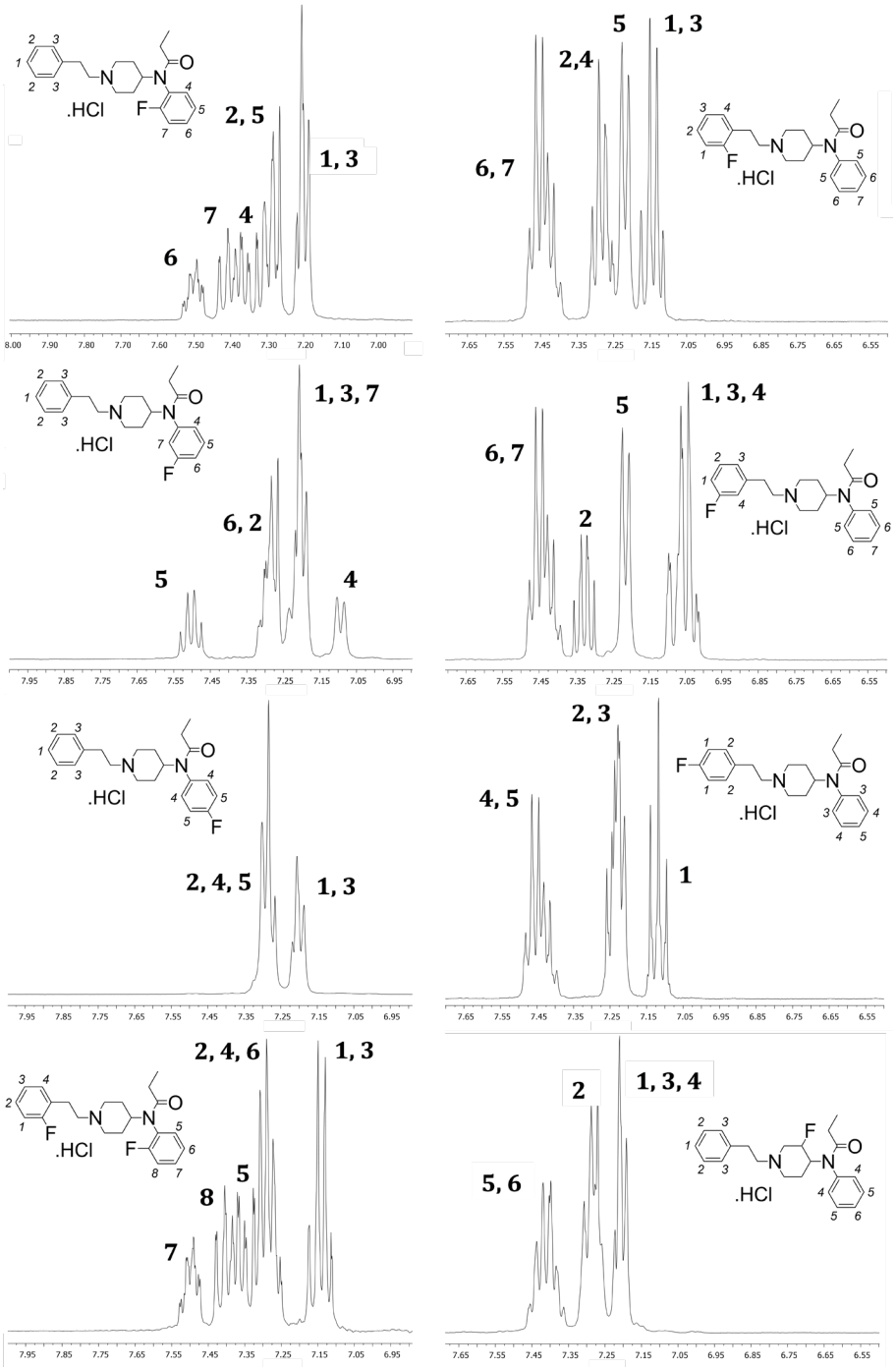

Figure 8. Aromatic region (7.025 – 7.750 ppm) of the high-field (400 MHz) ^{1}H NMR spectra of fluorofentanyls (**1a-c**, **5a-c**, **6** and **7**) in DMSO-d_6.

Although ^{1}H NMR allowed the identification of fluorofentanyl regioisomers, and potentially their quantification, its effectiveness may be limited in adulterated mixtures where signal overlaps are likely. The use of ^{19}F NMR circumvents this issue – fluorine atoms do not typically occur in the drugs and adulterants commonly mixed with fentanyl, such as heroin,

acetaminophen and caffeine. ^{19}F NMR would therefore allow the quantification of fluorofentanyls in mixtures.

Overlaid ^{19}F NMR spectra of the target fluorofentanyls are shown in **Figure 9** and chemical shifts of each fluorine signal are reported in **Table 2**. The same pattern was observed for both the (**1a-c**) and (**5a-c**) series: due to the electron-donating effect of the substituent (amide or alkyl chain), *ortho* isomers were more shielded than *para*-isomers, which were more shielded than *meta* isomers. In 3-fluorofentanyl (**7**), the fluorine is located on an aliphatic ring and thus extremely shielded (-199.4 ppm). All target compounds produced fully resolved signals using high-field ^{19}F NMR and could easily be identified and separated in a potential mixture **(Figure 9a**). This shows that, confronted with a suspected fluorofentanyl sample, ^{19}F NMR can be used to tell which ring is substituted and at which position. Difluorinated compound (**6**) produced two signals, equivalent to those of 2'-fluorofentanyl (**5a**) and *ortho*-fluorofentanyl (**1a**) (**Figure 9b**). This simplifies the identification of potential new difluorinated compounds, as a simple ^{19}F NMR analysis and comparison to reference chemical shifts can help determine the fluorine substitution pattern, without the need for more complex and time-consuming 2D analysis.

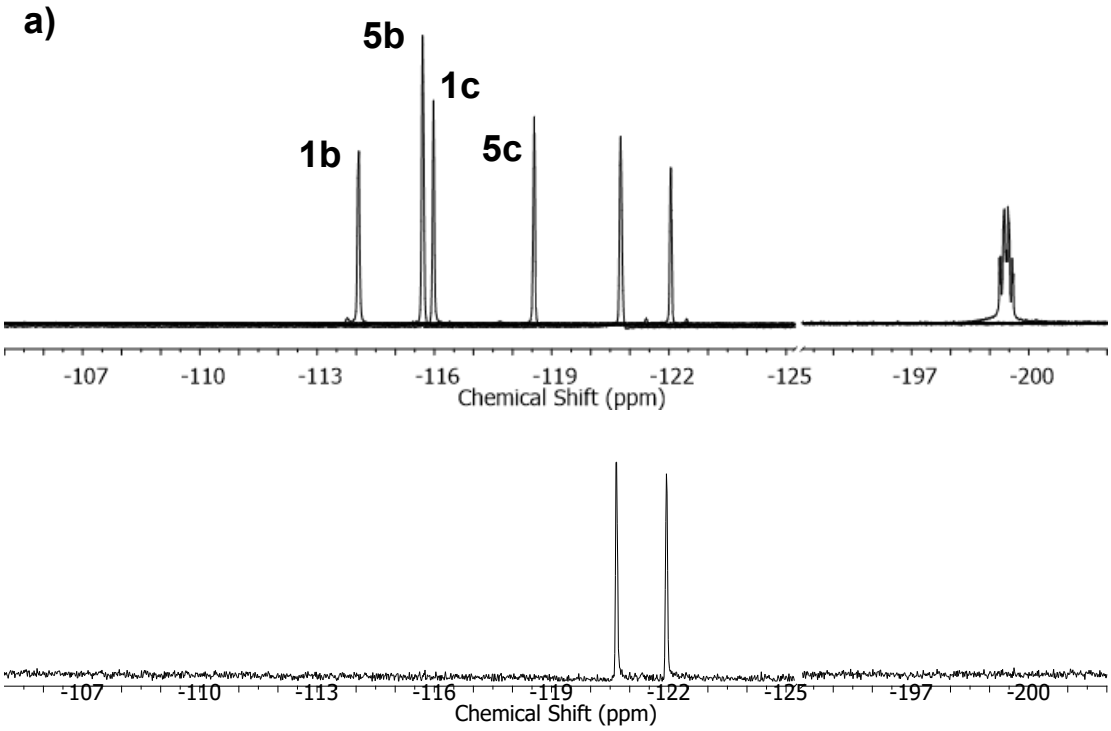

Figure 9. (a) Overlay of high-field (376 MHz) ^{19}F NMR target fluorofentanyls (**1a-c**, **5a-c**, **7**); (b) High-field (376 MHz) ^{19}F NMR of compound (**6**).

Low-field ^{19}F NMR detection of fluorofentanyls was also investigated. Low-field benchtop NMR instruments have the advantage of being much more affordable than their high-field counterparts, easier to use and potentially field-deployable. Previous studies have reported the reliable identification of illicit drugs [35] and fentanyl derivatives [36] by low-field ^{1}H NMR. **Figure 10** shows an overlay of the low-field ^{19}F NMR spectra of target fluorofentanyls. The use of benchtop NMR resulted in a loss of resolution: two signals coincide (**5b** and **1c**) but are still partially resolved. Benchtop ^{19}F NMR proved suitable for the discrimination of fluorinated regioisomers of fentanyl (except **5b** and **1c**). These also co-elute by TLC, so unequivocal identification of these derivatives would require a combination of the ^{19}F NMR approach with ^{1}H NMR or GC-MS analysis.

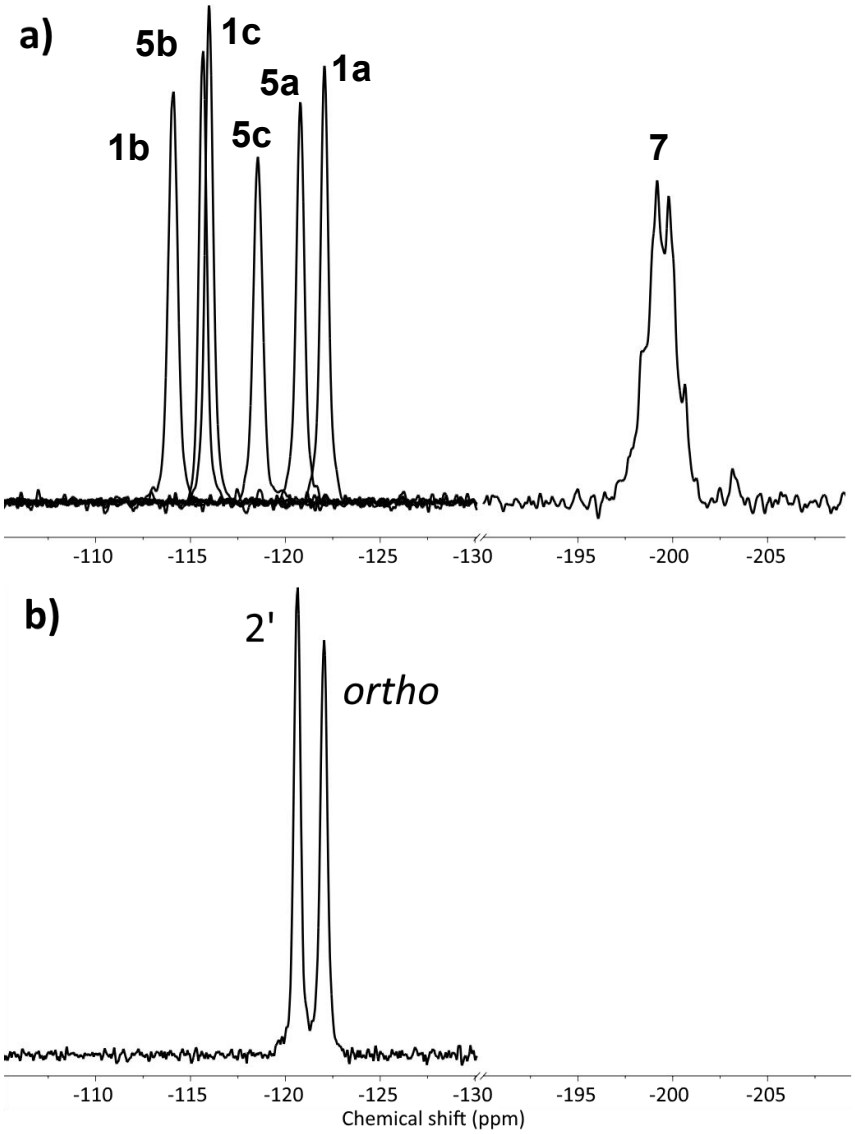

Figure 10. (a) Overlay of low-field (56.76 MHz) ^{19}F NMR spectra of target fluorofentanyls (**1a-c**, **5a-c**, **7**) (-105 to -130 ppm; -190 to -210 ppm); (b) Low-field (56.76 MHz) ^{19}F NMR spectrum of compound (**6**) (-105 to -130 ppm).

3.7 Low-Field ^{19}F NMR validation

A quantitative low-field ^{19}F NMR method was developed and validated in accordance with the ICH guidelines (see **Table 2**). [47] Calibration standards were prepared and all fentalogues demonstrated a linear response (r^2 = 0.994–0.999) over a 5–15 mg mL^{-1} range with satisfactory repeatability (RSD = 0.9 – 9.7 %, n = 6). The LOD and LOQ were 74 – 400 µg/mL and 290 – 1340 µg/mL respectively.

The accuracy of the method was determined using a percentage recovery study (see Supplementary Information, **Table S7**). Spiked samples were prepared in triplicates at three concentration levels over a range of 80–120% of a target concentration (10 mg/mL). Acceptable recoveries (100 ± 3%) were obtained for all analytes.

Finally, the possibility to detect a fluorinated fentanyl in a mixture with heroin using this method was tested. A solution containing 42 mg/mL heroin (**19**) and 1 mg/mL *ortho*-fluorofentanyl (**3a**) was prepared. This corresponds to a **1a**/**19** ratio of 2.4% *w/w*. The solution was first analyzed by ^{1}H NMR using the Pulsar benchtop NMR spectrometer. The resulting spectrum appeared to be that of pure heroin (**19**, see **Figure 11a**). When compared against a previously developed low-field ^{1}H NMR drug database, [35] which includes *ortho*-fluorofentanyl (**1a**), heroin was the only component identified in the sample, while (**1a**) was not detected. Increasing the number of scans in increments from 16 to 4096 yielded the same result.

^{19}F NMR analysis using the developed method revealed the presence of the fentanyl, within LOQ, demonstrating the applicability of this method to adulterated heroin street samples (see **Figure 11b**). It should be noted that none of the adulterants commonly mixed with heroin bear a fluorine atom. Although detection of a fluorine signal in a heroin sample, at a chemical shift consistent with a fluorofentanyl analogue, does not constitute a formal identification, it should be a cause for suspicion which leads to the use of other confirmatory methods (e.g. GC-MS analysis).

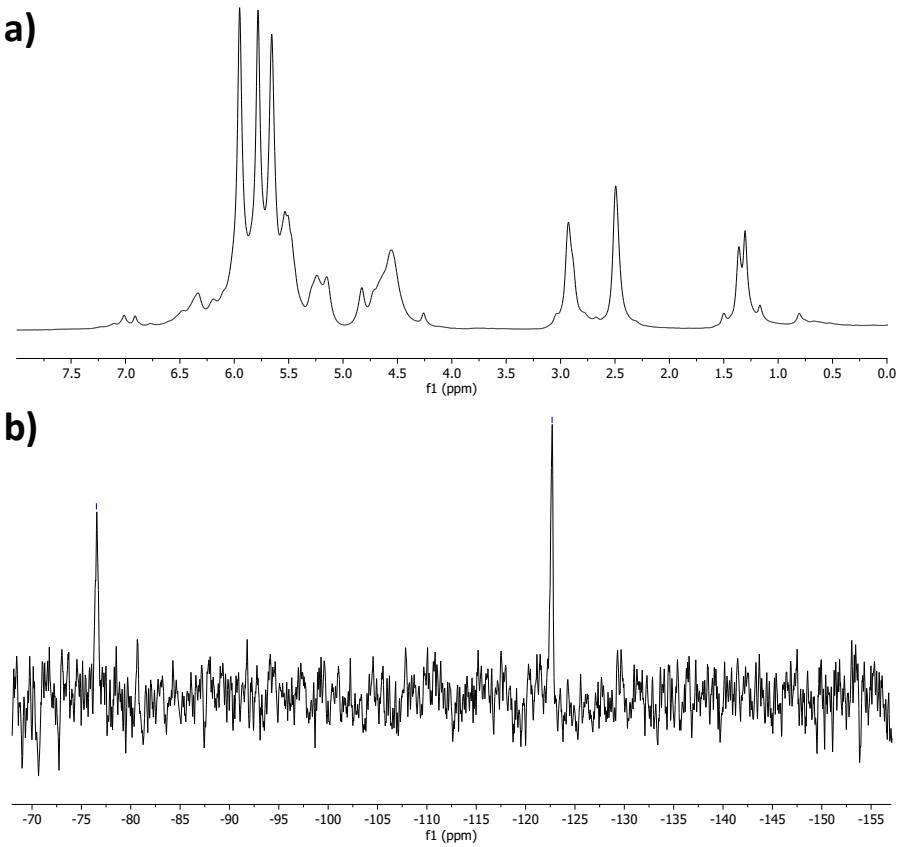

Figure 11. (a) Low-field ^{1}H NMR spectrum of an *ortho*-fluorofentanyl (**1a**)/heroin (**19**) mixture (2.4% *w/w*); (b) Low-field ^{19}F NMR spectrum of an *ortho*-fluorofentanyl (**1a**)/heroin (**19**) mixture (with TFA as internal standard).

4. Conclusion

In conclusion, it was shown that fluorinated regioisomers of fentanyl cannot be fully discriminated using presumptive colour tests, TLC or GC-MS. GC-MS afforded partial discrimination, but the co-elution of compounds with the same major *m/z* fragments prevented a conclusive identification. A GC-MS quantification method was developed, as it could still be useful for single-component samples or samples containing non co-eluting compounds. ^{19}F benchtop NMR was proposed as an orthogonal method for the identification and quantification of fluorinated regioisomers of fentanyl. Most fentalogues investigated, except (**1c**) and (**5b**), produced distinct signals in low-field ^{19}F NMR and a quantification method was developed using this technique. Benchtop NMR is a simpler, faster and cheaper method than GC-MS. Despite its lower sensitivity than GC-MS, benchtop ^{19}F NMR showed sufficiently low LODs and LOQs (74 – 400 µg/mL and 290 – 1340 µg/mL respectively), and it was shown that it could

detect *ortho*-fluorofentanyl (**1a**) in a 2.4% *w/w* heroin mixture, a common challenge for the detection of fentanyl analogues.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] J. Mounteney, I. Giraudon, G. Denissov, P. Griffiths. Fentanyls: Are we missing the signs? Highly potent and on the rise in Europe, Int J Drug Policy. 26 (2015) 626-631. http://doi.org/10.1016/j.drugpo.2015.04.003

[2] J. Suzuki, S. El-Haddad. A review: Fentanyl and non-pharmaceutical fentanyls, Drug Alcohol Depend. 171 (2017) 107-116. http://doi.org/10.1016/j.drugalcdep.2016.11.033

[3] P. Armenian, K. T. Vo, J. Barr-Walker, K. L. Lynch. Fentanyl, fentanyl analogs and novel synthetic opioids: A comprehensive review, Neuropharmacology. 134 (2018) 121-132. https://doi.org/10.1016/j.neuropharm.2017.10.016

[4] L. Scholl, P. Seth, M. Kariisa, N. Wilson, G. Baldwin. Drug and opioid-involved overdose deaths — United States, 2013–2017, MMWR Morb Mortal Wkly Rep. 67 (2019) 1419–1427. http://dx.doi.org/10.15585/mmwr.mm675152e1

[5] S. N. Lucyk, L. S. Nelson. Novel Synthetic Opioids: An Opioid Epidemic Within an Opioid Epidemic, Ann Emerg Med. 69 (2017) 91-93. http://doi.org/10.1016/j.annemergmed.2016.08.445

[6] P. Armenian, A. Olson, A. Anaya, A. Kurtz, R. Ruegner, R. R. Gerona. Fentanyl and a Novel Synthetic Opioid U-47700 Masquerading as Street "Norco" in Central California: A Case Report, Ann Emerg Med. 69 (2017) 87-90. http://doi.org/10.1016/j.annemergmed.2016.06.014

[7] R. G. Frank, H. A. Pollack. Addressing the Fentanyl Threat to Public Health, N Engl J Med. 376 (2017) 605-607. http://doi.org/10.1056/NEJMp1615145

[8] L. N. Rodda, J. L. Pilgrim, M. Di Rago, K. Crump, D. Gerostamoulos, O. H. Drummer. A Cluster of Fentanyl-Laced Heroin Deaths in 2015 in Melbourne, Australia, J Anal Toxicol. 41 (2017) 318-324. http://doi.org/10.1093/jat/bkx013

[9] R. T. DeRienz, D. D. Baker, N. E. Kelly, A. M. Mullins, R. Y. Barnett, J. M. Hobbs, J. A. Daniels, K. E. Harshbarger, A. M. Ortiz. Child Fatalities Due to Heroin/Fentanyl Exposure: What the Case History Missed, J Anal Toxicol. 42 (2018) 581-585. http://doi.org/10.1093/jat/bky052

[10] N. Baldwin, R. Gray, A. Goel, E. Wood, J. A. Buxton, L. M. Rieb. Fentanyl and heroin contained in seized illicit drugs and overdose-related deaths in British Columbia, Canada: An observational analysis, Drug Alcohol Depend. 185 (2018) 322-327. http://doi.org/10.1016/j.drugalcdep.2017.12.032

[11] United Nations Office on Drugs and Crime, January 2020 – United Kingdom: ACMD report on the misuse of fentanyl and fentanyl analogues as global number of opioid NPS rises. https://www.unodc.org/LSS/Announcement/Details/94dc6286-16bb-4e7a-9429-65d28918b332, 2020 (accessed 15 July 2020).

[12] A. Helland, W. R. Brede, L. S. Michelsen, P. O. M. Gundersen, H. Aarset, J. E. Skjølås, L. Slørdal. Two Hospitalizations and One Death After Exposure to Ortho-Fluorofentanyl, J Anal Toxicol. 41 (2017) 708-709. http://doi.org/10.1093/jat/bkx050

[13] Finnish Medicines Agency, 2-Fluorofentanyl (N-(2-fluorophenyl)-N-[1-(2-phenylethyl)piperidin-4-yl]-propanamide); 3-Fluorofentanyl (N-(3-fluorophenyl)-N-[1-(2-phenylethyl)piperidin-4-yl]-propanamide). http://ec.europa.eu/growth/tools-databases/tris/es/index.cfm/search/?trisaction=search.detail&year=2017&num=438&iLang=EN, 2017 (accessed 14 August 2019).

[14] Réseau Européen d'Information sur les Drogues et les Toxicomanies, Harms and harm reduction workbook. https://en.ofdt.fr/BDD/publications/docs/France2017-3-4-Hhr-EN.pdf, 2017 (accessed 14 August 2019).

[15] Advisory Council on the Misuse of Drugs, Misuse of fentanyl and fentanyl analogues. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/855893/ACMD_Report_-_Misuse_of_fentanyl_and_fentanyl_analogues.pdf, 2020 (accessed 15 Jan 2020).

[16] M. Bäckberg, O. Beck, K.-H. Jönsson, A. Helander. Opioid intoxications involving butyrfentanyl, 4-fluorobutyrfentanyl, and fentanyl from the Swedish STRIDA project, J Clin Toxicol. 53 (2015) 609-617. http://doi.org/10.3109/15563650.2015.1054505

[17] M. Rojkiewicz, M. Majchrzak, R. Celiński, P. Kuś, M. Sajewicz. Identification and physicochemical characterization of 4-fluorobutyrfentanyl (1-((4-fluorophenyl)(1-phenethylpiperidin-4-yl)amino)butan-1-one, 4-FBF) in seized materials and post-mortem biological samples, Drug Test Anal. 9 (2017) 405-414. http://doi.org/10.1002/dta.2135

[18] A. Helander, M. Backberg, P. Signell, O. Beck. Intoxications involving acrylfentanyl and other novel designer fentanyls - results from the Swedish STRIDA project, Clin Toxicol (Phila). 55 (2017) 589-599. 10.1080/15563650.2017.1303141

[19] F. E. Dussy, S. Hangartner, C. Hamberg, C. Berchtold, U. Scherer, G. Schlotterbeck, D. Wyler, T. A. Briellmann. An Acute Ocfentanil Fatality: A Case Report with Postmortem Concentrations, J Anal Toxicol. 40 (2016) 761-766. http://doi.org/10.1093/jat/bkw096

[20] C. Liu, T. Li, Y. Han, Z. Hua, W. Jia, Z. Qian. The identification and analytical characterization of 2,2'-difluorofentanyl, Drug Test Anal. 10 (2018) 774-780. http://doi.org/10.1002/dta.2264

[21] V. Spahn, G. Del Vecchio, D. Labuz, A. Rodriguez-Gaztelumendi, N. Massaly, J. Temp, V. Durmaz, P. Sabri, M. Reidelbach, H. Machelska, M. Weber, C. Stein. A nontoxic pain killer designed by modeling of pathological receptor conformations, Science. 355 (2017) 966-969. http://doi.org/10.1126/science.aai8636

[22] E. Kohyama, T. Chikumoto, H. Tada, K. Kitaichi, T. Ito. Analytical differentiation of quinolinyl- and isoquinolinyl-substituted 1-(5-fluoropentyl)-1H-indole-3-carboxylates: 5F-PB-22 and its ten isomers, Forensic Toxicol. 35 (2017) 56-65. http://doi.org/10.1007/s11419-016-0334-9

[23] T. Chikumoto, R. Furukawa, E. Kohyama, K. Suenami, H. Nagai, H. Tada, H. Kawashima, N. Kadomura, M. Soda, K. Kitaichi, T. Ito. Liquid chromatography–mass spectrometry studies on the isomeric 1-fluorobenzyl-3-naphthoyl-indoles: FUB-JWH-018 and five isomers, Forensic Toxicol. 37 (2019) 113-120. 10.1007/s11419-018-0442-9

[24] M. Kusano, K. Zaitsu, H. Nakayama, J. Nakajima, K. Hisatsune, T. Moriyasu, S. Matsuta, M. Katagi, H. Tsuchihashi, A. Ishii. Positional isomer differentiation of synthetic cannabinoid JWH-081 by GC-MS/MS, J Mass Spectrom. 50 (2015) 586-591. http://doi.org/10.1002/jms.3565

[25] Y. Abiedalla, L. W. Smith, K. M. Abdel-Hay, L. Neel, T. S. Belal, A. Thaxton-Weissenfluh, F. Smith, J. DeRuiter, C. R. Clark. Spectroscopic differentiation and chromatographic separation of regioisomeric indole aldehydes: Synthetic cannabinoids precursors, Forensic Chem. 12 (2019) 78-90. https://doi.org/10.1016/j.forc.2018.12.004

[26] Y. Nakazono, K. Tsujikawa, K. Kuwayama, T. Kanamori, Y. T. Iwata, K. Miyamoto, F. Kasuya, H. Inoue. Differentiation of regioisomeric fluoroamphetamine analogs by gas chromatography–mass spectrometry and liquid chromatography–tandem mass spectrometry, Forensic Toxicol. 31 (2013) 241-250. http://doi.org/10.1007/s11419-013-0184-7

[27] S. Negishi, Y. Nakazono, Y. T. Iwata, T. Kanamori, K. Tsujikawa, K. Kuwayama, T. Yamamuro, K. Miyamoto, T. Yamashita, F. Kasuya, H. Inoue. Differentiation of regioisomeric chloroamphetamine analogs using gas chromatography–chemical ionization-tandem mass spectrometry, Forensic Toxicol. 33 (2015) 338-347. http://doi.org/10.1007/s11419-015-0280-y

[28] A. Takeda, T. Tagami, A. Asada, T. Doi, M. Kawaguchi, Y. Satsuki, Y. Sawabe. Regioisomeric separation of ring-substituted cathinones by liquid chromatography–mass spectrometry with a naphthylethyl column, Forensic Toxicol. 35 (2017). http://doi.org/10.1007/s11419-016-0351-8

[29] E. L. Stuhmer, V. L. McGuffin, R. Waddell Smith. Discrimination of seized drug positional isomers based on statistical comparison of electron-ionization mass spectra, Forensic Chem. 20 (2020) 100261. https://doi.org/10.1016/j.forc.2020.100261

[30] J. Bonetti. Mass spectral differentiation of positional isomers using multivariate statistics, Forensic Chem. 9 (2018) 50-61. https://doi.org/10.1016/j.forc.2018.06.001

[31] J. T. Davidson, G. P. Jackson. The differentiation of 2,5-dimethoxy-N-(N-methoxybenzyl)phenethylamine (NBOMe) isomers using GC retention indices and multivariate analysis of ion abundances in electron ionization mass spectra, Forensic Chem. 14 (2019) 100160. https://doi.org/10.1016/j.forc.2019.100160

[32] N. Gilbert, R. E. Mewis, O. B. Sutcliffe. Classification of fentanyl analogues through principal component analysis (PCA) and hierarchical clustering of GC–MS data, Forensic Chem. 21 (2020) 100287. https://doi.org/10.1016/j.forc.2020.100287

[33] J. H. Hussain, N. Gilbert, A. Costello, C. J. Schofield, E. K. Kemsley, O. B. Sutcliffe, R. E. Mewis. Quantification of MDMA in seized tablets using benchtop 1H NMR spectroscopy in the absence of internal standards, Forensic Chem. 20 (2020) 100263. https://doi.org/10.1016/j.forc.2020.100263

[34] G. Assemat, F. Dubocq, S. Balayssac, C. Lamoureux, M. Malet-Martino, V. Gilard. Screening of "spice" herbal mixtures: From high-field to low-field proton NMR, Forensic Sci Int. 279 (2017) 88-95. https://doi.org/10.1016/j.forsciint.2017.08.006

[35] L. H. Antonides, R. M. Brignall, A. Costello, J. Ellison, S. E. Firth, N. Gilbert, B. J. Groom, S. J. Hudson, M. C. Hulme, J. Marron, Z. A. Pullen, T. B. R. Robertson, C. J. Schofield, D. C. Williamson, E. K. Kemsley, O. B. Sutcliffe, R. E. Mewis. Rapid Identification of Novel Psychoactive and Other Controlled Substances Using Low-Field (1)H NMR Spectroscopy, ACS Omega. 4 (2019) 7103-7112. http://doi.org/10.1021/acsomega.9b00302

[36] J. Duffy, A. Urbas, M. Niemitz, K. Lippa, I. Marginean. Differentiation of fentanyl analogues by low-field NMR spectroscopy, Anal Chim Acta. 1049 (2019) 161-169. http://doi.org/10.1016/j.aca.2018.12.014

[37] D. B. G. Williams, M. Lawton. Drying of Organic Solvents: Quantitative Evaluation of the Efficiency of Several Desiccants, J Org Chem. 75 (2010) 8351-8354. http://doi.org/10.1021/jo101589h

[38] G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottlieb, A. Nudelman, B. M. Stoltz, J. E. Bercaw, K. I. Goldberg. NMR Chemical Shifts of Trace Impurities: Common Laboratory Solvents, Organics, and Gases in Deuterated Solvents Relevant to the Organometallic Chemist, Organometallics. 29 (2010) 2176-2179. http://doi.org/10.1021/om100106e

[39] F. Yang, X. Yu, C. Liu, C.-X. Qu, Z. Gong, H.-D. Liu, F.-H. Li, H.-M. Wang, D.-F. He, F. Yi, C. Song, C.-L. Tian, K.-H. Xiao, J.-Y. Wang, J.-P. Sun. Phospho-selective mechanisms of arrestin conformations and functions revealed by unnatural amino acid incorporation and 19F-NMR, Nat Commun. 6 (2015) 8202. http://doi.org/10.1038/ncomms9202

[40] G. F. Pauli, S.-N. Chen, C. Simmler, D. C. Lankin, T. Gödecke, B. U. Jaki, J. B. Friesen, J. B. McAlpine, J. G. Napolitano. Importance of purity evaluation and the potential of quantitative 1H NMR as a purity assay, J Med Chem. 57 (2014) 9220-9231. 10.1021/jm500734a

[41] N. Gilbert, L. H. Antonides, C. J. Schofield, A. Costello, B. Kilkelly, A. R. Cain, P. R. V. Dalziel, K. Horner, R. E. Mewis, O. B. Sutcliffe. Hitting the Jackpot – development of gas chromatography–mass spectrometry (GC–MS) and other rapid screening methods for the analysis of 18 fentanyl-derived synthetic opioids, Drug Test Anal. 12 (2020) 798-811. http://doi.org/10.1002/dta.2771

[42] G. McLaughlin, N. Morris, P. V. Kavanagh, J. D. Power, J. O'Brien, B. Talbot, S. P. Elliott, J. Wallach, K. Hoang, H. Morris, S. D. Brandt. Test purchase, synthesis, and characterization of 2-methoxydiphenidine (MXP) and differentiation from its meta- and para-substituted isomers, Drug Test Anal. 8 (2016) 98-109. http://doi.org/10.1002/dta.1800

[43] United Nations Office on Drugs and Crime, Rapid testing methods of drugs of abuse. https://www.unodc.org/documents/scientific/Rapid_Testing_Methods_of_Drugs_of_Abuse_E.pdf, 1995 (accessed 06 July 2019).

[44] K.-A. Kovar, M. Laudszun, Chemistry and reaction mechanisms of rapid tests for drugs of abuse and precursors chemicals. https://www.unodc.org/pdf/scientific/SCITEC6.pdf, 1989 (accessed 06 July 2019).

[45] M. J. Kangas, D. Symonsbergen, A. E. Holmes. A new possible alternative colorimetric drug detection test for fentanyl, Organic & Medicinal Chem IJ. 4 (2017) 555645.

[46] P. M. Geyer, M. C. Hulme, J. P. B. Irving, P. D. Thompson, R. N. Ashton, R. J. Lee, L. Johnson, J. Marron, C. E. Banks, O. B. Sutcliffe. Guilty by dissociation—development of gas chromatography–mass spectrometry (GC-MS) and other rapid screening methods for the analysis of 13 diphenidine-derived new psychoactive substances (NPSs), Anal Bioanal Chem. 408 (2016) 8467-8481. http://doi.org/10.1007/s00216-016-9969-y

[47] International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, Validation of analytical procedures: Text and methodology. http://www.ich.org/products/guidelines/quality/quality-single/article/validation-of-analytical-procedures-text-and-methodology.html, 2005 (accessed 06 July 2019).

Table 1. GC-MS validation data for the quantification of fluorofentanyls (**1a-c, 5a-c, 6, 7**), fentanyl (**18**), heroin (**19**), acetaminophen (**20**) and caffeine (**21**). See **Figure 7** for representative chromatogram.

Analyte	SIM ions	t_R (min)	RRT[a]	R_s	A_s	N (plates)	H (x10^{-5} m)	r^2	LOQ[n] (ng/mL)	LOD[o] (ng/mL)	Precision (%RSD, n = 6)				
											5.0 µg/mL	10.0 µg/mL	15.0 µg/mL	20.0 µg/mL	25.0 µg/mL
20	151.0, **109.0**, 80.0	4.88	0.39	–	6.1	418362	7.17	0.993[b]	2100	620	7.4	4.7	4.3	5.9	3.3
21	**194.1**, 109.0, 82.0	5.69	0.46	33.0	1.8	501999	5.98	0.997[c]	110	34	4.0	2.1	1.9	2.5	2.4
Eicosane (IS)	**57.1**, 71.1, 85.1	6.49	0.52	27.8	1.1	871966	3.44	–	–	–	–	–	–	–	–
19	369.2, **327.2**, 268.1	11.51	0.93	109.0	1.0	553506	5.42	0.998[d]	230	68	5.3	4.8	1.4	2.1	1.1
1b	**263.0**, 207.0, 164.0	11.79	0.95	4.3	1.0	564224	5.32	0.999[e]	40	12	2.8	0.3	2.4	1.3	1.1
6	**263.0**, 207.0, 164.0	11.87	0.95	1.1	1.0	514321	5.83	0.999[f]	44	13	1.1	1.6	1.5	1.6	1.1
1c	**263.0**, 207.0, 164.0	11.99	0.96	1.7	1.0	506423	5.92	0.998[g]	45	13	2.1	1.0	2.4	1.2	1.2
1a	**263.0**, 207.0, 164.0	12.14	0.98	2.1	1.1	459280	6.53	0.999[i]	57	17	3.1	1.2	1.4	1.2	1.2
5a	**245.0**, 189.0, 146.0	12.16	0.98	0	1.0	533666	5.62	0.999[h]	31	9.0	1.2	0.6	1.3	1.5	0.9
5b	**245.0**, 189.0, 146.0	12.24	0.98	1.6	1.1	450960	6.65	0.999[j]	57	17	3.7	1.9	1.6	2.2	1.7
5c	**245.0**, 189.0, 146.0	12.27	0.99	0	1.2	433778	6.92	0.998[k]	45	13	2.2	1.3	2.1	1.0	1.4
18	**245.1**, 189.1, 146.0	12.44	1.00	2.4	1.1	468388	6.40	0.999[l]	55	17	3.9	2.4	1.0	2.5	1.3
7	**263.0**, 207.0, 186.1	12.47	1.00	0	1.1	464368	6.46	0.999[m]	67	20	1.9	1.6	1.2	1.3	1.5

Key: [a] Relative retention time (in relation to fentanyl); [b] y=0.1438x-0.6305; [c] y=0.1920x-0.0628; [d] y=0.0597x – 0.0761; [e] y=0.2547x-0.0635; [f] y=0.1804x-0.0761; [g] y=0.2546x-0.1061; [h] y=0.2463x-0.1634; [i] y=0.2070x-0.0634; [j] y=0.2295x-0.2382; [k] y=0.2442x-0.0907; [l] y=0.2173x-0.4763; [m] y=0.2420x-0.2908; [n] Limit of detection (determined using a signal-to-noise ratio of 3:1); [o] Limit of quantification (determined using a signal-to-noise ratio of 10:1).

Table 2. Low-field ^{19}F NMR validation data for the quantification of fluorofentanyls (**1a-c, 5a-c, 6, 7**). See **Figure 10** for representative spectra.

Analyte	Chemical shift (ppm)[a]	r^2	LOD[k] (µg/mL)	LOQ[l] (µg/mL)	Precision (%RSD, n = 6)				
					5.0 mg/mL	8.0 mg/mL	10.0 mg/mL	12.0 mg/mL	15.0 mg/mL
1b	-114.064	0.999[b]	110	350	3.3	2.2	2.4	1.6	1.4
5b	-115.698	0.999[c]	100	350	4.1	2.8	2.8	2.0	1.6
1c	-115.977	0.996[d]	76	250	4.8	4.7	2.5	2.3	3.0
5c	-118.556	0.995[e]	130	440	5.3	2.5	4.2	1.8	2.3
5a	-120.762	0.999[f]	86	290	4.3	3.3	0.9	2.4	1.1
1a	-122.048	0.995[g]	74	250	5.8	4.8	2.6	4.1	2.4
7	-199.434	0.996[h]	400	1340	9.7	4.5	2.7	5.2	4.7
6 (2')	-120.657	0.994[i]	110	360	3.1	4.0	3.5	4.0	2.9
6 (*o*)	-122.047	0.994[j]	120	390	6.1	4.5	4.7	4.3	2.2

Key: [a] Referenced to trifluoroacetic acid (TFA); [b] y=1711.1x+570.8; [c] y=1629.4x+33.7; [d] y=1937.8x-188.0; [e] y=1386.0x+1129.4; [f] y=1441.0x+456.0; [g] y=1798.8x-1537.8; [h] y=1779.7x+69.9; [i] y=1467.3x+390.7; [j] y=1436.3x+303.2; [k] Limit of detection (determined using a signal-to-noise ratio of 3:1); [l] Limit of quantification (determined using a signal-to-noise ratio of 10:1).